UNIVERSIDADE ESTADUAL DE LONDRINA

MAIARA PIVA

ATIVIDADES ANALGÉSICA, ANTI-INFLAMATÓRIA E ANTIOXIDANTE DA *TRANS*-CHALCONA EM CAMUNDONGOS: PARTICIPAÇÃO DOS CANAIS TRPV1, TRPA1 E DA VIA DE SINALIZAÇÃO NF-κB

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Tese apresentada à Universidade Estadual de Londrina - UEL, como requisito parcial para a obtenção do título de Doutora em Patologia Experimental.

Orientador: Prof. Dr. Waldiceu Verri Jr

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Prof. Dr. Waldiceu Verri Jr.
Universidade Estadual de Londrina - UEL

Prof. Dr. Phileno Pinge Filho
Universidade Estadual de Londrina - UEL

Dra. Marília F. Manchope
Julius-Maximilians-Universistät (JMU)

Dra. Maria Isabel Lovo Martins
Universidade Estadual de Londrina - UEL

Dr. Glauco Akelinghton Freire Vitiello
Universidade Estadual de Londrina - UEL

Londrina, 17 de junho de 2025.

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RESUMO

PIVA, Maiara. **Atividades analgésica, anti-inflamatória e antioxidante da *trans*-Chalcona em camundongos:** participação dos canais TRPV1, TRPA1 e da via de sinalização NF-κB. 2025. 119 f. Tese (Doutorado em Patologia Experimental) – Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, 2025.

Cerca de 30% da população mundial adulta sofre com condições que causam dor, mas suas opções farmacológicas possuem eficiência limitada e efeitos adversos consideráveis, indicando a necessidade de novas alternativas mais efetivas e seguras. A *trans*-Chalcona (*t*C) é um flavonoide de cadeia aberta precursor de outros flavonoides, que possui efeitos antioxidantes e anti-inflamatórios. O objetivo deste trabalho foi investigar o potencial analgésico, anti-inflamatório e antioxidante do flavonoide *t*C em modelos de estudo de dor e inflamação em camundongos. A *t*C foi administrada via oral 30 minutos antes dos estímulos. A analgesia foi investigada após estímulo com ácido acético, fenil-*p*-benzoquinona (PBQ), formalina, adjuvante completo de Freund (CFA), capsaicina, isotiocianato de alila (AITC) e superóxido de potássio (KO_2), enquanto os parâmetros inflamatórios foram induzidos pelo KO_2, um doador de ânion superóxido. A dor manifesta por contorções abdominais foi induzida por ácido acético, PBQ e KO_2, a *t*C inibiu os três estímulos. A dor manifesta observada pelo tempo lambendo a pata e número de sacudidas foi induzida pela formalina, CFA, capsaicina, AITC e também foi inibida pela *t*C. A capsaicina é um agonista do canal iônico TRPV1 e o AITC é um agonista de TRPA1, que foram utilizados para demonstrar a capacidade da *t*C de modular as vias de sinalização induzidas por esses canais, tanto *in vivo* como *in vitro*, indicando o mecanismo de ação envolvido na atividade analgésica do flavonoide. A eficácia da *t*C na inibição da dor e inflamação induzidas pelo ânion superóxido foi demonstrada através da inibição das citocinas IL-1β TNF-α, IL-33, IL-6 e da via de sinalização NF-κB. A *t*C também inibiu o estresse oxidativo, ação observada pelo aumento na produção de antioxidantes, redução na produção de ânion superóxido e peroxidação lipídica, devido, pelo menos parcialmente, à regulação positiva da expressão de Nrf2 e à regulação negativa da expressão de $Gp91^{phox}$ e Cox-2. A *t*C também preveniu a sensibilização dos neurônios nociceptivos TRPV1+ e TRPA1+ pelo ânion superóxido. Desta forma, a tC demonstrou-se eficaz como analgésico, anti-inflamatório e antioxidante com efeito multialvos em camundongos.

Palavras-chave: flavonoide; analgésico; dor; ROS; TRPA1; TRPV1.

ABSTRACT

PIVA, Maiara. **Analgesic, anti-inflammatory and antioxidant activities of *trans*-Chalcone in mice:** the role of TRPV1 and TRPA1 channels and the NF-κB signaling pathway. (2025) 119 p. Doctoral thesis in Experimental Pathology. Center of Biological Sciences, Londrina State University, Londrina, 2025.

Approximately 30% of the adult population worldwide suffers from some pain-related condition. Nonetheless, current pharmacological options have limited efficiency and considerable adverse effects, pointing out the need for new, more effective and safer alternatives. *Trans*-chalcone (tC) is an open-chain flavonoid precursor of other flavonoids. *t*C has antioxidant and anti-inflammatory effects. The aim of this study was to investigate the analgesic, anti-inflammatory and antioxidant potential of the flavonoid *t*C on pain and Inflammation in mice. *t*C was administered orally 30 min before stimulus. Overt pain-like behavior was triggered by acetic acid, phenyl-*p*-benzoquinone (PBQ), formalin, complete Freund's adjuvant (CFA), capsaicin, allyl isothiocyanate (AITC) and potassium superoxide (KO_2). Inflammation was induced by KO_2, a superoxide anion donor. Overt pain-like behavior by abdominal writhing was induced by acetic acid, PBQ, and KO_2. *t*C (10 mg/kg) inhibited the pain induced by acetic acid and PBQ, while *t*C (30 mg/kg) inhibited all three stimuli. Paw licking time and number of flinches were induced by formalin, CFA, capsaicin, and AITC, which were inhibited by *t*C. Capsaicin is an agonist for TRPV1 ionic channel and AITC is a TRPA1 agonist. They were used to demonstrate *t*C ability to modulate the signaling pathways induced by these channels, both in vivo and in vitro, indicating the mechanism involved in *t*C analgesic effects. The efficacy of *t*C preventing pain and inflammation caused by superoxide anion was demonstrated by the inhibition of the cytokines IL-1β, TNF-α, IL-33 and IL-6, and the inhibition of the NF-κB signaling pathway. *t*C also inhibited oxidative stress, observed by increased antioxidant production, reduced superoxide anion production and lipid peroxidation, due, at least partially, to the upregulation of Nrf2 expression and the downregulation of $Gp91^{phox}$ and Cox-2 expression. *t*C also prevented the sensitization of TRPV1+ and TRPA1+ nociceptive neurons by superoxide anion. Thus, *t*C proved to be effective as an analgesic, anti-inflammatory and antioxidant with multitarget effects in mice.

Keywords: flavonoid; analgesic; pain; ROS; TRPA1; TRPV1.

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SUMÁRIO

1 INTRODUÇÃO

A dor possui um papel fisiológico importante ao alertar o indivíduo sobre ameaças reais ou potenciais, como traumas mecânicos, temperaturas extremas e exposição a substâncias químicas nocivas ou patógenos. A exposição contínua a esses elementos resulta no aumento da sensibilidade a eles, processo intimamente relacionado a sinalização mediada por espécies reativas e inflamação, que pode evoluir para um quadro de dor patológica potencialmente debilitante.

Atualmente, as opções disponíveis para o alívio da dor, como anti-inflamatórios, antidepressivos e opioides, apresentam eficácia limitada à longo prazo e perfil de segurança insatisfatório, tornando a perspectiva de utilizar tratamentos com baixa toxicidade e ação analgésica, anti-inflamatória e antioxidante, uma possibilidade atrativa do ponto de vista farmacêutico.

Os flavonoides compõem um grupo de compostos naturais que compartilham uma mesma base estrutural molecular e apresentam propriedades antioxidantes e anti-inflamatórias. A *t*C é um flavonoide de cadeia aberta, precursora de outros flavonoides e estruturalmente atípica, já que não possui radicais hidroxila em sua composição, sua atividade antioxidante é independente dos efeitos bioquímicos inerentes à uma estrutura molecular antioxidante *per se*. Somada a sua características estrutural intrigante, a ausência de estudos focados em possíveis efeitos analgésicos da *t*C, a tornam um alvo de pesquisa promissor.

2 REVISÃO DA LITERATURA

Cerca de 30% da população adulta global sofre com condições que causam dor (Zimmer *et al.*, 2022; Macchia *et al.*, 2024). O manejo farmacológico da dor aguda conta com alternativas como o paracetamol, os anti-inflamatórios não esteroidais (AINEs), medicamentos que atuam nos receptores opioides e monoamínicos, como tramadol e tapentadol, e diferentes combinações entre eles (Amaechi *et al.*, 2021). Entretanto, esses tratamentos apresentam eficácia limitada contra a dor e potenciais efeitos adversos preocupantes como hepatotoxicidade, úlceras gastrointestinais, hipertensão, insuficiência renal e dependência (Aminoshariae e Khan, 2015; Vonkeman e van de Laar, 2010; Benyamin *et al.*, 2008).

Como alternativas farmacológicas que visam minimizar os efeitos adversos associados às opções de medicamentos comumente usados para a dor, podemos citar o uso tópico de AINES (Ahmed *et al.*, 2015), ainda que a aplicação não consiga atingir os mesmos resultados terapêuticos da administração sistêmica (Khankhel *et al.*, 2024). As formulações tópicas também possibilitam o uso de substâncias incompatíveis com a administração sistêmica, como o anestésico lidocaína e o agonista de canal iônico capsaicina (Wilhelm *et al.*, 2010; Maihofner e Heskamp, 2013). Ainda, a agência regulamentadora de medicamentos dos EUA, a FDA (*Food and Drug Administration*), aprovou o uso da suzetrigina no início deste ano, caracterizando a primeira nova classe de analgésicos aprovada nos EUA em mais de 20 anos e o primeiro medicamento oral não opioide a ser aprovado para dor aguda (May, 2025). O novo fármaco gera analgesia ao inibir a atividade do canal de sódio dependente de voltagem Nav1.8, localizado principalmente no sistema nervoso periférico (Jones *et al.*, 2023; Vaelli *et al.*, 2024).

Os exemplos supracitados ilustram a importância da compreensão dos mecanismos que regem a sinalização da dor e da busca por novas alternativas farmacêuticas para o alívio dos sintomas dolorosos.

2.1 A DOR

A Associação Internacional de Estudo da Dor (IASP – do inglês *International Association for the Study of Pain*) define dor como uma “experiência sensitiva e emocional desagradável associada ou relacionada a lesão real ou

potencial dos tecidos". Isso significa que a dor vai além da nocicepção, ou seja, além da sinalização que ocorre no sistema nervoso desencadeada por estímulos potencialmente nocivos: se trata de uma experiência pessoal, podendo variar de acordo com a influência de fatores biológicos, psicológicos e sociais (Raja *et al.*, 2020). Tendo em vista que o presente trabalho não aborda fatores psicológicos e sociais, com fins didáticos, o termo dor será limitado à esfera nociceptiva.

2.1.1 O Sistema Nervoso

Nosso entendimento atual da arquitetura do Sistema Nervoso tem origem nos estudos pioneiros de Santiago Ramón y Cajal, considerado o "pai da neurociência moderna", que identificou cada célula nervosa como uma entidade autônoma, estabelecendo contato – não continuidade – com outras células nervosas. Suas observações, meticulosamente registradas em desenhos icônicos da neuroanatomia microscópica, conferiram a Ramón y Cajal o Prêmio Nobel de Fisiologia ou Medicina em 1906 (Katz-Sidlow, 1998; Sotelo, 2003). O Sistema Nervoso é dividido didaticamente em Sistema Nervoso Central (SNC), que compreende o encéfalo e a medula espinal, e Sistema Nervoso Periférico (SNP), que consiste em todo o tecido neural fora do SNC, incluindo nervos e gânglios.

As fibras nervosas periféricas são divididas didaticamente em 4 subclasses, de acordo com seu diâmetro, velocidade de condução e extensão da mielinização: 1.) Fibras Aα, que possuem o maior diâmetro, entre 13 e 20 μm, velocidade de condução entre 80 e 120 m/s e são altamente mielinizadas; 2.) Fibras Aβ: com diâmetro entre 6-12μm, condução entre 35-75 m/s, mielinização moderada; Fibras Aδ: 1 – 5 μm de diâmetro, velocidade de condução entre 5 e 30 m/s, com fina mielinização; e 4.) Fibras c: com diâmetro de 0,2 – 1,5 μm, condução lenta de 0,5 – 2 m/s e amielínicas (Russell, 1980; Waxman, 1980; West e Wolstencroft, 1983).

A comunicação entre o SNC e o SNP é realizada por meio de sinapses entre neurônios de primeira ordem, presentes no SNP e neurônios de segunda ordem, presentes no SNC. Outra divisão didática das fibras nervosas (axônios) refere-se à direção na qual esse sinal é enviado, classificadas em fibras aferentes ou eferentes. As fibras aferentes transportam sinais de receptores para o SNC (medula espinal ou cérebro), enquanto as fibras eferentes fazem o caminho oposto, transportando o sinal enviado do SNC para os membros periféricos, músculos, glândulas etc. para a

apresentação da reação adequada (Baars e Gage, 2010). As fibras aferentes primárias possuem uma morfologia característica, chamada pseudounipolar, na qual os terminais central e periférico emanam de uma haste axonal comum a partir do corpo celular neuronal intraganglionar, que incluem os gânglios da raiz dorsal (DRG), gânglios trigêmeos (GT) e outros gânglios dos nervos cranianos (Basbaum *et al.*, 2009; Meltzer *et al.*, 2021).

A sinalização da dor, transmitida da periferia para o sistema nervoso central pelo axônio do nociceptor aferente primário, é didaticamente dividida em quatro etapas: transdução, transmissão, modulação e percepção. A transdução consiste na detecção do estímulo nocivo pelos nociceptores e sua transformação em potencial de ação. A molécula de transdução de sinal mais comum é o cálcio ionizado (Ca^{2+}) (Berridge *et al.*, 1998). A transmissão consiste na condução do impulso até o corno dorsal da medula espinal, onde o estímulo sofre modulação e é enviado ao cérebro, onde finalmente é percebido como dor (Osterweis *et al.*, 1987).

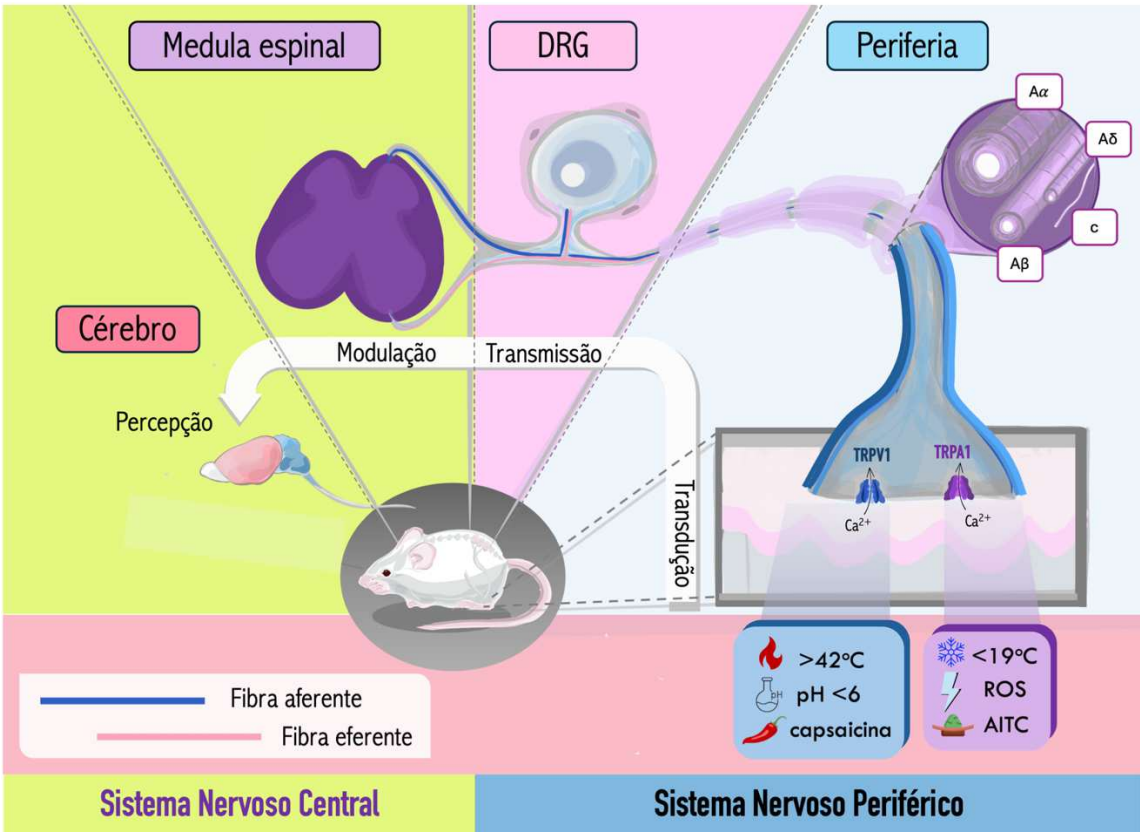

Figura 1. *Representação esquemática da sinalização da dor.* O estímulo nocivo (térmico, físico, químico ou biológico) ativa a abertura de canais iônicos, como TRPV1 e TRPA1, responsáveis pela transdução do sinal em potencial de ação. O potencial percorre as fibras aferentes (Aδ ou c) até o corno dorsal da medula espinal, onde é modulado e, via sinapse entre o neurônio de primeira ordem (que possui o corpo neuronal no DRG) e o neurônio de segunda ordem (que possui o corpo neuronal no SNC), é enviado até o cérebro, onde ocorre a percepção da dor (Fonte: imagem de autoria própria).

Dentre os possíveis estímulos que incitam a sinalização da dor, estão os estímulos térmicos (como calor ou frio nocivos), mecânicos (como pressão intensa e lesões traumáticas), químicos (como agonistas de receptores nociceptivos) e biológicos (como bactérias e componentes estruturais de patógenos), que são detectados por uma subpopulação de fibras nervosas periféricas chamadas de nociceptivas que, ao promover o reconhecimento dos estímulos nocivos, rege uma resposta adaptativa que funciona como um mecanismo de defesa visando prevenir maiores danos através de comportamentos nocifensivos (Klaumann *et al.*, 2008; Chiu *et al*., 2013; Grace *et al.*, 2014; Rosenbaum *et al.,* 2022).

Apesar de ser um tema controverso, o consenso atual é de que os neurônios aferentes envolvidos na sinalização da dor compreendem dois tipos de terminações nervosas: mecanorreceptores de alto limiar, responsivos a estímulos

mecânicos e térmicos, conduzidos por fibras A mielinizadas, principalmente Aδ, e que transmitem sinais rapidamente; e nociceptores fibras do tipo C polimodais, que são capazes de reagir a uma variedade de estímulos nocivos, incluindo mecânicos, térmicos e químicos, são amielínicas e apresentam baixa velocidade de condução (Cao e Zhang, 2008; Yam *et al.*, 2018; Mears e Mears; 2023).

Como a transdução da dor é mediada pela abertura de canais iônicos, que induz a despolarização neuronal e gera o potencial de ação a ser modulado e percebido como dor, os canais que controlam o tráfego iônico são de suma importância na via de sinalização nociceptiva. Os transdutores de dor incluem: os receptores purinérgicos não seletivos P2X dependentes de ATP; canais iônicos de potencial receptor transitório (TRP); canais iônicos sensíveis a ácido (ASICs); canais PIEZO mecanossensíveis; canais de sódio dependentes de voltagem (Nav); canais de cálcio dependentes de voltagem (Cav); entre outros (Consens e Manning, 1969; Caterina *et al.*, 1997; Jasti *et al.*, 2007; Coste *et al.*, 2010; Giniatullin, 2020). Pese a importância das diferentes classes de canais iônicos na transmissão da dor, o presente trabalho adotou como alvo de investigação canais TRP, que serão abordados mais detalhadamente.

2.2 CANAIS TRP

A superfamília multigênica TRP (receptor de potencial transitório, do inglês *Transient Receptor Potential*), é formada por 28 membros que codificam proteínas integrais de membrana e funcionam como canais iônicos permeáveis a uma ampla gama de cátions, como Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}. Os canais TRP são sensíveis a sinais celulares e ambientais de caráter sensorial, incluindo calor, frio, dor, estresse, visão e paladar, e podem ser ativados por uma série de estímulos (Consens e Manning, 1969; Vennekens *et al.*, 2012).

Estruturalmente similares, os canais TRP são constituídos por quatro subunidades que compartilham uma base tridimensional com seis segmentos transmembranares (S1 a S6), dois domínios citoplasmáticos variáveis (terminais N e C) e uma pequena alça formadora do poro (P) do receptor, localizada entre os segmentos S5 e S6 (Figura 2). Esses canais são classificados em sete subfamílias, de acordo com a homologia em suas sequências de aminoácidos: TRPA (Anquirina), TRPC (Canônica), TRPM (Melastatina), TRPML (Mucolipina), TRPN (NO-mecano-

potencial, NOMP), TRPP (Policistina), TRPV (Vaniloide) (Montell *et al.*, 2002; Clapham, 2007; Pedersen *et al.*, 2005; Catterall e Swanson, 2015; Duitama *et al.*, 2020; Zhang *et al.*, 2023).

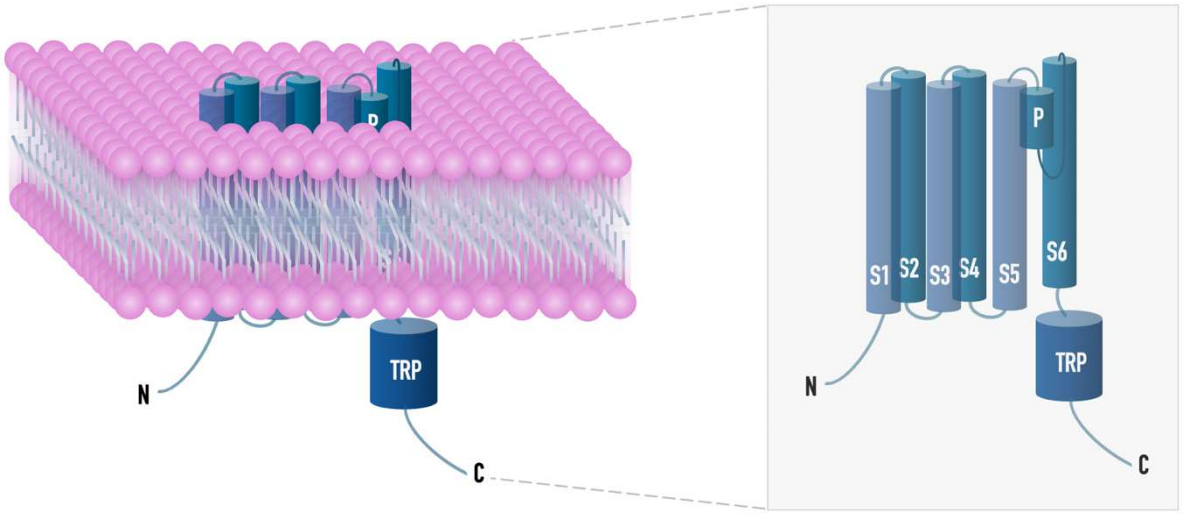

Figura 2. *Subunidade estrutural comum entre os canais iônicos TRP* (Fonte: imagem de autoria própria).

2.2.1 TRPV1

O TRPV1 pode ser ativado por calor superior a 42 – 43 °C, pH ácido (<6), pressão mecânica prejudicial e a compostos químicos nocivos. Embora a capacidade da capsaicina – o principal componente que confere a pungência das pimentas – de ativar os nervos sensoriais tenha sido descrita por Jancsó *et al.* (1967) ainda na década de 1960, a identificação do TRPV1 como o receptor que é ativado por ela e resulta a sensação de dor por ardência só ocorreu décadas depois, em estudo publicado por Caterina *et al.* (1997). A descoberta foi possível graças a uma estratégia de clonagem da expressão baseada no influxo de cálcio para isolar um cDNA funcional que codificasse o receptor de capsaicina de neurônios sensoriais. O trabalho também demonstrou que doses baixas de capsaicina eram capazes de sensibilizar o canal TRPV1 ao calor nocivo e à acidificação extracelular (Caterina *et al.*, 1997).

De maneira geral, o TRPV1 é identificado em neurônios do DRG de pequeno diâmetro, que dão origem às fibras c amielinizados e Aδ finamente mielinizados (Ma e Quirion, 2007).

A identificação do TRPV1 forneceu a base teórica que explica a

eficácia da utilização de modelos de dor manifesta como ferramenta na triagem de drogas com potencial analgésico. Como exemplo, podemos citar a injeção intraperitoneal de ácido acético, que gera contorções abdominais passiveis de inibição por substâncias que modulem alguma etapa da sinalização de dor mediada por TRPV1 (Verri *et al.*, 2008; Pavão-de-Souza *et al*., 2012). Pesem as evidências que já ligavam o canal TRPV1 à dor gerada por acidificação, a demonstração dos efeitos do ácido acético especificamente foi realizada por Liu e colaboradores (2013), que utilizaram animais *knock out* (*k.o.*) para TRPA1 ou TRPV1, e observaram que, em animais *k.o.* para TRPA1, a resposta ao ácido acético era normal, mas em animais *k.o.* para TRPV1, a dor manifesta estava completamente ausente, posicionando TRPV1 como o principal mediador dos efeitos pró-algesia de ácidos fracos com o ácido acético (Liu *et al.*, 2013).

