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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Renata da Silva Pereira Saccol

**EFEITO DA QUERCETINA NA SINALIZAÇÃO PURINÉRGICA E NO
METABOLISMO OXIDATIVO-INFLAMATÓRIO EM MODELO DE
ARTRITE**

Santa Maria, RS, Brasil
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Tese apresentada ao Curso de Pós-Graduação em Ciências Farmacêuticas, Área de Concentração em Análises Clínicas e Toxicológicas, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de **Doutora em Ciências Farmacêuticas**

Orientador: Prof^ª. Dra. Daniela Bitencourt Rosa Leal

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DEDICATÓRIA

Dedico este tese a toda a minha família que eu amo muito, em especial, ao meu filho Henrique, que é o grande amor da minha vida.

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RESUMO

EFEITO DA QUERCETINA NA SINALIZAÇÃO PURINÉRGICA E NO METABOLISMO OXIDATIVO-INFLAMATÓRIO EM MODELO DE ARTRITE

AUTORA: Renata da Silva Pereira

ORIENTADOR: Prof^ª. Dr^ª. Daniela Bitencourt Rosa Leal

A artrite reumatoide (AR) é uma doença inflamatória, autoimune, crônica, com envolvimento predominante da membrana sinovial das pequenas articulações e destruição progressiva de cartilagem e osso e eventualmente são observadas manifestações extra-articulares, como danos hepáticos, renais e vasculares. O sistema de sinalização purinérgica desempenha um importante papel na modulação das respostas inflamatórias e imunes, através das biomoléculas extracelulares, como os nucleotídeos de adenina, e seu derivado adenosina, cujas concentrações extracelulares são controladas por ação de ectoenzimas (E-NTPDase, E-5'-nucleotidase e E-ADA) presentes em superfícies de diversas células. Além disso, inúmeros estudos demonstraram o envolvimento do estresse oxidativo na patogênese das artropatias inflamatórias, como a AR. A quercetina é um flavonoide presente em diversos alimentos e com propriedades anti-inflamatórias e antioxidantes bem estabelecidas em diferentes modelos experimentais de doenças crônicas. No presente trabalho, investigou-se o efeito da quercetina na sinalização purinérgica e no metabolismo oxidativo-inflamatório em um modelo de artrite induzida por adjuvante (AIA). Ratos *Wistar* fêmeas foram divididos em grupos com e sem indução de artrite. Foram realizados testes de escore de artrite, edema de pata e hiperalgesia termal antes da indução. O adjuvante completo de Freund (CFA) foi injetado na pata traseira e, após 15 dias da indução, para a confirmação, estes testes foram repetidos. No 15º dia iniciou-se o tratamento com salina e quercetina nas doses de 5, 25 e 50 mg/kg, durante 45 dias. No fim do tratamento, os testes para comprovação da artrite foram novamente repetidos, além da atividade das ectoenzimas em linfócitos. Avaliações dos parâmetros do estresse oxidativo foram analisadas em soro, plasma e em tecido hepático e renal, além da dosagem das enzimas hepáticas no soro, da atividade da mieloperoxidase no plasma, bem como das citocinas IFN- γ e IL-4 e marcadores de dano ao DNA. Os resultados demonstraram que o modelo foi capaz de gerar um processo inflamatório, devido ao aumento do escore da artrite, do edema de pata e da diminuição da hiperalgesia termal. Observou-se também um aumento da atividade da E-NTPDase e diminuição da E-ADA em linfócitos, além de um aumento nos níveis de AST, da atividade da mieloperoxidase, da secreção do IFN- γ e da IL-4, bem como dos níveis de EROs em soro, fígado e rim, TBARS em plasma, aumento do dano ao DNA e diminuição das enzimas antioxidantes catalase (CAT) e superóxido dismutase (SOD). O tratamento com a quercetina foi capaz de reduzir os sinais e sintomas do processo inflamatório, além de alterar as atividades das ectoenzimas, reduzir os níveis de AST e os níveis de dano ao DNA, e ainda de proteger contra os danos causados pelo estresse oxidativo. Assim, podemos sugerir que a quercetina evidenciou excelente efeito anti-inflamatório, e antioxidante, mostrando ser um candidato promissor para a terapia adjuvante para o tratamento da artrite reumatoide.

Palavras-chave: Artrite. Quercetina. Sinalização Purinérgica. Estresse oxidativo.

ABSTRACT

EFFECT OF QUERCETIN ON PURINERGIC SIGNALLING AND OXIDATIVE-INFLAMMATORY METABOLISM IN ARTHRITIS MODEL

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ADVISOR: Prof^ª. Dr^ª. Daniela Bitencourt Rosa Leal

Rheumatoid arthritis (RA) is a chronic, autoimmune, inflammatory disease with predominant involvement of the small joints, progressive destruction of cartilage and bone, and extra-articular manifestations such as liver, kidney, and vascular damage. The purinergic signaling system plays an important role in the modulation of inflammatory and immune responses through extracellular biomolecules, such as adenine nucleotides, and its adenosine derivative, whose extracellular concentrations are controlled by ectoenzymes (E-NTPDase, E-5' nucleotidase, and E-ADA) present on the surface of several cells. Besides, numerous studies have demonstrated the involvement of oxidative stress in the pathogenesis of inflammatory arthropathies, such as RA. Quercetin is a flavonoid present in several foods and with well-established anti-inflammatory and antioxidant properties in different experimental models of chronic diseases. In the present study, the effect of quercetin on purinergic signaling and oxidative inflammatory metabolism in model of adjuvant-induced arthritis (AIA) was investigated. Female Wistar rats were divided into groups with and without induction of arthritis. Arthritis score, paw edema, and thermal hyperalgesia were performed before induction. In the arthritis groups, the complete Freund's adjuvant (CFA) was then injected into the hind paw and, after 15 days induction, for confirmation, these tests were repeated. On the 15th day, saline and quercetin treatments started at doses of 5, 25 and 50 mg / kg for 45 days. At the end of the treatment, the tests for evidence of arthritis were repeated, in addition to the activity of ectoenzymes in lymphocytes. Oxidative stress parameters were analyzed in serum, plasma, and hepatic and renal tissue, as well as serum hepatic enzymes, plasma myeloperoxidase activity, IFN- γ and IL-4 levels in serum, and DNA damage markers. The results demonstrated that an inflammatory process was generated, as seen by the increase in the arthritis score and paw edema and the reduction of the thermal hyperalgesia. There was also an increase in E-NTPDase activity and a decrease in E-ADA activity in lymphocytes, in addition to an increase in AST levels, myeloperoxidase activity, IFN- γ and IL-4 secretion, as well as levels of EROs in serum, liver, and kidney EROs, plasma T-BARS, increased DNA damage, and decreased antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD). The treatment with quercetin reduced the signs and symptoms of arthritis, besides altering the activities of the ectoenzymes, reducing the levels of AST and DNA damage, and protect against damage caused by oxidative stress. Thus, we can suggest that quercetin showed an excellent anti-inflammatory and antioxidant effect, proving to be a promising candidate for adjuvant therapy for the treatment of rheumatoid arthritis.

Keywords: Arthritis. Quercetin. Purinergic Signaling. Oxidative stress.

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LISTA DE ABREVIATURAS E SIGLAS

ACR	Colégio Americano de Reumatologia
ACPA	Anticorpos Anti-Proteína/Peptídeo Citrulinado
ADO	Adenosina
ADP	Adenosina Difosfato
AIA	Artrite Induzida por Adjuvante
AIC	Artrite Induzida por Colágeno
AIDS	Síndrome da Imunodeficiência Adquirida
AINHS	Anti-inflamatórios Não Hormonais
ALT	Alanina Aminotransferase
AMP	Adenosina Monofosfato
ANTI-CCP	Antipeptídeo Cíclico Citrulinado
AR	Artrite Reumatoide
ATP	Adenosina Trifosfato
AST	Aspartato Aminotransferase
CAT	Catalase
CFA	Adjuvante Completo de Freund
COXs	Cicloxigenases
Cu-Zn-SOD	Cobre-Zinco-SOD
DCV	Doença Cardiovascular
DMCDs	Drogas Modificadoras do Curso da Doença
DNA	Ácido Desoxirribonucleico
dsDNA	DNA de Fita Dupla
EAM	Manifestações Extra-Articulares
E-ADA	Ecto-Adenosina Desaminase
EDTA	Ácido Etilenodiamino Tetra-Acético
E-NPP	Nucleosídeo Pirofosfato/Fosfodiesterase
E-NTPDase	Ecto-nucleosídeo Trifosfato Difosfohidrolase
EROs	Espécies reativas de oxigênio
EULAR	Liga Europeia contra o Reumatismo
FAL	Fosfatase Alcalina
FR	Fator reumatoide
GPI	Glicosil-Fosfatidilinositol
GSH	Glutationa Reduzida
GPx	Glutationa Peroxidase
GST	Glutationa S-Transferase
H ₂ O ₂	Peróxido de Hidrogênio
HIV	Vírus da Imunodeficiência Humana
HLA	Antígeno Leucocitário Humano
HNO ₂	Ácido Nitroso
HO	Hidroxila
HOCl	Ácido Hipocloroso
IFN- γ	Interferon Gama
IL-2	Interleucina 2
IL-4	Interleucina 4
IL-1 β	Interleucina 1- beta
IL-5	Interleucina 5
IL-17	Interleucina 17

IL-18	Interleucina 18
iNOS	Óxido Nítrico Sintase Indutível
LES	Lúpus Eritematoso Sistêmico
MDA	Malondialdeído
MnSOD	Manganês SOD
NF-κB	Fator Nuclear Kappa β
N ₂ O ₃	Óxido Nítrico
NO	Óxido Nítrico
NO ₂	Nitritos
NO ₃	Nitratos
O ₂	Superóxido
ONOO	Peroxinitritos
PADI4	Peptidilarginina Deiminase 4
PGIA	Artrite Induzida por Proteoglicano
PUFA	Ácidos Graxos Poliinsaturados
Q3GA	3-O-β-D- Glucuronidato
RM	Ressonância magnética
RO	Alcoxila
ROO	Peroxila
SE	Epítipo compartilhado
SH	Grupos Tióis
SOD	Superóxido Dismutase
TBARS	Substâncias Reativas ao Ácido Tiobarbitúrico
Th	Células T Auxiliares
TNF-α	Fator de Necrose Tumoral Alfa
US	Ultrassonografia

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APRESENTAÇÃO

A presente tese foi redigida de acordo com as normas vigentes no Manual de Dissertações e Teses (MDT de 2015) e no Regulamento do PPG em Ciências Farmacêuticas, da Universidade Federal de Santa Maria, sendo organizada da seguinte maneira: Introdução, Revisão Bibliográfica, Objetivos, Manuscritos, Discussão, Conclusão, Perspectivas, Referências e Anexos.

A seção Introdução apresenta o embasamento teórico que abrange uma breve revisão da literatura e a justificativa para o desenvolvimento do trabalho.

A seção Revisão Bibliográfica traz o embasamento teórico através da revisão da literatura dos temas relevantes desta tese.

Os resultados que fazem parte desta tese estão apresentados sob a forma de três manuscritos (Manuscrito I, Manuscrito II e Manuscrito III), os quais se encontram no item Manuscritos e, representam a íntegra deste estudo. Os itens Materiais e métodos, Resultados, Discussão e Referências específicas encontram-se compondo cada manuscrito.

As seções Discussão e Conclusão, encontrados no final desta tese, apresentam interpretações e comentários gerais a respeito dos resultados contidos nos manuscritos.

A seção Referências lista somente às citações utilizadas nos itens Introdução, Revisão Bibliográfica e Discussão desta tese.

O item Anexo contém as cartas de aprovação da Comissão de Ética no Uso de Animais da UFSM e as cartas de submissão dos referidos manuscritos.

1 INTRODUÇÃO

A artrite reumatoide (AR) é uma artropatia inflamatória, crônica, autoimune, que afeta predominantemente as articulações sinoviais, e é caracterizada pela degradação, erosão e destruição da cartilagem e ossos (SOKKA et al., 2009; MA et al., 2018). A prevalência desta doença é de aproximadamente 0,5 a 1,0% na população mundial (KLEIN; KAROUZAKIS; GAY, 2018) e no Brasil varia de 0,2 a 1,0% (GOMES et al., 2017). Os pacientes com AR apresentam um aumento da mortalidade em comparação com a população geral, em particular devido a doenças cardiovasculares (DCV) (MARADIT-KREMER et al., 2005; BERGSTRÖM et al., 2018).

