NAYARA ANITELLI ARTERO

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EFEITO ANALGÉSICO E ANTI-INFLAMATÓRIO DA RESOLVINA D1 E RESOLVINA D2 EM CAMUNDONGOS

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Orientador: Prof. Drº Waldiceu Aparecido Verri Júnior

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Londrina, 24 de fevereiro de 2023.

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Carl Sagan

RESUMO

ARTERO, Nayara Anitelli. **Mediadores lipídicos pró-resolução em modelo de artrite crônica induzida por dióxido de titânio e em modelo agudo induzido por ânion superóxido:** efeito analgésico e anti-inflamatório da resolvina D1 e resolvina D2 em camundongos. 2023. 93 f. Tese (Doutorado em Ciências da Saúde) - Universidade Estadual de Londrina, Londrina, 2023.

A artrite é definida como doença crônica que pode vir a afetar as articulações. Acompanha formação de edema articular, dor, rigidez e deformidades. Diversos fatores podem induzir artrite como por exemplo o dióxido de titânio presente nas próteses articulares. Espécies reativas de oxigênio podem induzir processo inflamatório e dor através da estimulação de mediadores inflamatórios e sensibilização dos neurônios via ativação de canais iônicos, como receptor de potencial transitório vanilóide subtipo 1 (TRPV1). Os mediadores lipídicos especializados pró-resolução (MLPRs) são uma classe parcialmente definida por sua função de limitar o acúmulo de neutrófilos no tecido, regular citocinas pró-inflamatórias e estimular a fagocitose dos macrófagos. Atuam no desfecho do processo inflamatório, através de ações resolutivas, estimulando a liberação de óxido nítrico endotelial. Portanto, esse trabalho demonstrou os efeitos analgésicos e anti-inflamatórios da RvD1 e RvD2 em modelo de artrite crônica induzida por TiO2 e em modelo agudo induzido por KO2. No modelo de TiO2, os camundongos Swiss machos (n=6 por grupo), foram estimulados com injeção intra-articular de 3 mg/ articulação de TiO2 para induzir artrite crônica e 24 horas depois foram tratados com RvD1 e RvD2 (1, 3, 10ng/ animal intraperitoneal [i.p]). A RvD1 na dose de 3 ng diminuiu a hiperalgesia mecânica e térmica, edema articular induzido e o recrutamento de leucócitos para a cavidade intra-articular. O tratamento com RvD1 reduziu o desequilíbrio das patas traseiras através do static weight bearing (SWB) e não apresentou danos toxicológicos no tratamento por 30 dias. A RvD2 na dose de 10 ng, diminuiu a hiperalgesia mecânica e térmica, o edema articular, danos histológicos e a migração induzida por TiO2. Reduziu o desequilíbrio das patas traseiras através do SWB, demonstrando sua ação analgésica. Para o modelo de KO2, utilizamos camundongos tratados com 3 doses de RvD2 [1, 3, 10ng/animal, i.p] ou veículo [1% etanol em salina, i.p], 30 minutos antes do estímulo inflamatório com KO2 [30 µg/animal, intraplantar (i.pl.)]. A hiperalgesia mecânica foi determinada por versão eletrônica. A hiperalgesia térmica foi através do teste de Hargreaves e placa quente. A dor manifesta foi avaliada pelo número de contorções abdominais, sacudidas da pata (flinches) e tempo de lambida expressos ao longo de 20 e 30 minutos. A migração leucocitária foi determinada a partir da contagem das células presentes no tecido plantar dos animais 7h após o estímulo. A presença da citocina IL-1b foi avaliada por ELISA. O tratamento com RvD2 foi capaz de diminuir hiperalgesia mecânica apresentando analgesia em todos os tempos, além de diminuir a hiperalgesia térmica.Houve redução nos comportamentos de dor espontânea como número de contorcções abdominais e sacudidas de patas, redução no tempo de lambida de pata. Ademais, observamos redução na produção da citocina IL-1b e o recrutamento de leucócitos no tecido plantar induzidos pelo KO2. Portanto a RvD1 e RvD2 são abordagens terapêuticas interessantes no contexto da dor articular crônica e inflamação aguda por KO2, demonstrando potencial efeito analgésico e anti-inflamatório em ambos os modelos.

Palavras-chave: Inflamação; dor; lipídeo; prótese; ânion superóxido.

ABSTRACT

ARTERO, Nayara Anitelli. **Proresolution lipid mediators in a chronic arthritis model induced by titanium dioxide and in an acute model induced by superoxide anion:** analgesic and anti-inflammatory effect of Resolven D1 and Resolven D2 in mice. 2023. 93 p. Thesis (Doctorate in Health Sciences) - State University of Londrina, Londrina, 2023.

Arthritis is defined as a chronic disease that can affect the joints. It accompanies formation of joint edema, pain, stiffness and deformities. Several factors can induce arthritis, such as the titanium dioxide present in joint prostheses. Reactive oxygen species can induce inflammation and pain by stimulating inflammatory mediators and sensitization of neurons via activation of ion channels, such as transient receptor potential vanilloid subtype 1 (TRPV1). Specialized pro-resolving lipid mediators (MLPRs) are a class partially defined by their role in limiting tissue neutrophil accumulation, regulating pro-inflammatory cytokines, and stimulating macrophage phagocytosis. They act in the outcome of the inflammatory process, through resolving actions, stimulating the release of endothelial nitric oxide. Therefore, this work demonstrated the analgesic and anti-inflammatory effects of RvD1 and RvD2 in a model of chronic arthritis induced by TiO2 and in an acute model induced by KO2. In the TiO2 model, male Swiss mice (n=6 per group) were stimulated with an intra-articular injection of 3 mg/joint of TiO2 to induce chronic arthritis and 24 hours later were treated with RvD1 and RvD2 (1, 3, 10ng/animal intraperitoneal [i.p]). RvD1 at a dose of 3 ng decreased mechanical and thermal hyperalgesia, induced joint edema and leukocyte recruitment to the intra-articular cavity. Treatment with RvD1 reduced the imbalance of the hind legs through static weight bearing (SWB) and showed no toxicological damage in the treatment for 30 days. RvD2 at a dose of 10 ng decreased mechanical and thermal hyperalgesia, joint swelling, histological damage and migration induced by TiO2. It reduced the imbalance of the hind legs through SWB, demonstrating its analgesic action. For the KO2 model, we used mice treated with 3 doses of RvD2 [1, 3, 10ng/animal, i.p] or vehicle [1% ethanol in saline, i.p], 30 minutes before the inflammatory stimulus with KO2 [30 µg/animal , intraplantar (i.pl.)]. Mechanical hyperalgesia was determined by electronic version. Thermal hyperalgesia was assessed using the Hargreaves and hot plate test. Manifest pain was assessed by the number of abdominal contortions, paw flicks (flinches) and licking time expressed over 20 and 30 minutes. the stimulus. The presence of the cytokine IL-1b was evaluated by ELISA. Treatment with RvD2 was able to decrease mechanical hyperalgesia, presenting analgesia at all times, in addition to decreasing thermal hyperalgesia. There was a reduction in spontaneous pain behaviors such as number of abdominal contortions and paw shaking, reduction in paw licking time. Furthermore, we observed a reduction in the production of the cytokine IL-1b and the recruitment of leukocytes in the plantar tissue induced by KO2. Therefore, RvD1 and RvD2 are interesting therapeutic approaches in the context of chronic joint pain and acute inflammation by KO2, demonstrating potential analgesic and anti-inflammatory effects in both models.

Key words: inflammation; pain; lipid; prosthesis; superoxide anion.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

AR	Artrite reumatóide
HPETE	Ácido12-hidroperoxiéicosatetraenóico
KO_2	Superóxido de potássio
AINES	Anti-inflamatórios não esteróides
DHA	Ácido docosahexaenóico
EPA	Ácido eicosapentaenóico
CEUA	Comissão de Ética no Uso de Animais
TiO_2	Dióxido de titânio
EROs	Espécies reativas de oxigênio
ERNs	Espécies reativas de nitrogênio
NADPH	Fosfato de dinucleotídeo de nicotinamida e adenina
TNF-α	Fator de necrose tumoral alfa
GPX	Glutationa peroxidase
GO	Glutationa oxidase
IP	Intraperitoneal
MLPRs	Mediadores lipídicos especializados pró-resolução
MPO	Mieloperoxidase
NPs	Nanopartículas
NO	Óxido nítrico
PAMPs	Padrões Moleculares Associados à Patógenos
DAMPs	Padrões Moleculares Associados à Danos
RRPs	Receptores de Reconhecimento de Padrões
TLRs	Receptores do Tipo Toll
NLRs	Receptores do Tipo Nod
RvDs	Resolvinas
SOD	Superóxido dismutase
SNC	Sistema nervoso central
NaCl	Cloreto de sódio

SUMÁRIO

1.INTRODUÇÃO

A inflamação é uma resposta fisiológica protetora do organismo, a partir da ativação do sistema imune frente a uma variedade de estímulos, incluindo lesões teciduais e infecções (1,2). A resposta inflamatória controlada é um evento favorável (na proteção contra infecções ou inflamações por trauma, por exemplo), porém quando exacerbada pode tornar-se prejudicial ao organismo. Sendo assim, o estado inflamatório patológico é assumido (3). As consequências associadas à inflamação tais como recrutamento de leucócitos, edema e dor são importantes para proteção dos tecidos, porém a inflamação excessiva e contínua leva ao dano tecidual, dor crônica e disfunção orgânica. Sendo assim, o conhecimento dos mecanismos de inflamação é primordial para tratar doenças inflamatórias, promover a proteção do hospedeiro e a homeostase(4).

A dor é um sintoma presente na maioria das doenças inflamatórias, podendo até mesmo levar ao desenvolvimento da depressão ou perda da função de tecidos ou órgãos afetados. (5). A dor inflamatória, por exemplo, se origina quando ocorre interação entre o tecido lesado e os neurônios sensoriais periféricos (por exemplo, na dor aguda ou articular) (6), (7).

O estresse oxidativo é um desequilíbrio entre a produção de radicais livres e metabolitos reativos, os chamados oxidantes ou espécies reativas de oxigênio (EROs), e sua eliminação é realizada através de mecanismos de proteção conhecidos como antioxidantes (8). As EROs também são produzidas no local da inflamação, o que contribui para a manifestação da dor por interagirem diretamente com neurônios nociceptores (9). Um exemplo conhecido de EROs é o ânion superóxido, que pode ser gerado pelo fosfato de dinucleotídeo de nicotinamida e adenina (NADPH) oxidase (10). É uma forma comum de ERO e pode vir a gerar nocicepção através da via de sinalização conhecida como NFKB, aumentando consequentemente os mediadores inflamatórios naquele organismo (11). Outro processo doloroso são as lesões articulares por exemplo, que podem ser causadas por uma série de fatores, contendo uma inflamação no local que irá evoluir para um processo crônico, sendo seguida pela evolução da dor.

Mesmo com os medicamentos existentes direcionados para tentar armenizar e diminuir a dor, ainda apresentam efeitos adversos maléficos que dificultam o tratamento, levando a progressão da doença e da dor. O controle da dor e da inflamação necessita de melhor visibilidade na área da saúde, pois, é um agravante da população que necessita de melhora para voltar a ter uma qualidade de vida mais digna. Sendo assim, a dor e inflamação se enquadra na grande lista de problemas de saúde pública, já que pode levar a diversos quadros negativos quando não controlada corretamente.

1.1 REVISÃO DE LITERATURA

1.2 Inflamação

A resposta inflamatória é uma rápida reação fisiológica do organismo frente a um dano tecidual como uma invasão microbiana, viral ou até mesmo um trauma. Geralmente ocorre de forma competente para eliminar os agentes nocivos e remover tecidos mortos gerados após uma resposta eficaz. Respostas inflamatórias agudas descontroladas, ou episódios recorrentes de inflamação aguda sobreposta à inflamação crônica, estão associadas a danos nos tecidos que podem causar desconforto e comprometer severamente a função normal dos tecidos (12).

A invasão de um determinado patógeno ou um dano celular podem ser reconhecidos através de mecanismos da resposta imune inata. O sistema imunológico apresenta os denominados Receptores de Reconhecimento de Padrões (RRPs), que reconhecem Padrões Moleculares Associados à Patógenos (PAMPs) (presentes em patógenos) e/ou Padrões Moleculares Associados à Danos (DAMPs) (resultantes de alterações ou danos em moléculas do organismo), os quais são ativados, resultando em vários eventos relacionados à imunidade inata e adaptativa: como a ativação de vias transcricionais que resultarão na produção de mediadores pró-inflamatórios; na indução do recrutamento leucocitário; na fagocitose e atividade microbicida feita pelos fagócitos; na produção de anticorpos e como último exemplo, na geração de células imunes de memória (13). Em relação aos fatores de transcrição, tem-se a ativação do fator nuclear κB (NF-κB) que induz a produção de diversas citocinas inflamatórias como fator de necrose tumoral α (TNF-α), interleucina 1β (IL-1β) e 6 (IL-6) (14,15).

Após o reconhecimento do ataque alterações vasculares e celulares ocorrem para que a eliminação do agente agressor ocorra. Estas alterações foram propostas pelo romano Aulus Cornelius Celsus no século I d.C., o qual foi o primeiro a descrever os quatro principais sinais de inflamação: rubor (vermelhidão), tumor (inchaço), calor e dor (16). Rudolf Virchow propôs um

quinto sinal do processo inflamatório conhecido como perda de função (functio laesa), que seria decorrente de um processo inflamatório mais prolongado (17).

De fato, os efeitos associados à inflamação como edema, recrutamento de leucócitos e dor são importantes para proteção dos tecidos, porém a inflamação exacerbada e contínua leva ao dano tecidual, dor crônica e disfunção do órgão. Portanto, o conhecimento dos mecanismos de inflamação é essencial para tratar doenças inflamatórias, promover a proteção do hospedeiro e a homeostase (18). Logo no início do processo inflamatório, cerca de 6 a 24 horas após estímulo nocivo, as células que predominam no local são os neutrófilos, pois já estão presentes na corrente sanguínea e respondem as quimiocinas que ajudam na migração desses neutrófilos pelo endotélio vascular (19). Em 24 a 48 horas, o tipo celular que predomina naquele local será o monócito, que após a circulação no sangue até o tecido por diapedese, vai se diferenciar em macrófago que possuirá fenótipos diferentes, dependendo da fase inflamatória que estiver. Por exemplo, na fase de início encontram-se os macrófagos M1, com perfil pró-inflamatório. Já na fase resolutiva encontram-se os do tipo M2, caracterizado por seu perfil anti-inflamatório e de resolução (20, 21).

O processo inflamatório pode ser dividido em dois tipos, o agudo e/ou crônico. A inflamação aguda normalmente é causada por uma infecção viral ou bacteriana, traumas, necrose no tecido, queimaduras ou qualquer material estranho presente no órgão ou tecido. O sistema imune inato é ativado na tentativa de combater o agente agressor (22). No local, a inflamação aguda é caracterizada por promover dilatação e aumento da permeabilidade vascular, acompanhada do aumento do fluxo sanguíneo, resultando no recrutamento de leucócitos, incluindo neutrófilos, monócitos, macrófagos e linfócitos (23). Os mediadores químicos possuem papel extremamente importante durante todo o percurso do processo inflamatório, sendo eles os produzidos por células e aqueles que são produzidos a partir de elementos presentes no plasma. Os mediadores derivados da atividade celular são: serotonina, histamina, citocinas, quimiocinas, óxido nítrico (NO) e mediadores lipídicos (prostanóides, leucotrienos, fator de ativação plaquetária, mediadores como lipoxinas (24) e resolvinas. Aqueles derivados de componentes presentes no plasma incluem

bradicinina, membros do sistema complemento e da cascata de coagulação) (17).

Outros compostos como as espécies reativas de oxigênio (EROs) são produzidas durante o processo de fagocitose pelos macrófagos e neutrófilos. Isso ocorre após à ativação da NADPH oxidase, que vai reduzir o O2, formando o anion superoxido (O2•-), fazendo com que forme outras EROs através da dismutação espontânea, reagindo com as espécies reativas de nitrogênio (ERNs) ou enzimas presentes nos neutrófilos, como por exemplo a mieloperoxidase (MPO). Consequentemente, os agentes nocivos são eliminados no fagolisossomo das células fagocíticas e as EROs podem ser controladas pelos sistemas enzimáticos endógenos, como a catalase, superóxido dismutase (SOD), glutationa peroxidase (GPX) e glutationa oxidase (GO) (17). As EROs possuem papel importante na inflamação, pois a produção desses radicais livres ajuda a potencializar o processo inflamatório, levando a ativação de vias de sinalização como NF-KB, levando também a ativação de fatores de transcrição, promovendo danos as membranas lipídicas ou material genético (25-28).

A inflamação crônica é uma inflamação de duração prolongada (semanas ou meses) em que a lesão tecidual, a persistência do agente agressor e as tentativas de reparo coexistem em diferentes combinações. Pode seguir a partir da inflamação aguda ou pode começar de forma insidiosa, como uma resposta de baixo grau sem qualquer manifestação de reação aguda cronologicamente anterior (29). Diversas doenças são bem caracterizadas por possuir um perfil crônico, como por exemplo a artrite, doenças autoimunes, doenças intestinais, infecções bacterianas e doenças neurodegenerativas (30). A inflamação crônica é resultado de um dano tecidual ainda maior, remodelação tecidual ineficiente e/ ou cicatrização tecidual inadequada, como por exemplo através da deposição excessiva de matriz extracelular. A prolongação da inflamação acontece devida a produção tardia, insuficiente ou até mesmo inexistente dos mediadores lipídicos pró-resolução (MLPR) que dificulta a resolução do processo inflamatório (31).

A inflamação excessiva é amplamente reconhecida como um componente unificador em muitas doenças crônicas, incluindo doenças como síndrome metabólica e doenças neurológicas e, portanto, é uma preocupação de saúde

pública (32).