2.2.2 TRPA1

A exemplo da capsaicina no canal TRPV1, a identificação do canal TRPA1 como responsável pela sinalização nociceptiva induzida pela aplicação tópica do óleo de mostarda (isotiocianato de alila - AITC) na pele também foi realizada pelo grupo de pesquisa de David Julius, neurocientista que, junto a seu colega Ardem Patapoutian – que identificou os canais Piezo –, recebeu o Prêmio Nobel em Fisiologia ou Medicina em 2021. O grupo observou que o AITC ativa terminações nervosas sensoriais através de sua ação agonista no canal TRPA1, produzindo dor, inflamação e hipersensibilidade robusta a estímulos térmicos e mecânicos (Jordt *et al.*, 2004). TRPA1 também atua na nocicepção induzida por espécies reativas de oxigênio (ROS), pruritógenos e baixas temperaturas (<19ºC), e é normalmente co-expresso com TRPV1 em neurônios DRG (Jordt *et al.,* 2004; Story *et al.,* 2003; Kobayashi *et al.,* 2005).

A co-expressão dos canais TRPV1 e TRPA1 resulta em considerável associação entre eles, especialmente em relação à dor e à inflamação neurogênicas, geradas por ativação direta de nociceptores que desencadeia a liberação de glutamato e peptídeos neuromoduladores de seus terminais centrais no corno dorsal da medula espinhal, bem como de peptídeos pró-inflamatórios, como a substância P e o peptídeo relacionado ao gene da calcitonina (CGRP), na medula espinal e/ou nos terminais periféricos. A liberação desses neuropeptídeos periféricos promove o

extravasamento de proteínas plasmáticas e a adesão de leucócitos a vênulas pós-capilares (mediada por substância P), vasodilatação arteriolar (mediada por CGRP), hiperalgesia/alodinia e sensibilização periférica (hiperalgesia primária no local da aplicação com hiperalgesia secundária sensível a toques sutis em uma área adjacente) comumente observadas após a administração de capsaicina ou AITC (Holzer, 1991; LaMotte *et al.*, 1991; Szallasi e Blumberg, 1999; Ren e Dubner, 1999; Simone *et al.*, 1991).

Cabe ressaltar que a interação entre a sinalização de dor e o processo inflamatório não ocorre apenas na dor neurogênica. Estímulos pró-inflamatórios periféricos também podem gerar estímulos nociceptivos, como a produção de ROS, geração de calor e acidificação do meio, promovendo a sensibilização periférica. De acordo com a intensidade e duração do estímulo nociceptivo, a sinalização da dor enviada ao SNC, que é mediada por células imunes residentes, denominadas micróglia, que produzem mediadores inflamatórios como a interleucina 1 beta (IL-1β), o fator de necrose tumoral-α (TNF-α), o fator neurotrópico derivado do cérebro (BDNF) e a prostaglandina E_2 (PGE_2), sensibilizam tanto neurônios de primeira como de segunda ordem, modificando o fenótipo destes neurônios, alterando também suas propriedades de transdução, condução e transmissão, contribuindo para o surgimento da hipersensibilidade, da dor espontânea, podendo chegar a induzir a sensibilização central, ou espinal, fenômeno que contribui para a cronificação da dor (Scholz e Woolf, 2002; Pinho-Ribeiro *et al.*, 2017).

2.3 Inflamação

A dor já era associada a resposta imune décadas antes de Cristo, quando Cornilius Celsus a identificou como um dos sinais cardinais da inflamação, assim como o rubor, calor, edema, e associada posteriormente, a perda de função. A dor possui um papel tanto de proteção ao alertar o indivíduo, a fim de evadir do estímulo indutor da dor, como de adaptação visando proteger o tecido acometido durante o processo de reparo (Grace *et al.*, 2014). Intimamente relacionada, a resposta inflamatória nos sistemas nervoso central e periférico pode atuar no desenvolvimento e/ou persistência de diversos estados patológicos dolorosos (Watkins *et al.*, 2003).

De maneira geral, o processo inflamatório pode ser classificado como agudo ou crônico, e costuma ser divido em três fases distintas que compreendem: o aumento da permeabilidade vascular, resultante da dilatação de pequenos vasos, que leva ao aumento do fluxo sanguíneo (causando rubor), da permeabilidade da microvasculatura com vazamento de proteínas plasmáticas (causando edema) e leucócitos da circulação, iniciando a infiltração de leucócitos da microcirculação, causando seu acúmulo no foco da lesão e sua ativação para eliminar o agente agressor, eventualmente promovendo a remodelação e reparo tecidual (Kumar *et al.*, 2008).

Esses processos são regulados por receptores que detectam a classe de ameaça e induzem a produção mediadores inflamatórios e lipídicos adequados para combatê-la. Ao ser desencadeado pela invasão de patógenos estranhos ou dano tecidual, o sistema imune inato é imediatamente ativado em resposta a moléculas portadoras de padrões moleculares associados a patógenos (PAMPs) ou padrões moleculares associados a danos (DAMPs), respectivamente (Janeway, 1989; Seong e Matzinger, 2004; Li e Wu, 2021).

Como reguladores iniciais da inflamação, metabólitos do ácido araquidônico, moléculas de adesão, citocinas, quimiocinas e o fator de ativação plaquetária causam liberação de outros mediadores dando início a quimiotaxia (Patil *et al.*, 2019). Esses mediadores inflamatórios liberados pelas células imunes atuam ainda nos nociceptores e resultam na redução de seu limiar de ativação, acarretando a sensibilização periférica, um dos principais causadores da dor no local da inflamação (Jain *et al.*, 2024). Entre os principais mediadores inflamatórios envolvidos nesta etapa podemos citar as citocinas pró-inflamatórias, como TNF-α, IL-1β e IL-6, e mediadores lipídicos, como prostaglandinas (PGs) e leucotrienos (LTs) (Yao e Narumiya, 2019). Tais eventos dão início ao processo inflamatório agudo (com duração de horas a dias) para eliminar os patógenos e/ou os tecidos danificados (Kumar, *et al.*, 2008).

O recrutamento de granulócitos – inicialmente neutrófilos – para o tecido lesado atua tanto na defesa do hospedeiro através da eliminação de patógenos, os neutrófilos desempenham um papel crucial na fisiopatologia da inflamação estéril (Linnerz e Hall, 2020; Phillipson e Kubes, 2011). Seu recrutamento tem início com o rolamento em células endoteliais ativadas por meio de interações entre as selectinas P/E e seus ligantes, incluindo o ligante-1 da glicoproteína P-selectina (PSGL-1) (Phillipson e Kubes, 2011). As integrinas ativadas, principalmente αLβ2 e αMβ2, se

ligam a molécula de adesão intercelular-1 (ICAM-1), promovendo o rolamento lento, a adesão e o rastejamento dos neutrófilos. A sinalização no local do dano produz quimioatraentes, que guiam os neutrófilos rastejantes durante a transmigração através da barreira das células endoteliais. Os neutrófilos aderentes também podem auxiliar na adesão plaquetária e na formação de microtrombos, levando à oclusão microvascular e agravando condições inflamatórias (Kim et al., 2015; Li *et al*., 2019).

Ao passo que os PAMPs, DAMPs, patógenos e tecidos danificados são eliminados, o processo de resolução é induzido, inibindo a sinalização pró-inflamatória, promovendo a eliminação das quimiocinas e dos granulócitos recrutados. (Sugimoto *et al.*, 2016; Yao e Narumiya, 2019).

2.3.1 Citocinas

As citocinas são proteínas solúveis com baixo peso molecular (≈ 6 – x70 kDa) responsáveis pela regulação dinâmica da maturação, crescimento e capacidade de resposta das células imunes, podendo ser secretadas por uma variedade de células (como linfócitos, macrófagos, células natural killer (NK), mastócitos etc.) (Liu *et al.,* 2021). Cabe ressaltar que um único tipo de citocina pode ser secretada por diferentes tipos de células e também pode atuar em vários tipos de células, o que confere a essa classe de mediadores múltiplas atividades biológicas (Sprague e Khalil, 2009).

As citocinas podem ser agrupadas como fatores de necrose tumoral (TNFs), interleucinas, linfocinas, monocinas, interferons (IFNs), fatores estimuladores de colônias (CSFs) e fatores de crescimento transformadores (TGFs). As citocinas costumam ser classificadas de acordo com seu papel no processo inflamatório, agrupadas como pró-inflamatórias: IL-1β, IL-6, IL-8, IL-12, TNF-α e interferons, entre outras, pois facilitam as reações inflamatórias e tendem a estimular células imunocompetentes; ou anti-inflamatórias: IL-4, IL-6, IL-10, IL-11, IL-13, antagonista do receptor de IL-1 (IL-1RA) e TGF-β, que inibem a inflamação e suprimem as células imunes (Boshtam *et al.*, 2017; Sprague e Khalil, 2009). É valido ressaltar que algumas citocinas podem apresentar papeis duplos, como a IL-6, a depender do contexto em que estão inseridas.

2.4 Estresse Oxidativo

A geração de ROS intracelular pode ser classificada em duas categorias: dos processos biológicos que liberam ROS como um subproduto, ou um produto residual, de várias outras reações necessárias, ou dos processos que geram ROS intencionalmente, seja na síntese ou degradação molecular, como parte de uma via de transdução de sinal, ou como parte de um mecanismo de defesa celular (Morgan e Liu, 2011).

Durante o processo inflamatório, a expressão da NADPH oxidase fagocítica NOX2 ($gp91^{phox}$) depende e é induzida por NF-κB. As NADPH oxidases utilizam NADPH para produzir superóxido, que é usado tanto na defesa imunológica como nos processos de sinalização celular (Anrather *et al.,* 2006). Na defesa contra patógenos infecciosos, nos compartimentos fagossômicos, o superóxido é convertido pela superóxido dismutase e mieloperoxidase em ácido hipocloroso, um potente microbicida (Quinn *et al.*, 2006).

Na inflamação, o superóxido é produzido a uma taxa que sobrecarrega a capacidade do sistema de defesa da enzima SOD endógena de removê-lo. Esse desequilíbrio resulta em lesões mediadas por superóxido (Wang *et al.*, 2004; Muscoli *et al.,* 2003).

Os metabólitos do ácido araquidônico apresentam papel duplo, tanto na promoção da inflamação como na geração de espécies reativas. A enzima ciclooxigenase-2 (COX-2, também conhecida como prostaglandina G/H sintase 2) que converte ácido araquidônico em prostaglandina H2 (PGH2). Durante a segunda etapa da reação que produz PGH2, o superóxido também é gerado. Portanto, o superóxido é um produto secundário dessa reação que também induz o estresse oxidativo (Deng *et al.*, 2003; Marnett *et al.*, 1999).

2.5 Via NF-κB

A via de sinalização mediada pelo fator de transcrição nuclear kappa B (NFκB) é de especial interesse no contexto inflamatório e na ativação neuronal. Ela foi a primeira família de fatores de transcrição descoberta nas regiões sinápticas do cérebro em 1989 (Korner *et al.*, 1989). Inicialmente, seu papel na resposta imune era o foco da maior parte dos estudos, mas desde então seu papel na função das células

neuronais e gliais na transdução de eventos sinápticos em sinalização nuclear vem sendo reconhecido, bem como em processos relacionados ao desenvolvimento neuronal, plasticidade e degeneração (Kaltschmidt *et al.,* 1994; Moynagh *et al.*, 1993; Sparacio *et al.*, 1992; Chou *et al.*, 2011).

A família NF-κB consiste em cinco fatores de transcrição: p50, p52, p65 (RelA), RelB e c-Rel. Ao formar homodímeros ou heterodímeros, esses fatores de transcrição compartilham um domínio de ligação e dimerização de DNA N-terminal e induzem a expressão de genes pró-inflamatórios, podendo induzir, por exemplo, a produção de IL-1β e TNF-α, COX-2 etc (Li e Verma, 2002).

A atividade biológica celular mediada por NF-κB ocorre por meio de sua ligação a região promotora no núcleo (Singh e Singh, 2020). Normalmente é encontrada em diversos tipos celulares em sua forma citoplasmática latente, complexado a proteínas inibitórias, e sua ação dependente do contexto do tecido, podendo induzir diferentes respostas em ambientes fisiológicos ou patológicos, sofrendo influência de fatores neurotróficos, neurotransmissores, atividade elétrica, sinalização mediada por citocinas e estresse oxidativo (Kaltschmidt *et al.*, 1994).

Em contexto pró-inflamatório, a sinalização por NF-κB possui duas vias bem caracterizadas, a canônica e a não-canônica. Em uma breve visão geral, na via canônica o fator de transcrição NF-κB está normalmente localizado no citoplasma como um heterodímero, formado principalmente por p50/p65 (RelA, também denominado p65), associado a proteínas IκB, que mascaram o domínio de ligação ao DNA e as mantêm sequestradas no citoplasma. Ao passo que mediadores pró-inflamatórios induzem a fosforilação do IκB, causam sua ubiquitinação e consequente degradação, então o NF-kB ativo é translocado para o núcleo e desencadeia a transcrição dos genes-alvo. Por outro lado, a via não-canônica, que visa predominantemente a ativação do complexo p52/RelB NF-κB, é independente da degradação de IκB, ocorrendo devido à fosforilação de IKK-α (IκB quinase-α) mediada pela cinase indutora de NF-κB (NIK), que desencadeia a fosforilação e o processamento de p100, uma molécula que funciona tanto como precursora de p52 quanto como inibidora específica de RelB, permitindo assim a ativação não-canônica da via NF-κB (Lingappan, 2018; Morgan e Liu, 2011; Sun, 2011).

2.6 FLAVONOIDES E *TRANS*-CHALCONA

Flavonoides são compostos derivados de plantas que compartilham uma estrutura básica, seu núcleo flavana, composto por três anéis denominados A, B e C. De maneira geral, os padrões de substituição e a posição dos grupos hidroxila (-OH) nestes anéis indicam os mecanismos de ação de cada flavonoide. Suas características de segurança observadas em estudos pré-clínicos e clínicos, associadas a seus promissores efeitos antioxidantes, analgésicos e anti-inflamatórios tornaram os flavonoides alvo de um número crescente de pesquisas (Ferraz *et al.,* 2020). As chalconas, mais especificamente, pertencem a uma classe dos flavonoides presentes em vegetais, frutas, chás e outras plantas, principalmente nas pétalas das flores, possuindo importante papel na polinização das plantas devido a sua coloração amarela atrativa para insetos e pássaros (Silva *et al.*, 2021). Além disso, as chalconas compõem um dos maiores grupos de produtos naturais bioativos com potencial anticâncer, anti-inflamatório, antimicrobiano, antioxidante e antiparasitário (Jasim *et al.*, 2021).

A *trans*-Chalcona (1–3-diphenyl-2-propen-1-one) é um flavonoide de cadeia aberta, encontrado de forma abundante em plantas comestíveis (Karimi-Sales *et al.*, 2018). Como flavonoide e precursora de outros flavonoides, a *t*C possui uma peculiaridade estrutural, pois não apresenta caráter químico antioxidante direto, observado pela ausência de radicais hidroxila em sua estrutura e comprovado por Martinez *et al.* (2017a). Pese tal característica, sua administração *in vivo* é capaz de gerar efeitos antioxidantes, tornando-a um alvo de estudo promissor para investigar a fundo as propriedades antioxidantes dos flavonoides além da ação antioxidante inerente à uma estrutura molecular antioxidante *per se* (Martinez *et al*., 2017a).

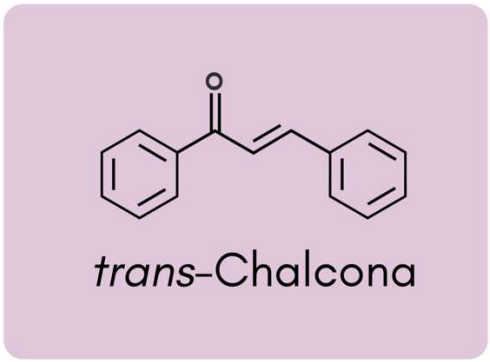

Figura 3. *Estrutura química da trans-Chalcona (tC).*

Os efeitos anti-inflamatórios da *t*C foram observados em modelos de dano cutâneo induzido pela luz UV (Martinez *et al.*, 2017a e 2017b), colite (Guazelli *et al.*, 2023), artrite gotosa induzida por MSU (Staurengo-Ferrari *et al.*, 2018) e artrite reumatoide induzida por CFA (Jabbar *et al.*, 2024). Lamoke *et al.* (2011) identificaram ainda o efeito da *trans*-Chalcona na redução da neovascularização patológica da retina isquêmica, atribuída à diminuição na expressão proteica do fator de crescimento endotelial vascular (VEGF) e da molécula de adesão intercelular 1 (ICAM-1), efeito correlacionado com a inibição da ativação do transdutor de sinal dos ativadores transcricionais e ativador da transcrição 3 (STAT3) e da via de sinalização do NF-κB.

A capacidade anti-inflamatória da *t*C é reflexo de sua capacidade de modular a liberação de citocinas, como observado nos dois modelos de artrite publicados com a *t*C, e de regular vias de sinalização ligadas ao processo inflamatório, em concordância com os achados do grupo de Lamoke (2011) supracitado. No modelo de artrite gotosa investigado por Staurengo-Ferrari *et al.* (2018), o tratamento com *t*C foi capaz de modular as citocinas IL-1β, TNF-α, IL-6, encontradas inibidas, e o TGF-β, estimulado após o tratamento. O trabalho observou ainda a capacidade da *t*C de atuar diretamente em células de defesa, de inibir a via de sinalização NF-κB e a montagem do inflamassoma NLRP3. No modelo de artrite reumatoide induzida por CFA, publicado recentemente por Jabbar e colaboradores (2024), foi encontrada a modulação negativa da expressão gênica das citocinas IL-1β, IL-6, IL-17 e TNF-α associada à melhora observada após o tratamento com *t*C, enquanto a expressão de IL-10 foi induzida pelo tratamento. A expressão das enzimas COX-2 e óxido nítrico sintase induzível (iNOS), de cunho pró-inflamatório e pró-oxidativo, respectivamente, também foi regulada negativamente nos grupos tratados com *t*C (Jabbar *et al.*, 2024).

Em relação a possíveis propriedades analgésicas da *t*C, os únicos indícios prévios presentes na literatura são a redução na hiperalgesia por estímulo mecânico e térmico no modelo de artrite gotosa (Staurengo-Ferrari *et al.*, 2018) e por estímulo térmico no modelo de atrite induzida por CFA (Jabbar *et al.*, 2024). Em outra esfera, as chalconas naturais, de modo geral, foram identificadas como potenciais inibidores do canal TRPV1, característica atribuída a sua estrutura química relativamente simples, o que favoreceria sua modificação química de forma conveniente, tornando-as alvos atraentes como ligantes específicos para o tratamento da dor periférica (Benso *et al.*, 2019). Ainda, outro flavonoide da classe das chalconas, a hesperidina metil chalcona (HMC), preveniu a dor muscular de início tardio causada

por exercícios não habituais em camundongos. Os mecanismos descritos envolvem o combate ao estresse oxidativo e a inflamação, além da redução da atividade dos neurônios nociceptivos TRPV1+ e TRPA1+ (Cortez *et al.*, 2025), indicativos da capacidade das chalconas de modular a sinalização da dor via TRPV1 e TRPA1.

Em suma, a estrutura química atípica da *t*C para um flavonoide, proporciona uma ferramenta de estudo conceitualmente relevante, pois permite investigar os mecanismos de ação de um flavonoide além de suas ações antioxidantes *per se.* Além disso, a *t*C demonstrou perfil de segurança promissor, atividades anti-inflamatória e antioxidante que, por si só, a tornam atrativa como potencial tratamento anti-inflamatório. Adicionalmente, a ausência de investigações prévias com foco no potencial analgésico da *t*C, abre um campo de estudo novo e promissor envolvendo tanto a dor e seus mecanismos regentes, como sua relação com a atividade antioxidante peculiar do flavonoide.

Portanto, o presente trabalho investigou a hipótese de que a TC possui efeitos analgésicos, anti-inflamatórios e antioxidantes resultantes de ação multialvos, incluindo efeito modulador de receptores nociceptores (TRPV1 e TRPA1).

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar a capacidade analgésica da *trans*-Chalcona em modelos de dor manifesta e sua eficácia contra a dor, inflamação e dano oxidativo induzidos pelo ânion superóxido, identificando os mecanismos de ação envolvidos nos processos.

3.2 OBJETIVOS ESPECÍFICOS

- ⇒ Avaliar a capacidade da *t*C de reduzir a dor manifesta induzida por ácido acético, fenil-*p*-benzoquinona, formalina e adjuvante completo de Freund
- ⇒ Identificar se os nociceptores TRPV1 e TRPA1 estão envolvidos na ação analgésica da *t*C através dos modelos de dor manifesta induzida por seus agonistas, capsaicina e AITC, respectivamente
- ⇒ Avaliar se a *t*C é capaz de modular a resposta neuronal via TRPV1 e TRPA1 *in vitro*
- ⇒ Avaliar a capacidade da *t*C de inibir a dor e a inflamação induzidas pelo KO_2
- ⇒ Analisar os efeitos da *t*C no recrutamento de leucócitos intraplantar induzido pelo KO_2
- ⇒ Avaliar os efeitos da *t*C na sinalização oxidativa no tecido plantar estimulado com KO_2
- ⇒ Avaliar os efeitos da *t*C na produção das citocinas IL-1β, TNF-α, IL-33 e IL-6 no tecido plantar após estímulo com KO_2
- ⇒ Avaliar os efeitos da *t*C na expressão gênica de moduladores inflamatórios no tecido plantar após estímulo com KO_2
- ⇒ Investigar os efeitos da *t*C na ativação da via de sinalização NF-κB

desencadeada pelo KO_2

⇒ Analisar os efeitos da *t*C no influxo de cálcio após estímulo com os agonistas capsaicina ou AITC em neurônios nociceptivos $TRPV1^+$ e $TRPA1^+$ sensibilizados *in vivo* pelo KO_2

4 ARTIGO 1

trans-Chalcone alleviates overt pain-like behavior through the inhibition of TRPV1 and TRPA1 ionic channels

Trabalho realizado no Laboratório de Dor, Inflamação, Neuropatia e Câncer (LADINC), da Universidade Estadual de Londrina, seguindo as normas do periódico Journal of Natural Products – ISSN 0163-3864 – F.I.: 3.4 [2023], Qualis A1 CAPES Ciências Biológicas II.

trans-Chalcone alleviates overt pain-like behavior through the inhibition of TRPV1 and TRPA1 ionic channels

*Maiara Piva [1], Sergio M. Borghi [1,2], Kelly Megumi Yaekashi [1], Thais Gabriela de Oliveira da Silva Pereira [1], Mariana M. Bertozzi [4], Ketlem C. Andrade [1], Cássia Calixto-Campos [1], Victor Fattori [3], Rubia Casagrande [4], Waldiceu A. Verri [1]**

[1]Department of Immunology, Parasitology and General Pathology, Center of Biological Sciences, Londrina State University, Londrina, Paraná, Brazil 86057-970; maiarapiva@gmail.com; sergio_borghi@yahoo.com.br; kelly.megumi.yaekashi@uel.br; thais.gabriela@uel.br; waverri@uel.br

[2]Center for Research in Health Sciences, University of Northern Paraná, Londrina, Paraná, Brazil

[3]Departament of Vascular Biology Program, Department of Surgery, Boston Children's Hospital-Harvard Medical School, Karp Research Building, 300 Longwood Ave, 02115, Boston, Massachusetts, United States

[4]Departament of Pharmaceutical Sciences, Center of Health Sciences, Londrina State University, Londrina, Paraná, Brazil 86038-440; mariana.mbertozzi@uel.br; rubiacasa@uel.br;

*Corresponder: Prof. Waldiceu A. Verri Jr., Biological Science Center, Londrina State University. Rod. Celso Garcia Cid Pr 445 KM 380, Londrina, Paraná, Brazil. P.O. box 10.011, zip code 86057-970. Phone: +55 43 3371-4979, Faz: +55 43 3371-4387, waldiceujr@yahoo.com.br or waverri@uel.br;

ABSTRACT

Pain is a debilitating condition that affects around 30% of the adult population worldwide. Current pharmacological options for pain management present limited efficacy and concerning adverse effects. Therefore, alternatives with compelling results and favorable safety profile represent an important medical gap. *trans*-Chalcone (*t*C) is an open-chain flavonoid with broad antioxidant and anti-inflammatory properties that prevents mechanical and thermal peripheric sensitization in inflammatory arthritic experimental models, although its analgesic potential was yet to be the focus of pre-clinical research. Peripheric pain signaling is mediated by different classes of ionic channels. Among them, members of the transient receptor potential (TRP) family of ionic channels have been widely associated with pain transmission and modulation. The aim of our study was to investigate if the flavonoid *t*C can promote analgesia, assessing overt pain-like behavior induced by different nociceptive stimuli, and to determine if *t*C can regulate TRPV1 (TRP vanilloid 1) and TRPA1 (TRP ankyrin 1) ionic channels effects following nociceptive activation. Initially, the writhing response to intraperitoneal acetic acid or phenyl-*p*-benzoquinone (PBQ) was appraised, followed by assessment of intraplantar (i.pl.) overt pain-like behavior triggered by formalin, complete Freund's adjuvant (CFA), capsaicin, or allyl isothiocyanate (AITC). *t*C (3, 10 or 30 mg/kg) was administered orally (p.o.) by gavage 30 minutes before the nociceptive stimulus. Abdominal contortions induced by acetic acid were analyzed for 20min and the dose of 10 mg/kg was selected for the following experiments. *t*C was able to reduce contortions triggered by acetic acid and PBQ, as well as the paw-induced overt pain-like behavior, *i.e.* number of flinches and time spent licking the injected paw, triggered by formalin and CFA, indicating *t*C ample analgesic potential. Inhibition of TRPV1 ionic channel by the flavonoid was investigated using its agonist, capsaicin, to induce nociception. Likewise, the TRPA1 agonist AITC was injected to observe *t*C antinociceptive effects. Our results demonstrate that the flavonoid presents broad analgesic effects, inhibiting pain-like displays mediated by both of the targeted nociceptive ionic channels. Additionally, *t*C effects on dorsal root ganglia (DRG)-derived neurons were investigated *in vitro*, where pre-treatment with *t*C (3 μM) significantly reduced calcium influx on TRPV1- and TRPA1-expressing neurons after stimuli with capsaicin and AITC, respectively, compared to vehicle-treated neurons. In sum, we demonstrate for the first time *t*C wide-ranging analgesic properties. Its effects

are, at least in part, caused by TRPV1 and TRPA1 nociceptors inhibition in mice. Our results, added to the previously described safety profile on this flavonoid, are encouraging for further clinical investigation of these newly discovered antinociceptive effects.