Além de afetar o sistema articular, esta doença pode atingir sistemas extra-articulares (CHOY, 2012). As manifestações extra-articulares podem ser: amiloidose, vasculite reumatoide, comprometimento pulmonar e neurológico, além de anormalidades cardíacas, musculares, renais e hepáticas (OLIVEIRA et al., 2015). Estas manifestações desempenham um papel importante como preditor de mortalidade precoce em pacientes com AR (BONGARTZ et al., 2007; COJOCARU et al., 2010).

Uma das características registradas na patogênese da AR é a sinovite persistente, que é resultante do influxo de células imunes nas articulações. Neste cenário, as células T efetoras juntamente com as células B e outras células efectoras inatas, formam uma rede complexa que promove a produção de citocinas pró-inflamatórias (MCINNES; SCHETT, 2007; SCHETT et al., 2013; CHEN et al., 2018). As células T CD4⁺ (Th) desempenham um papel importante no desenvolvimento da AR. Durante a ativação de células T em um ambiente específico de citocinas, essas células T CD4⁺ são polarizadas em subpopulações em fenótipos de células Th, incluindo Th1, Th2, Th17 e células T reguladoras (Treg), que são definidas por seus padrões individuais de produção de citocinas e das suas funções efectoras. As células Th1 são pró-inflamatórias e secretam interferon (IFN- γ), fator de necrose tumoral (TNF- α) e interleucina (IL -1 β), que promovem o processo inflamatório e dano articular (MU et al., 2009; NIU et al., 2017), enquanto as células Th2 secretam interleucina-4 (IL-4) e interleucina-5 (IL-5) e induzem a imunidade humoral (FARHADI et al., 2014).

No processo inflamatório decorrente da artrite, as respostas imunes e inflamatórias ativadas geram um desequilíbrio entre as citocinas pró-inflamatórias e anti-inflamatórias favorecendo assim a indução da autoimunidade (BURNSTOCK; KNIGHT, 2004). Entre os mediadores que sinalizam as respostas a diversas condições fisiológicas ou patológicas está o sistema purinérgico. O sistema purinérgico é constituído por enzimas, nucleotídeos,

nucleosídeos e receptores purinérgicos. As biomoléculas extracelulares, como os nucleotídeos de adenina, adenosina trifosfato (ATP), adenosina difosfato (ADP) e adenosina monofosfato (AMP), e seu derivado nucleosídeo, adenosina, os quais são secretados por leucócitos, plaquetas e células endoteliais danificadas, medeiam diversos efeitos biológicos, incluindo a contração do músculo liso, a neurotransmissão, a resposta imune, a inflamação e a agregação plaquetária (RALEVIC e BURNSTOCK, 1998).

As enzimas atuam na regulação dos níveis extracelulares dos nucleotídeos e nucleosídeos. A enzima ectonucleosídeo trifosfato difosfohidrolase (E-NTPDase, CD39, EC 3.6.1.5) catalisa a desfosforilação sequencial do nucleosídeo trifosfato de adenosina (ATP \rightarrow ADP \rightarrow AMP), enquanto a enzima 5'-nucleotidase (CD73, EC 3.1.3.5) catalisa a desfosforilação do nucleosídeo monofosfato (AMP \rightarrow ADO) e a enzima adenosina desaminase (E-ADA, EC 3.5.4.4) a desaminação da adenosina (ADO) em inosina (ZIMMERMANN, 2001; COLGAN et al., 2006; YEGUTIKIN, 2008).

Outro aspecto importante que deve ser considerado em doenças com inflamação persistente é a presença do estresse oxidativo, que contribui para o dano tecidual, e consequentemente para a sua cronicidade. O acúmulo das espécies reativas de oxigênio (EROs) danifica proteínas, lipídios e DNA, e também pode servir como importante moléculas sinalizadoras intracelulares que amplificam a resposta inflamatória proliferativa sinovial (FILIPPIN et al., 2008; HIRAO et al., 2012). Os danos causados pelo estresse oxidativo podem ser protegidos por sistemas enzimáticos antioxidantes, representados pelas enzimas superóxido dismutase (SOD), catalase (CAT) e glutatona peroxidase (GPx) e por sistemas não enzimáticos, como a vitamina C, a vitamina E, os carotenoides e os flavonoides (GUTTERIDGE; HALLIWELL, 2010; VASILJEVIC et al., 2016).

Dentre todos os flavonoides, destaca-se, a quercetina, que é encontrada em vegetais, frutas, grãos e nozes (HARNLY et al., 2006; GARCIA MATEOS et al., 2013; HALEAGRAHARA et al., 2017). Ela apresenta significativos efeitos antioxidantes e citoprotetores (CHOI et al., 2003; AFIFI et al., 2018) e várias ações farmacológicas, incluindo efeitos anti-inflamatórios, hepatoprotetores, renoprotetores e neuroprotetores (CAI et al., 2013; AFIFI et al., 2018). Estudos demonstram que a quercetina apresenta efeitos antioxidantes e anti-inflamatórios, incluindo a inibição de células imunes, tais como macrófagos, e da secreção de citocinas inflamatórias, tais como interferon- γ (IFN- γ), fator de necrose tumoral alfa (TNF- α) e a interleucina-2 (IL-2) (JAVADI et al., 2016).

Sendo assim, torna-se necessário a busca de estratégias terapêuticas a fim de melhorar a qualidade de vida dos pacientes com AR. Desta forma, é de interesse científico e clínico,

avaliar se a quercetina, através de suas ações, anti-inflamatória e antioxidante, tem um efeito positivo no tratamento desta doença autoimune. Para isto, é necessário esclarecer os mecanismos moleculares envolvidos nestes efeitos, inclusive aqueles relacionados à sinalização purinérgica e ao estresse oxidativo. Desta forma, a partir deste estudo, espera-se contribuir para a busca de uma terapia complementar que possa beneficiar os pacientes com AR.

2 REVISÃO BIBLIOGRÁFICA

2.1 ARTRITE REUMATOIDE

A AR é a doença autoimune reumatoide mais prevalente. É caracterizada pela inflamação sinovial crônica e dano progressivo da cartilagem articular e do osso (SMOLEN; ALETAHA; MCINNES, 2016; MCINNES; SCHETT, 2017; REYES-CASTILHO; MUÑOZ-VALLE; LIAMAS-COVARRUBIAS et al., 2018). A característica principal da AR é o acometimento simétrico das pequenas e das grandes articulações, com maior frequência de envolvimento das mãos e dos pés. O caráter crônico e destrutivo da doença pode levar a importante limitação funcional, com perda de capacidade laboral e de qualidade de vida, a menos que o diagnóstico seja feito em fase inicial da doença e o tratamento determine melhora clínica (MOTA et al., 2012).

A prevalência desta doença é predominante no sexo feminino, com tendência a surgir após a quarta década de vida, com pico de incidência na quinta década (BRENOL et al., 2007). As taxas de mortalidade em pacientes com AR são mais elevadas do que na população em geral (as razões de mortalidade variam de 1,28 a 2,98) (KITAS; GABRIEL, 2011; NOGUEIRA et al., 2015) e a expectativa de vida é reduzida em até 3 a 5 anos, especialmente em pacientes que desenvolvem efeitos adversos relacionados com o tratamento, incluindo infecções, tumores e toxicidade gastrointestinal de fármacos usados em terapia de AR (KOOTTA et al., 1977; DAVIS; MATTESON, 2012; NOGUEIRA et al., 2015).

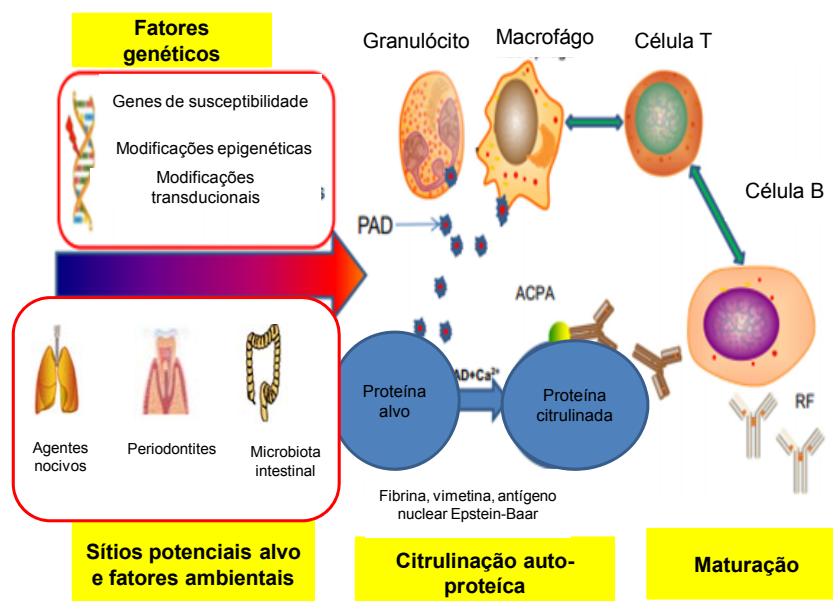
2.1.1 Etiologia

A AR se origina de uma combinação de fatores genéticos e fatores ambientais, que quando presentes aumenta a suscetibilidade para o desenvolvimento das manifestações clínicas. Os fatores genéticos estão ligados a uma série de genes que carregam informações relacionadas com a AR. Esses genes são especialmente aqueles que regulam os genes do antígeno leucocitário humano (HLA), e alguns outros fatores, como promotores de citocinas, genes de sinalização de células T, dentre outros fatores (KOURILOVITCH; GALARZA-MALDONAD; ORTIZ-PRADO, 2014). O fator genético contribui com 60% da susceptibilidade da AR. Os fatores genéticos estão fortemente associados à positividade do anticorpo antipeptídeo cíclico citrulinado (anti-CCP) e à resposta do paciente ao tratamento (TURESSON; MATTESON, 2006; TAN et al., 2010; GOELDNER et al., 2011).

Os agentes infecciosos e os seus produtos também têm sido associados à AR, por exemplo: *Escherichia coli*, vírus Epstein-Barr, citomegalovírus, espécies de *Proteus* e proteínas de choque térmico. No entanto, o mecanismo de ação ainda permanece ambíguo, mas algumas hipóteses são feitas em relação ao mimetismo molecular. Da mesma forma, a AR também está ligada a doença periodontal, pois a bactéria, *Porphyromonas gingivalis* expressa a enzima peptidilarginina deiminase 4 (PADI4), o que resulta em citrulinização de proteínas (ALAM et al., 2017).

O tabagismo tem sido implicado como um potencial gatilho ambiental, estando associado à incidência de AR em indivíduos com suscetibilidade ao alelo HLA, denominado epítipo compartilhado (SE) (ENTEZAMI et al., 2011). Foi observado que o cigarro foi capaz de acelerar as reações de citrulinização em proteínas pulmonares, consequentemente aumentando os níveis de autoanticorpos (KLARESLOG; PADYUKOV; ALFREDDSON, et al., 2007). Na figura 1 estão ilustrados os fatores de risco que desencadeiam a AR.

Figura 1 - Fatores de riscos desencadeantes da AR



Fonte: Adaptado de Guo et al. (2018).

2.1.2 Patogênese

A AR é resultante da ação das células T e B autorreativas, que levam à sinovite, a infiltração celular e a um processo desorganizado de destruição e remodelação óssea (GOELDNER et al., 2011). A membrana sinovial é a principal fonte de citocinas pró-inflamatórias e proteases e, em conjunto com osteoclastos e condrócitos, promove a destruição articular. As projeções de tecido proliferativo penetram na cavidade articular, invadindo a cartilagem e o tecido ósseo, formando o *pannus*, característico da AR (FIRESTEIN, 2003; GOELDNER et al., 2011).

O evento inicial da doença é o processo inflamatório que ocorre na membrana sinovial com infiltrado de linfócitos e macrófagos (HARRIS, 1997). O osteoclasto, uma célula multinucleada que possui capacidade de reabsorver tecido ósseo (HERMAN et al., 2008), é uma das principais células envolvidas no processo de erosão óssea inflamatória (GOLDRING, 2003). A ativação e o recrutamento de tais células são influenciados por citocinas e mediadores inflamatórios (GOLDRING, 2002).