Os patologistas dividem a resposta inflamatória aguda em iniciação e resolução (32) A resolução se dá por meio de sinais que em conjunto contribuem para reverter o acúmulo de granulócitos no sítio inflamatório e reprogramar o tecido nos níveis celular e molecular, levando a um estado de “homeostase adaptada” (32).

Os MLPR são produtos da transformação dos ácidos graxos essenciais ômega-6 e ômega-3, como ácido araquidônico, ácido eicosapentaenóico (EPA) e ácido docosaexaenóico (DHA). São subdivididos nas famílias das lipoxinas (única proveniente do ômega-6), resolvinas (Rv), protectinas e maresinas (MaR). Desempenham um papel extremamente importante na resolução do processo inflamatório e também estão envolvidos na defesa do hospedeiro contra microrganismos e lesões teciduais, bem como no remodelamento tecidual (33).

1.3 Dor inflamatória

Um importante mecanismo que serve como alerta ao indivíduo frente uma lesão ou trauma ao seu organismo é a dor (34). A Associação Internacional para o Estudo da Dor (IASP) define a dor como: “uma experiência sensorial e emocional desagradável que é associada a lesões reais ou potenciais. A dor é subjetiva, pois cada indivíduo aprende a utilizar este termo por meio de suas experiências” (35). A dor é um sintoma presente na maioria das doenças inflamatórias, podendo até mesmo levar ao desenvolvimento da depressão ou perda da função de tecidos ou órgãos afetados.

As vias sensoriais são formadas por uma cadeia neuronal específica constituídas pelo neurônio de 1ª ordem originado na periferia, responsável por detectar o estímulo e transmitir o impulso em direção ao Sistema Nervoso Central (SNC). Já o neurônio de 2ª ordem está localizado no corno dorsal da medula espinhal que propagará o impulso; e o neurônio de 3ª ordem está presente no tálamo, que se projeta ao córtex somatossensorial no cérebro a informação dolorosa (46).

A percepção da dor está intimamente ligada à inflamação, uma resposta biológica complexa do sistema somatossensorial, juntamente com o sistema imunológico, neuronal, autônomo e circulatório a injúrias nos tecidos por patógenos ou traumas (36).

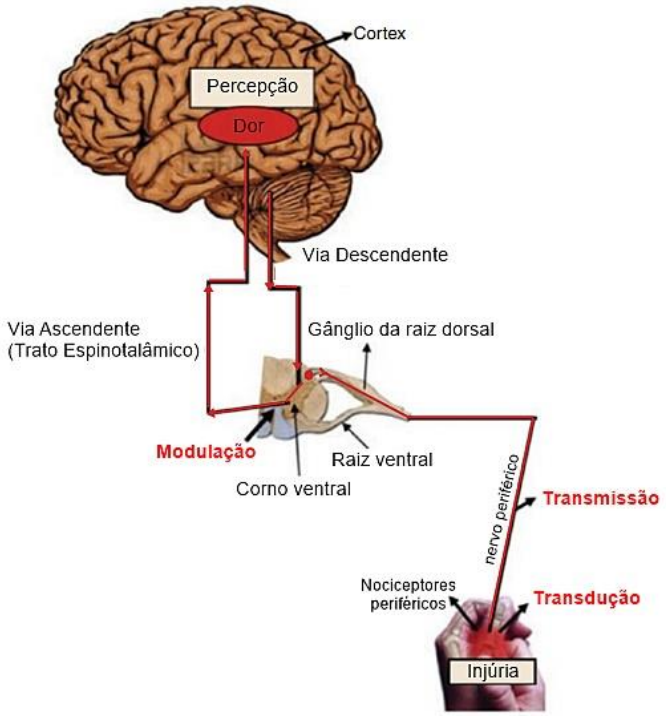
A identificação dos estímulos em diferentes intensidades e proporções ocorre por estimulação de receptores periféricos específicos, conhecidos como os nociceptores (37,38). Os neurônios nociceptores em sua maioria são os do tipo fibra Aδ, que são neurônios mielinizados e de condução mais rápida, respondendo principalmente a sensibilidade à dor mecânica, e C não-mielinizados e de condução lenta, respondendo especialmente a sensibilidade à dor térmica (39) entre outros estímulos.

A classificação da dor pode ser feita de acordo com seus mecanismos patofisiológicos. Tal como a dor nociceptiva e inflamatória, que tem papel adaptativo protetor. A dor nociceptiva tende a eliminar o agente nocivo. Por exemplo, uma fonte de calor induz um reflexo motor de retirada do elemento acometido, protegendo assim o indivíduo de um dano tecidual pela temperatura extrema. A dor inflamatória ocorre pela interação entre o tecido lesionado, que produz mediadores inflamatórios com neurônios sensoriais periféricos que inervam a região. Também ajuda na recuperação do indivíduo desencorajando o contato físico com a região ou até o movimento do membro lesionado. Em contrapartida, a dor neuropática é causada por uma lesão ao tecido nervoso periférico ou central e tem características mal-adptativas, o que gera um funcionamento anormal do sistema sensorial. Outro tipo de dor mal-adaptativa ocorre quando existe relato de dor pelo paciente, porém não existe mais dano ou processo inflamatório ativo, como ocorre na Artrite reumatóide (AR) (40,41).

O estímulo doloroso e nocivo é percebido por meio da ativação de diferentes tipos de receptores e canais iônicos presentes na terminação nervosa periférica do neurônio primário, como canais de potencial transitório (TRP), canais iônicos voltagem dependente, receptores de mediadores inflamatórios como de lipídeos e citocinas. Os canais do tipo TRP são ativados por diferentes tipos de estímulos como temperatura, agentes irritantes e ligantes endógenos. Por exemplo, o canal de potencial transitório da subfamília Vaniloide 1 (TRPV1)

é um canal de cálcio não-seletivo que é ativado pela capsaicina, um conhecido componente da pimenta, com temperatura nocivas (> 42ºC), baixo pH e ligantes endógenos como por exemplo, os leucotrienos derivados da ação da lipoxigenase sobre o ácido araquidônico. (43-45).

Figura 1: Sistema da percepção da dor pela da coluna dorsal



(RANG E DALE, 2016; FATTORI *et al.,* 2019; BADJWA e KALILI, 2019; GRUBB S., PASKANVAS G., 2019)

1.4 Modelo de dor inflamatória induzida por KO_2

Diversos mecanismos são capazes de manter a concentração de EROs dentro dos limites fisiológicos. O desbalanço entre o aumento de espécies reativas e a diminuição de defesas antioxidantes resultam no estresse oxidativo

(47). Os radicais livres como as espécies reativas de nitrogênio (ERNs) e oxigênio (EROs) exercem um importante papel no processo inflamatório. Um exemplo conhecido é o ânion superóxido, que pode ser gerado pelo fosfato de dinucleotídeo de nicotinamida e adenina (NADPH) oxidase (48). A primeira ERO a ser gerada pela NADPH oxidase é o ânion superóxido e sua regulação acontece através da enzima SOD. É uma forma comum de ERO e pode vir a gerar nocicepção através da via de sinalização conhecida como NFKB, aumentando consequentemente os mediadores inflamatórios naquele organismo (49).

O ânion superóxido (O_{2}^{-}) e seus derivados por exemplo, tem uma produção intimamente ligada a tolerância por diversos fatores como opioides, dor inflamatória, neurogênica e neuropática (50). Existe evidências de que um doador de ânion superóxido, o KO_2 induz hiperalgesia térmica através do teste de Hargreaves em 20 minutos e 60 minutos após injeção na pata (51,50). Produz hiperalgesia mecânica, térmica e comportamento de dor em modelo animal com camundongos (53).

Em nosso laboratório, já foi demonstrado através da padronização do modelo com KO_2 por Maioli e colaboradores (54). As respostas inflamatórias induzidas por KO_2 avaliadas em camundongos foram: hiperalgesia mecânica, hiperalgesia térmica, formação de edema de pata, atividade da mieloperoxidase, comportamentos evidentes do tipo dor, recrutamento de leucócitos, estresse oxidativo e expressão de mRNA da ciclooxigenase-2.

1.5 Modelo artrite induzida por dióxido de titânio (TiO_2)

A destruição das principais articulações responsáveis pelos movimentos de amplitude corporal é causada por diversos motivos, sendo eles de origem autoimune, inflamatória, traumática ou infecciosa, levando ao quadro conhecido como artrite. A partir dessa condição, pacientes podem vir a necessitar da substituição parcial ou total dessa articulação por algum tipo de prótese (55).

A definição da artrite é dada como uma doença aguda ou crônica que pode vir a afetar uma ou mais articulações. Esse quadro pode estar acompanhado de formação de edema articular, dor, rigidez e deformidades nas articulações. A destruição articular pode ocorrer de várias formas, como por exemplo: deposição de cristais (gota), alterações degenerativas (osteoartrite), autoimune como artrite reumatóide, anormalidades metabólicas como a hemocromatose, infecciosa como artrite séptica ou até mesmo defeitos congênitos na cartilagem ou tecido ósseo (56). Com um processo inflamatório progressivo combinado com essas outras condições, a dor, destruição da articulação e diminuição da função e consequentemente diminuição da qualidade de vida do paciente é inevitável (57).

A artroplastia é o nome dado ao procedimento cirúrgico que substitui totalmente ou parcialmente uma articulação. É um importante procedimento que pode vir a restaurar a qualidade de vida do paciente, aliviando a dor e restaurando a função articular que estava danificada. Mesmo com a importância da artroplastia, este procedimento pode falhar em 15% dos casos, chegando a 40% segundo estudos anteriores (58,59). O TiO_2 é destaque entre os biomateriais utilizados para o desenho de próteses. No entanto, esse agente pode causar inflamação articular e falha em próteses e implantes (60,61).

Um dos implantes mais utilizados no mundo para uso biomédico por ter excelentes propriedades como alta biocompatibilidade, grande resistência a tração, alta resistência a corrosão e flexibilidade é o titânio (62). O TiO_2 é um pó branco inodoro (63), geralmente usado em uma variedade de produtos como pinturas, revestimentos, cerâmicas, protetores solares e como aditivos em produtos farmacêuticos, corantes alimentares (64) e próteses articulares (65). Para ser considerado biocompatível, o biomaterial tem que realizar sua função sem desencadear qualquer efeito local ou sistêmico indesejado, gerando uma resposta celular ou tecidual mais favorável e apropriada ao paciente que se encontra naquele momento e melhorar o desempenho clínico dessa terapêutica em questão (66). No entanto, as propriedades que se encontram na superfície desse biomaterial não garantem uma fixação estável entre o implante e o tecido ao redor, o que pode ocasionar a formação de uma camada de fibrose, atrapalhando a transmissão de carga na interface do osso com o implante, onde

micros movimentos podem vir a proporcionar uma falha no implante colocado (67). O TiO_2 foi classificado pela Agência Internacional de Pesquisa sobre o câncer como um cancerígeno de grupo 2B (68). Outro instituto a classificar o TiO_2 como um produto possivelmente perigoso foi o Instituto Nacional de Segurança e saúde, que incluiu as nanopartículas de TiO_2 na lista de produtos com pontenciais cancerígenos ocupacionais (69). No tecido humano, o titânio acumulado é caracterizado por um pigmento preto acumulado no local, acompanhado por um processo fibroso, podendo progredir para uma necrose ou uma reação granulomatosa (70).

Gonçalves e Girard, mostraram que as nanopartículas (NP) de TiO_2 são capazes de atrair e consequentemente gerar influxo de neutrófilos, tal como levar à produção local de vários mediadores pró-inflamatórios, como diversas quimiocinas, como a MIP-1β, no modelo *in vivo* de bolsa de ar murino (71). Houve também uma comprovação de que as NP de TiO_2 de fato podem gerar o quadro de artrite, o que ocorreu em um caso clínico mostrado por Dorner e colaboradores (72) em 2006, onde uma paciente sem histórico de artrite familiar desenvolveu artrite relacionada a uma prótese de TiO_2 e vanádio. Em relação aos dados citados acima e outros mais encontrados na literatura, foi observado que as NP de TiO_2 podem sim levar a indução da citotoxidade, influxo de neutrófilos, inflamação crônica, necrose e formação de artrite. Em nosso laboratório, foi padronizado o modelo de artrite induzido por TiO_2, que mostrou induzir hiperalgesia articular, edema, estresse oxidativo, liberação de citocinas pró-inflamatórios – como TNF- α**,** IL- 1 β e IL-6 – e ativação da via de sinalização RANKL/RANK/OPG. Neste caso, pode ocorrer a destruição da cartilagem e o aumento na reabsorção óssea, levando à destruição articular (73).

Sendo assim, a artrite crônica induzida por TiO_2 é um modelo favorável para a pesquisa médica, proporcionando a compreensão dos mecanismos celulares e moleculares relacionado ao processo inflamatório asséptico mediado por partículas liberadas no espaço periprotético.

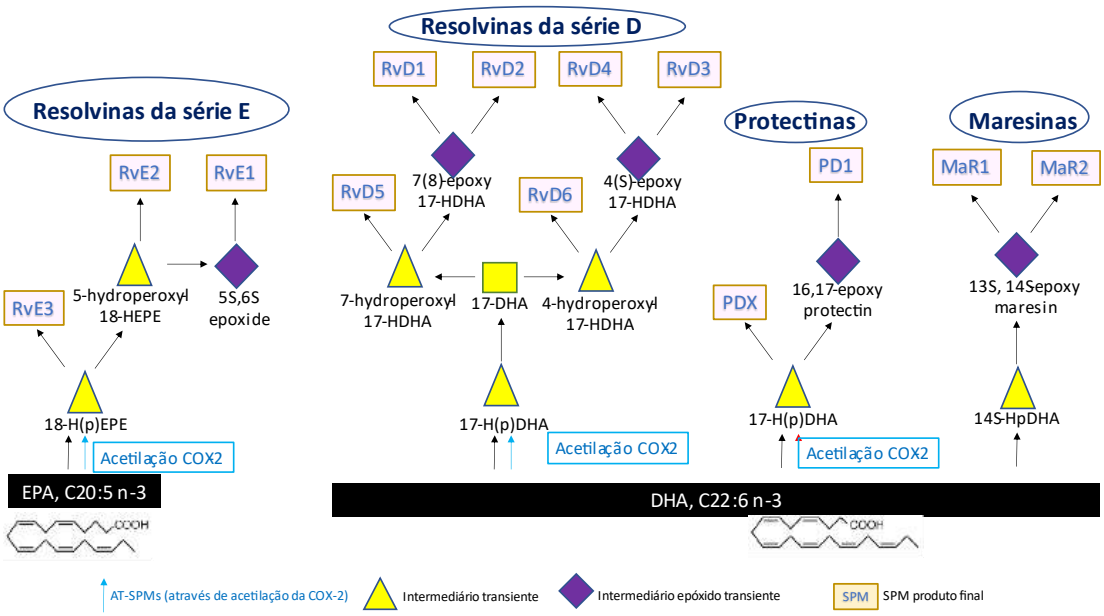
1.6 Mediadores lipídicos especializados pró-resolução (Resolvina D1 e D2)

Diversos fármacos são utilizados para o tratamento da inflamação e controlam pontos chaves, como a inibição de enzimas, inibição de efeitos pró-inflamatórios, bloqueio de receptores, entre outros. Os principais fármacos utilizados são os anti-inflamatórios não esteróides (AINES), o qual o mecanismo atua inibindo as isoformas da enzima cicloxigenase (COX 1 e 2) e também os glicocorticóides, que inibem a transcrição de genes pró-inflamatórios e induzem a transcrição de genes antiinflamatórios (74).

Os mediadores lipídicos especializados pró-resolução (MLPRs) são uma classe de mediadores, parcialmente definidos por sua função de limitar o acúmulo de neutrófilos no tecido, contrarregular citocinas pró-inflamatórias e estimular a fagocitose dos macrófagos. Durante o processo da eferocitose, os fagócitos geram os mediadores especializados pró-resolvinas (MLPRs) que ajudam a inibir a ativação neutrofílica, aumentando a expressão de células apoptóticas do receptor de quimiocina CC (CCR5) para depuração de quimiocina e consequentemente promover a morte bacteriana. Além dos fagócitos, as células linfóides tem funções vitais na defesa do organismo. Expressando receptores de MLPR, podendo servir como efetores celulares desses mediadores (75).

Os MLPRs atuam no desfecho do processo inflamatório, através de ações resolutivas, estimulando a liberação de óxido nítrico endotelial e prostaciclina, aumentando, portanto, a fagocitose dos macrófagos, eferocitose, depuração microbiana e efluxo dos leucócitos para os linfonodos. Também desempenham papel fundamental na defesa do organismo e consequentemente na remodelação do tecido (76,32). Durante a fase de recuperação da inflamação, foram identificadas novas famílias de mediadores lipídicos antiinflamatórios endógenos como as resolvinas (RvDs) e lipoxinas. As RvDs são classificadas nas séries D ou E e são derivadas dos ω-3-PUFAs ácido docosahexaenóico (DHA) e ácido eicosapentaenóico (EPA), respectivamente. Já as lipoxinas são derivadas do ω-6-PUFA ácido araquidônico (AA) (77).

Figura 2: Biossíntese da produção dos mediadores lipídicos pró resolução Resolvinas da série D.



Modificado de: O mediador lipídico pró-resolução Maresina-1 inibe neurônios CGRP-positivos: controle da dor inflamatória e alternativa não hormonal para tratamento da endometriose (Victor Fattori).

O grupo D das Resolvinas é composto por uma série de metabólitos da lipoxigenase do DHA, nos quais as RvD1 e RvD2 vem se tornando mais conhecida na literatura. A 15-lipoxigenase (ALOX15) ou ciclooxigenase-2 (COX-2) é modificada juntamente com a aspirina (COX-2) e converte DHA em 17-hidroxi DHA (17-HDHA) e depois 5-lipoxigenase (ALOX5) metabolizam 17-HDHA em RvD1, RvD2, RvD3 e RvD4 (53). As Resolvinas desse estudo são RvD1 e RvD2, que apresentaram diversos efeitos benéficos em estudos diferentes, como reduzir a inflamação e o estresse oxidativo nos danos da retina em paciente com diabetes mellitus (78), redução do processo inflamatório e infiltrado de células inflamatórias, promoveu a calcificação ao redor do ápice radicular e cicatrização de lesões ósseas periapicais (79). Demonstrou papel resolutivo na inflamação causada por queimaduras em modelo de ratos Wistar, suprimindo a necrose secundária, apresentado por Inoue e colaboradores (80). As ações eficientes in vivo de RvD1 e RvD2 são demonstradas em muitas outras doenças, como em vias aéreas (76), sistemas dérmico, renal e ocular, e em processos que incluem dor, fibrose e cicatrização de feridas (81,82).

