Universidade Estadual de Londrina

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EFEITO ANALGÉSICO E ANTI-INFLAMATÓRIO DA NARINGENINA EM MODELO DE ARTRITE INDUZIDO POR DIÓXIDO DE TITÂNIO E INFLAMAÇÃO POR ÂNION SUPERÓXIDO

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Dissertação de mestrado apresesentada 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, 21 de fevereiro de 2017.

"O sucesso é ir de fracasso em fracasso sem perder entusiasmo."

(Winston Churchill)

MANCHOPE, Marília Fernandes. **Efeito analgésico e anti-inflamatório da naringenina em modelo de artrite induzido por dióxido de titânio e inflamação por ânion superóxido**. 2017. 95 f. Dissertação (Mestrado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2017.

RESUMO

Flavonoides são compostos polifenólicos encontrados amplamente na dieta humana. A naringenina é um flavonoide pertencente a classe das flavanonas, encontrada em frutas cítricas como toranja, laranja e limão. Ademais, a naringenina é uma molécula pleiotropica com atividade anti-inflamatória. Portanto, nosso objetivo foi avaliar o efeito da naringenina em modelo crônico de artrite induzido pelo dióxido de titânio (TiO_2) e modelo agudo de dor inflamatória induzido pelo superóxido de potássio (KO_2) – um doador de ânion superóxido. A hiperalgesia mecânica, hiperalgesia térmica e edema foram avaliados por um analgesímetro digital, placa quente e paquímetro. Leucócitos totais foram contados em câmara de Neubauer e subsequentemente foram feitas lâminas para a contagem diferencial de polimorfonuclear e mononuclear. Ensaios colorimétricos foram usados para avaliar a atividade da mieloperoxidase (MPO), estresse oxidativo (GSH, capacidade antioxidante total [FRAP], lipoperoxidação e produção de ânion superóxido) e toxicidade. Produção de citocinas (TNFα, IL-10) e ativação do NFκB foram avaliados por ELISA. A expressão de RNAm foi determinada por RT-qPCR para os genes gp91^{phox}, TNFα, pro-IL-1β, IL-33, COX-2, preproET-1, Nrf-2, HO-1, RANKL, RANK e OPG. A toxicidade do tratamento com naringenina em induzir dano gástrico, hepático e renal foi avaliado pela atividade da MPO no estômago, níveis plasmáticos de aspartato aminotransferase (AST), alanina aminotransferase (ALT), ureia e creatinina. Em relação ao modelo crônico, no primeiro dia após o estímulo intra-articular com TiO_2 foi realizado uma curva dose-resposta de naringenina e os animais foram tratados diariamente com naringenina (16.7, 50 e 150 mg/kg, via oral [v.o.]) e a hiperalgesia mecânica e edema foram mensurados após 1, 3, 5, 7, e 24 h após o tratamento e seguidamente de dois em dois dias até o 30º dia. Verificamos que a dose de 50 mg/kg inibiu a hiperalgesia mecânica, edema, migração de leucócitos totais – polimorfonuclear e mononuclear – estresse oxidativo (expressão de RNAm gp91^{phox}, produção de ânion superóxido e lipoperoxidação), expressão de RNAm de citocinas pró-inflamatórias (TNFα, pro-IL-1β e IL-33), ativação do NFκB e modulou o sistema RANKL/RANK/OPG. Posteriormente, avaliamos a ação da naringenina (16.7, 50 e 150 mg/kg, v.o.) no modelo agudo de dor induzido pelo estímulo intraplantar de KO_2. A dose de 50 mg/kg de naringenina inibiu o número de contorções abdominais avaliado por 20 min, número de sacudidas da pata e o tempo gasto lambendo a pata em 30 min. A hiperalgesia mecânica e hiperalgesia térmica foram avaliados 0,5, 1, 3, 5, e 7 h após o estímulo intraplantar com KO_2. A dose de 50 mg/kg de naringenina inibiu a hiperalgesia mecânica e hiperalgesia térmica dependente da ativação da via de sinalização NO–GMPc–PKG–canais K_{ATP}. Ademais essa dose também inibiu a MPO na pata após 7 h do estímulo com KO_2, estresse oxidativo (GSH, FRAP, lipoperoxidação, produção de ânion superóxido e expressão de RNAm gp91^{phox}), produção de citocinas (TNFα, IL-10 e expressão de RNAm IL-33), expressão de RNAm para COX-2, preproET-1, e ativou a via Nrf2/HO-1 após 3 h do estímulo com KO_2. Com base no exposto acima, naringenina

apresenta efeito analgésico e anti-inflamatório em modelos de artrite crônica e dor inflamatória aguda. Com o estudo foi possível contribuir para a compreensão dos mecanismos de ação que permeiam atividade dessa molécula e reforça a ação pleiotropica da naringenina.

Palavras-chave: Artrite. Ânion superóxido. Dor. Flavonoides. Naringenina.

MANCHOPE, Marília Fernandes. **Naringenin inhibitis pain and inflammation in titanium dioxide-induced arthritis and superoxide anion-indcued inflammation.**. 2017. 95 p. Dissertation (Master's degree Experimental Phatology) – Universidade Estadual de Londrina, Londrina, 2017.

ABSTRACT

Flavonoids are polyphenolic compound found widely in human diet. Naringenin is a flavonoid which belongs to flavanones class found in citric fruits, including lemon, orange, tangerine and grapefruit. Naringenin is a pleotropic molecule with anti-inflammatory activity. Therefore, we aim to investigate the effect of naringenin in two models: the first model was chronic arthritis – titanium dioxide-induced – and the second was acute inflammatory pain – potassium superoxide-induced – in mice. The mechanical hyperalgesia, thermal hyperalgesia, and edema were assessed by an electronic anesthesiometer, hot plate, and caliper. Total leukocytes were counted in Neubauer chamber, and after differential count in polymorphonuclear and mononuclear was performed in slides. Colorimetric assays were performed to evaluated myeloperoxidase (MPO) activity, oxidative stress (GSH, total antioxidant capacity [FRAP], lipid peroxidation, superoxide anion production), and toxicity. Cytokines production (TNFα and IL-10), and NFκB activation were evaluated by ELISA. The expression of gp91^{phox}, COX-2, preproET-1, Nrf-2, HO-1, IL-33, TNFα, pro-IL-1β, RANKL, RANK e OPG mRNA were determined by RT-qPCR. The naringenin-induced toxicity in gastric, hepatic and kidney tissue were evaluated by MPO activity in stomach and plasma concentration of aminotransferase (AST), alanine aminotransferase (ALT), urea and creatinine. About the chronic model, mice were treated daily during 30 days with curve dose-response of naringenin (16.7, 50 or 150 mg/kg, per oral [p.o.]) after 24h after intra-articular injection of TiO_2 and mechanical hyperalgesia and knee edema were evaluated in the first day 1, 3, 5, 7, e 24 h after naringenin treatment and day every other day up 30th day. The dose of 50 mg/kg of naringenin inhibited mechanical hyperalgesia, knee edema, total leukocytes recruitment – polymorphonuclear and mononuclear – oxidative stress (gp91^{phox} mRNA expression, superoxide anion production, and lipid peroxidation), pro-inflammatory cytokines (TNFα, pro-IL-1β e IL-33) mRNA expression, NFκB activation, and modulated RANKL/RANK/OPG system 30 days after TiO_2 injection. In a second moment, we evaluated the effect of naringenin (16.7, 50 or 150 mg/kg, p.o.) treatment in KO_2-induced acute inflammatory pain. The dose of 50 mg/kg of naringenin inhibited number of abdominal writhing in 20 min., and number of paw flinches and time spent licking the paw in 30 min. Mechanical hyperalgesia and thermal hyperalgesia were evaluated 0.5, 1, 3, 5, and 7h after KO_2 intraplantar injection. The dose of 50 mg/kg of naringenin inhibited mechanical hyperalgesia and thermal hyperalgesia depends on naringenin activated NO–GMPc–PKG–K_{ATP} channel signaling pathway. Moreover, the dose of 50 mg/kg of naringenin also inhibited MPO activity 7h after KO_2 injection, and oxidative stress (GSH, FRAP, lipid peroxidation, superoxide anion production), cytokines production (TNFα, IL-10 and IL-33 expression mRNA), COX-2 and preproET-1 mRNA expression, and activated Nrf2/HO-1 pathway 3h after KO_2 injection. Take this into account, naringenin presents analgesic and anti-inflammatory effects in models of chronic arthritis and

acute inflammatory pain. These data contribute to understanding of mechanism of action of this molecule, reinforcing the pleotropic action of naringenin.

Key words: Arthritis. Superoxide anion. Pain. Flavonoids. Naringenin.

LISTA DE ABREVIATURAS E SIGLAS

a.C.	Antes de cristo
ARE	Região promotora de elementos de resposta antioxidante
CCl_4	Tetracloreto de carbono
COX-2	Ciclo-oxiganese 2
d.C.	Depois de cristo
DAMPs	Molécula padrão associada ao dano
EGF	Fator de crescimento epidermal
ERK	Quinase regulada por sinal extracelular
EROs	Espécies reativas do oxigênio
ET-1	Endotelina-1
fMLP	N-formilmetionina-leucil-fenilalanina
GMPc	Monofosfato cíclico de guanosina
GSH	Glutationa reduzida
GSSG	Glutationa oxidada
HO-1	Heme-oxigenase 1
ICAM-1	Molécula de adesão intracelular-1
IL-1β	Interleucina-1β
IL-6	Interleucina-6
IL-8	Interleucina-8
JNK	Quinase c-Jun N-terminal
K_{ATP}	Canal de potássio sensível ao ATP
Keap-1	Proteína 1 associada a ECH similar a Kelch KO_2
KO_2	Superóxido de potássio
LPS	Lipopolissacarídeo
LTB_4	Leucotrieno B_4
MAPK	Proteína quinase ativada por mitógenos
NADPH	Fosfato de dinucleotídeo de nicotinamida e adenina
NFκB	Fator de transcrição nuclear κB
NLR	Receptor semelhante a NOD
NO	Óxido nítrico
Nrf2	Fator 2 relacionado ao fator de transcrição nuclear eritróide 2
PAMPs	Molécula padrão associada à membrana de patógenos

PGE_2	Prostaglandina E_2
PGI_2	Prostaciclina
PI_3K	Fosfatidilinositol 3-quinase
PKA	Proteína quinase A
PKC	Proteína quinase C
PKG	Proteína quinase G
PRRs	Receptor de reconhecimento do padrão
RANK	Receptor ativador do NFκB
RANKL	Ligante receptor ativador do NFκB
SOD	Superóxido dismutase
TiO_2	Dióxido de titânio
TLR	Receptor semelhante a toll
TNFα	Fator de necrose tumoral α
TNFR1	Receptor 1 do fator de necrose tumoral

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1. INTRODUÇÃO

1.1. INFLAMAÇÃO

Os sinais da inflamação já foram descritos a muito tempo. De fato, hieróglifos realizados pela civilização suméria datados de 2700 a.C. representam os sinais do processo inflamatório – vermelhidão, calor, edema e dor. Nessa mesma linha, os sinais do processo inflamatório também foram futuramente descritos por Celcius no primeiro século d.C. (ROCHA E SILVA, 1994). O processo inflamatório é uma resposta adaptativa do organismo desencadeada por estímulo e/ou condição nociva, como uma infecção e/ou injuria tecidual (MEDZHITOV, 2008). Nesse sentido, o processo inflamatório é essencial para a sobrevivência do organismo e tem como objetivo orquestrar a defesa do indivíduo frente ao estímulo e/ou condição nociva e seguidamente reestabelece a homeostase. Diante disso, é possivel sub-dividir o processo inflamatório em duas fases: iniciação e resolução (MADERNA; GODSON, 2009). Por outro lado, quando o processo inflamatório é exacerbado ou persistente pode ocasionar destruição tecidual, fibrose e posteriormente levar ao 5º sinal da inflamação – perda de função do tecido e/ou órgão acometido (MADERNA; GODSON, 2009).

A molécula padrão associada à membrana de patógenos (PAMPs) e molécula padrão associada ao dano (DAMPs) são reconhecidos pelo receptor de reconhecimento do padrão (PRRs) – como receptor semelhante a toll (TLR), receptor semelhante a NOD (NLR) – presentes nas células residentes, como macrófagos e mastócitos, dando início ao processo inflamatório (MEDZHITOV, 2008; TAKEUCHI; AKIRA, 2010). A ativação de PPRs promove ativação do fator de transcrição nuclear κB (NFκB) e consequente produção de citocinas pró-inflamatórias, como fator de necrose tumoral α (TNFα), interleucina (IL)-1β e IL-6 (MEDZHITOV, 2008; TAKEUCHI; AKIRA, 2010). Em sequência, essas citocinas descritas acima induzem a produção de outros mediadores pró-inflamatórios como aminas vasoativas, quimiocinas, proteína do complemento C5a, leucotrieno B_4 (LTB_4), prostaglandina E_2 (PGE_2) e endotelinas, os quais colaboram para a amplificação do processo inflamatório (CUNHA; CACINI; FERREIRA, 1986; FERREIRA; ROMITELLI; DE NUCCI, 1989; GUERRERO et al., 2008; PARADA et al., 2001; RIBEIRO et al., 1997; SPECTOR; WILLOUGHBY, 1964; TING et al., 2008;

VERRI et al., 2006b). As aminas vasoativas, como serotonia e histamina, e lipídeos como PGE_2 e prostaciclina (PGI_2) são responsáveis pelo aumento do calibre do leito vascular e aumento no fluxo sanguíneo local. E depois, ocorre aumento na permeabilidade vascular e extravasamento de líquido rico em proteínas que contribui para formação do edema. Em consequência ocorre a lentificação do fluxo sanguíneo (estase). A evolução do processo de estase altera as condições hemodinâmicas e promove a marginalização leucocitária, em especial neutrófilos (ABBAS; KUMAR; FAUSTO, 2016; BOTTARO et al., 1986; SPECTOR; WILLOUGHBY, 1964; THEOHARIDES et al., 2007). Em conjunto os mediadores citados acima também ativam o endotélio e promovem a quimitaxia de neutrófilos, para o foco inflamatório. Os neutrófilos sofrem quimiotaxia precisamente para o foco inflamatório por intermédio da participação hierárquica de diferentes moléculas quimioatraentes (FOXMAN; CAMPBELL; BUTCHER, 1997). Por exemplo, no modelo de inflamação estéril hepática induzido por injúria térmica focal os neutrófilos interagem com o endotélio ativado por meio de selectinas e integrinas e são guiados por intermédio de um gradiente vascular de CXCL2 (MCDONALD et al., 2010). É relevante que os neutrófilos possuem diferentes mecanismos de adesão ao endotélio que variam de acordo com o estímulo, na inflamação estéril a molécula de adesão intracelular-1 (ICAM-1) liga-se a integrina mac-1, por outro lado na inflamação infecciosa liga-se ao CD44 (MCDONALD et al., 2010). Após a adesão firme ao endotélio os neutrófilos transmigram pelo endotélio para o tecido onde são guiados por moléculas quimioatraentes finais como proteína do complemento C5a e N-formilmetionina-leucil-fenilalanina (fMLP) até o foco inflamatório (FOXMAN; CAMPBELL; BUTCHER, 1997; MCDONALD et al., 2010).

Outro conjunto de mediadores importantes no processo inflamatório são as espécies reativas do oxigênio (EROs). Elas podem ser geradas a partir da mitocôndria e de sistemas enzimáticos (HOLMSTRÖM; FINKEL, 2014), como da fosfato de dinucleotídeo de nicotinamida e adenina (NADPH) oxidase (BABIOR, 2004). As EROs são consideradas como os anti-heróis dentro da literatura científica. Apesar de serem reconhecidas como vilãs por causarem dano tecidual as EROs são cruciais para ativação de determinadas vias de sinalização (HOLMSTRÖM; FINKEL, 2014). Por exemplo, o fator de crescimento fator de crescimento epidermal (EGF) induz a fosforilação de resíduos de tirosina a partir da ação da enzima tirosina fosfatase, a ativação desta enzima ocorre pela ação das

EROs em resíduos específicos de cisteína. Posteriomente, a enzima tirosina fosfatase é inativada por produtos de oxidação, como glutationa oxidada (GSSH) (forma oxidada da glutationa reduzida [GSH]), lipídos peroxidados, entre outros (TRUONG; CARROLL, 2012). Isto demonstra o refinamento na regulação de vias de sinalização dependente das EROs, em que o sistema funciona de maneira reversível sendo que as EROs promovem a ativação de seus alvos e posterior inativação (HOLMSTRÖM; FINKEL, 2014).

Em relação a ação das EROs no processo inflamatório, mediadores pró-inflamatórios como TNFα e endotelina-1 (ET-1) induzem a produção do ânion superóxido pela NADPH oxidase (KILPATRICK et al., 2010; LÓPEZ-SEPÚLVEDA et al., 2011). Após, as EROs ativam quinases como proteína quinase ativada por mitógenos (MAPK) e fosfatidilinositol 3-quinase (PI_3K) e fatores de transcrição como NFκB (GLOIRE; PIETTE, 2009; TORRES; FORMAN, 2003; ZHANG et al., 2016). Focando na ação das EROs no NFκB, essa via de sinalização é conhecida por ser sensível ao estado redox celular (TOLEDANO; LEONARD, 1991). Posteriormente a ubitinação e degradação do IκB, a subunidade p65 do NFκB é fosforilada em resíduos específicos de serina pela proteína quinase A (PKA). A atividdade da PKA sobre o NFκB é aumentada pela ação de EROs e em consequência ocorre aumento na atividade do NFκB (JAMALUDDIN et al., 2007). Ademais, o tratamento com a molécula com antioxidante N-acetilcisteína inibiu a associação do NFκB na região promotora do gene IL-8 (JAMALUDDIN et al., 2007). Evidenciando a ação do ânion superóxido na sinalização do NFκB, o tratamento com um mimético da enzima superóxido dismutase (SOD) – M40403 – inibiu a ativação do NFκB bem como a produção das citocinas TNFα e IL-6 (NDENGELE et al., 2005).

Outro fator de transcrição importante dentro do processo inflamatório é o fator 2 relacionado ao fator de transcrição nuclear eritróide 2 (Nrf2). Este fator de transcrição é considerado um dos sistemas citoprotetores mais importantes adquiridos pelos vertebrados ao longo da evolução (GAO; DOAN; HYBERTSON, 2014; MAHER; YAMAMOTO, 2010). Polimorfismos de única base no gene Nrf2 são relacionados com a progressão de doenças (CHO; MARZEC; KLEEBERGER, 2015), por exemplo a subistituição de uma base na região promotora do gene confere maior susceptibilidade a lesões pulmonares (MARZEC et al., 2007). Nesse sentido, o Nrf2 tem papel importante no controle do processo inflamatório. Em condições fisiológicas o Nrf2 está associado ao Keap-1 e esse complexo é constantemente marcado para

degradação proteassomal. Por outro lado, o ataque eletrofílico a molécula Keap-1 em resíduos específicos de cisteina promove a dissociação do complexo Keap-1/Nrf2 (HUANG; NGUYEN; PICKETT, 2002; NITURE; JAIN; JAISWAL, 2009). Em sequência o Nrf2 é fosforilado e este fator de transcrição se transloca para região promotora do núcleo denominada ARE (Elementos de resposta antioxidadante). Ação do Nrf2 nesta região promotora ARE promove a transcrição de genes relacionados com a resposta citoprotetora (GAO; DOAN; HYBERTSON, 2014; HUANG; NGUYEN; PICKETT, 2002). Por exemplo, a heme-oxigenase 1 (HO-1) é um dos genes alvos do Nrf2, e esta enzima apresenta atividade antioxidante e anti-inflamatória (GAO; DOAN; HYBERTSON, 2014; STEINER et al., 2001). Esta enzima catalisa a conversão do grupamento heme em monóxido de carbono, ferro e biliverdina. Posteriomente a biliverdina é convertida em bilirrubina pela biliverdina redutase, sendo essas duas moléculas capazes de neutralizarem as EROs produzidas durante o processo inflamatório (PAE et al., 2010). Além do mais, a ativação da via da HO-1 com produção de biliverdina e monóxido de carbono é capaz de inibir a migração de neutrófilos para o foco inflamatório (FREITAS et al., 2006) e produção de citocinas pró-inflamatórias como TNFα, IL-1β e IL-6 (SO et al., 2008).

