Universidade Estadual de Londrina

VICTOR FATTORI

MECANISMOS DE INIBIÇÃO DA INFLAMAÇÃO E DOR PELA CURCUMINA NOS MODELOS DE ARTRITE INDUZIDA POR DIÓXIDO DE TITÂNION E INFLAMAÇÃO INDUZIDA POR ÂNION SUPERÓXIDO EM CAMUNDONGOS

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Dissertação de mestrado apresentada ao programa de pós-graduação em Patologia Experimental da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Mestre em Patologia Experimental

Orientador: Prof. Dr. Waldiceu Aparecido Verri Junior.

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Londrina, 14 de março de 2016.

Dedico a minha esposa Natasha, meu irmão Vinicius e meus pais.

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“Demore o tempo que for para você decidir o que quer da vida, e depois que decidir, não recue ante nenhum pretexto, pois o mundo tentará te dissuadir”

Profeta Zaratustra, de Friedrich Nietzsche.

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RESUMO

Os turmericos (*Curcuma longa*) têm sido utilizados pela Medicina Ayevedica e chinesa por milhares de anos para o tratamento de diversas doenças, como asma, úlcera e artrite. Cucumina, o principal constituinte derivado do rizoma da planta, é uma molécula pleitrópica e possui atividade anti-inflamatória, em parte, através da inibição do NF-κB. Tendo em vista essas características, curcumina é uma alternativa promissora para o tratamento da dor. Desse modo, nosso objetivo foi avaliar a atividade da curcumina em modelo agudo de dor induzido pelo superóxido de potássio (KO_2), um doador de ânion superóxido ($O_2^{\bullet-}$) e modelo de artrite induzido por dióxido de titânio. Para avaliação da dor manifesta, foi contabilizado o número total de contorções abdominais (durante 20 minutos) ou número total de sacudidas de patas e o tempo lambida (durante 30 minutos). A hiperalgesia mecânica e térmica foi avaliada 0,5; 1; 3; 5 e 7 h após o estímulo utilizando um analgesímetro digital e placa quente, respectivamente. A produção de citocinas (IL-1β, TNF-α e IL-10) e atividade do NF-κB foram avaliados por ELISA, 3 h após o estímulo. A produção de $O_2^{\bullet-}$ foi avaliada através do ensaio nitroblue tetrazolium, e a atividade antioxidante total pelo ensaio ABTS, ambos 3 h após o estímulo. A atividade da mieloperoxidade e recrutamento de leucócitos foram avaliados 7 h após o estímulo. A expressão do mRNA de Nrf2, HO-1 e $gp91^{phox}$ foram avaliados por RT-qPCR. O tratamento com curcumina 10 mg/kg reduziu dor manifesta induzida por $O_2^{\bullet-}$ de maneira dose dependente, além de inibir a hiperalgesia mecânica e térmica em todos os intervalos avaliados. A produção de citocinas induzidas pelo $O_2^{\bullet-}$ (IL-1β e TNF-α) bem como a atividade do NF-κB foram reduzidas pela curcumina. Esses efeitos foram acompanhandos pelo aumento da produção da citocina anti-inflamatória IL-10. Nós demonstramos, que o tratamento com curcumina 10 mg/kg foi capaz de aumentar a expressão do mRNA para o fator de transcrição Nrf2 e seu alvo HO-1. Além disso, o tratamento com curcumina aboliu a produção do $O_2^{\bullet-}$, restaurou a atividade antioxidante total e inibiu o recrutamento de leucócitos. Em um segundo momento, avaliamos o efeito da curcumina em modelo de artrite induzido dióxido de titânio. A hiperalgesia mecânica e edema foram avaliados 1, 3, 5, 7 e 24h após o estímulo e de dois em dois dias até o 30º dia após o estímulo. Nós observamos que o tratamento com curcumina na dose de 100 mg/kg foi efetiva na redução da dor, edema, recrutamento de neutrófilos. Como resultado disso houve menor degradação de proteoglicanos e maior preservação da articulação. Tendo isso como esteio, nós demonstramos que a curcumina apresenta atividades anti-inflamatórias e analgésica em modelos de dor crônica e dessa forma contribuirmos para o entendimento do mecanismo de ação dessa molécula reforçando sua atividade pleiotrópica.

Palavras chaves: Ânion superóxido. Artrite. Curcumina. Dor. Dióxido de Titânio. IL-33. NF-κB. Nrf2. Proteoglicanos

FATTORI, Victor. **Curcumin inhibits pain and inflammation in superoxide anion-iduced acute pain and titanium dioxide-induced arthritis**. 2015. 120 p. Dissertation (Master's degree in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2016.

ABSTRACT

Ayurvedic and Chinese medicine have used turmeric (*Curcuma longa*) as a medicinal herb for thousands of years to treat different diseases, such as asthma, ulcer, and arthritis. Curcumin, the main bioactive constituent derived from the rhizome of turmeric, is pleiotropic molecule and has its anti-inflammatory properties, at least in part, through inhibition of NF-κB. Due these features, curcumin is a promising alternative to treat inflammatory pain. Therefore, we aim of to evaluate the activity of curcumin in two models: the first model was acute pain – potassium superoxide (KO_2)–induced – and the second model was arthritis – titanium dioxide-induced – in mice. Experimental pain was induced by administration of 30 µg (i.pl.) or 1 mg (i.p.) of radical superoxide anion ($O_2^{\bullet-}$) donor KO_2. Overt pain-like behaviours were quantified by counting the number of abdominal writhings over 20 minutes, or the number of paw flinches and the time spent licking the paw over 30 minutes. Mechanical and thermal hyperalgesia was evaluated in the paw 0.5, 1, 3, 5 and 7 h after stimulus. Cytokine levels (IL-1β, TNF-α, and IL-10) and NF-κB activity in the paw tissue were measured by ELISA 3 h after stimulus. $O_2^{\bullet-}$ production was evaluated by nitroblue tetrazolium (NBT) assay, and the antioxidant capacity was evaluated by measuring the ABTS radical scavenging ability, both in samples colected 3 h after stimulus. Myeloperoxidase activity and total leukocyte recruitment was measured collected 7 h after stimulus in paw skin samples and peritoneal wash, respectively. Nrf2, HO-1 and $gp91^{phox}$ mRNA expression in paw skin was determined 3 h after stimulus by real time PCR. We observed that curcumin inhibited overt pain-like behaviours induced by $O_2^{\bullet-}$ in a dose-dependent manner, and inhibited mechanical and thermal hyperalgesia in all mesuared intervals as well. $O_2^{\bullet-}$-induced production of pro-inflammatory cytokines (IL-1β and TNF-α) and activity of NF-κB were supressed by curcumin, and these effects were acompanied by an enhanced production of the anti-inflammatory cytokine IL-10. We found increased expression of mRNA for transcription factor Nrf2 and HO-1 in curcumin-treated animals. Curcumin restored antioxidant capacity, abolished $O_2^{\bullet-}$ production and myeloperoxidase activity, and reduced $gp91^{phox}$ mRNA expression. In arthritis model, mechanical threshold and edema were evaluated after 1, 3, 5, 7, 24h and then each two days until 30^{th} day after titanium dioxide. We observed that curcumin at 100 mg/kg reduced titanium dioxide-induced pain, edema and neutrophils recruitment. As consequence of that, curcumin reduced proteoglycan degradation and avoid joint lesion. Taking this into account, we demonstrate that curcumin provided anti-inflammatory and analgesic properties in models of acute and chronic pain, and this may contribute to understanding the mechanisms of action of this molecule reinforcing, its pleoiotropic activity and supporting its use in clinical trials.

Keywords: Arthritis. Curcumin. IL-33. NF-κB. Nrf2 – Pain. Proteoglycan. Superoxide anion. Titanium Dioxide

LISTA DE ILUSTRAÇÕES

LISTA DE TABELA

LISTA DE ABREVIATURAS E SIGLAS

ABTS	Ácido 2,2′-azino-bis-3-ethilbenzthiazolino-6-sulphonico
AEC	Antes da Era Comum
ASIC	Canal Iônico Sensível a Ácidez
cGMP	Guanosina Monofosfato
COX	Ciclo-oxigenase
DAMP	Molécula Padrão Associada a Dano
EC	Era Comum
ELAM	Molécula de Adesão Leucocitária Endotelial
ERN	Espécies Reativas de Nitrogênio
ERO	Espécies Reativas de Oxigênio
FoxO3a	Fator de Transcrição Forkhead subfamília O
fMLP	N-Formilmetil-Leucyl-Fenilalanina
GABA	Ácido Gama-Aminobutírico
GSH	Glutationa Reduzida
HO-1	Heme Oxigenase-1
ICAM-1	Molécula de Adesão Intracelular-1
IL	Interleucina
Keap-1	Proteína 1 associada a ECH similar a Kelch
KO_2	Superóxido de Potássio
MPO	Mieloperoxidase
mRNA	RNA mensageiro
NADPH	Fosfato de Dinucleotídio de Nicotinamida e Adenina
Nav	Canal de Sódio Voltagem-Resistente
NBT	Nitroazul de Tetrazólio
NF-κB	Fator Nuclear de Cadeia Leve kappa de Células B Ativadas (Fator de Transcrição Nuclear kappa B)
Nrf2	Fator 2 Relacionado ao Fator de Transcrição Nuclear Eritróide 2
NLR	Receptor Semelhante a Nod
PAMP	Molécula Padrão Associada à Membrana de Patógenos
PGE_2	Prostaglandina E_2
PKA	Proteína Kinase A
PKC	Proteína Kinase C

PKG	Proteína Kinase G
PRR	Receptor de Reconhecimento de Padrão
RANK	Receptor Ativador do NF-κB
RANKL	Ligante do Receptor Ativador do NF-κB
SOD	Superóxido Dismutase
RLR	Receptor Semelhante a Rig
SPM	Mediadores Lipídicos Pró-Resolução
TLR	Receptor Semelhante a Toll
TNF-α	Fator de Necrose Tumoral alfa
TRPA1	Receptor de Potencial Transitório Subfamília A, membro 1
TRPV1	Receptor de Potencial Transitório Subfamília V, membro 1
VAS	Escala Analógica Visual
VCAM-1	Molécula de Adesão Vascular-1

SUMÁRIO

1 INTRODUÇÃO

1.1 INFLAMAÇÃO

Inflamação é uma das condições médicas com registros mais antigos. Isso é evidenciado com a descoberta de hierógrafos datados 2700 AEC desenvolvidos pela civilização sumeriana, e que representavam os sinais e sintomas da inflamação: vermelhidão, calor, edema e dor. Esses mesmos sinais e sintomas foram descritos e eternizados pelas letras do romano Aulus Cornelius Celsus, no primeiro século EC (ROCHA E SILVA, 1978). A inflamação aguda é uma resposta apropriada para uma defesa efetiva do hospedeiro e manutenção da homeostase, e é hodiernamente subdividida em duas fases: iniciação e resolução (MADERNA; GODSON, 2009). Entretanto, se ocorrer de forma excessiva pode levar a destruição do tecido, fibrose e com eventual perda de função do órgão afetado (MADERNA; GODSON, 2009). Em face de limitar tal resposta, mecanismos compensatórios foram desenvolvidos e mantidos ao longo do processo evolutivo, tais como: produção de citocinas anti-inflamatórias e agentes antioxidantes (LAWRENCE; WILLOUGHBY; GILROY, 2002; NITURE; KHATRI; JAISWAL, 2014). O NF-κB e Nrf2 são fatores de transcrição que têm papel chave nessa regulação, e serão discutidos com mais detalhes posteriormente.

O processo inflamatório é iniciado, geralmente, após o reconhecimento de PAMPs ou DAMPs por PRRs (TLR, NLR e RLR, por exemplo) presentens em células residentes, especialmente macrófagos (IWASAKI; MEDZHITOV, 2010; TAKEUCHI; AKIRA, 2010). Esse reconhecimento tem como resultado final a ativação do NF-κB e consequente produção de citocinas pró-inflamatórias, tais como IL-1β, TNF-α, e IL-6 (IWASAKI; MEDZHITOV, 2010; TAKEUCHI; AKIRA, 2010). Outros mediadores como quimiocionas (CXCL1 [KC em murinos] e CXCL2), proteína do complemento C5a LTB_4, PGE_2 e endotelinas (CUNHA, F. Q.; CACINI; FERREIRA, 1986; FERREIRA; ROMITELLI; DE NUCCI, 1989; GUERRERO et al., 2008; RIBEIRO et al., 1997; TING et al., 2008; VERRI; CUNHA; PARADA; WEI; et al., 2006), colaboram para amplificação do processo. Em conjunto, esses mediadores são reponsáveis por ativar o endotélio e guiar precisamente, inicialmente, neutrófilos para o foco inflamatório. Na última década, o processo de recrutamento de neutrófilos tem sido estudado elegantemente (MCDONALD et al., 2010; SREERAMKUMAR et al., 2014). É um processo que depende da

característica inicial do estímulo nóxico e segue uma cascata temporal, espacial e hierárquica de mediadores (MCDONALD et al., 2010; SREERAMKUMAR et al., 2014).O recrutamento de neutrófilos em sinusóides do fígado em modelo de inflamação estéril são dependentes da integrina Mac-1 e ICAM-1 (MCDONALD et al., 2010). Em contraste, após estímulo com *E. coli* o recrutamento de neutrófilos é dependente de CD44 em detrimento de Mac-1, revelando diferentes mecanismos de recrutamento de neutrófilos em modelos de inflamação estéril e não estéril (MCDONALD et al., 2010). Neutrófilos tendem a responder melhor à moleculas quimioatraentes finais como fMLP e C5a quando comparado à moleculas iniciais como IL-8 e LTB_4 (FOXMAN; CAMPBELL; BUTCHER, 1997). Corroborando esse fato, em modelo de inflamação estéril camundongos knockout para receptor de fMLP, mas não CXCR2, apresentaram redução significativa de neutrófilos no foco necrótico, o que demonstra que o receptor de fMLP guia localização precisa de neutrófilos em áreas de necrose, fortificando a ideia de hierarquia de mediadores para o recrutamento de neutrófilos (MCDONALD et al., 2010). Os neutrófilos na circulação normalmente têm uma vida útil curta e rapidamente sofrem apoptose constitutiva. No entanto, o microambiente inflamatório estimula a expressão de genes relacionados com a longevidade, como FoxO3a (JONSSON; ALLEN; PENG, 2005), além de promover ativação do NF-κB e contribuir para produção de mais citocinas pró-inflamatórias e EROs (WRIGHT et al., 2010).

A questão temporal também é relevante na resolução do processo inflamatório, o qual é visto como ativo e altamente regulado (SERHAN et al., 2015). A presença de neutrófilos apoptóticos associada a produção de PGE_2 inicia a troca na classe de mediadores lipídicos de pró-inflamatórios para pró-resolução (LEVY et al., 2001). Esses neutrófilos apoptóticos são fagocitados por macrófagos não flogísticos, em um processo conhecido como eferocitose. Os principais mediadores pró-resolução produzidos são as lipoxinas, maresinas e resolvinas (SERHAN et al., 2015). Em conjunto, essas e outras moléculas atuam na redução e no reparo dos danos provocados durante a inflamação aguda e promovem a resolução do processo de forma ativa.

1.1.1 NF-κB

A cascata de sinalização do NF-κB consiste, basicamente, em uma série de elementos regulatórios que culminam na ativação kinases responsáveis pela

fosforilação, ubiquitinação e degração de proteínas inibidoras desse fator de transcrição. O NF-κB é composto pelo agrupamento de complexos homo e heterodiméricos (HAYDEN; GHOSH, 2008). Nove dos 15 potenciais dímeros apresentam atividade transcripcional, ou seja, são capazes de se ligar na região promotora de genes transcritos pelo NF-κB, região essa denominada locais κB. O dímero de maior importância é o formado pelas subunidades p50-p65 (SEN; SMALE, 2010). Assim como toda via de sinalização, sua modulação negativa é essencial para manutenção da homeostase. O inibidor do NF-κB é o IκB, o qual é composto das subunidades α, β e ε. Esse fator tem a função de inibir os sinais de localização nuclear do heterodímero p50-p65 e impedir que ocorra a transcrição de genes relacionados ao NF-κB. A ativação desse fator de transcrição pode ocorrer por duas vias: canônica e não-canônica, e para efeito de discussão, iremos focar na primeira devido seu mecanismo *upstream* de sinalização ser dependende da transdução de sinal via PRR, TNFR e/ou IL-1R (HAYDEN; GHOSH, 2008). Para que haja ativação do NF-κB é necessário que seu inibidor (IκB) seja degrado. Para tanto, a presença de kinases é indispensável. Como citado anteriormente, a estimulação dos PRR que ocorre pelos sinais produzidos durante a lesão tecidual e/ou infecção leva à ativação do NF-κB através da degradação de IκB. O complexo de kinases do IκB (IKK), formado pelas subunidades catalíticas IKKα, IKKβ, e pela subunidade regulatória IKKγ (NEMO), inicia o processo de fosforilação do IκB, em sua subunidade α em dois resíduos específicos de serina (KARIN; BEN-NERIAH, 2000), propiciando a ubquitinação desse inibidor e consequente sua degradação via proteossomo. Isso permite que o heterodímero p50-p65 seja translocado até o núcleo da célula e induza a transcrição de genes pró-inflamatórios (Figura 1 A) (HAYDEN; GHOSH, 2008; SEN; SMALE, 2010).

As EROs são capazes de oxidar diversas proteínas, especialmente com resíduos de cisteína (tióis), essa característica permite a ativar diversas vias de sinalização, entre elas a do NF-κB (GLOIRE; PIETTE, 2009). De fato, a via do NF-κB é conhecida como sensível ao estado redox da célula (TOLEDANO; LEONARD, 1991). Após ubiquitinição e degradação do IκBα, resíduos específicos de serina da subunidade p65 são fosforilados por PKA. Isso permite a interação de p65 com a molécula coativadora do NF-κB (mas não apenas) CBP (proteína ligadora de CREB). Essa fosforilação por PKA é elevada na presença de EROs e isso possibita o aumento da atividade do NF-κB. De fato, tratamento com antioxidantes diminui a

associação de p65 com CBP na região promotora do gene da IL-8, por exemplo (JAMALUDDIN et al., 2007). Focando no ânion superóxido, tratamento com M40403 (molécula mimética da enzima SOD) inibiu a ativação do NF-κB, relacionada com diminuição da degradação do IκB, sugerindo a participação do ânion superóxido da regulação redox desse fator de transcrição (NDENGELE et al., 2005)

De modo a controlar a resposta inflamatória, o NF-κB estimula a transcrição de genes anti-inflamatórios, como por exemplo a citocina IL-10 (HAYDEN; GHOSH, 2008; SEN; SMALE, 2010), proteínas com função ubquitina-ligase (A20) (CHEN, 2005), seu inibidor IκB (LAWRENCE, 2009), moléculas antioxidantes como catalase, GSH e SOD, entre outros (HAYDEN; GHOSH, 2008; SEN; SMALE, 2010).

1.1.2 Nrf2

O fator de transcrição Nrf2 é um dos sistemas citoprotetores mais importantes adquirido pelos vertebrados ao longo da evolução (GAO, B.; DOAN; HYBERTSON, 2014). De fato, a substuição de um único nucleotídeo (SNP) na região promotora de genes transcritos pelo Nrf2 é capaz de conferir ao indivíduo maior susceptibilidade a lesão pulmonar (MARZEC et al., 2007). O Keap-1 é um uma molécula rica em resíduos de cisteína e com atividade ubiquitina-ligase e consequentemente é o inibidor dessa via de sinalização (ITOH et al., 1997). Portanto, sob condições fisiológicas o Nrf2 está associado ao Keap-1, constantemente ubquitinado e degrado via proteassomo (Figura 1C). Entretanto, após modificações em Keap-1 decorrentes de ataque eletrofílico em resíduos tióis específicos de cisteína, associado a fosforilação em resíduos específicos de serina do Nrf2, occore a dissociação do Keap-1 e estabilização do fator de transcrição. O Nrf2 estável é capaz de se dimerizar com proteína sMaf (*small* Maf) e com consequente translocação para núcleo (HUANG; NGUYEN; PICKETT, 2002; LI et al., 2008; NITURE; JAIN; JAISWAL, 2009) em genes com regiões promotoras denominadas ARE (Elementos de Resposta Antioxidante) para que haja aumento da expressão de genes relacionados a resposta citoprotetora (Figura 1D) (TURPAEV, 2013).

Um dos alvos mais interessantes do Nrf2 é a enzima HO-1 (TURPAEV, 2013). HO-1 apresenta atividade anti-inflamatória, antioxidante e antinociceptiva (STEINER et al., 2001; TURPAEV, 2013). Para exercer sua atividade antioxidante, HO-1 cataliza a reação do heme que leva a formação de monóxido de carbono, ferro e biliverdina. A enzima biliverdina redutase é responsável pela conversão da

biliverdina em bilirrubina, a qual tem potente atividade antioxidante, e com participação das EROs há a conversão de bilirruibina em biliverdina novamente. Desse modo esse ciclo garante a neutralização das EROs e provê atividade antioxidante para HO-1(PAE et al., 2010). Essa via ainda é capaz de inibir a migração e adesão de neutrófilos para o foco inflamatório (FREITAS et al., 2006), que são fundamentais para o desenvolvimento e manutenção de estados hiperalgésicos (CUNHA, T. M. et al., 2008). Além disso, HO-1 é capaz de ativar a via de canais de potássio sensíveis a cGMP/PKG/ATP (KAPITULNIK, 2004). Essa via é reponsável por aumentar o limiar de ativação dos nociceptores e consequentemente diminuir a despolarização provendo atividade antinociceptiva (STAURENGO-FERRARI et al., 2014; ZARPELON; SOUZA; et al., 2013).

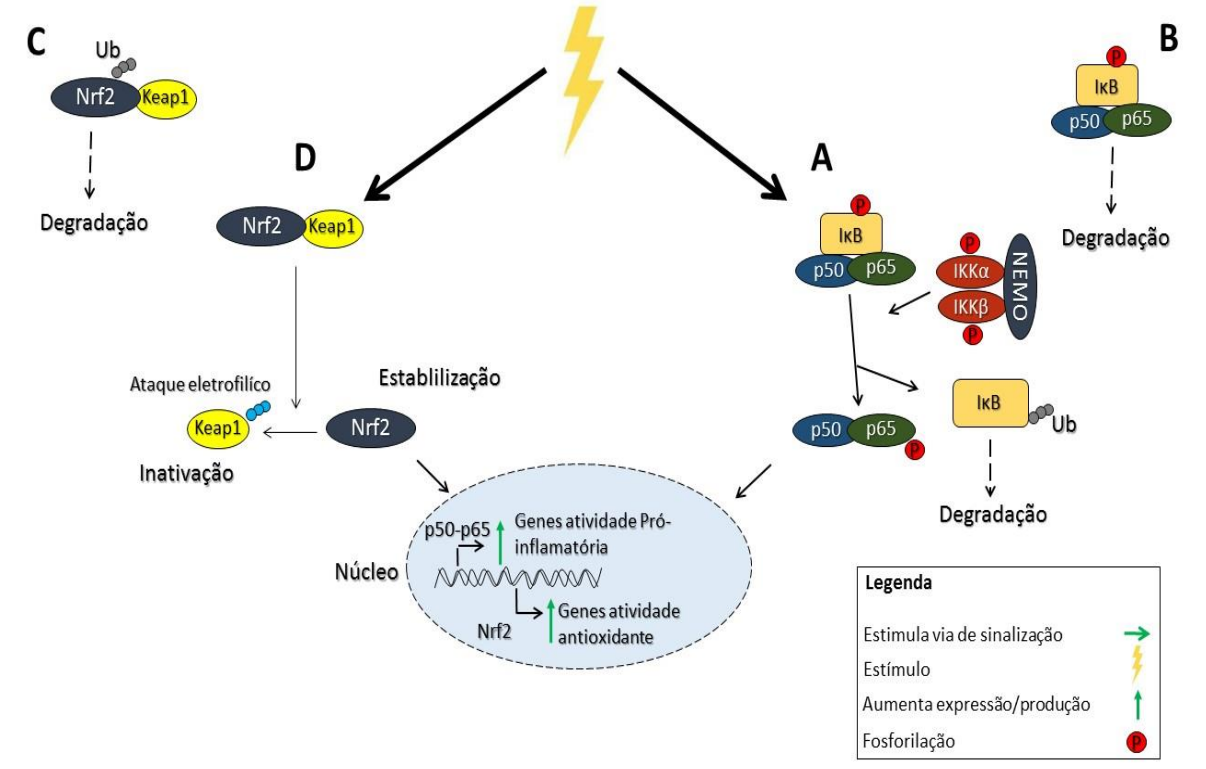

Figura 1 (A-D). Vias de sinalização NF-κB e Nrf2. Após um estímulo lesivo, vias de sinalização são ativadas para transcrição de genes relacionado a atividade pró-inflamatória (A) e citoprotetora (C). “B” e “D” representam a consequencia da via sem estímulo (HAYDEN; GHOSH, 2008; TURPAEV, 2013).

1.2 Dor

O sistema nervoso detecta e interpreta uma vasta gama de estímulos térmicos e mecânicos, bem como irritantes químicos ambientais ou endógenos.