Os efeitos do RvD1 na inflamação crônica doença também têm atraído a atenção nos últimos anos. Lima-Garcia e colaboradores demonstraram que o RvD1 pode aliviar a dor na artrite induzida por adjuvante em ratos (83). Sun e

colaboradores também relataram que o RvD1 pode suprimir a formação de pannus na artrite reumatóide. Em um estudo com camundongos propensos a diabetes, a RvD1 diminuiu o acúmulo de macrófagos no tecido adiposo, regulando a produção de citocinas e melhorando a sensibilidade à insulina (84). Trabalhos recentes demonstraram que a RvD1 possui atividades analgésicas quando administrado localmente ou sistemicamente em vários modelos de dor animal. A RvD1 reduziu a dor na mesma proporção que a morfina e os inibidores de COX-2 (nomeadamente NS-398) em doses muito mais baixas e não alterou a nocicepção basal, ao contrário do anestésico (por exemplo, lidocaína) comumente usada para controlar a dor durante a cirurgia (85,86).

A RvD2 possui diversos achados na literatura mostrando seu potencial grande efeito anti-inflamatório e analgésico. Usando diferentes modelos de distrofia muscular de Duchenne em camundongos, Dort e colaboradores demonstraram que a Resolvina D2 atinge tanto a inflamação e miogênese levando ao aumento da função muscular em comparação com os glicocorticóides. Em comparação, foi demonstrado que esse lipídeo possui melhor função terapeutica que a utilizada até hoje como padrão ouro para essa patologia. No estudo de Suzuki e colaboradores, foi examinado o efeito antidepressivo de RvD2 no comportamento semelhante à depressão induzida por dor crônica. O efeito antidepressivo de RvD2 foi examinado usando o teste de suspensão da cauda. A dor crônica e o quadro semelhante à depressão foi atenuado pela injeção intracerebroventricular de RvD2 (10 ng) (87).

O tratamento com RvD1 e RvD2 diminuiu respostas inflamatórias em modelo de fibrose cística, reduzindo significativamente a liberação de quimiocinas e citocinas selecionadas, incluindo IL-8 e TNF-α (88). Uma vez que lipídeos regulam potentemente mecanismos e mediadores, foi de grande interesse investigar se este SPM poderia controlar a dor crônica associada à inflamação.

Sendo assim, esse trabalho teve como papel, demonstrar os efeitos analgésico e antiinflamatório da RvD1 e RvD2 em modelo de artrite crônica induzida por TiO_2 e em modelo agudo induzido por KO_2 em camundongos, como nunca demonstrado antes na literatura.

2. OBJETIVOS

2.1 Objetivo geral

Avaliar o efeito analgésico e antiinflamatório da RvD1 e RvD2 na dor articular por meio do modelo de artrite crônica induzida por TiO_2 e o efeito analgésico e anti-inflamatório da RvD2 no modelo de inflamação aguda por KO_2 em camundongos.

2.2 Objetivos Específicos Do Modelo De Artrite crônica induzida por TiO_2

- Avaliar o efeito analgésico da RvD1 e RvD2 através da hiperalgesia mecânica;

- Avaliar o efeito anti-inflamatório da RvD1 e RvD2 na formação de edema articular;

- Avaliar o efeito analgésico da RvD1 e RvD2 através da hiperalgesia térmica;

- Avaliar o efeito analgésico da RvD1 e RvD2 na distribuição estática do peso corpóreo nas patas traseiras;

- Avaliar o efeito anti-inflamatório da RvD1 e RvD2 na migração leucocitária;

- Avaliar o efeito anti-inflamatório da RvD2 nos danos histopatológicos e infiltrado leucocitário na artrite crônica induzida por TiO_2;

- Avaliar os danos toxicológicos da RvD1 no tratamento durante 30 dias.

2.3 Objetivos Específicos Do Modelo agudo induzido por KO_2

- Avaliar o efeito analgésico da RvD2 através da hiperalgesia mecânica;

- Avaliar o efeito analgésico da RvD2 através da hiperalgesia térmica;

- Avaliar o efeito analgésico da RvD2 na distribuição estática do peso corpóreo nas patas traseiras;

- Avaliar o efeito analgésico da RvD2 na dor manifesta induzida por KO_2.

- Avaliar o efeito da citocina IL- 1 β.

3. MATERIAIS E MÉTODOS

3.1 Animais

Para os experimentos, foram utilizados camundongos Swiss machos, pesando de 20-25g. Os animais foram provenientes do Biotério Central da Universidade Estadual de Londrina (UEL) e foram mantidos no Biotério do Departamento de Ciências Patológicas da Universidade Estadual de Londrina por pelo menos dois dias antes dos experimentos, onde foi utilizado o ciclo de claro/escuro (12/12 h) e temperatura controlada (21°). Os animais foram divididos em gaiolas de polipropileno padrão, com a medida de 41 X 34 X 16 CM (Insight®) no biotério de acordo com os grupos experimentais (máximo de 12 animais por gaiola), com livre acesso à ração e água, foram ambientados nas condições experimentais com pelo menos 1 hora de antecedência em relação aos experimentos. Os procedimentos de cuidado e manuseio de animais estão todos de acordo com as diretrizes da Associação Internacional de Estudo da Dor (IASP). O projeto de pesquisa foi registrado sob número 11147.2016.40 (TiO_2) e 003.2021 (KO_2), aprovado pela Comissão de Ética no Uso de Animais (CEUA) da UEL.

3.2 Drogas e Reagentes

Os fármacos e reagentes utilizados no presente estudo foram: RvD1 e RvD2 (Cayman Chemical, MI, USA); solução salina (NaCl 0,9%) proveniente de Frenesius Kabi Brasil Ltda (Aquiraz, CE, Brasil); isoflurano proveniente de Abbott Laboratories (Abbott Park, IL, EUA); álcool absoluto (Dinâmica Química Contemporânea - São Paulo), superóxido de potássio (KO_2) 96,5% da Alfa Aesar (Ward Hill, MA, USA). O dióxido de titânio (TiO_2) foi adquirido da Synth (Diadema, SP, Brasil) e o tamanho da partícula foi <1 μm com uma média de 862,2 nm, conforme determinado pela análise de distribuição de tamanho (Malvern Instruments Ltd, UK).

3.3 Protocolos experimentais

3.3.1 Modelo de artrite crônica induzida por TiO_2

A primeira etapa experimental foi realizada com os animais (n = 6 por grupo de experimento) submetidos a uma curva de dose-resposta com o tratamento com os lipídeos RvD1 e RvD2. Os animais foram estimulados com injeção intra-articular (i.a) com 3 mg de TiO_2 suspenso em 10 μL de solução salina estéril 0,9% por articulação

femuro-tibial de acordo com padronização de Borghi e colaboradores (61). A hiperalgesia mecânica e edema foram avaliados 24 h após injeção de TiO_2 para avaliação da indução do modelo de artrite crônica. Logo após 24 h, os animais foram tratados com RvD1 e RvD2 nas doses de 1, 3, ou 10 ng/animal via intraperitoneal (i.p) e a hiperalgesia mecânica e o edema foram avaliados a partir de 1, 3, 5, 7 e 24 horas após o tratamento com os lipídeos no primeiro dia. Posteriormente, a hiperalgesia mecânica e o edema foram avaliados a cada 2 dias após o tratamento com RvD1 e RvD2 até o 30º dia. Imediatamente após as medidas no 30º dia, os animais foram anestesiados e eutanasiados, e o lavado articular do joelho foi coletado para a avaliar a migração de leucócitos. A dose mais eficaz da RvD1 foi a de 3ng/animal e a da RvD2 foi a de 10 ng/animal, escolhida com base nos resultados da hiperalgesia mecânica e/ou edema articular para seguir com os próximos experimentos. A segunda parte do delineamento foi realizada com os experimentos de hiperalgesia térmica com teste de Hargreaves e o teste da distribuição das patas traseiras através do Static Weight Bearing (SWB), onde as medidas foram feitas a cada 4 dias, durante os 30 dias de tratamento com as melhores doses de cada lipídeo. Após último dia, coletavam-se as amostras para realização das análises.

3.3.2 Modelo de inflamação aguda induzido por KO_2

Os animais utilizados para esse modelo agudo de inflamação (23) foram tratados com a dose de 1, 3 e 10 ng de RvD2 ou veículo (salina isotônica), por via intraperitoneal (100 µL/animal), 30 minutos antes da injeção intraplantar de KO_2 (30 µg/ 20 µL / pata). A hiperalgesia mecânica foi realizada pelo método de Von Frey com um medidor eletrônico e a hiperalgesia térmica determinada por Teste de Placa nos tempos: zero (basal), 30 minutos, 1 hora, 3 horas, 5 horas e 7 horas após a injeção intraplantar de KO_2. A dose mais eficaz da RvD2 foi a de 10 ng/animal e sendo assim, foi escolhida para a análise de hiperalgesia térmica feita por Teste de Hargreaves e comportamento de dor manifesta (contorções abdominais, sacudidas e tempo gasto lambendo a pata). Para o Teste de Hargreaves, as medidas também foram feitas nos tempos zero (basal), 30 minutos, 1 hora, 3 horas, 5 horas e 7 horas após a injeção intraplantar de KO_2 (30 µg/ 20 µL / pata). Para a avaliação da resposta de limiar basal, avaliada através de testes de hiperalgesia mecânica (versão eletrônica dos filamentos de von frey) e térmica (placa quente e teste de Hargreaves), os animais foram apenas tratados com RvD2 (10 ng, i.p.) ou veículo (salina isotônica, i.p.), meia hora antes do início das medidas, as quais foram feitas nos tempos 0 (basal), 30 minutos, 1 hora, 3 horas, 5 horas e 7 horas**.** Para o teste de contorção abdominal, os animais foram tratados com 10 ng de RvD2 ou

veículo (salina isotônica) via intraperitoneal meia hora antes da injeção intraperitoneal de KO_2 (1 mg / 100 µL), cujo comportamento foi avaliado ao longo de 20 minutos. Para determinação do comportamento de dor manifesta observado em sacudidas e tempo gasto lambendo a pata, os animais foram tratados com 10 ng de RvD2 ou veículo (salina isotônica) via intraperitoneal meia hora antes da injeção intraplantar de KO_2 (30 µg/ 20 µL / pata), cujo comportamento foi avaliado ao longo de 30 minutos. Para o teste de citocinas, os animais foram tratados com RvD2 (10 ng, i.p.) ou veículo (salina isotônica, i.p.), meia hora antes do estímulo com KO_2, e após 3 horas, o tecido da pata foi coletado para realização da análise nos tecidos.

3.4 Hiperalgesia Mecânica pela versão eletrônica dos filamentos de von Frey

Foi avaliada como já descrito pela literatura (89) e realizada em ambos os modelos (TiO_2 e KO_2). Resumidamente, em uma sala silenciosa, os animais foram colocados individualmente em gaiolas de acrílico (12 x 10 x 17 cm) com um piso de grade de arame, 15 a 30 minutos antes do teste de adaptação ambiental. A aplicação de força foi realizada apenas quando os animais estavam com as quatro patas na grade, não exibiam movimentos exploratórios ou defecação, e não estavam descansando sobre as patas. Um teste eletrônico de medição de pressão constituído por um transdutor de força de mão equipado com uma ponta de polipropileno (Anestesiômetro eletrônico von Frey; Insight, Ribeirão Preto, SP) foi utilizado para avaliar a nocicepção articular mecânica. Para este modelo, uma ponta grande (4,15 mm2) foi adaptada à sonda. Uma força perpendicular crescente foi aplicada na área central da superfície plantar da pata traseira para induzir um movimento de flexão da articulação femorotibial seguida de retirada da pata. Um espelho inclinado abaixo da grade forneceu uma visão clara da pata traseira. O aparelho eletrônico de medição de pressão registrou automaticamente a intensidade da força aplicada quando a pata foi retirada. O limiar mecânico induzido pela flexão foi expresso em gramas (g).

3.5 Edema articular

O edema da articulação femorotibial (modelo de TiO_2) foi avaliado através de medições dos diâmetros transversais utilizando o medidor de espessura de um calibre (Digmatic Caliper, Mitutoyo Corporation, Kanagawa, Japão). Foram realizadas duas medidas, uma frontal e outra lateral no joelho. Os valores de espessura da articulação femorotibial foram expressos como a diferença entre os diâmetros medidos antes (valor basal) e após a injeção intra-articular TiO_2 em milímetros (mm).

3.6 Avaliação da Hiperalgesia térmica articular avaliada pelo Hargreaves

A hiperalgesia térmica da região articular femorotibial (modelo TiO_2) e região da pata (modelo KO_2) foi avaliada com o auxílio do analgesímetro Hargreaves (Insight, Ribeirão Preto, SP). Resumidamente, em uma sala silenciosa, os animais foram colocados individualmente em gaiolas de acrílico (12 x 10 x 17 cm) com um piso de vidro, 15 a 30 minutos antes do teste de adaptação ambiental. A aplicação de calor foi realizada apenas quando os animais estavam com as quatro patas na base de vidro, não exibiam movimentos exploratórios ou defecação, e não estavam descansando sobre as patas. A pata padronizada para a medida foi a pata direita, devida a proximidade com a área de indução com o TiO_2 e KO_2. Foi inserida a força térmica de infravermelho de 30 µg na região central da pata do animal, e analisado o tempo em que o animal continuava na mesma posição com a incidência de calor. A partir do momento em que o animal apresentava o primeiro movimento, o aparelho finalizava a medida em segundos. Só se deve fazer a inserção de calor por no máximo 20 segundos, sendo este o ponto de corte (90).

3.7 Avaliação da Hiperalgesia térmica através da placa quente

O Teste de placa quente (± 52ºC) (54) foi realizado no modelo de KO_2, sendo os limiares basais determinados antes do tratamento com o lipídeo RvD2 e após injeção do estímulo na pata direita padronizada. Após o estímulo, as medidas foram feitas nos tempos: 30 minutos, 1 hora, 3 horas, 5 horas e 7 horas. O tempo máximo de permanência é de 20 segundos, para evitar a ocorrência de danos ao tecido plantar dos animais.

3.8 Análise comportamental de dor manifesta (contorção abdominal, sacudidas de pata e tempo gasto de lambida de pata)

Cada animal do modelo de KO_2 foi colocado em uma cuba de vidro e a intensidade dos comportamentos nociceptivos foi quantificada através da contagem do número total de contorções que ocorreram de 0 a 20 minutos após a injeção de KO_2 (54). A resposta de contorção considerada foi aquela em que houve a contorção dos músculos abdominais associada a um estiramento das patas traseiras. Os resultados foram expressos como o número total de contorções abdominais num período de 20 minutos. Os outros testes de dor manifestam foram determinados pelo número de movimentos de retirada/sacudida da pata *(flinches*) e o tempo total de lambidas da pata

(*licking*) que ocorrem de 0 a 30 minutos após a injeção de KO_2 (30 µg/pata, i.pl.). Os resultados foram expressos como o número total de sacudidas de pata e tempo de lambida num período de 30 minutos.

3.9 Alterações na distribuição do peso da pata utilizando *Static Weight Bearing*

Para o modelo de TiO_2, os animais foram condicionados individualmente em um aparato de acrílico posicionado para frente com as patas dianteiras apoiadas a frente, e as patas traseiras apoiadas sobre um sensor de medida de peso (g). O teste se inicia quando o animal está imóvel, a partir disso é realizada a medida de peso por 10 segundos. Ao concluir este período, tem-se a medida de peso da pata esquerda e direita, sendo a direita relativa ao estímulo. Este teste avalia a distribuição de peso entre as patas traseiras do camundongo. Enquanto o animal sem estímulo distribui o peso igualmente entre as duas patas, a razão da distribuição de peso entre a pata estimulada e a não estimulada é uma medida do nível de desconforto na pata estimulada. Por meio de medida contínua do peso suportado por cada pata, o Static Weight Bearing (SWB) (91) da Bioseb permite a medida objetiva da dor espontânea por acessar o equilíbrio postural. Esse teste foi realizado em ambos os modelos (TiO_2 e KO_2).

3.10 Migração leucocitária

As contagens total e diferencial de leucócitos recrutados para o espaço intra-articular no modelo de TiO_2 foram determinadas como descrito na literatura (61). Resumidamente, as cavidades da articulação do joelho foram lavadas com solução salina contendo ácido etilenodiaminotetracético (EDTA), que foi recuperada para avaliar a contagem de células total e diferencial. As contagens de células totais foram realizadas na câmara de Neubauer utilizando a solução Turk e as contagens de células diferenciais (100 células por campo) foram coradas com o kit panóptico rápido (Laborclin, Pinhais, PR, Brasil) sob microscopia óptica (Olympus CX31RTSF, Tóquio, Japão). Os resultados foram expressos como leucócitos totais, células polimorfonucleares ou mononucleares (células x 10^3/joelho).