Keywords: Flavonoid, analgesic, nociception, pain management.

INTRODUCTION

Pain is a significant public health issue that affects about 30% of the adult population globally, impairing daily activities, sleep, and interpersonal relationships [1, 2, 3]. Pain management relies on analgesics, which include several classes of medications, such as nonsteroidal anti-inflammatory drugs, antidepressants, antiepileptics, local anesthetics, opioids, and acetaminophen [4]. Despite ongoing efforts, acute pain relief remains suboptimal due to considerable adverse effects, insufficient efficacy and tolerance development, highlighting the need for novel analgesic options with higher safety profiles. A valuable experimental tool to screen potential analgesic drugs is the assessment of overt pain-like behavior [5].

To understand overt pain-like behavior, it is imperative to differentiate sensation and perception: the sensation refers to the actual detection of noxious stimuli and can occur much faster than the brain's perception of that nociceptive input, which in turn can be altered by context and multiple other related factors, both psychological and physiological, to finally be interpreted as pain [6]. The cells responsible for the detection of external stimuli sensation are first-order sensory neurons, also known as primary afferents, and they convey that information over long distances through action potentials [6]. It is accepted that overt pain-like behavior occurs due to the interaction of a noxious stimulus to nociceptors present on primary afferent peripheral axons, generating an almost immediate response as the ionic influx generates action potentials, characterizing the nociceptive signaling [7]. Therefore, these behavioral displays can be identified and measured to screen potentially analgesic drugs [8].

Two members of the transient receptor potential (TRP) family, TRPV1 (TRP-vanilloid 1) and TRPA1 (TRP-ankyrin 1), are amongst the currently known ionic channels that mediate nociception [9]. TRPV1 is a multistate channel present on

nociceptors, acting as a molecular transducer which, at negative holding potential activation, allows calcium and sodium influx, thereby inducing cell depolarization [10]. Similarly, the TRPA1 ionic channel is involved in inflammatory and immune responses, as well as the conversion of physical and chemical stimuli in irritative (itching) or pain sensations [11]. It has a pronounced sensitivity to oxidative stress [12]. TRPV1 and TRPA1 receptors are often co-expressed in nociceptive neurons, but their modulation and crosstalk remain a puzzling issue in pain research [13].

Trans-Chalcone (*t*C; $C_{15}H_{12}O$; benzylideneacetophenone) is a flavonoid precursor composed by a core structure that can be described as the backbone of flavonoids, presenting a relatively flexible and simple chemical structure [14]. Addicionally, *t*C occurs naturally in several plant sources, including Licorice (*Glycyrrhiza sp.*), *Angelica sp.*, Turmeric (*Curcuma longa*), *Passiflora sp.*, Kava-Kava (*Piper methysticum*), *Aniba riparia Didymocarpus corchorijolia* and Hawthorn (*Crataegus sp.*) [15, 16].

*t*C has previously demonstrated anti-inflammatory, antioxidant, antiparasitic, and antineoplasic properties [17, 18, 19, 20, 21, 22, 23]. So far, *t*C effects regarding pain have only been observed through the reduction of mechanical hyperalgesia in a murine model of gouty arthritis, action credited to the inhibition of nuclear factor-κB (NF-κB) pathway activation and the impairment of NLRP3 inflammasome assembly [24], and by the inhibition of thermal hyperalgesia induced by complete Freund adjuvant (CFA) [25]. Nonetheless, oral treatment with *t*C inhibits leukocyte recruitment, pro-inflammatory transcription factors and their downstream signaling pathways, effectively targeting cytokines and oxidative stress, which are pathological features related to pain [15, 17, 24, 26]. Considering *t*C atypical chemical structure for a flavonoid, due to the lack of hydroxyl radicals, which results in no chemically-related direct antioxidant activity [19], *t*C

antioxidant function is particularly remarkable, reducing lipid peroxidation and the related H_2O_2 induction of glutathione related enzymes in hepatocellular carcinoma [27]. In models of inflammatory diseases, *t*C impairs the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and improves antioxidant capacity by stimulating intrinsic mechanism of defense against oxidative stress, such as nuclear factor erythroid 2–related factor 2 (Nrf2)/antioxidant responsive elements (ARE) [26, 24, 20, 19]. Additionally, due to their simple and modular scaffold, chalcone derivatives have been previously proposed as non-canonical ligands for TRPV1 [28].

In the present study we sought to investigate potential analgesic properties of *t*C in experimental models of overt pain-like behavior in mice, focusing on the flavonoid effects on the ionic channels TRPV1 and TRPA1, evaluating afferent nociceptors activation *in vitro* and *in vivo*.

RESULTS

tC inhibits acetic acid- and phenyl-p-benzoquinone (PBQ)- induced overt pain-like behavior

Initially, we investigated the analgesic effects of *t*C (3-30 mg/kg) on overt pain-like behavior induced by acetic acid (0.8%) and determined the optimal dose of the flavonoid for the following experiments. The dose-response indicated that the lower dose of *t*C (3 mg/kg) did not induce a significant reduction of acetic acid-induced abdominal contortions (16,6% inhibition compared to vehicle group) (Fig 1C). In the other hand, the doses of 10 and 30 mg/kg had similar significant inhibitory effects on the number of abdominal contortions, of 58,8% and 66,5%, respectively. The activity of the dose of 30 mg/kg of *t*C started at 8 minutes, which was earlier than what was observed for the dose of 10 mg/kg (data not shown). Considering that no significant difference was noticed between the doses of 10 and 30 mg/kg of *t*C (p-value = 0.7803) (Fig 1C), the dose of 10 mg/kg was chosen for the following experiments. This selected dose was also efficient in significantly inhibiting the writhing response induced by PBQ when compared to vehicle-treated mice (54,6% inhibition) (Fig 1D).

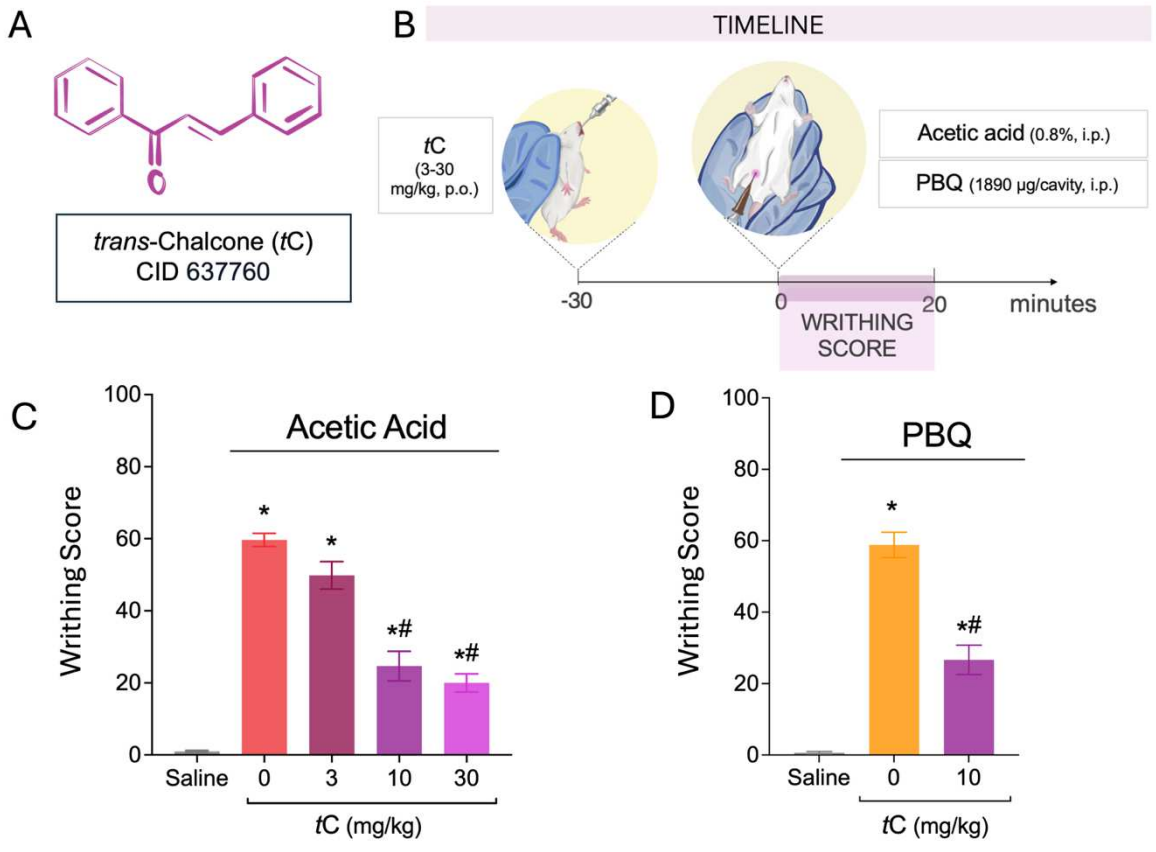

Fig 1. *tC inhibits acetic acid- and PBQ-induced overt pain-like behavior.* (A) Chemical structure of *trans*-Chalcone. (B) Timeline for writhing response assays. Mice were treated with *t*C (3 − 30 mg/kg) or vehicle (saline 20% Tween-80) 30 min before acetic acid (C), or PBQ (D) intraperitoneal (i.p.) stimuli. The vehicle of acetic acid was saline and the vehicle of PBQ was 2% DMSO in saline. The cumulative number of abdominal contortions (writhing score) was evaluated over 20 min for both assays. Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments. Shapiro-Wilk and Brown-Forsythe tests were used to assess normality and equality of group variances, respectively. Statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison test (**Table 1**). *$p \leq 0.05$ compared to saline group; $^{\#}p \leq 0.05$ compared to stimulus group.

tC inhibits formalin-induced overt pain-like behavior

After stablishing the best dose of *t*C for the present experimental condition, the formalin test was performed. As it can be observed in Figs 2B and 2C, the dose of 10 mg/kg of *t*C significantly inhibited the first (44%) and second phases (48%) of formalin-induced paw flinches (Fig 2B), as well as the first (38%) and second phases (46%) of formalin-induced paw licking time (Fig 2C), suggesting *t*C can target the transient depolarization and excitation of primary afferent neurons and later inflammatory response.

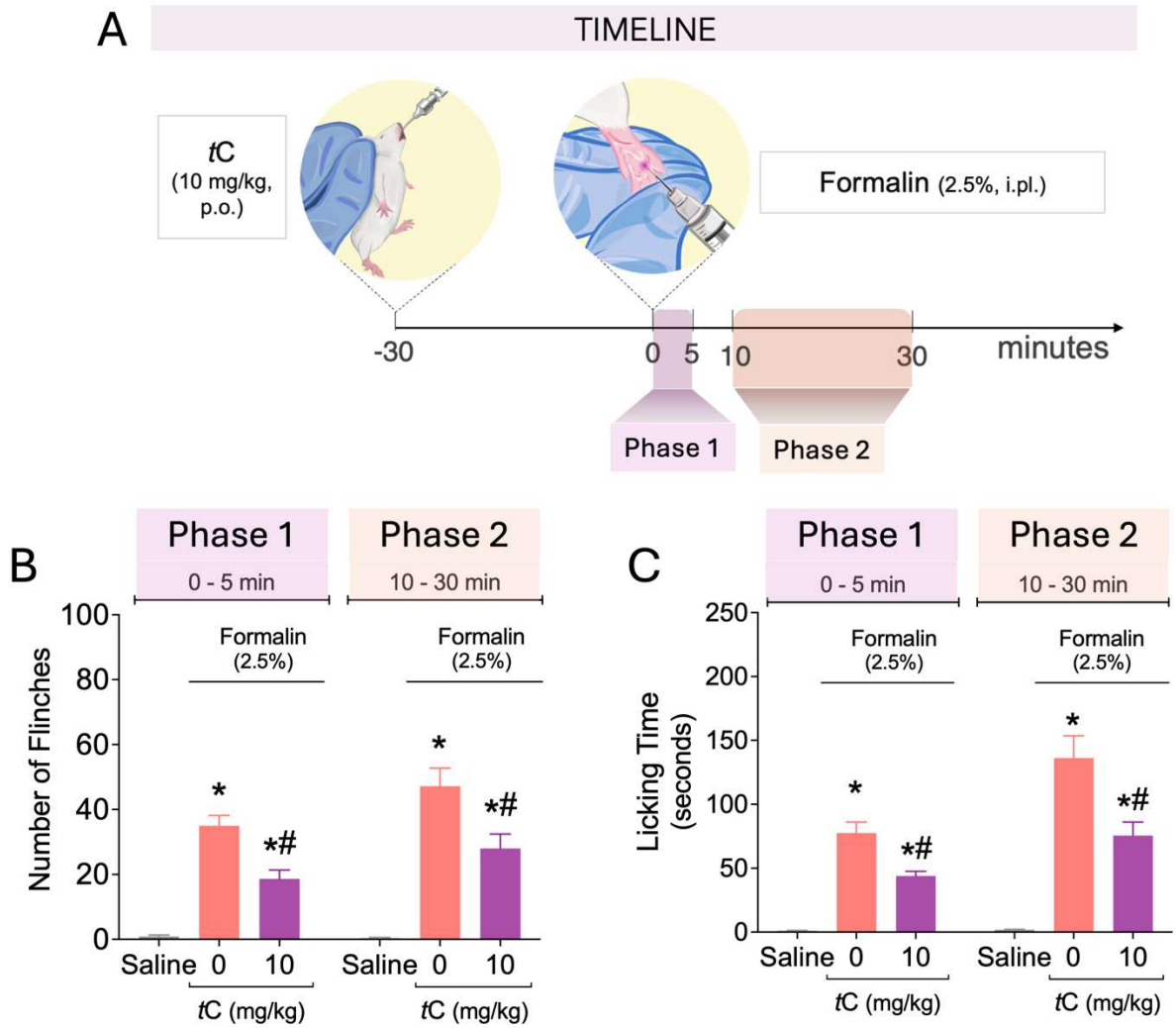

Fig 2. *tC inhibits formalin-induced paw flinching and licking.* (A) Experimental timeline. Mice were treated orally with vehicle (20% Tween-80 Saline) or *t*C (10 mg/kg) 30 minutes before the stimulus with formalin into the right hind paw. The total number of flinches (B) and total paw licking time (C) were initially evaluated for 5 minutes (0-5) at Phase 1, then for 20 minutes (10-30) at Phase 2 after the injection of formalin. Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments. Shapiro-Wilk test was used to assess normality, and statistical analyses were performed by Two-way ANOVA followed by Tukey's multiple comparison test (**Table 1**). *$p \leq 0.05$ compared to saline group; $^{\#}p \leq 0.05$ compared to stimulus group.

tC inhibits CFA-induced overt pain-like behavior

The following nociceptive test was triggered by the water-in-oil emulsion containing heat-killed *Mycobacterium tuberculosis* called complete Freund's adjuvant (CFA). The immunoadjuvant induced both of the analyzed behavioral parameters and the pre-treatment with *t*C also inhibited both the number of flinches (46% - Fig 3B) and

the time spent licking the induced paw (43% - Fig 3C) during the 30 minutes of assessment following CFA-injection.

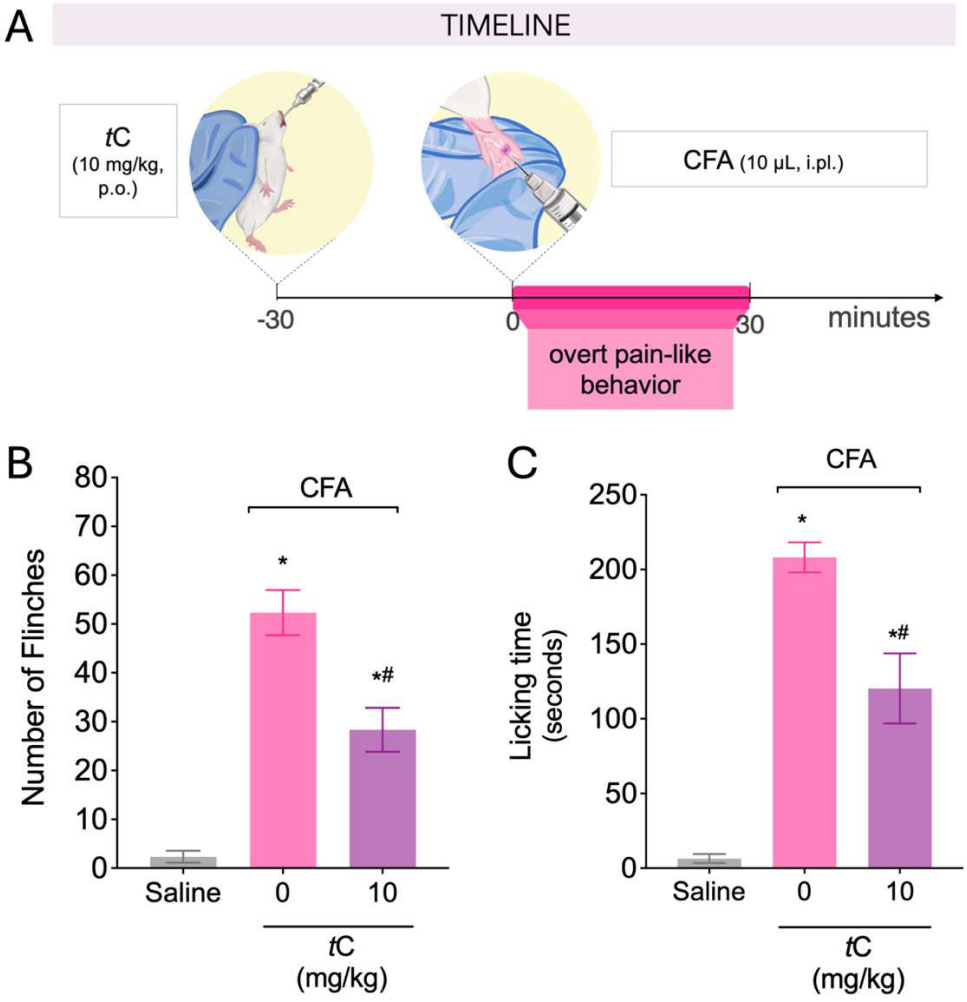

Fig 3. *tC inhibits CFA-induced paw flinching and licking time.* (A) Experimental timeline. Mice were treated orally with vehicle (20% Tween-80 Saline) or *t*C (10 mg/kg) 30 minutes before the stimulus with CFA. The total number of flinches (B) and total paw licking time (in seconds) (C) were evaluated for 30 minutes after CFA stimulus. Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments. Shapiro-Wilk and Brown-Forsythe tests were used to assess normality and equality of group variances, respectively. Statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison test (**Table 1**). *$p \leq 0.05$ compared to saline group; $^{\#}p \leq 0.05$ compared to stimulus group.

tC inhibits capsaicin- and AITC-induced overt pain-like behavior

To assess whether *t*C effects is related to the inhibition of TRPV1 and/or TRPA1 ionic channels, in the next set of experiments the ionic channels agonists were used to trigger overt pain-like behavior. Therefore, we evaluated if the pre-treatment with *t*C could inhibit the number of paw flinches and licking time triggered by capsaicin, the TRPV1 agonist. Our results demonstrate that *t*C significantly inhibited Capsaicin-induced flinches in 44% (Fig 4B) and licking time in 37,5% (Fig 4C) during the 5 minutes of assessment. Likewise, the number of flinches and time spent licking the paw after the injection of the TRPA1 agonist allyl isothiocyanate (AITC) were inhibited in 35,1% (Fig 4D) and 52% (Fig 4E), respectively.

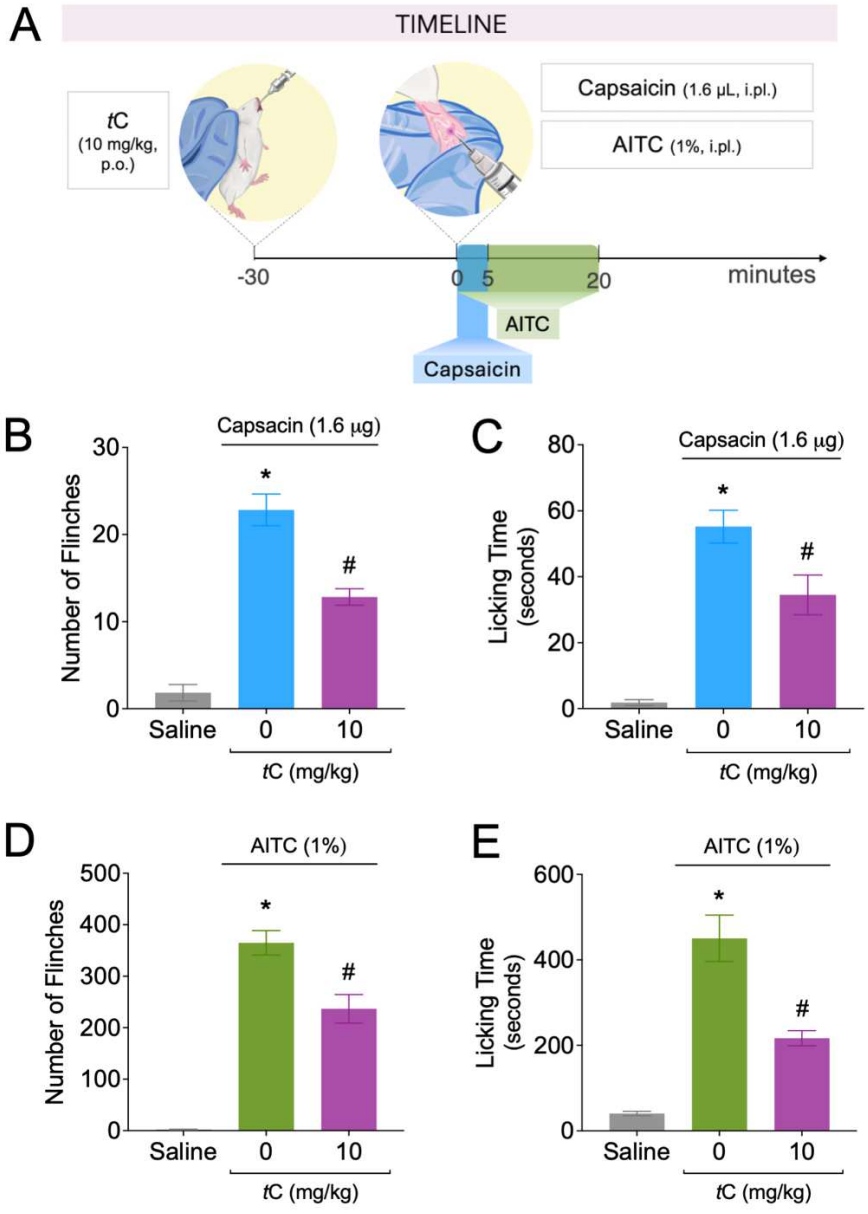

Fig 4. *tC inhibits capsaicin- and AITC-induced overt pain-like behavior.* (A) Experimental timeline. Time spent licking the induced paw (B) and total number of paw flinches (C) were

counted during 5 min after i.pl. injection of capsaicin (1.6 µg/ 25 µL/paw). Likewise, time spent licking the induced paw (D) and total number of paw flinches (E) were counted during 20 min after AITC (1% v/v [10 M] in 20 µL). Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments. Shapiro-Wilk and Brown-Forsythe tests were used to assess normality and equality of group variances, respectively. Statistical analyses were performed by one-way ANOVA followed by Tukey's multiple comparison test (**Table 2**). *$p \leq 0.05$ compared to saline group; $^{\#}p \leq 0.05$ compared to stimulus group.

tC reduces TRPV1- and TRPA1-expressing neuron activation in vitro

We also investigated whether *t*C could inhibit TRPV1- and/or TRPA1-expressing ($TRPV1^+$ and $TRPA1^+$) neurons activation. For this purpose, cultured dorsal root ganglia (DRG)-derived neurons were stimulated with capsaicin (Fig 5) or AITC (Fig 6). Pre-treatment with *t*C (3 µM) effectively inhibited capsaicin-induced calcium influx compared to vehicle-treated DRG neurons, which can be observed through the reduced fluorescence intensity (20%) after adding the TRPV1 agonist (Figs 5A and 5C) and the number of neurons activated by capsaicin, which was 30% lower than the control group (Fig 5D).