A progressão temporal do processo inflamatório é importante e altamente regulada. O processo de resolução é ativo e orquestrado por mediadores lipídicos pró-resolução, como lipoxinas, resolvinas, maresinas e protectinas (SERHAN, 2014). A PGE_2 age nos neutrófilos e induz a expressão da enzima 15-lipoxigenase e troca de classe de mediador lipídico pró-inflamatório para pró-resolução, como a lipoxina A_4 (LEVY et al., 2001). Os mediadores lipídicos pró-resolução promovem a ativação de macrófagos não-flogísticos, os quais realizam a eferocitose dos corpos apoptóticos de neutrófilos. Os sinais da resolução do processo inflamatório incluem a redução de citocinas pró-inflamatórias, retirada dos neutrófilos da superfície epitelial, fagocitose dos neutrófilos em apoptose e remoção dos *debris* inflamatórios e infecciosos (SERHAN, 2014). Por outro lado, a falha na resolução do processo inflamatório eleva a quantidade de neutrófilos no tecido e produção de mediadores pró-inflamatórios como prostaglandinas, leucotrienos e citocinas que pode contribuir para cronificação do processo inflamatório (ARNARDOTTIR et al., 2016; HSIAO et al., 2015; LEVY et al., 2001). Algumas doenças de cunho inflamatório já têm sido correlacionadas com a falha nos

mecanismos de resolução. De fato, pacientes com enfisema pulmonar apresentam aumento da enzima 15-prostaglandina desidrogenase que promove a degradação de mediadores lipídicos (HSIAO et al., 2015), tal fato reduz os mediadores lipídicos pró-resolução, como as resolvinas (CROASDELL et al., 2015). O modelo de artrite induzido pelo soro artritogênico K/BxN pode ser auto-resolutivo ou ter a resolução atrasada de acordo com o protocolo de indução do modelo. Durante a artrite auto-resolutiva induzida pelo soro K/BxN a fase de resolução é caracterizada pelo aumento da série D das resolvinas (Rvs) como RvD1, RvD2, RvD3 e RvD4. Por outro lado, no protocolo de artrite com atraso na resolução a RvD3 está reduzida e o tratamento destes animais com RvD3 inibe a migração de leucócitos, produção de mediadores lípidicos pró-inflamatórios (PGE_2, LTB_4), edema e score clínico. Corroborando, pacientes com artrite reumatoide apresentam baixos níveis de RvD3 no soro quando comparado a indivíduos saudáveis (ARNARDOTTIR et al., 2016).

1.2. Dor Inflamatória

A dor é uma experiência sensorial e emocional desagradável que tem como objetivo alertar o indivíduo sobre um dano real ou potencial e desencadear uma resposta protetiva adequada (IASP, 2012; JULIUS; BASBAUM, 2001). A dor de origem inflamatória ocorre pela interação entre o tecido lesado e os neurônios sensoriais periféricos, isto ocorre por exemplo na dor inflamatória aguda, articular, entre outras (IASP, 2012; JI et al., 2011). O processo doloroso é clinicamente relevante, haja vista a capacidade do indivíduo em identificar a existência de um processo danoso por meio da dor. Tendo em vista a relação entre o componente emocional e a dor, fatores como humor, atenção e expectativa da intensidade dolorosa interferem na percepção da dor (BUSHNELL; ČEKO; LOW, 2013; WIECH et al., 2014). Por outro lado, a dor pode ultrapassar o seu papel de alertar o indivíduo e tornar-se crônica e deliberante (JULIUS; BASBAUM, 2001), isto ocasiona redução na qualidade de vida do indivíduo.

A percepção do estímulo doloroso ocorre devido aos neurônios sensoriais – nociceptores. Os fibras nociceptivas são divididos no tipo A – subdivididas em Aα, Aβ e Aδ – e tipo C (BRAZ et al., 2014). As fibras Aα e Aβ são de largo diâmetro, possuem maior quantidade de mielina, a maioria destas fibras conduzem estímulos inócuos como toque leve e propriocepção com velocidade 35-

75 m/s (FERREIRA et al., 2009; JULIUS; BASBAUM, 2001). A ativação de fibras Aα e Aβ recruta interneurônios inibitórios no corno dorsal da medula espinal que inibem a entrada ou quantidade de informação nociceptiva na medula espinal referente ao local inervado. Esse fato explica porque um estímulo inócuo pode reduzir a informação nociceptiva quando ambos ocorrerem no mesmo local (JULIUS; BASBAUM, 2001). As fibras de médio diâmetro Aδ são levemente mielinizadas e possuem velocidade de condução de 5-30 m/s, podem ser divididas em: 1) mecanoceptoras – respondem preferencialmente a estímulos mecânicos intensos e potencialmente danosos; 2) polimodais – respondem a estímulos mecânicos, térmicos e químicos. A ativação de fibras Aδ gera sensação dolorosa intensa em alfinetada ou pontada de forma transiente. Além disso, os mecanoceptoras podem aumentar sua despolarização frente a estímulos térmicos intensos (FEIN, 2012; JULIUS; BASBAUM, 2001). As fibras do tipo C são de pequeno diâmetro, amielinizadas e apresentam velocidade de condução 0,5 a 2 m/s. Estas fibras do tipo C são polimodais e responsáveis por dor em queimação (FEIN, 2012; JULIUS; BASBAUM, 2001). O corpo desses neurônios está no gânglio da raiz dorsal e as projeções destes vão para as lâminas no corno dorsal da medula espinal (BRAZ et al., 2014; FEIN, 2012; JULIUS; BASBAUM, 2001).

O sistema sensorial converte o estímulo do ambiente em sinal elétrico. Assim, após a ativação do nociceptor primário, este transmite a informação periférica até o corno dorsal da medula espinal onde faz sinapse com neurônios de segunda ordem (secundários) após a percepção do estímulo ocorre pela transmissão do sinal para o córtex somatossensorial local onde ocorre a interpretação do estímulo nociceptivo. Ademais, as projeções que envolvem sinapses na região da amídala são responsáveis pelo componente emocional da sensação dolorosa (FEIN, 2012; JULIUS; BASBAUM, 2001; MILLAN, 1999; REICHLING; LEVINE, 2009; SCHOLZ; WOOLF, 2002; WOOLF; SALTER, 2000).

A dor de origem inflamatória é decorrente da sensibilização neuronal que ocorre por aumento na excitabilidade do nociceptor periférico frente a ação de mediadores pró-inflamatórios em seus respectivos receptores no terminal periférico do nociceptor. Diversos mediadores pró-inflamatórios podem induzir a sensibilização neuronal, pode-se destacar as citocinas IL-33, TNFα, e IL-1β, aminas vasoativas, como histamina e serotonina, prostaglandinas, como PGE_2 e PGI_2 (BINSHTOK et al., 2008; FERREIRA; NAKAMURA; DE ABREU CASTRO, 1978; JI; XU; GAO, 2014;

JIN; GEREAU, 2006; LIU et al., 2016; PARADA et al., 2001; SACHS et al., 2009; VERRI et al., 2006a). Este aumento na excitabilidade dos neurônios aferentes contribui para o aumento na sensibilidade dolorosa, que pode ser dividida em duas categorias: 1) hiperalgesia – resposta exacerbada a um estímulo doloroso; 2) alodinia – resposta nociceptiva a estímulos previamente não dolorosos (FERREIRA et al., 2009).

A presença de neutrófilos no tecido tem estrita relação com a dor inflamatória. De fato, a presença de neutrófilos tem papel essencial na manutenção da dor infamatória (CUNHA et al., 2008). Os neutrófilos recrutados liberam mediadores pró-inflamatórios como IL-33, TNFα, e IL-1β, e PGE_2 , os quais irão agir em seus respectivos receptores no terminal periférico do nociceptor (BINSHTOK et al., 2008; JIN; GEREAU, 2006; LIU et al., 2016; PINHO-RIBEIRO; VERRI; CHIU, 2017; SACHS et al., 2009). Ativação desses receptores levará a ativação de quinases como proteína quinase C (PKC), PKA e MAPK p38, que por sua vez irão fosforilar os canais de sódio Nav 1.8 (FERREIRA et al., 2009; PINHO-RIBEIRO; VERRI; CHIU, 2017). O aumento da condutância pelo canal de sódio no nociceptor irá reduzir o limiar de ativação neuronal e consequentemente ocasionar a sensibilização (PINHO-RIBEIRO; VERRI; CHIU, 2017). Demonstrando a importância dos neutrófilos para a hiperalgegia, o tratamento com o inibidor de adesão leucocitária fucoidina inibidiu a hiepralgesia mecânica induzida pela carragenina inibindo a migração de neutrófilos (CUNHA et al., 2008).

O ânion superóxido tem sido relacionado com o desenvolvimento da dor inflamatória. O tratamento com tempol – mimético da enzima SOD – inibe a hiperalgesia mecânica na inflamação aguda induzida pela carragenina (KHATTAB, 2006). Corroborando, o ânion superóxido é importante para o desenvolvimento da hiperalgesia térmica associada a inflamação aguda (NDENGELE et al., 2008; WANG, 2004). E nesse mesmo sentido, nosso grupo demonstrou que o ânion superóxido doado pelo superóxido de potássio (KO_2) produz comportamentos de dor manifesta – como contorções abdominais e sacudidas da pata – hiperalgesia mecânica e hiperalgesia térmica (MAIOLI et al., 2015) A dor inflamatória induzida pelo KO_2 é dependente da ativação do NFκB (PINHO-RIBEIRO et al., 2016c). Em decorrência da ativação do NFκB, outros mediadores pró-inflamatórios como ET-1 e TNFα são produzidos e também participam na dor inflamatória induzida pelo KO_2 (SERAFIM et al., 2015; YAMACITA-BORIN et al., 2015). Por exemplo, o tratamento

com PDTC (inibidor seletivo do NFκB), bosentan (antagonista misto para os receptores de endotelina), etanercept (receptor solúvel de TNFα) e animais *knockout* para TNFR1 inibem a dor manifesta, hiperalgesia mecânica, hiperalgesia térmica, recrutamento de neutrófilos, produção de citocinas pró-inflamatórias - TNFα e IL-1β - e estresse oxidativo (PINHO-RIBEIRO et al., 2016c; SERAFIM et al., 2015; YAMACITA-BORIN et al., 2015). Nesse sentido, existe uma estrita relação entre o ânion superóxido, ativação do NFκB e produção de mediadores pró-inflamatórios, demonstrando o importante papel dessa molécula no desenvolvimento do processo inflamatório e doloroso.

1.3. Modelo de Artrite Crônica Induzido por TiO_2

Inflamação articular crônica ocasiona alterações morfológicas caracterizadas pela destruição da superfície de suporte de peso da articulação. Este cenário poderá levar à substituição parcial ou total da articulação, sendo este procedimento conhecido como artroplastia. A artroplastia é um procedimento de grande sucesso na medicina moderna, uma vez que restabelece a mobilidade, reduz a dor e de maneira geral, aumenta a qualidade de vida de milhões de pessoas (COBELLI et al., 2011).

Artroplastia de joelho e quadril são indicados para o tratamento de osteoartrite, artrite reumatoide, fraturas e necrose avascular (SIDDIQUI et al., 2012; SOEVER et al., 2010). As primeiras substituições de joelho foram realizadas nas décadas de 70 e 80, sendo atualmente considerado um tratamento eficaz e de baixo custo para casos em estágio terminal de artrite (CARR et al., 2012). Só em 2010 cerca de 7 milhões de estadunidenses já haviam realizado a substituição total do joelho ou quadril e acredita-se que até 2030 cerca de 11 milhões de estadunidenses irão realizar substituição do joelho ou quadril, tornando a artroplastia um dos procedimentos cirúrgicos eletivos mais comum nos Estados Unidos (MARADIT KREMERS et al., 2015). As próteses utilizadas na artroplastia podem ser de materiais como: aço inoxidável; liga metálica de cobalto, crômio e molibdênio; titânio e ligas metálicas de titânio (como titânio, alumínio e vanádio); polietileno e polimetilmetacrilato. É necessário que os materiais protéticos sejam biocompatíveis e possuam propriedades mecânicas apropriadas para suportar as forças aplicadas na prótese (LEE; GOODMAN, 2008).

Apesar do sucesso da artroplastia que contribui para a melhora da qualidade de vida do paciente, em torno de 10-15% dos procedimentos de artroplastia irão falhar, sendo o processo de osteólise um dos principais fatores que induzem a falha da substituição da articulação. (GOODMAN, 2007; HARRIS, 2001; LOONEY et al., 2006; SARGEANT; GOSWAMI, 2006; SUNDFELDT et al., 2006). A incidência da osteólise tem sido relatada em torno de 5-20% dos procedimentos de artroplastia (HARRIS, 2001; PURDUE et al., 2006), no entanto existe estudo que apresenta taxas superiores a 40% (DORR et al., 1997).

As partículas protéticas liberadas são responsáveis pelo início do processo inflamatório estéril (COBELLI et al., 2011), na década de 70 foram reportados os primeiros casos clínicos de partículas protéticas induzindo erosão cística óssea (HARRIS et al., 1976). As principais partículas provenientes do desgaste da prótese metálica são os óxidos metálicos, hidróxidos metálicos e fosfatos metálicos. Quando comparado com as partículas protética de polietileno, as partículas metálicas liberadas são marcadamente menores (<50 nm contra >0,1 μm) e mais numerosas (COBB; SCHMALZREID, 2006).

As nanopartículas metálicas liberadas da prótese ativam os macrófagos residentes no espaço periprotético e consequentemente há a fagocitose das nanopartículas metálicas (ST PIERRE et al., 2010). Os macrófagos ativados produzem quantidades crescentes de mediadores químicos, como ligante receptor ativador do NFκB (RANKL), citocinas pró-inflamatórias como fator TNFα, IL-1β e IL-6 bem como produção de EROs. Estes promovem a ativação do NFκB, que contribuirá diretamente para a manutenção do processo inflamatório estéril no tecido periprotético (COBELLI et al., 2011; WANG et al., 2010; WOOLEY et al., 2002). Ademais, este microambiente pró-inflamatório induz a osteoclastogênese, bem como pronunciada ativação de osteoclastos. De fato, RANKL (DANKS et al., 2016) ou TNFα e IL-6 (O'BRIEN et al., 2016) induzem ativação de osteoclastos de maneira dependente ou independente do receptor ativador do NFκB (RANK), respectivamente. O conjunto destes fatores promoverá a reabsorção óssea e consequente progressão do processo de osteólise (COBELLI et al., 2011; WOOLEY et al., 2002). A ativação do sistema imune pelas nanopartículas metálicas resultará na rejeição da prótese e necessidade de novo procedimento cirúrgico, o que pode não ser possível dependendo da condição de saúde e idade do paciente.

O dióxido de titânio (TiO_2) é um pó branco e inodoro utilizado na confecção de próteses ortopédicas, mas também é amplamente empregado como pigmento de tintas, corante alimentar, protetor solar e cremes cosméticos (GURR et al., 2005). O titânio acumula-se em tecidos como pulmão, pele ou sinóvia. O acúmulo de titânio nos tecidos é caracterizado pela deposição de pigmento preto, acompanhado de fibrose, necrose ou reação granulomatosa (MORAN et al., 1991). O TiO_2 é genotóxico, pode penetrar na pele e induzir dano oxidativo ao DNA (DUNFORD et al., 1997). Além do mais, pode permanecer nos tecidos e órgãos e promover dano tecidual em variado grau (CHEN et al., 2009; WANG et al., 2009). A exposição broncoalveolar ao TiO_2 induz resposta inflamatória e a magnitude da resposta gerada dependente da quantidade e formato de partículas administradas (BAISCH et al., 2014; SILVA et al., 2013). As partículas de titânio induzem artrite, fato alicerçado pelo caso clínico de um paciente sem histórico familiar prévio que desenvolveu artrite. O desenvolvimento da artrite neste paciente foi induzido pela liberação de partículas provenientes do implante metálico de titânio e vanádio (DÖRNER et al., 2006).

Com base no exposto acima, nosso laboratório padronizou um modelo de artrite crônica induzido por TiO_2. O TiO_2 induz hiperalgesia articular, edema, estresse oxidativo, liberação de citocinas pró-inflamatórios – como IL-33, TNFα, IL-1β, e IL-6 – e ativação da via de sinalização RANKL/RANK. Este cenário promove a destruição da cartilagem bem como aumento na reabsorção óssea e consequentemente destruição articular (BORGHI et al., em revisão). Portanto a artrite crônica induzido por TiO_2 é um ótimo modelo na medicina translacional que possibilita a compreenção dos mecanimos celulares e moleculares relacionado ao processo inflamatório aséptico mediado por partículas liberadas no espaço periprotético.

1.4. NARINGENINA

Naringenina (4',5,7-Trihdroxyflavanone) é um flavonoide pertecente a classe das flavanonas encontrado principalmente em frutas cítricas como toranja, tangerina, limão entre outros (LEE et al., 2001). Os flavonoides são compostos polifenólicos amplamente encontrados em frutas, legumes, sementes, ervas, especiarias e grãos integrais. (GRAF; MILBURY; BLUMBERG, 2005; PANDEY;

RIZVI, 2009; VERRI et al., 2012). Sobre a função destas moléculas polifenólicas nas plantas citadas anteriormente, os flavonoides são metabolitos secundários de plantas vasculares, geralmente estão relacionados a defesa antioxidante contra radiação ultravioleta ou contra micro-organismos, incluindo bactérias, fungos e vírus (GRAF; MILBURY; BLUMBERG, 2005). Além do mais, a estrutura molecular altamente reativa é capaz de doar hidrogênio e eletróns dos flavonoides e coferem a capacidade destas moléculas neutralizarem as EROs (VERRI et al., 2012). Essas características dos flavonoides demonstram potencial uso destas moléculas na medicina.

Uma importante característica da naringenina é atividade pleiotropica desta molécula incluindo efeito anti-inflamatório e analgésico. Em relação aos efeitos do tratamento com naringenina relacionados a produção de mediadores lipídicos pró-inflamatórios, naringenina inibe a expressão de (ciclo-oxiganese 2) COX-2 (JAYARAMAN et al., 2012; PARK; KIM; CHOI, 2012), atividade da fosfolipase A_2 – enzima responsável pela liberação de ácido araquidônico – (LÄTTIG et al., 2007) por conseguinte reduz a produção de mediadores lipídicos pró-inflamatórios (AL-REJAIE et al., 2013). De fato, naringenina inibiu dose-dependente a produção de PGE_2 na colite ulcerativa induzido por ácido acético (AL-REJAIE et al., 2013). Em adição, a naringenina inibe outras vias importantes no processo inflamatório, como NFκB através da inibição a ligação da subunidade p65 do NFκB ao DNA no modelo de asma induzido por ovoalbumina (SHI et al., 2009), e a via das MAPK através da inibição da fosforilação da quinase regulada por sinal extracelular (ERK), quinase c-Jun N-terminal (JNK) e p38 em células do epitélio brônquico humano estimuladas com lipopolissacarídeo (LPS) (YU et al., 2015). Corroborando, dados demonstraram que a naringenina inibe a fosforilação da subunidade p65 do NFκB e ERK na lesão renal induzido por daunorrubicina (KARUPPAGOUNDER et al., 2015). No sentido da inibição do NFκB e MAPK, a naringenina inibe a produção de citocinas pró-inflamatórias como TNF-α, IL-1β, IL-6 (AL-REJAIE et al., 2013; PINHO-RIBEIRO et al., 2016a, 2016b; VAFEIADOU et al., 2009)

A produção de mediadores pró-inflamatórios como citocinas hiperalgésicas TNF-α, IL-1β, IL-33 e mediadores lipídicos como PGE_2 tem importante papel na dor inflamatória (BINSHTOK et al., 2008; FERREIRA; NAKAMURA; DE ABREU CASTRO, 1978; JIN; GEREAU, 2006; LIU et al., 2016; SACHS et al., 2009; VERRI et al., 2006a). Nessa linha, a naringenina inibe a

hiperalgesia mecânica induzida pela carragenina (PINHO-RIBEIRO et al., 2016b) e LPS (PINHO-RIBEIRO et al., 2016a) através da inibição da produção das citocinas TNF-α, IL-1β e IL-33 (PINHO-RIBEIRO et al., 2016a, 2016b). Ademais, a naringenina inibe a hiperalgesia mecânica induzida pela PGE_2 (PINHO-RIBEIRO et al., 2016b). Fármacos utilizados na clínica como dipirona e opioides ativam a via sinalização analgésica NO–GMPc–PKG–canais K_{ATP} (CURY et al., 2011). Dados públicados pelo nosso grupo demontraram ainda que a naringenina ativa a via de sinalização NO–GMPc–PKG–canais K_{ATP} e inibe a hiperalgesia mecânica induzida pela carragenina (PINHO-RIBEIRO et al., 2016b).