Quando intenso, esses estímulos são capazes de gerar dor aguda. A dor é um sintoma presente em muitas doenças e é uma das maiores causas de procura ao atendimento médico. Durante o processo evolutivo, a percepção da dor, ou nocicepção, foi um mecanismo que se manteve por conferir ao indivíduo a capacidade de autopreservação, identificando situações que possam causar danos ou mesmo controlar as consequências de uma lesão já ocorrida. Esse caráter evolutivo é evidenciado em indivíduos portadores da doença conhecida como “insensibilidade congênita à dor”, ou “analgesia congênita”, já que raramente atingem a idade adulta, por não disporem desses mecanismos inatos protetores. A compreensão dos mecanismos envolvendo a dor, especialmente as crônicas, é essencial, tendo em vista seu impacto na qualidade de vida das pessoas afetadas. Tal a influência da dor na qualidade de vida, sua presença constante é um fator de risco consistente para pensamentos e comportamentos suicidas (CALATI et al., 2015).

1.2.1 Fibras Nociceptivas

A percepção de um estímulo como doloroso só é possível graças a presença de neurônios sensoriais, denominados de nociceptores. Nesse sentido, a nocicepção pode ser considerada uma modalidade sensorial tanto quanto visão ou olfato, já que um determinado estímulo de determinada intensidade e proporção pode ser detectado por células especializadas para tanto. Existem dois tipos de fibras nociceptivas: A e C, sendo que a primeira se subdivide em Aα, Aβ e Aδ (BRAZ et al., 2014). As fibras do tipo Aα e Aβ apresentam axônios com maior quantidade de mielina e respondem a estímulos inócuos na pele, músculos e articulações. As fibras do tipo Aδ são finamente mielinizadas e respondem a estímulos mecânicos, térmicos e químicos. As fibras do tipo C não aparesentam mielina e respondem também a estímulos térmicos, químicos e mecânicos, são denonimadas de fibras “sileciosas” e são as responsáveis pela resposta a estímulos como capsaicina (presente na pimenta) (TODD, 2010). O corpo celular desses neurônios estão no gânglio da raiz dorsal (DRG) e suas projeções vão para lâminas do corno dorsal da medula espinal, variando de acordo com o tipo de fibra (BRAZ et al., 2014; TODD, 2010). As fibras C e Aδ conduzem informações para áreas nociceptivas específicas nas lâminas I e II da superfície do corno dorsal da medula espinal e também para neurônios de espectro dinâmico amplo na lâmina V, os quais codificam tanto informações de

estímulos inócuos quanto nocivos. Por outro lado, fibras amplamente mielinizadas Aβ transmitem informações como toque leve ou estímulos mecânicos inócuos para estruturas no corno dorsal da medula espinal nas lâminas III e IV (TODD, 2010).

1.2.2 Fisiologia Da Dor – Aspectos Gerais

O entendimento da fisiologia da dor no mundo moderno se inicia com René Descartes em seu livro “Treatise of Man”, 1664. Para isso, Descartes utilizou seu icônico diagrama de um garoto próximo a uma fogueira (Figura 2). Ele propôs que o fogo ativa fibras em locais periféricos e tal informação seria transmitida para o cérebro, onde há o reconhecimento de tal estímulo como doloroso. Essa informação desencadeia uma resposta de retirada do pé de próximo do estímulo nóxico (Figura 2). Para Descarte, a dor era apenas uma consequência de uma ativação linear periférica para o cerébro (MOAYEDI; DAVIS, 2013), definição essa que não é errônea, apenas incompleta. Muito se evoluiu desde então. Isso é exemplificado com a definição de dor pelo comitê de taxonomia da Associação Internacional para o Estudo da Dor (IASP): “uma experiência sensorial e emocional desagradável que é associada a lesões reais ou potenciais ou descrita em termos de tais lesões”. Desse modo, cada indivíduo interpreta a dor de acordo com suas experiência, o que torna inconcebível a dissociação do caráter emocional para seu entendimento. Outros fatores interferem no limiar nociceptivo e sua magnitude, e que não podem ser ignorados ou subjulgados, dentre eles estão estado de humor, atenção (BUSHNELL; CEKO; LOW, 2013) e expectativas em relação à intensidade do estímulo (WIECH et al., 2014).

O entendimento conteporâneo da fisiologia da dor inclui sensibilização perífericа de nociceptores, sensibilização espinal, plasticidade neuronal e mudança de fenótipo de céulas do sistema imune (MOGIL; YU; BASBAUM, 2000; REICHLING; LEVINE, 2009; SCHOLZ; WOOLF, 2002; WOOLF; SALTER, 2000). Após ser gerado perifericamente por um estímulo nociceptivo, o mecanismo ascendende envolve o impulso nervoso percorrer o neurônio nociceptivo de primeira ordem até o corno dorsal da medula espinal, ou até o núcleo trigeminal, onde estes neurônios fazem sinapses com as terminações dos neurônios nociceptivos de segunda ordem. A informação é transmitida ao neurônio nociceptivo de segunda ordem por neurotransmissores excitatórios (peptídeo relacionado ao gene da calcitonina [CGRP], glutamato e a substância P, por exemplo) que são liberados pelo

neurônio nociceptivo primário na fenda sináptica (BRAZ et al., 2014; SCHOLZ; WOOLF, 2002). O mecanismos inibitório da dor envolve a liberação de mediadores inbitórios (GABA, canabinóides e β-endorfina, por exemplo) de modo a limitar a despolarização dos nociceptores ativados (MILLAN, 2002). Basicamente o processo de nocicepção envolve transdução, condução, transmissão e percepção. A transdução é a conversão de um estímulo nóxico em atividade elétrica decorrente da despolarização periférica dos nociceptores. Esse processo é estimulado por canais ionicos expressos na membrana dos nociceptores (Tabela 1). A condução é a passagem do potencial de ação pelos axônios para terminações centrais, e a transmissão é a transferência sináptica de neutrotransmissores na fenda sináptica de um neurônio nociceptivo para o outro. Por fim, a percepção é o fornecimento da informação referente ao estímulo inicial (através de sinapses no córtex somatossensorial) sobre a localização e a intensidade do estímulo nóxico. Outras projeções envolvem sinapses na região da amídala, contribuindo para o componente emocional do processo doloroso (MILLAN, 1999; MOGIL et al., 2000; REICHLING; LEVINE, 2009; SCHOLZ; WOOLF, 2002; TODD, 2010; WOOLF; SALTER, 2000).

1.2.2.1 Dor inflamatória

A dor de origem inflamatória resulta do aumento da excitabilidade (sensibilização) dos nociceptores periféricos decorrente da estimulação de mediadores pró-inflamatórios. Diversos mediadores podem contribuir para a sensibilização dos nociceptores (Tabela 1). Nesse sentido, alguns termos utilizados na prática clínica relacionada à dor inflamatória como alodinia (dor decorrente de um estímulo inócuo) e hiperalgesia (resposta exacerbada a um estímulo doloroso) que também são utilizados na prática experimental em animais. A exacerbação ou persistência da experiência da dor está presente em diversas doenças e representa a principal causa da procura por atendimento médico.

A presença de neutrófilos é um denominador comum no que se refere a dor inflamatória. Eles tem papel fundamental na manutenção da dor inflamatória (CUNHA, T. M. et al., 2008). Neutrófilos recrutados produzem citocinas pró-inflamatórias como IL-1β, TNF-α e IL-33 (VERRI; CUNHA; PARADA; POOLE; et al., 2006). Após um estímulo lesivo uma cascata de citocinas pró-inflamatórias precede a liberação de aminas simpáticas e PGE_2 que levam a sensibilização dos nociceptores. Essa cascata inicia com a liberação da alarmina IL-33 (ZARPELON;

CUNHA; et al., 2013) a qual estimula a produção sequencial de TNF-α→IL6→IL-1β→PGE_2; e TNF-α→CXCL1→IL-1β→aminas simpáticas (CUNHA, T. M. et al., 2005; VERRI; CUNHA; PARADA; POOLE; et al., 2006). Essas moléculas, ao se ligarem em seus receptores ativam kinases (PKA e PKC, por exemplo), que por sua vez fosforilam canais iônicos (TRPV1, TRPA1, Nav1.8; por exemplo) e ativam os nociceptores (BRAZ et al., 2014; SCHOLZ; WOOLF, 2002). Outros mediadores como endotelina (VERRI et al., 2009; ZARPELON; CUNHA; et al., 2013; ZARPELON et al., 2012), LTB_4 (GUERRERO et al., 2008), proteína do complemento C5a (TING et al., 2008) e EROs, em especial ânion superóxido (JANES; NEUMANN; SALVEMINI, 2012; MAIOLI et al., 2015; WANG et al., 2004) liberados por neutrófilos contribuem de forma siginificativa para a hiperalgesia. Desse modo, a depleção de neutrófilos (anticorpo anti-neutrófilo), inibição da migração dessas células (fucoidan), ou utilização de antagonistas de endotelinas reduzem a migração de neutrófilos e a hiperalgesia (CUNHA, T. M. et al., 2008; GUERRERO et al., 2008; TING et al., 2008; VERRI et al., 2009).

A compreensão atual ainda envolve os nociceptores como moléculas ativas na defesa do hospedeiro. Um fato que possibilitou um grande avanço nesse sentido foi a descoberta da expressão de PRR (TLR 2, 3, 4 e 7; e NLR por exemplo) associado à capcidade de liberar DAMP (CHIU et al., 2013; LIU et al., 2010; QI et al., 2011). Isso sugere que os nociceptores são capazes de reconhecer estímulos lesivos como bactérias, responder a esses estímulos e auxiliar na ativação de células do sistema imune. Um dos principais DAMPs liberados e reconhecidos por nociceptores é o ATP (Tabela 1). Seu mecanismo *downstream* envolve a ativação de componentes da plataforma inflamassoma neuronal e de células do sistema imune, que culmina na liberação da forma ativa da IL-1β e IL-18 (MARIATHASAN et al., 2006). Interessantemente, neurônios expressam os componentes dessa plataforma, sendo capaz, não só de reconhecer, mas também de liberar essas citocinas em sua forma ativa (DE RIVERO VACCARI et al., 2008). Além disso, o fato de *S. aureus* ser capaz de modular a resposta de nociceptores e causar dor (CHIU et al., 2013), reforça a ideia de os nociceptores participarem ativamente da montagem da resposta imune.

Figura 2. Descrição de René Descartes do processo de percepção da dor. "Partículas de calor" (letra “A”) ativam um local da pele (letra “B”) conectado a uma válvula no cérebro (letras “d” e “e”) por uma fibra fina (letra “C”). Tal atividade abre a válvula, permitindo que o espírito do animal possa fluir a partir de uma cavidade (letra “F”) para os músculos, levando-os a recuar do estímulo, virar a cabeça e os olhos para a parte do corpo afetada, e mover a mão e girar o corpo de forma protetora afastando-se do fogo (Figura adaptada de (MOAYEDI; DAVIS, 2013).

285

Tabela 1. Principais mediadores e estímulos pró-nociceptivos e seus receptores

Mediador/Estímulo	**Receptor**
IL-1β	IL-1R
TNF-α	TNFR
Bradicinina	B1 e B2
LTB_4	BLT1 e BLT2
PGE_2	EP_3
ATP	$P2X_2$, $P2X_3$ e $P2X_7$
C5a	C5aR
Endotelina-1	ET_A e ET_B
Calor/ pH baixo	TRPV1
Mecânico/Irritantes	TRPA1
Frio	TRPM8
Frio	Nav1.8
pH baixo	ASIC

(BRAZ et al., 2014; BURNSTOCK, 2009; GUERRERO et al., 2008; MOGIL et al., 2000; NAKAMURA; FERREIRA, 1987; SCHOLZ; WOOLF, 2002; VERRI; CUNHA; PARADA; POOLE; et al., 2006; WOOLF; SALTER, 2000)

286

1.3 Espécies Reativas De Oxigênio E NADPH Oxidase

O oxigênio é uma molécula fundamental para existência de vida complexa no planeta. Seu acúmulo na atmosfera levou ao desenvolvimento de organismos chamados aeróbios. Apesar de indispensável, seus efeitos acompanham uma paradoxal toxicidade decorrente da formação de EROs. De fato, após o aparecimento do oxigênio diversos micro-organismos sucumbiram frente essa adversidade por não disporem de mecanismos de defesa adequados para tal. Mantendo o paradóxo, as EROs desempenham papel fundamental na homeostase, apresentando toxicidade quando não há controle adequado sobre sua produção ou detoxificação. As EROs são formadas após a transferência de elétrons na cadeia fosforilativa (mitocôndria) ou através do sistema da NADPH oxidase (JONES, 2008), o qual é composto por proteínas que se encontram dissociadas na célula em repouso. Esses componentes são a $p40^{phox}$, a $p47^{phox}$ e a $p67^{phox}$, agrupadas em um sistema protéico citoplasmático de 240kDa. Também há o citocromo b558, composto pelas proteínas $p22^{phox}$ e $gp91^{phox}$ localizadas nas membranas das vesículas secretórias e dos grânulos específicos citoplasmáticos. Outras proteínas de baixa massa molecular, ligantes de nucleotídeo guanina: a Rac 1 e 2 e Rap1a, também participam do processo (BEDARD; KRAUSE, 2007; JONES, 2008). Quando ativo, o sistema multienzimático NADPH oxidase é responsável pela transferência de elétrons do NADPH para o oxigênio molecular, formando o ânion superóxido (BEDARD; KRAUSE, 2007), o qual é produzido em grande escala por neutrófilos e tem proeminente atividade microbicida (BABIOR; KIPNES; CURNUTTE, 1973). Em condições fisiológicas, o ânion superóxido é dismutado pela SOD em peróxido de hidrogênio e esse é transformado em duas (2) molécula de água e uma (1) de oxigênio (JONES, 2008).

As EROs são capazes de oxidar diversas proteínas, especialmente com resíduos de cisteína (tióis), essa característica permite ativar diversas vias de sinalização, entre elas a do NF-κB (GLOIRE; PIETTE, 2009). De fato, a via do NF-κB é conhecida como sensível ao estado redox da célula (TOLEDANO; LEONARD, 1991). Após ubiquitinição e degradação do IκBα, resíduos específicos de serina da subunidade p65 são fosforilados por PKA. Isso permite a interação de p65 com a molécula coativadora do NF-κB (mas não apenas) CBP (proteína ligadora de CREB). Essa fosforilação por PKA é elevada na presença de EROs e isso possibita o aumento da atividade do NF-κB. De fato, tratamento com antioxidantes diminui a

associação de p65 com CBP na região promotora do gene da IL-8, por exemplo (JAMALUDDIN et al., 2007). Focando no ânion superóxido, tratamento com M40403 (molécula mimética da enzima SOD) inibiu a ativação do NF-κB, relacionada com diminuição da degradação do IκB, sugerindo a participação do ânion superóxido da regulação redox desse fator de transcrição (NDENGELE et al., 2005)

1.3.1 Relação Entre Ânion Superóxido E Dor

Existe uma linha tênue entre ânion superóxido e dor. Desse modo, é necessário que haja uma sintonia fina para o controle adequado da produção de EROs, especialmente do ânion superóxido. Em relação a dor, o ânion superóxido é importante para o desenvolvimento de hiperalgesia térmica associada com inflamação aguda e crônica (NDENGELE et al., 2008; WANG et al., 2004); e promoção de hiperalgesia mecânica (KIM; CHUNG; CHUNG, 2008). Associado a isso, o ânion superóxido é capaz de ativar canais TRPA1 (FERNANDES et al., 2013), que são essenciais para dor (Tabela 1). Corroborando, o tratamento com Tempol (molécula mimética da enzima SOD) foi capaz de inibir hiperalgesia mecânica e inflamação em modelo de dor induzido por carragenina (KHATTAB, 2006).

Adicionalmente à sua atividade hiperalgésica, nosso grupo demonstrou – utilizando o superóxido de potássio como um doador de ânion superóxido – que o ânion superóxido é capaz de produzir comportamentos de dor manifesta, como contorções abdominais e sacudidas de pata (MAIOLI et al., 2015). Além disso, o ânion superóxido (injetado perifericamente) é capaz contribuir para sensibilização espinal por aumentar a expressão de endotelina-1, TNF-α e IL-1β (SERAFIM et al., 2015; YAMACITA-BORIN et al., 2015). Corroborando esse fato, inibição desses mediadores utilizando o bosentan (antagonista misto de receptor de endotelina), etanercept (receptor solúvel de TNF-α) e camundongos knockout para TNFR1 apresentaram redução de comportamentos relacionados a dor, recrutamento de neutrófilos, estresse oxidativo e produção de citocinas pró-inflamatórias (SERAFIM et al., 2015; YAMACITA-BORIN et al., 2015). Esses dados apontam para a interação fundamental entre o ânion superóxido e diversos mediadores e fatores de transcrição, o que sugere o papel crucial dessa molécula no desenvolvimento de dor e inflamação. Assim, estratégias que visem sua inibição extremamente atrativas.

1.4 ARTRITE

Artrite é um nome generalizado para doenças que afetam as articulações, dentre elas se destacam: osteoartrite, artrite reumatoide (RA), artrite séptica e gota (MARTIN; HARPER, 2010; SMOLEN; ALETAHA; REDLICH, 2012). No Brasil, a prevalência de RA varia entre 0,2% e 1%, dependendo da região (DE AZEVEDO; FERRAZ; CICONELLI, 2008). As doenças artríticas impactam significantemente nos cofres públicos. De fato, em um estudo envolvendo 192 pacientes com RA, 47 deles se aposentaram precocemente devido incapacidade físicas provenientes da doença. Para essa população, o impacto orçamentário público foi de US$ 2.423,51 por paciente por ano (DE AZEVEDO et al., 2008). A dor, especificamente alodinia, é o sintoma que converge a artrite. Desse modo, pacientes acometidos com os diversos tipos de artrites apresentam redução brusca da qualidade de vida, já que atividades normais diárias, como se movimentar, são excruciantes.

A revolução industrial foi o salto inicial para uma série de transformações tecnológicas que sepultou regimes arcaicos de produção entorno do mundo. Isso possibilitou uma nova era tecnológica. Com o avanço tecnológico, o prolongamento da vida humana foi possível, devido a cura/erradicação de várias doenças (poliomielite por exemplo) e com o paradoxal, porém inevitável, surgimento de doenças que outrora não havia. Acompanhado a isso, o avanço tecnológico propiciou a implementação de intervenções médicas para aumentar a qualidade de vida de pacientes afetados com diversas doenças; dentre elas o uso de próteses para substituir completamente ou parcialmente um membro. Em particular para artrite, um desses procedimentos é a artroplastia – substituição total da articulação por próteses (LEE; GOODMAN, 2008) – uma intervenção necessária devido destruição das articulações acometidas. De fato, pacientes submetidos a atroplastia apresentam melhor qualidade de vida quando comparados ao que não foram submetidos à intervenção (DA SILVA et al., 2014; MARICONDA et al., 2011). Para fabricação das próteses é utilizada uma variedade de materiais, incluindo aço inoxidável, ligas de cobalto molibdénio, titânio e ligas de titânio (tal como titânio alumínio e vanádio), polietileno entre outros; em uma tentativa de encontrar materiais que são biocompatíveis e possuem as propriedades mecânicas necessárias para suportar as forças aplicadas para a prótese. Muitos destes materiais permanecem em uso hoje em dia (LEE; GOODMAN, 2008).

Infelizmente, 10-15% dos pacientes que utilizam próteses apresentam osteólise decorrente de detritos liberados pelos materiais prostéticos (GOODMAN, 2007; HARRIS, 2001). Os relatos de osteólise datam de 1970 (CHARNLEY, 1975) e tem sido associada com todos materiais utilizados em materiais prostéticos (GOODMAN, 2007; HARRIS, 2001). A inflamação crônica oriunda do uso de prótese está relacionada com a fagocitose de partículas liberadas por esses materiais, causando necrose e osteólise (GOODMAN; MA, 2010). Dois principais mecanismos colaboram para a destruição óssea: (I) a osteoclastogênese que ocorre decorrente da sinalização RANK-RANKL (HAYNES et al., 2001) e (II) a fagocitose de partículas liberadas pelos materiais prostéticos, causando liberação de enzimas proteolíticas e citocinas pró-inflamatórias (GOODMAN; MA, 2010; TAKEI et al., 2000).

Em particular, uma paciente, sem histórico de artrite familiar, desenvolveu artrite relacionada a implante após a implementação de uma prótese. O biomaterial em questão era uma liga de titânio e vanádio (ISO standard 5832-3:1996) (DORNER et al., 2006). Após incubação de células mononucleares sanguíneas derivadas da paciente com dióxido de titânio, foi observado aumento da produção de TNF-α, uma citocina fundamental na fisiopatologia da artrite (DORNER et al., 2006). De fato, a exposição pulmonar a dióxido de titânio em ratos, leva ao aumento da produção de citocinas pró-inflamatórias (como IL-1β, TNF-α e IL-6) acompanhado com maior influxo de leucócitos e destruição tecidual (GUSTAFSSON et al., 2011). Corroborando esses resultados, incubação com dióxido de titânio é capaz de produzir dano no DNA e aumentar a atividade do NF-κB (PRASAD, R. Y. et al., 2014), além de ser capaz de aumentar liberação do citocromo C no citoplasma das células culminando na ativação de caspase-3 e consequente apoptose dos hepatócitos (EL-SAID et al., 2014). Esses resultados sugerem que as partículas de dióxido de titânio são capazes de induzir estresse oxidativo, citotoxidade, genotoxicidade, inflamação crônica e em última instância apoptose e necrose celular; associado a isso um relato de artrite induzida por implante. Contudo, os benefícios decorrentes da utilização de próteses são extraordinários, necessitando, portanto, de um controle da quantidade de dióxido de titânio implementado nesses biomateriais acompanhado do desenvolvimento ou aplicação de moléculas com atividade biológica capazes de controlar os efeitos deletérios provenientes da liberação dessas partículas.

1.5 CURCUMINA

A Medicina Tradicional ainda é tida como uma fonte próspera de compostos com atividades farmacológicas para a medicina conteporânea. Os extratos de plantas têm sido utilizado por milhares de anos por praticantes de Medicina Tradicional Chinesa e Indiana (Ayurvedica), mas apesar disso a aplicação desses compostos ainda é vista com ceticismo por parte de praticantes da medicina ocidental. Uma das principais característica e que tornam os compostos derivados de produtos naturais extremamente atrativos, reside no fato de eles terem sidos testados “clinicamente” durante milhares de anos por civilizações praticantes de medicinas alternativas. A curcumina apresenta mais de 100 diferentes alvos moleculares (AGGARWAL, B. B.; HARIKUMAR, 2009), e é um dos compostos que se destaca dentre os derivados de produtos naturais. Sua importância farmacológica é evidenciada através de inúmeras moléculas análogas e derivadas de sua estrutura (AGRAWAL; MISHRA, 2010; MARTINEZ-CIFUENTES et al., 2015; PRASAD, S.; TYAGI, 2015; ZHAO; LIU; LIANG, 2013).

A curcumina [(E,E)-1,7-bis(4-hydroxi-3-metoxifenil)-1,6-heptadieno-3,5-diona] (Figura 3) é um composto de coloração amarela e o constituinte presente em maior concentração no extrato do rizoma da planta *Curcuma longa* (açafrão). O extrato da cúrcuma comercialmente disponível contém cerca de 77% de curcumina pura, 17% dimetoxicurcumina e 3% bis-dimetoxicurcumina (SANDUR et al., 2007). Além de ser utilizada como condimento alimentar, tem sido amplamente utilizada por praticante da Medicina Tradicional Chinesa e Ayuvedica para o tratamento de diversas enfermidades, como disenterias, flatulência, úlcera, feridas e artrite (AGGARWAL, B. B.; SUNG, 2009). Essa molécula possui atividade anti-inflamatória, antioxidante, anticancerígena, antiviral e antibacteriana (AGGARWAL, B. B.; HARIKUMAR, 2009; AGGARWAL, B. B.; SUNG, 2009), além de ser extremamente segura. Administração de curcumina por 4 semanas nas doses de 1-2 g/kg não demonstrou efeitos tóxicos siginificativos. A LD_{50} foi de 12,2 g/kg (ARORA et al., 1971). Em humanos, a dose máxima utilizada e que não apresentou efeitos tóxicos foi de 8000 mg por dia (CHENG et al., 2001). A curcumina tem atividade pleiotrópica, e aparentemente, não apresenta um alvo específico. Ao invés disso, seu efeito farmacológico está relacionado a um sinergismo envolvendo modulação de diversas moléculas em diferentes vias de sinalização (Figura 3) (ZHOU, H.; BEEVERS; HUANG, 2011). Esse pleiotropismo é proporcionado por 3 importantes funcionalidades em sua

molécula: (1) o anel aromático orto-metilado com um grupamento fenol, (2) a presença de uma dicetona α, β insaturada e que possibilita (3) a formação de uma molécula tautômera (co-existente) ceto-enol (ANAND et al., 2008). Essas características únicas propiciam a ligação da curcumina com uma gama de alvos moleculares. Sua atividade anti-inflamatória está relacionada interação direta com diversos mediadores, como TNF-α, COX-1 e COX-2, por exemplo. A curcumina foi capaz de reduzir a sinalização entre o TNF-α e seu receptor (WUA et al., 2010). Essa interação foi possível já que a curcumina se liga (através de ligações iônicas) em diversos resísuos específicos de aminoácidos (cisteína [Cys129], leucina [Leu89] e ácido aspártico [Asp105]) presentes na molécula do TNF-α (WUA et al., 2010). Em relação a COX-1 e COX-2, a curcumina se liga, entre outros, em resíduo específico de serina (Ser535) e tirosina (Tyr355), respectivamente (SELVAM et al., 2005). Além disso, a curcumina inibe atividade do NF-κB e consequentemente produção de citocinas pró-inflamatórias (AGGARWAL, S. et al., 2006), reduz a expressão de moléculas de adesão (SUYENAGA et al., 2014), tem capacidade de inibir a polimerização de actina em neutrófilos (LARMONIER et al., 2011) e reduz estresse oxidativo, aumentando por exemplo atividade do fator de transcrição Nrf2 e consequentemente HO-1 (GAO, S. et al., 2013; HEEBA; MAHMOUD; EL HANAFY, 2012). Além disso, a curcumina é capaz de reduzir osteoclastogênese através da inibição da sinalização entre RANK e RANKL (OH; KYUNG; CHOI, 2008; ZHOU, T. et al., 2013) (Figura 3).