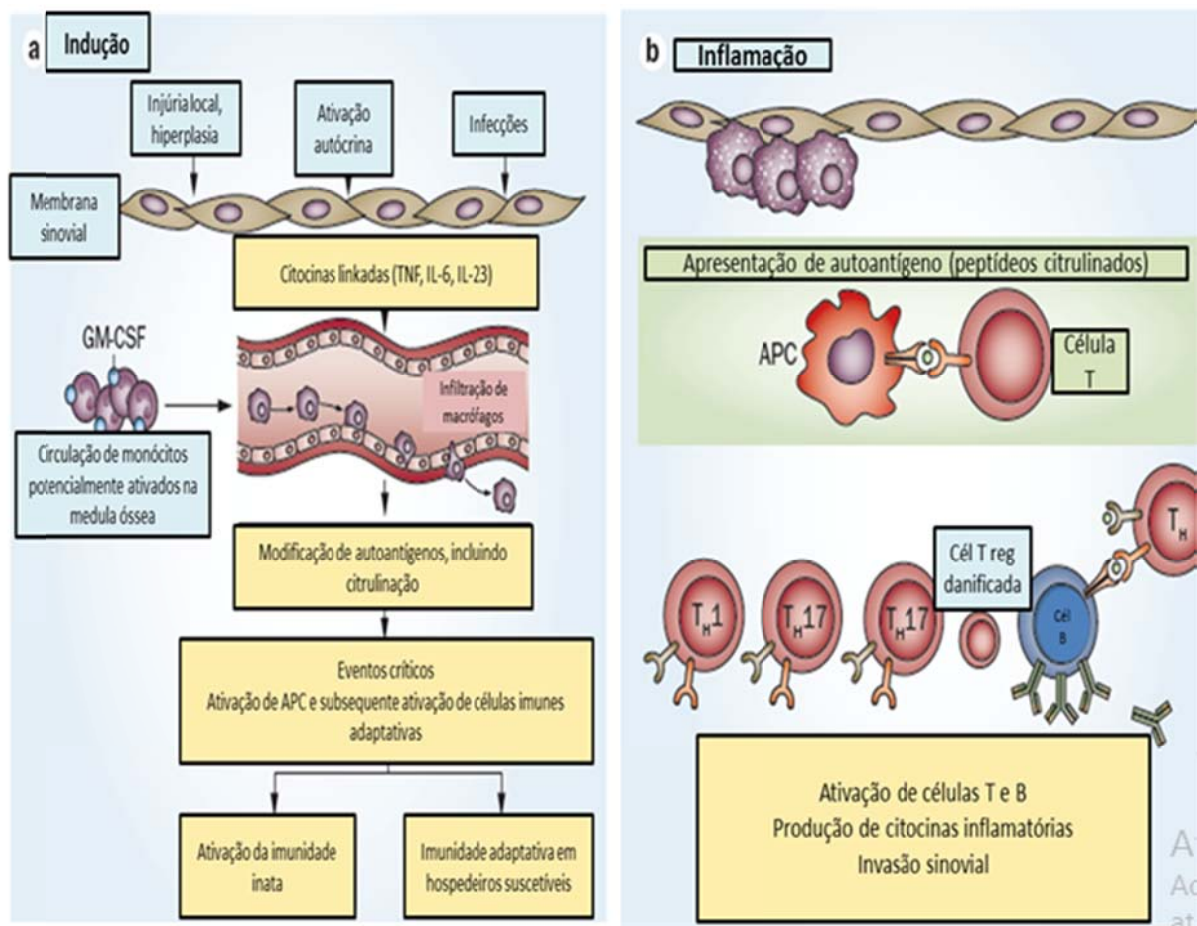
O padrão de resposta Th1 ou Th17 é caracterizado por apresentar linfócitos T autorreativos, os quais produzem mediadores inflamatórios que resultam nas deformidades articulares e ósseas (TU et al., 2012; LIM et al., 2016). Também ocorre o predomínio na diferenciação de linfócitos Th1 em relação aos Th2 desencadeando a superestimulação dos macrófagos, que passam a produzir IL-1 e TNF- α , ambas pró-inflamatórias, que promovem ativação endotelial e celular, gerando degradação da cartilagem (BRENNAN; McINNES, 2008; CHOY, 2012; MERINI et al., 2012; TU et al., 2012; SILVA, 2013; SCHINNERLING et al., 2017).

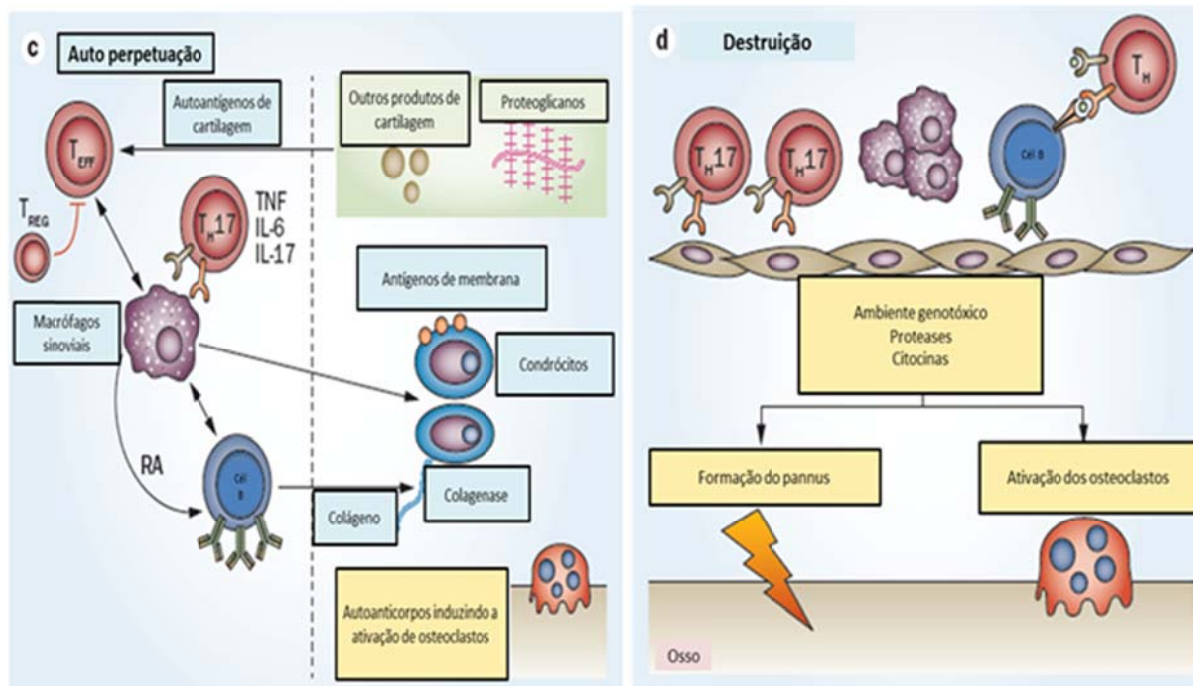
O fator de necrose tumoral (TNF- α) ocupa um lugar de destaque no processo erosivo articular, através da ativação dos osteoclastos (MIOSSEC, 2004). A hiperplasia sinovial é o principal contribuinte para a formação do tecido *pannus* invasivo. Foi observado o acúmulo de células T na sinóvia, o que levou à hipótese de que uma reação inflamatória dependente de células T a um antígeno desconhecido que está ligado à AR (BURMESTER; FEIST; DÖRNER, 2014). As células B também têm um grande papel de patogenicidade a partir da apresentação do autoantígeno seguido de produção de autoanticorpos que conduz a formação de complexos imunes e liberação de citocinas (MARTINEZ-GAMBOA et al., 2006; BURMESTER; FEIST; DÖRNER, 2014).

Em resumo, a AR é caracterizada por diversas etapas, como demonstrado na figura 2. A ativação inicial do sistema imunológico conduzindo uma cascata inflamatória é a fase de

indução (a), que é seguida por uma fase de inflamação (b), onde os autoantígenos e as proteínas citrulinadas são apresentadas estabelecendo uma patologia crônica perpetuando os processos inflamatórios em articulações e em alguns sítios extra-articulares, após a fase de autoperpetuação (c), em que os autoanticorpos induzem a ativação de osteoclastos e por fim a fase de destruição (d), em que os fibroblastos sinoviais e osteoclastos são ativados por citocinas pró-inflamatórias, tais como IL-6 e TNF- α , fazendo com que ocorra a destruição do osso e da cartilagem (BURMESTER; FEIST; DÖRNER, 2014).

Figura 2 - Desenvolvimento gradual da AR





Fonte: Adaptado de BURMESTER et al. (2014). APC (células apresentadoras de antígeno), GM-CSF (fator estimulante de colônias de granulócitos e macrófagos), AR (artrite reumatoide), TEF (células T efetoras), TH (células T helper), TH1 (células T helper 1), TH17 (células T helper 17), Treg (células T reguladoras).

2.1.3 Manifestações extra-articulares

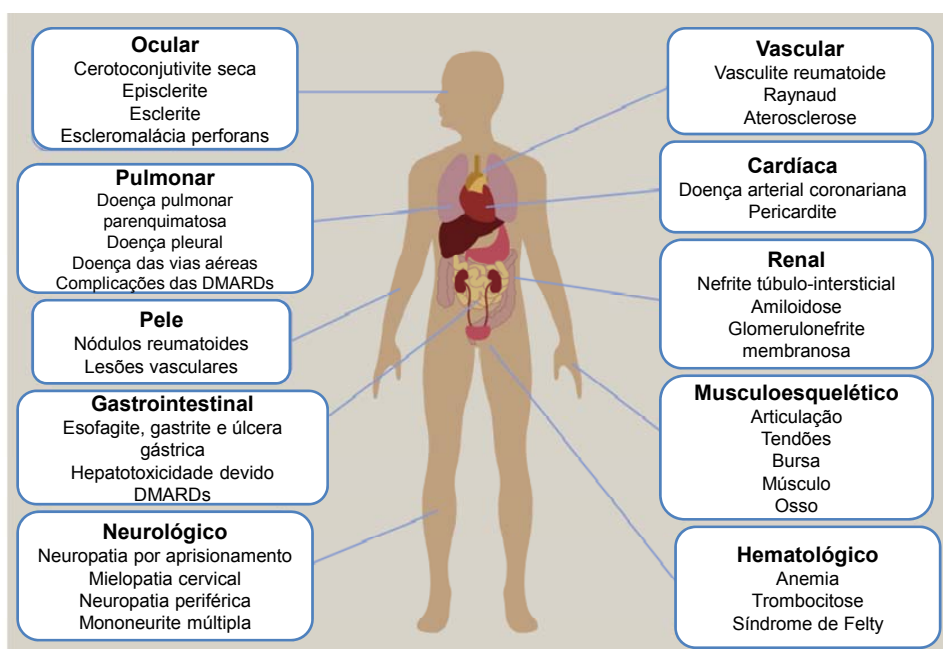
A AR é uma doença autoimune sistêmica que afeta predominantemente o sistema musculoesquelético, entretanto, pode frequentemente manifestar-se em outros tecidos como: os sistemas gastrointestinal, dermatológico, cardiovascular, respiratório, ocular e renal. Estas características clínicas são denominadas como manifestações extra-articulares (MEA) (TURESSON,; JACOBSSON; BERGSTROM, 1999; COJOCARU et al., 2010; CHANDRASHEKARA et al., 2017) e estão associadas com curso mais grave da doença e com o aumento da taxa de mortalidade (SIHVONEN et al., 2004; TURESSON et al., 2006; CHANDRASHEKARA et al., 2017).

As MEA ocorrem em cerca de 40% dos pacientes com AR no início ou durante o curso de sua doença (CIMMINO et al., 2000; COJOCARU et al., 2010) e são consideradas manifestações sérias, devendo ser tratadas e monitoradas (YOUNG; KODURI, 2007; COJOCARU et al., 2010).

Os exemplos mais comuns encontrados na literatura de MEA são: nódulos reumatóides, pericardite, pleurite, derrame pleural, doença pulmonar intersticial, hipertensão

da artéria pulmonar, Síndrome de Caplan, Síndrome de Felty, anemia, trombocitose, neuropatia, esclerite, episclerite, Síndrome de Sicca, glomerulonefrite, úlceras cutâneas, vasculites, amiloidose, Síndrome de Sjogren, entre outras (TURESSON et al., 2000; TURESSON et al., 2003; TURESSON et al., 2007; MOURA et al., 2012). Na figura 3 podemos visualizar os principais órgãos acometidos pela MEA.