Outro mecanismo importante inerente as ações da naringenina é ativação do fator de transcrição Nrf2 (LOU et al., 2014; RAMPRASATH et al., 2014). A expressão de enzimas citoprotetoras está relacionado a ativação do fator de transcrição Nrf2 (GAO; DOAN; HYBERTSON, 2014; MAHER; YAMAMOTO, 2010). Nesse sentido, a naringenina induz a ativação do Nrf2 e inibe o estresse oxidativo (ESMAEILI; ALILOU, 2014; RAMPRASATH et al., 2014). Por exemplo, o tratamento com naringenina induz da ativação do Nrf2/HO-1 e inibe a lesão hepática induzida pelo tetracloreto de carbono (CCl_4) (ESMAEILI; ALILOU, 2014). Essas características relacionadas aos mecanismos de ação nos fizeram avaliar a eficácia do tratamento com naringenina frente a dois modelos: 1) modelo de artrite crônica induzido por TiO_2; 2) modelo agudo de dor inflamatória induzido pelo KO_2 um doador de ânion superóxido.

2. OBJETIVOS

2.1. OBJETIVO GERAL

Avaliar o efeito analgésico e anti-inflamatório da naringenina em modelo de artrite induzido por TiO_2 e inflamação aguda induzido por ânion superóxido.

2.1.1. Objetivos Especificos – Modelo Crônico

- Avaliar o efeito da naringenina na hiperalgesia mecânica e edema induzidos por TiO_2 em modelo de artrite crônica;
- Avaliar o efeito da naringenina no recrutamento de leucócitos para a cavidade articular do joelho induzido por TiO_2 em modelo de artrite crônica;
- Avaliar o efeito do tratamento crônico na toxicidade gástrica, hepática e renal com naringenina em modelo de artrite crônica induzido por TiO_2;
- Avaliar o efeito da naringenina no estresse oxidativo induzido por TiO_2 em modelo de artrite crônica;
- Avaliar o efeito da naringenina na expressão de RNAm das citocina IL-33, TNFα e pro-IL-1β na articulação induzido por TiO_2 em modelo de artrite crônica;
- Avaliar o efeito da naringenina na ativação do NFκB na articulação induzido pelo TiO_2 em modelo de artrite crônica;
- Avaliar o efeito da naringenina no sistema RANKL/RANK/OPG na articulação em modelo de artrite crônica induzido por TiO_2.

2.1.2. Objetivos Específicos – Modelo Agudo

- Avaliar o efeito da naringenina na dor manifesta induzida por KO_2 um doador de ânion superóxido;
- Avaliar o efeito da naringenina na hiperalgesia mecânica, hiperalgesia térmica e atividade da enzima mieloperoxidase na inflamação aguda induzida por KO_2 um doador de ânion superóxido;
- Avaliar se o efeito analgésico da naringenina é depende da ativação da via de sinalização NO–GMPc–PKG– canais K_{ATP} na inflamação aguda induzida por KO_2 um doador de ânion superóxido;
- Avaliar o efeito da naringenina no estresse oxidativo no tecido cutâneo plantar na inflamação aguda induzida por KO_2 um doador de ânion superóxido;

- Avaliar o efeito da naringenina dos níveis das citocinas TNFα e IL-10 e expressão de RNam para IL-33 no tecido cutâneo plantar na inflamação aguda induzida por KO_2 um doador de ânion superóxido;
- Avaliar o efeito da naringenina na expressão de RNAm para COX-2, preproET-1 Nrf-2 e HO-1 no tecido cutâneo plantar na inflamação aguda induzida por KO_2 um doador de ânion superóxido.

3. EDITORIAL – PUBLICADO (ONCOTARGET)

O presente editorial foi realizado no Laboratório de Dor, Inflamação Neuropatia E Câncer, da Universidade Estadual de Londrina e segue as normas da revista Oncotarget. O presente editorial intitula-se “Naringenin: an analgesic and anti-inflammatory citrus flavanone” (doi: 10.18632/oncotarget.14084).

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Editorial: Neuroscience

Naringenin: an analgesic and anti-inflammatory citrus flavanone

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In this editorial, we discuss recent evidence from our research group on the analgesic and anti-inflammatory mechanisms of the flavonoid naringenin (4',5,7-tryhidroxy-flavanone). Flavonoids are polyphenolic compounds found in human diet [1]. Naringenin belongs to flavanone class of flavonoids, and it is mainly found in citrus fruits including, lemon, orange, tangerine and grapefruit [1-5]. The antioxidant activity is the most recognized effect of flavonoids, which depends, for instance, on hydrogen donation and electron stabilization in the phenolic rings [1]. Naringenin presents therapeutic effect in several models of inflammatory pain [2, 3, 5]. Naringenin inhibits the pain-like behavior induced by inflammatory stimuli such as phenyl-p-benzoquinone, acetic acid, formalin, complete Freund´s adjuvant, capsaicin, carrageenan [2], superoxide anion [3], and LPS [5]. Moreover, naringenin inhibits UVB irradiation-induced skin inflammatory edema, cytokine production, myeloperoxidase activity, matrix metalloproteinase-9 activity, and oxidative stress [4].

Pathogen (PAMPs) and damage (DAMPs) associated molecular patterns and inflammatory mediators activate resident macrophages. Resident macrophages produce chemotactic molecules to recruit leukocytes to the inflammatory foci, mainly neutrophils in the early events of inflammation. Activated macrophages and neutrophils induce oxidative stress by producing superoxide anion and other reactive oxygen (ROS) and nitrogen species. Naringenin inhibits leukocyte recruitment [2-5] and production of superoxide anion [3-5], whilst increases GSH [2-4], and antioxidant capacity [3-5]. Naringenin also acts on macrophages inducing Nrf2 activation, a nuclear factor that induces antioxidant and anti-inflammatory responses, inducing HO-1 expression [3]. PAMPs, DAMPs and ROS induce NFκB activation in macrophages resulting in the production of pro-hyperalgesic cytokine such as IL-33, TNFα, IL-1β and IL-6. Pro-hyperalgesic cytokines induce the production of lipid mediators such as PGE_2 that sensitize the nociceptor neurons. Naringenin inhibits LPS- and carrageenan-induced NFκB activation *in vivo* [2] and *in vitro* [5], which contributes to naringenin inhibition of IL-33 [2], TNFα [3-5], IL-1β [2, 4, 5] and IL-6 [4,5] production and expression of cyclooxygenase-2 mRNA [3] (Figure 1).

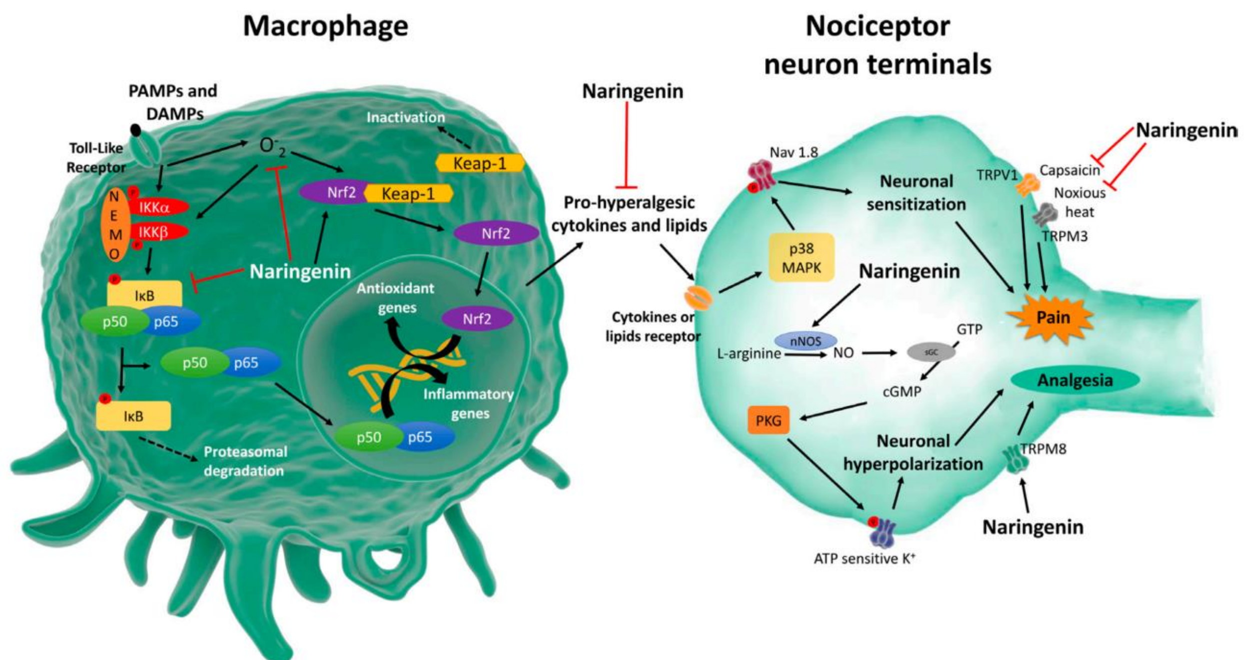

Figure 1: Schematic summary of naringenin analgesic and anti-inflammatory mechanisms.

The ligand-gated and voltage-gated ion channels are essential to the role of nociceptor neurons to sense noxious stimuli [6]. Inflammatory cells-released pro-hyperalgesic cytokines and lipids that bind to their receptors expressed in nociceptor neuron terminals activating intracellular signaling pathways resulting in the modulation of ion channels activity including Nav1.8, TRPV1, and TRPA1. As a consequence, there is nociceptor neuron activation and/or sensitization to nociceptive stimuli, thus, generating pain [6]. The evidence that naringenin inhibits NFκB activation and induces Nrf2 activation is in line with indirect effects over nociceptor neuron activity since inhibiting NFκB and inducing Nrf2 reduce cytokine production and oxidative stress. Thus, naringenin inhibits the production of nociceptive molecules in non-neuronal cells, which will result in reduced activation of nociceptor neurons. For instance, naringenin inhibits NFκB-dependent TNFα and IL-1β production by macrophages [5]. These cytokines induce nociceptive neuron sensitization via p38 MAPK phosphorylation of Nav1.8 sodium channels [6] (Figure 1).

Evidence also support that naringenin directly modulates nociceptor neuron activity. High concentrations of naringenin reduces TRPV1 activation [7], which corroborates the naringenin inhibition of capsaicin-induced overt pain like-behavior and mechanical hyperalgesia [2]. At much lower concentrations compared to TRPV1, naringenin blocks TRPM3 ion channel, a noxious heat sensor channel [7]. Naringenin can also activate TRP channels such as TRPM8, which has been described to induce analgesia [7]. Thus, naringenin regulates TRP channels expressed by nociceptor neurons such as TRPV1, TRPM3 and TRPM8 to induce analgesia (Figure 1).

Nitric oxide (NO) mediates the analgesic effect of opioids, and some non-steroidal anti-inflammatory drugs such as dipyrone [8]. The analgesic effect of NO depends on the induction of the production of second messenger cGMP by activating soluble guanylate cyclase (sGC). Then, the cGMP-dependent protein kinase (PKG) is activated and phosphorylates ATP sensitive K^+ channel to induce potassium influx hyperpolarizing the nociceptor neurons, thus, inhibiting excitatory nociceptive synaptic transmission [8]. Naringenin inhibits mechanical hyperalgesia [2, 3], thermal hyperalgesia [3] and neutrophil recruitment [2] by activating the NO-cGMP-PKG-ATP sensitive K^+ channel signaling pathway (Figure 1) since these effects of naringenin were reduced by the respective inhibitors L-NAME, ODQ, KT5833 and glibenclamide [2, 3].

Concluding, naringenin acts by mechanisms involving the inhibition of leukocyte recruitment [2-5], oxidative stress [2-5], NFκB activation [2, 5] and pro-hyperalgesic cytokine production [2-5] on the immune cells such as macrophages. Nevertheless, naringenin also modulates TRP channels such as TRVP1, TRPM3 and TRPM8 reducing pain [7], and activates a NO signaling pathway that induces nociceptor neuron hyperpolarization [2, 3]. Therefore, naringenin treatment is a promising analgesic, anti-inflammatory and antioxidant compound, requiring further investigation in preclinical models and clinical settings.

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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 “Naringenin mitigates titanium dioxide (TiO_2)-induced chronic arthritis in mice: Role of oxidative stress, cytokines, NFκB, and RANKL/RANK/OPG system”.

Naringenin mitigates titanium dioxide (TiO_2)-induced chronic arthritis in mice: Role of oxidative stress, cytokines, NFκB, and RANKL/RANK/OPG system

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ABSTRACT

Arthroplasty is a procedure in advanced cases of inflammatory arthritis including osteoarthritis and rheumatoid arthritis, and also in cases of fractures and osteonecrosis. Unfortunately, 10-15% of the patients respond against the wear debris released by prosthetic biomaterials such as titanium dioxide (TiO_2) inducing osteolysis and failure in joint replacement. The prosthesis-released wear debris provokes an aseptic inflammatory process inducing bone resorption and joint destruction. Thus, we aim to investigate the effect and mechanisms of naringenin in the TiO_2-induced chronic arthritis. Mice were treated daily during 30 days with naringenin (16.7, 50, or 150 mg/kg) by per oral (p.o.) route starting 24 h after the intra-articular (i.a.) injection of 3 mg/joint of TiO_2. Naringenin inhibited TiO_2-induced mechanical hyperalgesia, knee edema, leukocytes recruitment, oxidative stress (gp91^{phox} mRNA expression, superoxide anion production, and lipid peroxidation), cytokine mRNA expression (IL-33, TNFα, and pro-IL-1β), NFκB activation, as well as modulated RANKL/RANK/OPG system in inflamed joint. In addition, chronic treatment with naringenin did not induce stomach, liver, or renal damage. Therefore, we demonstrated that naringenin mitigates pain and inflammatory damages in a model of implant-related arthritis induced by TiO_2.

KEYWORDS: Arthritis; Implant; Arthroplasiy; Pain, Aseptic inflammation.

1. Introduction

Arthroplasty is a successful procedure in advanced cases of inflammatory arthritis including osteoarthritis and rheumatoid arthritis, and also in cases of fractures and osteonecrosis.1,2 In fact, about 7 million of Americans had had total knee or hip replacements in 2010 and until 2030 about 11 million of Americans will have total knee or hip replacements.3 In this sense, arthroplasty is an efficient and successful procedure with low cost relative for terminals stages of arthritis.4 The prosthesis is design with materials such as cobalt chrome molybdenum (CoCrMo) alloys, titanium and titanium alloys, polyethylene, and polymethylmethacrylate.5 The prosthetic material must be biocompatible and presents mechanical properties that supports strength applied in the prosthesis.5 Despite arthroplasty successful procedure, about 10-15% of the patients respond against the wear debris released by biomaterials of the prosthesis inducing osteolysis and failure in joint replacement.6–10

The prosthesis-released wear debris induces an aseptic inflammatory process.11 In fact, in the 1970s were reported the first clinical case of prosthesis-released wear debris inducing bone cystic erosion.12 The metallic prosthesis releases wear debris including metallic oxides, metallic hydroxide, and metallic phosphates. As consequence of that, resident cells produces increasing amounts of inflammatory mediators including receptor activator of nuclear factor kappa-B ligand (RANKL),13 tumor necrosis factor (TNF)α, interleucine (IL)-1β, IL-613,14 and reactive oxygen species (ROS).15 In this sense, RANKL16 or TNFα and IL-617 induce osteoclastogenesis activating osteoclast differentiation in a receptor activator of nuclear factor kappa-B (RANK)-dependent- or independent-manner favoring bone resorption by osteoclast in periprosthetic tissue.11,14 As consequence of bone resorption, there is prosthesis rejection and a revision surgery will be required.18 Also, this inflammatory milieu induces NFκB activation that maintains and perpetuates the aseptic inflammatory process in the periprosthetic tissue.11,13,14

The titanium dioxide (TiO_2) is a white and odorless powder used in designing implants in orthopedic and dentistry filds.19,20 Of note, a patient without familiar history of rheumatic diseases developed implant-related arthritis due to titanium replacement.21 In fact, incubation of TiO_2 with patient's peripherical mononuclear blood cells induces TNFα release.21 The TiO_2 accumulates in tissue including lungs, skin, and synovial cavity is characterized by a black pigment deposition accompanied fibrosis, necrosis, and granulomatous reaction.22 In addition, TiO_2 could be trapped in tissues and organs promoting damage in varied degree.23,24 Given that, our laboratory standardized a model of TiO_2-induced chronic arthritis in mice, and TiO_2 induces articular pain, knee edema, oxidative stress, inflammatory cytokines production including IL-33, TNFα, IL-1β, and IL-6, increasing RANKL/RANK signaling pathway ultimately leading to joint destruction (Borghi et al., under review). Thus, Borghi and coworkers demonstrated that TiO_2-induced chronic arthritis is a useful model in translational medicine to the study of cellular and molecular mechanisms that involves TiO_2-triggered joint inflammation and bone destruction.

The flavonoid naringenin (4',5,7-tryhidroxy-flavanone) is a polyphenolic compound found in the human diet,25 mainly in citrus fruit including lemon, orange, tangerine, and grapefruit.26 Naringenin intake has been associated to reduce the risk of several chronic diseases,27 including collagen-induced arthritis.28 Human intake of 135 mg of naringenin p.o. is rapidly absorbed and plasma concentration is detected in 20 min as its conjugated forms. The elimination half-life is 2.31 ± 0.40 h occurring through urinary excretion and its elimination is complete in 24 h.29 For instance, naringenin inhibits TNFα30–32, IL-1β31–33 and IL-631,32 release in several models of inflammatory pain. Moreover, naringenin inhibits RANKL-induced osteoclastogenesis and bone resorption through suppression of p38 MAPK phosphorylation inhibiting osteolysis in titanium particles-induced calvarial osteolysis.34 Considering the benefits of prosthesis to the patients' life quality, new pharmacological

approaches are needed to control inflammatory response triggered by prosthesis wear debris. Naringenin treatment could be a conceivable approach to inhibit implant-related joint inflammation. Thus, in the present study, we evaluated the therapeutic effect and mechanisms of naringenin in the pathogenesis of TiO_2-induced chronic arthritis.

Results and Discussion

Naringenin Inhibits TiO_2-Induced Mechanical Hyperalgesia and Edema. Mice were treated with naringenin (16.7, 50, or 150 mg/kg) by per oral (p.o.) route starting 24 h after the intra-articular (i.a.) injection of 3 mg/joint of TiO_2. Mice were treated daily during 30 days, 1 h before the measurements of articular mechanical hyperalgesia and edema. The saline group did not present articular mechanical hyperalgesia (Figure 1a) and edema (Figure 1b). The i.a. injection of TiO_2 induced articular mechanical hyperalgesia (Figure 1a) and edema (Figure 1b) from 1-24 h in the first day and subsequently from the 2nd to 30th day. All doses of naringenin inhibited TiO_2-induced articular mechanical hyperalgesia in the first day and subsequently from the 2nd to 30th day. The dose of 16.7 mg/kg inhibited TiO_2-induced articular mechanical hyperalgesia from 1 to 5 h after the treatment in the first day and up to 30 days. The dose of 50 mg/kg of naringenin inhibited TiO_2-induced articular mechanical hyperalgesia from 1 to 7 h after the treatment in the first day and subsequently from the 2nd to 30th day. In addition, the dose of 50 mg/kg of naringenin was statistically different from the other doses. The dose of 150 mg/kg of naringenin inhibited TiO_2-induced articular mechanical hyperalgesia only 5 h after the treatment in the first day and up to 30 days (Figure 1a). Only the dose of 50 mg/kg of naringenin inhibited TiO_2-induced articular edema after 5 h after the treatment in the first day and from the 4th to 30th day. In addition, the dose of 50 mg/kg of naringenin was statistically different of the other doses (Figure 2b). Chronic arthritis-induced pain and edema decrease patient's quality of life.35 Naringenin presents analgesic and anti-

inflammatory effects in different pain models.30,32,33 Part of this effect lies in the direct modulation of sensory neurons. In fact, naringenins modulates transient potential receptor (TRP), blocking TRPV1 and TRPM3, and is an agonist of TRPM8.36,37 These effects ultimately lead to analgesia. Moreover, corroborating its action on sensory neurons, naringenin activates the analgesic NO−cGMP−PKG−K_{ATP} channel signaling pathway30,33 such as other analgesic drugs, including opioids and dipyrone.38 These data indicate that chronic treatment with naringenin presents consistent inhibition of mechanical hyperalgesia and edema in the TiO_2-induced chronic arthritis.