A curcumina tem sido amplamente utilizada em diversos ensaios clínicos (GOEL; KUNNUMAKKARA; AGGARWAL, 2008), porém iremos nos ater nos ensaios clínicos que avaliaram sua atividade analgésica. O primeiro ensaio clínico demonstrando sua atividade analgésica foi em 1980 em pacientes com artrite reumatóide (DEODHAR; SETHI; SRIMAL, 1980). O tratamento com curcumina foi capaz de reduzir edema e rigidez matinal das articulações, e aumentar o tempo de caminhada dos pacientes sem queixas de dor (DEODHAR et al., 1980). Em outro ensaio clínico, também em pacientes com artrite reumatóide, a curcumina foi capaz de reduzir a dor reportada pelos pacientes, edema da articulações e sensibilidade no local (CHANDRAN; GOEL, 2012). Nesse sentido, um medicamento derivado de curcumina foi desenvolvido: Meriva®. Em diferentes ensaios clínicos em pacientes com osteoartrite, o Meriva® reduziu a dor reportada pelos pacientes (escala de VAS), rigidez matinal das articulações, produção de IL-6, IL-1β e sVCAM (BELCARO

et al., 2010; DI PIERRO et al., 2013). Essas características (Figura 3) nos conduziram a avaliar a eficácia da curcumina frente a dois modelos: um modelo dor dor agudo induzido por ânion superóxido e em segundo momento em um modelo de dor crônica de artrite induzido por dióxido de titânio (TiO_2).

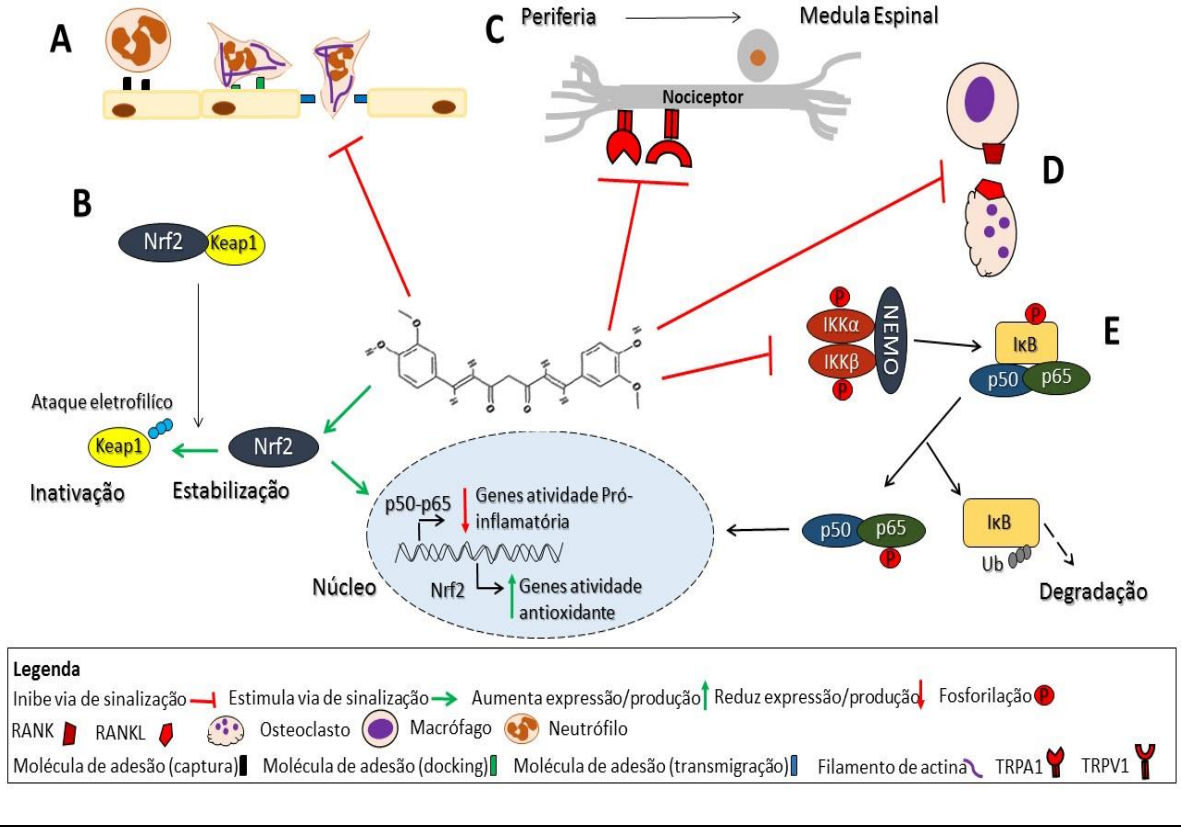

Figura 3. Alvos moleculares da curcumina. A curcumina inibe o recrutamento de neutrófilos e a expressão de moléculas de adesão (A). Além disso é capaz de aumentar a atividade do Nrf2 e seus genes alvos, como a HO-1 (B); e inibir a sinalização entre RANK e RANKL, diminuindo a osteólise (D); e inibir a tividade do NF-κB e consequentemente reduz a produção de mediadores pró-inflamatórios (E). Adicionalmente, a curcumina tem a capacidade de dessessibilizar os nociceptores (C) por inibir a atividade de canais iônicos associados a reconhecimento de estímulos dolorosos (Tabela 1). Em conjunto, essas características propiciam uma proeminente atividade anti-inflamatória e anti-nociceptiva para a curcumina (AGGARWAL, B. B.; HARIKUMAR, 2009; AGGARWAL, B. B.; SUNG, 2009; LEAMY et al., 2011; OH et al., 2008; YEON et al., 2010).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o efeito da curcumina em modelos de dor agudo (induzido por ânion superóxido) e dor crônica (atrite induzida por dióxido de titânio).

2.1.1 Objetivos Específicos (Modelo Crônico)

-Determinar a melhor dose de curcumina em modelo de hiperalgesia mecânica e edema; e degradação de proteoglicanos

-Avaliar o efeito da curcuina sobre atividade da mieloperoxidase

2.1.2 Objetivos Específicos (Modelo Agudo)

-Avaliar o efeito da curcumina, através de ensaio de dose-resposta, em modelo de dor manifesta

-Avaliar o efeito da curcumina em modelo de hiperalgesia mecânica e térmica

-Avaliar o efeito da curcumina sobre o recrutamento de leucócitos

-Avaliar o efeito da curcumina sobre parâmetros relacionados ao estresse oxidativo, Nrf2 e HO-1

-Avaliar o efeito da curcumina sobre produção de citocinas e atividade do NF-κB

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3 ARTIGO 1 PUBLICADO (PHARMACOLOGICAL RESEARCH)

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Neutrophils and Arthritis: Role in Disease and Pharmacological Perspectives

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Abstract

The inflammatory response in the joint can induce an intense accumulation of leukocytes in the tissue that frequently results in severe local damage and loss of function. Neutrophils are essential cells to combat many pathogens, but their arsenal can contribute or aggravate articular inflammation. Here we summarized some aspects of neutrophil biology, their role in inflammation and indicated how the modulation of neutrophil functions could be useful for the treatment of different forms of arthritis.

Keywords: Neutrophil, phagocytosis; neutrophil extracellular traps; arthritis; neutrophil recruitment; infection; tissue damage

1) Introduction

Rheumatic diseases, represented by varied forms of arthritis and other musculoskeletal disorders, affect millions of people around the world and are currently one of the most studied diseases in many centers of research [1]. There are variable and well-established models to study different types of arthritis, most of them in mice, which give us valuable information about the mechanisms that regulate the production of mediators of inflammation, cellular infiltration in the joint and tissue damage and dysfunction. Furthermore, these proof-of-concept models are useful for pre-clinical studies during the development of new anti-inflammatory and anti-rheumatic drugs [2]. However, although useful, these models do not represent the authentic pathogenesis in humans, which can cause failures during translational studies. Thus, there are many challenges for the study of arthritis, especially on basic research, and being conscious of the limitation of arthritis models is only the first step for a better interpretation of the findings.

Neutrophils are crucial cells that have significant roles in virtually all inflammatory diseases, ranging from acute, chronic, autoimmune, infectious, and non-infectious conditions [3]. The most known effector functions of neutrophils are related to their role in innate immunity since different chemoattractants quickly recruit neutrophils from the bloodstream. However, recent research points out neutrophils as active cells during adaptive immunity, facilitating the recruitment and activation of antigen-presenting cells or due to a direct interaction with T cells [4,5].

Neutrophils have an arsenal of antimicrobial molecules that are essential to combat several microorganisms. In this regard, the neutropenia or malfunctioning of neutrophils is associated with the development of opportunistic infectious diseases. On the other hand, several of the neutrophil-derived molecules that are crucial for the control of pathogens are detrimental to the host tissue. Especially in autoimmune and autoinflammatory disorders, the accumulation of neutrophils causes tissue damage and dysfunction, sometimes irreversible [4]. Thus, the development of compounds that interfere on neutrophil biology is useful for the treatment of many inflammatory diseases. Particularly in rheumatology, the infiltration of neutrophils is directly associated with the worsening of the clinical condition, including cartilage and bone destruction and pain in the different forms of arthritis [6]. Here, we explored the recent findings in the field of neutrophil biology, its association with arthritis and possible pharmacological approaches focusing on the biology of neutrophils.

2) Neutrophil biology

Neutrophils represent one of the most important effector cells during innate immune response. The mechanisms by which neutrophils deal with infection and their contribution to tissue damage are discussed below in separated sections. In humans, these cells represent the most abundant leukocytes in the blood, a variable that changes considerably among the species [7]. Importantly, there is a rapid mobilization of stored neutrophils from the bone marrow to the circulation during infectious/inflammatory conditions [8,9]. Independent on the species, neutrophils quickly migrate from blood to the tissue under different chemoattractants during inflammation, where they have an important role in the control of infections and contribute to tissue repair. During the maturation of neutrophils, much of their arsenal of molecules filled different types of granules, including primary or azurophilic, secondary or specific, and tertiary or gelatinase. Section 4 addresses the content of each granule in detail (Killing mechanisms of neutrophils in host defense) [For review see 10,11]. Adult humans produce billions of neutrophils daily mainly by the actions of the cytokine Granulocyte-Colony Stimulating Factor (G-CSF) on progenitor stem cells. In fact, the infusion of G-CSF is clinically used to increase the neutrophil count in the peripheral blood of patients undergoing myelosuppressive chemotherapy for acute myeloid leukemia or severe chronic neutropenia [12]. Currently, a paradigm in the field of neutrophil biology is its lifespan, considered short when compared to other leukocytes. Using *in vivo* labeling neutrophils, Pillay and colleagues found that human neutrophils present a lifespan of 5.4 days on circulation while mouse neutrophils survive up to 12.5 hours in physiologic conditions [13].

Some studies indicate that there is a heterogeneous population of neutrophils that could represent different phenotypes of these cells during an inflammatory response. Those variations depend on their morphology, cell surface markers, secreted molecules and density [For review see 14]. However, these subsets of neutrophils could be only a natural variation during their lifespan, considering the immature, mature and aged neutrophils and according to their state of activation. *In vitro* studies suggest that aged neutrophils decrease their ability to migrate and produce less pro-inflammatory molecules [15]. However, it was recently demonstrated that aged neutrophils in circulation display pro-inflammatory activity in mice [16]. Interestingly, neutrophil aging depends on the microbiota, since microbiota-depleted mice have reduced the circulation of aged neutrophils. Moreover, the decrease of a commensal population by the treatment with antibiotics ameliorates organ damage in mouse models of inflammation [16].

Senescent neutrophils express high levels of CXCR4 (the ligand CXCL12 is highly expressed by bone marrow stromal cells), a process that is essential for the home back to the bone marrow where the apoptotic neutrophils are phagocytosed by macrophages, ending their life cycle [9] (Figure 1). *In vitro*, peripheral blood human neutrophils increase their expression of CXCR4 around 3 h at 37^{o}C, an event that anticipates the apoptosis [17]. Importantly, there is an established concept that the apoptosis of neutrophils on inflammatory milieu is an important signal for the resolution of inflammation. The induction of neutrophil apoptosis by administration of H_2O_2 leads to the resolution of joint inflammation in antigen-induced arthritis in mice [18]. Mechanistically, the efferocytosis of apoptotic neutrophils by macrophages changes the profile of lipid mediators produced by these last cells, leading to the production of molecules with anti-inflammatory and pro-resolution properties, so-called specialized pro-resolving mediators (SPM) [For review see 19] (Figure 1).

In addition to the neutrophil function in innate immunity, recent evidence points out their significant contribution to an adequate activation of adaptive immune response. Stored molecules in neutrophils, such as myeloperoxidase and proteinase-3, are potential autoantigens in some diseases and can lead to the development of anti-neutrophil cytoplasmic autoantibodies (ANCA) that mediate the chronic inflammation of some vasculitis [20]. Moreover, some studies demonstrate that neutrophils can express MHC-II, the co-stimulatory molecules CD86, CD40, and CCR7, a chemokine receptor that enables cells to migrate towards lymph node through lymphatic vessels, acting as antigen presenting cells. These conditions support the idea of the existence of neutrophil-like antigen-presenting cells, suggesting that they could have a strong relationship with T cells [21-24]. On the other hand, TLR-activated neutrophils cross-talk with NK cells by promoting their activation, which, in turn, drive the maturation of dendritic cells for a proper antigen presentation to T cells [25]. Moreover, the previous migration of neutrophils could facilitate the infiltration of T cells towards the site of infection. In a mouse model of Influenza virus infection, the early migrated neutrophils to trachea create a trail of CXCL12 in the tissue that attract $CD8^+$ T lymphocytes locally, crucial cells to deal with this disease [5]. These findings strength the multirole of neutrophils in immunity, suggesting neutrophils may differentiate into different subsets defined by distinct phenotypic and functional profiles depending on the disease, a so-called neutrophils plasticity [26]. Altogether, deepening on the basic knowledge of neutrophil biology will certainly help the better understanding of the pathogenesis of several diseases and will favor the optimization of future treatments.

3) Mechanisms of neutrophil recruitment

As previously mentioned, the physiological control of neutrophil availability in the circulation depends on the chemokine receptor CXCR4 expressed on their surface that regulates their maintenance, egress or regress from/to bone marrow [27] (Figure 1). However, during inflammation, the recruitment of neutrophils from the bloodstream to inflamed tissue is well orchestrated by a plethora of soluble molecules and by interaction with endothelial cells [For review see 28]. Among the chemoattractants, ELR^+ (glutamic acid–leucine–arginine) CXC chemokines constitute the most important class of mediators involved in neutrophil recruitment [29] (Figure 2A). In this regard, several attempts have been performed to block their receptors, mainly CXCR2, in different types of inflammatory diseases [30]. However, the expression of chemokine receptor on the neutrophil surface can vary along the inflammatory response. For instance, in a mouse model of severe sepsis, neutrophils lose the expression of CXCR2 in a mechanism dependent on the signaling molecules PI3Kγ and GRK2, impairing their recruitment to the site of infection by the previously produced chemokines resulting in a lessened control of bacterial growth [31,32]. In the same model, neutrophils can express CCR2, a non-canonical receptor for these cells, which is functional and causes neutrophil migration due to binding to its ligands [33]. In addition to chemokines, LTB_4, C5a, and especially formyl-peptide (fMLP) are fundamental to a precise guidance of neutrophils to inflammatory foci [34,35].

Along the vascular bed in proximity to inflamed tissue, chemokines are concentrated on endothelial cells by binding to glycosaminoglycans (GAGs), such as heparin, heparan sulfate, and dermatan sulfate. GAGs permit a close interaction between chemokines and leukocytes during the rolling and adhesion processes, potentiating cell migration [29] (Figure 2A). Thus, the blockade of GAG-binding sites for chemokines could be useful to regulate the migration of leukocytes. In mice, the injection of modified chemokines that are still able to bind to GAGs but lost their capacity to activate their receptors impaired the recruitment of neutrophils to the tissue when stimulated by exogenous or endogenous-produced chemokines. As a result, there was reduced tissue inflammation, including articular damage in mouse models of arthritis [36,37].

Currently, much attention is given to the mechanisms that regulate the so-called reverse transmigration of neutrophils, where migrated neutrophils return to blood vessels (Figure 1). This phenomenon occurs in inflamed tissue and could contribute to inflammation in distant organs [38]. However, a possible contribution to the resolution of inflammation cannot be discarded, especially in acute inflammation, as observed in zebrafish models

[39,40]. Mechanistically, the decreased expression of the junctional adhesion molecule C (JAM-C) between endothelial cells facilitates the reverse transmigration of neutrophils [38]. A recent work showed that LTB_4 produced during cremaster muscle under ischemia and reperfusion injury drives the neutrophil elastase release by neutrophils that induce the proteolytic cleavage of JAM-C, promoting the reverse transmigration and potentiate remote tissue inflammation [41] (Figure 1).

4) Killing mechanisms of neutrophils in host defense

Impairments in neutrophils quantity or function render the host susceptible to a variety of potentially life-threatening microbial agents, which highlight the fundamental role of these cells in immune system homeostasis. Although neutrophils have a well-recognized role as phagocytic cells, their vast microbicidal mechanisms and phenotypes demonstrate they are far away from only phagocytic cells. Neutrophils killing activity occurs by two distinct mechanisms. The first one is phagocytosis, which involves intracellular degranulation of vesicles containing proteases or ROS production into the phagolysosome. The second mechanism is the release of intracellular content (neutrophils extracellular traps, NETs) (Figure 2B). The process of forming NETs induces a unique form of death known as NETosis [3]**.** Nevertheless, neutrophils can continue living after forming NETs for an unknown period *in vivo* depending on NETosis rout.

Neutrophils recognize opsonized targets to phagocyte through opsonin receptors such as Fcγ receptors, C-type lectins or complement receptors. A complex interplay of membrane lipids, intracellular signaling cascades, and cytoskeletal rearrangements mediate this process [10]. In opposition to macrophages, neutrophils contain a collection of pre-formed granules, and the lysosome that fuses with the phagosome even before internalization is complete [42]**.** Neutrophils present three types of granules [For review see 10,11]: (a) azurophilic or primary granules that contain cathepsin G, elastase, and proteinase 3, and myeloperoxidase (MPO). MPO highlights among these enzymes because it reaches up to 5% of total weight of neutrophils and produces the microbicidal molecule hypochlorite, a strong oxidant, upon reaction with H_2O_2 and Cl^- [43]**.** Moreover, MPO is also required for NETosis since neutrophils of MPO-deficient patients do not release NETs. Nevertheless, even low MPO activity is sufficient for NETs formation because pharmacological inhibitors or neutrophils from patients with partial MPO activity undergo NETosis [44]. (b) The second type of granule is the specific or secondary that contain lactoferrin, neutrophil gelatinase-associated lipocalin and about two-thirds of the lysozyme and, the third one is (c) gelatinase or tertiary that are

granules containing gelatinase [10,11]. Alongside with phagocytosis, NADPH oxidase activation is indispensable, which is a tightly regulated process and triggered upon recognition of agents in the neutrophils cell surface [42,45]**.** Assembly of an active oxidase on phagosomes is necessary for generation of superoxide anion and derivative ROS, which kill engulfed microorganisms [10]. Apart from the microbicidal mechanism, superoxide anion can activate transient receptor potential (TRP) channels [46] and it is crucial to induce pain-like behaviors [47-51] (Figure 2B)**.**

One killing mechanism in particular expanded the current view on the repertoire of neutrophils killing *modus operandi* and have been a focus of investigation over the decade since its discovery [52]**.** The composition of NETs includes chromatin, histones, and enzymes from neutrophils granules [52]. NETs trap microbial agents limiting their action and dissemination. Furthermore, an array of antimicrobial enzymes (e.g. MPO, elastase and serine proteases) from granules facilitates the extracellular destruction of microorganisms entrapped within the NETs. Neutrophils release NETs via two central mechanisms [53,54]. The major route is the slow lytic cell death mechanism (120–240 min) – so-called suicidal NETosis – in which there is release of intracellular content and cell death, a process requiring the activation of NADPH oxidase and production of ROS [52,55,56]. The other pathway – coined as non-suicidal NETosis – involves quick (5–60 min) and NADPH oxidase-independent release of vesicles containing uncondensed chromatin and granule proteins in the extracellular space, where they assemble into NETs. The non-suicidal NETosis induces NETs formation without signs of neutrophil lysis and death [57,58]. For instance, NETosis has protective role in gout [59], and in viral [60]**,** bacterial [52,61] and fungal infections [62]. Therefore, this is a critical defense mechanism, although not always desirable since the release of intracellular contents may drawback an innate immune response and worsening of installed diseases (we will further discuss this issue in the next topic, focusing on RA) (Figure 2B; 2C).

5) Relationship between neutrophils' granules enzymes and joint tissue destruction in arthritis

In addition to the protective role of neutrophils during infections, these cells also have a role in tissue destruction in arthritis. In this section, we will discuss the mechanisms of neutrophils that contribute to joint lesions in arthritis.

Neutrophils can be activated in RA by immune complexes (such as rheumatoid factor) both within the synovial fluid and deposited on the articular cartilage surface [63]. These complexes engage Fcγ receptors and thereby trigger neutrophil activation, which releases

ROS and extracellular granule contents due to frustrated phagocytosis. Increased degranulation, ROS production and delayed apoptosis of neutrophils correlate with the intensity of synovial inflammation and the destructive capacity of joint neutrophils in RA, summarizing that granules' components and ROS act synergistically to degrade articular cartilage [63-65]**.** In fact, the basal levels of total ROS and superoxide anion are significantly elevated in neutrophils from RA patients compared with neutrophils from healthy individuals [66,67]**.** Furthermore, the stimulation of joint tissue with neutrophilic products hypochlorite and H_2O_2 inhibit the synthesis of proteoglycan [68,69]**.** Hypochlorite also mediates the activation of neutrophilic collagenase and gelatinase, which contributes to joint destruction [69] (Figure 2C). In agreement with these findings, treatment with antioxidant molecules such as quercetin [70,71] and curcumin [72,73] ameliorate clinical index and joint destruction in experimental models of arthritis, in part, by inhibiting oxidative stress, and neutrophils enzymes activity.

Initially, NETosis was seen as an antimicrobial mechanism, but recent data suggest that NETosis could contribute to the pathophysiology of autoimmune diseases. The citrullination of proteins constitutes the major posttranslational modification that generates novel antigens recognized by antibodies (anti-citrullinated protein autoantibodies) in patients with autoimmune diseases, especially in RA [74]. As aforementioned in the section above, histones are components of NETs and are targets of hypercitrullination catalyzed by peptidylarginine deiminase 4 in an irreversible reaction [75]. NETosis seems to be the primary source of citrullinated antigens [76] and as a matter of fact, NETosis aggravates RA disease [77,78] and systemic lupus erythematosus [79,80]. In synovial fluid of patients with RA, NETosis contributes to the release of peptidylarginine deiminase 4 and consequently generation of citrullinated antigens [81] (Figure 2C).

NETs formation and its importance in diseases remain intriguing. Future discoveries will aid our knowledge in NETs biology and will contribute to the understanding of its role in the development of chronic inflammatory diseases. Apparently, NADPH oxidase-dependent mechanism that activates NETosis seems more relevant in chronic diseases than NADPH oxidase-independent mechanism. Of note, neutrophils from patients with the chronic granulomatous disease who carry mutations that impair the function of Nox2 enzyme, fail to form NETs, and gene therapy restores this ability in NADPH oxidase-competent neutrophils *in vitro* [82]. Likewise, mice lacking $p47^{phox}$ (a subunit of NADPH oxidase) fail to form NETs and resolve *A. fumigatus*-induced pneumonia, one of the leading causes of morbidity and mortality in chronic granulomatous disease [83]. Despite that, it is still necessary to unveil

which NETs formation pathway is involved to which disease, and then seek for new therapeutic interventions aiming to stimulate or not NETs formation and perhaps stimulate a particular pathway of NETs formation ("suicidal" or "nonsuicidal"), accordingly with the disease.