3.11 Processamento Histológico

As amostras da articulação femorotibial do modelo de TiO_2 foram coletadas, fixadas em formalina tamponada e submetidas ao processamento histológico, para a

desidratação com banhos graduados de álcool (Dinâmica Química Contemporânea - São Paulo), diafinização com banhos de xilol (Synth - Diadema-SP), impregnação em parafina (Synth - Diadema-SP) (59). Foram realizadas secções de 7 micrômetros no micrótomo. As lâminas com os cortes foram submetidas as técnicas de coloração de hematoxilina (Laborclin - Pinhais-PR) e eosina (Laborclin - Pinhais-PR), e tricômio de Mallory – composto por um trio de corantes, sendo eles a hematoxilina (Laborclin - Pinhais-PR), vermelho de Mallory (Laborclin - Pinhais-PR), e verde Luz (Laborclin - Pinhais-PR), sendo este antecipado por um banho de ácido fosfomobilidico (Sigma-Aldrich – Jurubatuba-SP). Através da coloração de Hematoxilina e Eosina (HE), foi possível observar o tecido, e realizar a análise por meio de um score clínico, onde foram avaliados a hiperplasia sinovial, neovascularização e o infiltrado inflamatório, sendo estes pontuados de 0 a 3, demonstrando um grau de degradação tecidual conforme o nível crescente, sendo 0 para nenhuma alteração e 3 para a alteração mais graves, podendo ser nesse caso um aumento significativo do tecido hiperplásico, de novos vasos e de infiltrado inflamatório que prejudicam o tecido.

3.12 Ensaio de Toxicidade no estômago (mieloperoxidase)

Realizado através da atividade de mieloperoxidase (MPO), usando um ensaio colorimétrico, considerando-se um marcador confiável da migração de neutrófilos para os tecidos. As amostras do estômago foram coletadas em tampão K2HPO4 50 mM (pH 6,0) contendo 0,5% de brometo de hexadecil trimetilamônio (HTAB) e mantidos a -86°C até o uso. As amostras congeladas foram homogeneizadas usando um tecido turrax (Tissue-Tearor 985370, BioSpec Products, Bartlesville, OK, EUA), centrifugado (2 min, 16,000g, 4°C) e o sobrenadante resultante foianalisado usando um espectrofotômetro (Multiskan GO Microplate Spectrophotometer, ThermoScientific, Vantaa, Finlândia) para determinação da atividade MPO a 450 nm. A atividade MPO das amostras foi comparada a uma curva padrão de neutrófilos. Em resumo, 15 µL de amostra foram misturados com 200 µL de tampão de fosfato 50 mM (pH 6,0), contendo 0,167 mg / mL de dicloridrato de O-dianisidina e 0,0005% de peróxido de hidrogênio. Os resultados foram apresentados como atividade MPO (número de neutrófilos X 106/mg de tecido).

3.13 Ensaio de Toxicidade hepática e renal

O sangue foi colhido em microtubos contendo 50 µL de EDTA anticoagulante (5.000 UI / mL) e centrifugados (200 g, 10 min, 4°C) e o plasma foi separado. Para determinar as atividades enzimáticas de AST e ALT como indicadores de hepatotoxicidade e níveis de ureia e creatinina como indicadores de nefrotoxicidade, as amostras de plasma foram processadas de acordo com as instruções do fabricante (Labtest Diagnóstico S. A.,

Brasil). Os resultados foram apresentados como U/mL (AST e ALT) ou mg/dL (ureia e creatinina) do plasma.

3.14 Análise estatística

Foi feita através do software Prisma 8.0. A normalidade dos dados foi verificada através do teste Shapiro-Wilk. Para dados normais, foram utilizados testes ANOVA de uma (*one-way*) ou duas (*two-way*) vias, seguidos pelo pós-teste Tukey ou pós-teste Sidak. Para dados sem distribuição normal, foi realizado o teste não paramétrico Kruskal-Wallis seguido do teste Dunn's. Para todas as análises, as diferenças consideradas significativas apresentam $P < 0,05$.

4. ARTIGO 1 PARA PUBLICAÇÃO

O presente trabalho foi realizado no laboratório de Dor, Inflamação, Neuropatia e Câncer, da Universidade Estadual de Londrina e segue as normas da revista: Inflammopharmacology

Resolvin D1 ameliorates pain and inflammation in the mouse model of tio2-induced chronic arthritis

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Abstract

Arthritis is characterized by being an acute or chronic disease that affects one or more joints. It can be induced by inflammatory, autoimmune and infectious diseases that lead to the destruction of bone surfaces in major joints. Arthroplasty is a surgical procedure that replaces all or part of a dysfunctional joint with a prosthesis. Unfortunately, after the arthroplasty procedure, about 10-15% have joint replacement failure, which can reach 40%, and may be caused by infection and aseptic loosening. Titanium dioxide (TiO2) is a white powder normally used in products such as medicine, food and orthopedic prostheses. It was observed that TiO2 nanoparticles (NPs) penetrated into tissues causing damage. The discovery of specialized pro-resolution mediators marks the beginning of the physiology and pharmacology of resolution. RvD1 is a new treatment option for several inflammatory diseases. RvD1 assessment has been observed in several treatments of a variety of disorders. Here, we show the role of SPM RvD1 in the resolution of the inflammatory process and pain, using the model of chronic arthritis induced by TiO2 in mice. RvD1 decreases mechanical and thermal hyperalgesia, joint swelling and relative paw weight ratio. Decreased the migration of inflammatory cells to the knee joint and did not induce kidney, liver or stomach damage. Despite the need for further studies to provide the exact mechanisms by which RvD1 acts to reduce inflammation and pain, it was possible to observe that RvD1 attenuates mechanical and thermal hyperalgesia, edema formation and leukocyte recruitment, showing possible analgesic and anti-inflammatory effects -inflammatory

Keywords: Arthritis, inflammation, prothesis, Titanium dioxide, specialized pro-resolution mediators

Introduction

The definition of arthritis is characterized by being an acute or chronic disease that affects one or more joints. It may include joint swelling, joint deformities, stiffness and pain (HARTH and NIELSON, 2019). It is induced by inflammatory, autoimmune and infectious diseases that lead to the destruction of bone surfaces in major joints. Arthroplasty is a surgical procedure that replaces all or part of a dysfunctional joint with a prosthesis (LEE and GOODMAN, 2008). Many patients suffering from osteoarthritis, inflammatory arthritis (such as rheumatoid arthritis (RA), post-traumatic arthritis, and tumors (ULRICH, 2008) may require replacement arthroplasty (SOEVER, 2010). Therefore, arthroplasty is an important procedure for the terminal stages of arthritis (CARR, 2012). Even after the arthroplasty procedure, about 10-15% of joint replacement failure can reach 40% according to previous studies (HARRIS, 2001; PURDUE, 2006). This arthroplasty failure can be caused by infection and aseptic loosening (SUN, 2015). Regarding aseptic loosening, debris released by prosthesis biomaterials inducing osteolysis causing arthroplasty failure (SUN, 2015; COBELLI, 2011; HAYNES, 2001).

Wear debris released from prosthesis-activated immune cells in periprosthetic tissue l leading to an aseptic inflammatory response. This wear and tear debris are rapidly phagocytosed by resident cells that produce nuclear factor kappa-B (COBELLI, 2011), tumor necrosis factor (TNF)α, interleukin (IL)-1β, IL-6 (COBB, 2006), and reactive oxygen species (ROS) (FREEMAN, 2009; WOOLEY, 1996). The activation and differentiation of osteoclasts are induced by this active microenvironment inducing bone resorption. Arthritis is induced by inflammatory, autoimmune and infectious diseases that lead to destruction of bone surfaces in major joints. Arthroplasty is a surgical procedure that replaces all or part of a dysfunctional joint with a prosthesis. replacement arthroplasty (SOEVER, 2010). According to the Agency for Healthcare Research and Quality, in 2017, more than 754.000 arthroplasties were performed in the United States (KURT, 2007). It is expected that approximately 3.48 million arthroplasties will be performed by the year of 2030 (SUN,02015). Thus, arthroplasty is an important procedure for arthritis endpoints (CARR, 2012). Despite the importance of arthroplasty, about 10-15% of joint replacement failure can reach 40% according to previous studies (HARRIS, 2001; PURDUE, 2006). Arthroplasty failure can be caused by infection and aseptic loosening (SUN, 2015). Tissue damage occurs through titanium particles that can detach from prostheses due to the wear process. These particles induce the activation of the immune

response, activating local resident immune cells, such as macrophages, which will promote the phagocytosis of these wear residues (ST. PIERRE, 2010). Wear debris are rapidly phagocytosed by resident cells that produce nuclear factor κ-B ligand receptor activator (RANKL) [9], tumor necrosis factor (TNF)α, interleukin (IL)-1β, IL-6 [11], and reactive oxygen species (ROS) (FREEMAN, 2009; WOOLEY, 1996).

Titanium dioxide (TiO_2) is a white powder (GONÇALVES e GIRARD, 2011) normally used in various products as additives in drugs, dyes and orthopedic prostheses (GURR, 2005). It has been observed that nanoparticles (NPs) of TiO_2 have penetrated the stratum corneum of the skin causing damage (WU, 2009). The accumulation of titanium in human tissues is known and characterized by the deposition of a black pigment, accompanied by a fibrous process, necrosis or granulomatous reactions (MORAN, 1991). Reactive species of Cytotoxic TiO_2-induced oxygen ROS (ROS) were found in human epidermal cells (SHUKLA, 2011). Furthermore, TiO_2 induced a recruitment of leukocytes in the lungs of mice with MIP-1α, KC, TNFα and IL-β (AMBALAVANAN, 2013). Furthermore, TiO_2 induced a prolonged and immediate reactive oxygen species (ROS) in microglia *in vitro* (LONG, 2007). Regarding the role of TiO_2 in prostheses, Borghi et al, 2018 showed that intra-articular injection of TiO_2 NPs induced arthritis chronic inducing neutrophil influx, oxidative stress, cytokine production. Manchope et al also demonstrated that TiO_2 induces mechanical hyperalgesia, edema formation and tissue degradation.

For resolution of inflammatory exudates to occur, omega-3 fatty acids are used to produce structurally distinct families of signaling molecules known as resolvins, protectins, and maresins, collectively referred to as specialized pro-resolution mediators (SPMs). SPMs are agonists with the potential to stimulate key cell resolution events by limiting polymorphonuclear neutrophil infiltration and increasing macrophage clearance from apoptotic cells (TABAS, GLASS, 2006).

The class of Resolvin D (RvD) comprises a series of lipoxygenase metabolites of docosahexaenoic acid (DHA) and in which RvD1, the molecule studied in our article, receives more attention. Aspirin-modified 15-lipoxygenase (ALOX15) cyclooxygenase-2 (COX-2) converts DHA to 17-hydroxy DHA (17-HDHA) and then 5-lipoxygenase (ALOX5) metabolizes 17-HDHA to RvD1, RvD2, RvD3, and RvD4. The discovery of specialized pro-resolution mediators marks the beginning of the physiology and pharmacology of resolution (SERHAN, 2017). RvD1 has been chosen as a new treatment

option for several inflammatory diseases. The evaluation of RvD1 has been observed in several treatments of a variety of disorders, such as neurodegenerative diseases (KRASHIA, 2019), acute kidney injury (LUAN, 2020), fibrotic/electrical remodeling (HIRAM, 2021), colitis-associated cancers (ZHONG, 2018) and Gouty Arthritis (ZANINELLI, 2022).

Therefore, this article discusses the role of the SPM RvD1 in the resolution of the inflammatory process and pain, using the model of chronic arthritis induced by TiO_2 in mice, which may help us to understand the role of this lipid in the resolution of this disease for the tissues affected.

Material and Methods

Animals

For the experiments, male Swiss mice weighing 20-25g were used. The animals came from the Central Animal Facility of the State University of Londrina (UEL) and were kept in the Animal Facility of the Department of Pathological Sciences of the State University of Londrina for at least two days before the experiments, where the light/dark cycle was used (12 /12 h) and controlled temperature (21°). Animal care and handling procedures are all in accordance with International Association for the Study of Pain (IASP) guidelines. The research project was registered under number 11147.2016.40.

Drugs

The drugs and reagents used in the present study were: RvD1 (Cayman Chemical, MI, USA); saline solution (0.9% NaCl) from Frenesius Kabi Brasil Ltda (Aquiraz, CE, Brazil); isoflurane from Abbott Laboratories (Abbott Park, IL, USA); absolute alcohol (Dinâmica Química Contemporânea - São Paulo), Titanium dioxide (TiO2) was purchased from Synth (Diadema, SP, Brazil) and the particle size was <1 μm with an average of 862.2 nm as determined by size distribution analysis (Malvern Instruments Ltd, UK).

Experimental procedures

The experiments were carried out in two parts, starting with the animals (n = 6 per experimental group) submitted to a dose-response curve with the treatment with the lipid RvD1. The animals were stimulated with intra-articular injection (i.a.) with 3 mg of TiO2 suspended in 10 μL of 0.9% sterile saline solution per femorotibial joint according to the standardization of [24]. Mechanical hyperalgesia and edema were evaluated 24 h after TiO2 injection to assess the induction of the chronic arthritis model. Soon after 24 h, the animals were treated with RvD1 at doses of 1, 3, 10 ng/animal intraperitoneally (i.p.) and mechanical hyperalgesia and edema were evaluated from 1, 3, 5, 7 and 24 hours after treatment with RvD1 on the first day [13]. Subsequently, mechanical hyperalgesia and edema were assessed every 2 days after treatment with RvD1 until day 30. Immediately after measurements on the 30th day, the animals were anesthetized and euthanized, and the knee joint cavities were washed. Samples were collected to assess leukocyte migration. The most effective dose of RvD1 was 3 and 10 ng/animal and the chosen one was 3ng/ animal, chosen based on the results of mechanical hyperalgesia to proceed with the next experiments. All other experiments were performed with the 30-day stimulus model and, after the last day, samples were collected for analysis.

Evaluation of Mechanical knee Joint Hyperalgesia

Mechanical joint hyperalgesia of the femorotibial joint was evaluated as already described in the literature (GUERRERO, 2006). Briefly, in a quiet room, the animals were placed individually in acrylic cages (12 x 10 x 17 cm) with a wire grid floor, 15 to 30 minutes before the environmental adaptation test. Force application was performed only when the animals had all four paws on the grid, were not exhibiting exploratory movements or defecation, and were not resting on their paws. An electronic pressure measurement test consisting of a hand force transducer equipped with a polypropylene tip (von Frey electronic anesthesiometer; Insight, Ribeirão Preto, SP) was used to assess mechanical joint nociception. For this model, a large tip (4.15 mm2) was fitted to the probe. An increasing perpendicular force was applied to the central area of the plantar surface of the hind paw to induce a movement of flexion of the femorotibial joint followed by withdrawal of the paw. A mirror tilted below the grille provided a clear view of the hind leg. The electronic pressure measurement device automatically recorded the intensity of the applied force when the paw was withdrawn. The test was repeated until subsequent measurements were consistent (ie, the variation between these measurements was less than 1 g). The mechanical threshold induced by flexion was expressed in grams (g).

Assessment of joint swelling

Femorotibial joint swelling was assessed by measuring cross-sectional diameters using a caliper (Digmatic Caliper, Mitutoyo Corporation, Kanagawa, Japan). Two measurements were taken, one frontal and one lateral on the knee. Femorotibial joint thickness values were expressed as the difference between diameters measured before (baseline) and after intra-articular TiO2 injection in millimeters (mm) (MAIOLI, 2015).

Assessment of leukocyte migration

Total and differential counts of leukocytes recruited to the intra-articular space were determined as described in the literature (ZANINELLI, 2022). Briefly, knee joint cavities were flushed with saline containing ethylenediaminetetraacetic acid (EDTA), which was retrieved to assess total and differential cell counts. Total cell counts were performed in the Neubauer chamber using the Turk solution and differential cell counts (100 cells per field) were stained with the panoptic kit (Laborclin, Pinhais, PR, Brazil) under light microscopy (Olympus CX31RTSF, Tokyo, Japan). Results were expressed as total leukocytes, polymorphonuclear and mononuclear cells (cells x 103/knee).

Stomach Toxicity Assay (myeloperoxidase)

Performed through myeloperoxidase (MPO) activity, using a colorimetric assay, considered a reliable marker of neutrophil migration into tissues. Stomach samples were

collected in 50 mM K2HPO4 buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide (HTAB) and kept at -86°C until use. Frozen samples were homogenized using a tissue turrax (Tissue-Tearor 985370, BioSpec Products, Bartlesville, OK, USA), centrifuged (2 min, 16,000g, 4°C) and the resulting supernatant was analyzed using a spectrophotometer (Multiskan GO Microplate Spectrophotometer, ThermoScientific, Vantaa, Finland) for determination of MPO activity at 450 nm. The MPO activity of the samples was compared to a standard curve of neutrophils. Briefly, 15 µL of sample was mixed with 200 µL of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/mL O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. Results were presented as MPO activity (number of neutrophils X 106/mg tissue).

Hepatic and Renal Toxicity Assay

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

Static Weight Bearing

The animal was individually conditioned in an acrylic apparatus positioned forward with the front paws resting in front, and the hind paws resting on a weight measurement sensor (g). The test starts when the animal is immobile, after which the weight measurement is performed for 10 seconds. At the end of this period, the weight of the left and right paws is measured, with the right paw relative to the stimulus. This test evaluates the weight distribution between the hind legs of the mouse. While the unstimulated animal distributes weight equally between the two paws, the ratio of weight distribution between the stimulated and unstimulated paw is a measure of the level of discomfort in the stimulated paw. Through continuous measurement of the weight supported by each paw, Bioseb's Static Weight Bearing allows the objective measurement of spontaneous pain by accessing postural balance.

Evaluation of joint thermal hyperalgesia assessed by Hargreaves

Thermal hyperalgesia in the femorotibial joint region was assessed using a Hargreaves Apparatus (Ugo Basile, Gemonio, VA, Italy). Briefly, in a quiet room, the animals were placed individually in acrylic cages (12 x 10 x 17 cm) with a glass floor, 15 to 30 minutes before the environmental adaptation test. Heat application was performed only when the animals had all four paws on the glass base, were not exhibiting exploratory movements or defecation, and were not resting on their paws. The paw standardized for measurement

was the right paw, due to its proximity to the TiO2 induction area. An infrared thermal force of 30 μ was inserted in the central region of the animal's paw, and the time in which the animal remained in the same position with the incidence of heat was analyzed. From the moment the animal made the first movement, the device completed the measurement in seconds. Only insert heat for a maximum of 20 seconds, which is the cut-off point.