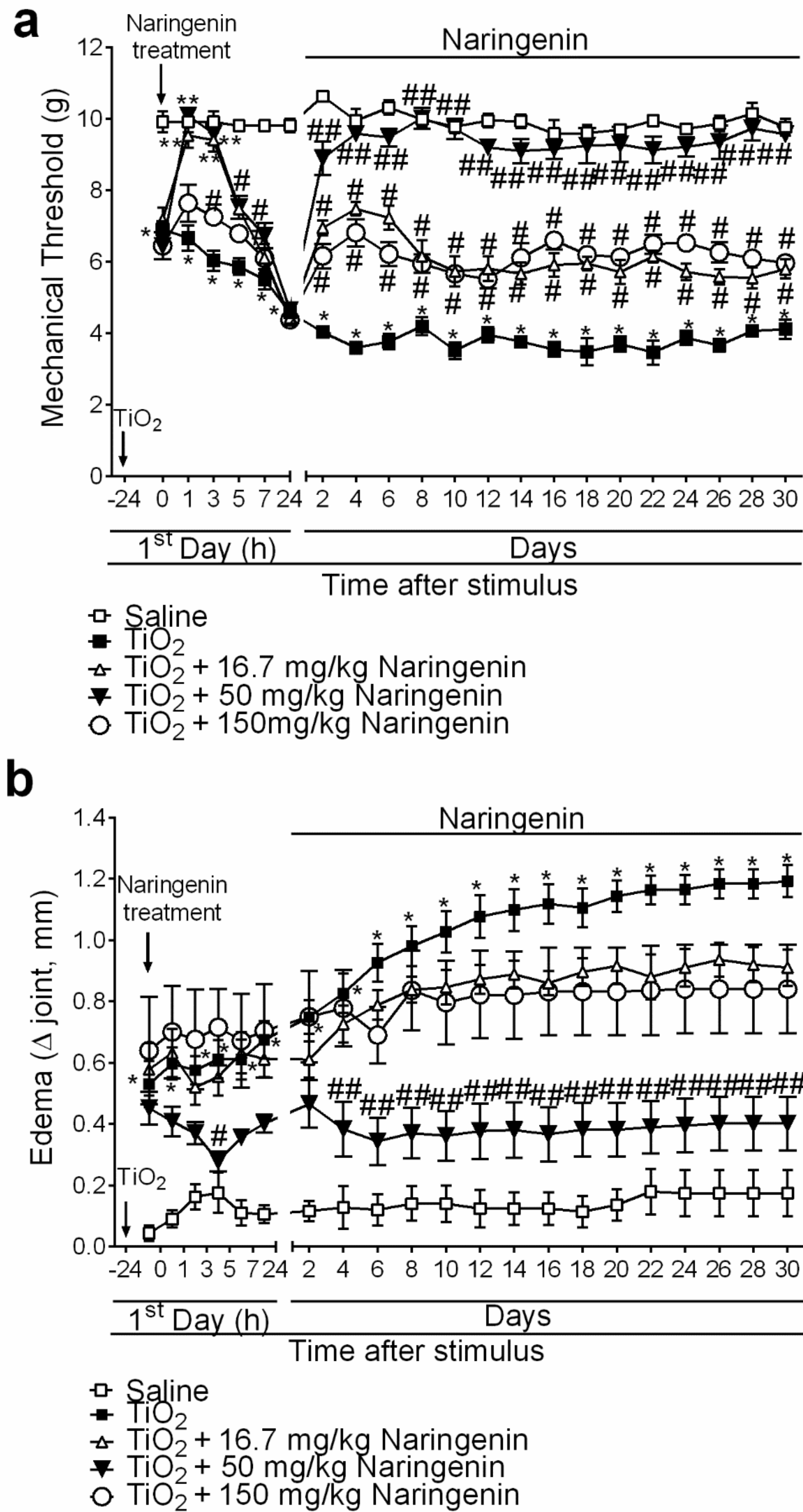

Figure 1. Naringenin inhibits TiO_2-induced articular mechanical hyperalgesia and edema. Mice were treated daily during 30 days with naringenin (16.7, 50, or 150 mg/kg, p.o.) 24 h

after intra-articular injection of TiO_2 (3 mg/joint). (a) Articular mechanical hyperalgesia and (b) edema were measured initially from 1-24 h in the first day and subsequently every other day until the 30th day after TiO_2 injection. Results are presented as mean ± SEM of six mice per group per experiment and are representative of two separate experiments. [*$p < 0.05$ compared to the saline group; #$p < 0.05$ compared to the TiO_2 group; **$p < 0.05$ compared to the TiO_2 and naringenin (150 mg/kg) groups; ##$p < 0.05$ compared to the TiO_2 and naringenin (16.7 and 150 mg/kg) groups (repeated measures two-way ANOVA followed by Tukey's *post hoc*)].

Naringenin Inhibits TiO_2-Induced Recruitment of Total Leukocytes, Polymorphonuclear, And Mononuclear. Mice were treated daily during 30 days with naringenin (16.7, 50 or 150 mg/kg) by p.o. route starting 24 h after the i.a. injection of 3 mg/joint of TiO_2. Knee joint wash was harvested and the number of total leukocyte (Figure 2a), polymorphonuclear (Figure 2b), and mononuclear (Figure 2c) cells were counted 30 days after TiO_2 injection. The doses of 16.7 and 50 mg/kg of naringenin inhibited TiO_2-induced recruitment of total leukocytes to the knee joint (Figure 2a), polymorphonuclear (Figure 2b) and mononuclear (Figure 2c) cells. Therefore, the dose of 50 mg/kg was chosen for the next set of experiments considering its efficacy in inhibiting mechanical hyperalgesia, knee joint edema (Figure 1), and leukocytes recruitment (Figure 2). Neutrophils- and macrophages-derived inflammatory mediators are a well-recognized components of inflammatory hyperalgesia.39 In fact, TiO_2 activates neutrophils40 and macrophages21 which release pro-inflammatory cytokines, including TNFα,21 IL-1β,41 IL-642, and IL-840. In addition, IL-8 induces neutrophils chemotaxis43 and their survival in inflamed joints are enhanced.44 In the inflammatory foci neutrophils are the major cells and their vast microbicidal array contribute to the destruction of cartilage and bone directly by releasing NETs or the pro-inflammatory

cytokines IL-33, IL-1β, and TNF-α, or indirectly by activating osteoclasts through RANK-RANKL signaling pathway.45,46 Herein, we observed that naringenin reduced leukocytes recruitment, which is in agreement with previous studies.30–33 For instance, naringenin reduces TNFα-induced ICAM-1 expression in human endothelial cells,47and ICAM-1 expressed by endothelial cells is important to leukocytes recruitment.48–50 Therefore, the inhibition of TiO_2-induced leukocytes recruitment by naringenin is an important feature.

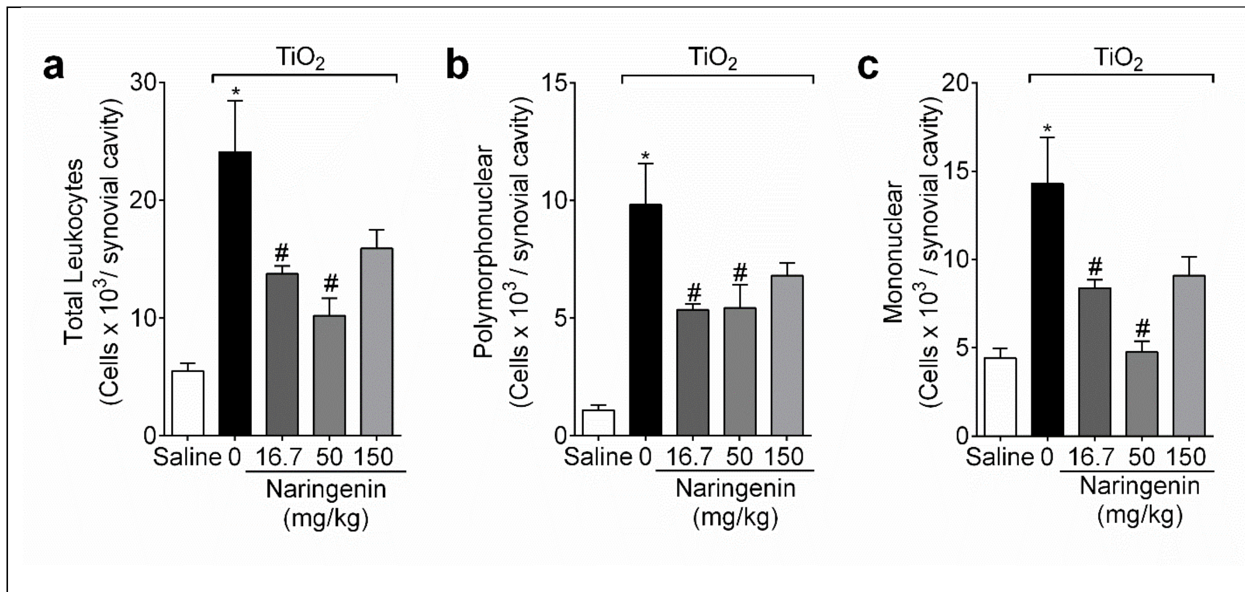

Figure 2. Naringenin inhibits TiO_2-induced recruitment of total leukocytes, polymorphonuclear, and mononuclear. Mice were treated daily during 30 days with naringenin (16.7, 50, or 150 mg/kg, p.o.) 24 h after intra-articular injection of TiO_2 (3 mg/ joint). (a) Total leukocytes, (b) polymorphonuclear, and (e) mononuclear cells counts in knee joint washes were evaluated 30 days after TiO_2 injection. Results are presented as mean ± SEM of six mice per group per experiment and are representative of two separate experiments. [*$p < 0.05$ compared to the saline group; #$p < 0.05$ compared to the TiO_2 group (one-way ANOVA followed by Tukey's *post hoc*)].

Naringenin Does Not Induce Stomach, Liver, or Kidney Damage. Mice were treated daily during 30 days with the dose of 50 mg/kg of naringenin by p.o. route starting 24 h after

the i.a. injection of 3 mg/joint of TiO_2. Stomach and plasma samples were harvested to determine myeloperoxidase (MPO) activity in stomach samples and the plasma concentration of aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, and creatinine (Figure 3) as previously described.51–53 Indomethacin-treated mice (2.5 mg/kg, i.p., diluted in tris/HCl buffer, for 7 days) were used as positive control for stomach injury. Acetaminophen-treated mice were used as positive control for liver injury (650 mg/kg, orally, diluted in sterile saline, 200 mg/mL, once). Diclofenac-treated mice (200 mg/kg, orally, diluted in sterile saline, once) were used as positive control for kidney injury. The treatment with the dose of 50 mg/kg of naringenin did not alter gastric MPO activity (Figure 3a), the plasma concentration of AST (Figure 3b), ALT (Figure 3c), urea (Figure 3d), and creatinine (Figure 3e). Indomethacin (a nonselective cyclooxygenase inhibitor with known gastrointestinal side effect) induced significant increase of gastric MPO activity (Figure 3a). Acetaminophen (an analgesic and antipyretic drug known to induce liver damage) induced significant increase of AST (Figure 3b) and ALT (Figure 3c) plasma concentration. Diclofenac (a nonselective cyclooxygenase inhibitor that induces kidney damage) induced significant increase of urea (Figure 3d) and creatinine (Figure 3e) plasma concentration corroborating previous findings.51–53 Current treatment for chronic pain is based in opioids and non-steroidal anti-inflammatory drugs.54 Focusing on arthritis, this treatment lies on the use of glucocorticoids, non-steroidal anti-inflammatory drugs, and disease modifying anti-rheumatic drugs.55 These drugs often induce side effects including gastrointestinal complications, cardiovascular effects, nausea, and others.56 Here, we provided evidence that naringenin is a safe drug given that long-term treatment did not induce gastric (Figure 3a), liver (Figure 3b-c), or kidney (Figure 3d-e) damage. Of note *in vitro* cell viability assay demonstrated that naringenin presents low toxicity when compared to other flavonoids, even at high concentration such as 200 μM.57 In agreement with the data above, naringenin

treatment for 7 days does not induce liver and stomach damage.33 The treatment with positive control groups following previously established protocols demonstrates that the assays used can detect the selective tissue lesion markers51–53 and the lack of tissue lesion markers alteration upon 30 days of naringenin treatment demonstrated it did not induce gastric, liver, or renal damage.

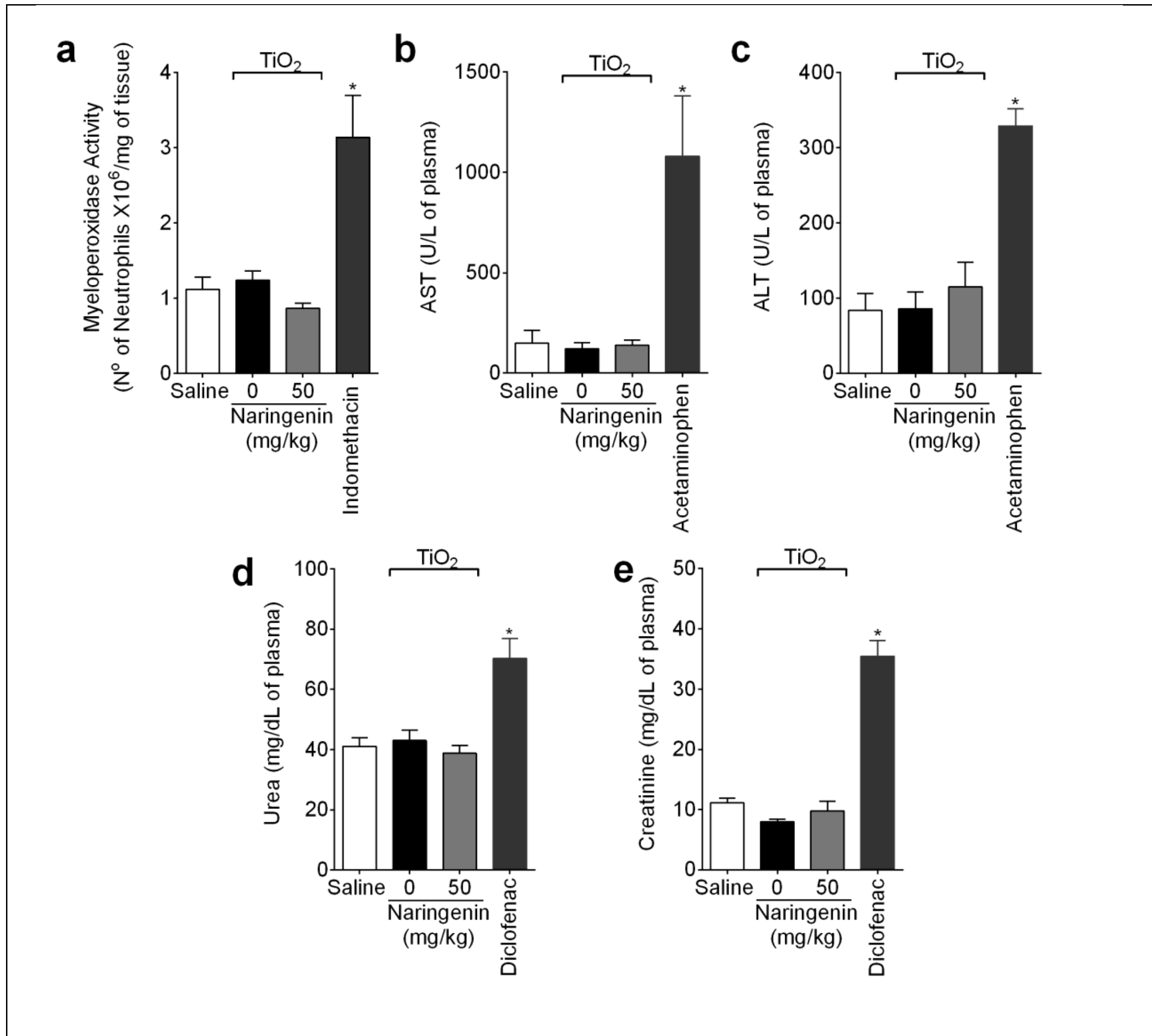

Figure 3. Naringenin does not induce stomach, liver, or kidney damage. Mice were treated daily during 30 days with naringenin (50 mg/kg, p.o.); or indomethacin (2.5 mg/kg, i.p., diluted in tris/HCl buffer) during 7 days; or acetaminophen (650 mg/kg, ip, diluted in saline, 200 mg/mL); or diclofenac (200 mg/kg, p.o., diluted in saline) were administrated only once. (a) MPO activity in the stomach; (b) AST and (c) ALT plasmatic levels; (d) urea and (e)

creatinine plasmatic levels were determined. Results are presented as mean ± SEM of six mice per group per experiment and are representative of two separate experiments. [*$p < 0.05$ compared to the saline, TiO_2, and naringenin (50 mg/kg, p.o.) groups (one-way ANOVA followed by Tukey's *post hoc*)].

Naringenin Inhibits TiO_2-Induced Oxidative Stress. Next, we wonder about the effect of naringenin in knee joint oxidative stress, considering that TiO_2-induced chronic arthritis induces superoxide anion production by increasing gp91^{phox} expression in knee joint (Borghi et al., under review). Mice were treated daily during 30 days with the dose of 50 mg/kg of naringenin by p.o. route starting 24 h after the i.a. injection of 3 mg/joint of TiO_2. Joint was harvested 30 days after stimulus for determination of gp91^{phox} mRNA expression (Figure 4a), superoxide anion production (Figure 4b), and lipid peroxidation (Figure 4c). TiO_2 induced an increase of gp91^{phox} expression (Figure 4a) a NOX2 component involved in superoxide anion production,58 which was inhibited by naringenin. In agreement, naringenin also inhibited TiO_2-induced superoxide anion production (Figure 4b) and lipid peroxidation (Figure 4c). ROS production induces oxidative damage in biomolecules, including lipdis. ROS attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids, producing a wide variety of oxidation products, including malondialdehyde (MDA), an oxidation product of omega-3 and omega-6 fatty acids and commonly used biomarker for oxidative stress.59 For instance, TiO_2 induces the production of ROS in others models not related to arthritis.60–64 Indeed, TiO_2 under UVA irradiation induces ROS production increasing MDA levels in human keratinocyte cell line HaCaT.64 In addition, TiO_2 enhances neutrophils phagocytosis in Syk (spleen tyrosine kinase)-dependent manner40 and as consequence activates NADPH oxidase generating ROS.65 These mechanisms provide deleterious consequences in the inflammatory milieu and perpetuate a loop that leads to joint

destruction, given that NADPH oxidase, ROS, and MPO are required for neutrophils undergo NETosis.46 The release of NETs seems important in implant-related arthritis considering that TiO_2 triggers histone citrullination and NETs release.66 Focusing on oxidative stress, injection of a superoxide anion donor induces inflammatory pain in a NFκB-dependent manner67 suggesting that there is a close relationship between oxidative stress, inflammation, and pain. For instance, superoxide anion-induced pain and oxidative stress are inhibited in TNFR1 knockout mice, etanercept-68, and PDTC-treated mice.67 Therefore, strategies aimed at inhibiting oxidative stress is likely to be a highly attractive. Naringenin possesses *in vitro* and *in vivo* antioxidant activity.30,33,57 In fact, *in vitro* data show that naringenin presents scavenger ability at 40 μM and molecular docking demonstrated that naringenin reduces NADPH oxidase activation by inhibiting PKC-mediated $p47^{phox}$ phosphorylation by interacting with Gly-253 and Leu-251 amino acid residues.57 In addition, *in vivo* data show that naringenin also increases Nrf2 activation, a transcription factor that are related to expression of antioxidant molecules such as GSH30,33 suggesting that naringenin not only reduces ROS but also increases antioxidant defense. In agreement with the data above, naringenin inhibits oxidative stress in superoxide anion-30 and carrageenan-induced33 inflammatory pain, streptozotocin-induced diabetic neuropathy,69 and UVB radiation-induced oxidative stress in hairless mice.31 On the other hand, these effects of naringenin seems to impair neutrophils microbicidal activity. For instance, naringenin inhibits the killing of *Staphylococcus* aureus by neutrophils.70 Therefore, given the relationship between oxidative stress and pain, the inhibition of the parameters herein evaluated is an important mechanism of naringenin to mitigates aseptic inflammatory pain.