It is also important to mention that neutrophils contribute to bone resorption through osteoclast activation (Figure 2C). For instance, lipopolysaccharide [84] activates TLR4 inducing receptor activator of NF-κB ligand (RANKL) expression by peripheral blood human neutrophils and neutrophils recruited to murine air pouch. The co-culture of these neutrophils with human monocyte-derived osteoclasts and murine RAW 264.7 cells stimulates bone resorption. Importantly, confocal microscopy reveals neutrophil-osteoclast contact and even invagination. Furthermore, synovial fluid neutrophils of patients with exacerbation of RA activates osteoclastogenesis in co-culture system, which corroborates their high expression of RANKL in the synovial fluid [85]. Further demonstrating the interactions between neutrophils and osteoclasts in RA, the neutrophil chemoattractant chemokine CXCL2 induces RANKL production by osteoclast precursors in a JNK- and NF-κB-dependent manner as well as osteoclastogenesis. The CXCR1/2 receptor antagonist repertaxin reduced RANKL-induced osteoclastogenesis, and the administration of CXCL2 in mice induces bone erosion. In agreement with these *in vitro* and murine evidence, there are high titers of CXCL2 in the blood and synovial fluid of RA patients [86].

6) Balancing the role of neutrophils in infectious and non-infectious arthritis

Inhibition of neutrophil recruitment is a double edge sword in arthritic diseases. In one hand, inhibiting neutrophil recruitment in infectious arthritis is detrimental. On the other hand, inhibiting neutrophil recruitment in non-infectious arthritis reduces the disease severity. For instance, in septic arthritis, neutrophils control bacterial load limiting infection. In RA, neutrophils are the main infiltrating cells in the synovial fluid, and their turnover can exceed 10^9 cells per day in a 30 mL joint effusion [87]. In both non-infectious and infections arthritis neutrophils contribute to tissue lesion. In addition to this dichotomy, neutrophils also have a dual role in gout arthritis as discussed below.

Neutrophils in the circulation usually have a short lifespan and rapidly undergo constitutive apoptosis. However, during migration toward inflammatory sites, activated neutrophils encounter changes in the inflammatory microenvironment that stimulate expression of genes related to longevity. One of these changes lies in the increased activity of the transcription factor Foxo3a that ensures neutrophil survival during inflammatory arthritis

[64]**.** FOXO transcription factors have been found to play a critical role in the regulation of proliferation, apoptosis and oxidative stress [88]. A milder disease courses in patients carrying the *FOXO3A* minor allele (G, but not T haplotype) of rs12212067 [89] and increased expression of FoxO3 in polymorphonuclear cells in RA [90]. Accordingly, Foxo3a-deficient mice resist to K/BxN serum transfer-induced arthritis due enhanced FAS ligand expression and the consequent early neutrophil apoptosis [64]. This evidence suggests that delayed apoptosis of neutrophils is an important component in the development of RA, and therefore, inhibition of these pathway/or facilitating neutrophil apoptosis and inhibiting neutrophil recruitment are possible approaches to reduce inflammatory arthritis. Monosodium urate (MSU) crystal deposition in joints causes gouty arthritis with the participation of components of the innate immune system and mononuclear phagocytes, which are critical orchestrators of the inflammatory response. Nevertheless, neutrophils have a prominent role in the tissue lesion especially in gout attack in which the neutrophil counts increase drastically [91]. Seminal studies in crystal-driven gout in canines demonstrated the fundamental role of neutrophils in the acute phase of gout. In these studies, neutrophil depletion significantly suppressed inflammation suggesting that its inhibition could be a new target for gouty arthritis [84,92]. The recruited neutrophils also produce IL-1β, TNF-α, IL-8, and IL-6 contributing to increasing inflammation [91,93]. In this regard, the blockade of the chemokine receptor CXCR2 reduces the infiltration of neutrophil, joint inflammation and pain induced by MSU crystals in mice [94]. In fact, neutrophils contribute to inflammatory pain through the production of hyperalgesic molecules including leukotrienes and prostaglandins in response to a broad range of endogenous stimuli such as endothelin-1, LTB4, IL-1□ and complement system molecules such as C5a [95-98] (Figure 2D). On the other hand, the inhibition of neutrophil recruitment may be prejudicial in gouty arthritis. NETs form dense aggregates that proteolytically degrade MSU crystals. Furthermore, the subsequent phagocytosis of MSU degraded components by macrophages ultimately lead to a reduction of inflammatory process [59]. This mechanism accounts for the resolution of the acute phase of gout. Therefore, in gouty arthritis neutrophils have a dual role of contributing to tissue lesion, but also to limiting inflammation triggers. It is likely that focusing only in inhibiting neutrophil chemotaxis in gouty arthritis is not the definitive therapeutic approach, but rather reducing the harmful actions maintaining the beneficial effects of neutrophils is more appropriate. For instance, determining the mechanisms regulating the fate of neutrophils as to whether they will form NETs or not seems an important approach to enhance the degradation of MSU crystals.

Septic arthritis is one of the most dangerous joint diseases due to the rapid disease development course, severe joint lesion, sequels to the joint function and pain and even septic shock-induced death despite the use of antibiotics. The primary etiologic agent of septic arthritis is *Staphylococcus aureus*, an extracellular growing bacterium [99]. There is evidence that both drug resistant *S. aureus* such as methicillin-resistant *Staphylococcus aureus* (MRSA) and non-drug resistant *S. aureus* cause septic arthritis. The MRSA is a cause of septic arthritis in hospitalized patients, and non-drug resistant *S. aureus* causes septic arthritis in cases of spontaneous development within the community [100,101]. A contributing factor for *S. aureus*-induced arthritis is the immune suppression induced by drug, diseases and aging [100].

Neutrophils depletion may increase pro-inflammatory cytokine production (e.g. TNF-α and IL-6) in septic arthritis, which would seem a contradiction if we consider the pro-inflammatory role of neutrophils. However, the increasing cytokine production by neutrophil depletion is related to an increase in disease severity related to increasing the bacterial load. Failure in controlling bacteria proliferation results in increasing activation of tissue resident cells that produce higher amounts of cytokines and chemokines. Therefore, neutrophils play a protective role due to their phagocytic ability and are critical cells for the outcome during the early stages of *S. aureus* infection although they also contribute to tissue lesion [102] (Figure 2B, 2C). In agreement with that, mice develop more frequent and severe clinical septic arthritis upon anti-inflammatory therapy. Neither treatment with IL-1 receptor antagonist (IL-1Ra) [103] nor inhibition of the transcription factors NF-κB and AP-1 alone or in combination with antibiotics ameliorate *S. aureus*-induced arthritis [104]. These data may be due to the counteracting effect of increased bacterial load due to diminished bactericidal response. A bias of these studies is that *S. aureus* was injected systemically causing septic arthritis alongside with sepsis, which is a condition with a fundamental role of neutrophil recruitment toward the inflammatory foci to host survival [32,105]. Therefore, remains the question whether the inhibition of neutrophil would benefit the host with *S. aureus* infection in the joint cavity representing the condition of established septic arthritis only and not the events that would lead to septic arthritis.

The ability of the host to respond to bacterial infection is essential since neutrophils are an important part of the host protective system. In fact, patients with rheumatoid arthritis are 4- to 15-fold more susceptible to septic arthritis than the general population [100]. A likely explanation is that the presence of joint lesions facilitates its colonization together with a reduced immune response due chronic treatment with disease modifying drugs,

corticosteroids, non-steroidal anti-inflammatory drugs and biologic therapies that cause patient immune suppression.

An important point to keep in mind is that the role of neutrophils in disease context may vary as a consequence of the inflammatory milieu. For instance, we have shown that in a model of antigen-induced arthritis in mice, TNF-α induces the expression of ST2 (IL-33R) rendering neutrophils chemoattractable by IL-33. Corroborating, TNF-α induces ST2 expression and IL-33 responsiveness of human neutrophils, and neutrophils of RA patients under infliximab treatment present lessen ST2 expression and IL-33 responsiveness [106]. Therefore, determining the neutrophil phenotype under varied infectious and non-infectious inflammatory disease may open a venue to selectively targeting neutrophil phenotype related to a particular group of diseases without increasing the susceptibility to another group of diseases.

Septic arthritis is one of the most dangerous joint diseases due to the rapid disease development course, severe joint lesion, sequels to the joint function and pain and even septic shock-induced death despite the use of antibiotics. The primary etiologic agent of septic arthritis is *Staphylococcus aureus*, an extracellular growing bacterium [99]. There is evidence that both drug resistant *S. aureus* such as methicillin-resistant *Staphylococcus aureus* (MRSA) and non-drug resistant *S. aureus* cause septic arthritis. The MRSA is a cause of septic arthritis in hospitalized patients, and non-drug resistant *S. aureus* causes septic arthritis in cases of spontaneous development within the community [100,101]. A contributing factor for *S. aureus*-induced arthritis is the immune suppression induced by drug, diseases and aging [100].

Neutrophils depletion may increase pro-inflammatory cytokine production (e.g. TNF-α and IL-6) in septic arthritis, which would seem a contradiction if we consider the pro-inflammatory role of neutrophils. However, the increasing cytokine production by neutrophil depletion is related to an increase in disease severity related to increasing the bacterial load. Failure in controlling bacteria proliferation results in increasing activation of tissue resident cells that produce higher amounts of cytokines and chemokines. Therefore, neutrophils play a protective role due to their phagocytic ability and are critical cells for the outcome during the early stages of *S. aureus* infection although they also contribute to tissue lesion [102] (Figure 2B, 2C). In agreement with that, mice develop more frequent and severe clinical septic arthritis upon anti-inflammatory therapy. Neither treatment with IL-1 receptor antagonist (IL-1Ra) [103] nor inhibition of the transcription factors NF-κB and AP-1 alone or in combination with antibiotics ameliorate *S. aureus*-induced arthritis [104]. These data may be due to the

counteracting effect of increased bacterial load due to diminished bactericidal response. A bias of these studies is that *S. aureus* was injected systemically causing septic arthritis alongside with sepsis, which is a condition with a fundamental role of neutrophil recruitment toward the inflammatory foci to host survival [32,105]. Therefore, remains the question whether the inhibition of neutrophil would benefit the host with *S. aureus* infection in the joint cavity representing the condition of established septic arthritis only and not the events that would lead to septic arthritis.

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7) Pharmacological approaches to modulate the neutrophil recruitment and activity in animal models of arthritis

Varied animal models of inflammatory arthritis show that neutrophils are the first immune cells that migrate toward arthritic joint, and that early measures of joint inflammation correlate with neutrophil infiltration [64,106-109]. The pharmacological approaches that aim neutrophil inhibition (Table 1) highlight its importance in RA. Taking into account that the inhibition of neutrophil recruitment is prejudicial to the host in septic arthritis and sometimes in gout arthritis, in this section, we will highlight pharmacological approaches targeting neutrophil recruitment and activity presenting therapeutic benefit in RA. We also provided

information on the pharmacological/genetics approaches related to infectious arthritis models and their consequence to the host (Table 2).

Chemokines, especially CXCL1 and CXCL2 [110], provide a luminal gradient that guides neutrophils towards inflammatory foci. This role of chemokines supports chemokine receptors as useful targets to modulate neutrophil recruitment. In a model of antigen challenge in immunized mice, treatment with DF2162, an allosteric antagonist of CXCR1 and CXCR2, decreases neutrophils recruitment and MPO activity, and consequently hyperalgesia (exacerbated pain response) and TNF-α production [111,112]. Strengthening the therapeutic potential of antagonizing chemokines receptors, in two models of arthritis, two selective CXCR1/2 antagonists (SCH563705 and repertaxin) reduce disease activity, clinical score, paw swelling, pro-inflammatory cytokine production and neutrophil recruitment [113,114].

Alongside with chemokines, C5a fragment (a peptide of complement system) bounds to C5aR expressed by neutrophils and acts as chemoattractant and activator of these cells depending on its concentration. At low concentration C5a acts as a chemoattractant while, at higher concentration, C5a can elicit neutrophil production of superoxide anion and enzyme release [115]**.** Inhibition of C5a-C5aR signaling through antibody anti-C5aR [116,117] or in C5aR knockout mice [118,119] reduces the clinical and histological score, neutrophil recruitment, neutrophils enzymes activity (MPO and MMP-9), pro-inflammatory cytokine production and bone erosion in models of experimental arthritis. Additionally, the importance of this signaling lies on C5aR and FcγR activation in neutrophils that induce the release of LTB_4 and IL-1β. These mediators contribute to promotion and progression of arthritis [118].

Considering the vast enzymatic arsenal of neutrophils, inhibition of their degranulation would be an interesting approach in RA. Pentoxifylline, a nonspecific phosphodiesterase inhibitor and a reducer of TNF-α mRNA half-life, attenuates neutrophil oxidative burst and decrease pro-inflammatory mediator synthesis [120]. Treatment with pentoxifylline diminishes neutrophil recruitment and TNF-α production in ovalbumin immunized and challenged mice [121] and in uric acid crystal-induced joint inflammation in mice [122]. In agreement with that, treatment with pentoxifylline reduces arthritic index, neutrophil recruitment, TNF-α production, and prevents proteoglycan loss in a model of antigen-induced arthritis [123].

The murine K/BxN serum transfer model closely resembles human arthritis [124]. In this model, mice lacking 5-LOX and 15-LOX (key enzymes in leukotrienes and lipoxins production) or a 5-LOX pharmacological inhibitor (L-739,010) ameliorate arthritis by decreasing clinical index, bone and cartilage erosion alongside with neutrophils recruitment

[125,126]. Likewise, neutrophil-derived LTB_4 signaling through either BLT1 or BLT2 accounts for the development of K/BxN serum transfer model of arthritis [127,128]. Signaling of neutrophils through BLT1 drives the production of IL-1β, which amplifies arthritis severity by inducing the production of neutrophil chemoattractants by synovial cells [129]. In agreement with that, antagonizing the LTB_4 receptor BLT1 [130] or using BLT12/2 or BLT1/BLT2 double-knockout mice result in protection from the development of collagen-induced arthritis [131]. In an inverse pathway, this leukotriene system can also be triggered by cytokines and modulate neutrophil recruitment. For instance, IL-15 induces IL-18 maturation in synovial fluid neutrophils and peripheral blood neutrophils of RA patients, but not in peripheral blood neutrophils of healthy human volunteers. IL-15 induces IL-18-dependent production of LTB_4 by RA neutrophils and induces neutrophil migration in ovalbumin immunized and challenged mice by the same mechanism [132]. Targeting IL-15 with IL-15 soluble receptor also reduces pain in ovalbumin immunized and challenged mice [133]. In fact, HuMax-IL-15 (a human anti-IL-15 monoclonal antibody) reaches clinical trial and ameliorates disease activity, inhibits IL-15-induced release of IFN-γ and reduces CD69 expression by synovial mononuclear cells in patients with RA [134].

Regarding infectious arthritis such as Lyme's disease (a *Borrelia burgdorferi* infection), 5-LOX deficiency is prejudicial to the host, contributing to earlier joint swelling, inability to resolve arthritis and to worsening of arthritis score [135]. *B. burgdorferi* also induces IL-15 (which induces LTB_4 production [132]) production by neutrophils and mononuclear cells [136]. Importantly, targeting IL-15 with antibody and soluble receptor prevents *B. burgdorferi* vaccination and infection-induced paw edema, cellular infiltration in the joints (neutrophils, macrophages, and lymphocytes) and histopathologic indications of arthritis, including hyperplasia, hypertrophy, and villus formation of the synovium [137]. These set of data demonstrate the fundamental role of neutrophils in animal models of arthritis and may provide insight into the human inflammatory disease. Figure 2 summarizes the molecules we discussed and additional molecules with a role in non-infectious arthritis models. It is also important to point out that are important molecules that modulate diseases involving joint inflammation, but that do not seem to have as central mechanism targeting neutrophils. For instance, there are therapies targeting activation of B cells, T cells, NK cells, innate lymphoid cells (known as group 3 ILCs) and osteoclasts, as reviewed by others [138-141].

8) Perspectives of therapies targeting neutrophils in arthritis

Neutrophils represent a promising pharmacological target in RA considering the role of innate immunity in addition to adaptive immunity in RA physiopathology [142], the high counts of neutrophils in inflamed synovial fluid and the contribution of neutrophils to tissue lesion in RA. Of all cells implicated in the physiopathology arthritic diseases, neutrophils possess the greatest cytotoxic potential, owing to their ability to release degrading enzymes, the release of NETs-containing enzymes, pro-inflammatory cytokines, reactive oxygen species and regulation of osteoclastogenesis. Thus, to improve comprehension, we highlighted some examples in Figure 2 of potential targets of neutrophils function and recruitment, and in Table 2 examples of compounds undergoing clinical trials that target the neutrophil.

The current treatment of RA patients includes glucocorticoids, non-steroidal anti-inflammatory drugs and disease modifying anti-rheumatic drugs. Biological agents such as monoclonal antibodies and recombinant proteins that target TNF-α, CD20, CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), IL-1 receptor, as well as therapies based on the blockade of T-cell and B-cell functions have shown efficacy to control physical signs and pain [143,144]. For instance, methotrexate is a compound that blocks folic acid metabolism and widely used in clinic settings. Its benefits in RA include stimulation of neutrophil apoptosis [145], inhibition of NF-κB pathway [146], reduction adhesion molecules expression, LTB_4 production [147], and consequently neutrophils recruitment, and ROS production [148]. Anti-TNF-α therapies are also widely used for the treatment of RA patients. Its benefits include reduction in adhesion molecule [149,150], and neutrophils recruitment and activation, accompanied by a reduction of NF-κB activity and pro-inflammatory cytokines such as IL-6, IL-1β and IL-8 [149,151]**.** The current clinical practice has these molecules as targets, but their side effects and high costs usually limit their applicability in the chronic treatment of RA [143]. The development of adaptive immunity against the biologic therapies regularly occurs limiting their use. Some RA patients are refractory to even high doses of glucocorticoids or immunosuppressive agents [143,152]. Paradoxically, corticosteroids inhibit many neutrophil functions, but also delay its apoptosis, an effect that needs further investigation for a full understanding of its underlying mechanism and relevance *in vivo* [153]. Soluble ST2 and IL-33 are presented in higher concentrations in plasma/serum of patients with RA compared to healthy volunteers. Furthermore, the IL-33/ST2 signaling pathway has a pro-inflammatory role in RA [106,154,155]. Assessing neutrophils in peripheral blood of patients with RA, we observed that neutrophils derived from RA patients

1453 treated only with methotrexate present higher expression of ST2 compared to healthy donors
1454 or patients under treatment with methotrexate plus infliximab. Thus, after TNF-α stimulus,
1455 neutrophils from healthy donors increase the expression of ST2. This finding supported the
1456 clinical relevance of IL-33 in RA and provided an important novel mechanism by which anti-
1457 TNF-α therapy ameliorates inflammation [106]. Likewise, RA patients treated with
1458 Etanercept have significant reduction of serum IL-33 levels, which showed significant
1459 correlations with the number of tender joints, VAS scale and C-reactive protein [156].
1460 Therefore, targeting IL-33 may provide very promising therapy to RA and certainly deserves
1461 further investigation in clinical trials.

1462 The use of molecules from natural sources represents a promising land to treat RA (but
1463 not only), by acting as primary therapy or at least as therapeutic adjuvants to reduce the daily
1464 doses of conventional drugs that RA patients receive [152,157,158]. Importantly, there are
1465 molecules from natural sources with fewer side effects than current therapies alongside with
1466 low toxicity, allowing utilization of high concentrations. Curcumin and quercetin highlight
1467 between these molecules. Curcumin is an extremely pleiotropic compound and presents more
1468 than 100 different targets [159]. Both quercetin [160] and curcumin [161] block neutrophils
1469 recruitment through inhibition of cellular signaling responsible for actin polymerization in
1470 association with down-regulation of adhesion molecules [162,163]. Curcumin also possesses
1471 bactericidal activity, which could be useful in cases of infectious arthritis [164,165]. The
1472 potential of curcumin in patients with RA was first reported back in 1980 with reduction of
1473 joint swelling, morning stiffness, and improvement of walking time [166]. A clinical trial with
1474 patients with RA also shows that curcumin reduces reported pain, tenderness and swelling of
1475 joint [167]. Corroborating, Meriva® (a curcumin-based medicine) demonstrates efficacy in
1476 clinical trials with patients with osteoarthritis by reducing reported pain (VAS scale) [168]. In
1477 another clinical trial, treatment with Meriva® reduces stiffness and physical signs (treadmill
1478 test), alongside with IL-1β, IL-6, and sVCAM-1 production [169].

1479 Another natural product-derived molecule that reaches clinical trials is Maritech®,
1480 which is a fucoidan-based medicine – a molecule that binds to the adhesion molecule L-
1481 selectin inhibiting its function. Treatment with Maritech® reduces reported joint pain,
1482 stiffness and difficulty with physical activities in patients with osteoarthritis in phase I and II
1483 open label study [170]. In an accompanying study in healthy volunteers, treatment with
1484 Maritech® enhances serum antioxidant capacity and reduces IL-6 levels [171]. Associated
1485 with that, treatment with Maritech® did not change cholesterol levels, liver, renal, and

hematopoietic functions during three months treatment; demonstrating that it is a safe drug [170].

Growing evidence point to agonist of receptors that leads to resolution of inflammatory process as a promising therapy for inflammatory diseases. Resolution of inflammation is a highly ordered active process, tightly regulated by many lipid mediators, enzymatically derived from essential polyunsaturated fatty acids (PUFA), and are termed specialized pro-resolving lipid mediators (SPMs). Lipoxin A4 (LXA4, an agonist of ALX/FPR2) and resolvins (Rv, an agonist of ALX/FPR2) are the most studied SPMs [172]. For instance, treatment with RvE1 reduces IL-8-induced neutrophil chemotaxis [173] and similarly, treatment with LXA_4 reduces neutrophil adherence and transmigration [174]. Isolated SPMs efficacy lies, in part, by inhibition of neutrophils recruitment to inflammatory foci. In fact, SPMs and PUFA have been tested in models of arthritis [109,175-177] and clinical trials [178], respectively. Using K/BxN serum transfer model, ALX/FPR2 KO mice exhibit exacerbated disease severity suggesting an endogenous role of SPMs in limiting disease development [175]**.** Administration of LXA_4 inhibits edema and neutrophil influx accompanied with a reduction of preproET-1 mRNA, KC/CXCL1, LTB_4 and TNF-α levels [109]. Resolvin D1 (RvD1) is a potent regulator of pain and neutrophils recruitment and binds to ALX/FPR2 receptor [179]. Treatment with RvD1 reduces pain and tissue damage, proving to be more potent than either steroid or non-steroidal anti-inflammatory drugs (NSAIDs). Its mechanism lies in the reduction of NF-κB and COX-2 expression in the spinal cord, and, in arthritic joints, inhibition of TNF-α and IL-1β production [177]. Regarding polyunsaturated fatty acids (PUFAs), patients with RA, who use omega-3 fatty acids reduce the use of NSAIDs and cardiovascular risk factors, and increase the period of disease remission compared to patients who did not use omega-3 fatty acids [180]. Randomized controlled clinical trials using omega-3 fatty acids in RA reported improvement in several clinical outcomes including reduced duration of morning stiffness, reduced number of tender or swollen joints, reduced reported joint pain, increased grip strength and decreased use of NSAIDs [178]. In mononuclear cells from healthy donors and stimulated with LPS, supplementation with omega-3 fatty acids results in reduced levels of TNF-α and IL-1β [181,182]. A meta-analysis of omega-3 fatty acids treatment and pain demonstrates that supplementation with omega-3 fatty acids for 3-4 months reduces patient-reported joint pain intensity, minutes of morning stiffness, the number of painful/tender joints, and NSAIDs consumption [183].

SPMs present anti-inflammatory activity without immunosuppressive activity, which may be a beneficial feature in infectious arthritis to control painful symptoms and bacterial load. There is evidence in different experimental models demonstrating that treatment with Rv enhances the clearance of *E. coli* and *S. aureus* [184,185]. Aligned with that, treatment with Rv increases the clearance of these bacteria by stimulating non-phlogistic phagocytosis, enhancing bactericidal activity of antibiotic, which ultimately results in lowering antibiotic requirement and contributes to diminishing resistance [185]. Regarding isolated SPM, RvE1 reaches clinical trials as RX-10045® for dry eye syndrome, and produces dose-dependent, statistically significant improvements on the primary endpoints of signs and symptoms of dry eye (Clinicaltrials.gov identifier: NCT00799552) [186,187]. Thus, these set of data demonstrates robust clinical trials are evidencing the efficacy of omega-3 fatty acids PUFAs in RA. Associated with animal data, and that SPMs present therapeutic effectiveness in picogram ranges [172], supporting and encouraging the use of isolated SPMs in clinical trials for patients with infectious or non-infectious arthritis.