Figura 3 - Diagrama das manifestações extra-articulares da AR



Fonte: Adaptado de Gulati; Farah; Mouyis (2018).

2.1.4 Diagnóstico

O diagnóstico da AR é estabelecido considerando-se achados clínicos e exames complementares. Até o ano de 2010, utilizavam-se os critérios clínicos de classificação do “Colégio Americano de Reumatologia” (ACR) de 1987. No entanto, em 2010 um grupo de trabalho conjunto da ACR e da Liga Europeia contra o Reumatismo (EULAR) desenvolveu uma nova abordagem para a classificação da AR. Este novo sistema tem como objetivo, identificar, entre os pacientes que apresentam um quadro recente de artrite inflamatória, os fatores que melhor discriminam os pacientes com alto risco para evoluir com doença

persistente ou erosiva versus aqueles que não apresentam este risco, assim podendo ser identificados os pacientes que precisam de início precoce de terapia (MOTA et al., 2010).

De acordo com o ACR (1987), eram analisados 7 critérios, como: 1- rigidez matinal, 2- artrite de três ou mais articulações, 3 – artrite de mão ou punho, 4- artrite simétrica, 5- nódulos reumatoides, 6- fator reumatoide e 7- alterações radiográficas. Destes 7 critérios, quatro eram necessários para classificar o paciente com AR e deveriam estar presentes por pelo menos seis semanas. Porém, aqueles pacientes que apresentarem dois ou três critérios não são excluídos da possibilidade do futuro desenvolvimento da doença (FELLET E SCOTTON, 2004).

Os novos critérios classificatórios ACR/EULAR podem ser aplicados a qualquer paciente, no entanto, devem atender dois requisitos básicos, deve haver evidência de sinovite clínica ativa no momento do exame em pelo menos uma articulação e os critérios só são aplicáveis aos pacientes para os quais a sinovite não possa ser mais bem explicada por outros diagnósticos (MOTA et al., 2013).

Os critérios propostos são baseados em um sistema de pontuação através de um escore de soma direta. As manifestações são divididas em quatro domínios: acometimento articular, sorologia, duração dos sintomas e provas de atividade inflamatória. Para a contagem de articulações acometidas pode-se utilizar métodos de imagem, como ultrassonografia (US) e ressonância magnética (RM), em caso de dúvida. Para classificar o paciente com AR é necessário uma pontuação ≥ 6 .

O diagnóstico precoce e o início imediato do tratamento são fundamentais para o controle da doença, além de prevenir a incapacidade funcional, a lesão articular e diminuição da dor, a fim de melhorar a qualidade de vida dos pacientes, já que a remissão completa raramente é alcançada (LAURINDO et al., 2004; FELLET; SCOTTON, 2004).

Os marcadores sorológicos utilizados são o fator reumatoide e o anticorpo anti-peptídeo citrulinado cíclico (anti-CCP). O fator reumatóide (FR) é um anticorpo dirigido contra a porção Fc da imunoglobulina G. Individualmente, o valor diagnóstico do FR é limitado, pois de 30 a 50% dos pacientes no início da patologia podem ser soronegativos para esse autoanticorpo (RENAUDINEAU et al., 2005; MOTA et al., 2013). O FR pode ser positivo em diversas outras condições, que não a AR, assim a negatividade do FR não exclui o diagnóstico da AR e a sua positividade também deve ser interpretada com cautela (MOTA al., 2013).

O anti-CCP pertence a um grupo de autoanticorpos capazes de reagir com vários peptídeos citrulinados em diversas proteínas, tais como: filagrina, fibrina, vimentina e enolase

alfa, e por esta razão, indicado como anticorpos anti-proteína / peptídeo citrulinados (ACPA) (ALESSANDRI et al., 2008). Este marcador é o mais específico na AR, sendo detectado mais precocemente na evolução da AR e pode ser usado como indicador de progressão e prognóstico da doença (ANJOS et al., 2009; MOTA et al., 2013).

2.1.5 Tratamento

O objetivo do tratamento de pacientes com AR é reduzir a dor e a inflamação e melhorar a qualidade de vida e evitar a progressão da doença e perda da função articular (ALAM et al., 2017). Atualmente, a terapêutica do paciente varia de acordo com o estágio da doença, sua atividade e gravidade. Para o controle da dor e do processo inflamatório articular faz-se o uso de analgésicos e anti-inflamatórios não hormonais (AINHs), associados ou não a doses baixas de glicocorticoides (CARRILHO, 2009; MOTA et al., 2011).

As drogas modificadoras do curso da doença (DMCDs) são um grupo de medicamentos com potencial para reduzir e prevenir o dano articular e ainda preservar a integridade e funcionalidade das articulações (O'DELL, 2004), podendo ser indicadas para o paciente a partir da definição do diagnóstico. Alguns exemplos são: hidroxicloroquina, metotrexato, sulfassalazina, leflunomida, dentre outras, sozinhas ou em combinação com drogas biológicas (LAURINDO et al., 2004; BÉRTOLO et al., 2009). O metotrexato é o fármaco de escolha para o manejo da AR, sendo eficiente na redução dos sinais e sintomas de atividade da doença, na melhora do estado funcional e na redução da progressão das lesões (SOLIMAN et al., 2011; ALAM et al., 2017).

O avanço da fisiopatologia da AR permitiu na identificação de mediadores biológicos que estão ligados diretamente com esta doença, como as citocinas, receptores de membrana, moléculas de adesão, e linfócitos T e B (O'DELL, 2010). Este avanço permitiu o desenvolvimento de uma classe importante de fármacos biológicos ou imunobiológicos, conhecido como agentes biológicos ou drogas modificadoras do curso da doença (DMCD-biológicas) (MOTA et al., 2015).

As DMCD biológicas que se encontram aprovadas pela Agência Nacional de Vigilância Sanitária (ANVISA) para uso no Brasil são: Anti-TNF (adalimumabe, certolizumabe, etanercepte, infliximabe e golimumabe), depletor de linfócito B (rituximabe), bloqueador da coestimulação do linfócito T (abatacepte), bloqueador do receptor de IL-6 (tocilizumabe) e moléculas que inibem as vias de sinalização intracelular pela inibição de células imunes (tofacitinib) (MOTA et al., 2018).

2.2 MODELOS EXPERIMENTAIS DE ARTRITE

Os modelos de AR são desenvolvidos em uma variedade de espécies animais, mas, ratos e camundongos são usados mais comumente para avaliar a progressão e patogênese da AR. Os modelos de roedores são amplamente utilizados por causa do baixo custo, homogeneidade genética e facilidade de manuseio (CHOUDHARY; BHATT; PRABHAVALKAR, 2018). Além disso, estudos em modelos experimentais resultam em um melhor entendimento do papel de diferentes tipos celulares e mediadores inflamatórios envolvidos no desenvolvimento da artrite (LOPES et al., 2011). Ademais, acrescenta-se a dificuldade da realização de estudos em pacientes por razões éticas ou técnicas (ASQUITH et al., 2009).

Já foram descritos vários modelos que mimetizam parte das características da doença em humanos, entre os quais o mais conhecido é a artrite induzida por adjuvante (AIA) e por proteoglicano (PGIA) (PRAKKEN et al., 2003). O primeiro modelo desenvolvido para o estudo da artrite foi a AIA, que foi descrito em 1956 (PEARSON, 1956), sendo um dos modelos de artrite experimental mais amplamente utilizados, em que a indução da poliartrite e se dá através de uma única injeção intradérmica com micobactérias mortas pelo calor em adjuvante completo de Freund (CFA) (BAGGOT et al., 1998).

A presença de micobactérias em CFA sugere uma causa infecciosa da poliartrite. No entanto, fármacos anti-inflamatórios, mas não antibióticos, são bem sucedidos em atenuar a gravidade da doença. Isto levou à utilização da AIA como modelo de rastreamento de agentes com potencial papel anti-inflamatório (BENDELE et al., 1999; PRAKKEN et al., 2003). O início da artrite clínica é visível após 10-14 dias da indução com o CFA. O primeiro sinal histopatológico observado é a infiltração de células mononucleares no tecido sinovial (PRAKKEN et al., 2003). Este infiltrado inflamatório nas lesões articulares contém células T ativadas por antígenos contra o *Mycobacterium tuberculosis* (WILBRINK et al., 1992; VAN EDEN; WASKMAN, 2003). As citocinas da resposta do tipo Th1, tais como interferon γ (INF- γ) e fator de necrose tumoral α (TNF- α), são expressas no início da AIA juntamente com as citocinas características de ativação dos macrófagos, como a IL-1 β (VAN EDEN; WASKMAN, 2003).

Os linfócitos desempenham um papel central no desencadeamento da artrite adjuvante. Células T estão envolvidas na imunopatogênese da resposta mediada por células para o antígeno do microrganismo *Mycobacterium tuberculosis*, induzindo um quadro clínico

completo de AIA, evidenciando a hipótese que agentes microbianos são gatilhos para o processo autoimune da AR em indivíduos susceptíveis (PRAKKEN et al., 2003).

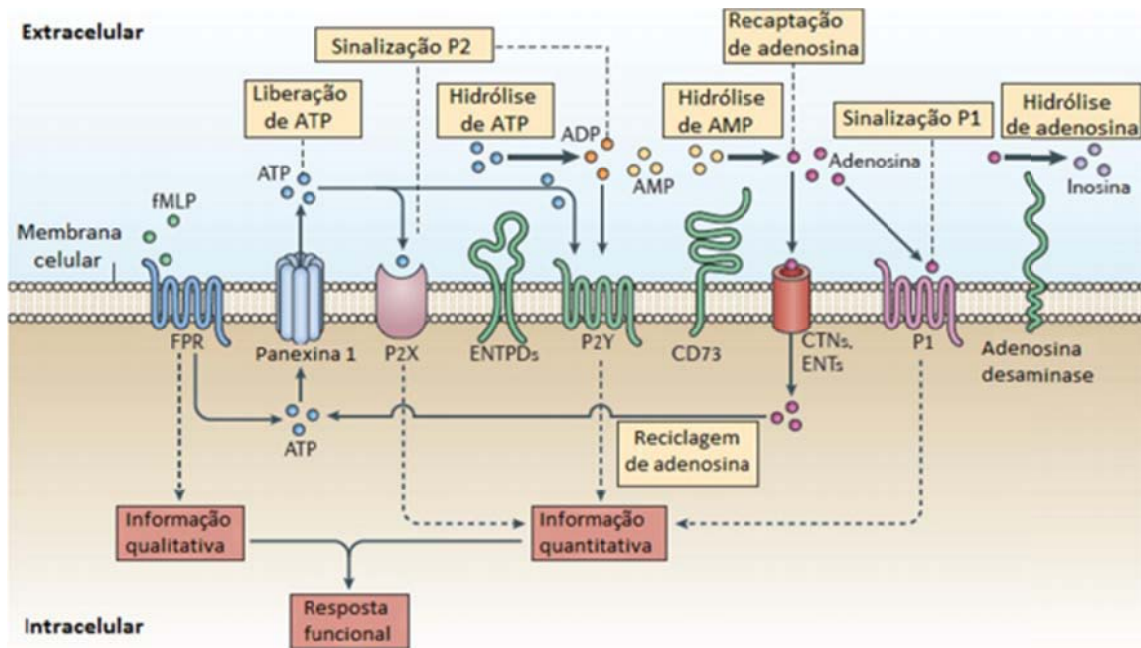
A PGIA foi descrita posteriormente em camundongos BALB/c pela imunização com proteoglicano de cartilagem humana ou murina (MIKECZ et al., 1990). Após a imunização parenteral, os primeiros sinais clínicos aparecem ao redor do 40º dia e parecem ser resultantes da ativação de linfócitos TCD4+ (BARDOS et al., 2003). Recentemente, os mecanismos inflamatórios vêm sendo desvendados nesse modelo e têm relação com a produção de IFN- γ (DOODES et al., 2010; GLANT et al., 2011; OJEDA et al., 2011). A artrite induzida por colágeno (AIC) apresenta muitas semelhanças com a AR humana. As duas principais características do modelo de AIC são, a quebra de tolerância e a geração de autoanticorpos contra colágeno do tipo II, proteínas citrulinadas (ACPA) e de fator reumatoide IgG (ASQUITH et al., 2009).