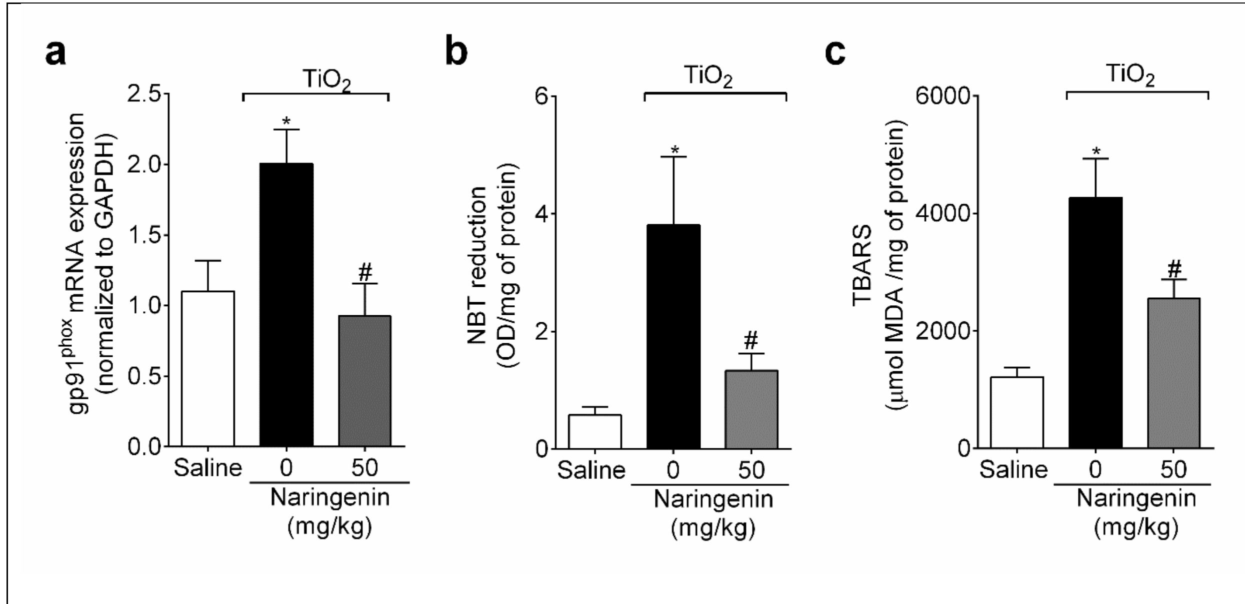

Figure 4. Naringenin inhibits TiO_2-induced oxidative stress. Mice were treated daily during 30 days with naringenin (50 mg/kg, p.o.) 24 h after TiO_2 i.a. injection (3 mg/joint). (a) gp91^{phox} mRNA expression, (b) superoxide anion production, and (c) TBARS levels were determined 30 days after TiO_2 injection. Results are presented as mean ± SEM of six mice per group per experiment and are representative of two separate experiments. [$*p < 0.05$ compared to the saline group; $\#p < 0.05$ compared to the TiO_2 group (one-way ANOVA followed by Tukey's *post hoc*)].

Naringenin Inhibits TiO_2-Induced Cytokines mRNA Expression. TiO_2 induces the production of cytokines21,23,40,41,71 that modulates inflammation and pain.39 In fact, TiO_2 induces the release of cytokines including IL-33, (Fattori et al., under preparation) TNF-α, IL-1β, and IL-6 in chronic arthritis (Borghi et al., under review). Mice were treated daily during 30 days with naringenin p.o. route starting 24 h after the i.a. injection of 3 mg/joint of TiO_2. Joint was harvested 30 days after stimulus for determination of cytokines (IL-33, TNF-α, and pro-IL-1β) mRNA expression by RT-qPCR (Figure 5). We observed that treatment with naringenin inhibited TiO_2-induced expression of IL-33 (Figure 5a), TNF-α (Figure 5b), and pro-IL-1β (Figure 5c). Interestingly, dorsal root ganglion sensory neurons can internalize

TiO_2 nanoparticles and increase the production of ROS and IL-1β, collaborating with pain-state.72 Also, in a long-term exposure can trigger or aggravate neurogenerative diseases. Mechanistically, IL-1β maturation occurs through several types of inflammasome73 and TiO_2 induces IL-1β release through NLRP3 activation.41 The inhibition of pro-inflammatory cytokines herein observed is important given that IL-33,74 TNFα,75 and IL-1β76 activate sensory neurons. Also, intra-articular injection of IL-33 induces mechanical hyperalgesia, which is dependent on the sequential release of TNF-α, IL-1β, INF-γ, ET-1, and PGE_2.77 In inflammatory arthritis increasing amounts of hyperalgesic cytokines including IL-33, TNFα, and IL-1β contributes to pathophysiology of joint inflammation and pain.77,78 Therefore, the inhibition of TiO_2-induced cytokine mRNA expression by naringenin might account to its anti-inflammatory and analgesic mechanisms.

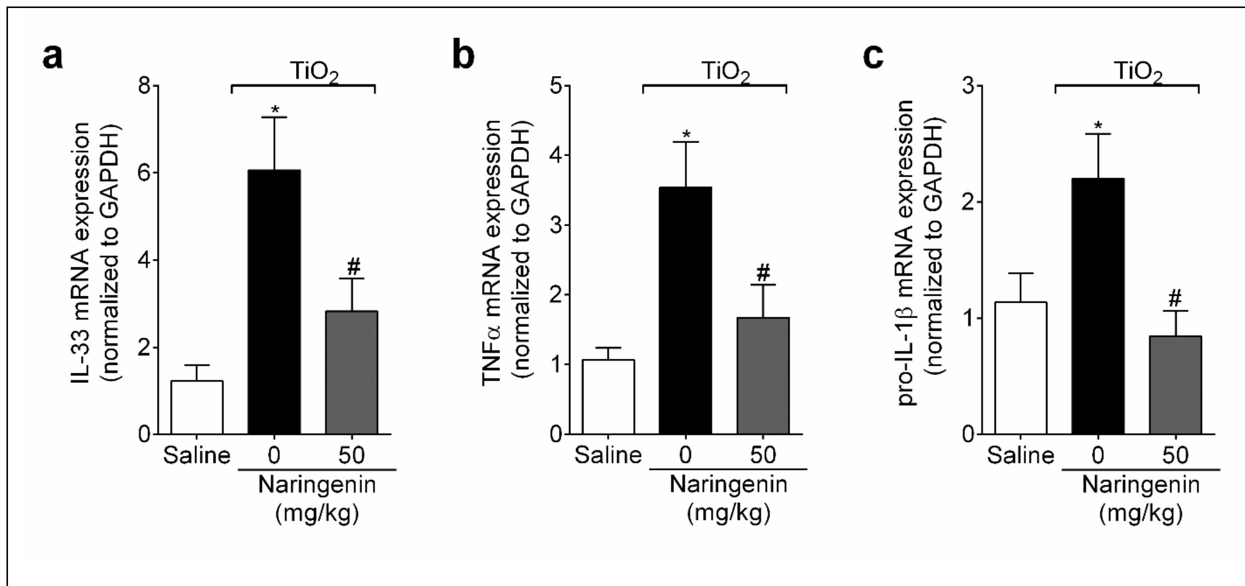

Figure 5. Naringenin inhibits TiO_2-induced cytokines mRNA expression. Mice were treated daily during 30 days with naringenin (50 mg/kg, p.o.) 24 h after TiO_2 i.a. injection (3 mg/joint). (a) IL-33, (b) TNF-α, and (c) pro-IL-1β mRNA expression were determined by RT-qPCR 30 days after TiO_2 injection. Results are presented as mean ± SEM of six mice per group per experiment and are representative of two separate experiments. [*$p < 0.05$ compared to the saline group; #$p < 0.05$ compared to the TiO_2 group (one-way ANOVA

followed by Tukey's *post hoc*)].

Naringenin Inhibits TiO_2-Induced NFκB Activation. NFκB activation plays an essential role in the development and progression of inflammatory arthritis once it is direct involved in the production of pro-inflammatory mediators in the inflamed joint.79 In fact, bioaccumulated TiO_2 in brain of rats induces NFκB activation and TNFα release.71 Also, i*n vitro* evidence in HepG2 cells suggest that TiO_2 activates NFκB signaling pathway and possesses genotoxic effect.80 Given that, we wonder whether TiO_2 induces joint NFκB activation and the modulation of this pathway by naringenin. Mice were treated daily during 30 days with naringenin p.o. route starting 24 h after the i.a. injection of 3 mg/joint of TiO_2. Joint was harvested 30 days after stimulus for determination of NFκB activation by ELISA (Figure 6). We observed that naringenin inhibited TiO_2-induced NFκB activation (Figure 6), which explains the inhibition of hyperalgesic cytokines mRNA expression including IL-33, TNF-α, and pro-IL-1β (Figure 5). For instance, TiO_2-induced IL-33 (Fattori, under preparation) TNFα, and IL-1β13,14 contributes to NFκB activation81–83 that maintains and perpetuates the aseptic inflammatory process in the periprosthetic tissue.11,13,14 In agreement with our data, naringenin also inhibits *in vivo* and *in vitro* carrageenan-33 and LPS-induced32 NFκB activation. Therefore, the inhibition of TiO_2-induced NFκB activation by naringenin might account to its anti-inflammatory and analgesic mechanisms.

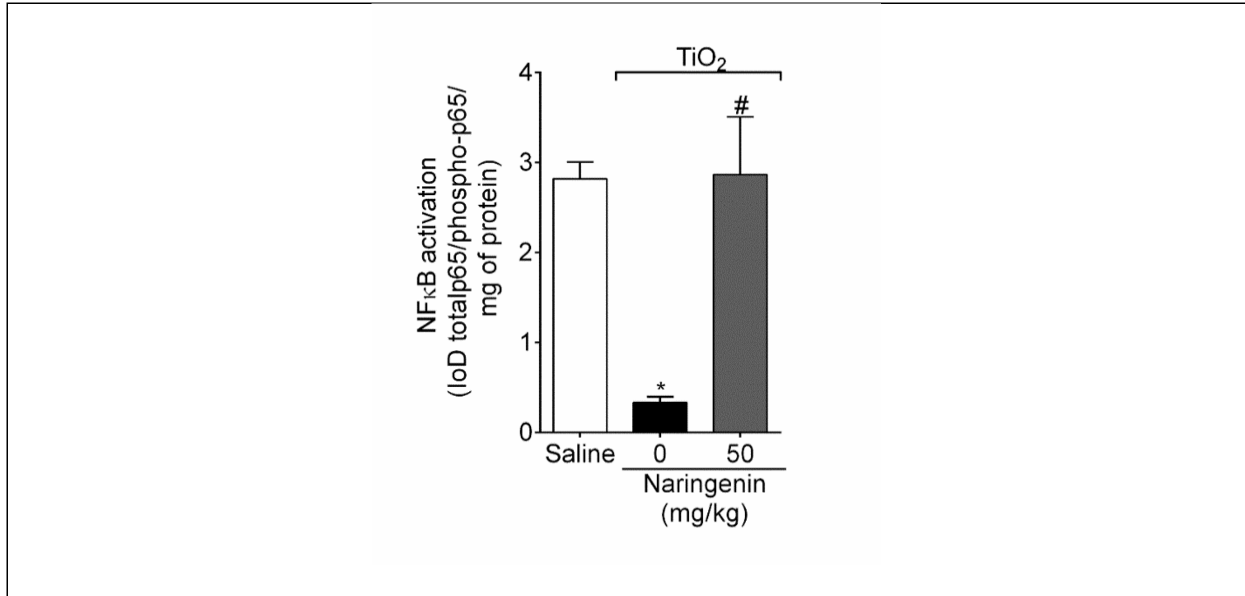

Figure 6. Naringenin inhibits TiO_2-induced NFκB activation. Mice were treated daily during 30 days with naringenin (50 mg/kg, p.o.) 24 h after TiO_2 i.a. injection (3 mg/joint). NFκB activation determined 30 days after TiO_2 injection. Results are presented as mean ± SEM of six mice per group per experiment and are representative of two separate experiments. [*$p <$ 0.05 compared to the saline group; #$p < 0.05$ compared to the TiO_2 group (one-way ANOVA followed by Tukey's *post hoc*)].

Naringenin Modulates RANKL/RANK/OPG System in TiO_2-Induced Chronic Arthritis. The levels of RANKL, RANK, and osteoprotegerin (OPG) regulate bone resorption.84 RANKL binds to RANK inducing osteoclast-mediated bone resorption. In fact, bone erosion and osteoclast activation were reduced in RANK knockout mice.17 On the other hand, OPG binds to RANKL that inhibits RANK-induced bone resorption and acts as a negative regulator of bone resorption.84 Thus the ratio between of RANKL/OPG is an important determinant of bone mass.84 RANKL/RANK induces osteoclasts progenitors activation and changes to multinucleated actives osteoclasts.85 In fact, neutrophil-osteoclast interaction can induce osteoclast differentiation into a large and TRAP (tartrate-resistant acid phosphatase)-positive cell contributing to bone ressorbtion.86 In addition, NFκB signaling

pathway possesses important role in RANKL-induced osteoclast formation given that NFκB selective inhibitors such as SC-514 (IKKβ inhibitor) and NBD (IKKγ inhibitor) block osteoclast differentiation.87 Also, cytokines such as TNFα and IL-6 induce osteoclastogenesis in osteoclast medium culture with synovial cells from RANK knockout mice, showing a RANK-independent manner to induces osteoclast formation.17 In this sense, TiO_2 induces osteoclast activation and bone resorption in chronic arthritis (Borghi et al., under review) through dependent-16 or independent-RANK-induced17 osteoclast activation. Herein, in order to assess the effect of naringenin on RANKL/RANK/OPG system in TiO_2-induced chronic arthritis, mice were treated daily during 30 days with naringenin p.o. route starting 24 h after the i.a. injection of 3 mg/joint of TiO_2. Joint was harvested 30 days after stimulus for determination of RANKL, RANK, and OPG mRNA expression by RT-qPCR (Figure 7). TiO_2 increased RANKL (Figure 7a) expression without altering RANK (Figure 7b). On the other hand, treatment with naringenin inhibited TiO_2-induced RANKL (Figure 7a) and decreased RANK expression (Figure 7b). TiO_2 did not alter OPG expression (Figure 7c). Naringenin induced OPG expression (Figure 7c) in TiO_2-induced chronic arthritis. In agreement, naringenin inhibits bone resorption in titanium particles-induced calvarial osteolysis.34 Mechanistically, naringenin inhibits F-actin ring polarization of osteoclasts, which is an essential step for bone resorption. Also, naringenin interferers in the RANKL/RANK/OPG system by reducing RANKL-induced p38 MAPK activation in osteoclasts.34 Moreover, naringenin inhibits TNFα30–32 and IL-631,32production and might inhibits bone resorption through RANK-independent manner. Given the importance of osteoclasts in bone resorption, these data suggest that naringenin may be promising flavonoid to control TiO_2-induced osteoclastogenesis and bone resorption.

In conclusion, the present data suggest that naringenin mitigates TiO_2-induced inflammatory pain, knee edema, and leukocyte recruitment by inhibiting oxidative stress,

cytokine mRNA expression, NFκB activation, as well as modulating RANKL/RANK/OPG system. Naringenin is a pleiotropic molecule and its analgesic effect in chronic arthritis involves targeting several signaling pathways and cells. Therefore, naringenin represents a promising therapeutic approach to mitigate complications related to implant-induced aseptic inflammation.

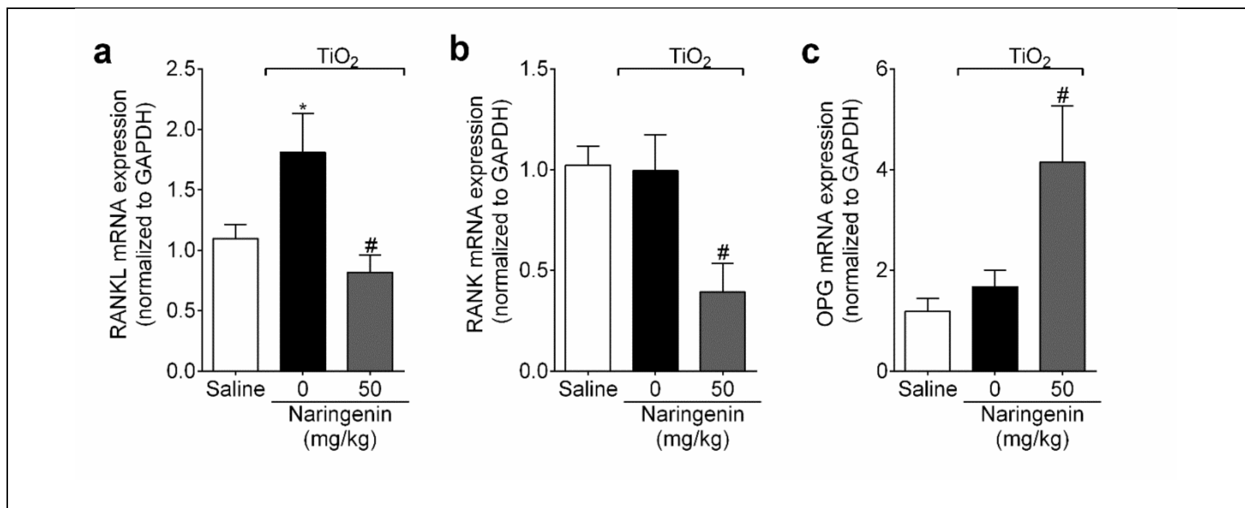

Figure 7. Naringenin modulates RANKL/RANK/OPG system in TiO2-induced chronic arthritis. Mice were treated daily during 30 days with naringenin (50 mg/kg, p.o.) 24 h after TiO_2 i.a. injection (3 mg/joint). (a) RANKL, (b) RANK, and (c) OPG mRNA expression were determined 30 days after TiO_2 injection. Results are presented as mean ± SEM of six mice per group for qPCR analysis per experiment and are representative of two separate experiments. [*$p < 0.05$ compared to the saline group; #$p < 0.05$ compared to the TiO_2 group (one-way ANOVA followed by Tukey's test)].

Experimental Section

General Experimental Procedures. In the first series of experiments, mice ($n = 6$ per group per experiment) were submitted to a dose-response experiment of naringenin treatment. Mice were stimulated i.a. with 3 mg of TiO_2 (suspended in 10 μL of sterile saline solution 0.9%) per knee joint (Borghi et al., under review). Mechanical hyperalgesia and edema were

evaluated 24 h after i.a. administration of TiO_2 to assess the arthritis model induction. Subsequently after 24 h, mice were treated with naringenin30 (16.7, 50, or 150 mg/kg, p.o.) and mechanical hyperalgesia and edema were evaluated from 1, 3, 5, 7, and 24 h hours after naringenin treatment in the first day (Borghi et al., under review). After that, mechanical hyperalgesia and edema were evaluated every other day 1h after naringenin treatment until the 30th day. Immediately after the measurements on 30th day, mice were anesthetized and euthanized, and washes knee joint cavities samples were harvested for leukocytes migration evaluation. The naringenin dose of 50 mg/kg was chosen based on the results hyperalgesia, edema, and leukocytes recruitment. Stomach and blood samples were harvested to evaluate stomach myeloperoxidase (MPO) activity, and the plasmatic levels of aspartate transaminase (AST), alanine transaminase (ALT), urea, and creatinine. Knee joint was harvested to evaluate oxidative stress (superoxide anion [nitroblue tetrazolium reduction levels], lipid peroxidation [TBARS assay], and $gp91^{phox}$ mRNA expression [RT-qPCR]), NFκB activation (ELISA), pro-inflammatory cytokines mRNA expression (IL-33, TNFα, and proIL-1β), and RANKL/RANK/OPG signaling pathway (RT-qPCR).