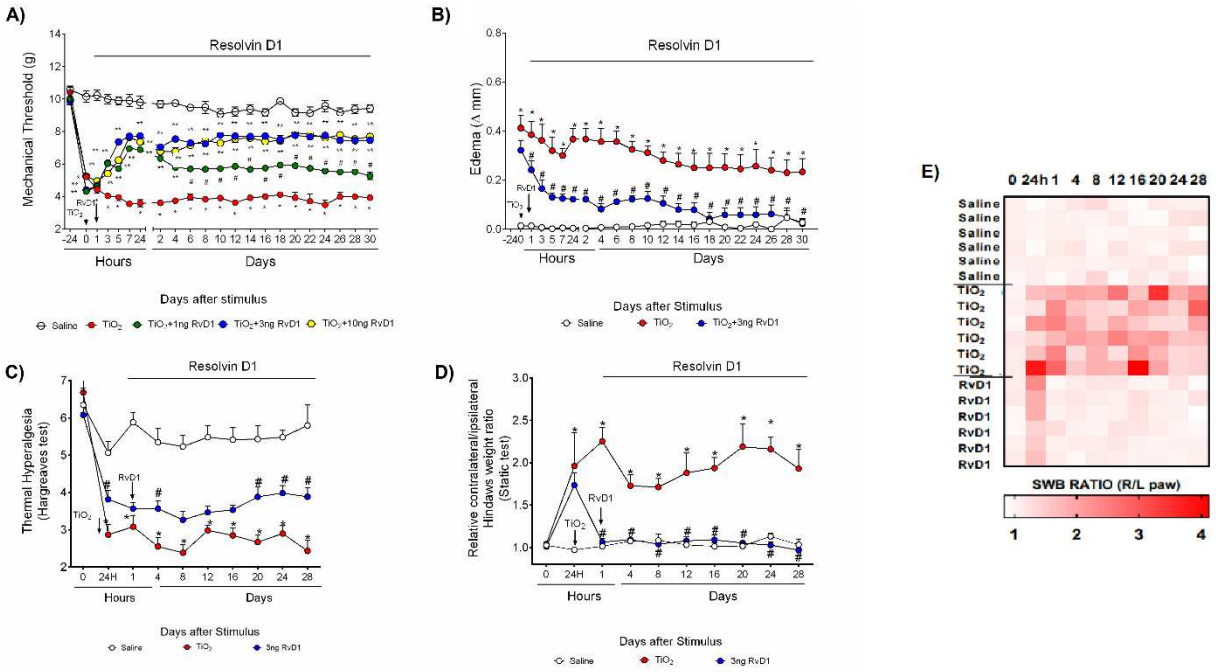
Statistical analysis

Performed using GraphPad Prism 8.0 software. Data normality was verified using the Shapiro-Wilk test. For normal data, one-way or two-way ANOVA tests were used, followed by the Tukey post-test or the Sidak post-test. For data without normal distribution, the non-parametric Kruskal-Wallis test was performed followed by the Dunn's test. For all analyses, differences considered significant have $P < 0.05$.

Results

Figure 1: RvD1 decreases mechanical and thermal hyperalgesia, articular edema and the ratio of relative weight of the paws in the model of chronic arthritis induced by TiO_2

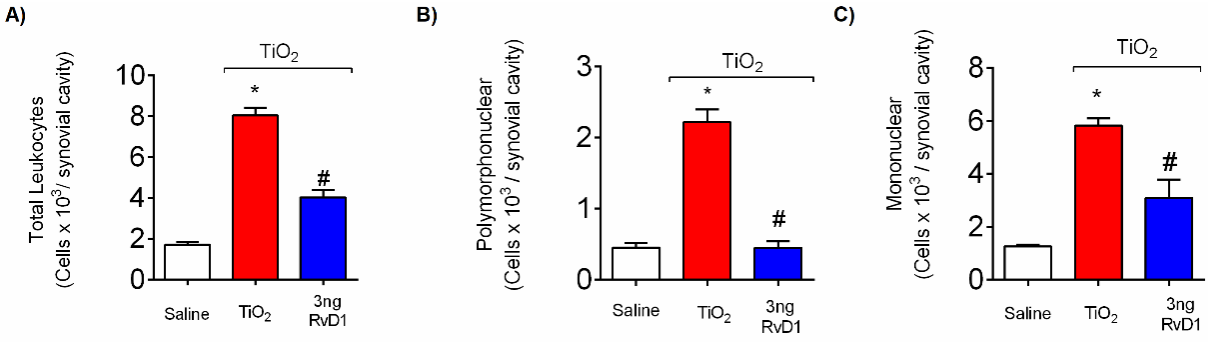
The experiment started with a dose-response curve for treatment with RvD1 (1, 3, 10 ng/animal, intraperitoneal [i.p.]) starting 24 h after intra-articular (i.a.) TiO_2 (3 mg/joint). Mechanical hyperalgesia (Fig. 1A) was evaluated 0, 1, 5, 7 and 24 hours after treatments and on alternate days until the end of the 30th day. As the effect of RvD1 lasted up to 72 h after administration, it was defined that the treatment would be administered to the mice every 3 days. The 3ng dose was selected for the following experiments, as it was the most effective dose together with the 10ng dose, reducing the mechanical hyperalgesia induced by TiO_2. Decreases the formation of joint edema (Fig. 1B) at the chosen dose during the period of the TiO_2-induced arthritis model, as well as in Thermal Hyperalgesia in the Hargreaves test (Fig. 1C) and static paw weight distribution (Fig. 1D and E), which were evaluated at 0h, 24h, 1, 4, 8, 12, 16, 20, 24, and 28 days after the stimulus.



Mechanical hyperalgesia (Fig. 1A) and joint edema (Fig. 1B) were evaluated 0, 1, 3, 5, 7 and 24 hours after treatments and on alternate days until the end of the 30th day. Thermal hyperalgesia (Fig. 1C) and relative paw weight ratio (Fig. 1D) and his heat map (Fig. were assessed at 0h, 24h after stimulation, and E) then every 4 days on days 1, 4, 8, 12, 16, 20, 24, and 28 days after stimulation. With this, it was possible to evaluate the most effective treatment dose, 3 and 10 ng, which was able to reduce mechanical hyperalgesia, where the 3ng dose was chosen for the next experiments. Thermal hyperalgesia and the relative weight of the paws were performed with the chosen dose (3ng/animal). Results are presented as mean ± standard error (SEM) of 6 mice per experimental group. *$p<0.05$ in relation to the saline group, **$p<0.05$ compared to the TiO_2 group, #$p<0.05$ compared to the TiO_2 and other doses of RvD1 groups. (Two-way ANOVA followed by Tukey's post-test).

Figure 2: RvD1 decreases the migration of inflammatory cells to the knee joint in the model of chronic arthritis induced by TiO_2

After 30 days of treatment with a dose of 3ng/animal, joint lavage was collected for counting. Total recruitment (Fig. 2A), Mononuclear (Fig. 2B) and polymorphonuclear (Fig. 2C) leukocytes were evaluated by light microscopy at the end of the 30-day TiO_2 stimulation model. Results are presented as mean ± standard error (SEM) of 6 mice per experimental group. *p<0.05 in relation to the saline group, **p<0.05 in relation to the TiO_2 group, #p<0.05 in relation to the thio2 and other doses of RvD1 groups. (Two-way ANOVA followed by Tukey's post-test).

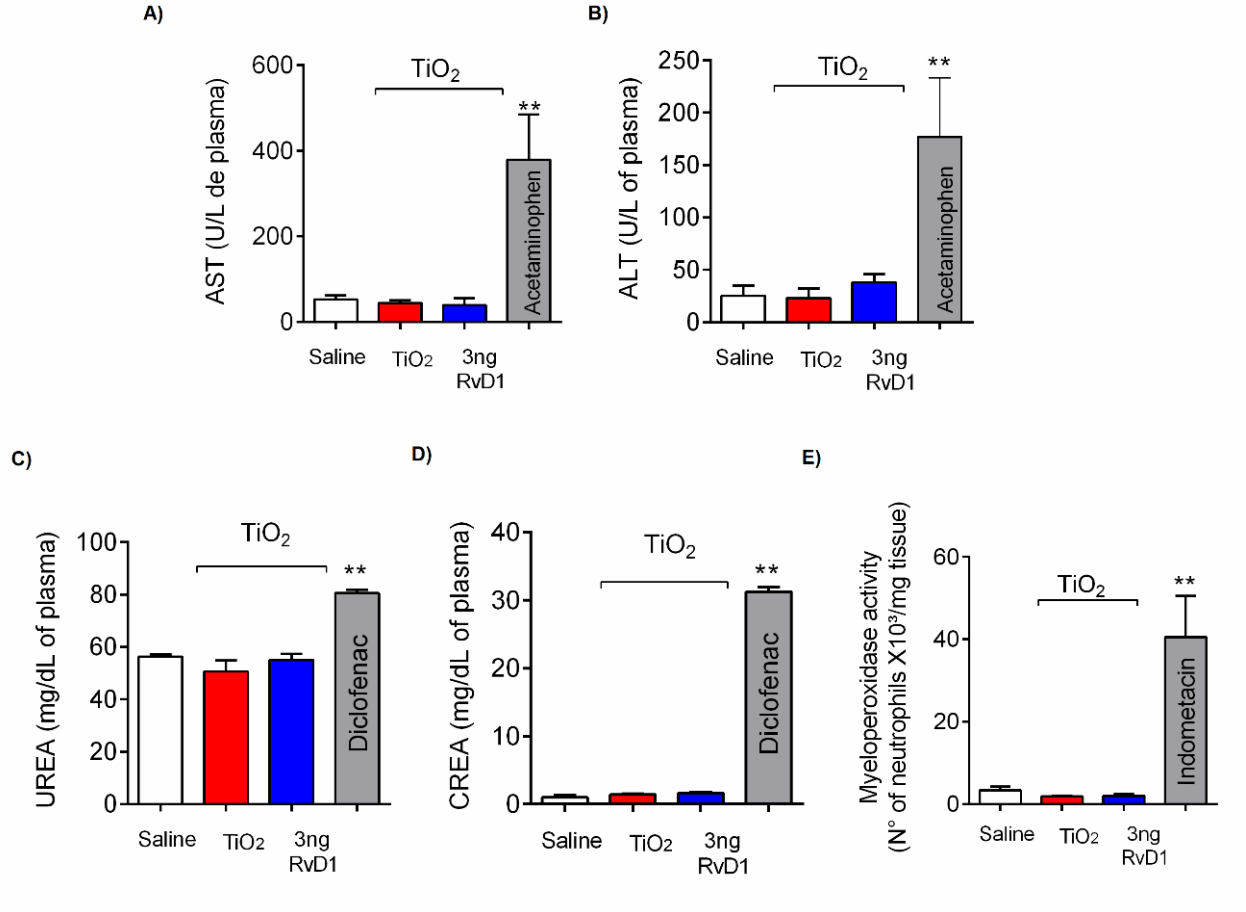


Animals were treated every 3 days for 30 days with RvD1 (1, 3, 10 ng/animal, intraperitoneal [i.p.]) starting 24 h after intra-articular (i.a) injection of TiO_2 (3 mg/joint). After 30 days of experiment, joint lavage was collected for counting. Total recruitment (Fig. 2A), polymorphonuclear (Fig. 2B) and mononuclear (Fig. 2C) leukocytes were evaluated by light microscopy at the end of the 30-day TiO_2 stimulation model. Results are presented as mean ± standard error (SEM) of 6 mice per experimental group. *p<0.05 in relation to saline group, **p<0.05 in relation to TiO_2 group, #p<0.05 in relation to the TiO_2 and other doses of RvD1 groups. (Two-way ANOVA followed by Tukey's post-test)

Figure 3: RvD1 does not induce kidney, liver or stomach damage.

Mice were treated daily for 30 days. Stomach and plasma samples were collected to determine plasma concentrations of aspartate aminotransferase (AST) (Fig 3A), alanine aminotransferase (ALT) (Fig 3B), urea (Fig 3C), creatinine (Fig 3D), and myeloperoxidase activity (MPO) (Fig 3E) in stomach samples as previously described [42]. Mice treated with indomethacin (2.5 mg/kg, i.p., diluted in tris/HCl buffer, for 7 days) were used as a positive control for stomach injury. Samples from mice treated with acetaminophen were used as a positive control for liver damage (650 mg/kg, orally, diluted in sterile saline, 200 mg/mL, once). Samples from mice treated with diclofenac (200 mg/kg, orally, diluted in sterile saline, once) were used as a positive control for kidney injury. Treatment with a dose of 3ng/animal of RvD1 did not change the plasma concentration of AST (Figure 3A) and ALT (Figure 3B), urea (Figure 3C) and creatinine (Figure 3D), and gastric MPO activity (Figure 3E). Thus, treatment with RvD1 for 30 days did not induce gastric lesions or liver and kidney damage. On the other hand,

indomethacin, acetaminophen, and diclofenac induced lesions in these organs, respectively.



The animals were treated 30 days daily with RvD1 (3ng/animal) for 30 days, or indomethacin (2.5 mg/kg, i.p., diluted in tris/HCl buffer) for 7 days; or acetaminophen (650 mg/kg, ip, diluted in saline, 200 mg/mL); or diclofenac (200 mg/kg, p.o., diluted in saline) were administered only once. (A) AST and (b) ALT plasma levels; (C) urea, (D) creatinine plasmatic levels and (E) MPO activity in the stomach; were determined. The results were presented as mean ± SEM of 6 mice per experimental group, were performed 2 times separately. * $p<0.05$ compared to the saline group. (One-way ANOVA followed by Tukey's post-test).

Discussion

Titanium is the main biomaterial used in orthopedic prosthesis and implants (LONG, 1998). Over the time, the wear of prosthesis leads to loosening and the release of TiO_2 debris, which induces inflammation due to the immune response evoked by immune cells against the particles of TiO_2. TiO_2 particles released to the peri-prosthetic tissue induce the activation of the phagocyte cells that either fail to phagocyte or fail to eliminate the debris phagocyted, thus producing and releasing elevated levels of pro-inflammatory mediators, such as inflammatory cytokines and ROS, and generating the aseptic inflammatory response (WOOLEY, 1996; WOOLEY, 2002; COBELLI, 2011; WEI, 2009; SHIMIZU, 2010). Resolvins are a family of specialized pro-resolvin lipid mediators (SPMs) generated during the acute inflammation, more specifically, in the resolution phase. Resolvin D1 (RvD1) is a resolving of class D that exhibits potent anti-inflammatory actions in several models of inflammation and pain (DUFFIELD, 2006; JI, 2011). The present study shows the anti-inflammatory and analgesic properties of the Resolvin D1 (RvD1) in reducing the mechanical and thermal hyperalgesia, edema, and leucocyte recruitment to the knee joint in the TiO_2-induced arthritis in mice.

RvD1 can modulate transient potential receptors (TRP) by inhibiting TRPA1, TRPV3 and TRPV4, but not TRPV1 (BANG, 2010). Consistent with data demonstrated by other models of inflammatory pain, we demonstrated that RvD1 reduced the mechanical hyperalgesia induced by TiO_2 at low doses (1-10 ng) which involves the activation of TRPA1/TRPV4 (ALESSANDRI-HABER, 2006; WEI, 2009; XU, 2010; LIMA-GARCIA, 2011). Still in relation to hyperalgesia, this time in thermal, in our study it was possible to observe a decrease in thermal hyperalgesia in the group of animals that received 10ng of RvD1 throughout the experiment, when compared with the group stimulated with TiO2. In the literature, an animal model of type 2 diabetes, the thermal hyperalgesia of the animals was evaluated by the Hargreaves method, where the animals treated with the lipid RvD1 also showed a decrease in latency in relation to the group that was only stimulated (SHEVALYE, 2015)

Edema formation is a hallmark of the inflammatory process. Here, we present that RvD1 was able to decrease joint edema caused by TiO2. Our data corroborate Liu et al., who demonstrated in a model of early brain injury, that RvD1 exerted a strong anti-inflammatory effect and markedly reduced neutrophil infiltration and cerebral edema

(LIU, 2021). Recruitment of leukocytes to the knee joint is a hallmark of joint disease. In this project, it was possible to observe that RvD1 was able to decrease leukocyte migration in total, polymorphonuclear and mononuclear parameters, as demonstrated by Zaninelli and collaborators, where treatment with RvD1 through two delivery routes, both intrathecal and intraperitoneal, as well as in this study, reduced MSU-induced recruitment of both total leukocytes and neutrophils and mononuclear cells (ZANINELLI, 2022).

An interesting fact is that the results observed in this study using RvD1 as an anti-inflammatory and analgesic treatment were achieved in concentrations of nanograms (ng), implicating the high potency of this SPM in treating inflammatory diseases such as arthritis.

Here we also demonstrated that RvD1 treatment did not induce adverse side effects normally presented for patients in chronic usage of NSAIDs, such as liver, kidney, and stomach injury (HOHMANN, 2013; FATTORI, 2017).

Regarding potential treatments for pain and inflammation of the prosthesis, current medications have side effects and are not effective in interrupting the disease process, which suggests that there is still a need for the development of new therapeutic approaches. Therefore, after data obtained in this work that coincide with data from the literature, it is to be expected that RvD1 is a possible treatment with better results for this type of disease, with fewer side effects and greater resolution in the chronic inflammation of joint arthritis caused by prostheses.

Conclusion

In conclusion, this study demonstrated the potential anti-inflammatory effects of RvD1 in TiO2-induced arthritis in mice. Although further studies must be performed to provide the exact mechanisms by which RvD1 acts to reduce inflammation and pain, here we observed that one possible explanation would be that RvD1 attenuates the mechanical and thermal hyperalgesia and edema formation by reducing the recruitment of leucocytes to the knee joint.