The interaction between platelets and neutrophils is an essential checkpoint to neutrophil recruitment [188]. An indispensable component is the selectin ligand PSGL-1, which signaling results in the redistribution of receptors (such as Mac-1 and CXCR2) that drives neutrophil migration toward tissues [189]. This interaction seems to be an important step to the formation of NETs as well [189,190]. Accordingly, in a model of collagen-induced arthritis, treatment with a soluble receptor of PSGL-1 reduces the clinical severity and TNF-α production [191], which might be explained by inhibition of NETs formation [189,190] Interestingly, mice that overexpress P-selectin increase the amount of NETs formation following the stimulation with platelet-activating factor (PAF) [190]. Another point that strengths the platelet-neutrophil cross-talk lies in the activation of neutrophils through receptor advanced glycation end products (RAGE) activation by high mobility group box 1 (HMGB-1) released from platelets [192]. In neutrophils, this induces autophagy and preferentially leads to death by NETosis instead of apoptosis [193,194]. In fact, mice that received platelets lacking HMGB-1 gene [194] or mice lacking RAGE [195] fail to release NETs. Although in conditions such as cancer, autophagy results in enhanced survival of neutrophils and NETs formation [195]. Future therapeutic intervention exploiting these mechanisms might involve shifting the balance toward the resolution of inflammation by promoting aggregation of NETs.

Over the past years, the multistep process of neutrophils recruitment from blood to tissue has been elegantly studied [34,35,189,196]. A reasonable future approach should

consist in selectively inhibiting neutrophils recruitment accordingly with the characteristic of initial noxious stimuli. Neutrophils tend to respond better to ending chemoattractant such as formyl-peptide (fMLP) and C5a than IL-8 and LTB_4 [35]. This spatial, temporal and hierarchic cascade of mediators involving neutrophil recruitment toward tissues prompts therapies aiming inhibition of a starter chemoattractant (initial targets, IL-8 and LTB_4) or ending chemoattractant (final targets, fMLP and C5a) by using selective antagonist targeting a specific step. Regarding adhesion molecules, neutrophils adhesion and recruitment within liver sinusoids in sterile inflammation depend on the integrin Mac-1 and ICAM-1 [34]. In contrast, when used *E. coli* as stimuli for neutrophil recruitment to the liver surface rather than a necrotic injury, the adhesion of neutrophils in sinusoids depends on CD44 rather than Mac-1, revealing different adhesion molecules for neutrophil recruitment in infection versus sterile inflammation [34]. Furthermore, the observation that neutrophils rapidly migrated away from high concentrations of CXCR2 ligands implies that the necrotactic stimulus must hierarchically override CXCR2 signaling. Mice lacking fMLP receptor, but not those lacking CXCR2, significantly attenuated neutrophils chemotaxis next to necrotic foci, demonstrating that fMLP receptor guides precise localization of neutrophils into areas of sterile tissue necrosis, and further corroborates this hierarchic cascade [34]. The last barrier to neutrophils transmigration to inflammatory foci requires a cross-talk between neutrophils and pericytes [For review see 197]. Pericytes contribute actively to the precise guidance of neutrophils by paving the road through modulation of basement membrane proteins and signalizing hotspots gaps that drive neutrophils into inflammatory foci [197]. The communication between ICAM-1 of pericytes and Mac-1 and LFA-1 of neutrophils, alongside to the enlargement of the gaps size by pericytes mediate this guidance [197-199]. Associated to that, pericytes recognize DAMPs and PAMPs [197]; produce chemokines, such as CXCL1, IL-8, and MIF; express ICAM-1 following TNF-α and IL-1β stimulation [199]; and enhance neutrophils survival [198] which further increase this cascade.

Taking into account the differential requirement of adhesion molecules in infectious and non-infectious recruitment, direct inhibition of selective adhesion molecules is a logic therapeutic approach. In agreement with that, treatment with anti-TNF-α and corticosteroids of RA patients down-regulate the expression of adhesion molecules such as VCAM-1, ICAM-1, and E-selectin [150,151,200,201]. Despite that, inhibition of integrin or selectin in varied clinical trials fails to improve symptoms in patients with RA [202-204] or psoriasis [205]. However, using alefacept (LFA-3–IgG1 fusion protein, a T cell-specific agent) on synovial inflammation in patients with psoriatic arthritis improves both clinical and histopathological

[206]. This issue remains poorly understood and even in animal models data are contradictory, since inhibition of adhesion molecules demonstrates efficacy [207,208], or no efficacy depending on which adhesion molecule was the target. For instance, anti-LFA-1 presents no effect [207] or aggravates the disease, in the case of mice lacking selectin [209]. In a mouse model of collagen-induced arthritis, treatment with PF-03475952 (an anti-CD44 monoclonal antibody) reduces the incidence of arthritis and clinical index [210]. In a clinical trial with patients with head and neck cancer, the efficacy of an immuno-conjugate (bivatuzumab mertansine) consisting of an anti-microtubule agent coupled to an anti-CD44v6 monoclonal antibody was tested. In this study, three patients showed a partial response; however, the treatment caused severe skin toxicity with a fatal outcome, leading to early termination of this trial [211]. Therefore, a better screening on the approach of adhesion molecule as the target is still necessary.

9) Concluding remarks

Enlightenment of the neutrophil recruitment cascade and understanding that the characteristic of initial noxious stimuli requires different adhesion molecules and chemoattractants might improve the knowledge by which neutrophils migrate toward the tissue. If a specific chemoattractant or receptor is not available at the appropriate time and place, the cascade comes to a halt, and the neutrophil inflammatory reaction collapses. Thus, targeting neutrophils recruitment through this multistep cascade, and controlling NETs formation (stimulating or not, or perhaps specific pathway “suicidal” or “nonsuicidal”), might represent prospering therapeutic approaches for non-infectious arthritis. Regarding infectious arthritis, therapies that enhance the microbicidal activity of neutrophils or macrophages alongside with anti-inflammatory benefits (as isolated SPMs and curcumin), seem a good option for the nearest future in an attempt to reduce antibiotic resistance. Therefore, controlling the inflammatory response is essential to avoid excessive tissue damage and consequent loss of function in infectious and non-infectious arthritis. Thus, the neutrophil seems a promising cellular target to achieve a balanced inflammatory response.

Conflict of interest

The authors declare no conflict of interest.

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Table 1. Examples of potential therapies targeting neutrophil recruitment and activation

Type	Compound	Stage/Model	Reported effect	Reference
Natural product-derived	Fucoidan	mBSA-immunised (mice)	Inhibition of neutrophil recruitment and mechanical hyperalgesia	[212]
		Collagen-induced (mice)	Reduction of adhesion molecules, TNF-α and INF-γ production and leukocyte infiltration	[213]
		Human Fibroblast-like synoviocytes (*in vitro)*	Attenuation of expression and secretion of metalloproteinase, accompanied by suppression of NF-κB and reduction in the viability, survival, and invasiveness of IL-1β-treated of RA Fibroblast-like synoviocytes	[214]
Soluble receptor	ST2	mBSA-immunised (mice)	Inhibition of neutrophil recruitment, reduction of pain, and IL-1β and TNF-α production	[106,215]
		Collagen-induced (mice)	Attenuation of diseases progression, reduction of TNF-α, IL-6 and IL-12 production, and inhibition of neutrophil recruitment	[216]
	PSGL-1	Ovalbumin-sensitized (mice)	Reduction of clinical severity and TNF-α production	[191]
Antibody	ST2	Collagen-	Attenuation of clinical	[217]

		induced (mice)	severity, joint destruction, reduction in neutrophils recruitment and RANKL mRNA expression	
	C5aR	Collagen-induced (mice)	Reduction of clinical and histological score, inhibition of neutrophil recruitment, TNF-α, IL-6, KC and MCP-1 production	[116]
Agonist of receptor	LXA_4 and BML-111 (lipoxin agonists)	Zymosan (mice)	Inhibition edema and neutrophil influx, preproET-1 mRNA, KC/CXCL1, LTB4 and TNF-α levels	[109]
	RvD1	CFA-induced (rats)	Reduction of hyperalgesia, TNF-α and IL-1β production, and joint and paw swelling	[177]
	RvE1 (RX-10045)	Clinical Trials (Dry eye syndrome)	Improvement in dryness, stinging, burning, ocular discomfort, and leukocyte recruitment (CAE staining)	[187]
Antagonism of receptor	LY293111Na (LTB4 antagonist)	Collagen-induced (mice)	Reduction of arthritic index and neutrophil recruitment	[130]
	SCH563705 (CXCR1/2 antagonist)	Anti-collagen antibody-induced (mice)	Reduction of disease activity, paw swelling, pro-inflammatory cytokine production, neutrophil recruitment	[114]
	DF2162 (CXCR1/2 allosteric antagonist)	Zymosan (mice)	Inhibition of neutrophil recruitment and mechanical hyperalgesia	[95]
		mBSA-immunised (mice)	Inhibition of neutrophil recruitment, TNF-α, CXCL1, and CXCL2 production	[112]

Table 2. Examples of emerging therapies in clinical trials targeting neutrophil recruitment and activation

Type	Compound	Disease	Reported effect	Reference
Natural product-derived	Meriva® (curcumin-based medicine)	Osteoarthritis	Reduction of reported pain (WOMAC scale), stiffness and physical signs (treadmill test); inhibition of IL-1β, IL-6, and sVCAM-1 production	[169]
		Osteoarthritis	Reduction of reported pain (VAS scale)	[168]
	Curcumin	RA	Reduction of reported pain, tenderness, swelling of joint and C-reactive protein	[167]
		RA	Reduction of joint swelling, morning stiffness, and improvement of walking time	[166].
	Maritech® (fucoidan-based medicine)	Osteoarthritis	Reduction of reported joint pain, stiffness, difficulty with physical activities	[170]
Supplementation	Omega-3 fatty acids	RA	Reduction of reported pain, use of NSAID and disease modifying anti-rheumatic drugs	[218]
			Reduction of reported pain, duration of morning stiffness, rheumatoid factor, joint pain and time to onset of fatigue	[219]
			Reduction of reported pain and use of NSAID	[220].

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Figure Legends

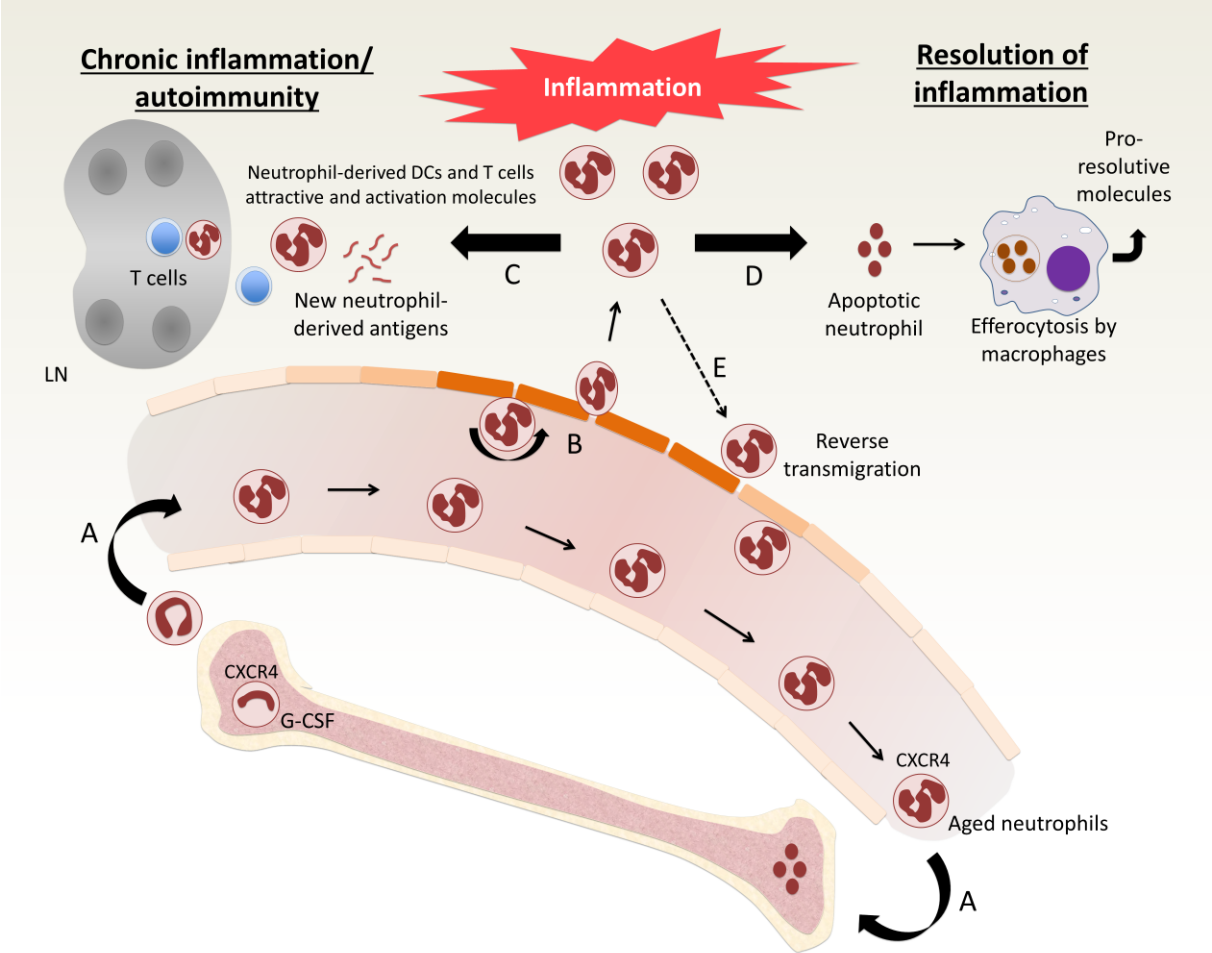

Figure 1: Schematic view of neutrophil circulation in homeostatic and inflammatory conditions. (A) New formed neutrophils in bone marrow reach blood vessels after the loss of CXCR4 on their surface. Aged circulating neutrophils express back its chemokine receptor that drives their return to bone marrow, where they became apoptotic and phagocytosed by resident macrophages. (B) In inflammatory condition, circulating neutrophils interact with active endothelial cells and reach the site of inflammation through a plethora of chemoattractants. (C) Neutrophils contribute to chronic inflammation and adaptive immunity through the release of new potential autoantigens (proteinase 3 and myeloperoxidase), by guiding dendritic cells and T cells to the site of inflammation or by direct interaction with T cells. (D) On the other hand, efferocytosis of apoptotic neutrophils by resident macrophages contributes to the resolution of inflammation by the release of several pro-resolutive molecules. (E) The reverse transmigration of neutrophil from inflammatory milieu could contribute to the resolution of acute inflammation or could favor the development of inflammation to distant organs. LN: lymph node; G-CSF: granulocyte-colony stimulating factor.

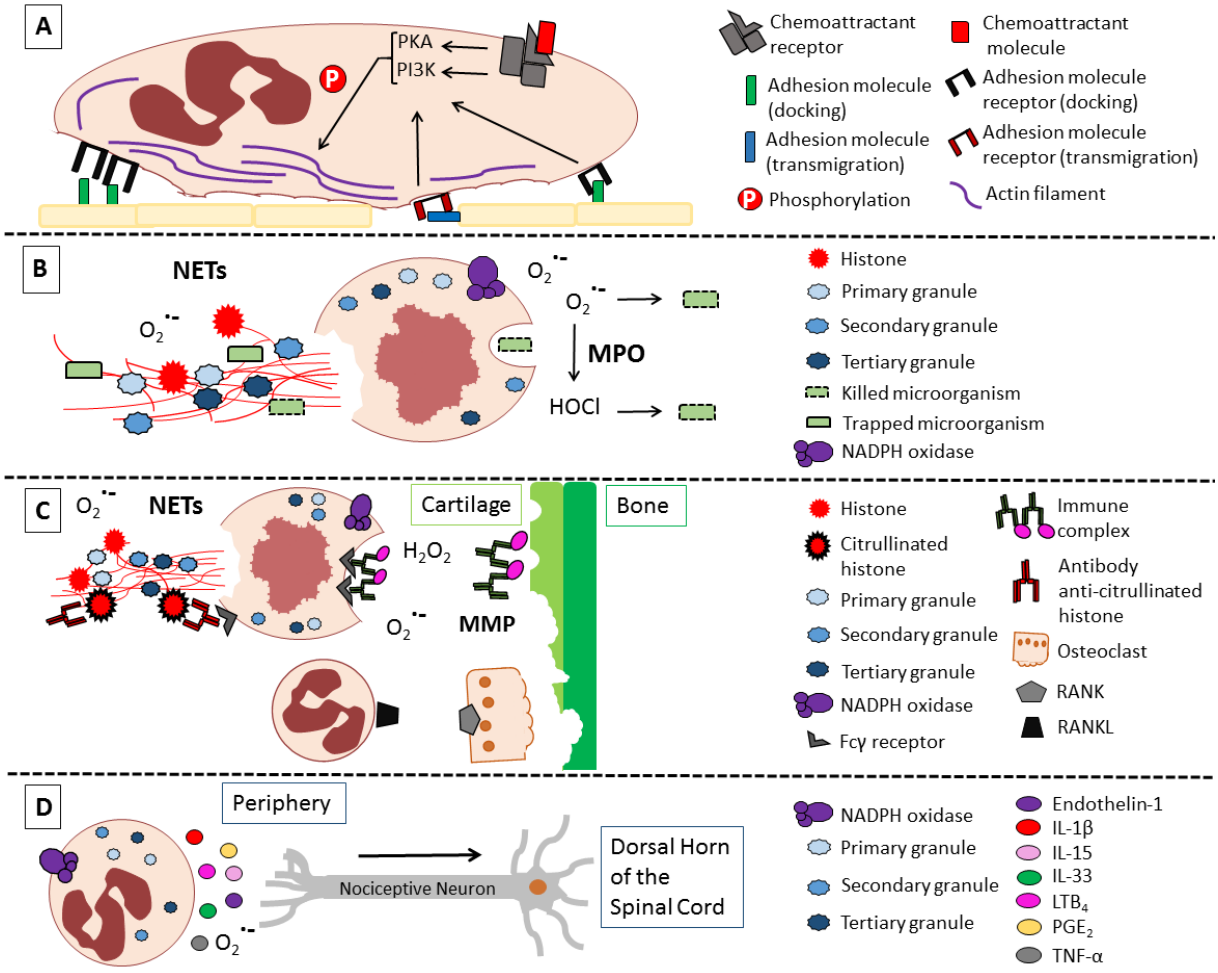

Figure 2: Neutrophils and arthritis: physiopathological mechanisms in their beneficial and detrimental roles in disease. (A) Schematic representation of the neutrophil recruitment mechanisms. Neutrophils interact with endothelial cells through adhesion molecules and chemokines linked to proteoglycan molecules. This interaction leads to activation of neutrophil intracellular second messengers and activation of actin filaments resulting in transmigration towards the inflammatory foci [28,29,35,129,196]. (B) Schematic representation of the neutrophil microbicidal mechanisms. Upon phagocytosis, the neutrophil preformed killing molecules stored in granules are released into the phagolysosome to encounter the microorganisms. Enzymes such as NADPH oxidase and MPO produce the microbicidal molecules superoxide anion and HOCl, respectively. Neutrophils can also release NETs, which entrap and kill microorganisms [10,11,52,61]. The release of NETs depends on cellular nucleus modifications leading to loss of nucleus integrity [221]. (C) Schematic representation of the neutrophil-dependent tissue lesion mechanisms. Immune complex and pro-inflammatory molecules activate neutrophils that produce ROS and release MMPs responsible for cartilage destruction. Neutrophils can also induce osteoclast differentiation and activation leading to bone erosion. NETs contribute forming citrullinated histones and as an immune consequence, there is the production of antibodies against citrullinated histones that account to the activation of neutrophils and other cells contributing to tissue damage [74,81,85,118,192]. (D) Schematic representation of the neutrophil-dependent pain mechanisms. Recruited neutrophils further produce hyperalgesic molecules capable of activating nociceptive neurons causing pain [51,95-98,222]. MPO: myeloperoxidase; MMP: matrix metalloproteinases; HOCl: hypochlorite; $O_2^{\bullet-}$: superoxide anion.

4 ARTIGO PARA PUBLICAÇÃO (JOURNAL OF NATURAL PRODUCTS)

O presente trabalho foi realizado no Laboratório de Dor, Inflamação, Neuropatia e Câncer, da Universidade Estadual de Londrina e segue as normas da revista Journal of Natural Products. Os resultados parciais estão descritos no artigo intitulado “Curcumin ameliorates titanium dioxide-induced arthritis by reducing pain, neutrophils recruitment and proteoglycan degradation”

Curcumin ameliorates titanium dioxide-induced arthritis by reducing pain, neutrophils recruitment and proteoglycan degradation

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ABSTRACT

Arthritic diseases affect millions of people worldwide and reduce substantially the quality of life. The inflammatory response in the joint induces intense neutrophils recruitment, which leads to cartilage and bone destructions. A usual outcome of patients with arthritic diseases is arthroplasty, a procedure known as total or partial replacement of affected articulations. One of the main biomaterial used to fabricate the prosthesis is titanium dioxide. 10 to 15% of patients respond against the debris released. In this sense, we aim to investigate the efficacy of curcumin in a titanium dioxide-induced arthritis. Mice were pretreated with curcumin at 10 and 100 mg/kg 1 h before titanium dioxide injection and after that, daily during 30 days. After 30 days of stimulus, the joint was collected for analysis. By using an electronic version of von Frey filaments, we investigated the effects of curcumin in pain intensity to a mechanical stimulus (mechanical threshold) after titanium dioxide injection. We observed that intra-articular injection of titanium dioxide increased pain and edema. Treatment with curcumin at 100 mg/kg reduced both parameters. Associated to that, curcumin reduced MPO activity, IL-33 production, and reduced proteoglycan degradation. Therefore, we demonstrated the efficacy of curcumin in a titanium dioxide-induced arthritis

Keywords: arthritis, curcumin, IL-33, implant, pain, titanium dioxide.

1. Introduction

Acute pain has protective function and acts as warn to the host for maintenance bodily integrity. Chronic pain, however, is a maladaptive response and represents a substantial and growing unmet medical need worldwide. The sensitization of nociceptive neurons is a common denominator of all types of pain leading to hyperalgesia (an increased response to a stimulus that is normally painful) and/or allodynia (pain due to a stimulus that does not normally provoke pain)[1]. Several mediators contribute to nociceptor sensitization or activation, such as prostaglandin (PG) E_2, sympathetic amines[2, 3], endothelin-1[4, 5], superoxide anion[6, 7], and the pro-inflammatory cytokines IL-33[8], IL-1β[9] and TNF-α [10]. Although hyperalgesia usually occurs in patients, it does not represent a major clinical problem. On the other hand, allodynia does, since impairs normal daily activities of the patients and brusquely reduces the quality of life.

Arthritis is a common denomination to diseases that affect joints. Among these diseases, highlight rheumatoid arthritis, gout, osteoarthritis and septic arthritis[11, 12]. A usual outcome of patients with arthritic diseases is arthroplasty, a procedure known as total or partial replacement of affected articulations[13]. Patients with implants present extraordinary benefits and increase of life quality[14, 15]. Unfortunately, 10 to 15% of patients respond against the debris released by biomaterials that constitute implants, which ultimately leads to osteolysis[16, 17]. One of these biomaterials is titanium dioxide. In a specific case, a patient without familiar history of arthritic diseases develops implant-related arthritis[18]. In fact, after incubation with titanium dioxide, mononuclear cells derived from patient presented increased levels of TNF-α production[18]. *In vitro* evidence in HepG2 cells suggest that titanium dioxide activates NF-κB signaling pathway and has genotoxic effects[19]. Corroborating these finding, injection of titanium dioxide increases neutrophils recruitment and IL-1β production[20]. Taking into account these finding, we have standardized a model of arthritis induced by titanium dioxide. In this model, intra-articular injection of titanium dioxide induces implant related arthritis, that leads to pain, edema, increases RANK-RANKL signaling pathway, enhances pro-inflammatory cytokines production and ultimately leads to joint destruction (Borghi et al. 2016, in preparation)

Although Traditional medicine remains as a good source of therapeutic compounds to contemporaneous medicine, Western medical establishment views their application with skepticism. Current treatment of patients with rheumatoid arthritis lies in glucocorticoids, non-steroidal anti-inflammatory drugs and disease modifying anti-rheumatic drugs.

Monoclonal antibodies and recombinant proteins usually lead to adaptive immunity against the drugs limiting their use [21, 22]. In this sense, the use of natural products-derived molecules represents a promising land to treat chronic pain, by acting as main therapy or at least as therapeutic adjuvants to reduce the daily doses of conventional drugs that these patients receive[23-25]. Curcumin [(E,E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a yellow pigment present in the rhizome of the plant *Curcuma longa* considered a promising compound. One of the greatest benefits of molecules derived from natural sources, such as curcumin, is that they have been tested "clinically" for thousands of years, since the traditional medicine practitioners in South Asia use these molecules to treat a variety of diseases, including arthritis[26]. Curcumin is a highly pleiotropic molecule with antifungal, antiviral, antitumor, and antioxidant activities[27] with more than 100 different molecular targets. The potential of curcumin in patients with RA was first reported back in 1980 with reduction of joint swelling, morning stiffness, and improvement of walking time[28]. Corroborating, in a clinical trial with osteoarthritic patients under treatment with Meriva® (a curcumin-based medicine) reduces reported pain (VAS scale)[29]**.** In another clinical trial, treatment with Meriva® reduces stiffness and physical signs (treadmill test); alongside with IL-1β, IL-6, and sVCAM-1 productio[30]. Recently, a meta-analysis study containing 606 patients with 306 under treatment with curcumin further corroborates these findings and states that curcumin has potential to be a novel alternative for pain relief [31]. We have previously demonstrated the analgesic effect of curcumin in acute model of pain induced by superoxide anion[7]. We observed that treatment with curcumin provides inhibits pain-like behaviors, reduces pro-inflammatory cytokines production and increases antioxidant capacity. Taking into account these aforementioned observations, we aim to investigate the potential effect of curcumin in titanium dioxide-induced arthritis.