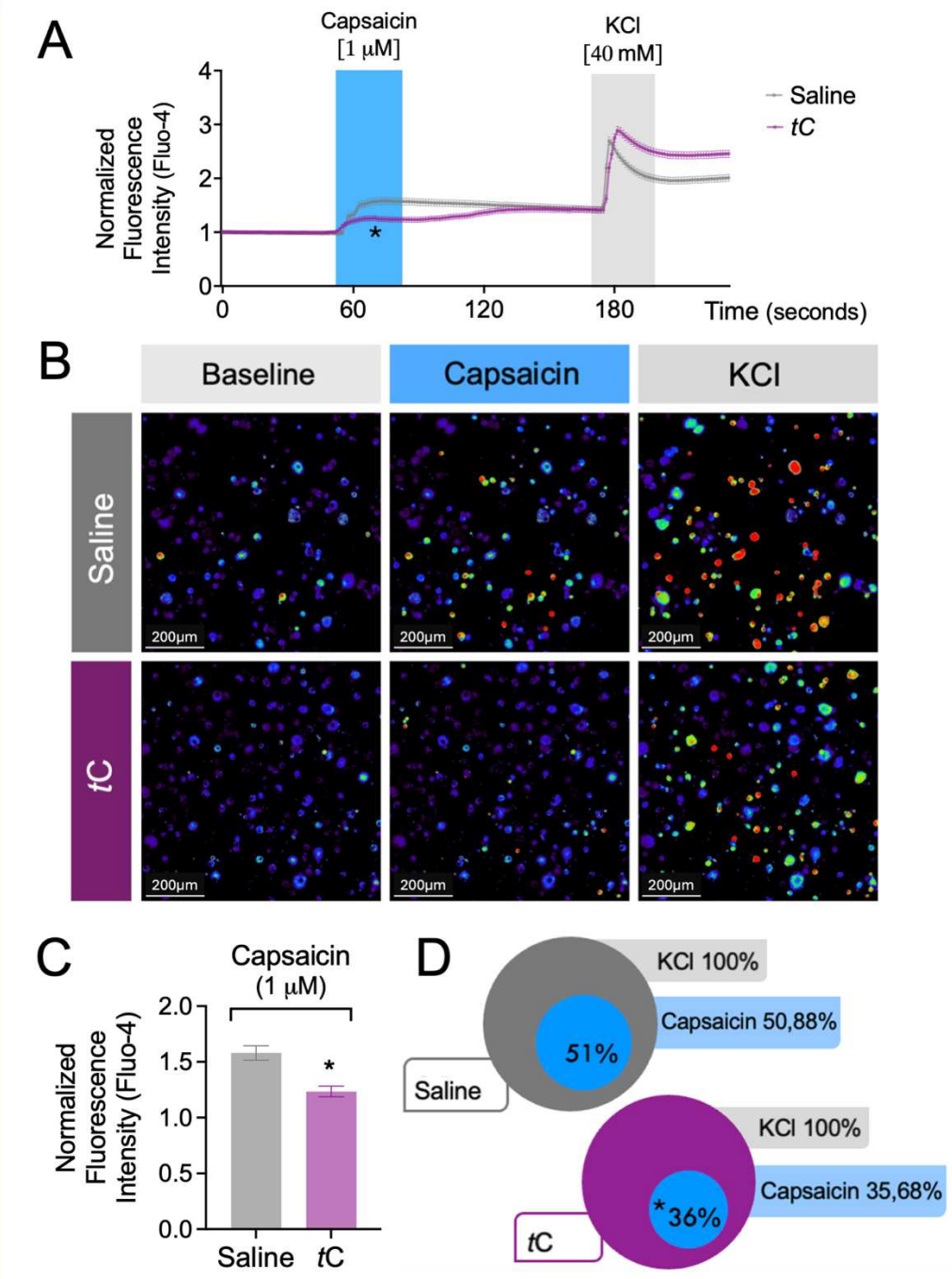

Fig 5. *tC inhibits TRPV1⁺ nociceptor activation in vitro.* To assess TRPV1⁺ neuron activation in vitro, plates containing naïve DRG neurons were pre-treated with *t*C (3 µM) or vehicle (0.01% DMSO) for 60 min. Plates were recorded for 4min: 1min of initial reading, followed by stimulus with capsaicin (1 µM in HBSS) for 2 min at the 60 s-mark, and with KCl for 1min at the 180 s-mark (40 mM, activates all neurons). (A) Normalized fluorescence intensity tracers throughout the 4 min of recording. (B) Representative images acquired at baseline, Capsaicin stimulus, and KCl activation, for *t*C and saline groups. Captured with 20x objective lens. Scale bars = 200 µm (C) Comparative mean fluorescence intensity between saline and *t*C groups after capsaicin stimulus. (D) Venn's diagram indicating the percentage of DRG-derived neurons with

increased calcium influx after capsaicin stimulus. Results are provided as means ± SEM of the cell sum for four culture dishes per group and are representative of two separate experiments. Saline group = total of 360 neurons; *t*C group = total of 399 neurons. Statistical analyses were performed by Mann Whitney test (**Table 2**). *p ≤ 0.05 compared to saline group.

Neurons pre-treated with *t*C (3 µM) and induced with the TRPA1 agonist showed a decrease in fluorescence intensity (24%) (Figs 6A and 6C) as well as the reduction in the number of neurons that underwent calcium influx after the addition of AITC (37,6%) to the cell culture when compared to vehicle-treated DRG neurons (Fig 6D).

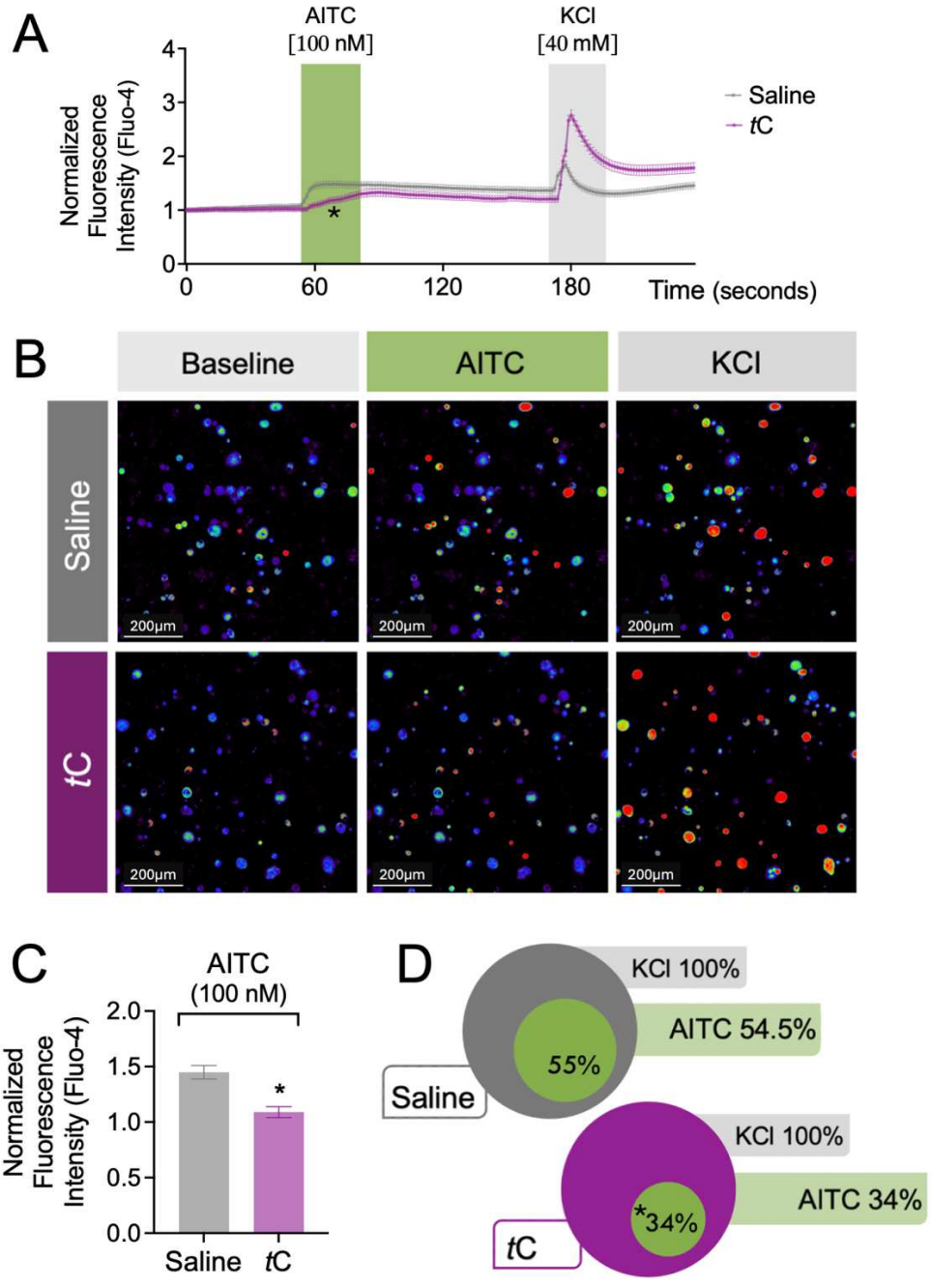

Fig 6. *tC inhibits TRPA1^{+} nociceptor activation in vitro.* To assess TRPA1 activation in vitro, plates containing naïve DRG neurons were pre-treated with *t*C (3 μM) or vehicle (0.01% DMSO) for 60 min. Fluo-4-labeled cells were recorded for 4 min: 1 min of initial reading, followed by stimulus with AITC (100 nM in HBSS) for 2 min at the 60 s-mark, and with KCl for 1 min at the 180s-mark (40 mM, activates all neurons). (A) Normalized fluorescence intensity tracers throughout the 4 min of recording. (B) Representative pictures acquired at baseline, AITC stimulus, and KCl activation, for *t*C and saline groups. Captured with 20x objective lens. Scale bars = 200 μm (C) Comparative mean fluorescence intensity between saline and *t*C groups after AITC stimulus. (D) Venn's diagram indicating the percentage of DRG-derived neurons with increased calcium influx after AITC stimulus. Results are provided as means ± SEM of the cell sum for four culture dishes per group and are representative of two separate experiments. Saline group = total of 252 neurons; *t*C group = total of 160 neurons. Statistical analyses were performed by Mann Whitney test (**Table 2**). *$p \leq 0.05$ compared to saline group.

DISCUSSION

The primary goal of our study was to determine whether *t*C could be effective as an analgesic. The writhing response to acetic acid and PBQ was the starting point to assess the flavonoid potential regulating nociception. Even though overt pain-like behavior induction by acetic acid and/or PBQ are widely used and effective to screen potential analgesics[29], the exact mechanism promoting nociceptive activation is yet to be pinpointed. The acetic acid-triggered writhing response appears to be mediated by TRPV1 inhibition[30], possibly due to an acid-induced extracellular pH reduction. The ionic channels involved in PBQ-induced overt pain-like behavior remain unknown. Moreover, these noxious chemicals induce peripheral and central nociceptor sensitization associated with the release of pro-inflammatory mediators[5,8], whereas the PBQ-triggered response seems to be mediated, at least partially, by IL-18 and IL-33, as the acetic acid response appears to be modulated by IL-33, but IL-18 independent[31].

The formalin test is arguably the best-characterized model for overt pain-like behavior testing, and its dual phase design is used to screen compounds with analgesic and/or anti-inflammatory potential due to the time-dependent sensitivity for different classes of analgesic drugs[32,33]. As the test comprises two phases, the initial phase (0-5 min) is accepted as the result of nociceptive activation and our results indicate *t*C ability to inhibit this signaling process. The second phase (10-30 min), which was also inhibited with *t*C pre-treatment, is mainly attributed to inflammation-driven mechanisms, relying on cytokine signaling and leukocyte recruitment as well as the activation of ionic channels[34]. IL-33, TNF-α, IL-1β, IL-8, and IL-6 cytokines have been shown to participate on the second phase of the formalin test[31,35]. The reduction observed in the second phase after *t*C treatment is coherent to previous findings in MSU-induced arthritis[24], the only evidence of *t*C effects in mechanical hyperalgesia inhibition described thus far. Addicionally, formalin-induced nociception occurs through TRPA1 ionic channel[36], providing the first evidence of TRPA1-mediated pain inhibition by *t*C.

Regarding CFA-induced nociception, the involvement of both TRPA1[37] and TRPV1[38] have been previously described. So, to confirm *t*C inhibitory effects on TRPV1 and TRPA1 ionic channels, suggested by acetic acid-, formalin-, and CFA-induced overt pain-like behavior reduction, respectively, the nociceptive response was induced *in vivo* by intraplantar injection with their agonists capsaicin (TRPV1) and AITC

(TRPA1). Both TRPV1 and TRPA1 expression have been observed similarly in axon terminals of mouse and monkey corneas, seen primarily in axons containing components of large dense vesicles, where they appeared to separate intracellular vesicular structures[39]. As for the type of fiber in which they were labelled, TRPA1 was observed in large-diameter NF200-positive Aδ axons, while TRPV1 was expressed in small diameter C fibers of distinct populations[39], reinforcing the role of these two receptors in peripheral nociception signaling. Our results confirmed *t*C inhibition of overt pain-like behavior triggered by capsaicin and AITC *in vivo.*

Under physiological conditions, once specific noxious stimulus to which TRPV1 and/or TRPA1 ionic channels are responsive come in contact with the sensory nerve fibers, they activate these receptors and allow ion influx to transduce the noxious stimulus into electrical activity, which is conducted to the DRG and spinal cord, where the signal is further propagated by synapse through the spinal cord to the brain. In response, peripheral release of neuropeptides (mainly substance P and CGRP) activate the immune cells, so acute pain and nociceptive-induced inflammation are generated[40,41,42]. Under pathological conditions, the continuous activation of neural nociceptive circuits induce functional plasticity like transcriptional and post-translational modifications, leading to changes in both neuronal excitability and synaptic transmission, structural remodeling and reorganization of neural networks[42]. Therefore, the possibility to inhibit both TRPV1 and TRPA1 ionic channels with one treatment is encouraging for acute pain as well as sustained painful conditions.

Seen that the nociceptive signaling involves large distances of signal propagation that can suffer the interference from different types of cells and factors, to ensure that *t*C was modulating nociceptors activity, primary cultures of axotomized DRG neurons from naïve mice were used to observe ionic channels activity by calcium influx. The *in vitro* experimental approach is important to narrow *t*C effects upon neuronal cells, demonstrating that its analgesic effects were, at least in part, resultant of the inhibition in $TRPV1^+$ and $TRPA1^+$ nociceptors activity. Moreover, these findings indicate *t*C multitargeted effects, previously shown by the reduction of cytokine production *in vitro* on bone marrow-derived macrophages[24], and now by the inhibition of calcium influx on $TRPV1^+$ and on $TRPA1^+$ neurons.

It is worth noticing that *t*C analgesic mechanisms might not be solely dependent on TRP channels inhibition. Previous studies demonstrated its anti-inflammatory properties by cytokines inhibition, such as IL-1β[25,43,19,24,45,46], TNF-α[25,19,20,24,43,46], IL-

6[25,24,46], IL-12[19], IL-17[25], IL-18[45,48], and IFN-γ[19]. *t*C was also able to upregulate IL-10[25,43] and TGF-β[24].

The flavonoid also shown the ability to inhibit: COX-2[25,20,42,46], NF-κB pathway activation[43,24,45,48,18], STAT3[18], NLRP3 inflammasome assembly[24,45], MPO activity[19,20,24], ICAM[45,18,47], VCAM[45], VEGF[18,47], MMP9[19,20], therefore affecting leukocyte activation and migration, as well as tissue repair. Moreover, *t*C was able to inhibit ROS[43,46] by reducing lipid peroxidation[19,20] and superoxide anion production[19,20,24], possibly due to gp91^{phox}[19,20,24], iNOS[25,44,46], and Keap1[46] downregulation.

tC antioxidant properties were observed on FRAP[20,24] and ABTS[20,24] assays, by catalase[19,20] and glutathione[19,20,24,45] modulation, possibly related to Nrf2[20,24,46,49], HO-1[20,24,46,49,50] and Sirt1[43,51] up regulation. Additionally, the flavonoid has indicated neuroprotective properties on experimental models for Alzheimer's[52] and Parkinson's[41] disease. *t*C has also demonstrated pharmacological advantages, such as improved efficacy and controlled release by microcapsule-delivery[53], its efficiency in topical formulation[20], and the design of a methodology for tC determination by spectrophotometry[54].

Here, we described for the first time the potential use of *t*C as an analgesic. *t*C induced analgesia in classical models of overt pain-like behavior, including abdominal contortions and formalin tests. This effect can be attributed, at least in part, by nociception inhibition of TRPV1 and TRPA1 ionic channels in afferent neurons.

CONCLUSIONS

The flavonoid *t*C has demonstrated multitargeted effects on multiple cell types: anti-inflammatory and antioxidant properties, as well as athero-, hepato- and neuroprotective effects.

We showed here, for the first time, that *t*C has analgesic properties related to the inhibition of TRPV1+ and TRPA1^{+} nociceptor neurons. These data were obtained by the combination of in vivo and in vitro approaches, including classical overt pain-like behavior models and calcium imaging of cultured mouse DRG-derived neurons.

By acting on multiple targets, therapeutic effects can be achieved by mild inhibition of complementary pathways, resulting in reduced side effects. Among *t*C advantages, its efficacy as an oral analgesic associated with a satisfactory safety profile in mice, its efficacy on different delivery systems, such as topical and microencapsulated, indicate a promising alternative to manage painful conditions, therefore should be considered for further investigation in preclinical and clinical studies to alleviate pain.

EXPERIMENTAL SECTION

Animals

Experiments were performed in 6-8 weeks old male Swiss mice, weighing 20–30 g, obtained from the Londrina State University animal facility (Londrina, PR, Brazil). Mice had access to water and food ad lib and were kept at controlled temperature (24°C ± 2) with 12 h light/dark cycles. Standard polypropylene cages measuring 41 x 34 x 16 cm (Insight®) were used to house groups of 6 animals per cage. Behavioral tests were carried out always between 9 a.m. and 5 p.m. and acclimatization in the testing room was prepared at least 2 hours before the beginning of the experiments. The experimenters were blinded to the different groups evaluated in the study. For sample collection, mice were euthanized by terminal anesthetization with isoflurane 5%, followed by decapitation procedure. The Londrina State University's Animal Ethics Committee (CEUA processes 24684.2016.72, from December 8, 2016, and 050.2024, from December 12, 2024) approved all procedures of this study. All efforts were made to minimize the number of animals used and their suffering. Animal studies are reported in compliance to the ARRIVE guidelines[55].

Experimental design

Mice were treated p.o. with 3, 10 or 30 mg/kg of *t*C (95%, Santa Cruz Biotechnology, Dallas, TX, USA) diluted in saline 20% tween-80 or vehicle (20% tween-80 in saline solution) 30 minutes before i.p. or i.pl. injections of the different inflammatory stimulus, including acetic acid (0.8%, i.p.), PBQ (1890 µg/kg, i.p.), formalin (2.5%, i.pl.), capsaicin (1.3 µg, i.pl.), and AITC (1%, i.pl.). The doses of *t*C used were based on previous studies of our group[19,24]. Overt pain-like behavior tests using acetic acid and PBQ-induced abdominal writhing as well as formalin, capsaicin, and AITC-induced paw flinching and licking as stimuli were applied to evaluate *t*C potential analgesic effects. Confocal microscopy calcium dynamics imaging in cultured DRG neurons of naïve mice was also used to evaluate whether *t*C may modulate TRPV1 and TRPA1 activity. Fig 7 presents the experimental design of the present

study.

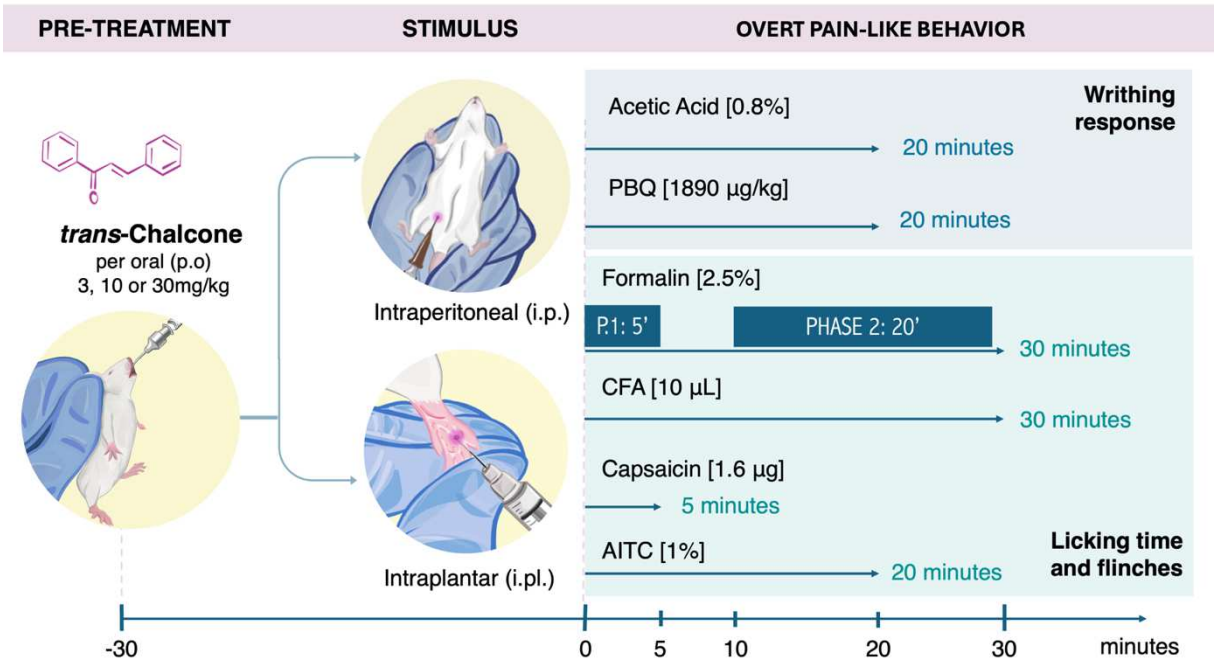

Fig 7. *Experimental design.* (A) For behavior experiments *t*C treatments were administered orally by gavage 30 minutes before stimuli. The doses of 3, 10 and 30 mg/kg were tested initially to observe *t*C effects on writhing response after acetic acid (0.8% in saline solution/200 µL per abdominal cavity) stimulus, where the 10mg/kg dose was chosen for the following experiments. Vehicles were prepared at the same time and equal volumes as treatments, but without adding *t*C. The writhing response to acetic acid and PBQ were assessed for 20 minutes after i.p. administration of the respective stimulus. For the formalin test, the time licking the injected paw and number of flinches were separated into two phases, the first 5 minutes accounted for phase 1, and from the 10th minute until the 30th minute after formalin i.pl. injection was the duration of phase 2. Capsaicin response was assessed for 5 minutes and AITC response for 20 minutes.

Overt pain-like behavior tests

Writhing response

The acetic acid- and PBQ-induced writhing models were performed as previously described[8]. Acetic acid (0.8%, v/v in saline), PBQ (DMSO 2%, v/v in saline, 1890 µg/kg), or vehicle were injected into the peritoneal cavities of mice 30 minutes after pre-treatment with *t*C (3-30 mg/kg, p.o.). The intensity of overt pain-like behavior is expressed as the cumulative writhing score over 20 minutes after stimulus.

Formalin test

The number of paw flinches and time licking the paw were determined between 0-30 minutes after 25 µL formalin i.pl. injection, as previously described[56,57,58]. The period was divided into intervals of 5 minutes, determining the first (0-5 min) and second (15-30 min) phases, which are characteristic of the neurogenic and inflammatory phases of the test, respectively. Results are presented as the number of flinches in the first and second phases.

CFA-induced overt pain-like behavior

Overt-pain-like behavior was determined by the number of paw flinches and time spent licking the stimulated hind paw, analyzed for 30 min after intraplantar injection with 10 *µ*L of CFA. These behaviors are considered indicative of nociception. Results were expressed by the total number of flinches and time spent licking over 30 min[34].

Capsaicin- and AITC-induced overt pain-like behavior

*t*C (10 mg/kg, p.o.) was administered 30 min before the nociceptive stimuli injection. Paw flinching and licking were evaluated over 5 min after i.pl. injection of capsaicin (1.6 µg/25 µL/paw), or 30 min after i.pl. injection of AITC (1%, 20 µL/paw)[59,60]. Results are expressed as the total number of flinches or time spent licking the paw (in seconds).

Calcium influx imaging by confocal microscopy

Naïve dorsal root ganglia (DRG) neurons were harvested bilaterally between L_4-L_6 vertebrae (Fig 8.1) into culture media with Dulbecco's modified Eagle medium (DMEM) high glucose 10% fetal bovine serum (FBS), mechanically and enzymatically disrupted by pipetting and collagenase A/dispase II solution, supplemented with 5 mM $CaCl_2$ (Collagenase A, Roche 10103578001, Dispase II, Roche 04942078001) incubation at 40ºC until complete dissociation (Fig 8.2). DRG cells were then centrifuged (1.500 rpm for 5 min) to obtain a monolayer of DRG axotomized neurons placed over a glass-bottom culture plate pre-coated with laminin (Fig 8.3). Cells were kept at 37ºC, 5% CO_2 overnight on neurobasal culture media (Invitrogen 21103-049) and supplemented with 2% B27 serum-free supplement (50x, Invitrogen 17504-044) 1% Pen/Strep (Cellgro 15140-163), 1% L-Glutamine (Invitrogen 25030-164), 0.004% ARA-C 0.004% glial-derived neurotrophic factor (GDNF) and 0.002% nerve growth

factor (NGF). Culture plates were seeded by duplicates and pre-treated with *t*C (3 µM) or vehicle (0,01% dimethylsulfoxide; DMSO) for one hour before stimuli (Fig 8.4). Cells were then loaded with 1.2 µM of Fluo-4AM probe for 40 minutes (Fig 8.5) (same concentrations for all experimental conditions), plates were washed, and the media was replaced by hanks' balanced salt solution (HBSS) with calcium and magnesium for neuron stimuli imaging in a confocal microscope (TCS SP8, Leica Microsystems). To assess TRPV1 and TRPA1 activation, DRG plates were recorded for 4 minutes, divided into 1 minute of initial reading (0-s mark, baseline values), followed by stimulation with capsaicin (1 µM, TRPV1 agonist) or AITC (100 nM, TRPA1 agonist) for 2 min at the 60-seconds mark and KCl (40 mM, a viability control for cells) for 1 minute at the 180-s mark (Fig 8.6). Calcium influx was analyzed based on the mean fluorescence intensity detected in each neuron with the LAS X Software along the time course (Leica Microsystems). Despite Fluo-4's green fluorescence range (Ex/Em of Ca^{2+}–bound formv494/506 nm), by using the color scale different levels of fluorescence intensity are converted into the observed color spectrum, where purple/blue represent low fluorescence and red is the maximum fluorescence intensity. Experiment recordings were obtained using the 20x objective lens.