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5. ARTIGO 2 PARA PUBLICAÇÃO

O presente trabalho foi realizado no laboratório de Dor, Inflamação, Neuropatia e Câncer, da Universidade Estadual de Londrina e segue as normas da revista: Inflammation research

The specialized pro-resolving mediator (SPM) resolvin D2 (RvD2) reduces inflammation and pain in a mouse model of titanium dioxide-induced chronic arthritis

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Abstract

Clinical conditions leading to arthroplasty include osteoarthritis, inflammatory arthritis (such as rheumatoid arthritis), osteonecrosis, joint dysplasia and tumors. However, even performing the arthroplasty process, about 10-15% of joint replacement fail. One of the most used implant materials in the world for biomedical purposes due to its excellent properties such as high biocompatibility, high corrosion resistance and flexibility is titanium. However, it has been shown that TiO_2 nanoparticles (NPs) can penetrate the skin as well as the stratum corneum, causing damage to the individual. Specialized pro-resolving mediators (SPMs) are a class of mediators which can be defined by the function of limiting the accumulation of neutrophils in the tissue. Resolvin D2 (RvD2) was able to decrease painful and inflammatory parameters in several scientific models around the world. Therefore, this article sought to investigate the role of this SPM in the TiO2-induced mouse model of chronic arthritis. Mice were stimulated with intra-articular injection of 3 mg/joint of TiO_2 to induce chronic arthritis and 24 hours later they were treated with RvD2 (1, 3, 10ng/animal intraperitoneal [i.p]. The mechanical and therma hyperagesia, joint edema, histological damage and leukocyte recruitment were evaluated after the treatment. RvD2 decreased mechanical and thermal hyperalgesia, TiO_2-induced joint edema, histological damage and leukocyte recruitment, showing to be a molecule with great potential to treat inflammatory and pain parameters.

Keyworks: Resolvin D2, Arthritis, Inflamation, Arthroplasty, Pain

Introduction

Several reasons can cause the destruction of the main joints of the body, such as inflammatory, autoimmune, infectious, or traumatic origin, leading the patient to develop arthritis. Therefore, there are cases in which patients undergo procedures that perform the total or partial replacement of the joint (1). Clinical conditions leading to arthroplasty include osteoarthritis, inflammatory arthritis (such as rheumatoid arthritis), osteonecrosis, joint dysplasia, post-traumatic arthritis, and tumors (2). However, even performing the arthroplasty process, about 10-15% of joint replacement fail, reaching up to 40%, according to previous studies (3,4).

One of the most used implant materials in the world for biomedical purposes due to its excellent properties such as high biocompatibility, high tensile strength, high corrosion resistance, and flexibility is titanium (5). Titanium dioxide (TiO_2) is a white powder (6), odorless, popularly used in pharmaceutical products, food coloring, hygiene and finally in orthopedic prostheses (7). Adverse responses to the biomaterials used in the manufacture of prostheses occur due to the stimulation of cells residing in the periprosthetic tissue through particles that result from the wear of the prosthesis itself. This tissue serves as an interface between the prosthesis and the bone and contains monocyte/macrophage lineage cells (8,9).

Despite its high popularity in the commercial environment, including sunscreens, it has been shown that nanoparticles (NPs) of TiO_2 can penetrate the skin as well as the stratum corneum, causing damage to the individual (10). When deposited in tissues such as the lungs, skin and synovium, NPs of TiO_2 transform into a characteristic black pigment, accompanied by fibrosis, necrosis, and granulomatous reactions (11). A clinical case reported by Dorner et al in 2006 (12) proved that TiO_2's NPs can in fact generate arthritis, in which the patient had no case of this disease in the family and developed joint inflammation with the use of a TiO_2 and vanadium prosthesis. In addition, TiO_2 also induced joint pain, edema formation, oxidative stress, and increased pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α in an animal model of chronic arthritis (13). Furthermore, TiO_2 caused cartilage damage, leukocyte recruitment, and NFkB activation (14).

Specialized pro-resolving mediators (SPMs) are a class of mediators which can be defined by the function of limiting the accumulation of neutrophils in the tissue, counter-regulating pro-inflammatory cytokines and stimulating macrophage phagocytosis (15).

Resolvin D2 (RvD2) was able to reduce pathological changes in kidney and liver tissues in a burn model in rats, decreasing the probability of failure of these affected organs (16). RvD2 promoted host defense mechanisms in a model of 2-hit sepsis and secondary lung infection by increasing Toll-like receptor-2 (TLR-2) gene expression and phagocytic capacity of alveolar macrophages/monocytes (17).

Considering the promising data regarding the protective effects of RvD2 in different inflammatory models, this article sought to investigate the role of this SPM in the TiO_2-induced mouse model of chronic arthritis.

Material and Methods

Animals

For the experiments, male Swiss mice weighing 20-25g were used. The animals came from the Central Animal Facility of the State University of Londrina (UEL) and were kept in the Animal Facility of the Department of Pathological Sciences of the State University of Londrina for at least two days before the experiments, where the light/dark cycle was used (12 /12 h) and controlled temperature (21°). Animal care and handling procedures are all in accordance with International Association for the Study of Pain (IASP) guidelines. The research project was registered under number 11147.2016.40.

Drugs

The drugs and reagents used in the present study were: RvD2 (Cayman Chemical, MI, USA); saline solution (0.9% NaCl) from Frenesius Kabi Brasil Ltda (Aquiraz, CE, Brazil); isoflurane from Abbott Laboratories (Abbott Park, IL, USA); absolute alcohol (Dinâmica Química Contemporânea - São Paulo), Titanium dioxide (TiO_2) was purchased from Synth (Diadema, SP, Brazil) and the particle size was <1 μm with an average of 862.2 nm as determined by size distribution analysis (Malvern Instruments Ltd, UK).

Experimental procedures

The first step of experiments was performed with the animals (n = 6 per experimental group) submitted to a dose-response curve with the treatment with the lipid RvD2. The animals were stimulated with intra-articular injection (i.a.) with 3 mg of TiO_2 suspended in 10 μL of 0.9% sterile saline solution per femorotibial joint according to the standardization of Borghi et al., 2017. Mechanical and thermal hyperalgesia and edema were evaluated 24 h after TiO_2 injection to assess the induction of the chronic arthritis model. Soon after 24 h, the animals were treated with RvD2 at doses of 1, 3, 10 ng/animal intraperitoneally (i.p.) and mechanical hyperalgesia and edema were evaluated from 1, 3, 5, 7 and 24 hours after treatment with RvD2 on the first day (13). The hind paw distribution test was performed using the Static Weight bearing (SWB) with the most effective dose every 4 days during the 30 days of treatment. Subsequently, mechanical hyperalgesia and edema were assessed every 2 days after treatment with RvD2 until day 30. Immediately after measurements on the 30th day, the animals were anesthetized and euthanized, and the knee joint cavities were washed. Samples were collected to assess leukocyte migration. The most effective dose of RvD2 was 10 ng/animal, chosen based on the results of mechanical hyperalgesia to proceed with the next experiments. All other experiments were performed with the 30-day stimulus model and, after the last day, samples were collected for analysis.

Mechanical hyperalgesia by Von Frey filaments

It was evaluated as described in the literature (18). Briefly, in a quiet room, the animals were placed individually in acrylic cages (12 x 10 x 17 cm) with a wire grid floor, 15 to 30 minutes before the environmental adaptation test. Force application was performed only when the animals had all four paws on the crate, did not exhibit exploratory movements or defecation, and were not resting on their paws. An electronic pressure measurement test consisting of a hand force transducer equipped with a polypropylene tip (Von Frey Electronic Anesthesiometer; Insight, Ribeirão Preto, SP) was used to assess mechanical joint nociception. For this model, a large tip (4.15 mm2) was fitted to the probe. An increasing perpendicular force was applied to the central area of the plantar surface of the hind paw to induce a movement of flexion of the femorotibial joint followed by withdrawal of the paw. A slanted mirror below the grille provided a clear view of the

hind paw. The electronic pressure measuring device automatically recorded the intensity of the force applied when the paw was withdrawn. The test was repeated until subsequent consistent measurements (ie, the variation between these measurements was less than 1 g). The mechanical threshold induced by flexion was expressed in grams (g).

Evaluation of joint edema

Femorotibial joint swelling was assessed by measuring the transverse diameters using a gauge (Digmatic Caliper, Mitutoyo Corporation, Kanagawa, Japan). Two measurements were taken, one frontal and one lateral at the knee. Femorotibial joint thickness values were expressed as the difference between the diameters measured before (baseline) and after intra-articular TiO_2 injection in millimeters (mm).

Thermal hyperalgesia evaluation evaluated by Hargreaves

Thermal hyperalgesia of the femorotibial joint region was evaluated with the aid of the Hargreaves algesimeter (Insight, Ribeirão Preto, SP). Briefly, in a quiet room, the animals were placed individually in acrylic cages (12 x 10 x 17 cm) with a glass floor, 15 to 30 minutes before the environmental adaptation test. The heat application was performed only when the animals had four legs at the base of glass, did not exhibit exploratory movements or defecation, and were not resting on their paws. The standardized paw for the measurement was the right paw, due to the proximity to the induction area with TiO_2. The infrared thermal force of 30 μ was insidious in the central region of the animal's paw and analyzed the time when the animal was still in the same position with the heat insistence. From the moment the animal had the first movement, the device finished the measurement in seconds. Heat should only be infused for a maximum of 20 seconds, which is the cut point of tissue damage (19).

Static Weight Bearing

The animal was individually conditioned in an acrylic device positioned forward with the front paws supported to the front, and the hind legs supported on a weight measurement sensor (g). The test starts at the moment the animal is immobile, from this

the weight measurement is performed for 10 seconds. At the end of this period, the weight measure of the left and right paws measured, with the right being relative to the stimulus. This test evaluates the weight distribution between the mouse's hind legs. While the unstimulated animal distributes the weight equally between the two legs, the ratio of weight distribution between the stimulated and unstimulated paw is a measure of the level of discomfort in the stimulated paw. By continuous measurement of the weight supported by each paw, the Static Weight Bearing (Bioseb) allows the objective measurement of spontaneous pain by accessing postural balance (19).

Leukocyte migration

The total and differential counts of leukocytes recruited to the intra-articular space were determined as described in the literature (2). Briefly, the knee joint cavities were washed with saline solution containing ethylenediaminetetraacetic acid (EDTA), which was recovered to assess total and differential cell counts. Total cell counts were performed in a Neubauer chamber using Turk solution and differential cell counts (100 cells per field) were stained with the panoptic kit (Laborclin, Pinhais, PR, Brazil) under light microscopy (Olympus CX31RTSF, Tokyo, Japan). Results were expressed as total leukocytes, polymorphonuclear and mononuclear cells (cells x 10^3/knee).

Histological processing

The samples of the femorotibial joint were collected, fixed in buffered formalin and submitted to histological processing, for dehydration with graduated alcohol baths (Dinâmica Química Contemporânea - São Paulo), diaphanization with xylol baths (Synth - Diadema-SP), impregnation in paraffin (Synth - Diadema-SP). Sections of 7 micrometers were performed on the microtome. The slides with the sections were submitted to the staining techniques of hematoxylin (Laborclin - Pinhais-PR) and eosin (Laborclin - Pinhais-PR), and Mallory's trichome - composed of a trio of dyes, being hematoxylin, Mallory red (Laborclin - Pinhais-PR), and Luz green (Laborclin - Pinhais-PR), the latter being anticipated by a phosphomobilitic acid bath (Sigma-Aldrich - Jurubatuba-SP). Through Hematoxylin and Eosin (HE) staining, it was possible to observe the tissue, and perform the analysis through a clinical score, where synovial

hyperplasia, neovascularization and inflammatory infiltrate were evaluated, being scored from 0 to 3, demonstrating a degree of tissue degradation according to the increasing level, with 0 for no alteration and 3 for the most severe alteration, in which case it may be a significant increase in hyperplastic tissue, new vessels and inflammatory infiltrate that damage the tissue. Through Mallory's Trichome staining, it was possible to observe cartilage degradation in the femorotibial joint, since the cartilaginous tissue comes from mesenchymal cells called chondrocytes, and these cells are easily stained by Mallory's trichrome. The analysis was performed with the help of the Image J program, which allowed measuring the percentage of cartilage degradation per area of the tibia and fibula separately.

Statistical analysis:

Data were analyzed using GraphPad Prism statistical software (GraphPad Software, Inc., USA-500.288, version 8.0). Results are expressed as mean ± SE of measurements taken on 6 mice/group per experiment and are representative of 2 separate experiments. One-way or two-way analysis of variance (ANOVA) was used according to the test to compare groups at one time point or groups and doses at all time points. The factors analyzed were treatment, time and interaction time versus treatment. When there was a significant interaction between time versus treatment, one-way ANOVA was performed followed by Tukey's t test for each time point. On the other hand, when nociceptive responses were presented as total values in the indicated time period, differences between responses were evaluated by one-way ANOVA followed by Tukey's t test. Statistical differences were significant at $P<0.05$.

Results

RvD2 decreases mechanical and thermal hyperalgesia, articular edema and the ratio of relative weight of the paws in the model of chronic arthritis induced by TiO_2

The experiment was initiated with a dose-response curve for treatment with RvD2 (1, 3, 10 ng/animal, intraperitoneal [i.p.]) starting 24 h after intra-articular (i.a) injection of the TiO_2 (3 mg/ articulation). Mechanical hyperalgesia (Fig. 1A) and articular edema (1B) was evaluated 0, 1, 5, 7 and 24 hours after treatments and on alternate days until the end of the 30th day. Since the effect of the RvD2 lasted for up to 72 h after administration, it was defined that the treatment would be given to the mice every 3 days. The 10ng dose was selected for the following experiments, since it was the most effective dose, reducing the mechanical hyperalgesia and edema induced by TiO_2. Thermal hyperalgesia in the Hargreaves test (Fig. 1C) and static paw weight distribution (Fig. 1D) and his heat map (Fig. 1E) were evaluated at 0h, 24h after stimulation, 1, 4, 8, 12, 16, 20, 24, and 28 days after the stimulus. RvD2 also reduced thermal hyperalgesia (Fig. 1C) and static weight distribution imbalance (Fig. 1D) caused by TiO_2-induced joint inflammation.

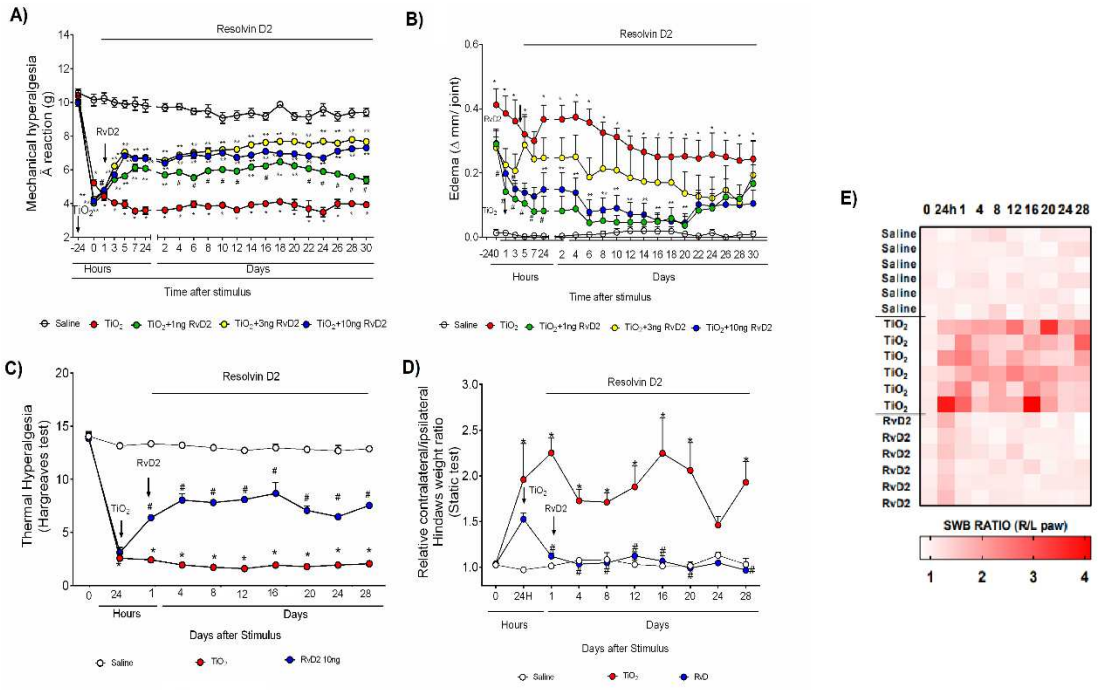

Figure 1: RvD2 decreases mechanical and thermal hyperalgesia, articular edema and the ratio of relative weight of the paws in the model of chronic arthritis induced by TiO2 Mechanical hyperalgesia (Fig. 1A) and edema (Fig. 1B) was evaluated 0, 1, 3, 5, 7 and 24 hours after treatments and on alternate days until the end of the 30th day. Thermal hyperalgesia (Fig. 1C) and relative paw weight ratio (Fig. 1D) and his heat map (Fig. 1E) were evaluated at 0h, 24h after stimulation, and then every 4 days on days 1, 4, 8, 12, 16, 20 , 24 and 28 days after stimulation. With this, it was possible to evaluate the most effective dose of treatment, 10 ng, which was able to reduce mechanical hyperalgesia, thermal hyperalgesia and relative weight ratio of the paws. Results were presented as mean ± standard error (SEM) of 6 mice per experimental group. *p<0.05 in relation to the saline group, **p<0.05 in relation to the TiO_2 group, #p<0.05 in relation to the thio2 groups and other doses of RvD2. (Two-way ANOVA followed by Tukey's post-test).

RvD2 reduces the histopathological changes caused by TiO_2 in the knee joint.

The histopathological analysis was performed. Fig. 2A presents the quantitative score of the histopathological changes caused by TiO_2 in the knee joint in which RvD2 as a post-treatment significantly reduced these changes. Figs. 2B, C and D show representative images of knee joint sections (saline, TiO_2 e RvD2). In figure 2b, it was possible to observe that the joint cavity of the negative control group is preserved and in excellent condition, compared to the group in image 2c, where the joint cavity has an evident leukocyte infiltrate, neovascularization and synovial hyperplasia. In Figure 2D, it is possible to observe all these parameters decreased, demonstrating the effect of RvD2. It is possible to observe an accumulation of TiO_2 in the tissue, characterized by a black pigment.