Há também, o modelo de artrite induzida por Zymosan, que é um polissacarídeo derivado da parede celular do fungo *Saccharomyces cerevisia*, cujo principal componente estrutural é a β -glucana que apresenta propriedades imunoestimulatórias (DERBOCIO et al., 2005). A injeção deste polissacarídeo na cavidade intra-articular do joelho dos ratos resulta em uma artrite inflamatória proliferativa com infiltração de células mononucleares, hipertrofia sinovial e formação do pannus com o pico da doença em cerca de 3 dias e diminuição da inflamação no 7º dia (KEYSTONE et al., 1977; ASQUITH et al., 2009). As principais limitações deste modelo são a natureza monoartrítica da doença e a competência técnica necessária para uma injeção intra-articular (ASQUITH et al., 2009). Existem ainda, os modelos de artrite manipulados geneticamente, como por exemplo: TNF- α modelo de rato transgênico de artrite inflamatória, modelo K/BxN, modelo SKG, DR4-CD4, entre outros (ASQUITH et al., 2009).

2.3 SISTEMA PURINÉRGICO

O sistema purinérgico envolve três principais componentes: nucleotídeos e nucleosídeos extracelulares, receptores purinérgicos e as ectoenzimas (YEGUTKIN, 2008). É caracterizado por ser uma via de sinalização importante em diversos tecidos, desencadeando múltiplos efeitos celulares, incluindo resposta imune, inflamação, dor, agregação plaquetária, vasodilatação mediada pelo endotélio, proliferação e morte celular (BURNSTOCK; KNIGHT, 2004) (Figura 4).

Figura 4 - Componentes da sinalização purinérgica



Fonte: Adaptada de JUNGER (2011).

Os nucleotídeos da adenina (ATP, ADP e AMP) e seu derivado nucleosídeo adenosina, os quais são secretados por leucócitos, plaquetas e células endoteliais danificadas sinalizam vias de grande importância que medeiam diversos efeitos biológicos, incluindo a contração do músculo liso, a neurotransmissão, a dor, a agregação plaquetária, a resposta imune e a inflamação (RALEVIC; BURNSTOCK, 1998).

Em condições fisiológicas, os nucleotídeos são encontrados no meio extracelular em baixas concentrações (DI VIRGILIO et al., 2001). Entretanto, o ATP em elevadas concentrações pode atuar como uma potente molécula citotóxica, capaz de levar à morte de diferentes classes de células, pela formação de grandes poros na membrana plasmática, com exceção daquelas que possuem alto poder de quebra do ATP em sua superfície (FILIPPINI et al., 1990). Foi observada uma alta concentração de ATP no local da inflamação, como consequência de sua liberação ativa ou passiva de mastócitos, linfócitos, macrófagos, células endoteliais e necróticas (DI VIRGILIO, 2007; SCHETINGER et al., 2008). Existem evidências que sugerem que o ATP liberado exerce um papel pró-inflamatório e induzir à liberação de citocinas através dos receptores P2X7 (DI VIRGILIO, 2007; SCHETINGER et al., 2008).

O ADP não possui um papel definido nos linfócitos (DI VIRGILIO et al., 2001), porém nas plaquetas, este nucleotídeo age como um importante indutor da agregação

plaquetária e da tromborregulação, podendo ser liberado na circulação sanguínea após danos teciduais (ZIMMERMANN, 1999). Em situações de disfunção ou dano vascular, o ADP é liberado do interior de grânulos existentes nas plaquetas, sendo então considerado o agonista mais importante do recrutamento plaquetário e o indutor da formação de trombos no interior de vasos (MARCUS et al., 2003).

O AMP é um metabólito intermediário da hidrólise do ATP (BARSOTTI; IPATA, 2004) que exerce a função de sinalizador em situações de desequilíbrio no metabolismo, servindo também como substrato para a formação da adenosina (CUNHA, 2001; LATINI; PEDATA, 2001). A adenosina é reconhecida por possuir propriedades anti-inflamatórias (CRONSTEIN, 1994), vasodilatadoras, neuroprotetoras (JACOBSON et al., 2006), e imunossupressoras (SPYCHALA; MITCHEL; BARANKIEWICZ, 1997), além de atuar como um potente inibidor da agregação plaquetária (BOROWIEC et al., 2006). Além disso, a adenosina age como reguladora endógena da inflamação, mediando a transição da inflamação aguda para a cicatrização. Alterações patológicas ou a manipulação farmacológica do metabolismo da adenosina ou da expressão e/ou função dos receptores da adenosina podem ter um papel tanto na patogênese como na terapia das doenças reumáticas (CRONSTEIN; SITKOVSKY, 2017).

A inosina é uma purina de ocorrência natural, formado pela quebra da adenosina e apresenta potencial papel anti-inflamatório, inibe a ativação plaquetária e possui efeito neuroprotetor (SITKOVSKY et al., 2004). Estudos *in vitro* evidenciaram que a inosina inibe a produção de citocinas pró-inflamatórias, como o TNF- α , IL-1 β e IFN- γ e reduz a ativação de neutrófilos (SITKOVSKY, 2003). Esses efeitos da inosina parecem ser mediados por receptores de adenosina (SITKOVSKY, 2003).

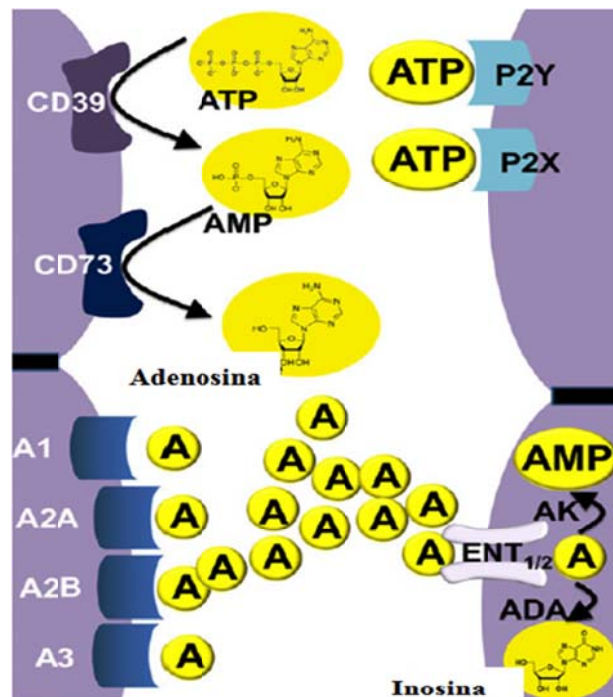
Em condições fisiológicas, os nucleotídeos e nucleosídeo da adenina são encontrados no meio extracelular em baixas concentrações não atravessando a membrana celular, mas podendo realizar suas ações biológicas através de receptores específicos presentes na superfície celular, denominados receptores purinérgicos (DI VIRGILIO et al., 2001).

Os receptores que ligam nucleotídeos e nucleosídeos são divididos em receptores de adenosina ou P1 (subdivididos em A1, A2A, A2B e A3) e receptores P2, que são subdivididos em dois grandes grupos, os receptores ionotrópicos P2X (P2X1-7), e os receptores metabotrópicos P2Y (P2Y1, P2Y2, P2Y4, P2Y6 e P2Y11). Os receptores P2X ligam-se preferencialmente ATP e/ou UTP e P2Y podem ser ativados por ATP, UTP, UDP, ITP e ainda nucleotídeos glicosados (BURNSTOCK, 2002; ROBSON et al., 2006).

Em especial, o receptor P2X7, que é expresso em quase todos os tecidos e órgãos (BURNSTOCK; KNIGHT, 2004), tendo maior distribuição nas células imunes (GU et al., 2000). Este receptor pode funcionar como um poro iônico não seletivo permitindo o efluxo de K^+ e Na^+ e influxo de Ca^{2+} (MORANDINI; SAVIO; COUTINHO-SILVA, 2014). A liberação de grande quantidade de ATP intracelular durante o processo inflamatório aumenta a sinalização purinérgica através da ativação do receptor P2X7 desencadeando eventos pró-inflamatórios. A liberação da principal citocina, a IL-1 β , durante o processo inflamatório, está associada com a atividade do receptor P2X7 (LISTER et al., 2007). Estudos relacionando a ativação do receptor P2X7 e a liberação e ativação de outras citocinas como IL-1 α , IL-2, IL-4, IL-6, IL-13, IL-18 e TNF- α também têm sido descritos (MEHTA et al., 2001; SMITH et al., 2001; LOOMIS et al., 2003; JACOB et al., 2013).

O controle dos níveis extracelulares dos nucleotídeos da adenina e da adenosina, bem como a consequente sinalização purinérgica por eles induzida através dos receptores, é fundamental na manutenção dos processos fisiológicos de sinalização purinérgica como secreção, inflamação, fluxo sanguíneo, dentre outros (ROBSON et al., 2006). Este controle é realizado por uma variedade de enzimas ancoradas à superfície celular ou localizadas no meio intersticial de forma solúvel (ZIMMERMANN et al., 2007) (Figura 5).

Figura 5 - Visão geral de nucleotídeos extracelulares e nucleosídeos



Fonte: Adaptado de Idzko et al. (2014).

2.3.1 Ectonucleotidases

As enzimas que hidrolisam nucleotídeos extracelulares são conhecidas como ectonucleotidases. Estas enzimas podem ser classificadas como família das E-NTPDases (ectonucleosídeo trifosfato difosfohidrolases ou apirase; EC 3.6.1.5; CD39), família E-NPP (ectonucleotídeo pirofosfatase/fosfodiesterase; EC 3.1.4.1), fosfatases alcalinas e ecto-5'-nucleotidase (EC 3.1.3.5), sendo amplamente distribuídas nos tecidos (ZIMMERMANN et al., 2000).

Há pelo menos oito membros diferentes da família da E-NTPDase (E-NTPDase 1-8), que foram descobertas e clonadas nos últimos anos (ZIMMERMANN, 2000; ROBSON et al., 2006; SCHETINGER et al., 2008). As NTPDases 1, 2, 3 e 8 são enzimas localizadas na superfície celular com um sítio catalítico voltado para o meio extracelular, enquanto que NTPDases 4-7 demonstram evidência de localização intracelular (VORHOFF et al., 2005; SCHETINGER et al., 2008).

A E-NTPDase 1 hidrolisa os substratos ATP e ADP com a mesma velocidade, a E-NTPDases 3 e 8 demonstraram uma preferência pela hidrólise do ATP em relação ao ADP. A E-NTPDase 2 hidrolisa preferencialmente o substrato ATP (ZIMMERMANN, 2001; KUKULSI et al., 2005). As E-NTPDases 5 e 6 exibem localização na superfície intracelular e são secretadas após expressão heteróloga, hidrolisando preferencialmente o ADP. As E-NTPDases 4 e 7 são de localização inteiramente intracelular (ROBSON et al., 2006). A E-NTPDase é uma nucleotidase e possui importante desempenho no controle da função linfocitária, incluindo o reconhecimento do antígeno e/ou ativação de atividades efetoras das células T-citotóxicas (FILIPPINI et al., 1990). Essa enzima foi identificada como antígeno de superfície CD39 das células linfoides, sendo que o aumento da sua expressão levaria a uma maior hidrólise de ATP e ADP nessas células (REZER et al., 2007).

A família das E-NPPs consiste em sete membros estruturalmente relacionados (E-NPP1 a E-NPP7) que foram numerados de acordo com a sua ordem de descobrimento. (YEGUTKIN, 2008). A E-NPP (EC 3.1.4.1) é responsável pela hidrólise de ligações 5'-fosfodiéster em nucleotídeos e seus derivados, em que purinas e pirimidinas servem como substratos, resultando na produção de nucleotídeo monofosfatado (AMP) (ZIMMERMANN, 2001). Esta enzima foi detectada em quase todos os tecidos (BOLLEN et al., 2000). As E-NPPs tem papel na apoptose induzida por ATP (CHOW et al., 1997). A apoptose mediada por ATP pode resultar tanto na ativação dos receptores P2X7 em mastócitos, plaquetas, macrófagos e linfócitos (GODING et al., 2003), quanto na geração de adenosina e ativação do

receptor purinérgico A₃ como foi mostrado para astrócitos, neuroblastoma e em células mesangiais (ZHAO et al., 2002).