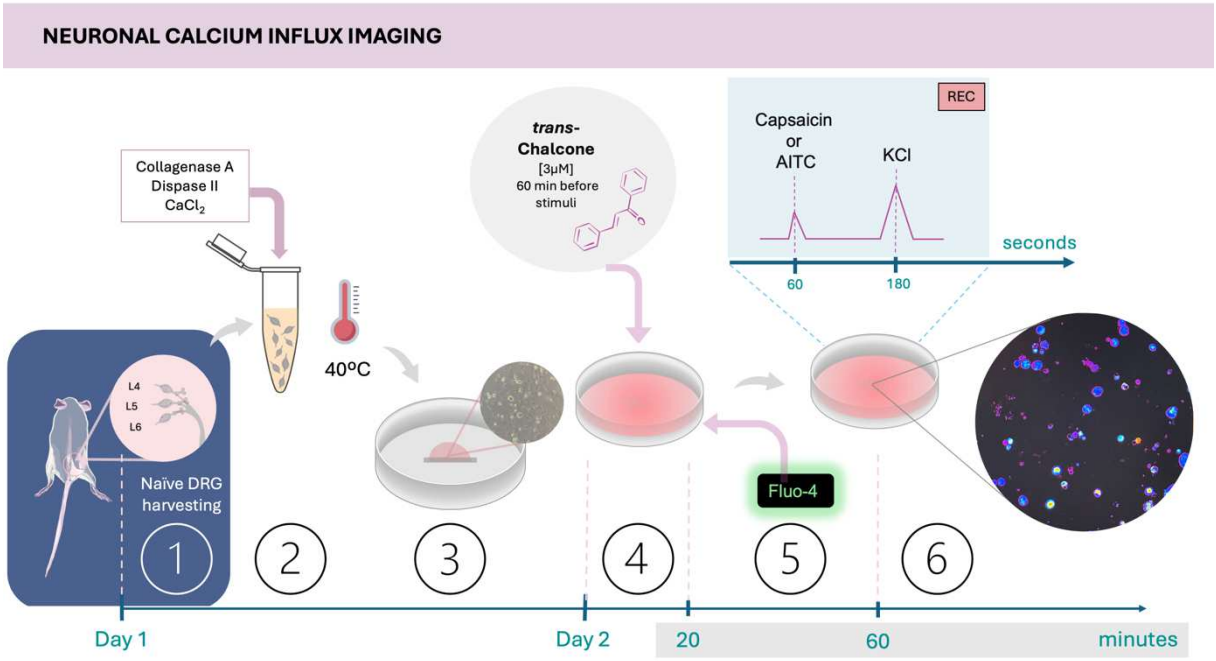

Fig 8. *Schematic representation of neuronal calcium influx.* 1. DRG from both sides of L4, L5 and L6 were collected from naïve mice. 2. Neuron somas were separated adding collagenase A, dispase II and the co-factor $CaCl_2$ and kept at 40ºC to carry out enzyme digestion of the DRG extracellular matrix. 3. Neuron somas were seeded on culture dishes previously coated

with laminin, neurobasal medium was added and cells were incubated overnight at 37ºC and 5% CO_2 for stabilization. 4. The next day, the medium was removed, cells were washed twice with HBSS to remove unattached cells, and fresh medium was added with *t*C or vehicle one hour prior to the assay. 5. Fluo-4 was added to the culture medium and incubated for 40 minutes. 6. Plates were washed twice with HBSS $Ca^{++}Mg^{++}$ to remove remaining treatments and fluorescent probe, and cultures were recorded for 4 minutes: at the 60 s-mark, Capsaicin or AITC was added, at the 180 s-mark, KCl was added.

Statistical analysis

Results are presented as means ± standard error means (SEM) of measurements of 6 mice per group per experiment and are representative of two separate experiments. Sample size was determined using G*Power 3.1[61] through repeated-measures analysis of variance (ANOVA), within between interaction and effect size f = 0.7, α probability error = 0.05, power of analysis (1-β probability error = 0.95), correlation between repeated measures = 0.5, and nonsphericity correction ε = 1. These denominators were chosen based on previous study of our group[24]. The method of block randomization was used to randomize subjects into groups which results in equal sample sizes at all time points for each assay. Data were submitted to Shapiro-Wilk normality test and Brown-Forsythe homogeneity of variance test before proceeding to parametric or non-parametric analysis, whereas data with p-value ≥ 0.05 for both tests were statistically analyzed by one- or two-way ANOVA followed by Tukey's test for multiple comparisons. For non-parametric data, statistical analyses were conducted by Kruskal-Wallis' test followed by Dunn's multiple comparisons test or Mann Whitney test, when comparing two groups. Statistical analyses were performed with GraphPad Prism 9 software (GraphPad Software Inc., San Diego, CA, USA). Results were considered significantly different if p-value ≤ 0.05.

Table 1. Statistical analysis for Figs 1-3.

Aceitc Acid and PBQ overt pain-like behavior			Normality		Equality of group variances		Statistical test		Post-test	
Result			Shapiro-Wilk test		Brown-Forsythe test		One-way ANOVA		Tukey's multiple comparisons test	
	Groups	n	W value	P value	F value	P value	F value	P value	Comparison	P value
Fig 1C	Saline	6	0.8268	0.101	2.522 (4, 25)	0.0664	67.59 (4, 25)	<0.0001	Saline vs. 0	<0.0001
	0	6	0.9607	0.8253					0 vs. 3	0.1437
	3	6	0.9301	0.5812					0 vs. 10	<0.0001
	10	6	0.982	0.5647					0 vs. 30	<0.0001
	30	6	0.9182	0.4928					10 vs 30	0.7803
Fig 1D	Saline	6	0.8216	0.0911	2.669 (2, 15)	0.102	85.90 (2, 15)	<0.0001	Saline vs. 0	<0.0001
	0	6	0.9005	0.3772					Saline vs. 10	<0.0001
	10	6	0.8907	0.3218					0 vs. 10	<0.0001

Formalin overt pain-like behavior			Normality		Statistical test		Post-test			
Result			Shapiro-Wilk test		Two-way ANOVA		Tukey's multiple comparisons test			
	Flinches	Groups	W value	P value	Time x Column Factor	P value	Comparison	P value	Comparison	P value
						0.236	0-5 minutes		10-30 minutes	
Fig 2B	0-5 minutes	3	0.9994	0.9516	Time	0.0361	Saline vs. 0	<0.0001	Saline vs. 0	<0.0001
	10-30 minutes	3	0.9516	0.8006	Column Factor	<0.0001	Saline vs. 10	0.0023	Saline vs. 10	<0.0001
					Subject	0.7933	0 vs. 10	0.0051	0 vs. 10	0.0011
	Licking time	Groups	W value	P value	Time x Column Factor	P value	Comparison	P value	Comparison	P value
						0.0131	0-5 minutes		10-30 minutes	
Fig 2C	0-5 minutes	3	0.9949	0.8633	Time	0.0005	Saline vs. 0	<0.0001	Saline vs. 0	<0.0001
	10-30 minutes	3	0.9969	0.8934	Column Factor	<0.0001	Saline vs. 10	0.0064	Saline vs. 10	<0.0001
					Subject	0.286	0 vs. 10	0.0379	0 vs. 10	0.0002

CFA overt pain-like behavior			Normality		Equality of group variances		Statistical test		Post-test	
Result			Shapiro-Wilk test		Brown-Forsythe test		One-way ANOVA		Tukey's multiple comparisons test	
	Group	n	W value	P value	F value	P value	F value	P value	Comparison	P value
Fig 3B	Saline	6	0.8139	0.078	2.197 (2, 15)	0.1457	43.43 (2, 15)	<0.0001	Saline vs. 0	<0.0001
	0	6	0.8641	0.2039					Saline vs. 10	0.0006
	10	6	0.9037	0.3963					0 vs. 10	0.0012
	Group	n	W value	P value	F value	P value	F value	P value	Comparison	P value
Fig 3C	Saline	6	0.875	0.2468	2.750 (2, 15)	0.0961	46.66 (2, 15)	<0.0001	Saline vs. 0	<0.0001
	0	6	0.9155	0.4739					Saline vs. 10	0.0002
	10	6	0.9167	0.4819					0 vs. 10	0.0021

Table 2. Statistical analysis for Figs 4-6.

Capsaicin and AITC overt pain-like behavior			Normality		Equality of group variances		Statistical test		Post-test	
Result			Shapiro-Wilk test		Brown-Forsythe test		One-way ANOVA		Tukey's multiple comparisons test	
	Group	n	W value	P value	F value	P value	F value	P value	Comparison	P value
Fig 4B	Saline	6	0.824	0.0956	1.646 (2, 15)	0.2258	34.97 (2, 15)	<0.0001	Saline vs. 0	<0.0001
	0	6	0.9441	0.692					Saline vs. 10	0.0004
	10	6	0.8716	0.2327					0 vs. 10	0.0151
Fig 4C	Saline	6	0.8232	0.0941	0.5339 (2, 15)	0.5971	64.27 (2, 15)	<0.0001	Saline vs. 0	<0.0001
	0	6	0.8916	0.3264					Saline vs. 10	<0.0001
	10	6	0.8862	0.2988					0 vs. 10	0.0002
Fig 4D	Saline	6	0.9006	0.3778	3.607 (2, 15)	0.0526	38.63 (2, 15)	<0.0001	Saline vs. 0	<0.0001
	0	6	0.8955	0.3481					Saline vs. 10	0.005
	10	6	0.7963	0.0544					0 vs. 10	0.0004
Fig 4E	Saline	6	0.9076	0.4207	2.692 (2, 15)	0.1003	75.78 (2, 15)	<0.0001	Saline vs. 0	<0.0001
	0	6	0.91	0.4362					Saline vs. 10	<0.0001
	10	6	0.8241	0.0957					0 vs. 10	0.0018
Calcium Influx - Capsaicin					Mann Whitney test					
Fig 5A	Group		neurons		Comparison				P value	
	Saline		360		*t*C vs. Saline				<0.0001	
	*t*C		399							
Fig 5C	Group		neurons		Comparison				P value	
	Saline		360		*t*C vs. Saline				<0.0001	
	*t*C		399							
Fig 5D	Group		n		Comparison				P value	
	Saline		4		*t*C vs. Saline				0.0286	
	*t*C		4							
Calcium Influx - AITC					Mann Whitney test					
Fig 6A	Group		neurons		Comparison				P value	
	Saline		252		*t*C vs. Saline				0.0076	
	*t*C		160							
Fig 6C	Group		neurons		Comparison				P value	
	Saline		252		*t*C vs. Saline				0.0056	
	*t*C		160							
Fig 6D	Group		n		Comparison				P value	
	Saline		4		*t*C vs. Saline				0.0286	
	*t*C		4							

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Conflict of interest

The authors declare no competing financial interest.

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5 ARTIGO 2

trans-Chalcone reduces pain, inflammation and oxidative stress triggered by superoxide anion

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trans-Chalcone reduces pain, inflammation and oxidative stress triggered by superoxide anion

Maiara Piva[a], Marília F. Manchope[a], Fernanda Barbosa-Costa[a], Beatriz H. S. Bianchini[a], Sérgio Borghi[a], Rubia Casagrande[b], Waldiceu Verri Jr [a]

a. Department of Immunology, Parasitology and General Pathology, Center for Biological Sciences, State University of Londrina, Londrina, Paraná, Brazil
b. Department of Pharmaceutical Sciences, Center of Health Science, Londrina State University, Londrina, 86038-440, PR, Brazil

Corresponding Author: Waldiceu Verri Jr – Biological Science Center, Londrina State University. Rod. Celso Garcia Cid Pr 445 KM 380, Londrina, Paraná, Brazil. P.O. box 10.011, zip code 86057-970. Phone: +55 43 3371-4979, Faz: +55 43 3371-4387, waldiceujr@yahoo.com.br or waverri@uel.br

ABSTRACT

Trans-Chalcone (tC) is an atypical flavonoid: its molecular structure does not bare hydroxyl groups to act as hydrogen donors and, therefore, is not able to directly scavenge reactive species. Nonetheless, tC reduces oxidative stress and promotes an antioxidative response in vivo. Such particular characteristics allow us to investigate the effects and mechanisms of a flavonoid beyond an inherent chemically antioxidant effect. Potassium superoxide (KO_2), an anion superoxide donor, was used to induce pain and inflammation in mice. Overt pain-like behavior, mechanical hyperalgesia, edema, leukocyte recruitment, oxidative response, IL-1β, TNF-α, IL-6 and IL-33 cytokine levels, NF-κB activation, Nrf2, $Gp91^{phox}$, Cox-2 expression, TRPV1- and TRPA1-expressing DRG neuron sensitization were assessed. *t*C (3-30mg/kg) was administered per oral 30 minutes before stimulus with KO_2. The dose of 30mg/kg of $_{tC}$ inhibited abdominal contortions and was selected for the following experiments. Mechanical hyperalgesia was inhibited in all timepoints assessed (0.5, 1, 3, 5 and 7 hours). *t*C also reduced edema and myeloperoxidase activity, indicative of leukocyte recruitment inhibition. Moreover, despite its molecular structure, *t*C induced antioxidant production (assessed by FRAP and ABTS) while reducing superoxide anion production and lipid peroxidation, due to, at least partially, the upregulation Nrf2 expression and downregulation of $Gp91^{phox}$ and Cox-2 expression. *t*C inhibited KO_2-induced NF-κB activation, IL-1β, TNF-α, Il-6, and IL-33 production. We also identified a reduction in $TRPV1^+$ and $TRPA1^+$ nociceptors activation after treatment with *t*C, observed in prior to stimulus (baseline activation) and after agonists addition, the prevention of neuron sensitization due to KO_2-induced oxidative damage. In sum, the ability of *t*C to impair neuron activation, inhibit cytokine pro-inflammatory signaling, reduce NF-κB activation, and induce an antioxidant response, resulting in peripheral sensitization inhibition, demonstrate a protective role of *t*C against oxidative stress in mice. Taken together, our data suggests further clinical investigation exploring *t*C prospect as an analgesic, anti-inflammatory and antioxidant potentially effective against conditions attributable to oxidative stress damage.

Keywords: analgesic; potassium superoxide; TRPV1; TRPA1; nociception; flavonoids.

HIGHLIGHTS

- *trans*-Chalcone reduces superoxide anion-induced pain and inflammation.
- *t*C inhibits inflammatory NF-κB pathway and cytokine production.
- *t*C induces antioxidant signaling via NRF2 expression.
- *t*C inhibits sensitization of TRPV1+ and TRPA1+ DRG neurons.

INTRODUCTION

Reactive oxygen species (ROS), such as the superoxide anion, are important regulators in both physiological and pathological conditions (Brieger *et al.*, 2012). Under physiological balance, they are necessary for cell signaling, but under pathological conditions they can be harmful, inducing oxidative damage, and leading to disease and cell death (Andrés *et al.*, 2023). Important pathological conditions such as atherosclerosis, chronic obstructive pulmonary disease, Alzheimer's disease and cancer are oxidative damage-induced conditions (Forman & Zhang, 2021). Therefore, the study of pain and inflammation induced by oxidative stress can be a valuable tool to better understand its mechanisms and discover new treatment alternatives.

Potassium superoxide (KO_2) is a superoxide anion donor known to induce pain and inflammation by nuclear factor-κB (NF-κB) activation (Lourenco-Gonzalez *et al.*, 2019; Fattori *et al.*, 2015; Pinho-Ribeiro *et al.*, 2016), production of tumor necrosis factor (TNF)-α (Manchope *et al.*, 2016; Yamacita-Borin *et al.*, 2015; Lourenco-Gonzalez *et al.*, 2019; Serafim *et al.*, 2015; Fattori *et al.*, 2015; Pinho-Ribeiro *et al.*, 2016; Maioli *et al.*, 2015; Borghi *et al.*, 2025), interleukin (IL)-1β (Lourenco-Gonzalez *et al.*, 2019, Serafim *et al.*, 2015, Fattori *et al.*, 2015, Pinho-Ribeiro *et al.*; 2016), IL-33 (Lourenco-Gonzalez *et al.*, 2019; Borghi *et al.*, 2025), and IL-10 [Serafim *et al.*, 2015; Fattori *et al.*, 2015; Pinho-Ribeiro *et al.*, 2016; Maioli *et al.*, 2015; Manchope *et al.*, 2016) cytokines. Inflammation starts with immune resident cells that detect superoxide-induced damage and release cytokines, which recruit and activate more immune cells, activate NF-κB pathway and induce inflammasome NLRP3 assembly, intensifying the response (Bauernfeind *et al.*, 2009).

Moreover, KO_2 promotes the upregulation of cyclooxygenase-2 (COX-2) mRNA expression (Manchope *et al.*, 2016), a key inflammatory enzyme that catalyzes arachidonic acid for prostaglandins generation (Marnett *et al.*, 1999). KO_2 induces superoxide production by the upregulation of $gp91^{phox}$, the heme binding subunit of NADPH oxidase, which mediates the final steps of electron transfer to molecular oxygen for superoxide ion generation (Maioli *et al.*, 2015; Borghi *et al.*, 2025; Manchope *et al.*, 2016; Yu *et al.*, 1998).

KO_2 induces overt pain-like behavior and nociceptive sensitization that generates hyperalgesia (Fattori *et al.*, 2015; Pinho-Ribeiro *et al.*, 2016; Manchope *et al.*, 2016; Borghi *et al.*, 2025). Sensitization is an increased excitability of nociceptors

due to a reduction in neuronal threshold that facilitates depolarization and, therefore, magnifies the response to noxious stimuli (Gold & Gerald, 2010).

Pain transduction is mediated by ionic channels opening, which induce neuronal depolarization and generate an action potential. The pain transducers include ATP-gated P2X3, transient receptor potential (TRP) ionic channels, acid sensitive ion channels (ASICs), mechanosensitive Piezo, voltage-gated sodium (Nav) channels, voltage-gated calcium (Cav) channels, among others (Giniatullin, 2020). TRP ionic channels are sensors for multiple noxious stimuli, such as changes in pH, temperature, osmolarity, and chemical agents (González-Ramírez *et al.*, 2017). The TRP ankyrin 1 (TRPA1) channel is particularly interesting for this study since it is responsible for the detection of ROS, pruritogens and cold temperature (Faris *et al.*, 2023). TRPA1 is often co-expressed with TRP vanilloid 1 (TRPV1) channels on DRG neurons (Story *et al.*, 2003; Kobayashi *et al.,* 2005). TRPV1 can be activated by heat superior to 43°C and acidic pH, and both TRPA1 and TRPV1 channels are responsive to harmful mechanical pressure and noxious chemical compounds (González-Ramírez *et al.*, 2017).

trans-Chalcone (*t*C – 1,3-difenil-2-propen1-ona) is open-chain flavonoid that presents anti-inflammatory properties in animal models of skin and hepatic inflammation (Martinez *et al.*, 2017a e 2017b; Karimi-Sales *et al.,* 2018; Singh *et al.*, 2016) as well as CFA-induced and gouty arthritis (Jabbar *et al.*, 2024; Staurengo-Ferrari *et al.*, 2018). Regarding possible analgesic effects, *t*C reduced mechanical hyperalgesia in a gouty arthritis model (Staurengo-Ferrari *et al.*, 2018) and thermal hyperalgesia induced by CFA (Jabbar *et al.*, 2024).

A neuroprotective role of *t*C in Alzheimer's and Parkinson's disease experimental models seem associated to the activation of AMPK, SIRT1-PGC1α pathway (Omidi-Shahsavandi *et al.*, 2023; Cheng *et al.*, 2024). Interestingly, unlike most flavonoids, *t*C chemical structure does not bare hydroxyl radicals, therefore, its antioxidant properties demonstrated in vivo are independent to an inherent structural characteristic (Martinez *et al.*, 2017). Additionally, *t*C is a flavonoid precursor that can be described as the flavonoids' backbone, ergo, conceptually, understanding the antioxidant activity of *t*C can give us valuable insight on the mechanisms driving flavonoids response to oxidative stress beyond an inherent scavenger activity.

Given *t*C promising properties and the importance of oxidative damage on pathological conditions, we sought to investigate the potential analgesic, anti-

inflammatory and neuroprotective effects of *t*C against superoxide anion-triggered pain and inflammation in mice, as well as the mechanisms involved in the process.

METHODS

The methodology was developed and conducted according to ARRIVE guidelines.

Experimental design

All treatments were administered orally (per oral, p.o.) by gavage. *t*C was diluted in saline solution with 20% Tween 80. Intraplantar (i.pl.) injection of KO_2 (30 μg diluted in 25 μL of saline solution per animal) were administered always at the right hind paw 30 minutes after treatment. Due to KO_2 fast inactivation (Maioli *et al.*, 2015), stimulus was injected between 30-180 seconds after saline dilution to improve consistent results. KO_2 salt was stored protected from light sources inside a desiccant. The characteristic yellow color was indicative of stability. *t*C analgesic effect was assessed by overt pain-like behavior. tC treatments (3, 10 or 30mg/kg) were administered 30 min before abdominal contortions induction by intraperitoneal (i.p.) injection with 1mg of KO_2 per animal. Mechanical hyperalgesia was assessed by electronic von Fray 0.5, 1, 3, 5 and 7 hours after KO_2 stimulus. Paw edema was measured 1, 3, 5 and 7 hours after KO_2 i.pl. injection. Myeloperoxidase activity (MPO) was measured 7 hours after KO_2 paw injection. Calcium imaging by confocal microscopy in cultured DRG-derived neurons collected 5 hours after KO_2 i.pl. injection was used to evaluate TRPV1- and TRPA1-expressing neurons activity. Superoxide anion production, lipid peroxidation levels, total antioxidant capacity (FRAP and ABTS assays) on paw tissue were collected 3 hours after KO_2 injection. The same timeframe and sample tissue were used for RT-qPCR, ELISA and Western Blot assays.

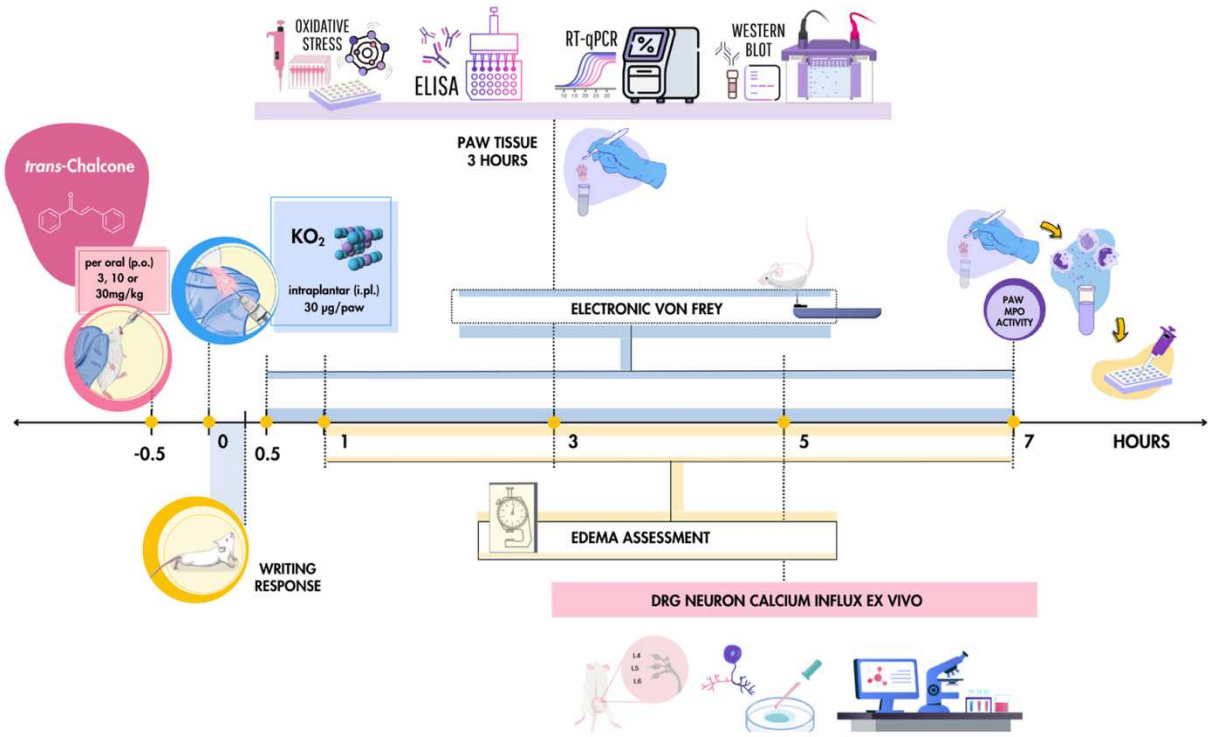

Fig 1. *Experimental design. t*C treatment was administered by gavage 30 min prior to KO_2 injection. The dose-response was carried out by the abdominal contortions assessed over 20 min after KO_2 (1mg/abdominal cavity) intraperitoneal (i.p.) administration. For all following experiments, animals were stimulated with 30 µg of KO_2 diluted in 25 µL of saline, injected subcutaneously into the plantar surface of the right hind paw. Mechanical hyperalgesia was measured using electronic von Frey 0.5, 1, 3, 5 and 7 hours after stimulus. Edema formation was measured 1, 3, 5 and 7 hours after stimulus. Leucocyte recruitment to the plantar tissue injected with KO_2 was evaluated by MPO activity 7 hours after i.pl. stimulus. Oxidative stress (TBARS and NBT), antioxidant capacity (FRAP and ABTS), mRNA expression (RT-qPCR), and protein expression (ELISA and Western Blot) were assessed using the injected plantar paw tissue collected 3 hours after i.pl. KO_2 injection. For the calcium influx *ex vivo* assessment, DRG were collected 5 hours after i.pl. KO_2 injection.