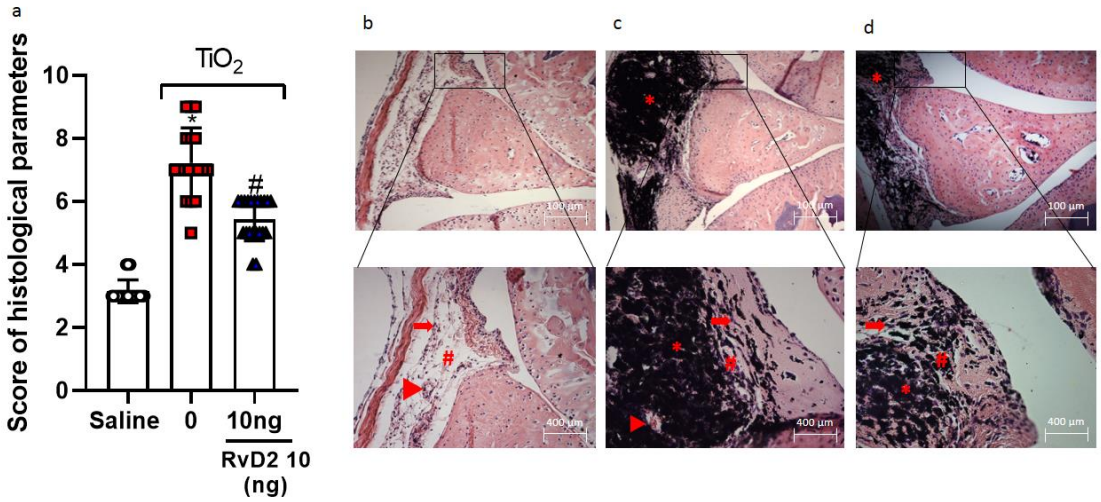

Figure 2: RvD2 decreases synovial hyperplasia, cell recruitment and neovascularization. Animals were treated every 3 days for 30 days with RvD2 (10 ng/animal, intraperitoneal [i.p.]) starting 24 h after intra-articular (i.a) TiO_2 injection (3 mg/joint). Panel (a) shows a graphical representation of the clinical score analysis, (b) the femorotibial space of mice belonging to the saline group, in (c) the femorotibial space of mice stimulated with TiO_2 and treated with vehicle (saline) and in (d) the femorotibial space of mice stimulated with TiO_2 and treated with RvD2. The arrow represents leukocyte recruitment, the arrowhead demonstrates neovascularization, the asterisk represents TiO_2, and the square represents synovial hyperplasia. Results were expressed as mean ± SEM, n = 6 animals per group per experiment, (* P <0.05 vs. saline, #P <0.05 compared to the TiO2 group; One-way ANOVA followed by the post-test Tukey).

RvD2 decreases leukocyte recruitment caused by TiO_2 arthritis.

After observing that RvD2 was able to decrease hyperalgesia in the TiO_2-induced chronic arthritis model, we evaluated its effect on inflammatory parameters. Joint edema was assessed every 2 days during 30 days of treatment. RvD2 decreased joint swelling caused by TiO_2 (Fig. 3A), as well as the recruitment of total (Fig. 3B), mononuclear (Fig. 3C), and polymorphonuclear (Fig. 3D) leukocytes. It was possible to verify an anti-inflammatory effect of this specialized lipid in this experiment.

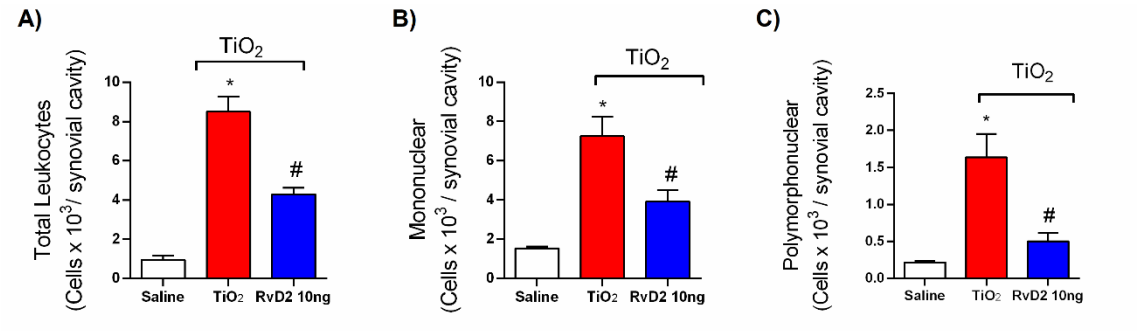

Figure 3: RvD2 decreases the migration of inflammatory cells to the knee joint in the titanium dioxide (TiO_2)-induced arthritis model. Animals were treated every 3 days for 30 days with RvD2 (10 ng/animal, intraperitoneal [i.p.]) starting 24 h after intra-articular (i.a) injection of TiO_2 (3 mg/joint). The recruitment of total (Fig. 3A), mononuclear (Fig. 3B) and polymorphonuclear (Fig. 3C) leukocytes were evaluated by light microscopy at the end of the 30-day model of TiO_2 stimulation. Results were presented as mean ± standard error (SEM) of 6 mice per experimental group. *$p<0.05$ in relation to the saline group and #$p<0.05$ in relation to the TiO_2 groups and other doses of RvD2. (Two-way ANOVA followed by Tukey's post-test).

Discussion

Arthroplasty is a surgical procedure to completely or partially replace a dysfunctional joint. This procedure is important to restore the patient's quality of life, alleviate pain and restore joint function. Despite the importance of arthroplasty, this procedure can fail in 15% of cases, reaching 40% according to previous studies (20,21). Titanium dioxide (TiO_2) is prominent among the biomaterials used for the design of prostheses. TiO_2 is one of the main biomaterials used in metallic prostheses and implants, but it is also the agent causing joint inflammation and failure in prostheses and implants [12,22].

In the present study, it was possible to observe that TiO_2 triggered several parameters in the joint tissue of the mice, showing that this model is very relevant to demonstrate how this component of prostheses can be harmful in some cases to the lives of patients. By treating the mice via intraperitoneal injection with the lipid RvD2, the data obtained here showed that this specialized pro-resolving mediator (SPM) attenuate pain in the mechanical and thermal hyperalgesia tests, edema formation and leukocyte recruitment. RvD2 was able to reduce the difference in the ratio of the relative weight of the paws and also decreased the detrimental histopathological changes caused by TiO_2 in the knee joint, such as leukocyte recruitment, neovascularization, and synovial hyperplasia.

Corroborating this study that demonstrated a decrease in the hyperalgesia and in the leukocyte infiltrate, several evidence in the literature showed the analgesic and anti-inflammatory activity of the lipid RvD2. Spite et al. (23) demonstrated in their work that this lipid generated short-term analgesia in cancer xenograft models, which led to a decrease in neutrophil infiltration and myeloperoxidase (MPO) activity. Using a cancer supernatant model, they demonstrated that RvD2 reduced cancer-derived cytokines/chemokines (TNF-a, IL-6, CXCL10 and MCP-1). Aseptic loosening causes arthroplasty failure, reducing the patient's quality of life, increasing pain and edema formation. TiO_2's nanoparticles (NPs) induce inflammation with leukocyte recruitment, and as seen in Manchope et al. cytokine expression (IL-33, TNFα, pro-IL-1β and IL-6), proving how harmful these particles can be by increasing pain and inflammation in periprosthetic tissue (24). In a model of inflamed obese adipose tissue, the SPMs RvD1 and RvD2 rescued impaired adiponectin expression and secretion, as well as decreased production of pro-inflammatory adipokines, including leptin, TNF-a, IL-6 and IL-1b, demonstrating strong anti-inflammatory potential of these SPMs (25). Still on the

potential analgesic and anti-inflammatory effects of SPMs, in a model with adult FVB mice that underwent unilateral ligation of the carotid artery (vascular model that induces a potent inflammatory response), after the administration of RvD2 and Maresin 1 (MaR1) was observed a reduction of cell proliferation and recruitment of neutrophils and macrophages, besides an increased polarization of M2 macrophages in the arterial wall (27).

Rheumatoid arthritis, for example, is characterized as a debilitating autoimmune disease, containing chronic inflammation of the main joints, presenting an increase in the volume of synovial fluid, large cell infiltrates of various cell types (such as neutrophils, macrophages, and dendritic cells) in the region of synovium, high levels of pro-inflammatory cytokines and eventual erosion and remodeling of cartilage and bone (26). Here, we can observe that treatment with RvD2 inhibited mechanical and thermal joint hyperalgesia, edema, and leukocyte recruitment (polymorphonuclear and mononuclear) induced by TiO_2. Viola and colleagues presented evidence of the imbalance between inflammatory and resolving lipid mediators during atheroprogression. The presence of RvD2 and MaR1 again successfully prevented atheroprogression, suggesting that resolving lipid mediators potentially represents an innovative strategy to resolve yet another different type of inflammation (28).

Conclusion

After several evidence of the potential anti-inflammatory and analgesic action of several SPMs shown so far, it is possible to conclude that RvD2 has great potential of being a possible efficient treatment for TiO_2-induced chronic arthritis.

Data Availability Statement: Data is available upon reasonable request to the corresponding Author.

Conflicts of Interest: The authors declare no conflict of interest.

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6. ARTIGO 3 PARA PUBLICAÇÃO

O presente trabalho foi realizado no laboratório de Dor, Inflamação, Neuropatia e Câncer, da Universidade Estadual de Londrina e segue as normas da revista: Plos one

Resolvin D2 attenuates superoxide anion-induced inflammatory pain by inhibiting IL-1β production

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Abstract

Potassium superoxide (KO_2) intraplantar (i.pl.) or intraperitoneal (i.p.) injection induces inflammatory pain. The aim of this study was to investigate the analgesic effects of resolvin D2 (RvD2) on acute inflammatory pain induced by KO_2. KO_2-induced mechanical and thermal hyperalgesia (hot plate and Hargreaves test) in mice were reduced by i.p. treatment with exogenous RvD2. Also, RvD2 decreased the abdominal contortions, number of paw flinches and time spent licking the paw induced by superoxide. These analgesics effects promoted by RvD2 were, at least in part, due to the inhibition of TRPV1 channel and the paw tissue levels of IL-1β RvD2. In conclusion, RvD2 treatment prevented the superoxide anion-induced inflammatory pain in mice by targeting the pro-inflammatory cytokine IL-1β.

Keyworks: KO_2, Resolvin D2, inflammation, pain,

Introduction

Reactive oxygen species (ROS) are produced during the regular cellular metabolism and its generation is controlled by the redox cycle (1), however some conditions such as acute or chronic inflammation can lead to uncontrolled production of ROS and oxidative stress which result in cell damage and pro-inflammatory effects (2,3). Elevated levels of ROS, mainly superoxide, have been implicated in the development and maintenance of pain (3,4,5). It has been showed by using inhibitors or ROS scavengers that superoxide is responsible for peripheral and central sensitization in models of inflammatory pain, neuropathic pain, and morphine-induced hyperalgesia and tolerance (2,6,37,4,5;9,10,11). We previously demonstrated that intraperitoneal or intraplantar (subcutaneously) injection in the hind paw of mice of potassium superoxide (KO_2), a superoxide anion donor, evokes an inflammatory response by several mechanisms including cytokine production, oxidative stress, leucocyte recruitment (12,13), spinal glial cells (10), activation of the transcription factors NF-κB (14,15) and Nrf-2 (14,16), TNF-α/TNFR1 pathway (17), and endothelin receptors ET_A and ET_B (18,19). Furthermore, KO_2 induces overt pain-like behavior (abdominal contortions and paw flinches and licking), and mechanical and thermal hyperalgesia through the NO−cGMP−PKG−K_{ATP} channel signaling pathway (12,16).

The resolution of inflammation is now understood as an active process starting during the acute phase of the inflammatory response in order to return the tissues to homeostasis thus preventing the detrimental effects of a prolonged inflammation (20,21). The Specialized Pro-Resolving Mediators (SPMs) comprises a family of important molecules synthesized from the omega-three polyunsaturated fatty acids (ω-3 PUFAs) derivatives eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that play a pivotal role in the resolution of inflammation (22). Resolvin D2 (RvD2) is a D-series Resolvin biosynthesized by polymorphonuclear (PMN) cells and/or macrophages through 17-lipoxygenation of DHA (23) that signals via its receptor GPR18 mostly expressed by phagocytic cells (24). It was demonstrated that RvD2 presented potent pro-resolving actions by enhancing macrophage phagocytosis in vitro and polymorphonuclear phagocytosis in vivo, reducing pro-inflammatory cytokines in a mouse model of sepsis (23) and increased phagocytosis-mediated bacterial clearance (25). Furthermore, intrathecal injection of RvD2 prevented inflammatory pain induced by formalin and reversed inflammatory pain induced by Complete Freund's Adjuvant (CFA) by inhibiting

the Transient Receptor Potential Vanilloid 1 (TRPV1) and Ankyrin 1 (TRPA1) channels (26). Moreover, low doses of RvD2 significantly decreased TRPV1 and TRPA1 activation on sensory neurons via lipid raft modification (27). In addition, intrathecal injection of RvD2 relieved pain and alleviated bladder overactivity in a rat model of interstitial cystitis/bladder pain syndrome (28), intrathecal or intravenous administration of RvD2 suppressed mechanical allodynia in a fibromyalgia-like model induced by reserpine in mice (29) and intracerebroventricular injection of RvD2 reversed chronic pain-induced depression-like behavior (30). Considering the resolutive effects of RvD2 in inflammation and pain, in this study the role of RvD2 in KO_2-induced inflammatory pain was investigated.

Material and Methods

Animals

Male Swiss mice (25±5 g) from Universidade Estadual de Londrina were housed in standard plastic cages with free access to food and water, with a 12:12-h light/dark cycle, at 21ºC. Care and handling procedures for the animals were in accordance with the International Association for the Study of Pain (IASP) guidelines and with the approval of the Ethics Committee of the Universidade Estadual de Londrina (process CEUA 003.2021).

Drugs

KO_2 (96.5%) was purchased from Alfa Aesar (Tewksbury, MA, USA). RvD2 was purchased from Cayman Chemical (Ann Arbor, MI, USA); RvD2 (Cayman Chemical, MI, USA); saline solution (NaCl 0.9%) from Frenesius Kabi Brasil Ltda (Aquiraz, CE, Brazil); isoflurane from Abbott Laboratories (Abbott Park, 19 IL, USA).

Experimental procedures

In the first set of experiments mice were treated with vehicle (2% ethanol in saline) or RvD2 at doses of 1, 3, or 10 ng/mice intraperitoneal (i.p.) 30 min before saline or KO_2

injection (30 μg/paw/intraplantar [i.pl.]) and the mechanical and thermal hyperalgesia were accessed for a dose-response screening. The dose of 10 ng of RvD2 was chosen for the other experiments. Next, the Hargreaves test was performed to better characterize the hyperalgesia to heat. Further, the changes in paw weight distribution were measured. After that, the overt pain-like behavior was evaluated through the observations of abdominal contortions and number of flinches and time spent licking the paw. Also, the production of the pro-inflammatory cytokine IL-1β was determined in paw tissue samples 3 h after KO_2 stimulus. The time points selected for measurements and tissue dissection after the injection of KO_2 as well the dose for both stimulus and RvD2 were based on previous studies of our laboratory (12).

Mechanical hyperalgesia

Mechanical hyperalgesia was assessed as previously reported (12). Briefly, in a quiet room, mice were placed in acrylic cages (12×10×17 cm) with wire grid floors at least 30 min before the measurements started. The test consisted of evoking a hindpaw reflex with a hand-held force transducer (electronic anesthesiometer; Insight Equipamentos, Brazil) adapted with a 0.5 mm2 polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hindpaw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements, and the intensity of pressure was recorded automatically. The animals were tested before (basal) and after treatment and stimuli, and the value for each interval was an average of 3 measurements. The results are reported as the change (Δ) in withdrawal threshold (in g), calculated by subtracting the basal mean measurements from the mean measurements obtained at 0.5, 1, 3, 5, or 7 h after the injection of KO_2 (30 μg/paw/i.pl.).

Hot plate test (Thermal hyperalgesia)

Mice were placed in a 10 cm wide glass cylinder on a hot plate (EFF 361, Insight Equipamentos) maintained at 52°C. Two control latencies at least 10 min apart were determined for each mouse. The normal latency (reaction time) was 12-20 s. The latency was also evaluated 0.5, 1, 3, 5, and 7 h after KO_2 injection (30 μg/paw). The reaction time

was scored when the animal lifted or licked the injected paw from the hot plate. A maximum latency (cutoff) was set at 30 s to avoid tissue damage (31).

Hargreaves test (Thermal hyperalgesia – infrared light)

Hyperalgesic responses to heat were determined as described by Hargreaves et al. (32), and a cutoff latency of 15 s was employed to prevent tissue damage in nonresponsive animals. Mice were individually placed in acrylic chambers. A mobile unit consisting of a high-intensity projector bulb was positioned to deliver a thermal stimulus directly to an individual hindpaw from beneath the chamber. The withdrawal latency period of injected and contralateral paws was determined to the nearest 0.1 s with an electronic clock circuit and thermocouple. If the animal failed to respond by 20 s, the test was terminated. Each point represents the delta change (s) in withdrawal latency (withdrawal latency of contralateral minus withdrawal latency of injected paw) at each time point. Results are expressed as paw withdrawal latency changes (s).

Writhing response test

Mice were placed individually in large glass cylinders and the overt pain-like behavior was quantified by counting the total number of writhing (abdominal contortions) occurring between 0 and 20 min after KO_2 injection (1mg/mouse/i.p.). The intensity of nociceptive behavior was expressed as the cumulative number of writhings over 20 min (12).

Paw flinches and time spent licking test

The number of paw flinches and time spent licking the stimulated paw were determined between 0 and 30 min after i.pl. injection of KO_2 (30 µg/paw). Results are reported as the cumulative number of paw flinches and time spent licking the paw over 30 min (12).