A ecto-5'-nucleotidase é uma enzima que catalisa a desfosforilação do AMP em adenosina (REGATEIRO et al., 2013), tendo sido identificada como CD73. O principal papel fisiológico desta enzima é a formação da adenosina a partir do AMP extracelular e subsequente ativação dos receptores P₁ (ZIMMERMAN, 2000; SCHETINGER et al., 2008). É uma enzima ancorada à membrana plasmática por glicosil-fosfatidilinositol (GPI), que representa um marcador de maturação para os linfócitos T e B, estando ausentes nas células imaturas (AIRAS et al., 1997). Esta enzima é expressa em uma variedade de tecidos como o hepático, renal, nervoso e também em diferentes tipos de células, entre as quais as plaquetas (ZIMMERMANN et al., 1992).

Vários estudos têm mostrado uma atividade alterada da enzima E-NTPDase em pacientes com diferentes condições patológicas como o diabetes (LUNKES et al., 2003), a esclerose múltipla (SPANVELLO et al., 2010), o infarto agudo do miocárdio (BAGATINI et al., 2008), na síndrome da imunodeficiência adquirida (AIDS) (LEAL et al., 2005), e em pacientes com AR (BECKER et al., 2010; JAQUES et al., 2013). Também foram observadas alterações na atividade desta enzima em modelos animais de artrite (CASTILHOS et al., 2015; SILVEIRA et al., 2016).

2.3.2 Adenosina desaminase

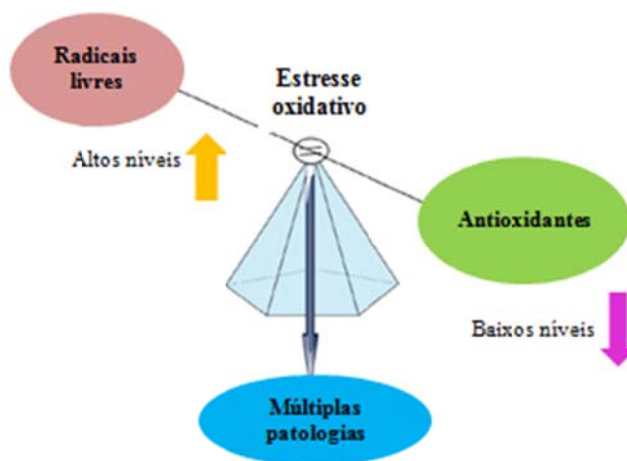
O produto final da quebra dos nucleotídeos, a adenosina, sofre a ação da adenosina desaminase (E-ADA, E.C.3.5.4.4). Esta enzima leva a desaminação irreversível da adenosina ou deoxiadenosina em seus respectivos produtos, a inosina e a amônia (BOURS et al., 2006). A ADA é amplamente distribuída em quase todos os tecidos de mamíferos, sendo conhecida como um marcador molecular de ativação de células T (FRANCO et al., 1997). Alterações em sua atividade têm sido consideradas indicadores de distúrbios imunológicos (POURSHARIFI et al., 2009). Além disso, a ativação excessiva do receptor purinérgico pelo aumento dos níveis de adenosina extracelular pode contribuir para a imunossupressão ao alterar o desenvolvimento e a função dos linfócitos (BOURS et al., 2006). A ADA possui um importante papel na resposta imune e inflamatória devido à regulação das concentrações de adenosina. A adenosina possui propriedades anti-inflamatórias e protetoras do tecido hospedeiro contra danos, tendo um importante papel na diferenciação e proliferação dos linfócitos (BALDISSERA et al., 2016).

A E-ADA é considerada um marcador não específico da imunidade mediada por células (YONEYAMA et al., 2003). Alterações na atividade da E-ADA tem sido associadas com doenças em que há envolvimento da imunidade celular. A adenosina exibe potentes ações anti-inflamatórias e imunossupressoras através da inibição da proliferação de células T, da secreção de citocinas e da migração de leucócitos (KOBIE et al., 2006). Além disso, a adenosina atua como um sinal de “feedback negativo” para evitar a estimulação imunológica mediada por ATP, prevenindo a inflamação descontrolada e diminuindo os danos colaterais para os tecidos saudáveis (GESSI et al., 2007).

2.4 ESTRESSE OXIDATIVO

O estresse oxidativo é um fenômeno celular ou condição que ocorre como resultado do desequilíbrio entre os níveis de antioxidantes e oxidantes (radicais livres ou espécies reativas) em favor dos oxidantes (IGHODARO; AKINLOYE, 2017) (Figura 6).

Figura 6 - Estresse oxidativo: Desequilíbrio entre radicais livres e antioxidantes



Fonte: Adaptado de Ighodaro; Akinloye, 2017

O estresse oxidativo desempenha um papel importante na patogênese da artrite reumatoide (HITCHON et al., 2004; DI DALMAZI et al., 2016). As células presentes na

articulação inflamada (macrófagos, neutrófilos, linfócitos e células endoteliais), uma vez isoladas e estimuladas, são capazes de produzir espécies reativas de oxigênio. Estes radicais, na presença de moléculas de lipídios, DNA, proteínas, carboidratos ou proteoglicanos, provocam lesão oxidativa (RAMOS; RAMOS; DOMINGUEZ, 2000). Os radicais livres têm sido implicados como mediadores de dano tecidual na artrite reumatoide, em conjunto com as citocinas pró-inflamatórias. Experimentalmente, já foi verificado que a excessiva produção de EROs pode levar a uma aceleração no dano à cartilagem articular e ativação de osteoclastos (GOLDRING, 2002; FILIPPIN et al., 2008), contribuindo para a progressão da doença (MATEEN et al., 2016).

2.4.1 Espécies Reativas

Os radicais livres podem ser definidos como espécies químicas reativas que possuem um único elétron desemparelhado em uma órbita externa (RILEY, 1994; RAHMAN, 2007). Existem vários tipos de radicais livres no sistema biológico, no entanto, aqueles derivados do oxigênio, que são conhecidos como espécies reativas de oxigênio (EROS), são de grande preocupação e interesse para os pesquisadores no campo da química dos radicais livres. O oxigênio é uma molécula altamente suscetível à formação de radicais livres devido à sua estrutura (IGHODARO; AKINLOYE, 2017).

Os mecanismos de geração de radicais livres ocorrem, normalmente, nas mitocôndrias, membranas celulares e no citoplasma, e podem ser favorecidos pelos íons de ferro e de cobre. A mitocôndria é a principal fonte geradora de radicais livres, através da cadeia transportadora de elétrons, durante a produção de energia a partir de combustíveis e do oxigênio. Outra fonte importante geradora de radicais livres são as enzimas NADPH oxidases, que são proteínas de membrana, que tem a função de transferir elétrons através das membranas celulares (BARBOSA et al., 2010).

As principais EROS distribuem-se em dois grupos, os radicalares: hidroxila (HO^\bullet), superóxido ($\text{O}_2^{\bullet-}$), peroxila (ROO^\bullet) e alcoxila (RO^\bullet), e os não-radicalares: oxigênio, peróxido de hidrogênio (H_2O_2) e ácido hipocloroso (HOCl). Dentre as ERNs incluem-se o óxido nítrico (NO^\bullet), óxido nitroso (N_2O_3), ácido nitroso (HNO_2), nitritos (NO_2^-), nitratos (NO_3^-) e peroxinitritos (ONOO^-) (HALLIWELL e GUTTERIDGE, 1999; DINIZ et al., 2015;).

As EROS podem ser prejudiciais e benéficas em sistemas biológicos dependendo do ambiente (LOPACZYNSKI; ZEISEL, 2001; GLADE, 2003; RAHMAN, 2007). Os efeitos benéficos das ROS envolvem, por exemplo, a defesa contra agentes infecciosos, e na sua

função em vários sistemas de sinalização celular. Em contraste, quando em altas concentrações, as EROS podem causar danos estruturais, incluindo lipídios e membranas, proteínas e ácidos nucleicos (POLI et al., 2004; RAHMAN, 2007). Os efeitos nocivos dos EROS são equilibrados pela ação dos antioxidantes (HALLIWELL, 1996; RAHMAN, 2007).

As EROS podem induzir a peroxidação lipídica e interromper a disposição da bicamada lipídica da membrana, podendo assim inativar receptores e enzimas ligados à membrana e aumentar a permeabilidade tecidual (GIRROTI, 1985; BIRBEN et al., 2012). A peroxidação lipídica envolve os processos de decomposição oxidativa de ácidos graxos poliinsaturados (PUFA) e de fosfolipídios de membrana, conduzindo à formação de misturas de hidroperóxido lipídico e produtos finais aldeídicos, tais como o malondialdeído (MDA). A peroxidação lipídica altera a permeabilidade e a fluidez das membranas celulares (OLSZEWSKA-SLONINA et al., 2011). Os danos celulares pelo estresse oxidativo através da quantificação do MDA pode ser analisado através do método de substâncias reativas ao ácido tiobarbitúrico (TBARS), técnica introduzida por JENTZSCH et al. (1995) e utilizada até hoje.

Apesar da presença do sistema de defesa antioxidantes da célula para combater os danos causados pelas EROS, o dano oxidativo se acumula durante o ciclo de vida e tem sido implicado no envelhecimento, e em doenças cardiovasculares, câncer, distúrbios neurodegenerativos, e em outras condições crônicas (RAHMAN, 2003; RAHMAN, 2007).

2.4.2 Antioxidantes

Os antioxidantes são substâncias que combatem os radicais livres e evitam os danos causados por eles. Estas substâncias podem reduzir significativamente os danos adversos causados por oxidantes, desintegrando-os antes que eles reajam com os alvos biológicos, prevenindo reações em cadeia ou impedindo a ativação de oxigênio em produtos altamente reativos (AZZI; DAVIES; KELLY, 2004; RATNAM et al., 2006).

As moléculas antioxidantes nos sistemas vivos agem em diferentes níveis. Esses níveis incluem a prevenção dos radicais, a eliminação dos radicais e a reparação dos danos. Com base na linha de defesa, os antioxidantes podem ser categorizados como antioxidantes de defesa de primeira linha, antioxidantes de defesa de segunda linha, antioxidantes de defesa de terceira linha e antioxidantes de defesa de quarta linha (IGHODARO; AKINLOYE, 2017).

Os antioxidantes de defesa de primeira linha atuam para suprimir ou prevenir a formação de radicais livres ou espécies reativas nas células. São muito rápidos em neutralizar qualquer molécula com potencial em se desenvolver em um radical livre com a capacidade

para induzir a produção de outros radicais. As três enzimas chaves são: superóxido dismutase, catalase e glutathione peroxidase. Os antioxidantes de defesa de segunda linha são responsáveis por eliminar os radicais ativos, inibindo a iniciação da cadeia e as quebras das reações de propagação da cadeia. A maioria dos antioxidantes incluindo ácido ascórbico, ácido úrico, glutathione, que são hidrofílicos, e alfa-tocoferol (vitamina E) e ubiquinol, que são lipofílicos pertencem a esta categoria (IGHODARO; AKINLOYE, 2017).

Os antioxidantes de defesa de terceira linha são responsáveis por reparar os danos causados pelos radicais livres às biomoléculas e por reconstituir a membrana celular danificada. É um grupo de enzimas para reparo do DNA, proteínas e lipídios danificados. Dentre elas, estão: os sistemas de enzimas de reparo de DNA (polimerases, glicosilases e nucleases) e enzimas proteolíticas (proteínases, proteases e peptidases) que estão localizadas no citosol e nas mitocôndrias das células (IGHODARO; AKINLOYE, 2017). E por fim, os antioxidantes de defesa de quarta linha envolvem basicamente um mecanismo de adaptação, no qual eles utilizam os sinais necessários para evitar a formação ou a reação dos radicais livres (NIKI, 1993; IGHODARO; AKINLOYE, 2017).

Entre as principais enzimas responsáveis pela defesa antioxidante enzimática do organismo destacam-se: a superóxido dismutase (SOD), a catalase (CAT) e a glutathione peroxidase (GPx). Através delas, as células tentam manter baixas as quantidades do radical superóxido e de peróxidos de hidrogênio, evitando a formação do radical hidroxil (BOVERIS; CADENAS, 1997). Além das defesas antioxidantes enzimáticas, possuem grande relevância os antioxidantes não enzimáticos. Deste grupo destacamos o papel dos grupamentos tióis (-SH) e das vitaminas C e E (VALKO et al., 2007) e dos compostos fenólicos, onde se destacam os flavonoides e os poliflavonoides (PIETTA, 2000).