2. General experimental procedures

2.1. Animals

Male Swiss mice (25-30 g) from the Universidade Estadual de Londrina, Paraná, Brasil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water and a light/dark cycle of 12:12 h and kept at 21º C. All behavioral tests were performed between 9 am and 5 pm in a temperature-controlled room. Animal care and handling procedures followed the International Association for the Study of Pain (IASP) guidelines. The Ethics Committee of the Universidade Estadual de Londrina approved this study (Process number 21934.2015.55). All efforts were made to minimize the number of animals used and their suffering. The experimenters were blinded to the treatments.

2.2. Experimental procedures

Mice were pretreated per oral (p.o.) with 10 or 100 mg/kg of curcumin or with vehicle (20% Tween 80 in saline) 1 h before single intra-articular (i.a.) injection of 3 mg of titanium dioxide and after that, mice were treated daily until the end of the experiment. During the specified time points mechanical threshold and edema were evaluated. 30 days after the stimulus, the following parameters were determined: myeloperoxidase activity, proteoglycan quantification, and cytokines production. The dose of titanium dioxide and time points for sample collection were determined in standardizing experiments in our laboratory (Borghi 2016, in preparation). Titanium dioxide was diluted in 10 µL of sterile saline immediately before application. Based on the results from mechanical hyperalgesia and edema, the dose of curcumin of 100 mg/kg was chosen and used in the next experiments.

2.3. Electronic pressure meter test

Electronic pressure meter test was evaluated by an electronic version of von Frey's filaments as reported previously[32]. In a quiet room, temperature controlled, mice were placed in acrylic cages (12 × 10 × 17 cm) with wire grid floors 15-30 min before the start of testing. The test consisted of evoking a hind paw flexion reflex with a handheld force transducer (electronic anesthesiometer, IITC Life Science, Woodland Hills, CA) adapted with a 4.15 mm^2 polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the plantar hind paw with a gradual increase in pressure. The gradual increase in pressure was manually performed in blinded experiments. The upper limit pressure was 15 g. The end-point was characterized by the removal of the paw followed by clear flinching

movements. After paw withdrawal, the intensity of the pressure was automatically recorded, and the final value for the response was obtained by averaging three measurements. The animals were tested before and after treatments. The flexion-elicited withdrawal threshold is expressed in grams (g).

2.4 Joint edema

The volume of the joint was measured in mm with a caliper (Mitutoyo, Suzano, SP, Brazil) before (zero time) the i.a. stimulus with titanium dioxide, and after the administration of stimulus in the indicated time points. The joint edema is expressed as Δmm/joint.

2.6. Myeloperoxidase (MPO) activity

The neutrophil recruitment and macrophage recruitment to the joint was evaluated by the MPO activity as previously described[7]. Briefly, mice were terminally anesthetized, and the joint samples were collected in 400 μL of 50 mM K2HPO4 buffer (pH 6.0) containing 0.5% HTAB and then homogenized in ice-cold Tissue-Tearor (Biospec). After that, homogenates were centrifuged (16100g, 2 min, 4 °C), and the supernatants were collected. For the MPO assay, aliquots of 30 μL of supernatant were placed in a 96-well plate and mixed with 200 μL of 50 mM K2HPO4 buffer (pH 6.0), containing 0.0167% ortho-dianisidine dihydrochloride and 0.05% H_2O_2. The absorbance was determined after 5 min at 450 nm (Multiskan GO microplate spectrophotometer, ThermoScientific, Vantaa, Finland). The MPO activity of samples was compared to a standard curve of neutrophils and presented as MPO activity.

2.7. Proteoglycan quantification assay

Proteoglycan contents were determined as described previously[33]. In brief, patella was carefully collected from each animal and fixed with formaldehyde (4%) overnight using a shaker. They were then transferred into a solution of formic acid (5%) and incubated for 4 h using a shaker for decalcification. Each patella was then placed into 100 ml papain digestion buffer consisting of a papain suspension (5 mg/mL) in calcium and magnesium-free PBS with 5 mM cysteine and 10 mM EDTA, pH 7.4. The samples were sealed and incubated in a humidified container in a 60˚C oven for 16 h. After reaching room temperature, the samples were centrifuged (1000 g × 10 min) to collect the condensation droplets. Next, 50 ml of the supernatants and of serial chondroitin sulfate solutions (standard curve; 50–1000 mg/mL) was placed into 96-well microtiter plates. The chondroitin sulfate STD solutions were also incubated with papain digestion buffer. Then, 300 mL of a 1,9-dimethylmethylene blue

(DMMB; 50 mg/L) solution was added to each well, and the absorbance at 525 nm was measured immediately in a plate reader. The GAG content was calculated from the standard curve. The DMMB solution was prepared by dissolving 50 mg DMMB in 5 ml ethanol and diluting to a volume of 1000 ml with 0.2% (w/v) sodium formate buffer, pH 3.5.

2.8. Cytokine measurement

Joint samples were collected 30 days after the injection of titanium dioxide, and homogenized in 500 µL of ice-cold buffer containing protease inhibitors, and centrifuged (3000 rpm × 10 min × 4 °C), and the supernatants used to measure IL-33 levels by an enzyme-linked immunosorbent assay (ELISA) using eBioscience kits. As a control, the concentration of this cytokine was determined in animals injected with saline. The results are expressed as picograms (pg) of cytokine/100 mg of tissue.

2.9. Statistical analysis

Results are presented as means ± SEM of measurements made on six mice in each group per experiment and are representative of two separate experiments. Two-way repeated measures analysis of variance (ANOVA) followed by Tukey's *post hoc* was used to compare all groups and doses at all times when responses were measured at different times after the stimulus injection. Differences between responses were evaluated by one-way ANOVA followed by Tukey's *post hoc* for data of single time point. Statistical differences were considered significant when $P < 0.05$.

3. Results

3.1. Curcumin at 100 mg/kg reduces pain and joint edema

We first sought to determine the dose of curcumin capable of reducing pain. Mice were pretreated with curcumin (p.o) at 10 and 100 mg/kg 1 h before single intra-articular (i.a.) injection of 3 mg of titanium dioxide and after that, mice were treated daily until the end of the experiment. 30 days after stimulus joint was collected for analysis. We observed that titanium dioxide was able to reduce mechanical threshold (Fig 1A) and increased joint edema (Fig 1B). On the other hand, treatment with curcumin at 100 mg/kg reduced pain by increasing mechanical threshold (Fig 1A) and reduced joint edema (Fig 1B) in the evaluated time points.

3.2. Curcumin at 100 mg/kg reduces proteoglycan degradation

Next, we look for investigating whether curcumin could reduce proteoglycan degradation. Mice were pretreated with curcumin (p.o) at 10 and 100 mg/kg 1 h before single intra-articular (i.a.) injection of 3 mg of titanium dioxide and after that, mice were treated daily until the end of the experiment. 30 days after stimulus joint was collected for analysis. What we found was that titanium dioxide increased proteoglycan degradation and treatment with curcumin at 100 mg/kg avoided proteoglycan degradation (Fig 2).

3.3. Curcumin at 100 mg/kg reduces myeloperoxidase activity

The next step was to investigate whether curcumin could reduce neutrophils recruitment. Mice were pretreated with curcumin (p.o) at 100 mg/kg 1 h before single intra-articular (i.a.) injection of 3 mg of titanium dioxide and after that, mice were treated daily until the end of the experiment. 30 days after stimulus joint was collected for analysis. We observed that titanium dioxide increased neutrophils recruitment estimated by increased myeloperoxidase activity and treatment with curcumin at 100 mg/kg reduced it (Fig 3).

3.4. Curcumin at 100 mg/kg reduces IL-33 production

To evaluate the effect of curcumin on cytokine production, we sought to determinate IL-33 production. Mice were pretreated with curcumin (p.o) at 100 mg/kg 1 h before single intra-articular (i.a.) injection of 3 mg of titanium dioxide and after that, mice were treated daily until the end of the experiment. 30 days after stimulus joint was collected for analysis. We observed that titanium dioxide increased the levels of IL-33 and treatment with curcumin at 100 mg/kg reduced IL-33 production (Fig 4).

4. Discussion

We have previously demonstrate that injection of titanium dioxide into joint induces arthritis and that treatment with quercetin ameliorates the impairment provided by titanium dioxide (Borghi 2016, in preparation). Regarding curcumin, we demonstrate that in acute model of pain, curcumin inhibits superoxide anion induced-pain-like behaviors by reducing NF-κB activity and as consequence pro-inflammatory cytokines and increasing Nrf2 expression and as consequence antioxidant defense system and IL-10 production[7]. In the present work, we further corroborated the analgesic effect of curcumin by demonstrating its efficacy in a chronic model, such as arthritis. Herein, we demonstrate that curcumin at 100 mg/kg reduced pain, edema, neutrophils recruitment and proteoglycan degradation in titanium dioxide-induced arthritis.

Chronic pain is a public health problem and has a negative impact on the quality life of people affected. Arthritic diseases are accompanied with chronic pain which is a consistent factor to suicidal thoughts and behaviors[34] and has a straight relationship with depression[35] since occurs alongside with allodynia which impairs normal daily activities. Therefore, seek for strategies that aim reduce pain should always remain in focus. A usual outcome of patients with arthritic diseases is arthroplasty, a procedure known as total or partial replacement of affected articulations[13]. Unfortunately, 10-15% of patients that wear prosthesis present osteolysis due prosthetics debris release [16, 17]. Reports of osteolysis date 1970 and have been associated with all biomaterials, including titanium dioxide[16, 17]. Generation of giant cells and "frustrated phagocytosis" in response to microparticles, or endosomal destabilization as a result of phagocytosis of nanoparticles, are two of the major pathways through which wear debris activate the NLRP3 inflammasome and lead to the release of the mature form of IL-1β[16, 20, 36, 37]. Ultimately, this innate immune response leads to activation of RANK-RANKL signaling pathway in osteoclasts and promotes bone erosion [16, 37].

IL-33 is a cytokine from IL-1 family and the only agonist of ST2[38]. We observed that titanium dioxide increased IL-33 levels and curcumin reduced it. In the absence of inflammation, IL-33 possess nuclear localization, which means that can act as an alarmin, and, therefore, is rapidly released in stressful conditions[38]. We have demonstrated that injection of IL-33 promotes mechanical hyperalgesia and increases pro-inflammatory cytokines such as IL-1β and TNF-α[8]. Corroborating, we also demonstrated that IL-33 is rapidly produced (30 minutes after carrageenan injection) and remains up to 5 hours, in neutrophils dependent manner, since treatment with fucoidan reduces IL-33 production[39].

Importantly, 2 hours after carrageenan injection, mice lacking ST2 presents reduced levels of TNF-α suggesting that IL-33 production is an upstream event to TNF-α[39] in the cascade of hyperalgesic cytokines[3]. Additionally to acute effects, an important point to keep in mind is that the IL-33/ST2 signaling pathway has a pro-nociceptive role in chronic diseases such as rheumatoid arthritis[40-42] and neuropathy[43]. In a model of neuropathic pain, we demonstrated that IL-33 production remains elevated during 21 days and that oligodentrocytes are the major source of IL-33[43]. Apart from its hyperalgesic effect *per se*, we demonstrated that neutrophils from peripheral blood of patients with rheumatoid arthritis treated only with methotrexate present higher expression of ST2 compared to healthy donors or patients under treatment with methotrexate plus infliximab. After TNF-α stimulus, neutrophils from healthy donors increased the expression of ST2. This finding added new evidence in the mechanisms of anti-TNF therapy by demonstrating that TNF-α primes neutrophils to respond to IL-33 chemotaxis[41]. Regarding bone loss, elevated levels of IL-33 reduces bone mineral density, increases leukocytes recruitment, and increases the level of pro-inflammatory cytokines such as IL-6 and INF-γ. This ultimately leads to bone erosion[44]. Corroborating, IL-33 increases the expression of RANKL in osteoclasts[45]. Importantly, mice lacking IL-33 did not change body weight and biomechanical strengh[44]. In this sense, inhibition of IL-33/ST2 signaling is a very promisor therapy since interacts with multiple pathways. To our knowledge, this is the first evidence of curcumin inhibiting IL-33 production. Curcumin inhibits IKK activity, and therefore avoids p65-p50 NF-κB heterodimer activation and consequent translocation to the nucleus[27]. This is a possible explation by which curcumin inhbited IL-33 levels herein observed.

Neutrophils play an important role in several chronic diseases, especially in arthritic diseases, which they are the major cells in the inflammatory infiltrate[22]. Herein, injection titanium dioxide enhanced MPO activity and curcumin was able to reduce. Neutrophils' vast microbicidal array contributes to the destruction of cartilage and bone directly[22] or indirectly by activation osteoclasts through RANK-RANKL signaling pathway[46]. The discovery that neutrophils release NETs added new weapons in this vast arsenal[47] and opened new avenues for the treatment of these diseases. The release of NETs triggers a robust autoimmune response and collaborates to the genesis of diseases such as systemic lupus erythematosus, rheumatoid arthritis, and gout[22]. In rheumatoid arthritis, NETosis seems the primary source of citrullinated antigens[48] which are generated by peptidyl arginine deiminase 4[49] and contributes to aggravate the disease[50, 51]. This mechanism seems important in implant-related arthritis since titanium dioxide triggers histone citrullination and NETs release[52]. Apart from NETs,

neutrophils are the major source of pro-inflammatory cytokines such as IL-33, IL-1β, and TNF-α in arthritic diseases[22, 41]. These cytokines have central role in the pathophysiology of arthritic diseases and pain states. Another striking feature is that titanium dioxide inhibits neutrophils apoptosis and increases IL-8 and IL-6 production[53]. Associated to that, titanium dioxide enhances neutrophils phagocytosis in Syk (spleen tyrosine kinase)-dependent manner[54] and as consequence activates NADPH oxidase which leads to augmentation of ROS production[55]. These mechanisms provide deleterious consequences in the inflammatory milieu and perpetuate a loop that leads to joint destruction, since NADPH oxidase, ROS[56], and MPO[57] are required for neutrophils undergo NETosis. Curcumin inhibits neutrophils actin polymerization[58] and reduces adhesion molecules expression[59], which results in inhibition of recruitment of these cells into inflammatory foci herein observed. Therefore, inhibition of neutrophils recruitment is a crucial step to reduce cartilage and bone destructions and – alongside with NF-κB inhibition – may explain why we observed a reduction in IL-33 production aligned with a reduction in proteoglycan degradation.

Curcumin is a highly pleiotropic molecule and its mechanism of action of curcumin depends on a synergism involving multiples signaling pathways e cells. In the present work, we demonstrated the analgesic effect of curcumin in implant-related arthritis model. Therefore, treatment with curcumin at 100 mg/kg reduced neutrophils recruitment into inflammatory foci. As a consequence of that, we observed reduced levels of IL-33, proteoglycan degradation, and finally reduced pain and edema induced by titanium dioxide injection.

Conflict of interest

The authors declare no conflict of interest.

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Figure captions

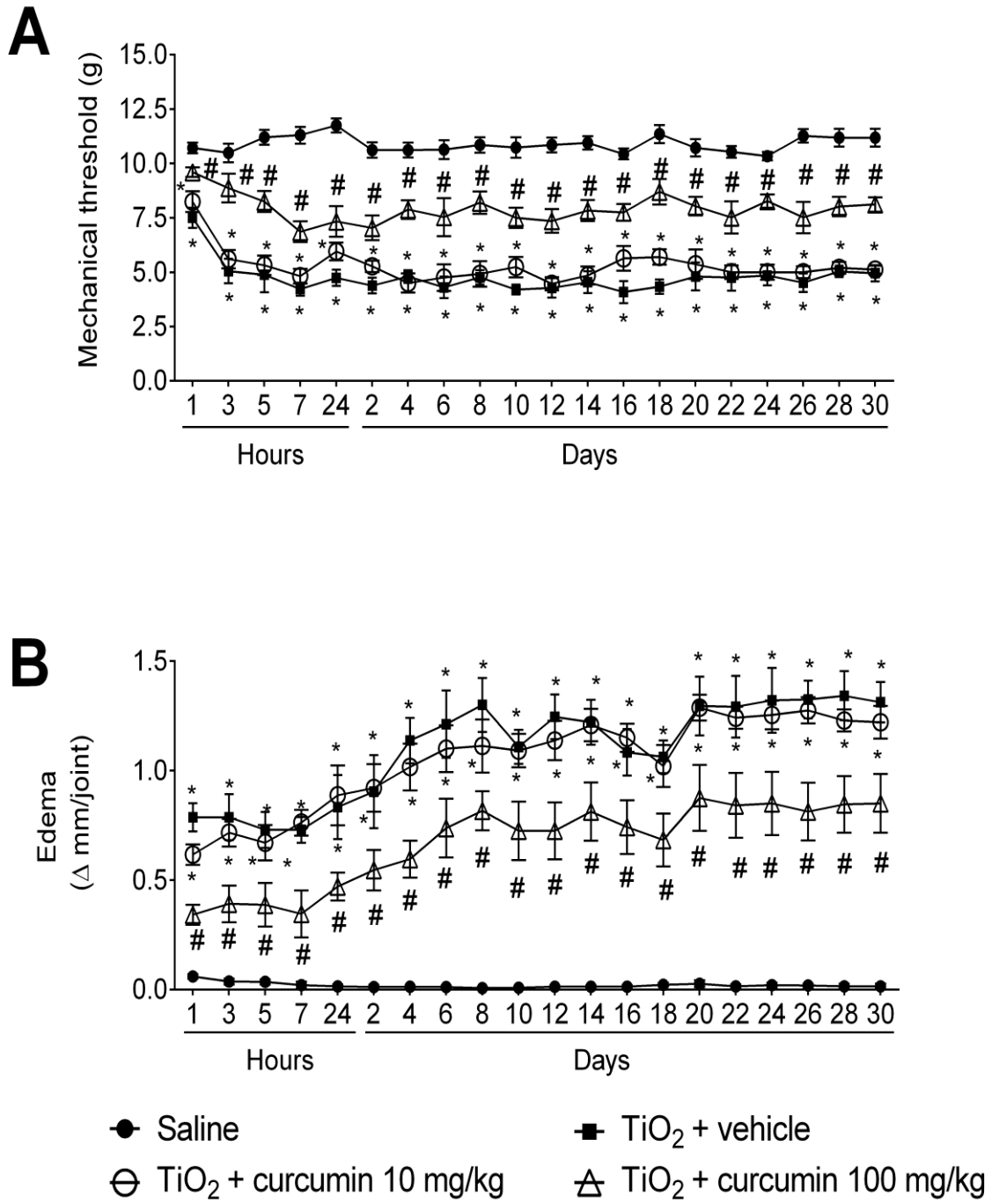

Figure 1. Curcumin at 100 mg/kg reduces pain and joint edema.
Mice were pretreated with curcumin (p.o) at 10 and 100 mg/kg 1 h before single intra-articular (i.a.) injection of 3 mg of titanium dioxide and after that, mice were treated daily until the end of the experiment. Mechanical threshold (A) and edema (B) were measured at the specified time points. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). *$p<0.05$ vs. saline group #$p<0.05$ vs. titanium dioxide group (two-way repeated measures ANOVA followed Tukey's *post hoc*)

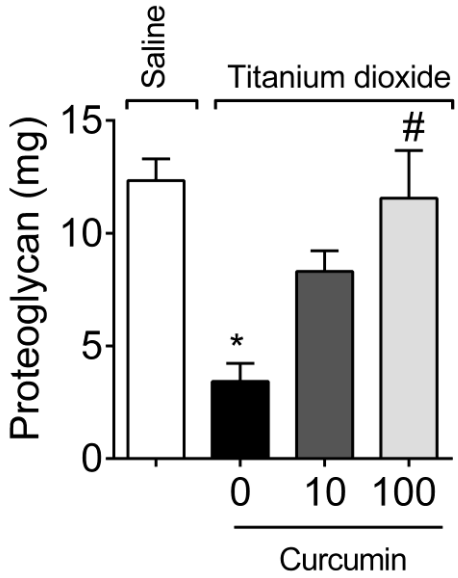

Figure 2. Curcumin at 100 mg/kg reduces proteoglycan degradation
Mice were pretreated with curcumin (p.o) at 10 and 100 mg/kg 1 h before single intra-articular (i.a.) injection of 3 mg of titanium dioxide and after that, mice were treated daily until the end of the experiment. 30 days after stimulus joint was collected for proteoglycan quantification. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). *p<0.05 vs. saline group #p<0.05 vs. titanium dioxide group (one-way ANOVA followed Tukey's *post hoc*)

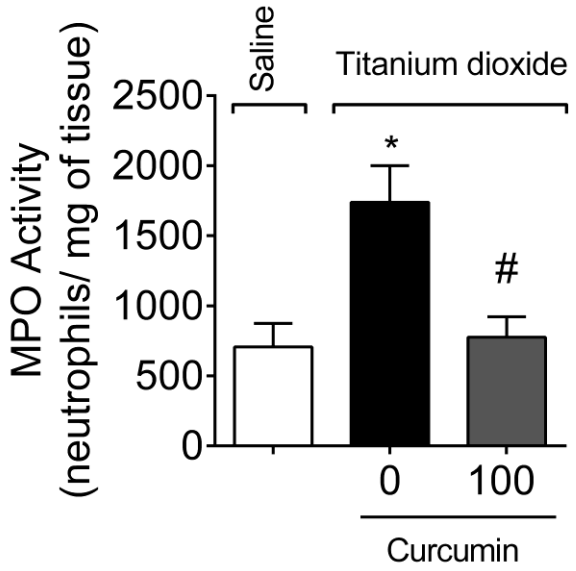

Figure 3. Curcumin at 100 mg/kg reduces myeloperoxidase activity
Mice were pretreated with curcumin (p.o) 100 mg/kg 1 h before single intra-articular (i.a.) injection of 3 mg of titanium dioxide and after that, mice were treated daily until the end of the experiment. 30 days after stimulus joint was collected for MPO activity. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). *p<0.05 vs. saline group #p<0.05 vs. titanium dioxide group (one-way ANOVA followed Tukey's *post hoc*)

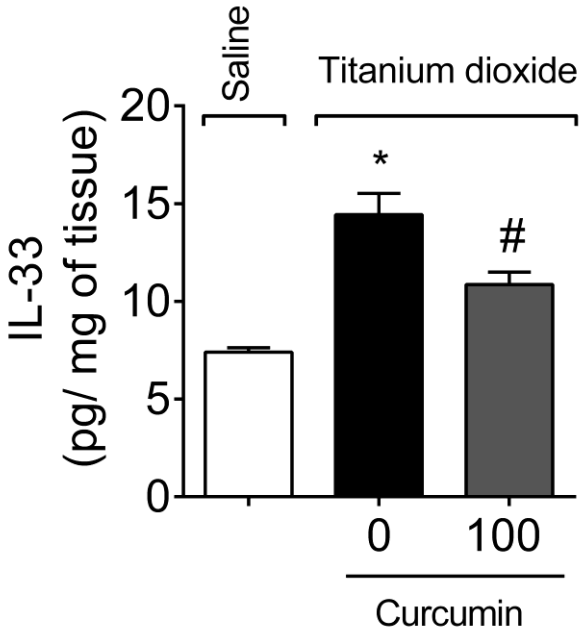

Figure 4. Curcumin at 100 mg/kg reduces IL-33 production
Mice were pretreated with curcumin (p.o) at 100 mg/kg 1 h before single intra-articular (i.a.) injection of 3 mg of titanium dioxide and after that, mice were treated daily until the end of the experiment. 30 days after stimulus joint was collected for dosage of IL-33 by ELISA. Results are expressed as mean ± SEM (n = 6 per group per experiment, representative of two separate experiments). *$p<0.05$ vs. saline group #$p<0.05$ vs. titanium dioxide group (one-way ANOVA followed Tukey's *post hoc*)

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5 ARTIGO 2 PUBLICADO (INFLAMMATION RESEARCH)

O presente trabalho foi realizado no Laboratório de Dor, Inflamação, Neuropatia e Câncer, da Universidade Estadual de Londrina e publicado na revista Inflammation Research. Os resultados estão descritos no artigo intitulado “Curcumin inhibits superoxide anion-induced pain-like behavior and leukocyte recruitment by increasing Nrf2 expression and reducing NF-κB activation” (doi: 10.1007/s00011-015-0885-y).

Curcumin inhibits superoxide anion-induced pain-like behavior and leukocyte recruitment by increasing Nrf2 expression and reducing NF-κB activation

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ABSTRACT

Objective: Evaluated the activity of curcumin in superoxide anion-induced pain-like behavior and leukocyte recruitment in mice.