Cytokine Measurement

Paw skin tissues were dissected into 500 µL of ice-cold saline and frozen at -80ºC until usage. On the assay day, samples were homogenized using a centrifuged (3000 rpm × 10 min × 4°C), and the resultant supernatants were used to determine IL-1β concentrations by enzyme-linked immunosorbent assay (ELISA) accordingly to manufacturer's instructions (eBioscience). The measurements were conducted at 450 nm. The results were expressed as picograms (pg) of cytokine/mg of paw tissue.

Statistical analysis

Results are reported as means±SE of measurements made on 6 mice/group per experiment and are representative of 2 separate experiments. One-way or Two-way analysis of variance (ANOVA) were used according to the test to compare groups in one time point or groups and doses at all time points. The analyzed factors were treatment, time, and time *vs* treatment interaction. When there was a significant time *vs* treatment interaction, one-way ANOVA followed by Tukey's t-test was performed for each time. On the other hand, when the nociceptive responses were presented as total values at the indicated time period, the differences between responses were evaluated by one-way ANOVA followed by Tukey's t-test. Statistical differences were significant at $P<0.05$.

Results

Resolvin D2 (RvD2) attenuates mechanical and thermal hyperalgesia induced by KO_2

We first performed a dose-response of RvD2 to determine the analgesic effect of this SPM upon superoxide-induced hyperalgesia. The doses of 3 and 10 ng of RvD2 significantly reduced the mechanical hyperalgesia induced by the KO_2 injection at all time points tested with the dose of 10 ng being significantly different of the dose of 1 ng at 0.5, 3, 5, and 7 h (Fig. 1A). The dose of 1 ng of RvD2 only decreased the hyperalgesia at 1, 3, and 5 h after KO_2 injection (Fig. 1A). KO_2 induced thermal hyperalgesia and the doses of 3 and 10 ng of RvD2 attenuated the pain at all time points (Fig. 1B). The

analgesic effect of the 10 ng dose was significant at 1-7 h compared to the lowest dose tested. The dose of 1 ng only diminished the thermal hyperalgesia at 3 and 5 h after KO_2 injection (Fig. 1B). According to these results the dose of 10 ng of RvD2 was chosen for the next experiments.

RvD2 normalizes hind leg weight distribution and thermal hyperalgesia by Hargreaves

Administration of RvD2 reduced the thermal hyperalgesia to the infrared stimulus 3, 5, and 7 hours after the KO_2 injection (Fig. 2A). It was observed that mice injected with KO_2 had the weight of their bodies switched to the left paw (contralateral paw to the stimulus) from 1-5 hours after the injection which culminate in a decrease of the right/left ratio while mice pretreated with RvD2 showed no alteration in the ratio (Fig. 2B).

RvD2 reduces KO_2-induced abdominal contortions (writhing), paw flinches, and time spent licking the paw

Superoxide injection induced writing (Fig. 3A), flinching of the paw (Fig. 3B), and time spent licking the paw (Fig. 3C) in the mice. Pre-treatment with RvD2 30 min before the i.p. stimulus with KO_2 decreased the number of abdominal contortions (Fig. 3A). Furthermore, RvD2 diminished the flinching of the paw (Fig. 3B) and time spent licking the paw (Fig. 3C) induced by the i.pl. injection of KO_2.

RvD2 inhibits KO_2-induced IL-1β production

IL-1β concentration in the paw tissue was measured to evaluate the mechanisms underlying the analgesic effect of RvD2. Treatment with RvD2 inhibited the IL-1β production (Fig. 4).

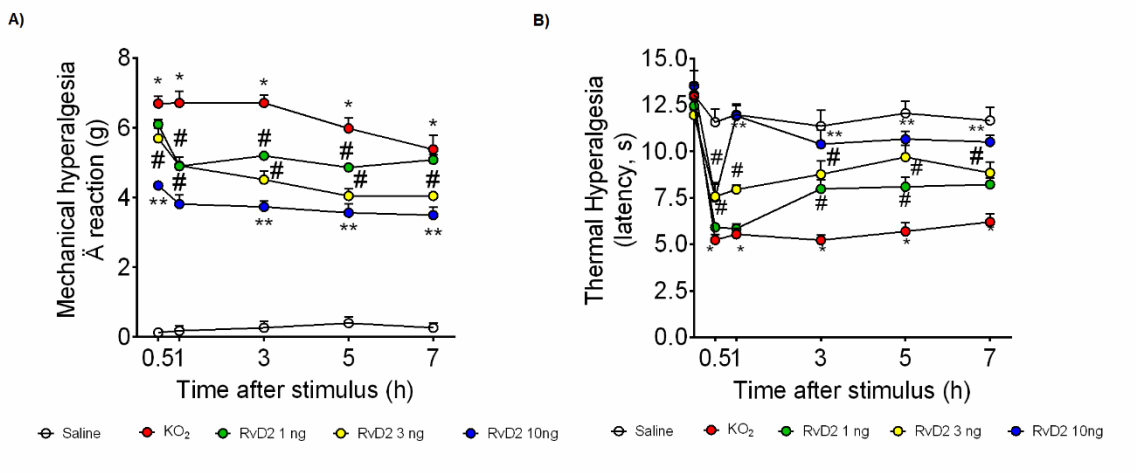

Fig. 1. KO_2-induced mechanical and thermal hyperalgesia are reduced by RvD2. Mechanical hyperalgesia (A) and thermal hyperalgesia (B) were evaluated 0.5, 1, 3, 5, and 7 h after intraplantar injection of KO_2 (30 μg/paw). Mechanical hyperalgesia is presented as Δ withdrawal threshold (in grams) calculated by subtracting the mean measurements at 0.5, 1, 3, 5, and 7 h after KO_2 stimulus from the baseline mean measurements (before stimulus) and thermal hyperalgesia is presented as latency (in seconds). Results are expressed as mean ± SEM, n = six mice per group per experiment, two independent experiments. ∗p < 0.05 vs. saline group, #p < 0.05 vs. KO_2 group, ∗∗p < 0.05 vs 1 ng; two-way repeated measures ANOVA followed by Tukey's post-test

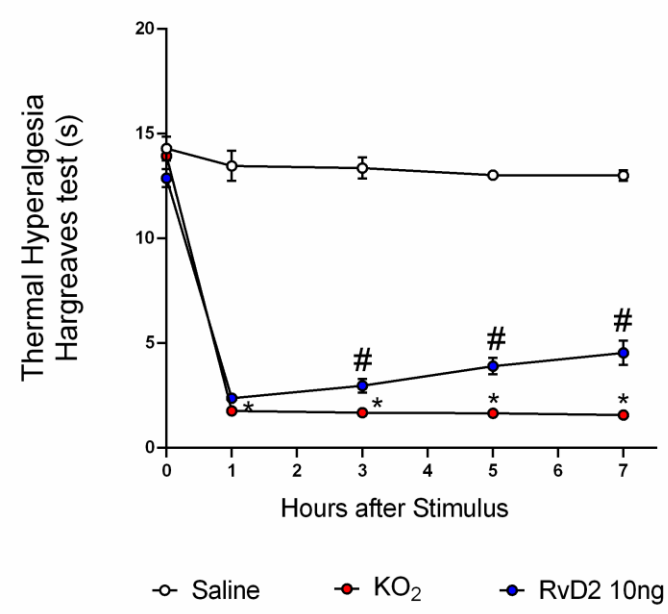

Fig. 2. RvD2 attenuates the hyperalgesia to the heat and the pain sensation induced by KO_2. Hargreaves test was another method to evaluate the thermal hyperalgesia. Measurements were performed at 1, 3, 5, and 7h after KO_2 stimulation (A). Results are presented as latency (in seconds). SWB test was used as a non-reflexive method of pain measurement (B). Results are presented as the weight ratio of Right/Left rear paw. Results are expressed as mean ± SEM, n = six

mice per group per experiment, two independent experiments. *p < 0.05 vs. saline group; #p < 0.05 vs. KO_2 group, two-way repeated measures ANOVA followed by Tukey's post-test.

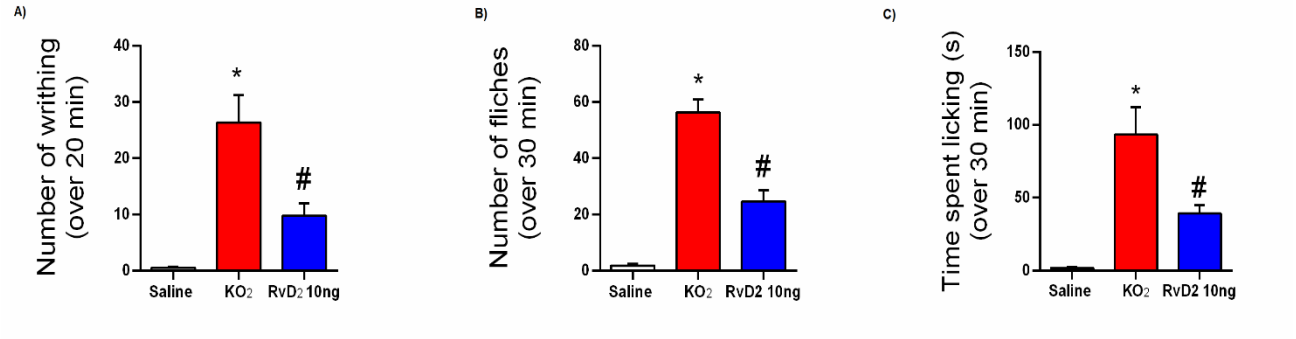

Fig. 3. RvD2 decreases writhing, flinching of the paw, and time spent licking the paw induced by superoxide anion. The number of abdominal contortions over 20 min (A) after intraperitoneal injection of KO_2 (1 mg/cavity) was evaluated. The number of paw flinches (B) and time spent licking the paw (C) were determined over 30 min after intraplantar injection of KO_2 (30 µg/paw). Results are shown as mean ± SEM, n = six mice per group per experiment, two independent experiments. ∗p < 0.05 vs. saline group; #p < 0.05 vs. KO_2 group, one-way ANOVA followed by Tukey's post-test.

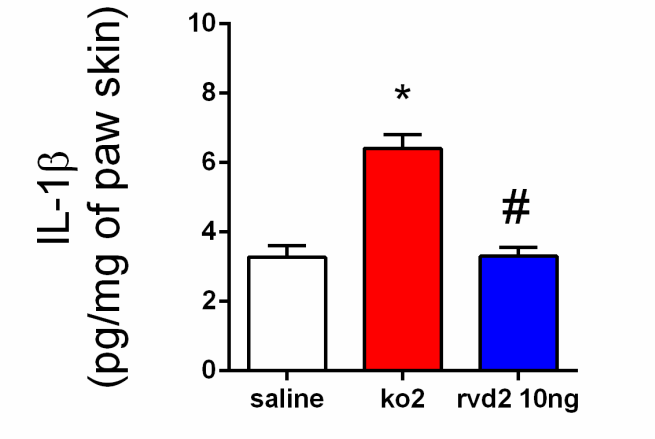

Fig. 4. RvD2 prevents KO_2-induced IL-1β production. Mice received RvD2 treatment 30 min before i.pl. injection of KO_2. Paw skin samples were collected 3 h after KO_2 injection and processed by ELISA. Results are mean ± SEM of 6 mice per group per experiment and are representative of 2 independent experiments. *p< 0.05 vs. saline group, #p< 0.05 vs. KO2 group. One-way ANOVA followed Tukey's post-test.

Discussion

During resolution phase of self-limited inflammation, ω-3 PUFAs are converted by recruited leucocytes to SPMs that act on targeted cells to promote its effects that limit inflammation and restore homeostasis (33). The effects of superoxide as a mediator of inflammation and pain have been previously described (2,3,12). Here, we demonstrated that i.p. injection of synthetic RvD2, a member of the resolvin family series-D, ameliorated mechanical and heat hyperalgesia, inhibited pain-like behaviors, and reduced the paw tissue IL-1β production induced by potassium superoxide (KO_2), a superoxide anion donor.

RvD2 possesses anti-inflammatory and analgesic effects by reducing oxidative stress and expression of VEGF, NF-κB, TNF-α, and MCP-1 (34) and inhibiting TRPV1 and TRPA1 channels 35,36) Our results suggest that RvD2 significantly reduced the mechanical hyperalgesia and overt pain-like by inhibiting the release of the inflammatory cytokine IL-1β. Besides, RvD2 treated mice showed no discomfort in the injured paw as evaluated by the incapacitance test. These results rise the hypothesis of a direct or indirect effect of RvD2 in the inhibition of the NLRP3 inflammasome since this mechanism is responsible for the conversion of pro-IL-1β in its active form. However, additional data are necessary to support this mechanism.

TRPV1 receptors are present in sensory neurons and can be activated by a variety of noxious stimuli leading to thermal hyperalgesia (37). Superoxide presents neuronal effects via activation of TRPA1 and TRPV1 (38) and can directly activate nociceptive neurons evoking pain behavior. In this sense, the above data suggests that superoxide anion induces thermal hyperalgesia via TRPV1 activation and RvD2 acts on these receptors in order to inhibit the hyperalgesia to heat.

Altogether, the data obtained in this study reveals that RvD2 may be a promising lead for the development of novel analgesic therapies. Although, further studies are important to better understand the mechanisms and implications of using exogenous SPMs for the treatment of inflammation and pain.

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7.Conclusão

Neste trabalho, demonstramos o potencial analgésico e anti-inflamatório da RvD1 e RvD2 em modelo de artrite crônica induzida por TiO_2 e modelo de inflamação aguda iniciada pelo ânion superóxido através da administração do seu doador, o KO_2. A RvD1 e RvD2 foi capaz de promover a diminuição da hiperalgesia mecânica e térmica, formação de edema, testes de dor manifesta (número de agitações da pata, tempo lambendo a pata e contorções abdominais), recrutamento leucocitário, diminuição de citocinas e parâmetros histológicos agravados pelo estímulo crônico. Isso sugere que a RvD1 e RvD2 tem efeitos anti-inflamatórios e analgésicos em modelos agudos (KO_2) e crônicos (TiO_2).

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Anexos

1:

Universidade Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 151/2016 **Londrina, 15 de Julho de 2016.**

Prezado Pesquisador,

Certificamos que o projeto intitulado **"Avaliação do efeito analgésico dos mediadores lipídicos pró-resolução resolvina D1 (RvD1) resolvina D2 (RvD2), resolvina D5 (RvD5), maresina 1 (Mar1), maresina 2 (Mar2), protectina (PD1), 15-epi-lipoxina A4 (ATLA4) e lipoxina A4 (LxA4) em modelo de artrite induzido por TiO_2"**, protocolo CEUA nº **11147.2016.40**, 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 **05/07/2016**.

O objetivo do projeto investigar os mecanismos dos mediadores lipídicos pró-resolução RvD1, RvD2, RvD5, Mar1, Mar2, PD1, ATLA4 e LxA4 em modelo de hiperalgesia induzido por dióxido de titânio (TiO_2). Os animais serão divididos em gaiolas de polipropileno padrão medindo 41 X 34 X 16 CM (Insight®) no biotério de acordo com os grupos experimentais (máximo de 12 animais por gaiola), com livre acesso à água e ração e serão adaptados aos ambientes e condições experimentais com pelo menos 1 hora de antecedência em relação aos experimentos. Os procedimentos de cuidado e manuseio de animais estarão de acordo com as diretrizes da Associação Internacional de Estudo da Dor (IASP). A utilização de fármacos analgésicos não é realizada uma vez que estes podem interferir na resposta analisada, e desta forma prejudicar a interpretação dos resultados. A eutanásia será realizada por Isofluorano 1,5 a 3% em O_2 e serão posteriormente decapitados. GI 2.

Vigência do Projeto	01/01/2017 a 01/12/2022
Espécie/linhagem	Camundongo heterogênico / Swiss
Nº de animais	672
Peso/Idade	20-25 g / 2 meses
Sexo	Machos
Origem	Biotério Central / UEL
Amostras a serem coletadas	Articulação

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,

Profa. Dra. Glaura Scantamburlo Alves Fernandes
Coordenadora 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 Coord. do Biotério Central/UEL; Chefe do Departamento de Ciências Patológicas e Diretor(a) do Centro de Ciências Biológicas

Campus Universitário: Rodovia Celso Garcia Cid (PR 445), km 380 - Fone (043) 3371-4000 PABX - Fax 3328-4440 - Caixa Postal 10.011 - CEP 86057-970 – Internet http://www.uel.br
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2:

Universidade
Estadual de Londrina

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

OF. CIRC. CEUA Nº 024/2021

Londrina, 26 de março de 2021.

Prezado (a) professor (a),

Certificamos que o projeto intitulado: **"Avaliação do Efeito e Mecanismos Anti-Inflamatórios e Analgésicos das Resolvinas D1, D2 e D5 (RvD1, RvD2 E RvD5), Maresinas 1 e 2 (MaR1 e MaR 2), da Protectina DX, da Lipoxina A4 e da 15-epi-Lipoxina A4 em Modelo de Dor Induzida pelo Superóxido de Potássio (KO_2) em Camundongos"** protocolo CEUA nº **003.2021** 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), e foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Londrina (CEUA/UEL) no dia 26/03/2021.

O projeto tem como objetivo avaliar o efeito dos mediadores lipídicos pró-resolução (SPMs) na dor inflamatória induzida pelo superóxido de potássio (KO_2 - um doador de ânion superóxido). **Grau de invasividade: 3**.

Finalidade	( ) Ensino (X) Pesquisa científica
Vigência da autorização	01/05/2021 a 30/04/2025
Espécie/ linhagem/ raça	Camundongo heterogênico/ Swiss
Nº de animais	**6640**
Peso/ Idade	25-25 g/ 1-2 meses
Sexo	Machos.
Origem	Biotério Central da Universidade Estadual de Londrina
Amostras a serem coletadas	Tecido subcutâneo plantar, medula espinal, gânglio da raiz dorsal, sangue, estômago.

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

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

Profª Drª Maria Fernanda Rodrigues Graciano
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Estadual de Londrina

Profª. Drª Maria Fernanda Rodrigues Graciano
Coordenadora da CEUA/UEL

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