A SOD é a enzima responsável pela dismutação do ânion superóxido. Possui duas isoformas, uma constitutiva e outra induzível. A Manganês-SOD (MnSOD) reside na mitocôndria e é induzível por citocinas através da via NF-KB e outros co-fatores. A cobre-zinco-SOD (Cu-Zn-SOD) é constitutiva e se localiza no citoplasma, lisossomos, núcleo e espaços entre a membrana interna e externa da mitocôndria e peroxissoma (FILIPPIN et al., 2008). Outra enzima antioxidante é a catalase, que é responsável pela conversão do peróxido de hidrogênio em água e oxigênio, protegendo as células dos efeitos nocivos do peróxido de hidrogênio acumulado (MATEEN et al., 2016).

Já, a glutathione é um tripeptídeo que existe no organismo sob as formas reduzida (GSH) ou oxidada (GSSG) sendo sintetizada principalmente no fígado. Atua direta ou indiretamente em muitos processos biológicos importantes, como na detoxificação de agentes

químicos e na eliminação de produtos da lipoperoxidação, sendo ainda é requerida para a síntese de DNA, proteínas e de algumas prostaglandinas (FERREIRA; MATSUBARA, 1997). A glutathione peroxidase (GPx) juntamente com a catalase são responsáveis pela dismutação do peróxido de hidrogênio. O peróxido de hidrogênio produzido no núcleo é transportado para o citoplasma por túbulos existentes no núcleo, onde a GPx faz a degradação. A GPx degrada além do peróxido de hidrogênio, outros peróxidos. É a primeira defesa mitocondrial do peróxido de hidrogênio e é regulada por p53 e por hipóxia (FILIPPIN et al., 2008).

Os tióis são as moléculas muito importantes no processo de antioxidação que contém grupos tióis (-SH) (GUMUSYAYLA et al., 2016). Os radicais livres causam a oxidação de grupos - SH em proteínas (FERREIRA; MATSUBARA, 1997). Os grupos - SH podem reduzir os elétrons para que o organismo possa ser protegido dos danos oxidativos causados por EROs (GUMUSYAYLA et al., 2016).

2.5 GENOTOXICIDADE E CITOTOXICIDADE

O estresse oxidativo causa danos em moléculas importantes do organismo, como as membranas celulares (peroxidação lipídica) e mutações no ácido desoxirribonucleíco (DNA). Essas alterações são conhecidas como genotoxicidade (FRONZA et al., 2011). Os agentes genotóxicos são caracterizados por possuírem atividade biológica primária, própria ou de metabólitos, capaz de alterar informações codificadas no DNA. A genotoxicidade ocorre quando a exposição a um agente tóxico leva à alteração da estrutura ou do conteúdo do cromossomo (clastogenicidade) ou da sequência de pares de bases do DNA (mutagenicidade) (MCGREGOR, 2000).

Os efeitos genotóxicos podem ocorrer frente a concentrações muito baixas de substâncias, podendo afetar a reprodução, vida embrionária, desenvolvimento, crescimento e sobrevivência de organismos, estando relacionados com carcinogênese, defeitos hereditários por mutações, teratogênese e doenças de fundo genéticas (LEE; STEINERT, 2003).

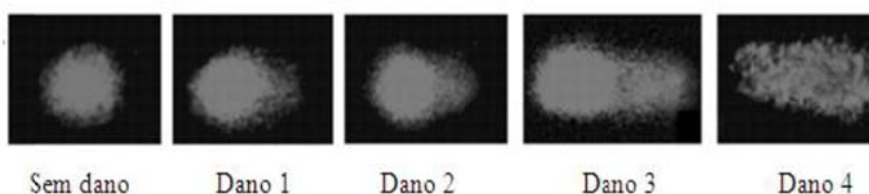
Os danos mais graves são aqueles causados ao DNA e ao RNA e que podem alterar a estrutura e função destas biomoléculas. Lesões no DNA podem bloquear os processos de replicação e transcrição, que quando não reparados ou reparados incorretamente, podem levar a mutações pontuais (alteração de um único par de bases do DNA ou um pequeno número de bases adjacentes) ou aberrações cromossômicas (alterações na estrutura ou número de cópias

de cromossomos), comprometendo a viabilidade da célula (GRIFFITHS et al., 2009; JACKSON; BARTEK, 2009).

O DNA pode ser alvo de radicais livres, principalmente o radical hidroxil, peróxido nítrico e aldeídos, e as alterações causadas por esses radicais são variáveis, sendo muitas vezes corrigidas por enzimas. Porém, quando esta reparação não ocorre o DNA fica exposto a mutações levando à instabilidade do genoma e possibilitando o aparecimento de doenças genéticas e até mesmo câncer em um quadro denominado genotoxicidade (RILEY, et al., 2008).

O ensaio de eletroforese em gel de célula única (ensaio cometa) é amplamente utilizado para a detecção de danos no DNA e reparo em uma variedade de células *in vitro* e *in vivo* (OLIVE; BANATH, 2006; IMANIKIA et al., 2016). Este ensaio consiste na lise celular, relaxamento do DNA e eletroforese, sendo possível observar após coloração, os fragmentos de DNA provindos da quebra causada pelo agente xenobiótico, que, ao serem submetidos à eletroforese “correm” formando uma cauda. Deve-se ter em mente que não existe célula sem dano no DNA, visto que o próprio metabolismo celular pode gerar em torno de 1000 lesões diárias no DNA/célula. O que se faz, rotineiramente é modular as condições técnicas (tempo de relaxamento e eletroforese) para que um mínimo de DNA migre da cabeça para a cauda do cometa nos controles negativos (TICE, 1995). O ensaio cometa tem a vantagem de ser rápido, sensível e relativamente um método barato (IMANIKIAA et al., 2016). Na figura 7 podemos visualizar a classificação do dano ao DNA pelo ensaio cometa.

Figura 7 - Classificação do dano ao DNA através da realização do ensaio cometa. Quanto maior o nível de dano, maior o comprimento da cauda e maior o número a ele atribuído. Classe 0 representa células sem dano e classe 4 o mais alto dano



Fonte: adaptado de TATSCH et al. (2011).

Em doenças inflamatórias crônicas, como a AR e o LES (lúpus eritematoso sistêmico), as EROS são liberados pelas células fagocíticas no local da lesão e atravessam as membranas celulares reagindo com o DNA nuclear (BASHIR et al., 1993). A natureza inflamatória da AR, particularmente nos períodos de exarcebação, sugere que possa haver um estado de

estresse oxidativo nesta doença (KOCYIGIT et al., 2005). Já foi proposto que o dano ao DNA induzido pelas EROS pode contribuir para aumentar as taxas de mutação, instabilidade do genoma, apoptose e regeneração do tecido associado, e proliferação celular (SARAN et al., 1999; KOCYIGIT et al., 2005).

No entanto, a citotoxicidade está relacionada ao grau em que um agente causa a ação destrutiva sobre as células (PETERS, 2013). Os estímulos tóxicos são capazes de ameaçar as funções metabólicas celulares, e conseqüentemente a resposta das células depende da toxicidade gerada, podendo adaptar-se ao ambiente a que estão submetidos (YANG et al., 2002). Os testes de toxicidade são realizados para determinar os potenciais riscos que novos produtos podem gerar na saúde e no meio ambiente (CASTANÕ E GÓMEZ, 2005).

Quant-iTPicoGreen[®] reagente dsDNA[™] é um ácido nucléico fluorescente utilizado para detecção e quantificação de DNA dupla fita (dsDNA) no plasma, indicando morte celular, pois para que o DNA dupla fita esteja livre no plasma a célula precisa ter rompido suas membranas (SWARUP, 2011). O *PicoGreen*[®] é uma sonda fluorescente que tem a capacidade de se ligar ao DNA formando um complexo (DRAGAN et al., 2010). As sondas fluorescentes podem interagir com os ácidos nucleicos desempenhando um papel importante nos estudos biofísicos das moléculas biológicas e seus complexos, proporcionando uma variedade de ensaios e técnicas (AHN et al., 1996).

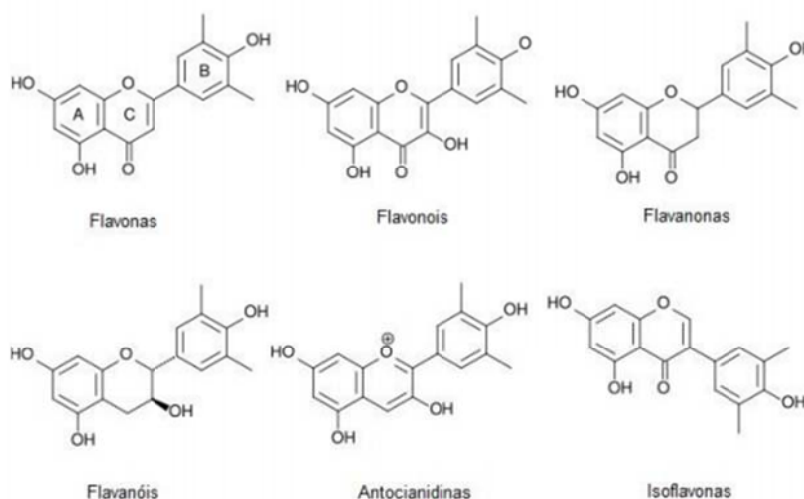
2.6 FLAVONOIDES

Os flavonoides são uma classe importante de compostos naturais conhecidos como polifenóis, que são metabolitos secundários que ocorrem naturalmente em plantas e encontrados em grande parte em alimentos e bebidas, como frutas, verduras, cereais, chá, café e vinho tinto (SELEEM et al., 2016). São classificados de acordo com a sua estrutura e a sua natureza química depende de sua classe estrutural, grau de hidroxilação, outras substituições e conjugações e ainda do grau de polimerização (HEIM; TAGLIAFERRO; BOBILYA, 2002; KUMAR; PANDEY, 2013).

Possuem três grupos hidroxila, dois dos quais estão no anel A, nas posições cinco e sete, e um localizado no anel B, na posição três. As ações bioquímicas dos flavonoides dependem da presença e da posição de vários grupos substituintes, que afetam o metabolismo de cada composto. Podem ser encontrados em formas livres ou ligadas: agliconas ou β -glicosídeos (MAJEWSKA; CZECZOT, 2009; KOZLOWSKA; SZOSTAK-WEIGEREK, 2014). As subclasses dos flavonoides incluem: flavonas, flavonois, flavanonas, flavanóis,

antocianidinas e isoflavonas (VEITCH; GRAYER, 2008; BOOTS; HAENEN; BAST, 2008; LAGO et al., 2014) (Figura 8).

Figura 8 - Principais subclasses dos flavonoides



Fonte: Adaptado de HOLLMAN; KATAN (1999)

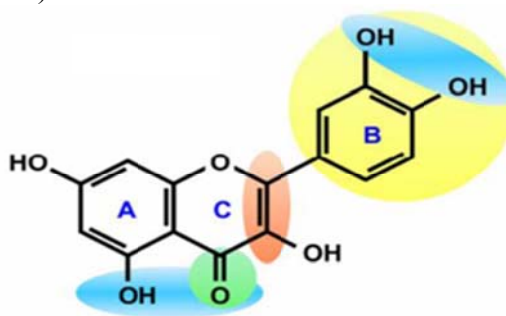
Estes compostos não podem ser sintetizados pelo metabolismo humano e, portanto, devem ser adquiridos através da alimentação. Estima-se que a ingestão diária varie entre 23 mg a 1 g de flavonoides (PETERSON; DRWYER, 1998). Um estudo realizado por ARABBI et al. (2004), mostrou que os alimentos comumente ingeridos pelos brasileiros e que são as principais fontes de flavonoides, são a laranja (70%), alface (9%) e tomate (2,5%), sendo que os principais flavonoides consumidos são os glicosídeos e a quercetina. A média de ingestão destes compostos no Brasil (17 a 88 anos) é de 79 mg/dia para as mulheres e 86 mg/dia para os homens.