Treatment: Curcumin 10 mg/kg subcutaneously 1h before stimulus.

Methods: KO_2 was used as superoxide anion donor. Overt pain-like behaviors were determined by the number of abdominal writhings, paw flinches and time spent licking the paw. Mechanical and thermal hyperalgesia were determined using an electronic anesthesiometer and hot plate, respectively. Cytokine concentration and NF-κB activity were determined by ELISA, antioxidant effect by nitrobluetretrazolium assay and ABTS radical scavenging ability. Myeloperoxidase activity was measured by colorimetric assay. The Nrf2, heme oxygenase-1 (HO-1) and $gp91^{phox}$ mRNA expression was determined by quantitative PCR. Data were analysed by ANOVA followed by Tukey's *post hoc* and considered significant when $P<0.05$

Results: Curcumin inhibited superoxide anion-induced overt pain-like behaviors as well as mechanical and thermal hyperalgesia. Curcumin also inhibited superoxide anion-induced leukocyte recruitment in the peritoneal cavity and in the paw skin inhibited myeloperoxidase activity, oxidative stress, IL-1β and TNF-α production and NF-κB activation as well as enhanced IL-10 production, and HO-1 and Nrf2 mRNA expression.

Conclusion: Curcumin inhibits superoxide anion-induced inflammatory pain-like behaviors and leukocyte recruitment by targeting inflammatory molecules and oxidative stress; and inducing antioxidant and anti-inflammatory pathways.

Keywords: curcumin, IL-10, NF-κB, Nrf2, pain, superoxide anion.

1. Introduction

Pain is a symptom present in several diseases affecting nearly one in every five patients and is the main cause of search for medical attendance. Therefore, it is considered an international health problem [1]. Nociceptive neuron sensitization is a process that facilitates depolarization. The sensitization of nociceptive neurons is a common denominator of all types of inflammatory pain leading to hyperalgesia (an increased response to a stimulus that is normally painful) and/or allodynia (pain due to a stimulus that does not normally provoke pain) [2]. Several mediators contribute to nociceptor sensitization or activation through membrane receptors, such as prostaglandin (PG) E_2, sympathetic amines, endothelin-1, and the pro-inflammatory cytokines IL-1β and TNF-α [3-5]. A cascade of pro-hyperalgesic cytokines precedes the release of prostaglandins and sympathetic amines [2]. In mice, this cascade initiates with the release of the alarmin IL-33 [6] that induces the production of TNF-α and keratinocyte-derived chemokine (KC/CXCL1). TNF-α and KC/CXCL1 trigger the production IL-1β that in turn stimulates the production of PGE_2 [5]. These cytokines further contribute to inflammatory hyperalgesia by promoting neutrophil recruitment toward the inflammatory site and inducing the production of PGE_2 and reactive oxygen species by migratory and tissue resident cells. Altogether culminating in sensitization of primary nociceptive neurons [7].

In addition to a hierarchy of cytokine release, there is also a temporal profile of inflammatory events. The inflammatory hyperalgesia depends on the sensitization of the nociceptors by hyperalgesic mediators. This nociceptor sensitization is not an immediate event; therefore, the peak of hyperalgesic mediator production occurs before the peak of hyperalgesia [2]. Similarly, neutrophil recruitment peaks approximately 6 h after cytokine injection. In fact, the peak of chemotactic cytokine production occurs before maximal neutrophil recruitment [8].

Reactive oxygen species (ROS) also contribute to inflammatory hyperalgesia, especially superoxide anion radical ($O_2^{\bullet-}$) [9, 10]. For instance, the anti-nociceptive effect of M40403 (SOD mimetic) depends on diminishing $O_2^{\bullet-}$ effects peripherally and centrally. Peripheral mechanisms triggered by $O_2^{\bullet-}$ are related to the release of pro-hyperalgesic cytokines, and central mechanisms are dependent on the reaction of $O_2^{\bullet-}$ with nitric oxide generating peroxynitrite. In turn, peroxynitrite nitrates endogenous MnSOD inactivating this enzyme and maintaining $O_2^{\bullet-}$ levels [10]. These processes promote maintenance of

inflammatory response and persistence of inflammatory pain. Furthermore, $O_2^{\bullet-}$ can promote mechanical hyperalgesia alone, e.g. without nerve injury and independently to react with nitric oxide as demonstrated in a model using antimycin A (mitochondrial complex III inhibitor) as $O_2^{\bullet-}$ generator. These data corroborate the notion that $O_2^{\bullet-}$ is an important hyperalgesic component as well as being a therapeutic target [11].

Non-steroidal anti-inflammatory drugs are widely used to treat inflammatory disorders and pain relief in clinical practice because of their fast onset of action and excellent curative effects. However, non-steroidal anti-inflammatory drugs induce side effects including ulceration in the gastrointestinal system [12] and platelet dysfunction [13]. In this sense, the research on novel anti-inflammatory drugs persists. Vegetables, fruits and drinks (e.g. wine and tea) of the human diet contain polyphenols. Daily doses of these compounds enhance life quality and decrease the risk of many inflammatory diseases, such as cardiovascular diseases [14]. Curcumin [(E,E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a yellow pigment present in the rhizome of the plant *Curcuma longa* considered a promising compound. Apart from its daily use in the kitchens as a condiment and spice, turmeric containing curcumin has been widely applied by the traditional medicine practitioners in South Asia for thousands of years to treat dysentery, ulcers, wounds, cough, fever and arthritis [15]. Furthermore, alimentary supplementation with curcumin seems to prevent neurodegenerative diseases such as Alzheimer's [16]. Curcumin is a highly pleiotropic molecule with antifungal [17], antiviral [18] antitumor [19] and antioxidant activities [20]. Importantly, curcumin presents low toxicity at dose regimens of up to 8 g daily [21]. Curcumin inhibits a plethora of molecular targets such as cyclooxygenase, lipoxygenase [22], matrix metalloproteinase 9 [23] and NF-κB [24]. In addition, curcumin downregulates the expression of adhesion molecules, and consequently reduces leukocyte recruitment during inflammation [25, 26] and contributes to up regulation of transcription factor Nrf2 [27, 28]. Curcumin exhibits poor bioavailability, but this issue has been substantially improved by various formulation strategies such as Meriva® (Indena SpA, Milan, Italy) documented in terms of comparative pharmacokinetics [29] and clinical efficacy as promising analgesic, anti-inflammatory and antioxidant medicine to several conditions [30-32].

Taking into account $O_2^{\bullet-}$ is an important mediator to inflammatory nociceptor sensitization; the analgesic effect and mechanisms of action of curcumin in KO_2 (an $O_2^{\bullet-}$ donor)-induced inflammation were investigated in the present study.

2. Material and Methods

2.1. Animals

Male Swiss mice (25-30 g) from the Universidade Estadual de Londrina, Paraná, Brasil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water and a light/dark cycle of 12:12 h and kept at 21º C. All behavioral testing were performed between 9 am and 5 pm in a temperature-controlled room. Animal care and handling procedures followed the International Association for the Study of Pain (IASP) guidelines. The Ethics Committee of the Universidade Estadual de Londrina approved this study (Process number 71.2012.68). All efforts were made to minimize the number of animals used and their suffering. The experimenters were blinded to the treatments.

2.2. Drugs

The following materials were obtained from the sources indicated: Curcumin (Santa Cruz Biotechnology, USA), KO_2 (Alfa Aesar, MA, USA), and ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonate)] (Sigma-Aldrich, St. Louis, MO, USA), NBT (nitroblue tetrazolium) (Amresco, Solon, OH, USA).

2.3. Experimental procedures

Mice were pretreated subcutaneously (s.c.) with 3, 10, or 30 mg/kg of curcumin or with vehicle (2% DMSO in saline) 1 h before intraplantar (i.pl.) or intraperitoneal (i.p.) injection of 30 μg or 1 mg of KO_2, respectively. All tests were performed injecting the stimulus in the paw except by writhing response. At indicated time points the following parameters were determined: writhing response, paw flinching and time spent licking the paw (Fig. 1), mechanical and thermal hyperalgesia (Fig. 2), myeloperoxidase activity in paw skin samples and peritoneal leukocyte recruitment (Fig. 3), cytokine (TNF-α, IL-1β and IL-10) production and NF-κB activation (Fig. 4), ABTS assay, $O_2^{\bullet-}$ quantification and $gp91^{phox}$ mRNA expression (Fig. 5), and Nrf2 and HO-1 mRNA expression (Fig. 6). The doses of KO_2 and time points for sample collection were determined in standardizing experiments in our laboratory, previous study of our laboratory and described in detail in the following sections [33]. KO_2 was diluted in saline immediately before application. Based on the results from behavioral tests, the dose of curcumin of 10 mg/kg was chosen and used in selected experiments.

2.4. Overt pain-like behavioral tests

Abdominal writhing was induced by i.p. injection of 1 mg of KO_2 [33]. Immediately after stimulus injection, each mouse was placed individually in a large glass cylinder, and the intensity of nociceptive behavior was quantified by counting the total number of writhings occurring between 0 and 20 min after stimulus injection. The writhing response consists of a contraction of the abdominal muscle together with a stretching of hind limbs. The intensity of the writhing response was expressed as the cumulative number of abdominal contortions over 20 min.

The number of paw flinches and the time spent licking the paw were determined between 0 and 30 min after i.pl. injection of 30 µg of KO_2. Each mouse was placed in a large glass cylinder immediately after stimulus injection. The intensity of nociceptive behavior was quantified by counting the total number of paw flinches and the time (seconds) spent licking ipsilateral paw [33].

2.5. Mechanical hyperalgesia test

Mechanical hyperalgesia was evaluated by electronic version of von Frey's filaments as reported previously [34]. In a quiet room, temperature controlled, mice were placed in acrylic cages (12 × 10 × 17 cm) with wire grid floors 15-30 min before the start of testing. The test consisted of evoking a hind paw flexion reflex with a handheld force transducer (electronic anesthesiometer, IITC Life Science, Woodland Hills, CA) adapted with a 0.5 mm^2 polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the plantar hind paw with a gradual increase in pressure. The gradual increase in pressure was manually performed in blinded experiments. The upper limit pressure was 15 g. The end-point was characterized by the removal of the paw followed by clear flinching movements. After paw withdrawal, the intensity of the pressure was automatically recorded, and the final value for the response was obtained by averaging three measurements. The animals were tested before and after treatments. The results are expressed by delta (Δ) withdrawal threshold (in grams) calculated by subtracting the mean measurements 0.5, 1, 3, 5 and 7 h after stimulus from the zero-time mean measurements [33].

2.6 Thermal hyperalgesia test

Heat thermal hyperalgesia was performed using a hot plate at 55°C ± 1ºC. The test was performed at same intervals on 2.5 [33]. The end-point was characterized by the removal of the paw followed by clear paw flinching or licking movements. The upper time was 20

seconds to avoid possible injury. The results are expressed by delta (Δ) withdraw latency (in seconds) calculated by subtracting the mean of zero-time measurements from the mean of measurements obtained 0.5, 1, 3, 5 and 7 h after stimulus [35].

2.7. Myeloperoxidase (MPO) assay

Neutrophil migration to the hind paw skin tissue of mice was evaluated using an MPO kinetic-colorimetric assay as described previously [33]. Samples of paw skin tissue were collected 7 h after stimulus in ice-cold 50 mM K_2HPO_4 buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) and kept at -80°C until use. Samples were homogenized, centrifuged (16,100 *g* × 4 min), and the resulting supernatant was assayed for MPO activity spectrophotometrically at 450 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland), with three readings in 1 min. The MPO activity of samples was compared to a standard curve of neutrophils. Briefly, 10 µL of sample was mixed with 200 µL of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/mL *o*-dianisidine dihydrochloride and 0.015% hydrogen peroxide. The results are presented as MPO activity (number of neutrophils x 10^4 per mg of tissue).

2.8. Leukocyte recruitment in the peritoneal cavity

Leukocyte recruitment to the peritoneal cavity was evaluated 7 h after i.p. injection of KO_2 (30 µg/cavity) [33]. Peritoneal cavities were washed with 200 µL of phosphate-buffered saline (PBS). Total leukocyte counts were performed in a Neubauer chamber after dilution in Turk's solution (2% acetic acid). Differential cell counts were performed using the Fast Panoptic Kit for histological analysis (Laborclin, Pinhais, PR, Brasil), and the values are expressed as the number of cells ($\times 10^6$) per cavity. Total and differential cell counts were performed under a light microscope (400× magnification, Olympus Optical Co., Hamburg, Germany).

2.9. ABTS assay

The ability of samples to resist oxidative damage was determined by their ability to scavenge the ABTS radical. The test was performed as described previously [36]. Samples were collected 3 h after i.pl. stimulus and homogenized immediately in ice-cold KCl buffer (500 µL, 1.15% weight/volume). The homogenates were centrifuged (200 *g* × 10 min × 4 °C), and the supernatants were used in the assay. Diluted ABTS solution (200 µL) was mixed with 10 µL of sample in each well. After 6 min of incubation (25 °C), the absorbance was

measured at 730 nm (Multiskan GO, Thermo Scientific). The results were equated against a standard Trolox curve (0.02 – 20 µmol).

2.10. $O_2^{\bullet-}$ production

The quantitation of $O_2^{\bullet-}$ production in tissue homogenates was performed using the NBT assay as described previously [36]. Skin samples were collected 3 h after the stimulus. Briefly, 50 µL of the homogenate was incubated with 100 µl of NBT (1 mg/mL) in 96-well plates at 37 °C for 1 h. The supernatant was carefully removed and the reduced formazan solubilized by adding 120 µL of 2 M KOH and 140 µL of DMSO. The NBT reduction was measured at 600 nm using a microplate spectrophotometer reader (Multiskan GO, Thermo Scientific). The tissue weight was used for data normalization, thus the results are expressed as NBT reduction (OD/mg of tissue).

2.11. Cytokine measurement

Skin samples were collected 3 hours after the injection of KO_2, and homogenized in 500 µL of ice-cold buffer containing protease inhibitors, and centrifuged (3000 rpm × 10 min × 4 °C), and the supernatants used to measure IL-1β, TNF-α and IL-10 levels by an enzyme-linked immunosorbent assay (ELISA) using eBioscience kits. As a control, the concentrations of these cytokines were determined in animals injected with saline. The results are expressed as picograms (pg) of cytokine/100 mg of tissue.

2.12. NF-κB activity

Skin samples were collected 3 h after stimulus and homogenized in ice-cold lysis buffer (Cell Signaling). The homogenates were centrifuged (200 *g* × 10 min × 4 °C), and the supernatants were used to assess the levels of total and phosphorylated NF-κB p65 subunit by ELISA using PathScan® kits (Cell Signaling) at 450 nm (Multiskan GO Thermo Scientific) according to the manufacturer's directions. The results are expressed as NF-κB activity (total-p65/phospho-p65 ratio).

2.13. Reverse transcription and quantitative polymerase chain reaction (RT-qPCR).

RT-qPCR was performed as previously described [36]. Skin samples were collected 3 h after stimulus and homogenized in trizol reagent, and total RNA was isolated according to manufacturer's directions. The purity of total RNA was measured with a spectrophotometer and the wavelength absorption ratio (260/280 nm) was between 1.8 and 2.0 for all

preparations. Reverse transcription of total RNA to cDNA, and qPCR were carried out using GoTaq® 2-Step RT-qPCR System (Promega) and specific primers (Applied Biosystems®). The relative gene expression was measured using the comparative $2^{-(\Delta\Delta Cq)}$ method. The primers used were: Nrf2, sense: 5'-TCACACGAGATGAGCTTAGGGCAA-3', antisense: 5'-TACAGTTCTGGGCGGCGACTTTAT-3'; $gp91^{phox}$, sense: 5'-AGCTATGAGGTGGTGATGTTAGTGG-3', antisense: 5'-CACAATATTTGTACCAGACAGACTTGAG-3'; Heme Oxygenase-1 (HO-1), sense: 5'-CCCAAAACTGGCCTGTAAAA-3', antisense: 5'-CGTGGTCAGTCAACATGGAT-3'; β-actin, sense: 5'-AGCTGCGTTTTACACCCTTT-3', antisense: 5'-AAGCCATGCCAATGTTGTCT-3'. The expression of β-actin mRNA was used as a reference gene to normalize data.

2.14. Statistical analysis

Results are presented as means ± SEM of measurements made on six mice in each group per experiment and are representative of two separate experiments. Two-way repeated measures analysis of variance (ANOVA) followed by Tukey's *post hoc* was used to compare all groups and doses at all times when responses were measured at different times after the stimulus injection. Differences between responses were evaluated by one-way ANOVA followed by Tukey's *post hoc* for data of single time point. Statistical differences were considered significant when $P < 0.05$.

3. Results

3.1. Curcumin reduces KO_2-induced overt pain-like behaviors

Mice received curcumin (3, 10, 30 mg/kg, s.c.) or vehicle (2% DMSO in saline) treatment 1 h before injection of KO_2 (1 mg, i.p.). The total number of writhing was determined between 0-20 min. Curcumin at the doses of 10 and 30 mg/kg reduced significantly the number of writhings (Fig. 1a), without differences between these two doses. Curcumin at 3 mg/kg was unable to show significantly inhibitory effect (Fig. 1a). Thus, the dose of 10 mg/kg of curcumin was chosen for the next experiments (Fig. 1b, 1c, 2-6). Curcumin also inhibited KO_2-induced paw flinches (Fig. 1b) and time spent licking the paw (Fig. 1c) between 0-30 min.

3.2. Curcumin reduces KO_2-induced mechanical and thermal hyperalgesia

Mice received curcumin (10 mg/kg, s.c.) or vehicle treatment 1 h before KO_2 injection (30 µg/paw) followed by evaluation of mechanical and thermal hyperalgesia. Curcumin decreased mechanical hyperalgesia at all time points compared to vehicle-treated animals (Fig. 2a). Pretreatment with curcumin also reduced thermal hyperalgesia (Fig. 2b).

3.3. Curcumin reduces KO_2-induced myeloperoxidase (MPO) activity

Mice received curcumin (10 mg/kg, s.c.) or vehicle treatment 1 h before KO_2 injection (30 µg/paw). KO_2 induced a significant increase of MPO activity in the paw skin 7 h after its injection. Curcumin pretreatment reduced KO_2-induced increase of MPO activity (Fig. 3a). Furthermore, the leukocyte recruitment in the peritoneal cavity was evaluated 7 h after KO_2 i.p. injection (30 µg/cavity). KO_2 increased the total number of leukocytes (Fig 3b), mononuclear cells (Fig 3c) and neutrophils (Fig 3d) whilst curcumin pretreatment reduced KO_2-induced recruitment of these cells.

3.4. Effect of curcumin on KO_2-induced cytokine production and NF-κB activation

KO_2 induced significant increase of TNF-α (Fig. 4a) and IL-1β (Fig. 4b) levels in the paw skin at 3 h after its injection. Curcumin reduced KO_2-induced production of the hyperalgesic cytokines TNF-α (Fig. 4a) and IL-1β (Fig. 4b). On the other hand, KO_2 induces a significant increase in IL-10 levels, an anti-hyperalgesic cytokine. Curcumin further increased the KO_2-induced IL-10 production in the paw skin (Fig. 4c). The total NF-κB p65 concentration remained constant, and the phosphorylated NF-κB p65 increased upon KO_2

stimulus resulting in decreased total NF-κB p65/phosphorylated NF-κB p65 ratio. In agreement with the curcumin inhibition of pro-hyperalgesic cytokine production, it also inhibited KO_2-induced NF-κB activation as observed by an increase of the total NF-κB p65/phosphorylated NF-κB p65 ratio (Fig. 4d).

3.5. Curcumin reduces KO_2-induced oxidative stress

$O_2^{\bullet-}$ donor depleted antioxidant capacity and increased $O_2^{\bullet-}$ production in the plantar tissue (Fig. 5A and 5B). On the other hand, curcumin impaired these deleterious effects, restoring the antioxidant capacity and inhibiting the increases in $O_2^{\bullet-}$ production (Fig. 5a and 5b). Corroborating these results, we demonstrated pretreatment with curcumin reduced the mRNA expression of $gp91^{phox}$, NADPH oxidase subunit (Fig. 5C).

3.6. Curcumin increases mRNA expression for Nrf2 and heme oxygenase-1 (HO-1)

KO_2 reduced while did not affect the mRNA expression for Nrf2 (Fig. 6a) and HO-1 (Fig. 6b) at 3 h, respectively. In turn, curcumin treatment enhanced Nrf2 mRNA expression (Fig. 6a) by 1.5 fold and HO-1 mRNA expression by 9.5 fold (Fig. 6b).

4. Discussion

Pain is one of the most common clinical signs of inflammation. Therapeutic approaches to this condition should target the mediators involved in its development, which include neuropeptides, eicosanoids, cytokines and reactive oxygen species (ROS). These mediators are closely related since cytokines including TNF-α and IL-1β induce the production of eicosanoids and $O_2^{\bullet-}$ while the latter induces the production of cytokines by activating the NF-κB pathway [9]. $O_2^{\bullet-}$ is important to the development of thermal hyperalgesia associated with acute and chronic inflammation [10, 37]; and promotes mechanical hyperalgesia [11]. Accordingly, treatment with Tempol (SOD mimetic) inhibited the hyperalgesia and inflammation in carrageenan-induced pain [38]. Hence, we demonstrated that KO_2 is a useful model to trigger a great variety of $O_2^{\bullet-}$ dependent nociceptive responses such as abdominal writhings, paw flinches and licking, and mechanical and thermal hyperalgesia [33]. Altogether, strategies that control $O_2^{\bullet-}$ overproduction and/or its deleterious effects, including oxidative stress and inflammation, seem promising therapeutic approaches for pain relief.

The mechanism of action of curcumin depends on a synergism involving weak targeting of varied proteins within related signaling networks [39]. In inflammatory pain context, curcumin inhibited overt pain-like behavior in formalin test [40], which has two components. The early phase (0-5 min) dependent on activation of nociceptors through TRPA1 receptors, which are important receptors in inflammatory pain and their increased activity, occurs in $O_2^{\bullet-}$ production dependent manner [41]. The late phase (15-30 min) depends on inflammatory cytokines production [42, 43]. In the present study, we observed that curcumin inhibited overt pain-like behaviors, and this may be due to the initial limitation of direct action $O_2^{\bullet-}$ on nociceptors and capacity of desensitizing TRPA1 receptors [44]. Importantly, curcumin inhibited both paw flinching and licking induced by $O_2^{\bullet-}$. At least in the formalin test, paw flinching is considered a peripheral and spinal response and paw licking presents the structural mechanisms of flinching plus supraspinal nociceptive structures [45, 46]. Therefore, it is likely $O_2^{\bullet-}$ activates peripheral, spinal and supraspinal responses, which are amenable to curcumin.

In addition to the direct effect on nociceptors, $O_2^{\bullet-}$ activates signaling pathways that culminate in the production of inflammatory mediators by resident cells promoting neutrophil recruitment to local of stimulus. In fact, $O_2^{\bullet-}$ contributes to neutrophil recruitment in inflammatory conditions [47]. Recruited neutrophils produce $O_2^{\bullet-}$, IL-1β, TNF-α and

prostaglandin E_2, and thus contribute to the maintenance of inflammatory hyperalgesia [7]. The MPO is an enzyme found in azurophilic granules of neutrophils and macrophages, and therefore, is an indirect marker of these cells [48]. MPO produces the microbicidal molecule hypochlorite, a strong oxidant, upon reaction with H_2O_2 and Cl^-. Curcumin inhibits the expression of adhesion molecules [25, 26], resulting in the reduction of cell counts in the inflammatory foci. In agreement, we observed that curcumin inhibited MPO activity in the paw skin, and total leukocytes, mononuclear cells and neutrophils in the peritoneal cavity.

The transcription factor NF-κB is a master regulator of both innate and adaptive immune responses. The phosphorylation of IκB (NF-κB inhibitor) allows NF-κB dimer to translocate from the cytoplasm to the nucleus where it regulates the transcription of a large number of genes, including pro-inflammatory cytokines and chemokines [49]. M40403 (SOD mimetic) decreased LPS-induced activation of NF-κB, and this effect was associated with diminished degradation of cytoplasmic IκB [50], suggesting that $O_2^{\bullet-}$ participates in redox-regulated NF-κB activation. Curcumin attenuates the development of asthma by inhibiting NF-κB activation [51]. Corroborating these data, we demonstrated that curcumin decreased $O_2^{\bullet-}$-induced NF-κB activation *in vivo* as observed by prevention of KO_2-induced decrease of the total NF-κB p65/phosphorylated NF-κB p65 ratio. Consequently, KO_2-induced IL-1β and TNF-α production was reduced by curcumin. These pro-inflammatory cytokines undergo rapid release and have a pivotal role in the development of hyperalgesia due the recruitment and activation of immune cells. $O_2^{\bullet-}$ also modulates the activity of TRPV1, an important component of thermal hyperalgesia [52] and curcumin has shown the capacity to antagonize TRPV1 [53]. In agreement with that, curcumin decreased KO_2-induced mechanical and thermal hyperalgesia.