Animals. Male Swiss mice weighing between 20-25 g from the Londrina State University, Londrina, Paraná, Brazil, were used in this study. Mice were housed in standard clear plastic cages with water and food *ad libitum*, light / dark cycle of 12 / 12h and controlled temperature (21°C). Mice were maintained in the vivarium of the Department of Pathology of Londrina State University for at least two days before experiments. Mice were used only once and were acclimatized to the testing room at least 1 hour before the experiments, which were conducted during the light cycle. Animal care and handling procedures were in accordance with the International Association for Study of Pain (IASP) guidelines and approved by the Londrina State University Ethics Committee on Animal Research and Welfare (process number

11849.2015.19). All efforts were made to minimize the number of animals used and their suffering.

Test Compounds. The compounds used in this study were saline solution (NaCl 0.9%; Frenesius Kabi Brasil Ltda, Aquiraz, CE, Brazil), Ethylenediaminetetraacetic acid disodium salt (EDTA; Synth, Diadema, SP, Brazil), and naringenin (Santa Cruz Biotechnology, Inc., 98%, Dalla, TX, USA). Titanium dioxide was purchased from Synth (Diadema, SP, Brazil) and particle size was < 1μm with an average of 862,2 nm as determined by size distribution analysis (Malvern Instruments Ltd, UK). Immediately before the injections, TiO_2 was suspended in sterile saline (10 μL) and naringenin was diluted in sterile saline solution. Naringenin (16.7, 50, or 150 mg/kg) and saline were administrated by p.o. in a volume of 100 μL.

Evaluation of Articular Mechanical Hyperalgesia. The articular mechanical hyperalgesia of the femorotibial joint was evaluated as previously described88. Briefly, in a quiet room, mice were placed individually in acrylic cages (12 x 10 x 17 cm) with a wire grid floor 15-30 minutes before the test for environmental adaption. Force application was performed only when animals were quiet, did not display exploratory movements or defecation, and were not resting on the paws. An electronic pressure-meter test consisting of a hand-held force transducer fitted with a polypropylene tip (electronic von Frey Anesthesiometer; Insight, Ribeirão Preto, SP, Brazil) was used to evaluate mechanical articular nociception. For this model, a large tip (4.15 mm^2) was adapted to the probe. An increasing perpendicular force was applied to the central area of the plantar surface of the hind paw to induce a flexion movement of the femorotibial joint followed by paw withdrawal. A tilted mirror below the grid provided a clear view of the hind paw. The electronic pressure-meter apparatus automatically recorded the intensity of the force applied when the paw was withdrawn. The test was repeated until the subsequent consistent measurements (i.e. the variation among these

measurements was less than 1 g) were obtained. The flexion-elicited mechanical threshold was expressed in grams (g).88

Articular Edema Measurements. Articular edema of the femorotibial joint was assessed through measurements of the transverse diameters using a caliper (Digmatic Caliper, Mitutoyo Corporation, Kanagawa, Japan). Thickness values of the femorotibial joint were expressed as the difference between the diameters measured before (basal value) and after TiO_2 intra-articular injection in millimeters (mm)89.

Leukocyte Migration Evaluation. The total and differential counts of recruited leukocytes to the intra-articular space were determined as previously described.45 Briefly, knee joint cavities were washed with saline containing Ethylenediaminetetraacetic acid (EDTA), which was recovered to evaluate total and differential cell counts. Total cell counts were performed in Neubauer chamber using Turk solution, and differential cell counts (100 cells per slide) were performed in slices stained with the panoptic kit (Laborclin, Pinhais, PR, Brazil) under a light microscope (Olympus CX31RTSF, Tokyo, Japan). Results were expressed as total leukocytes, polymorphonuclear, and mononuclear cells (cells x 10^3/ knee joint).

Myeloperoxidase Activity. Myeloperoxidase (MPO) activity was determined using a colorimetric assay, considering it is a reliable marker of the neutrophil migration to the tissues.90,91 Samples of the stomach were collected in 50 mM K_2HPO_4 buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide (HTAB) and kept at -86°C until use. Frozen samples were homogenized using a tissue turrax (Tissue-Tearor 985370, BioSpec Products, Bartlesville, OK, USA), centrifuged (2 min, 16,000 *g*, 4°C) and the resulting supernatant assayed using a spectrophotometer (Multiskan GO Microplate Spectrophotometer, ThermoScientific, Vantaa, Finland) for MPO activity determination at 450 nm. The MPO activity of samples was compared to a standard curve of neutrophils. Briefly, 15 μL of sample were mixed with 200 μL of 50 mM phosphate buffer (pH 6.0),

containing 0.167 mg/mL O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The results were presented as MPO activity (number of neutrophils X 10^6 / mg of tissue).

Liver and Kidney Toxicity Assays. Blood was harvested into microtubes containing 50 μL of the anticoagulant EDTA (5,000 IU/mL) and centrifuged (200 *g*, 10 min, 4 °C), and the plasma was separated. In other to determine enzymatic activities of AST and ALT as indicators of hepatotoxicity, and urea and creatinine levels as indicators of nephrotoxicity, plasma samples were processed according to the manufacturer's instructions (Labtest Diagnóstico S. A., Brazil). Results were presented as U/mL (AST and ALT) or mg/dL (urea and creatinine) of plasma.

Nitroblue Tetrazolium Reduction. The superoxide anion production was determined by the reduction of the redox dye Nitroblue tetrazolium (NBT).51 Knee joint frozen tissue from mice were homogenized with 500 μL of saline using an ultra-turrax (Tissue-Tearor 985370, BioSpec Products, Bartlesville, OK, USA), centrifuged (10 min, 3,300 *g*, 4 °C) and 50 μL of the homogenate was placed in 96-well plate, followed by the addition of 100 μL of nitro blue tetrazolium solution (1 mg/mL) (NBT, Sigma) and maintained at 37°C in warm bath for 5 minutes. The supernatant was removed, and the formazan precipitated was then solubilized by adding 120 μL of 2 M KOH and 120 μL of dimeltisulfoxide (DMSO). The optical density was measured using a microplate spectrophotometer reader (Multiskan GO Microplate Spectrophotometer, Thermoscientific, Vantaa, Finland) at 600 nm. The NBT reduction levels were corrected per the total protein concentration and the results were presented as NBT reduction (OD/mg of protein).

Lipid Peroxidation. Tissue lipid peroxidation was assessed by the levels of thiobarbituric acid reactive substances (TBARS).92 For this assay, TCA 10% was added to the homogenate and the mixture was centrifuged (3 min, 1000 *g*, 4 °C) to precipitate proteins. The protein-free supernatant was then separated and mixed with TBA (0.67%). The mixture was kept in a

water bath (15 min, 100°C). Malondialdehyde (MDA), an intermediate product of lipid peroxidation, was determined by the difference between absorbance at 535 and 572 nm using a microplate spectrophotometer reader. The TBARS were corrected per the total protein concentration and results were presented as TBARS (µmol of MDA/mg of protein).92

NFκB Activation. Mice were treated daily with vehicle or naringenin (50 mg/kg) after TiO_2 stimulus. Thirty days after the injection of TiO_2, mice were euthanized for the collection of the joint samples. The joint samples were homogenized in 400 μL of the appropriate buffer containing protease inhibitors. The homogenates were centrifuged (10 min, 16,100 *g*, 4 ºC), and the supernatants were used to assess the levels of total and phosphorylated NFκB p65 subunit by ELISA using PathScan® kits (Cell Signaling) at 450 nm (Multiskan GO Thermo Scientific) according to the manufacturer's directions. The total and phosphorylated NFκB p65 subunit were corrected per the total protein concentration and results are expressed as NFκB activation (IoD totalp65/phospho-p65 ratio/mg of protein)

RT-qPCR. Samples were homogenized in TRIzol reagent, and total RNA was extracted by using the SV Total RNA Isolation System (Promega). The purity of total RNA was measured using a spectrophotometer (Multiskan GO Microplate Spectrophotometer, Thermoscientific, Vantaa, Finland) and the wavelength absorption ratio (260/280) 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. qPCR reaction was performed in StepOnePlus™ Real-Time PCR System (Applied Biosystems®). The relative gene expression was measured using the comparative $2^{-(\Delta\Delta Cq)}$ method. The primers used were: gp91phox, sense: 5´-AGCTATGAGGTGG TGATGTTAGTGG-3´, antisense: 5´-CACAATATTTGTACCAGACAGACTTGAG-3´; IL-33, sense: 5´-TCCTTGCTTGGCAGTATCCA-3´, antisense: 5´-TGCTCAATGTGTCAACAGACG-3´; TNFα, sense: 5´-TCTCATCAGTTCTATGGCCC-3´, antisense: 5´-GGGAGTAGAC

AAGGTACAAC-3´; pro-IL-1β, sense: 5-´GAAATGCCACCTTTTGACAGTG-3´, antisense: 5´-TGGATGCTCTCATCAGGACAG-3´, RANK, sense: 5´-CTAATCCAGCAG GGAAGCAAAT-3´, antisense: 5´-GACACGGGCATAGAGTCAGTTC-3´; RANKL, sense: 5´-CAGAAGATGGCACTCACTGCA-3´, antisense: 5´-CACCATCGCTT TCTCTGCTCT-3´; OPG, sense: 5´-GGAACCCCAGAGCGAAATACA-3´, antisense: 5´-CCTGAAGAATGCCTCCTCACA-3´; glyceraldehyde 3-phosphate dehydrogenase (Gapdh), sense: 5´-CATACCAGGA AATGAGCTTG-3´, antisense: 5´-ATGACATCAAGAAGGTGGTG- The expression of Gapdh mRNA was used as reference gene, and the results were expressed as mRNA expression (normalized to Gapdh).

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 $P < 0.05$.

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Conflict of interest

The authors declare no conflict of interest.

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5. ARTIGO – PUBLICADO (PLOS ONE)

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RESEARCH ARTICLE

Naringenin Inhibits Superoxide Anion-Induced Inflammatory Pain: Role of Oxidative Stress, Cytokines, Nrf-2 and the NO-cGMP-PKG-K_{ATP}Channel Signaling Pathway

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Abstract

In the present study, the effect and mechanism of action of the flavonoid naringenin were evaluated in superoxide anion donor (KO_2)-induced inflammatory pain in mice. Naringenin reduced KO_2-induced overt-pain like behavior, mechanical hyperalgesia, and thermal hyperalgesia. The analgesic effect of naringenin depended on the activation of the NO−cGMP −PKG−ATP-sensitive potassium channel (K_{ATP}) signaling pathway. Naringenin also reduced KO_2-induced neutrophil recruitment (myeloperoxidase activity), tissue oxidative stress, and cytokine production. Furthermore, naringenin downregulated KO_2-induced mRNA expression of gp91phox, cyclooxygenase (COX)-2, and preproendothelin-1. Besides, naringenin upregulated KO_2-reduced nuclear factor (erythroid-derived 2)-like 2 (Nrf2) mRNA expression coupled with enhanced heme oxygenase (HO-1) mRNA expression. In conclusion, the present study demonstrates that the use of naringenin represents a potential therapeutic approach reducing superoxide anion-driven inflammatory pain. The antinociceptive, anti-inflammatory and antioxidant effects are mediated via activation of the NO−cGMP−PKG −K_{ATP} channel signaling involving the induction of Nrf2/HO-1 pathway.

Introduction

Pain is an unpleasant sensory and emotional experience, generally in association with tissue injury. During inflammation, pro-inflammatory mediators activate resident cells, recruited cells and nociceptors, thereby driving pain signaling. Nociceptive neurons do not express receptors for all inflammatory molecules, suggesting both direct and indirect activation and

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sensitization of nociceptors [1]. Increased levels of oxidative stress during the inflammatory response also contribute to nociception. For instance, reactive oxygen species (ROS) and reactive nitrogen species (RNS) can act directly and indirectly to induce nociceptor sensitization and activation [2–5]. The superoxide anion (O_2^-) is a common form of ROS that can drive nociception [5,6]. O_2^- reacts with nitric oxide (NO) generating peroxynitrite, which also contributes to nociception [3]. Superoxide dismutase (SOD), an enzymatic antioxidant, transforms superoxide anion in hydrogen peroxide, which may also induce nociception [2]. Therefore, O_2^- is a crucial ROS to the biological underpinnings driving nociception. O_2^- increases other pro-inflammatory effects, including increasing vascular permeability [7], inducing cytokine release [8,9] and increasing neutrophil recruitment [9,10], as well as provoking overt pain-like behavior and hyperalgesia [2–5]. In a physiological state, O_2^- levels remain under control by the action of the endogenous antioxidant systems, including SOD, and the endogenous antioxidant reduced glutathione (GSH) [9]. However, the imbalance between oxidants and antioxidants during inflammation leads to oxidative stress. This is important, as inhibiting the production of pro-inflammatory cytokines and ROS limit the development of inflammatory pain [6,11–13].

Naringenin (4',5,7-tryhidroxy-flavonone) is a flavonoid which belongs to flavonones class found in citric fruits, including lemon, orange, tangerine and grapefruit [14]. Naringenin inhibits the nociceptive responses in models of formalin-, acetic acid- and capsaicin-induced overt pain, as well as neuropathic pain [15–17]. Naringenin also inhibits inflammation by targeting cyclooxygenase (COX)-2 in ethanol-induced liver injury [18] and *in vitro* [19]. Moreover, naringenin inhibits phosphorylation of nuclear factor kappaB (NFκB) subunit p65 and mitogen-activated protein kinases (MAPK) in daunorubicin-induced nephrotoxicity [20] as well as inhibiting the EGFR-PI3K-Akt/ERK MAPK signaling pathway in human airway epithelial cells [21]. Naringenin also inhibits a number of aspects of oxidative stress, including lipid peroxidation and O_2^- production, as well as restoring GSH levels in UVB-induced oxidative stress in the skin of Hairless mice [22]. Furthermore, naringenin increases SOD in an experimental stroke model, highlighting its wide-acting induction of endogenous antioxidants [23]. In agreement with such antioxidant effects, naringenin also induces nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/ heme oxygenase (HO)-1 in CCl_4-induced hepatic inflammation [24]. Some flavonoids can induce antinociception by activating the NO–cGMP–PKG–ATP-sensitive potassium channel (K_{ATP}) signaling pathway [25–28]. Activating this signaling pathway is an important mechanism of action of a number of clinical analgesics, such as opioids [25], and some non-steroidal anti-inflammatory drugs including dipyrone [26], diclofenac [27], and indomethacin [28].

Given the above, the current study addresses the analgesic effects of naringenin in a model of O_2^--triggered inflammatory pain. It was also investigated as to whether naringenin's mechanism of action involves the NO–cGMP–PKG–K_{ATP} channel signaling pathway, the regulation of inflammatory mediators/enzymes and oxidative stress as well as the transcription factor Nrf2, and its downstream target, HO-1.

Materials and Methods

Animals

Male Swiss mice (25 ± 5 g) from Londrina State University were housed in standard plastic cages with free access to food and water, with a light/dark cycle of 12:12 h, at 21°C. All behavioral testing was performed between 9 a.m. and 5 p.m. in a temperature-controlled room. At the end of experiments, mice were anesthetized with isoflurane 3% to minimize suffering (Abbott Park, IL, USA) and killed by cervical dislocation followed by decapitation. The animal

condition was monitored daily and at indicated time points during the experiments. No unexpected animal deaths occurred during this study. Animals' care and handling procedures were in accordance with the International Association for Study of Pain (IASP) guidelines and with the approval of the Ethics Committee of the Londrina State University (process 133666.2013.71).

Drugs and reagents

Potassium superoxide (KO_2) (Alfa Aesar, 96,5%, Ward Hill, MA, USA). Naringenin (Santa Cruz Biotechnology, Inc., 98%, Dalla, TX, USA). Saline (NaCl 0,9%; Fresenius Kabi Brazil Ltda. Aquiraz, CE, Brazil). L-NAME (Research Biochemicals, Natick, MA, USA), KT5823 (Calbiochem, San Diego, CA, USA), ODQ [1H-(1,2,4)-oxadiazolol-(4,3-a)-quinoxalin-1-one, Tocris Cookson, Baldwin, MO, USA]. Glybenclamide, HTAB (Bromide, hexadecyl trimethyl ammonium), dihydrochloride O-dianisidine, GSH (reduced glutathione), EDTA sodium salt, ferric chloride hexahydrate, TPTZ (2,4,6-tripiridil-s-triazine) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-11 carboxylic) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DMSO 2% and Tween 80 (Química Moderna, Barueri, SP, Brazil).

Experimental procedures

Mice were pretreated per orally (po) with 16.7, 50, or 150 mg/kg of naringenin or with vehicle (saline) 1 h before intraplantar (ipl) or intraperitoneal (ip) injection of 30 μg or 1 mg of KO_2, respectively [6]. The writhing response was evaluated between 0–20 min after KO_2 ip injection and the paw flinching and licking nociceptive responses were quantified during 30 min after ipl injection of KO_2. Mechanical and thermal hyperalgesia were evaluated 0.5–7 h after KO_2. Inflammatory stimulation with KO_2 induced mechanical and thermal hyperalgesia only in the ipsilateral paw, in the side where the stimulus was injected. Myeloperoxidase (MPO) activity was evaluated 7 h after KO_2 administration. Mice were pretreated with inhibitors of the NO synthase (L-NAME; 90 mg/kg, ip, 1 h pre-treatment), guanylate cyclase (ODQ, 0,3 mg/kg, ip, 30 min pre-treatment), PKG (KT5823, 0,5 μg/animal, ip, 5 min pre-treatment), K_{ATP} channels (glibenclamide, 0,3 mg/kg, po, 45 min pre-treatment) before naringenin treatment (50 mg/kg, po). After 1 h mice received ipl injection of 30 μg of KO_2, and mechanical and thermal hyperalgesia were assessed 0.5–7 h thereafter. Evaluations of oxidative stress (GSH, FRAP, TBARS, NBT) and cytokine production (tumor necrosis factor [TNF]α and interleukin [IL]-10) were carried out, as well as RT-qPCR measures of $gp91^{phox}$, COX-2, prepro endothelin (ET)-1, Nrf2, HO-1, and IL-33, 3 h after KO_2 injection in samples of paw skin.

Overt pain-like behavioral tests

Abdominal writhing was induced by ip injection of 1 mg of KO_2 [6]. 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, and the intensity 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 0–30 min after ipl 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 the ipsilateral paw [6].

Mechanical hyperalgesia test

Mechanical hyperalgesia was evaluated by the electronic version of von Frey's test, as reported previously [29]. In a quiet, temperature controlled room, mice were placed in acrylic cages (12 x 10 x 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 [6].

Thermal hyperalgesia test

Heat thermal hyperalgesia was performed using a hot plate at 55 ± 1°C [6]. The end-point was characterized by the removal of the paw followed by clear paw flinching or licking movements. The upper time was 15 s to avoid possible injury. The results are expressed by total withdrawal latency (in seconds) of measurements obtained 0.5, 1, 3, 5 and 7 h after stimulus [30].

MPO assay

Neutrophil migration to the hind paw skin tissue was evaluated using an MPO kinetic-colorimetric assay, as described previously [6]. 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 (16,100g x 2 min, 4°C), 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, 15 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^{10} per g of tissue).

GSH measurement

Paw skin sample was collected and maintained at -80°C for at least 48 h. Sample was homogenized with 200 μL of 0.02 M EDTA. The homogenate was mixed with 25 μL of 50% trichloroacetic acid and was homogenized three times during 15 min. The mixture was centrifuged (15 min x 1500 *g* x 4°C). The supernatant was added to 200 μL of 0,2 M TRIS buffer, pH 8.2, and 10 μL of 0,01M DTNB. After 5 min, the absorbance was measured at 412 nm against a reagent blank with no supernatant. A standard curve was performed with standard GSH. The results are expressed as GSH nmol per mg paw [31].