Animals

Male Swiss mice, weighing 20-25 g, age 6-8 weeks, were provided by the central vivarium at Londrina State University, and housed under 12 hours light/dark cycles, climatized temperature (24± 1ºC) with food and water *ad lib*. For sample collection, mice were euthanized by terminal anesthetization with 5%, isoflurane, followed by decapitation procedure as confirmation. All procedures had previous approval of the Londrina State University's Animal Ethics Committee (CEUA processes

24684.2016.72, from December 8, 2016, and 050.2024, from December 12, 2024). No efforts were spared to minimize the number of animals used and their suffering.

Writhing response

KO_2-induced writhing model was performed as previously described (Verri *et al.*, 2008) KO_2 (1 mg diluted into 250 μL of saline solution per animal) or saline solution (sterile 0.9% sodium chloride) were injected into the peritoneal cavities of mice 30 minutes after pre-treatment with *t*C (3, 10 or 30 mg/ kg, p.o.). The intensity of overt pain-like behavior is expressed as the cumulative writhing score over 20 minutes after stimulus.

Mechanical hyperalgesia and edema

Mice were placed under individual glass domes of 20 cm diameter to limit movement without restriction or harm, placed above a wired surface to allow access to the induced area, and after sufficient climatization to the behavior experimental room and apparatus where no signs of distress were observed, the baseline measurements for mechanical threshold were taken using the electronic version of von Frey filaments, which detects (in grams) the upright pressure necessary to incite paw removal, applied to the plantar region of the right hind paw with a polypropylene tip. Measurements were repeated after 30 minutes, 1, 3, 5, and 7 hours after KO_2 injection. Results are expressed as the mean difference between baseline and each timepoint value (Δg) of six animals. After stablishing baseline, edema measurements were obtained 1, 3, 5 and 7 hours after KO_2 injection with the aid of a micrometer and are presented in millimeters (mm).

Myeloperoxidase (MPO) activity assessment

MPO activity was used as an indirect measure of neutrophils and macrophages migration to the stimulated paw tissue. Paw samples were collected 7 hours after KO_2 stimulus in 200 µL of K_2HPO_4 buffer solution (pH 6.0), containing 0.5% of HTAB, and were then homogenized with tissue homogenizer. After this step, samples were centrifuged (14,000 RPM x 4ºC x 2 min) and the supernatant was properly separated. Paw tissue homogenate supernatant was added to 200 µL of 50 mM phosphate buffer

solution (pH 6.0), containing 0.167 mg/mL of o-dianisidine dihydrochloride and 0.015% of hydrogen peroxide. Absorbances were recorded with a microplate spectrophotometer (Multiskan GO, Thermo Fischer Scientific) at 450 nm. MPO activity is expressed as MPO activity (number of neutrophils $x10^4$ / mg of tissue) compared to a neutrophil standard curve (Martinez *et al.*, 2017a).

Total antioxidant capacity

The 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and ferric reducing antioxidant power (FRAP) assays were performed to quantify sample's ability to resist oxidative damage. Paw tissue samples were homogenized in ice-cold PBS buffer containing KCl 1.15% and centrifuged at 200 x g in 4ºC for 10 min. The supernatants were transferred to new microtubes to perform the assays. On 96-well plates, ABTS reagent was added to the supernatant or standards for the calibration curve, allowed to incubate for 6 min at room temperature and the samples were read in a microplate spectrophotometer at 730 nm. To evaluate the FRAP levels, 150 µL of the FRAP reagent was added to the supernatant or standards for the calibration curve, and read at 595 nm (Multiskan GO, Thermo Fischer Scientific). Results were compared to their respective Trolox standard curve and expressed as Trolox equivalent/mg of tissue for both evaluations (Borghi *et al.*, 2013; Martinez *et al.*, 2020).

Lipid peroxidation and superoxide production

The thiobarbituric acid reactive substances TBARS test is an indicator of lipid peroxidation induced by free radicals. Once those radicals interact with lipids, they can cause destabilization and produce malondialdehyde (MDA), an intermediate product of lipid peroxidation. Therefore, the test detects the presence of MDA in samples, which react with thiobarbituric acid (TBA 10%) and, in the presence of $FeCl_3$ 1 mM, ascorbic acid 1 mM, 50% trichloroacetic acid (TCA) and TBA1% at 95ºC is altered to the color pink. Then, absorbance can be measured at 572 nm and 535 nm, and the results are expressed as 572 nm – 575 nm OD/mg of protein (Martinez *et al.*, 2020; Borghi *et al.,* 2016). Moreover, the determination of superoxide anion production was performed using the nitroblue tetrazolium (NBT) test as previously described adapted to microplates (Martinez *et al.*, 2017b; Martinez *et al.*, 2020). Briefly, samples

homogenates were incubated in microplates with 1 mg/ml NBT reagent for one hour at room temperature and protected from light. After incubation, the supernatant was removed, a 2 M KOH solution and pure dimethylsulfoxide (DMSO) were added to the microplates. The relative production of superoxide anion was measured spectrophotometrically at a 600 nm (Multiskan GO, Thermo Fisher Scientific). Results are expressed in OD/mg of protein.

Cytokine levels assessment

Cytokine levels were assessed by the enzyme-linked immunosorbent assay (ELISA) in paw samples collected 3 hours after KO_2 stimulus. Samples were collected in phosphate buffered saline solution, homogenized, centrifuged (3000 rpm x 4ºC x 15 minutes), and the supernatant was used to measure the levels of IL-1β, TNF-α, IL-6 and IL-33. The assays were conducted following manufacturer's instructions. The results were expressed as picograms (pg) of each cytokine per 100 mg of tissue.

Ca^{2+} influx imaging

The influx of calcium ions in afferent neurons is an indicator of their activity, enabling the comparison of influx thresholds between experimental groups. Mice were pre-treated with *t*C, and the right hind paw was stimulated 30 minutes later. After 5 hours, mice were terminally anesthetized, peripheral blood was manually removed after decapitation and the right side DRG (L4, L5 and L6) were harvested into culture Dulbecco's modified Eagle medium (DMEM) high glucose supplemented with 10% fetal bovine serum (FBS). DRG were enzymatically disrupted with collagenase A/dispase II solution, supplemented with 5 mM $CaCl_2$ (Collagenase A, Roche 10103578001, Dispase II, Roche 04942078001) and kept in incubation at 40ºC until DGR opened fibers were visualized, followed by pipetting and until complete dissociation. Cells were then centrifuged (1.500 rpm for 5 min) to obtain a monolayer of DRG axotomized neurons placed over a glass-bottom culture plate pre-coated with laminin. Cells were kept at 37ºC, 5% CO_2 overnight on neurobasal culture medium (Invitrogen 21103-049) supplemented with 2% B27 serum-free supplement (50x, Invitrogen 17504-044) 1% Pen/Strep (Cellgro 15140-163), 1% L-Glutamine (Invitrogen 25030-164), 0.004% ARA-C 0.004% glial-derived neurotrophic factor (GDNF), and

0.002% nerve growth factor (NGF). Four culture plates were seeded for each experimental group composed by 6 mice, and kept overnight at 37ºC, 5% CO_2 to stabilize. Culture plates were washed, and cell adherence was confirmed. Experimental sets were divided with one plate per group (Saline, KO_2 stimulus after vehicle treatment, and KO_2 stimulus after *t*C treatment). Cells were then loaded with 1.2 µM of Fluo-4-AM probe for 40 minutes, plates were washed, and the medium was replaced by hanks' balanced salt solution (HBSS) with calcium and magnesium for neuron stimuli imaging in a confocal microscope (TCS SP8, Leica Microsystems). To assess TRPV1 and TRPA1 activation, DRG plates were recorded for 4 minutes, divided into 1 minute of initial reading (0-s mark, baseline values), followed by stimulation with capsaicin (1 µM, TRPV1 agonist) or AITC (100 nM, TRPA1 agonist) for 2 min at the 60 -seconds mark and KCl (40 mM, a viability control for cells) for 1 minute at the 180 -s mark. Calcium influx was analyzed based on the mean fluorescence intensity detected in each neuron with the LAS X Software along the time course (Leica Microsystems). Despite Fluo-4's green fluorescence range (Ex/Em of Ca^{2+}–bound form 494/506 nm), by using the color scale different levels of fluorescence intensity are converted into the observed color spectrum, where purple/blue represent low fluorescence and red is the maximum fluorescence intensity. Experiment recordings were obtained using the 20x objective lens.

RT-qPCR

Paw samples were collected in lysis reagent (QIAzol, QIAGEN, ID: 79306), snap frozen in liquid nitrogen and stored at -80ºC. Total RNA was extracted following manufactures' instructions. Nucleic acid purity and integrity was assessed by spectrophotometry, adopting 1.6 - 2 cut off for Reading ratio 260/230 nm and 2.0 ± 0.1 for 260/280 nm ratio. QuantiTect Reverse Transcription kit (QIAGEN, 205314) was used to obtain cDNA, and QuantiNova SYBR Green (QIAGEN, 208056) kit was used for quantitative PCR analysis. Gene expression was obtained by the comparative $2^{-(\Delta\Delta Ct)}$ method after *Actin* housekeeping correction. Primer sequences are available on Table 1.

Table 1. Sense and antisense primer sequences used on qPCR.

TARGET	SENSE	ANTISENSE
gp91phox (*Cybb* gene)	5' AGC TAT GAG GTG GTG ATG TTA GTG G 3'	5' CAC AAT ATT TGT ACC AGA CAG ACT TGA G 3'
β-actin (*Actin* gene)	5' AGC TGC GTT TTA CAC CCT TT 3'	5' AAG CCA TGC CAA TGT TGT CT 3'
Nrf2 (*Nfe2l2* gene)	5' TCA CAC GAG ATG AGC TTA GGG CAA 3'	5' TAC AGT TCT GGG CGG CGA CTT TAT 3'
COX-2 (*Cox2* gene)	5' GTG GAA AAA CCT CGT CCA GA 3'	5' GCT CGG CTT CCA GTA TTG AG 3'
NFκB p65 (*Rela* gene)	5' CGC TGC GGA GCT TGT AGT 3'	5' GCA GGT GCG CGA CTC A 3'
Interleukin-33 (*Il33* gene)	5' TCC TTG CTT GGC AGT ATC CA 3'	5' TGC TCA ATG TGT CAA CAG ACG 3'
Interleukin-6 (*Il6* gene)	5' CTT GGG ACT GAT GCT GGT GAC A 3'	5' GCC TCC GAC TTG TGA AGT GGT A 3'

Fluorescent Western Blot

Western Blot assays were performed according to Zarpelon *et al.* (2016) adapted for fluorescent fotodocumentation. Briefly, paw samples were collected in lysis buffer (RIPA) with protease and phosphatase inhibitors. After mechanical dissociation, samples were centrifuged for 20min at 16,000 x g, the supernatant was transferred for a new tube for total protein quantitation. Samples were equalized, submitted to vertical electrophoresis and transferred to a nitrocellulose membrane by wet transfer. Transfer quality was confirmed with ponceau red, and membranes were blocked with 5% bovine serum albumin (BSA). Primary antibodies p-NFκB p65 mouse monoclonal (1:200, 27. Ser 536, sc-136548, Santa Cruz Biotechnology) and β-actin rabbit monoclonal (1:1000, D6A8, 8457, Cell Signaling), and secondary antibodies goat anti-mouse IgG Alexa Fluor™ 488 (1:200, A11001, Invitrogen) and goat anti-rabbit IgG Alexa Fluor™ 790 (1:200, A11367, Invitrogen), diluted in 3% BSA Tris-buffer saline (TBS), were selected for this study. Primary antibodies were incubated overnight under slow agitation. Membranes were than washed with TBS three times during 10min agitation, and fluorescent secondary antibodies were incubated for one hour, washing steps were repeated, membranes were fotoducumented (ImageQuant 800 - Cytiva), and quantified based on the fluorescence intensity of the target protein corrected by β-actin's fluorescence intensity. Relative expression is based on the saline group.

Statistical analysis

Sample size was estimated based on previous studies and confirmed with G*Power 3.1. The software GraphPad Prism 9.0 was used for statistical analysis and graphical representations. Pre-tests Shapiro-Wilk and Brown-Forsythe were used to verify normality and equality of group variances, respectively. To analyze multiple timepoints in normally distributed data, two-way ANOVA followed by Tukey's multiple comparisons test was performed. One-way ANOVA followed by Tukey was used in normal column analysis. Non-parametrical data was evaluated using Kruskal-Wallis followed by Dunn's multiple comparison test or Welch's ANOVA test followed by Dunnett's T3 multiple comparisons test. Groups were considered statistically different if p-value ≤ 0.05.

RESULTS

tC inhibits overt pain-like behavior, mechanical hyperalgesia, edema and leukocyte recruitment induced by superoxide anion

The first step of our study was to determine whether *t*C could reduce the pain phenotype induced by the superoxide donor. Overt pain-like behavior was quantified by the number of abdominal contortions induced by i.p. injection of KO_2 after treatment with different doses of *t*C (3-30 mg/kg) or vehicle. *t*C treatment reduced the writhing response (Fig. 2B) in a dose-dependent manner when compared to vehicle-treated mice, 56.6% inhibition 3 mg/kg, 62.3% inhibition with 10 mg/kg, and 74.6% for treatment with 30mg/kg of *t*C. However, only the highest dose presented statistically significant inhibition. Thus, the dose of 30 mg/kg was elected for further treatments with *t*C.

Accordingly, our results indicate that KO_2 rapidly induces hyperalgesia, observed 30 minutes after stimulus and sustained until at least the 7th hour (Fig. 2D), as the treatment with *t*C was able to reduce hyperalgesia in all timepoints analyzed (30 min = 56.1%, 1 hour = 68.2%, 3 hours = 70%, 5 hours = 83.2%, and 7 hours = 90.2% inhibition), further indicating nociception inhibition. Likewise, the flavonoid significantly reduced the extent of the edema (Fig. 2E), that was observed from the 1st (16%) until de 3rd hour (5.2%) after KO_2 injection.

As shown in Fig 2B, *t*C treatment was able to inhibit KO_2-induced leucocyte recruitment assessed 7 hours after the stimulus, reducing MPO activity in 46.7%.

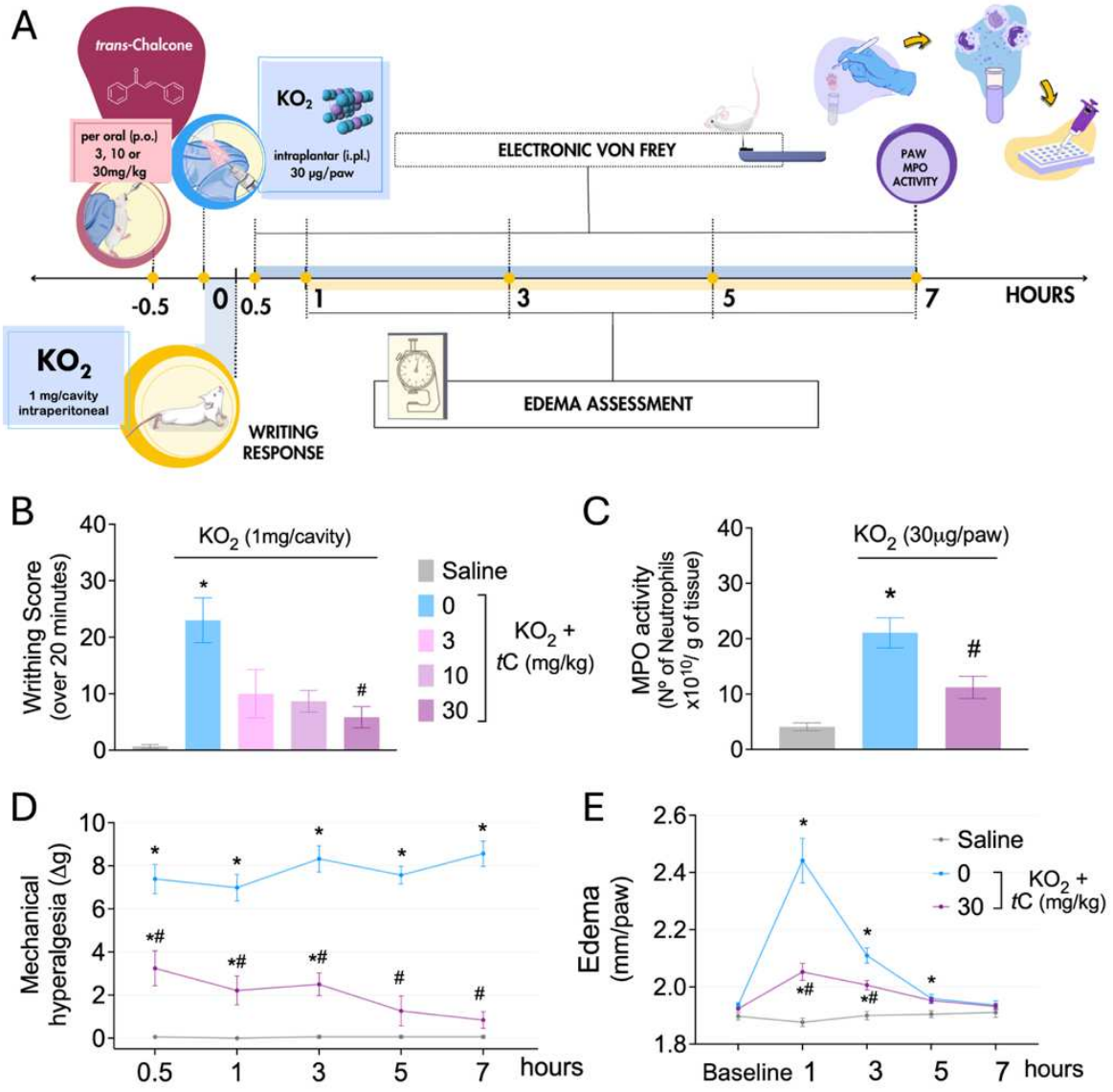

Fig 2. *tC inhibits writhing response, mechanical hyperalgesia, edema and leukocyte recruitment after KO_2 stimulus.* (A) Experimental timeline (B) Writhing response was induced by intraperitoneal (i.p.) administration of KO_2 (1 mg/cavity) 30 min after *t*C treatment (3-30 mg/kg) and assessed for 20 minutes – n = 6, Shapiro-Wilk was employed to verify normality. Statistical analyses were performed by Welch's ANOVA test followed by Dunnett's T3 multiple comparisons test. (C) MPO activity in the injected paw was measured 7 hours after stimulus – n = 5. Shapiro-Wilk and Brown-Forsythe were used to verify normality and equality of group variances. Statistical analyses were performed by One-way ANOVA followed by Tukey's multiple comparisons test. (D) Mechanical hyperalgesia was measured by electronic von Frey 0.5, 1, 3, 5 and 7 hours after KO_2 i.pl. injection. Shapiro-Wilk was used to verify normality. Statistical analyses were performed by Two-way ANOVA followed by Tukey's multiple comparisons test – n = 6, (E) Paw edema was measured after 1, 3, 5 and 7 hours from KO_2 stimulus. Shapiro-Wilk was used to verify normality. Statistical analyses were performed by

Two-way ANOVA followed by Tukey's multiple comparisons test – n = 6. Results are presented as means ± standard error mean (SEM) representative of two separate experiments *p-value ≤ 0.05 compared to saline group; #p-value ≤ 0.05 compared to stimulus group.

tC induces antioxidant response after KO_2 injection by the transcription factor Nrf2 (NF-E2 p45-related factor 2) upregulation

The following step in our study was to determine whether *t*C could inhibit oxidative stress and/or induce an antioxidant response to KO_2 stimulus. To do so, FRAP and ABTS assays were conducted. While KO_2 reduced the antioxidant capacity in the paw skin, *t*C was able to upregulate antioxidant levels, increasing in 69.6% ferric reducing power (Fig 3B) and in 42.9% the capacity to reduce the ABTS radical cation (Fig 3C). Moreover, mRNA copies for Nrf2 were upregulated by 109% (Fig 3D) on mice that received *t*C pre-treatment, indicating that the antioxidative response is induced, at least partially, by the transcription factor Nrf2 activation.

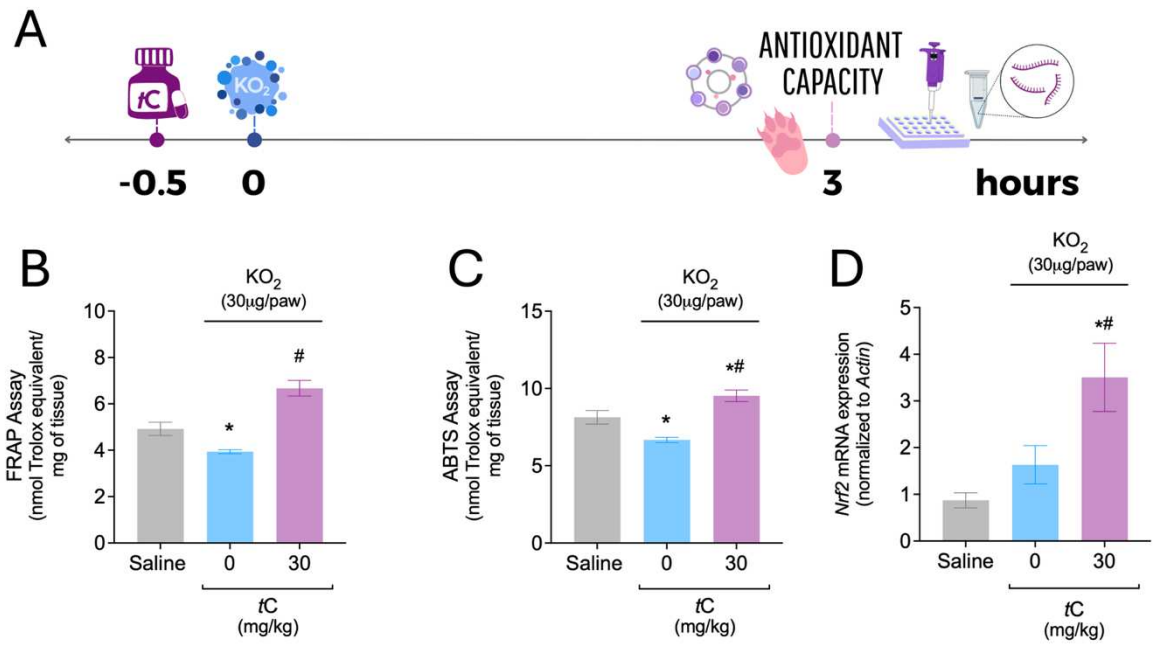

Fig 3. *tC induces antioxidant response against oxidative damage triggered by KO_2 in mice.* (A) Experimental timeline. Mice were pre-treated p.o. with *t*C 30min before KO_2 injection. The injected plantar paw tissue was collected 3 hours after the stimulus. (B) Paw skin FRAP (n = 6), (C) ABTS (n = 6), and (D) Nrf2 mRNA expression by RT-qPCR (n = 5) were assessed. Shapiro-Wilk and Brown-Forsythe tests were used to verify normality and equality of group variances. Statistical analyses were performed by One-way ANOVA followed by Tukey's multiple comparisons test. Results are presented as means ± SEM representative of two

separate experiments *p-value ≤ 0.05 compared to saline group; #p-value ≤ 0.05 compared to stimulus group.

tC reduces KO_2-triggered oxidative stress by NADPH oxidase inhibition

Regarding the flavonoid effects on paw tissue oxidative stress indicators after KO_2 stimulus, lipid peroxidation was reduced in 25.9% (Fig 4B) and superoxide anion levels were inhibited in 49.9% (Fig 4C) after *t*C pretreatment. These effects on reducing stress oxidation are, at least in part, a result of the downregulation of *Cybb* expression, the gene that encodes $gp91^{phox}$, one of the components of the NADPH oxidase complex, which generates superoxide anion. *Cybb* (Fig 4D) expression was reduced in 88.6% on the group that received *t*C pre-treatment.