NADPH oxidases are a family of enzyme complexes present in both phagocytic and non-phagocytic cells, which catalyze the transfer of electrons from NADPH to molecular oxygen, thereby generating $O_2^{\bullet-}$ and H_2O_2. $O_2^{\bullet-}$ contributes to nociceptor sensitization and as well as other ROS act as signaling molecule to trigger IL-1β production through activation of inflammasomes [54]. Therefore, modulating oxidative stress is a conceivable approach to diminish pain and inflammation [55, 56]. Curcumin presents prominent antioxidant activity and contributes to increasing GSH, catalase and SOD levels [57]. In fact, curcumin inhibited thermal hyperalgesia by controlling oxidative stress and normalizing peripheral and spinal antioxidant enzyme system, which contributed to reducing pro-inflammatory cytokines production in complete Freund's adjuvant (CFA) inflammation [58]. In addition, curcumin

scavenges $DPPH^{\bullet}$ and $DMPD^{\bullet+}$ radicals and improve ferric reducing antioxidant power (FRAP) *in vitro* [59]. The present data show that curcumin improves the antioxidant capacity *in vivo* by inhibiting $O_2^{\bullet-}$ production and maintaining the antioxidant defenses observed by re-establishment of ABTS radical scavenger activity.

Nrf2 is a potent transcriptional factor constantly ubiquitinated through Keap1 and degraded in the proteasome under regular conditions. In response to oxidative and electrophilic stress, Keap1 is inactivated, and Nrf2 translocates to nucleus and binds at a promoter sequence known as the antioxidant-responsive element (ARE) that is found in many cytoprotective genes. In general, target genes of Nrf2 are involved in glutathione synthesis and elimination of ROS, providing a protective function against oxidative stress [60, 61]. Evidence demonstrates that curcumin increases Nrf2 activity [27, 28], which lines up well with a 1.5-fold increase in Nrf2 mRNA expression found in our study. We found $O_2^{\bullet-}$ decreased mRNA expression of Nrf2, which might contribute to the impairment of an efficient endogenous antioxidant response. In line with this hypothesis, we found that curcumin potentiated the $O_2^{\bullet-}$-induced increased mRNA expression of heme oxygenase-1 (HO-1), a target of Nrf2 transcriptional activity [62]. The crosstalk mechanism between NF-κB and Nrf2 is puzzling and is still under elucidation. After $O_2^{\bullet-}$ producing stimulus such as PMA, the p65 subunit competes with Nrf2 for the coactivator CREB-binding protein. As a consequence, there is hypoacetylation blocking chromatin condensation, which suppresses Nrf2/ARE gene expression and dampens Nrf2 pathway activation [63]. This is a possible explanation for the KO_2-induced decrease of Nrf2 mRNA expression in the present study.

HO-1 is a ubiquitous inducible cellular stress enzyme and one of the target genes of Nrf2. The HO-1 major metabolic function is to catalyze the reaction of heme that leads to the formation of carbon monoxide, free iron and biliverdin [62]. This reaction allows biliverdin conversion into the strong antioxidant bilirubin, and ROS participate in the reaction that converts back bilirubin into biliverdin [64]. This cycle grants the neutralization of ROS and provides an antioxidant function to HO-1 [64]. Activation of cGMP/ PKG/ ATP-sensitive potassium channels lead to antinociceptive state [55, 65]. Co-treatment with an HO-1 inducer increased the antinociceptive effects of opioids and cannabinoids through activation of cGMP/ PKG/ ATP-sensitive potassium channels pathway in a model of CFA-induced pain [66]. On the other hand, the administration of HO-1 inhibitor increases mechanical hyperalgesia [67]. Several antioxidant molecules can increase HO-1 expression, and curcumin is one of them [27, 28, 68], a feature that we verified in our model with a 9.5-fold increase of expression.

Interleukin-10 (IL-10) is an anti-inflammatory and anti-hyperalgesic cytokine [2]. IL-10 deficiency enhances acute exercise-induced muscle hyperalgesia concomitantly with exacerbated oxidative stress [69]. On the other hand, increased nuclear levels of Nrf2 were accompanied by high levels of IL-10 in the plasma and the renal proximal tubules of treadmill-exercised rats [70]. In another model, hypochlorous acid-induced oxidative stress activated Nrf2 pathway and enhanced mRNA expression of IL-10 [71]. IL-10 was necessary to increase the expression of HO-1 in LPS-induced endotoxemia [72], suggesting the existence of an IL-10/HO-1 axis [72], which also may explain the prominent increase of HO-1 mRNA expression found in our study.

In conclusion, we found that curcumin reduced nociceptive responses induced by KO_2-induced $O_2^{\bullet-}$ through mechanisms including NF-κB inhibition and induction of Nrf2 mRNA expression, which contribute to inhibit *de novo* $O_2^{\bullet-}$ generation, IL-1β and TNF-α production, and enhance the levels of HO-1 and IL-10. This study also contributes to support that $O_2^{\bullet-}$ is a fundamental component of inflammatory hyperalgesia, further advances in the knowledge of the curcumin mechanisms of action, and supports the use of curcumin as an analgesic.

Conflict of interest

The authors declare no conflict of interest.

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Figures

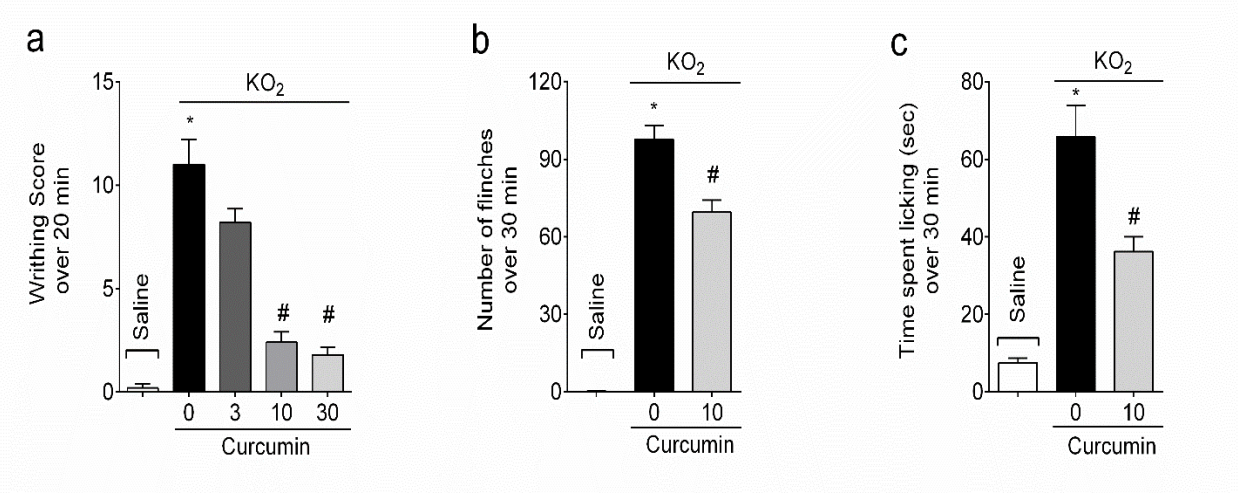


Fig. 1 Curcumin inhibits KO_2-induced overt pain-like behaviors

Mice were treated at doses 3, 10 and 30 mg/kg (s.c.) 1 h before i.pl. injection of 30 µg KO_2 or i.p. injection of 1 mg KO_2. Zero mg/kg of curcumin stands for vehicle group. Total number of writhing (a) was evaluated 0-20 min after i.p. injection of KO_2. The number of flinches (b) and time spent licking (c) the paw were evaluated 0- 30 min after i.pl. injection of KO_2. Results are expressed as mean ± SEM (n=6 per group per experiment, representative of two separate experiments). *p<0.05 vs. saline group #p<0.05 vs. KO_2 group. (one-way ANOVA followed Tukey's *post hoc*).

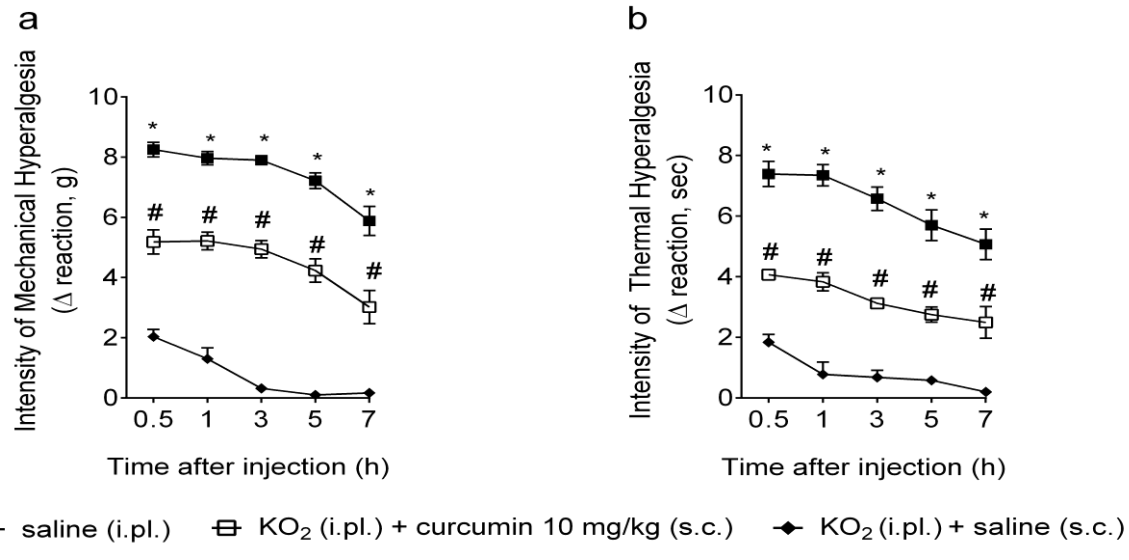


Fig. 2 Curcumin inhibits KO_2-induced mechanical and thermal hyperalgesia

Mice were treated with curcumin 10 mg/kg (s.c.) 1 h before i.pl. injection of 30 µg KO_2. The test was performed at 0.5, 1, 3, 5 and 7 h after stimulus for both mechanical (a) and thermal (b) hyperalgesia. Results are expressed as mean ± SEM (n=6 per group per experiment, representative of two separate experiments). *p<0.05 vs. saline group #p<0.05 vs. KO_2 group (two-way repeated measures ANOVA followed Tukey's *post hoc*).

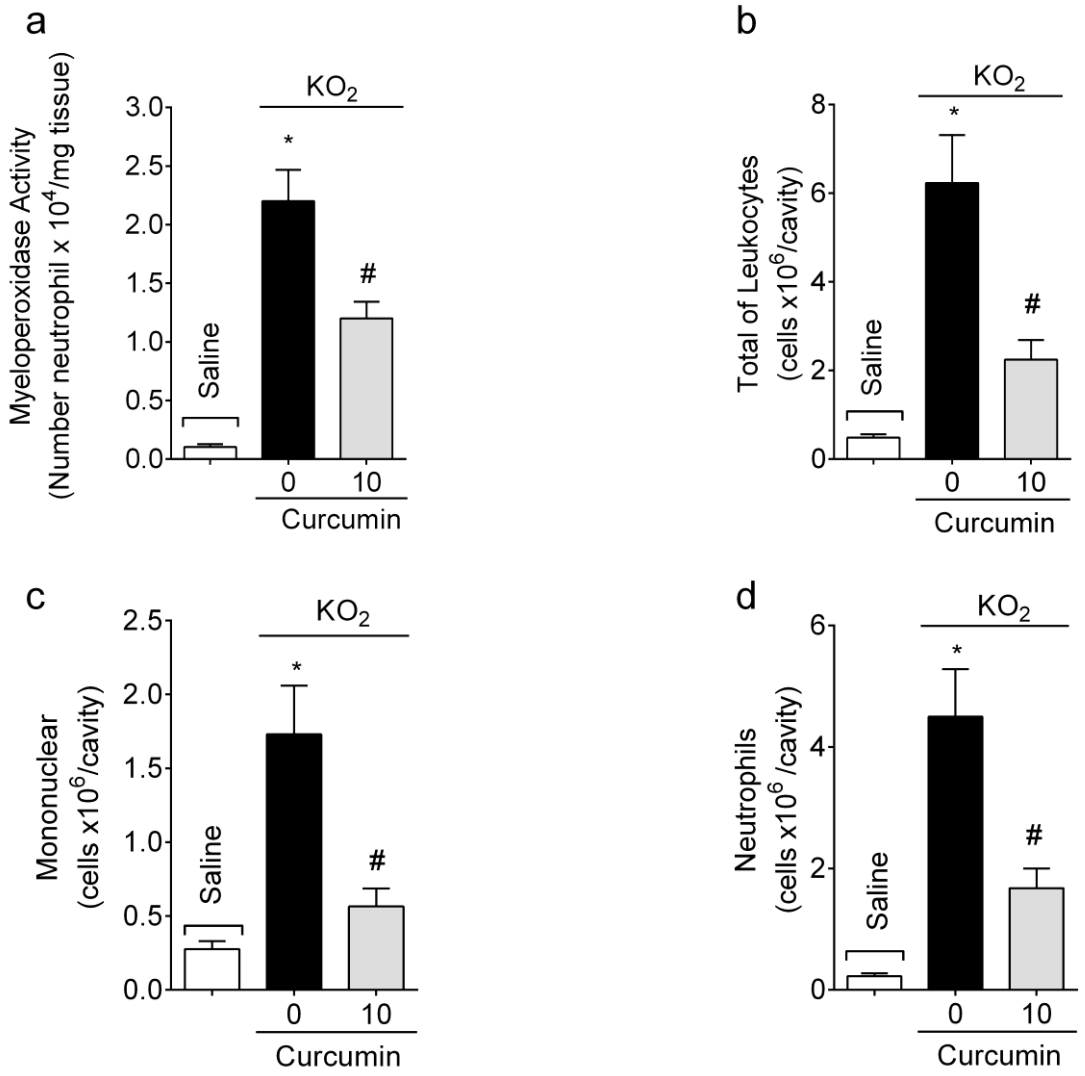


Fig. 3 Curcumin inhibits KO_2-induced leukocyte recruitment and myeloperoxidase activity
Mice were pretreated with curcumin 10 mg/kg (s.c.) 1 h before i.pl. or i.p. injection of 30 µg KO_2. Zero mg/kg of curcumin stands for vehicle group. Samples were collected 7 h after KO_2 injection. Myeloperoxidase activity was determined in paw skin samples (a). The total number of leukocyte (b), mononuclear cells (c) and neutrophils (d) were determined in peritoneal washes. Results are expressed as mean ± SEM (n=6 per group per experiment, representative of two separate experiments). *p<0.05 vs. saline group #p<0.05 vs. KO_2 group (one-way ANOVA followed Tukey's *post hoc*).

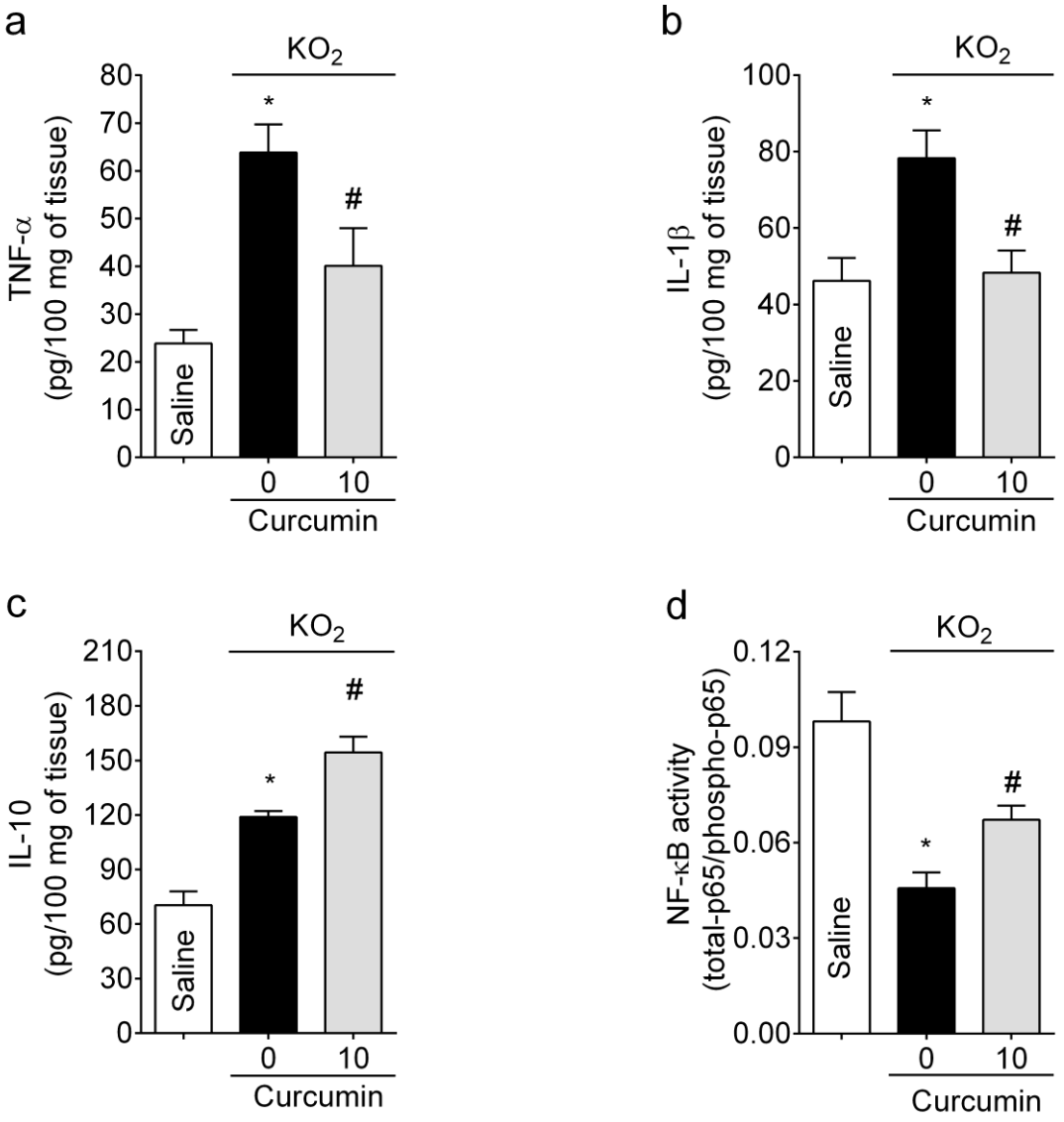
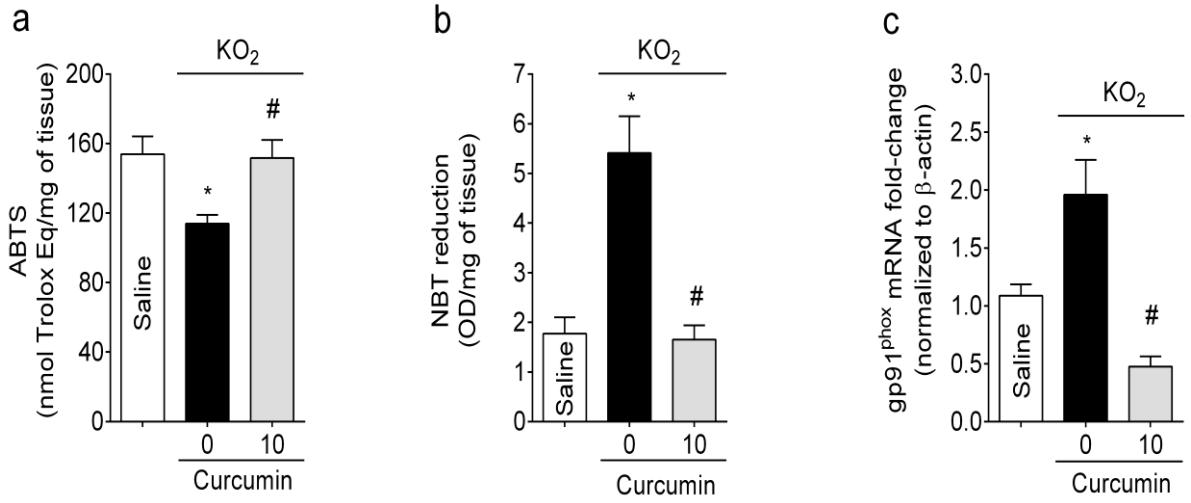
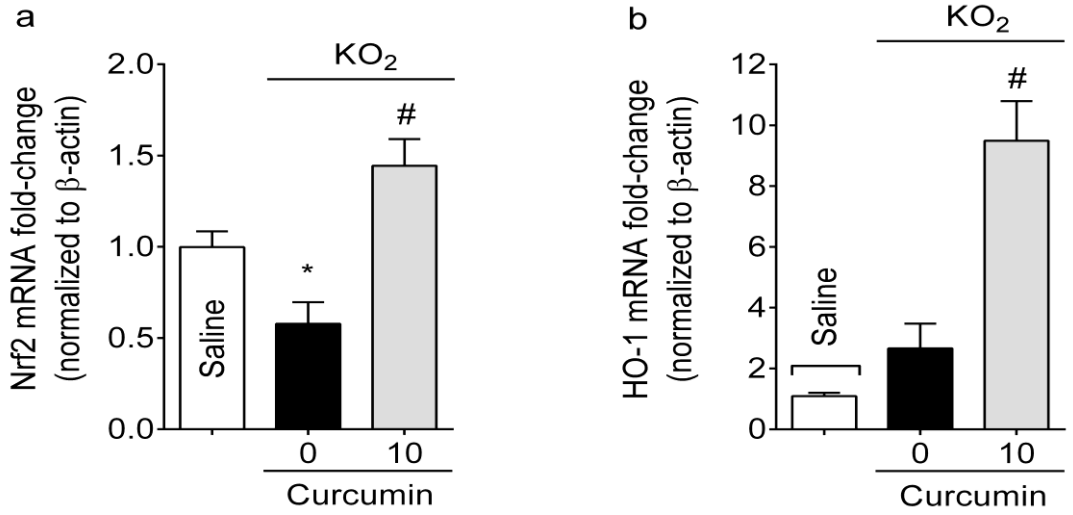


Fig. 4 Curcumin inhibits KO_2-induced cytokines production and NF-κB activation
Mice were treated with curcumin 10 mg/kg (s.c.) 1 h before i.pl. injection of 30 µg KO_2. Zero mg/kg of curcumin stands for vehicle group. Samples were collected 3 h after stimulus injection to determine TNF-α (a), IL-1β (b), IL-10 (c) levels and NF-κB activity (d). Results are expressed as mean ± SEM (n=6 per group per experiment, representative of two separate experiments). *$p<0.05$ vs. saline group #$p<0.05$ vs. KO_2 group (one-way ANOVA followed Tukey's *post hoc*).



3658

3659 **Fig. 5 Curcumin inhibits KO_2-induced oxidative stress**
3660 Mice were treated with curcumin 10 mg/kg (s.c.) 1 h before i.pl. injection of 30 µg KO_2. Zero mg/kg
3661 of curcumin stands for vehicle group. Sample were collected 3 h after stimulus to determine total
3662 antioxidant capacity (ABTS assay) (a), $O_2^{\bullet-}$ production (NBT assay) (b) and $gp91^{phox}$ mRNA
3663 expression (c). β-actin was used as a reference gene to normalize data mRNA expression. Results are
3664 expressed as mean ± SEM (n=6 per group per experiment, representative of two separate
3665 experiments). *p<0.05 vs. saline group #p<0.05 vs. KO_2 group (one-way ANOVA followed Tukey's
3666 *post hoc*).
3667



3668

3669 **Fig. 6 Curcumin increases KO_2-induced Nrf2 and HO-1 mRNA expression**
3670 Mice were treated with curcumin 10 mg/kg (s.c.) 1 h before i.pl. injection of 30 µg KO_2. Zero mg/kg
3671 of curcumin stands for vehicle group. Sample were collected 3 h after stimulus to determine Nrf2 (a)
3672 and HO-1 (b) mRNA expression. β-actin was used as a reference gene to normalize data mRNA
3673 expression. Results are expressed as mean ± SEM (n=6 per group per experiment, representative of
3674 two separate experiments). *p<0.05 vs. saline group #p<0.05 vs. KO_2 group (ANOVA followed
3675 Tukey's *post hoc*).
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6 CONCLUSÃO

O modelo de artrite induzido por dióxido de titânio e o modelo agudo de inflamação e dor induzido pelo ânion superóxido foram padronizados pelo nosso laboratório e se mostram como alternativas viáveis para os modelos pré-existestes de dor e inflamação. A curcumina, é uma molécula com potencial analgésico promissor, tendo em vista que atua em múltiplas vias de sinalização pussuindo mais de 100 diferentes alvos moleculares, entre eles: inibição do NF-κB e citocinas *downstream*, inibição da sinalização entre RANK-RANKL, aumento da atividade do Nrf2 e HO-1, inibição do recrutamento de neutrófilos etc; além de possuir baixa toxicidade. Sua efiácia é confirmada com o desenvolvimento de diversas moléculas análogas e principalmente de um medicamento tendo a curcumina como princípio ativo (Meriva®), o qual já utilizado em diversos ensaios clínicos com pacientes com câncer de mama, osteoartrite, psoríase, dor muscular de início tardio (DOMS) entre outras.