FRAP assay

Paw skin sample was collected and immediately homogenized with 500 μL of 1.15% KCl, and was centrifuged (10 min x 200 *g* x 4°C). The ability of the sample to resist oxidative damage

was determined using FRAP assays [32]. For the FRAP assay, 50 μL of supernatant was mixed with 150 μL of deionized water and 1.5 mL of freshly prepared FRAP reagent. The reaction mixture was incubated at 37°C for 30 min and the absorbance was measured at 595 nm. The results were equated against a Trolox standard curve (1.5–30 μmol/L, final concentrations). The results are expressed as Trolox equivalents per mg of paw.

Superoxide anion production

The quantitation of O_2^- production in tissue homogenates was performed using the NBT assay, as described previously [33]. Skin samples were collected 3h 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 1h. The supernatant was carefully removed and the reduced formazan solubilized by adding 120 μL of 2M 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 paw).

Lipid Peroxidation

Tissue lipid peroxidation was assessed by the levels of thiobarbituric acid reactive substances (TBARS) [34]. For this assay, TCA 10% was added to the homogenate and the mixture was centrifuged (1000 *g*, 3 min, 4°C) to precipitate proteins. The protein-free supernatant was then separated and mixed with TBA (0.67%). The mixture was kept in water bath (15 min, 100°C). Malondialdehyde (MDA), an intermediate product of lipid peroxidation, was determined by difference between absorbances at 535 and 572 nm using a microplate spectrophotometer reader. The results were presented as nmol of MDA per mg of paw [34].

Cytokine measurement

Paw skin samples were collected 3 h after the injection of KO_2, homogenized in 500 μL of ice-cold buffer containing protease inhibitors, centrifuged (3000 rpm x 10 min x 4°C) and the supernatants used to measure, TNFα 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 per mg of paw.

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

RT-qPCR was performed as previously described [33]. Paw skin samples were collected 3 h after stimulus and homogenized in trizol reagent, with the total RNA being isolated according to manufacturer's directions. The purity of total RNA was measured with a spectrophotometer with the wavelength absorption ratio (260/280 nm) being 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) following the manufacturer's instructions. The relative gene expression was measured using the comparative $2^{-(\Delta\Delta Cq)}$ method. The primers used were gp^{91phox}, sense: 5′ -AGCTATGAGGTGGTGATGTTAGTGG-3′ , antisense: 5′ -CACAATAT TTGTAC CAGACAGACTTGAG-3′ ; IL-33, sense: 5′ -TCCTTGCTTGGCAGTATCCA-3′ , antisense: 5′ -TGCTCAATGTGTCAACAGACG-3′ ; COX-2, sense: 5′ -GTGGAAAAACCTCGTC CAGA-3′ , antisense: 5′ -GCTCGGCTTC CAGTATTGAG-3′ ; preproET-1, sense: 5′ -TGT GTCTACTTCTGCCACCT-3′ , antisense: 5′ -CACCAGCTGCTGATAGATAC-3′ ; Nrf2, sense:

5′ −TCACACGAGATGAGCTTAGGGCAA−3′ , antisense: 5′ −TACAGTTCTGGG CGGCGACTT TAT−3′ ; HO-1, sense: 5′ −CCCAAAACTGGCCTGTAAAA−3′ , antisense: 5′ −CGTGGTCAGT CAACATGGAT−3′ ; β-actin, sense: 5′ −AGCTGCGTTT TACACCCTTT−3′ , antisense: 5′ − AA GCCATGCCAATGTTGTCT−3′ . The expression of β-actin mRNA was used as a reference gene to normalize data.

Statistical analysis

Results are presented as means ± SEM of measurements made on six mice in each group per experiment, and are representative of two independent experiments. Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times (curves), when the hyperalgesic responses were measured at different times after the administration or enforcement of the stimuli. The factors analyzed were treatment, time, and time versus treatment interaction. When there was a significant time versus treatment interaction, one-way ANOVA followed by Tukey's post hoc was performed on each occasion. On the other hand, when the hyperalgesic responses were measured once after the administration or enforcement of the stimuli, the difference between responses were evaluated by one-way ANOVA followed by Tukey's post hoc. Statistical differences were considered to be significant at $p < 0.05$.

Results

Naringenin inhibits KO_2-induced overt pain-like behavior

Mice received naringenin (16.7, 50,150 mg/kg, po) treatment 1h before injection of 1 mg of KO_2 ip for the assessment of the total number of writhings, or received 30 μg of KO_2 ipl for the evaluation of the total number of paw flinches and time spent licking the paw. The naringenin doses of 50 and 150 mg/kg inhibited KO_2-induced writhing response at a similar magnitude of effect, with the 3 mg/kg dose showing no statistically significant effect (Fig 1a). The dose of 50 mg/kg of naringenin was therefore selected for the next experiments. Naringenin also inhibited KO_2-induced paw flinches (Fig 1b) and time spent licking the paw (Fig 1c).

Naringenin inhibits KO_2-induced mechanical hyperalgesia, thermal hyperalgesia, and MPO activity

Mice received naringenin (50 mg/kg, po) treatment 1h before KO_2 injection (30 μg, ipl). Mechanical and thermal hyperalgesia were assessed 0.5, 1, 3, 5 and 7 h after KO_2 injection. Naringenin inhibited KO_2-induced mechanical and thermal hyperalgesia at all time points (Fig 2a and 2b). Naringenin also reduced KO_2-induced increased of MPO activity at 7 h (Fig 2c).

Naringenin inhibits KO_2-induced mechanical and thermal hyperalgesia by activating the NO−cGMP−PKG− K_{ATP} channel signaling pathway

Mice were treated with inhibitors of a) NO synthase (L-NAME; 90 mg/kg, ip, 1 h pre-treatment), b) guanylate cyclase (ODQ, 0,3 mg/kg, ip, 30 min pre-treatment), c) PKG (KT5823, 0,5 μg/animal, ip, 5 min pre-treatment), and d) the K_{ATP} channel (glibenclamide, 0,3 mg/kg, po, 45 min pre-treatment) before naringenin (50 mg/kg, po, 1 h before KO_2 injection) administration. After 1h naringenin treatment, mice received a KO_2 ipl injection. L-NAME (Fig 3a and 3b), ODQ (Fig 3c and 3d), KT5823 (Fig 3e and 3f), and glibenclamide (Fig 3g and 3h) inhibited the analgesic effect of naringenin in KO_2-induced mechanical and thermal hyperalgesia. Therefore, the anti-hyperalgesic mechanism of naringenin depends, at least in part, on activating the NO−cGMP−PKG− K_{ATP} channel signaling pathway.

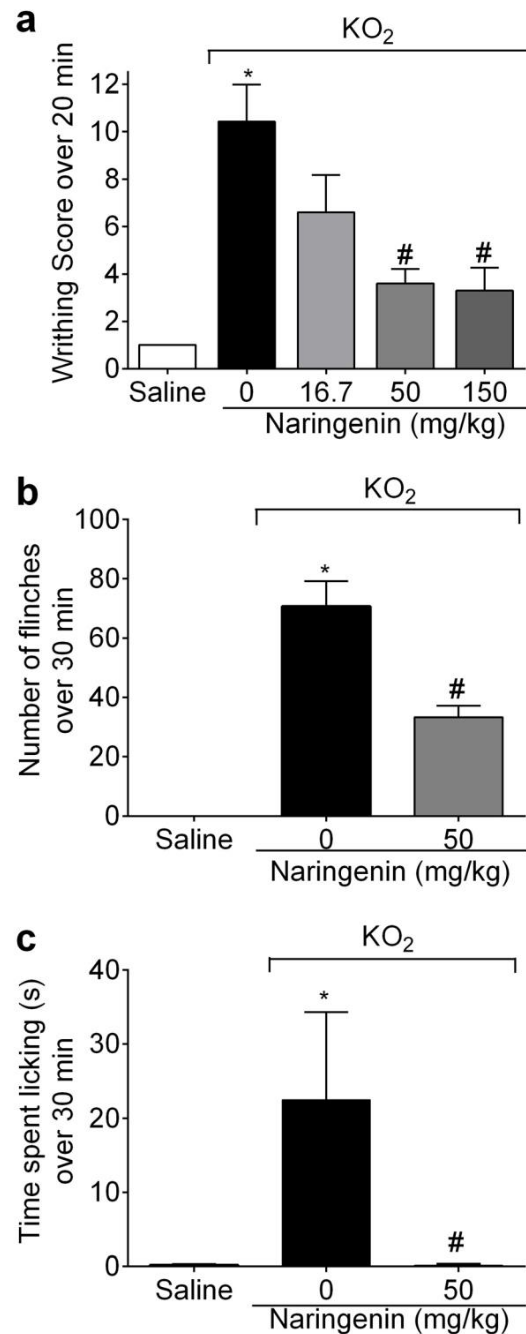

Fig 1. Naringenin inhibits KO_2-induced overt pain-like behavior. (a-c) Mice received naringenin (16.7, 50 and 150 mg/kg, po) treatment 1 h before ip injection of 1 mg of KO_2 or ipl injection of 30 μg of KO_2. The total number of writhings was evaluated 0–20 min after ip injection of KO_2. (b) The number of paw flinches and (c) time spent licking the paw were evaluated 0–30 min after ipl injection of KO_2. Results are mean ± SEM of 6 mice per group per experiment, and are representative of 2 independent experiments. *$p< 0.05$ vs. saline group, #$p< 0.05$ vs. KO_2 group. One-way ANOVA followed Tukey's post hoc.

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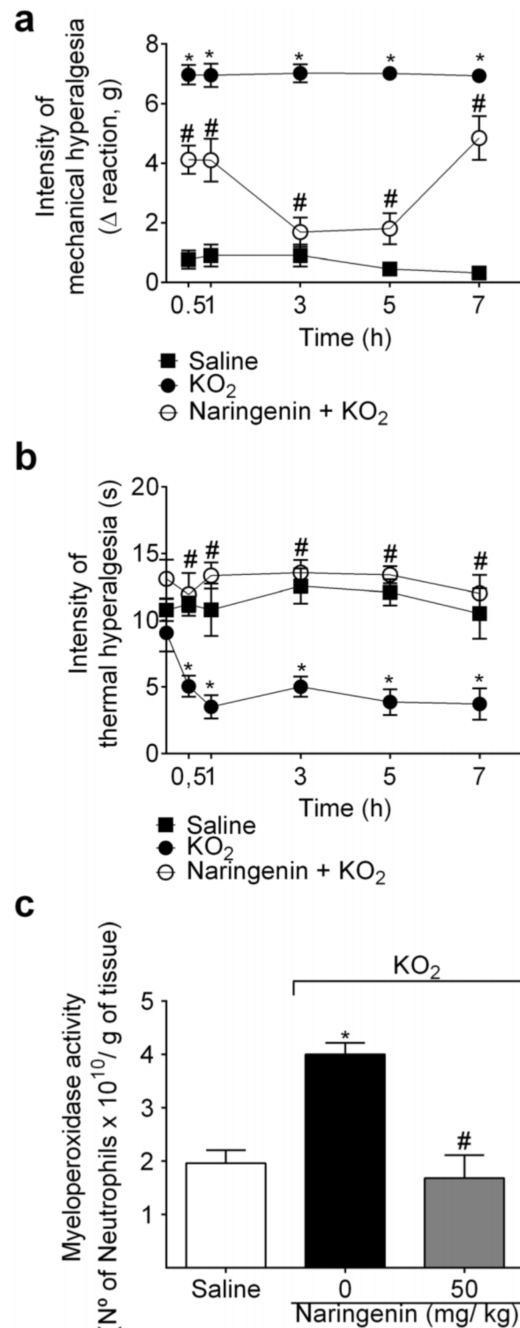

Fig 2. Naringenin inhibits KO_2-induced mechanical hyperalgesia, thermal hyperalgesia and myeloperoxidase (MPO) activity. (a-c) Mice received naringenin (50 mg/kg, po) treatment 1 h before ipl injection of 30 μg of KO_2. Mechanical (a) and thermal (b) hyperalgesia were evaluated between 0.5–7 h after ipl injection of KO_2. (c) At the 7th h after KO_2 injection, paw skin samples were collected for MPO activity assay. Results are mean ± SEM of 6 mice per group per experiment, and are representative of 2 independent experiments. *$p< 0.05$ vs. saline group, #$p< 0.05$ vs. KO_2 group. Repeated measures two-way ANOVA for hyperalgesia data and One-way ANOVA for MPO activity followed Tukey's post hoc.

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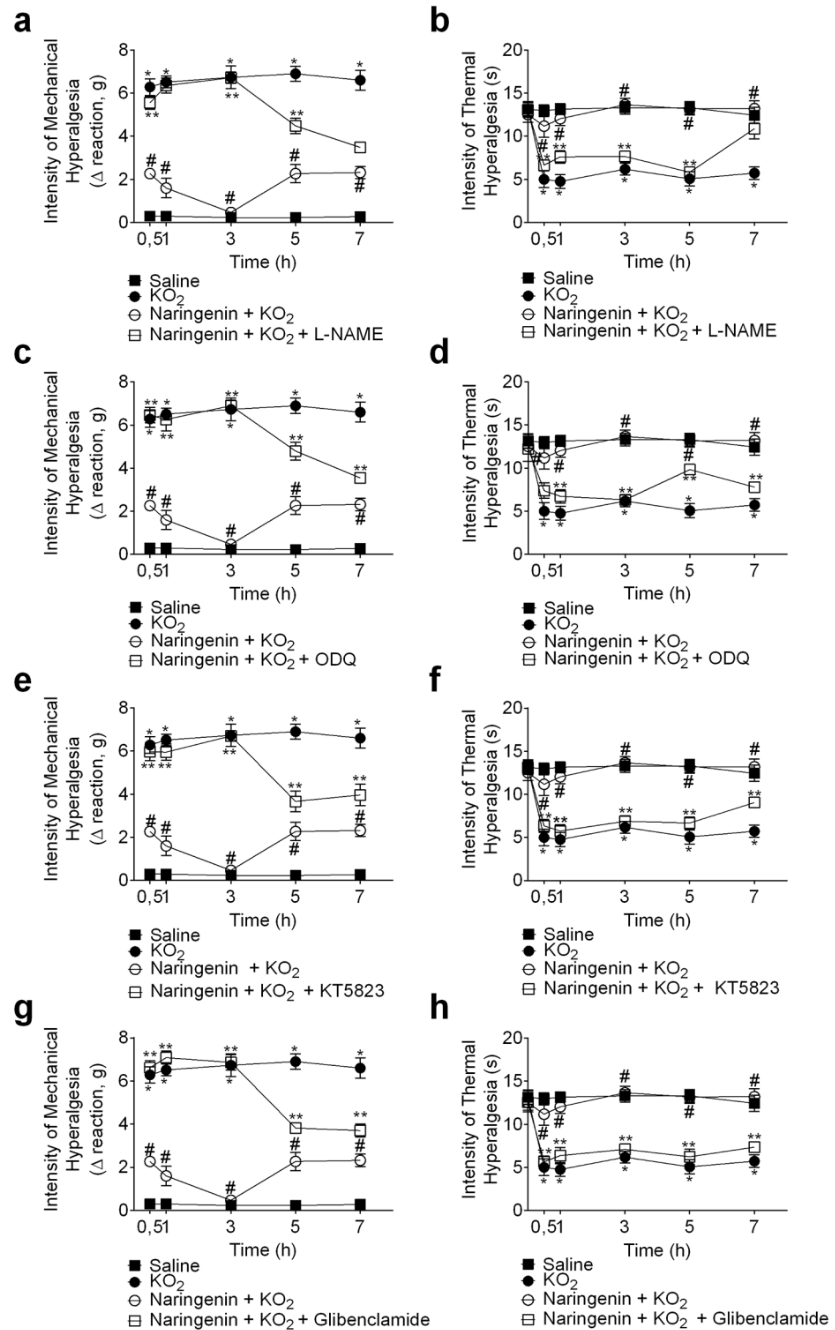

Fig 3. Naringenin inhibits KO_2-induced mechanical and thermal hyperalgesia by activating NO/cGMP/PKG/K_{ATP} channel signaling pathway. Mice received (a,b) L-NAME (NOS inhibitor, 90 mg/kg, ip, 1 h), (c,d) ODQ (guanylate cyclase inhibitor, 0,3 mg/kg, ip, 30 min), (e,f) KT5823 (PKG inhibitor, 0,5 μg/mice, ip, 5 min), or (g,h) glibenclamide (K_{ATP} channel inhibitor, 0,3 mg/kg, po, 45 min) treatment before administration of naringenin (50 mg/kg, po). (a-h) 1 h after naringenin administration, mice received an ipl injection of 30 μg of KO_2. Mechanical (a,c,e,g) and thermal (b,d,f,h) hyperalgesia were evaluated between 0.5–7 h after the ipl

injection of KO_2. Results are mean ± SEM of 6 mice per group per experiment, and are representative of 2 independent experiments. *$p < 0.05$ vs. saline group, #$p < 0.05$ vs. KO_2 group. Repeated measures two-way ANOVA followed Tukey's post hoc.

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Naringenin inhibits KO_2-induced oxidative stress

Mice received naringenin (50 mg/kg, po) treatment 1h before KO_2 injection (30 μg, ipl). Samples of paw skin were collected after 3 h for colorimetric assays and RT-qPCR. KO_2 depleted paw skin GSH (Fig 4a) and antioxidant capacity (Fig 4b); and increased lipid peroxidation (Fig 4c) and superoxide anion production (Fig 4d) in the paw skin. Naringenin treatment inhibited these deleterious effects of KO_2 by restoring GSH levels (Fig 4a), and antioxidant capacity (Fig 4b), and inhibiting lipid peroxidation (Fig 4c) and O_2^- production (Fig 4d). Corroborating the results on oxidative stress (Fig 4a–4d), naringenin also inhibited KO_2-induced $gp91^{phox}$ mRNA expression, a component of NADPH oxidase, an important source of O_2^- production (Fig 4e).

Naringenin inhibits KO_2-induced cytokine production and mRNA expression

The protocol of this section was the same as for Fig 4, with samples analyzed by ELISA and RT-qPCR. Naringenin treatment inhibited KO_2-induced TNFα (Fig 5a) and IL-10 (Fig 5b) production, and IL-33 mRNA expression (Fig 5c).

Naringenin inhibits KO_2-induced COX-2 mRNA expression

The protocol of this section was the same as for Fig 4, with samples analyzed by RT-qPCR. Naringenin treatment inhibited KO_2-induced COX-2 mRNA expression (Fig 6).

Naringenin inhibits KO_2-induced preproET-1 mRNA expression

The protocol of this section was the same as for Fig 4, and samples analyzed by RT-qPCR. Naringenin treatment inhibited KO_2-induced preproET-1 mRNA expression (Fig 7).

Naringenin increases Nrf2/HO-1 mRNA expression

The protocol of this section was the same as for Fig 4, with samples analyzed by RT-qPCR. Naringenin inhibited the KO_2-induced decrease in Nrf2 mRNA expression (Fig 8a), whilst enhancing KO_2-induced HO-1 mRNA expression (Fig 8b).

Discussion

Pain is a multisensory experience, which occurs in response to stimulation of the channels/ receptors that depolarizes nociceptor terminals, thereby generating an action potential, which activates synaptic transmission in the dorsal horn of spinal cord. Such processes induce a characteristic set of responses, including hyperalgesia (an increased response to a stimulus that normally provoke pain) and allodynia (pain due to a stimulus that does not normally provoke pain) [1].