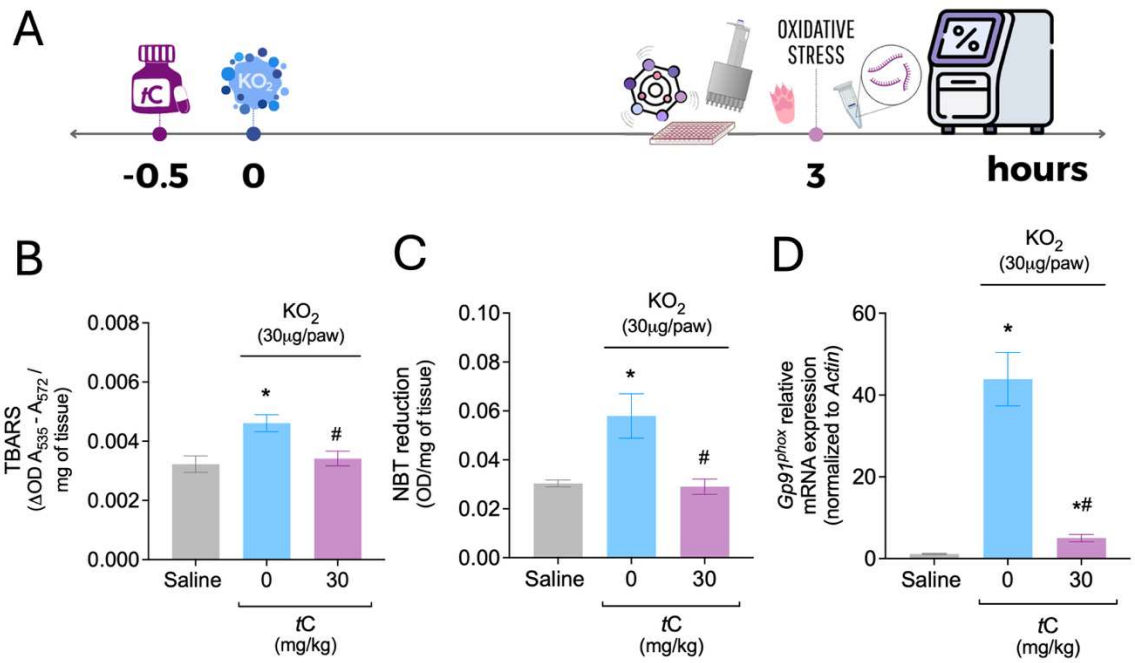

Fig 4. *tC reduces oxidative stress triggered by KO_2 in mice*. (A) Experimental timeline. Mice were pre-treated p.o. with *t*C 30min before KO_2 injection. The injected plantar paw tissue was collected 3 hours after the stimulus. (B) Paw skin TBARS (n = 6) (C), NBT (n = 5), and (D) $gp91^{phox}$ mRNA expression (*Cybb* gene) (n=5) were assessed. For TBARS and NBT, Shapiro-Wilk and Brown-Forsythe were used to verify normality and equality of group variances. Statistical analyses were performed by One-way ANOVA followed by Tukey's multiple comparisons test. To analyze $gp91^{phox}$ mRNA expression, Shapiro-Wilk was employed for normality assessment, and statistical analyses were performed with Welch's ANOVA test followed by Dunnett's T3 multiple comparisons test. Results are presented as means ± SEM

representative of two separate experiments *p-value ≤ 0.05 compared to saline group; #p-value ≤ 0.05 compared to stimulus group.

tC inhibits COX-2, IL-33 and IL-6 production triggered by KO_2

To begin assessing the effects of *t*C on superoxide anion-induced inflammation, the expression of cytokines IL-33 and IL-6 were investigated, as well as the proinflammatory enzyme cyclooxygenase 2 (COX-2) production. Our results demonstrate an upregulation in IL-33 protein levels (Fig 5C) and in in mRNA (Fig 5D) production triggered by KO_2, while treatment with *t*C reduced IL-33 protein levels in 28.3% and inhibited in 64.1% IL-33 mRNA expression (Figs 5C and 5D). Likewise, *t*C pretreatment was able to reduce IL-6 protein levels in 52.2% and inhibit its mRNA expression in 48.9%. Furthermore, *t*C treatment inhibited COX-2 (Fig 5B) mRNA expression triggered by the superoxide donor in 66.2%.

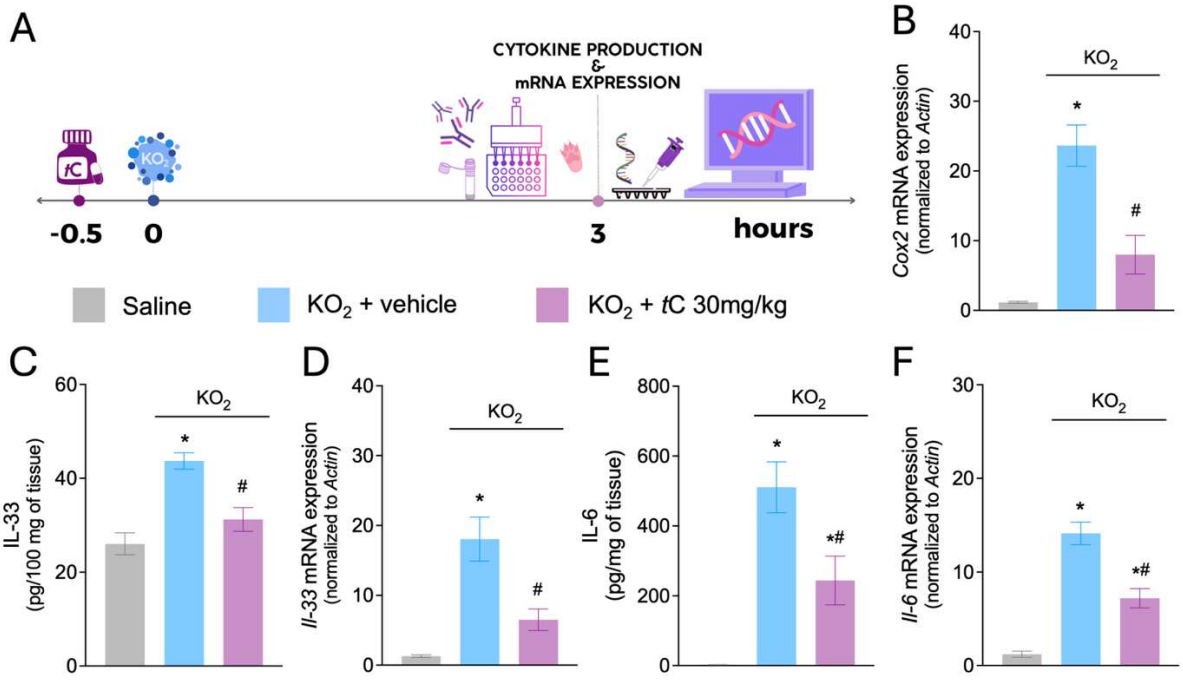

Fig 5. *tC inhibits KO_2-triggered inflammation in mice.* (A) Experimental timeline. Mice were pre-treated p.o. with *t*C 30min before KO_2 injection. The injected plantar paw tissue was collected 3 hours after the stimulus. (B) COX-2 mRNA expression – n = 5, (C) IL-33 production – n = 5, (D) IL-33 mRNA expression – n = 5, (E) IL-6 production – n = 5, (F) IL-6 mRNA expression – n = 5 were quantified by RT-qPCR and/or ELISA. Shapiro-Wilk and Brown-Forsythe were used to verify normality and equality of group variances. Statistical analyses were performed by One-way ANOVA followed by Tukey's multiple comparisons test. Results are presented as

means ± SEM representative of two separate experiments *p-value ≤ 0.05 compared to saline group; #p-value ≤ 0.05 compared to stimulus group.

tC inhibits NF-κB activation, TNF-α and IL-1β production induced by KO_2

The effects of tC on inflammation triggered by KO_2 were also observed on the NF-κB pathway activation. The expression of gene that encodes the p65 portion of NF-κB, called *RelA,* was downregulated in 27% (Fig 6D). Moreover, the active form of NF-κB p65 (phosphorylated-NF-κB p65) was inhibited by 42.5% (Figs 6B and 6C) on the group that received *t*C pretreatment.

Coherently, the levels of pro-inflammatory cytokines IL-1β and TNF-α were reduced on *t*C-treated mice. Under pro-inflammatory conditions, active NF-κB translocates into the nucleus and induces inflammatory cytokines production. Therefore, *t*C-mediated inhibition of NF-κB activation is, at least in part, responsible for the reduction observed in IL-1β (35.3% - Fig 6E) and TNF-α (37% - Fig 6F) levels.

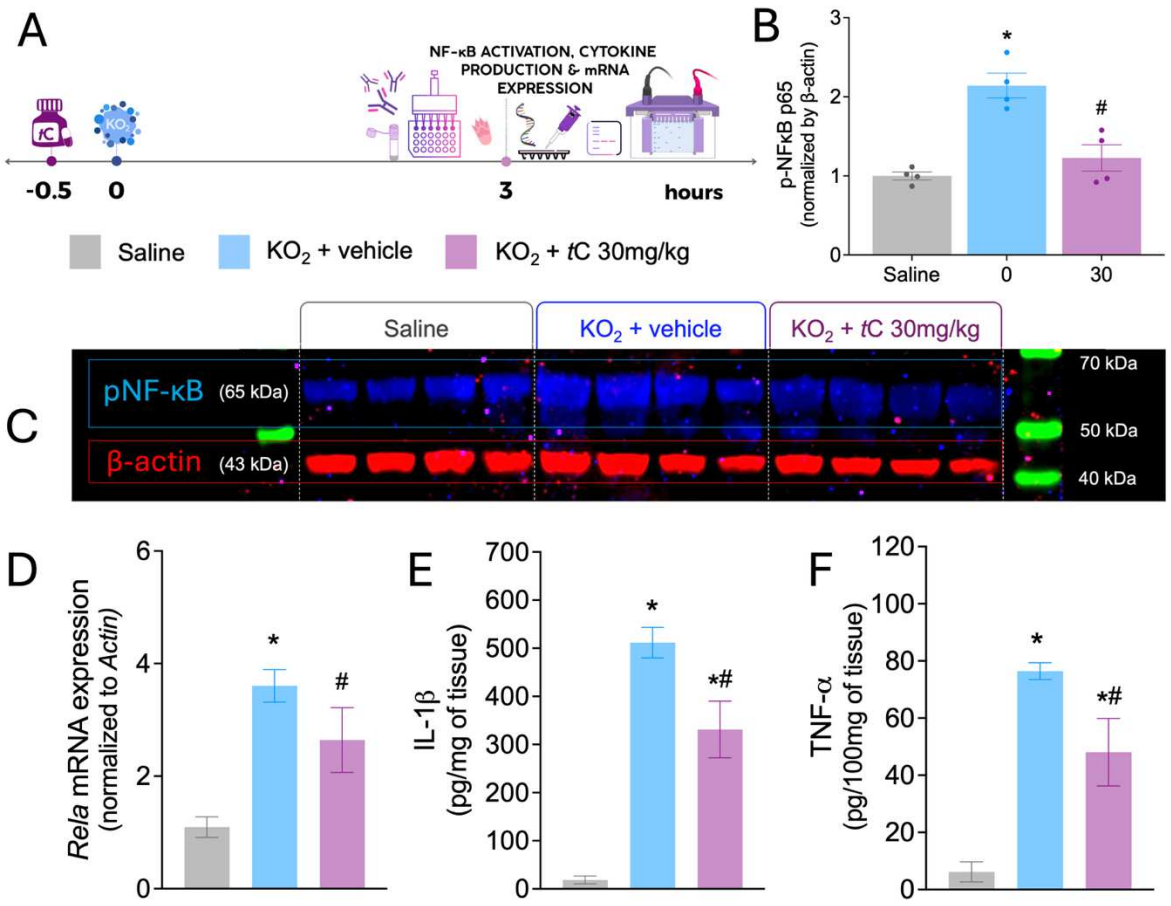

Fig 6. *tC inhibits pro-inflammatory cytokines signaling and NF-κB activation induced by KO_2.* (A) Experimental timeline. Mice were pre-treated p.o. with *t*C 30 min before KO_2 injection. The

injected plantar paw tissue was collected 3 hours after the stimulus. (B and C) p-NF-κB (blue) and β-actin (red) expression were assessed by Fluorescent Western Blot – n = 4. (D) mRNA expression of *RelA* (NF-κB p65) was measured via RT-qPCR – n = 6. (E) IL-1β and (F) TNF-α cytokine levels were investigated by ELISA. Shapiro-Wilk and Brown-Forsythe were used to verify normality and equality of group variances. Statistical analyses were performed by One-way ANOVA followed by Tukey's multiple comparisons test. Results are presented as means ± SEM representative of two separate experiments *p-value ≤ 0.05 compared to saline group; #p-value ≤ 0.05 compared to stimulus group.

tC inhibits TRPV1⁺ nociceptors sensitization

After observing *t*C effects on restoring superoxide anion-induced oxidative damage and inflammation, as well as the flavonoid effects inhibiting KO_2-triggered nociception and hyperalgesia, we investigated its effects on DRG-neurons pain signaling. To do so, calcium influx was assessed *ex vivo*, i. e. nociceptive neurons previously exposed to *t*C or vehicle pretreatment and following KO_2 or saline i.pl. injection were collected, processed and submitted to *in vitro* calcium influx imaging. Neuron population was determined by the expression of TRPV1 and TRPA1 nociceptive ionic channels. TRPV1-expressing neurons ($TRPV1^+$) were identified due to their sensitivity to capsaicin, a known TRPV1 agonist.

Our results showed that i.pl. KO_2 induced DRG-neuron sensitization, observed by the baseline increased fluorescence intensity of cultured neurons before capsaicin stimulus, 41% higher than the saline group (Fig 7E left). The *t*C pretreated group demonstrated a reduced baseline activation (26%) when compared to the vehicle treated group that also received KO_2 i.pl. stimulus (Fig 7E left). We also observed that *t*C pretreatment reduced the calcium influx on TRPV1+ neurons, observed by reduced fluorescence intensity in 36% after capsaicin-induced ionic channel activation (Fig 7E right), as well as the number of activated neurons, reduced on 50,5% (Venn's diagram Fig 7C).

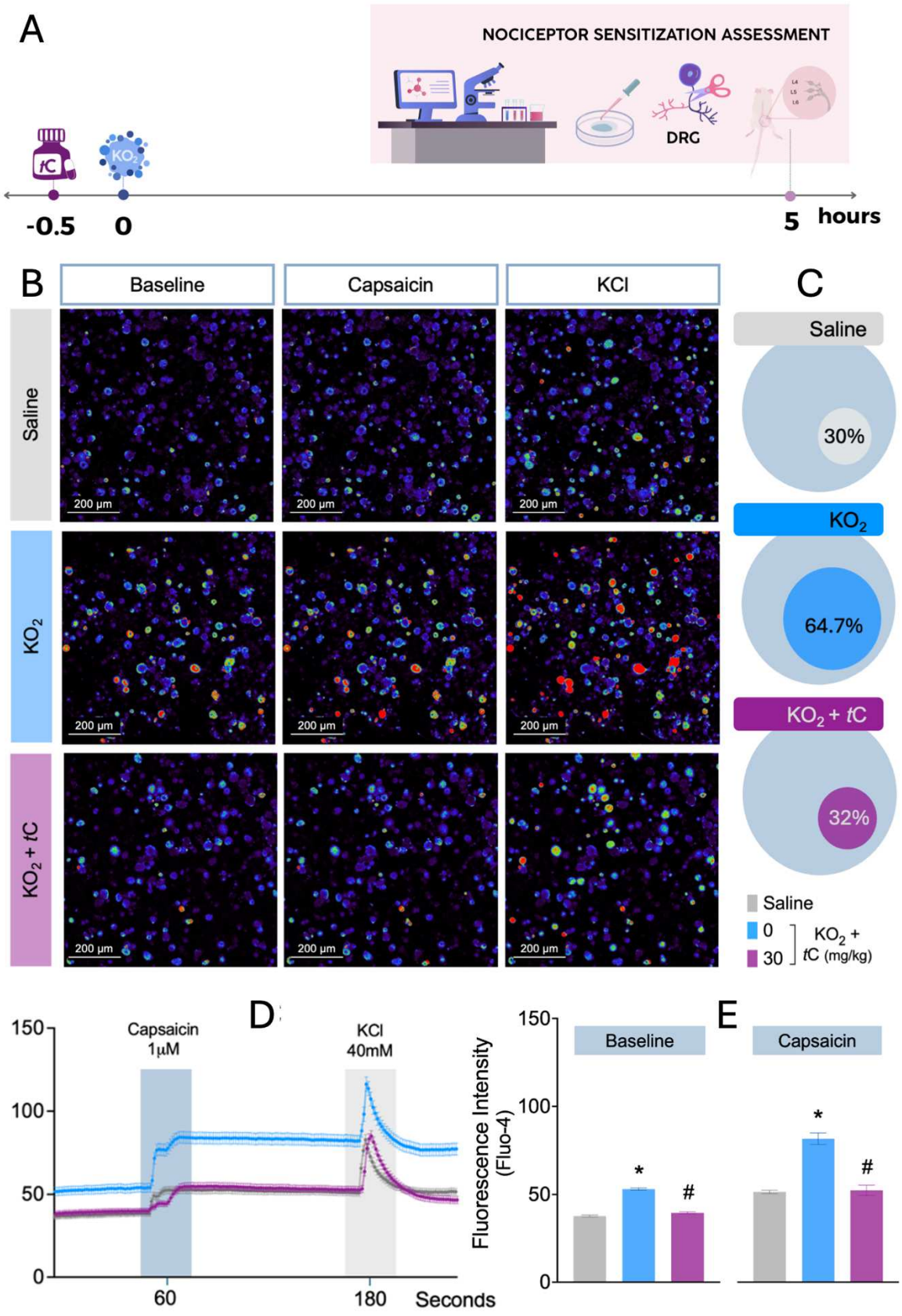

Fig 7. *tC inhibits KO2-induced TRPV1+ nociceptor activation.* (A) Experimental timeline. To assess the effects of *t*C on TRP1-positive neuron sensitization after KO_2 i.pl. injection, DRG neurons from the right side of L4, L5 and L6 were collected 5 hours after stimulus and

processed. Plates with Fluo-4-labeled cells were recorded for 4min: 1min of initial reading, followed by stimulus with capsaicin (TRPV1 agonist, 1 μM in HBSS) for 2 min at the 60 s-mark, and with KCl for 1 min at the 180 s-mark (40 mM, activates all neurons). (B) Representative pictures throughout the 4 min of data acquired at baseline, capsaicin stimulus, and KCl activation, for KO_2 + *t*C, KO_2 + vehicle (KO_2), and saline groups. (C) Venn's diagram indicating the percentage of DRG-derived neurons activated after capsaicin stimulus. (D) Fluorescence intensity tracers throughout the 4 min of recording. (E) Comparative mean fluorescence intensity between saline (317 neurons), KO_2 (308 neurons) and KO_2 + *t*C (352 neurons) groups at baseline and capsaicin-induction timepoints. Statistical analyses were performed with Kruskal-Wallis followed by Dunn's multiple comparison test. Results are expressed as mean ± SEM (D) or mean ± standard deviation (SD)(E) representative of two separate experiments. *p-value ≤ 0.05 compared to saline cells; $^{\#}$p-value ≤ 0.05 compared to *t*C treated cells.

tC inhibits TRPA1^{+} nociceptors sensitization

Similarly to our findings on TRPV1^{+} calcium influx triggered by KO_2 *in vivo*, vehicle-treated cells showed higher intracellular calcium levels compared to *t*C treated and saline groups before AITC stimulus (at baseline) (Fig 8E left), confirming that the intraplantar injection of KO_2 induced sensitization of DRG neurons. After the addition of the TRPA1 agonist, AITC, 73.4% of viable neurons showed an increase in calcium influx, whereas saline (52.3%) and *t*C-treated (48.7%) groups showed active calcium influx in about half of the viable neurons (Fig 8C – Venn's diagram). *t*C pretreatment inhibition of KO_2-triggered neuron sensitization was also observed on fluorescent intensity, which was 37.5% lower on KO_2-induced *t*C treated group than the vehicle treated group after AITC stimulus (Fig 8E right).

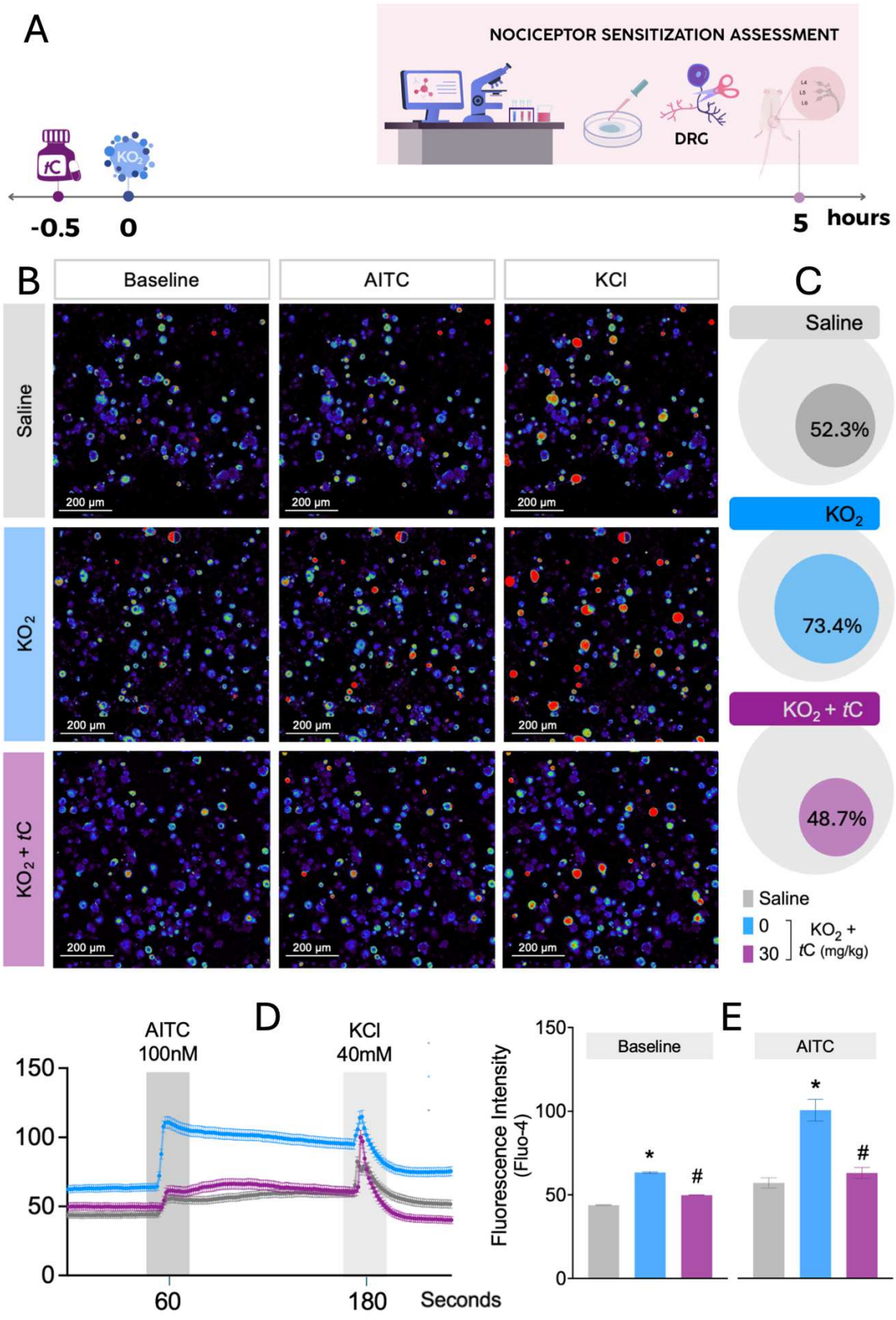

Fig 8. *tC inhibits KO_2-induced TRPA1$^+$ nociceptor activation.* (A) Experimental timeline. To assess the effects of *t*C on TRPV1-positive neuron sensitization after KO_2 i.pl. injection, DRG neurons from the right side of L4, L5 and L6 were collected 5 hours after stimulus and

processed. Plates with Fluo-4-labeled cells were recorded for 4min: 1min of initial reading, followed by stimulus with AITC (TRPA1 agonist, 100 nM in HBSS) for 2min at the 60 s-mark, and with KCl for 1min at the 180 s-mark (40 mM, activates all neurons). (B) Representative pictures throughout the 4 min of data acquired at baseline, AITC stimulus, and KCl activation, for KO_2 + *t*C, KO_2 + vehicle (KO_2), and saline groups. (C) Venn's diagram indicating the percentage of DRG-derived neurons activated after capsaicin stimulus. (D) Fluorescence intensity tracers throughout the 4 min of recording. (E) Comparative mean fluorescence intensity between saline (270 neurons), KO_2 (280 neurons) and KO_2 + *t*C (174 neurons) groups at baseline and AITC-induction timepoints. Statistical analyses by Kruskal-Wallis followed by Dunn's multiple comparison test. Results are expressed as mean ± SEM (D) or mean ± SD (E), representative of two separate experiments. *p-value $\leq$ 0.05 *vs.* saline cells; $^{\#}$p-value $\leq$ 0.05 *vs. t*C treated cells.

DISCUSSION

Our study evaluated *t*C potential against ROS-triggered pain, inflammation and oxidative stress. KO_2-induced overt pain-like behavior was inhibited by *t*C. It is accepted that overt pain-like behavior occurs due to the activation of nociceptors at the site of the stimulus, which initiate signaling transmission to primary afferent cell somas at the DRG, induce synaptic signaling at the spinal cord and send the nociceptive message to brain, where it can be perceived as pain (Prescott & Ratté, 2017).

Even though ROS induces nociception via TRPA1 ionic channel (Andersson *et al.*, 2008), a nociceptive stimulus can initiate pro-inflammatory signaling and sensitize other nociceptors (Aguggia, 2003). Hence, we investigated whether *t*C was able to inhibit neuron activation of TRPV1 and TRPA1 channels after KO_2 stimulus.

KO_2-induced neuron sensitization was observed by the increased number of neurons permissive to calcium influx at baseline and after neuron activation with capsaicin, inducing calcium influx in over 60% of viable neurons (Fig 7). Meanwhile, *t*C treatment presented a neuronal activation pattern similar to the saline group at baseline and after neuron activation with capsaicin, indicating a neuroprotective role of *t*C. Besides *t*C, other flavonoids have demonstrated the ability to inhibit TRPV1 signaling, including Vitexin (Borghi *et al.*, 2013), hesperidin methyl chalcone (HMC) (Pinho-Ribeiro *et al.*, 2015), and eriodictyol (Rossato *et al.*, 2011).

TRPA1 channels are sensible to ROS, which occurs through cysteine oxidation and disulfide formation between TRPA1 cysteine residues (Giorgi *et al.*, 2019). TRPA1 ionic channels were observed in Schwann cells, oligodendrocytes, astrocytes, primary afferent neurons, vascular endothelial cells, and other tissues that can relay nociceptive signals (Zhang *et al.*, 2023). Similarly to our findings on $TRPV1^+$ calcium influx triggered by KO_2 *in vivo*, *t*C inhibited AITC-induced calcium influx (Fig 8). Hence, the flavonoid appears to prevent further DRG-neurons sensitization by impairing the activation of TRPV1 and TRPA1 channels.

Coherently, the present study confirms *t*C analgesic properties against superoxide-triggered nociception on overt pain-like behavior but also demonstrates the flavonoid potential to inhibit nociceptive sensitization, both on $TRPV1^+$ and $TRPA1^+$ DRG neurons, along with the reduction of mechanical hyperalgesia and inflammation.

Inflammation presents itself with classic signs and symptoms, which include edema, erythema (redness), warmness, pain, and loss of function (stiffness and

immobility) (Punchard *et al.*, 2004). In addition, enhanced ROS generation may exacerbate those signs and symptoms, as the oxidative stress can induce the opening of endothelial cell-cell junctions, promoting the migration of inflammatory cells across the endothelial barrier. Along with leukocyte extravasation, the increased vascular permeability also enables fluid extravasation and accumulation at the injury site (Mittal *et al.*, 2014), resulting in two of the parameters we have analyzed: leukocyte recruitment and edema.

*t*C pretreatment reduced edema and leukocyte recruitment, the later was assessed indirectly by myeloperoxidase activity, an enzyme found mainly, but not exclusively, in lysosomal azurophilic granules of neutrophils recruited in the initial hours of acute tissue inflammation (Hanning *et al.*, 2023). Previous studies have shown *t*C potential to inhibit the adhesion molecules ICAM-1 (intercellular adhesion molecule 1), and VCAM-1 (vascular cell adhesion molecule 1) in a cardiac ischemia and reperfusion experimental study (Wang *et al.,* 2024), indicating that it could be a mechanism contributing to recruitment inhibition. TRPA1 inhibition by *t*C might also be mechanistically involved, seen as both genetic ablation and pharmacological inhibition of TRPA1 reduce skin edema (Liu *et al.*, 2013).

The effects of oxidative damage on mechanical hypersensitivity can be observed on chemotherapy-induced peripheral neuropathy, where the peripheral oxidative stress sensitizes mechanical nociceptors, while the spinal oxidative stress is able to maintain central sensitization that abnormally produces pain in response to Aβ fiber inputs (Shim *et al.*, 2019). Furthermore, intraganglionic ROS accumulation during peripheral inflammation has shown to contribute to pain and hyperalgesia in a TRPA1 dependent manner, and that ROS can further exacerbate pathological pain responses by upregulating TRPA1 expression (Zhang *et al.*, 2023). Addicionally, ROS appears to be required for rapid activation of central sensitization mechanisms on capsaicin-induced secondary (outside the primary capsaicin-injected site) mechanical hyperalgesia and allodynia (La *et al.*, 2017). Taken together, this data illustrates the importance of oxidative stress for the development and maintenance of hyperalgesia.

Pathological conditions such as atherosclerosis, chronic obstructive pulmonary disease, Alzheimer's disease and cancer are directly linked to oxidative damage (Forman & Zhang, 2021). ROS imbalance can be regulated by enzymatic and non-enzymatic mechanisms, whereas cellular glutathione, vitamins C and E, β-carotene, polyphenols and uric acid are examples of non-enzymatic low-molecular-weight

antioxidant compounds; and superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase can be named as antioxidant enzymes (Andrés *et al.*, 2023). Even though *t*C is not an antioxidant per se, its antioxidant effects in vivo (Martinez *et al.*, 2017a e 2017b; Staurengo-Ferrari *et al.*, 2018) appear to be the result of antioxidant enzymes upregulation.

We showed that KO_2 reduced the antioxidant capacity in the paw skin, and *t*C was able to upregulate antioxidant levels. This effect is, at least in part, mediated by the transcription factor Nrf2 upregulation, which, under oxidative stress or upon exposure to Nrf2 activators, can dissociate from the cytoplasmatic complex with Keap1 (Kelch-like ECH-associated protein) and translocate into the nucleus, regulating gene expression through the antioxidant response element (ARE) battery of genes (Ahmed *et al.*, 2017). Many antioxidant genes contain the ARE (TGCTGA(G/C)TCAGCA) in their promoters, such as superoxide dismutases, peroxiredoxins, glutathione transferases, metallothionein proteins, and the Nrf2 gene itself (On *et al.*, 2021).

In addition to the antioxidant response triggered by Nrf2, *t*C treatment also inhibited the expression of $gp91^{phox}$, a subunit of NADPH oxidase complex. Briefly, in the absence of pro-oxidative signaling, NADPH is dormant, and $gp91^{phox}$ forms a heterodimer with $p22^{phox}$ integrated to the cell membrane. When activated, $gp91^{phox}$ acts as the catalytic core of NADPH oxidase to produce superoxide (Takeya et al., 2003). Therefore, the inhibition of $gp91^{phox}$ is responsible, at least in part, for the reduction in lipid peroxidation and superoxide levels after *t*C treatment.

Beyond analgesic and antioxidant properties, *t*C treatment also modulates important inflammatory processes like cytokine production, COX-2 expression and NF-κB activation. Among the cytokines inhibited by the flavonoid, IL-33 seems to play an important role on superoxide-induced pain and inflammation. Borghi et al. (2025) recently showed on suppressor of tumorigenicity 2 (ST2) knock out mice that the IL-33/ST2 signaling pathway mediates, at least in part, KO_2-triggered inflammatory pain [Borghi *et al.*, 2015).

Moreover, IL-6, TNF-α and IL-1β were inhibited by *t*C treatment. Such inhibition, at least partially resultant of a decreased leukocyte recruitment, also impair the activation of NF-κB. A wide range of cellular processes are modulated by the transcription factor NF-κB: immunity, inflammation, cell development, growth and survival. Therefore, it is important to stress that its activation by oxidative species is

cell type and context specific (Lingappan, 2018; Siomek, 2022). Addicionally, the crosstalk between ROS and NF-κB signaling is a complex one, since certain NF-κB-regulated genes play a major role in regulating intracellular ROS levels, ROS also have shown inhibitory or stimulatory roles in NF-κB signaling (Morgan & Liu, 2011).

In a brief overview, the transcription factor NF-κB is normally localized in the cytoplasm as a heterodimer, mainly formed by p50/p65 (RelA, also named p65), associated with IκB proteins, which mask the DNA binding domain and keep them sequestered in the cytoplasm. Under pro-inflammatory signaling, IκB suffers phosphorylation, leading to its ubiquitination and degradation, and the active NF-kB translocates into the nucleus and activates the target genes transcription, thus characterizing the canonical NF-κB activation. On the other hand, the non-canonical pathway, that predominantly targets activation of the p52/RelB NFκB complex, is independent to IκB degradation, occurring due to NF-κB-inducing kinase (NIK)-mediated phosphorylation of IKK-α (IκB kinase-α), triggering p100 phosphorylation and processing, a molecule functioning as both the precursor of p52 and a RelB-specific inhibition, allowing the non-canonic NF-κB activation (Lingappan, 2018; Sun, 2011; Vicentini *et al.*, 2011). Both canonical and non-canonical activations of NF-κB by ROS have been described (Takada *et al.*, 2003; Powers *et al.*, 2011; Zhang *et al.*, 2021).

The activation of NF-κB mediates KO_2-induced pain and inflammation (Pinho-Ribeiro et al., 2016). KO_2 induced IL-1β, TNF-α, IL-6, and IL-33 upregulation and NF-κB activation, and *t*C effectively reduced cytokine levels, inhibited NF-κB activation and p65 expression, which also prevented cytokine production and de novo NF-κB activation, seen as ROS production is further increased by pro-inflammatory cytokines such as the IL-1β, which, in turn, is induced due to NF-κB activation and nuclear translocation under cellular pro-inflammatory conditions, illustrating the intricate crosstalk between these signaling pathways (Morgan & Liu, 2011).

It is important to stress that *t*C analgesic mechanisms might not be solely dependent on TRP channels or might not be directly impairing agonist-TRP channel binding. For instance, the flavonoid naringenin induces analgesia promoting neuronal nitric oxide (NO) production that, via NO-cGMP-PKG-ATP-sensitive potassium channel activation, allows potassium efflux-induced neuronal hyperpolarization, making the inside of the neuron more negative than the resting membrane potential, reducing neuronal excitability, and promoting analgesic effects (Cury *et al.*, 2011;

Manchope *et al.*, 2016). Also, potassium channel-activation can decrease sensory neurons sensitivity to capsaicin (Lengyel *et al.*, 2021), indicating an interaction between NO-induced analgesia and TRPV1 ionic channel inhibition. Nonetheless, further investigation is required to confirm or refute the involvement of this pathway on *t*C-mediated effects.

In sum, *t*C reduced nociceptive and inflammatory responses triggered by KO_2 through cytokines and NF-κB inhibition, which contributed to the inhibition of de novo IL-1β, TNF-α, IL-33 and IL-6 production. *t*C also induced Nrf2 mRNA expression and inhibited Cox-2, *RelA* and $gp91^{phox}$ expression, and prevented $TRPV1^{+}$ and $TRPA1^{+}$ nociceptors sensitization.

CONCLUSIONS

We investigated the efficacy of *t*C as an analgesic and anti-inflammatory alternative against pain and inflammation induced by superoxide anion in mice. Our results demonstrate that *t*C inhibits the activation of TRPV1- and TRPA1-expressing neurons and prevents neuron sensitization. Treatment with the flavonoid also reduces hyperalgesia, inflammation and oxidative stress. Altogether, *t*C effects against KO_2-triggered pain and inflammation provide information that support its use in clinical trials as a multitarget drug with analgesic, anti-inflammatory and antioxidative properties against pathological conditions attributable to oxidative damage.

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6 CONCLUSÃO

Nosso estudo identificou a capacidade do flavonoide *trans*-Chalcona de promover a analgesia em diferentes modelos de estudo de dor manifesta. Sua ação é, ao menos parcialmente, resultado da inibição do canais iônicos TRPV1 e TRPA1. A *t*C também reduzir a sensibilização neuronal, inibe a produção de citocinas pró-inflamatórias e a ativação do fator de transcrição NF-κB. Apesar da ausência de efeitos antioxidantes diretos, a *t*C é capaz de estimular a sinalização antioxidante e inibir a produção de espécies reativas. Os resultados obtidos demonstram que a *t*C é um flavonoide multialvos, capaz de modular as respostas nociceptiva e inflamatória a nível neuronal e de células de defesa.

Dentre os benefícios da *t*C, cabe citar sua versatilidade: a eficácia como analgésico administrado oralmente com ação sistêmica é desejável, ampliando a gama de condições dolorosas nas quais pode ser efetiva. A *t*C também é passível de microencapsulamento com a possibilidade de liberação controlada e potencialização, O mesmo é verdadeiro para sua formulação tópica, eficaz na redução dos danos causados pela irradiação UVB, mas ainda sem estudos referentes à possível ação analgésica de uso tópico. Essa estratégica terapêutica no campo da analgesia poderia ser promissora, tendo em vista a popularização dessa via no combate da dor, demonstrando sucesso com o uso tópico da capsaicina, da lidocaína e do mentol. A investigação da *t*C em modelos de dor associada a condições patologias, como a dor neuropática, também é promissora.

Desta forma, somados os efeitos encorajadores da *t*C em estudos pré-clínicos, existe potencial para ampliar as pesquisas pré-clínicas, bem como promover a investigação subsequente a nível clínico.

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ANEXOS

ANEXO A

Parecer CEUA **050.2024**

Universidade Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 129/2024

Londrina, 12 de dezembro de 2024.

Prezado(a) professor(a),

Certificamos que o projeto intitulado: **"Efeitos e mecanismos da *trans*-Chalcona em modelos murinos de dor e inflamação"** protocolo CEUA n° **050.2024** sob a responsabilidade de **Waldiceu Aparecido Verri Júnior** que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL) no dia **12/12/2024**.

Este projeto tem por objetivo investigar o potencial terapêutico, os mecanismos de ação e os efeitos farmacológicos da *trans*-Chalcona na dor e/ou na inflamação induzidas por ácido acético (AAc), fenil-p-benzoquinona (PBQ), Formalina, LPS, CFA, Carragenina, KO_2, AITC, Zimosan e Capsaicina, bem como o modelo de dor neuropática induzida pela constrição do nervo ciático (CCI). **Grau de invasividade**: **GI3.**

Finalidade	( ) Ensino (X) Pesquisa científica
Vigência da autorização	08/01/25 a 07/01/30
Espécie/ linhagem/ raça	Camundongo heterogênico / Swiss
N° de animais	**3696**
Peso/ Idade	20-25 g / 6-8 semanas
Sexo	Machos
Origem	Biotério Central da Universidade Estadual de Londrina
Amostras a serem coletadas	Pele da pata, plasma, lavado intraperitoneal, lavado intrta-articular, articulação, porção lombar da medula espinal, gânglios da raiz dorsal

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

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

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

Profª Drª Patrícia Chimin Perandini
Coordenadora da CEUA/UEL

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

ANEXO B

Parecer CEUA **24684.2016.72**

Universidade Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 249/2016 **Londrina, 08 de Dezembro de 2016.**

Prezado Pesquisador,

Certificamos que o projeto intitulado **"Avaliação do efeito terapêutico e mecanismo de ação da trans-chalcona em modelos de dor inflamatória em camundongos"**, protocolo CEUA nº **24684.2016.72**, sob a responsabilidade de **Waldiceu Aparecido Verri Junior**, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), foi **aprovado** pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL), em reunião realizada em **29/11/2016**.

O projeto irá testar o efeito da trans-chalcona e seus mecanismos de ação em modelos de dor e na inflamação experimental. Os materiais biológicos coletados dos animais do referente projeto não serão utilizados em outros projetos. Os animais serão acomodados em caixas padrão com no máximo 12 animais em cada caixa, em temperatura controlada, com exaustão de ar, ciclo claro/escuro, com livre acesso à ração e água. Os animais serão imobilizados manualmente por curtos períodos, somente para a administração dos estímulos inflamatórios e para o tratamento com o ácido vanílico e/ou outras drogas de acordo com os respectivos experimentos. Para eutanásia os animais serão anestesiados terminalmente com Isoflurano 1,5 a 3% em O2. GI 2.

Vigência do Projeto	01/01/2017 a 31/12/2019
Espécie/linhagem	Camundongo heterogênico / Swiss
Nº de animais	852
Peso/Idade	20-25g
Sexo	Machos
Origem	Biotério Central / UEL
Amostras a serem coletadas	Tecido plantar cutâneo, plasma, mucosa gástrica

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

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

Prof. Dr. João Waine Pinheiro
Vice-Coordenador da CEUA/UEL

Ilmo. Sr.
Prof. Dr. Waldiceu Aparecido Verri Junior
Coordenador do Projeto
Departamento de Ciências Patológicas / Centro de Ciências Biológicas
Com cópia para Coordenação do Biotério Central/UEL; Chefe do Departamento de Ciências Patológicas e Diretor(a) do Centro de Ciências Biológicas

ANEXO C

Artigos em colaboração

molecules

MDPI

Article

Hesperidin Methyl Chalcone Reduces the Arthritis Caused by TiO_2 in Mice: Targeting Inflammation, Oxidative Stress, Cytokine Production, and Nociceptor Sensory Neuron Activation

Nayara A. Artero [1], Marília F. Manchope [1], Thacyana T. Carvalho [1], Telma Saraiva-Santos [1], Mariana M. Bertozzi [1], Jessica A. Carneiro [1], Anelise Franciosi [1], Amanda M. Dionisio [1], Tiago H. Zaninelli [1], Victor Fattori [1], Camila R. Ferraz [1], Maiara Piva [1], Sandra S. Mizokami [1], Doumit Camilios-Neto [2], Rubia Casagrande [3] and Waldiceu A. Verri [1,*]

[1] Laboratory of Pain, Inflammation, Neuropathy and Cancer, Department of Pathology, Centre of Biological Sciences, Londrina State University, Londrina 86057-970, PR, Brazil
[2] Department of Biochemistry and Biotechnology, Centre of Exact Sciences, Londrina State University, Londrina 86057-970, PR, Brazil
[3] Department of Pharmaceutical Sciences, Centre of Health Science, Londrina State University, Londrina 86039-440, PR, Brazil
* Correspondence: waverri@uel.br; Tel.: +55-43-3371-4979

Abstract: Arthroplasty is an orthopedic surgical procedure that replaces a dysfunctional joint by an orthopedic prosthesis, thereby restoring joint function. Upon the use of the joint prosthesis, a wearing process begins, which releases components such as titanium dioxide (TiO_2) that trigger an immune response in the periprosthetic tissue, leading to arthritis, arthroplasty failure, and the need for revision. Flavonoids belong to a class of natural polyphenolic compounds that possess antioxidant and anti-inflammatory activities. Hesperidin methyl chalcone's (HMC) analgesic, anti-inflammatory, and antioxidant effects have been investigated in some models, but its activity against the arthritis caused by prosthesis-wearing molecules, such as TiO_2, has not been investigated. Mice were treated with HMC (100 mg/kg, intraperitoneally (i.p.)) 24 h after intra-articular injection of 3 mg/joint of TiO_2, which was used to induce chronic arthritis. HMC inhibited mechanical hyperalgesia, thermal hyperalgesia, joint edema, leukocyte recruitment, and oxidative stress in the knee joint (alterations in $gp91^{phox}$, GSH, superoxide anion, and lipid peroxidation) and in recruited leukocytes (total reactive oxygen species and GSH); reduced patellar proteoglycan degradation; and decreased pro-inflammatory cytokine production. HMC also reduced the activation of nociceptor-sensory $TRPV1^+$ and $TRPA1^+$ neurons. These effects occurred without renal, hepatic, or gastric damage. Thus, HMC reduces arthritis triggered by TiO_2, a component released upon wearing of prosthesis.

Keywords: inflammation; hesperidin methyl chalcone; titanium dioxide; prosthesis; arthritis; TRPV1; TRPA1; nociceptor sensory neuron; oxidative stress

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1. Introduction

Joint dysfunction can be induced by varied diseases, ranging from infections, chronic degeneration, tumors, and physical trauma [1]. The impairment of joint functionality and chronic pain drastically decrease mobility and the degree of engagement in common life interactions; thus, patients' overall quality of life is affected. In severe cases, there is a need for arthroplasty, which is a surgical procedure ranging from the partial to total replacement of a dysfunctional joint by a prosthesis [2]. From January 2008 to December 2015, 47,289 total knee and total hip arthroplasties were performed in Brazil [3]. From 2006 to 2007, 62,196 hospitalizations for arthroplasty occurred in Canada, with an overall incidence of 81.2 cases per 100,000 individuals annually [4]. According to the Agency for Healthcare

ANEXO C
Artigos em colaboração

World Journal of Stem Cells

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REVIEW

Stem cells and pain

Matheus Deroco Veloso da Silva, Maiara Piva, Geovana Martelossi-Cebinelli, Mariana Stinglin Rosa Ribas, Beatriz Hoffmann Salles Bianchini, Olivia K Heintz, Rubia Casagrande, Waldiceu A Verri Jr

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Matheus Deroco Veloso da Silva, Maiara Piva, Geovana Martelossi-Cebinelli, Mariana Stinglin Rosa Ribas, Beatriz Hoffmann Salles Bianchini, Department of Pathology, Laboratory of Pain, Inflammation, Neuropathy and Cancer, State University of Londrina, Londrina 86057-970, Paraná, Brazil

Olivia K Heintz, Morningside Graduate School of Biomedical Sciences, University of Massachusetts Chan Medical School, Worcester, MA 01655, United States

Rubia Casagrande, Department of Pharmaceutical Sciences, Center of Health Science, State University of Londrina, Londrina 86038-440, Paraná, Brazil

Waldiceu A Verri Jr, Department of Pathology, Laboratory of Pain, Inflammation, Neuropathy and Cancer, Center of Biological Sciences, State University of Londrina, Londrina 86057-970, Paraná, Brazil

Corresponding author: Waldiceu A Verri Jr, PhD, Associate Professor, Department of Pathology, Laboratory of Pain, Inflammation, Neuropathy and Cancer, Center of Biological Sciences, State University of Londrina, Rodovia Celso Garcia Cid/Pr 445 Km 380, Londrina 86057-970, Paraná, Brazil. waverri@uel.br

Abstract

Pain can be defined as an unpleasant sensory and emotional experience caused by either actual or potential tissue damage or even resemble that unpleasant experience. For years, science has sought to find treatment alternatives, with minimal side effects, to relieve pain. However, the currently available pharmacological options on the market show significant adverse events. Therefore, the search for a safer and highly efficient analgesic treatment has become a priority. Stem cells (SCs) are non-specialized cells with a high capacity for replication, self-renewal, and a wide range of differentiation possibilities. In this review, we provide evidence that the immune and neuromodulatory properties of SCs can be a valuable tool in the search for ideal treatment strategies for different types of pain. With the advantage of multiple administration routes and dosages, therapies based on SCs for pain relief have demonstrated meaningful results with few downsides. Nonetheless, there are still more questions than answers when it comes to the mechanisms and pathways of pain targeted by SCs. Thus, this is an evolving field that merits further investigation towards the development of SC-based analgesic therapies, and this review will approach all of these aspects.

Key Words: Inflammation; Neuropathy; Nociceptive; Pain; Pain treatment; Stem cells

ANEXO C
Artigos em colaboração

Inflammopharmacology
https://doi.org/10.1007/s10787-025-01762-6

Inflammopharmacology

ORIGINAL ARTICLE

Role of TRPV1$^+$ and TRPA1$^+$ nociceptive neurons in delayed-onset muscle soreness: inhibition by hesperidin methyl chalcone

Giovana B. Cortez[1,2] · Mariana M. Bertozzi[2] · Amanda M. Dionisio[1,2] · Maiara Piva[2] · Nayara R. Morelli[2] · Thacyana T. Carvalho[2] · Rubia Casagrande[1] · Waldiceu A. Verri Jr[1,2] · Sergio M. Borghi[1,2,3]

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Abstract

Objective Delayed-onset muscle soreness (DOMS) is a type of pain caused by muscle injury provoked by eccentric, high intensity, or long-duration exercise. Hesperidin methyl chalcone (HMC) is a flavonoid with analgesic and anti-inflammatory actions. We investigated the effects of HMC against DOMS.

Methods Mice received AMG9810 (100 nmol) or HC-030031 (10 µg) once intrathecally, or HMC twice (12 h plus 30 min before) intraperitoneally (1, 3, or 10 mg/kg) and were subjected to a single uninterrupted acute swimming session of 120 min to induce DOMS. Sham animals were subjected to swimming just for 30 s, and naïve mice were not exposed to water. Calcium imaging of dorsal root ganglia (DRG) neurons was used to assess nociceptive neuron activation. Muscle mechanical hyperalgesia was assessed 12–48 h later. Oxidative parameters (superoxide anion, lipid peroxidation, and antioxidant activity) and leukocyte recruitment (macrophages and neutrophils) were evaluated 2 and 24 h later, respectively.

Results DRG neurons from mice that underwent intense acute swimming showed higher levels of calcium at 24 h post-session relative to naïve mice. Capsaicin [transient receptor potential vanilloid 1 (TRPV1 agonist)] or AITC [transient receptor potential ankyrin 1 (TRPA1 agonist)] were used as agonists controls to identify the populations of responsive neurons positive for TRPV1/A1. KCl was used as a cell viability control. Counterproof pharmacologic functional tests targeting TRPV1 or TRPA1 with receptor antagonists reduce muscle mechanical hyperalgesia and DRG neuron increased activity. HMC (3 mg/kg) reduced muscle mechanical hyperalgesia, activation of DRG nociceptive neurons at 24 h post-swimming session and upon TRPV1 or TRPA1 agonists and inhibited oxidative stress and the recruitment of neutrophils and macrophages to muscle in DOMS mice.

Conclusions Thus, HMC prevented DOMS in mice caused by unaccustomed exercise. The underlying mechanisms of HMC involve targeting oxidative stress, inflammation, and reduced activity of TRPV1$^+$ and TRPA1$^+$ nociceptive neurons.

Keywords Hesperidin methyl chalcone · Delayed-onset muscle soreness · Intense acute swimming · Oxidative stress · And Leukocytes

✉ Waldiceu A. Verri Jr
waldiceujr@yahoo.com.br

✉ Sergio M. Borghi
sergio_borghi@yahoo.com.br

[1] Center of Health Science, Postgraduate Program in Health Sciences, State University of Londrina, Londrina, PR 86038-350, Brazil

[2] Department of Immunology, Parasitology and General Pathology, Center for Biological Sciences, State University of Londrina, Rod. Celso Garcia Cid, Pr 445, KM 380, P.O. box: 10.011, Londrina, PR 86057-970, Brazil

[3] Center for Research in Health Sciences, University of Northern Paraná, Londrina, PR 86041-140, Brazil

Abbreviations

AITC	Allyl isothiocyanate
ANOVA	Analysis of variance
ARE	Antioxidant responsive elements
CPK	Creatine phosphokinase
CCR2	C-C motif chemokine receptor 2
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxyde
DOMS	Delayed onset muscle soreness
DRG	Dorsal root ganglia
eGFP	Enhanced green fluorescent protein
FRAP	Ferric reducing ability potential
GDNF	Glial cell line-derived neurotrophic factor

Published online: 26 April 2025

ANEXO C

Artigos em colaboração

Journal of Neurochemistry JNC The Official Journal of the International Society for Neurochemistry

ORIGINAL ARTICLE

Perineuronal net in the extrinsic innervation of the distal colon of mice and its remodeling in ulcerative colitis

Matheus Deroco Veloso da Silva, Larissa da Silva Bonassa, Maiara Piva, Camila Regina Basso, Tiago Henrique Zaninelli, Camila Cristina Alves Machado, Fábio Goulart de Andrade, Carlos Alberto Miqueloto, Debora de Mello Gonçales Sant´Ana, Rubina Aktar, Madusha Peiris, Qasim Aziz, L. Ashley Blackshaw, Waldiceu A. Verri, Eduardo José de Almeida Araújo
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