Free radicals in biological systems are natural products during interactions between cells, as well as between tissues and organs. However, an imbalance between endogenous oxidants and antioxidants can alter cell homeostasis. Oxidants target proteins, thereby inducing cell damage and contributing to cell death [35]. Free radicals also cause inflammation [36]. Consequently, treatment with antioxidants is a promising approach to control a wide array of pathophysiological events. KO_2 is a O_2^- donor, and its injection resulting in nociceptive responses, such as

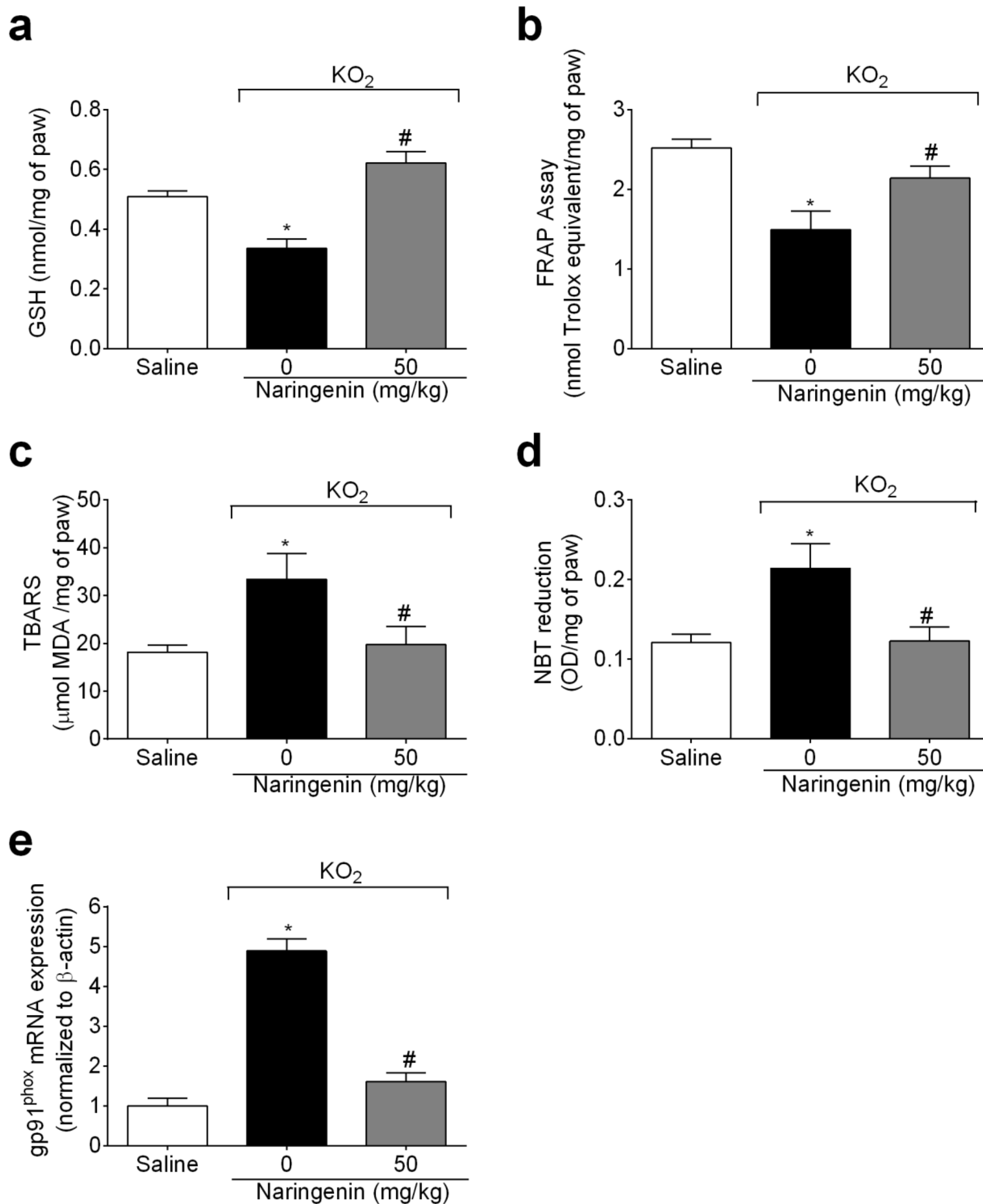

Fig 4. Naringenin inhibits KO_2-induced oxidative stress and gp91^{phox} mRNA expression. (a-e) Mice received naringenin (50 mg/kg, po) treatment 1 h before ipl injection of 30 μg of KO_2. Paw skin samples were collected 3 h after ipl KO_2 injection. Sample analyses were (a) GSH levels, (b) total antioxidant capacity (FRAP assay), (c) lipid peroxidation (TBARS assay), (d) O_2^- production (NBT assay), and (e) gp91^{phox} mRNA expression by RT-qPCR. β-actin was a reference gene to normalize mRNA expression data. Results are mean ± SEM of 6 mice per group per experiment, and are representative of 2 independent experiments. *p< 0.05 vs. saline group, #p< 0.05 vs. KO_2 group. One-way ANOVA followed Tukey's post hoc.

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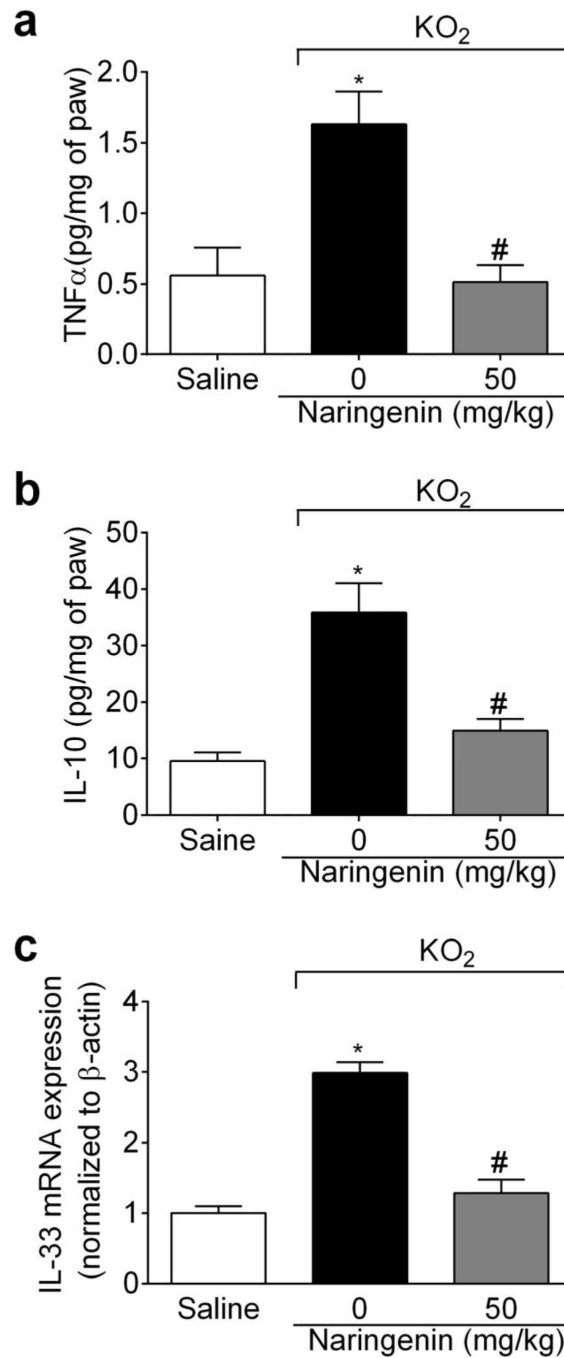

Fig 5. Naringenin inhibits KO_2-induced cytokine production and mRNA expression. (a-c) Mice received naringenin (50 mg/kg, po) treatment 1 h before ipl injection of 30 μg of KO_2. Paw skin samples were collected 3 h after ipl KO_2 injection. Sample analyses were (a) TNFα, and (b) IL-10 production by ELISA, and (c) IL-33 mRNA expression by RT-qPCR. β-actin was a reference gene to normalize mRNA expression data. Results are mean ± SEM of 6 mice per group per experiment, and are representative of 2 independent experiments. *$p< 0.05$ vs. saline group, #$p< 0.05$ vs. KO_2 group. One-way ANOVA followed Tukey's post hoc.

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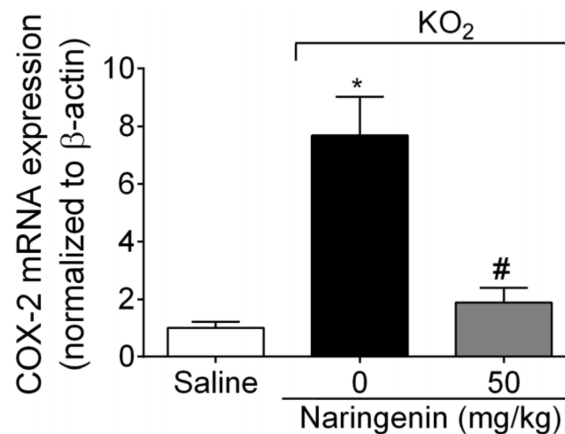

Fig 6. Naringenin inhibits KO_2-induced COX-2 mRNA expression. Mice received naringenin (50 mg/kg, po) treatment 1 h before ipl injection of 30 μg KO_2. Paw skin samples were collected 3 h after ipl KO_2 injection and analyzed for COX-2 mRNA expression by RT-qPCR. β-actin was a reference gene to normalize mRNA expression data. Results are mean ± SEM of 6 mice per group per experiment, and are representative of 2 independent experiments. *p< 0.05 vs. saline group, #p< 0.05 vs. KO_2 group. One-way ANOVA followed Tukey's post hoc.

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overt pain-like behavior, as well as mechanical and thermal hyperalgesia, which can be inhibited by analgesics [6]. Data indicates that O_2^--induced pain depends on both direct superoxide anion effects, and indirect effects, partly *via* the production of peroxynitrite [37]. O_2^- injection triggers inflammatory pain by mechanisms involving cytokine, COX-2 and ET-1 synthesis [6,38,39]. Oxidative stress is involved in the pain of varied inflammatory diseases such as rheumatoid arthritis [40,41], gout [42], delayed onset muscle soreness [43] and diabetes [44].

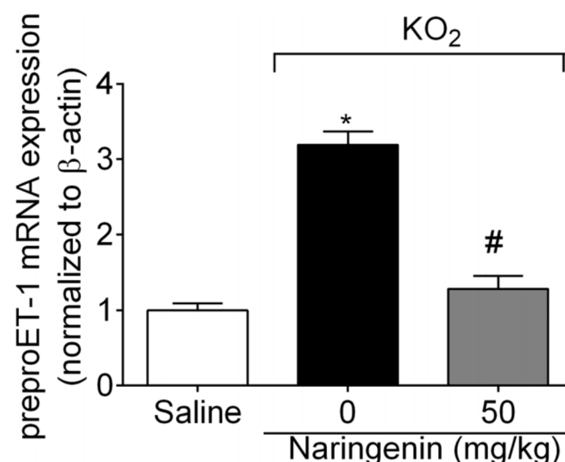

Fig 7. Naringenin inhibits KO_2-induced preproET-1 mRNA expression. Mice received naringenin (50 mg/kg, po) treatment 1 h before ipl injection of 30 μg of KO_2. Paw skin samples were collected 3 h after ipl KO_2 injection for preproET-1 mRNA expression analysis by RT-qPCR. β-actin was a reference gene to normalize mRNA expression data. Results are mean ± SEM of 6 mice per group per experiment, and are representative of 2 independent experiments. *p< 0.05 vs. saline group, #p< 0.05 vs. KO_2 group. One-way ANOVA followed Tukey's post hoc.

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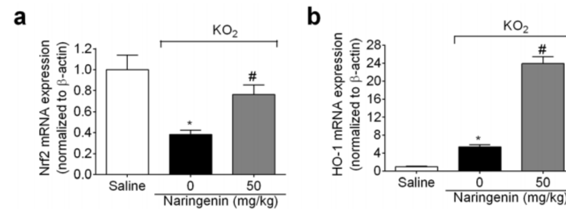

Fig 8. Naringenin increases Nrf2 and HO-1 mRNA expression. (a-b) Mice received naringenin (50 mg/kg, po) treatment 1 h before ipl injection of 30 μg KO_2. Paw skin samples were collected 3 h after ipl KO_2 injection for (a) Nrf2, and (b) HO-1 mRNA expression analysis by RT-qPCR. β-actin was a reference gene to normalize mRNA expression data. Results are mean ± SEM of 6 mice per group per experiment, and are representative of 2 independent experiments. *p< 0.05 vs. saline group, #p< 0.05 vs. KO_2 group. One-way ANOVA followed Tukey's post hoc.

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Therefore, KO_2-induced inflammatory pain is a useful model to study the action of analgesics that target oxidative stress-dependent events [12].

The therapeutic properties of naringenin include antinociception [15–17], as well as antioxidant [22,23,44–48] and anti-inflammatory activities [18,19,21,22]. However, the analgesic effect and mechanisms of action of naringenin in a model o oxidative stress-triggered inflammatory pain remained to be determined. Herein, we provide the first evidence that naringenin inhibits KO_2-induced overt-pain like behavior, MPO activity, mechanical hyperalgesia and thermal hyperalgesia via reduction of cytokine production and oxidative stress.

The activation of the NO−cGMP−PKG−K_{ATP} channel signaling pathway leads to nociceptor hyperpolarization, thereby reducing nociceptor neuronal transmission [25–28]. The present results show that naringenin inhibits the KO_2-induced mechanical and thermal hyperalgesia, at least in part, by activating the NO−cGMP−PKG−K_{ATP} channel signaling pathway. Free radical damage to membrane components leads to cellular dysfunction [49], which, by exposing neoepitopes, will contribute to an autoimmune response, which is receiving increasing interest as to its role in longer-term nociception. GSH is an important redox cellular system, with efficacy partly driven by the direct interactions of sulfhydryl groups (-SH) with ROS, thereby promoting a direct detox reaction [50]. Naringenin was able to restore GSH levels, as well as inhibiting other indicants of oxidative stress, such as FRAP, TBARS, and NBT in the paw skin. The KO_2 solution releases O_2^- for up to 10 min [6], however, NBT reduction (O_2^- production) occurred in paw skin samples collected 3 h after KO_2 injection. Therefore, O_2^- injection induces further O_2^-. This is corroborated by the results showing KO_2-induced the gp91^{phox} mRNA expression, which was inhibited by naringenin. The gp91^{phox} subunit of NADPH oxidase participates in the electron transfer to oxygen, thereby generating O_2^- [51]. O_2^--induced pain also depends on hyperalgesic cytokines, with TNFR1 deficiency reducing O_2^--induced pain and oxidative stress. TNFα-induced hyperalgesia depends on NADPH oxidase activation [38]. Not all cytokines are nociceptive, with IL-10 having anti-hyperalgesic effects [43]. IL-33 is a hyperalgesic cytokine that regulates the production of TNFα and IL-10 [52–55] Naringenin inhibited O_2^--induced production of TNFα and IL-10, as well as IL-33 mRNA expression.

Cytokine-induced inflammatory hyperalgesia in turn, depends, at least in part, on COX-2-dependent production of prostanoids such as prostaglandin E_2 and prostacyclin [1]. O_2^- also induces COX-2 mRNA expression, with celecoxib (a selective COX-2 inhibitor) diminishing O_2^--induced pain [6]. Naringenin inhibited O_2^--induced COX-2 mRNA expression. There is also a close relationship between cytokines and ET-1. Endothelin receptor antagonists inhibit cytokine-induced hyperalgesia and cytokines induce preproET-1 mRNA expression and ET-1

production [56,57]. Bosentan, an endothelin receptor antagonist, inhibits O_2^--induced hyperalgesia, and O_2^- induces preproET-1 mRNA expression [39]. Naringenin inhibited O_2^--induced prepro-ET-1 mRNA expression. Furthermore, activation of the ET receptors promotes oxidative stress and reduces the free radical scavenging ability [58]. Therefore, it is likely that ET-1 also contributes to the regulation of oxidative stress. In fact, bosentan inhibited O_2^--induced oxidative stress [39].

Consistent with the naringenin inhibition of cytokine production as well as COX-2 and preproET-1 mRNA expression, naringenin inhibits the activation of the pro-inflammatory transcription factor, NFκB, in several models of inflammation. For instance, naringenin inhibits NFκB activation in dextran sulphate sodium-induced colitis [59], ethanol-induced liver injury [18], streptozotocin-induced diabetes in mice [60] and rats [61], and experimental stroke [23]. Furthermore, naringenin also inhibits NFκB DNA-binding activity in ovalbumin-induced asthma [62] as well as in *in vitro* studies [63,64]. As such, it is not unlikely that naringenin inhibits KO_2-induced cytokines production, as well as COX-2 and preproET-1, by inhibiting NFκB induction and activity. Nrf2 is an important transcriptional regulator of the antioxidant response. In fact, Nrf2 activation is essential to the production of endogenous antioxidants such as GSH, thioredoxin system, HO-1, and NQO1 (NAD(P)H dehydrogenase, quinone 1) [65]. Keap-1 detects cellular environment changes such as oxidative stress, resulting in the activation of Nrf2, which readily translocates to the nucleus and upregulates downstream targets such as HO-1 [66]. Furthermore, the activation of the Nrf2/HO-1 pathway inhibits the production of inflammatory molecules such as TNFα [67–69], IL-6 [68,69] and IL-1β [67,68]. Nrf2 indirectly modulates NFκB activity [70], given that Nrf2 and NFκB compete for binding to the nuclear complex, coactivator p300/CBP (E1A binding protein p300/CREB-binding protein) [71]. Importantly, naringenin activates Nrf2 and is an agonist of the aryl hydrocarbon receptor, contributing to reducing the production of reactive oxygen species and inflammatory mediators, as well as modulating specific immune cell activity [47,72]. In the present study, naringenin inhibited the KO_2-induced decrease in Nrf2 mRNA expression and increased HO-1 mRNA expression. This mechanism might account for the analgesic effect of naringenin in KO_2-induced inflammation, by inhibiting pro-hyperalgesic cytokine production, and $gp91^{phox}$ as well as inducing antioxidant molecules.

The data presented here suggest that naringenin inhibits O_2^--induced inflammatory overt pain-like behavior, hyperalgesia, and neutrophil recruitment by activating the NO−cGMP −PKG−K_{ATP} channel signaling pathway, as well as inhibiting oxidative stress and cytokine production, coupled to decreasing the mRNA of $gp91^{phox}$, IL-33, COX-2, and preproET-1, and increasing Nrf2 and HO-1 mRNA expression. These data show for the first time that naringenin inhibits inflammatory pain triggered by O_2^- and its mechanisms. Therefore, suggesting that naringenin is a promising therapeutic approach as an analgesic, antioxidant and anti-inflammatory compound, requiring further investigation in nociception, as well as in the array of other medical conditions where these pathophysiological changes are also evident [73].

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Author Contributions

Conceived and designed the experiments: RC WAVJ. Performed the experiments: MFM CCC LCS ACZ FAPR. Analyzed the data: MFM CCC LCS ACZ FAPR SRG MMB RC WAVJ. Contributed reagents/materials/analysis tools: SRG MMB RC WAVJ. Wrote the paper: MFM CCC LCS ACZ FAPR SRG MMB RC WAVJ. Drafted the article or revised it critically for important intellectual content: MFM CCC LCS ACZ FAPR SRG MMB RC WAVJ. Final approval of the version to be published: MFM CCC LCS ACZ FAPR SRG MMB RC WAVJ. Agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: MFM CCC LCS ACZ FAPR SRG MMB RC WAVJ.

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CONCLUSÃO

Neste trabalho foram demonstrados os efeitos e mecanismos de ação do flavonoide naringenina em dois modelos: 1) modelo de artrite crônica induzido por dióxido de titânio; 2) modelo agudo de dor inflamatória induzido pelo superóxido de potássio – um doador de ânion superóxido. A naringenina é uma molécula pleiotropica que age em diversas vias de sinalização, e em conjunto conferem a ação analgésica a anti-inflamatória desta molécula. Naringenina inibe a migração de leucócitos, estresse oxidativo, ativação do NFκB e citocinas *downstream*, inibição da sinalização RANK/RANKL, em adição não possuiu efeito tóxico no modelo de artrite crônica induzido por dióxido de titânio. Em relação ao modelo agudo de dor inflamatória induzido pelo superóxido de potássio, a naringenina inibe a migração de leucócitos, estresse oxidativo, ativação do NFκB e citocinas *downstream*, aumento na atividade do Nrf2 e HO-1, e ativa a via analgésica NO–GMPc–PKG– canais K_{ATP}. Portanto, a naringenina é uma possível abordagem terapêutica para a artrite crônica relacionado ao implante induzindo inflamação estéril e dor inflamatória envolvendo ânion superóxido.

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