A absorção dos flavonoides alimentares liberados por mastigação dependerá das suas propriedades físico-químicas, tais como tamanho molecular, configuração, lipofilicidade, solubilidade e pKa. O flavonoide pode ser absorvido no trato gastrointestinal ou ir para o cólon antes da absorção. Após absorvidos, os flavonoides são conjugados no fígado em glucuronidato ou sulfato conjugado ou metabolizados em compostos fenólicos menores (KUMAR; PANDEY, 2013). Esses metabólitos circulam no sangue, sendo excretados na bile e na urina, tanto em ratos quanto em seres humanos (MANACH et al., 1998).

2.6.1 Quercetina

A quercetina (3,3',4',5,7-pentaidroxiflavona) é o principal flavonoide na dieta humana, amplamente distribuído no reino vegetal e abundante na alimentação humana (PETRY et al., 2007) (Figura 11). Pesquisas têm descrito várias aplicações terapêuticas da quercetina incluindo atividades antioxidante, anti-inflamatória, antitumoral e antiviral, bem como a capacidade de inibir a peroxidação lipídica e agregação plaquetária (AGUIRRE et al., 2011). (Figura 9).

Figura 9 - Estrutura da quercetina. O grupamento mais importante é o catecol (amarelo), outros grupamentos importantes: presença de insaturação no anel C (vermelho), presença de função 4-oxo no anel C (verde)



Fonte: Adaptado de Spencer et al.(2003).

O flavonoide quercetina é geralmente encontrado nos alimentos na forma glicosilada, como β -glicosidase. A natureza da glicosilação é conhecida por influenciar a eficiência de sua absorção (CRESPY et al., 1999; BEHLING et al., 2004). Após a ingestão, os glicosídeos da quercetina são rapidamente hidrolisados no intestino delgado pela atividade bacteriana no cólon para gerar a quercetina aglicona, que é posteriormente metabolizada nas chamadas reações de fase II nos derivados glucuronidados e/ou sulfatados (D'ANDREA, 2015). Vários metabólitos da quercetina são gerados após sua biotransformação, sendo os conjugados predominantes no plasma humano, o 3-O- β -D-glucoronidato (Q3GA) e quercetina-3'-sulfato (D'ANDREA, 2015). Após, esses metabólitos são processados no fígado e nos rins (ALRAWAIQ; ABDULLAH, 2014; BORGHI et al., 2018) e a sua excreção ocorre em até 48 horas, através principalmente da urina, respiração e uma parte pela bile (BEHLING et al., 2004).

Estudos *in vitro* sugerem que a quercetina tem um efeito anti-inflamatório, melhorando os sintomas clínicos e escore da artrite (CHOI et al., 2003). Em um estudo em humanos realizado por JAVADI et al. (2016), foi observado que a suplementação com a

quercetina melhorou a dor, a atividade da doença e a rigidez matinal. Acredita-se que a atividade antiinflamatória inclui a inibição de mediadores inflamatórios, como EROS e NO, a regulação da atividade das enzimas cicloxigenases (COXs) e óxido nítrico sintase indutível (iNOS), a redução dos níveis e expressão de citocinas e pela modulação de fatores de transcrição, como o fator nuclear (TUNON et al., 2009; RIBEIRO et al., 2015). O efeito antioxidante da quercetina se deve à capacidade deste flavonoide reduzir os níveis intracelulares de EROs em vários tipos de células e modular as enzimas desintoxicantes, tais como a SOD e a CAT (LI et al., 2014).

3 OBJETIVOS

3.1 OBJETIVO GERAL

Investigar o efeito do tratamento com a quercetina no sistema purinérgico e no metabolismo oxidativo-inflamatório em modelo de artrite induzida por adjuvante.

3.2 OBJETIVOS ESPECÍFICOS

Em fêmeas de ratos Wistar, com e sem artrite induzida por CFA, tratados com salina ou quercetina, pretendeu-se:

- Realizar a avaliação histopatológica das patas dos animais;
- Determinar a atividade da mieloperoxidase em plasma;
- Determinar as atividades das enzimas E-NTPDase e E-ADA em linfócitos;
- Quantificar as concentrações de nucleotídeos e nucleosídeo de adenina em soro;
- Determinar os níveis séricos das citocinas IFN- γ e IL-4;
- Verificar a atividade de enzimas marcadoras de danos teciduais, alanina aminotransferase (ALT), aspartato aminotransferase (AST) e fosfatase alcalina (FAL);
- Investigar os biomarcadores associados aos danos teciduais causados pelo estresse oxidativo no soro, fígado e rim;
- Verificar a atividade de antioxidantes SOD, CAT e os níveis de antioxidantes não-enzimáticos, tióis totais (-SH) e glutathiona reduzida (GSH) em soro, plasma, fígado e rim;
- Avaliar a atividade genoprotetora e citoprotetora da quercetina.

4 MANUSCRITOS

Os resultados que fazem parte desta tese apresentam-se sob a forma de três manuscritos, os quais se encontram aqui estruturados. Os itens Materiais e Métodos, Resultados, Discussão, e Referências específicas, encontram-se compondo os próprios manuscritos. Estes estão estruturados de acordo com as normas das revistas científicas para as quais formam submetidos, sendo: Cell Biochemistry & Function (Manuscrito I), Journal of Cellular Biochemistry (Manuscrito II) e Nutrition Research (Manuscrito III).

Manuscrito I: Role of quercetin in activity of E-NTPDase/E-ADA and cytokine secretion of complete Freund`s adjuvant-induced arthritis

Manuscrito II: Antioxidant, hepatoprotective and genoprotective effects of quercetin in a murine model of arthritis

Manuscrito III: Antioxidant effect of quercetin on the liver and kidney of rats submitted to CFA-induced arthritis

4.1 MANUSCRITO I

Submetido ao periódico Cell Biochemistry & Function



Effect of Quercetin on E-NTPDase/E-ADA Activities and Cytokine Secretion of Complete Freund's Adjuvant-Induced Arthritic Rats

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Manuscript ID	CBF-18-0060.R1
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Complete List of Authors:	Pereira, Renata; Universidade Federal de Santa Maria, da Silveira, Karine; Universidade Federal de Santa Maria, Adefegha, Adeniyi; Federal University of Technology, Akure, Nigeria, Biochemistry Department Manzoni , Alessandra ; Universidade Federal de Santa Maria, Microbiologia and Parasitologia da Silveira, Leonardo; Universidade Federal de Santa Maria, Coelho, Ana Paula; Universidade Federal de Santa Maria Castilhos, Lívia; Universidade Federal de Santa Maria, Microbiologia e Parasitologia Abdalla, Fátima Becker, Lara Martins, Nara; Universidade Federal de Santa Maria Oliveira, Juliana ; Federal University of Santa Maria, Chemistry Casali, Emerson; Universidade Federal do Rio Grande do Sul, Ciências Morfológicas Leal, Daniela Bitencourt; Universidade Federal de Santa Maria, Microbiologia e Parasitologia
Keywords:	quercetin, ectoenzymes, arthritis, cytokines, complete Freund's adjuvant
Abstract:	The effect of quercetin was assessed in animals induced with complete Freund's adjuvant (CFA). Arthritis scores, paw edema, latency, activities of myeloperoxidase, ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) and ecto-adenosine deaminase (E-ADA) in lymphocytes were determined. Furthermore, nucleotide and nucleoside levels as well as the secretion of pro- and anti-inflammatory cytokines were evaluated. Animals were treated with saline and quercetin in doses of 5 mg/kg, 25 mg/kg and 50 mg/kg for 45 days. The result revealed that quercetin (50 mg/kg) reduced arthritis score and paw edema but increased thermal hyperalgesia, which was observed. Histopathological analysis showed that all the doses of quercetin reduced infiltration of inflammatory cells. MPO activity was increased in the arthritis group; however, quercetin reduced this activity. E-NTPDase activity was increased in lymphocytes of arthritis rats and treatment with quercetin prevented this increase. However, E-ADA activity was reduced in the arthritis group and treatment with quercetin prevented the decreased activity of this enzyme in arthritis rat groups. Adenosine

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	levels were increased in arthritis and the levels were lowered with quercetin treatment. Quercetin treatment in arthritis groups decreased the elevated levels of cytokines in the arthritis control group. Thus, quercetin demonstrated an anti-inflammatory effect and this flavonoid may be a promising natural compound for the treatment of arthritis.

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3 **Effect of Quercetin on E-NTPDase/E-ADA Activities and Cytokine Secretion of**
4 **Complete Freund's Adjuvant-Induced Arthritic Rats**
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Abstract

The effect of quercetin was assessed in animals induced with complete Freund's adjuvant (CFA). Arthritis scores, paw edema, latency, activities of myeloperoxidase, ectonucleoside triphosphate diphosphohydrolase (E/NTPDase) and ecto/adenosine deaminase (E/ADA) in lymphocytes were determined. Furthermore, nucleotide and nucleoside levels as well as the secretion of pro/ and anti/inflammatory cytokines were evaluated. Animals were treated with saline and quercetin in doses of 5 mg/kg, 25 mg/kg and 50 mg/kg for 45 days. The result revealed that quercetin (50 mg/kg) reduced arthritis score and paw edema but increased thermal hyperalgesia, which was observed. Histopathological analysis showed that all the doses of quercetin reduced infiltration of inflammatory cells. MPO activity was increased in the arthritis group; however, quercetin reduced this activity. E/NTPDase activity was increased in lymphocytes of arthritis rats and treatment with quercetin prevented this increase. However, E/ADA activity was reduced in the arthritis group and treatment with quercetin prevented the decreased activity of this enzyme in arthritis rat groups. Adenosine levels were increased in arthritis and the levels were lowered with quercetin treatment. Quercetin treatment in arthritis groups decreased the elevated levels of cytokines in the arthritis control group. Thus, quercetin demonstrated an anti/inflammatory effect and this flavonoid may be a promising natural compound for the treatment of arthritis.

Significance of the study

Quercetin may represent a potential therapeutic compound in the treatment of rheumatoid arthritis. Findings from this study indicate that quercetin suppresses swelling and attenuates the underlying inflammatory responses. This is the first report where quercetin was shown to modulate the immune response to arthritis via attenuation of the purinergic system (E/NTPDase and E/ADA activities) and the levels of IFN/gamma and IL/4. Thus, this work is relevant to basic research and may hereafter be translated into clinical practice.

Keywords: Arthritis; Ectoenzymes; Cytokines; Quercetin; Complete Freund's adjuvant.

1. Introduction

Rheumatoid arthritis (RA) is a chronic auto/inflammatory disorder of the joints¹. It is characterized by irreversible joint damage and destruction of cartilage and bone.^{1,2} It affects approximately 1% of the world's population.³ Patients with RA often experience progressive disabilities with increased risk of death.⁴ In this pathology, an intense inflammatory process activates the immune response leading to an imbalance of pro/ and anti/inflammatory cytokines, thus favoring the induction of autoimmunity.⁵

CD4+ T helper (Th) cells play an important role in the development of RA. During the activation of T cells in a particular cytokine milieu, these naive CD4+ T cells are polarized into lineages of Th cells, including Th1, Th2, Th17, and regulatory T cells (Treg), which are defined by their individual cytokine production patterns and function.⁶ Th1 cells are pro/inflammatory and secrete interferon (IFN)/ γ , tumor necrosis factor (TNF)/ α , and interleukin (IL)/1 β , which promote the inflammatory process and joint damage,^{7,8} while Th2 cell/derived agents like IL/4 and IL/5 induce humoral immunity.⁶

Purinergic signaling plays an important role in modulating inflammatory and immune responses via extracellular biomolecules such as adenine nucleotides, adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), and adenosine.^{9,10} The levels of extracellular ATP, ADP, AMP, and adenosine are dynamically controlled during inflammation by the actions of enzymes expressed in immune cells.¹¹

Ectonucleoside triphosphate diphosphohydrolase (E/NTPDase) is a membrane/ bound enzyme involved in the breakdown of ATP and ADP to AMP, which is sequentially hydrolyzed by E/5'/nucleotidase to adenosine.¹² Ecto/adenosine deaminase (E/ADA) is responsible for catalysis of the irreversible deamination of adenosine and 2'/deoxyinosine, which contributes to the removal of adenosine from the extracellular compartment.¹³

Several epidemiological and experimental studies have shown that quercetin has antioxidant, anti/inflammatory, anti/proliferative, and pro/apoptotic effects.¹⁴ In addition, quercetin can inhibit secretion of inflammatory cytokines, such as IFN/ γ , TNF/ α , and IL/2,¹⁵ and can modulate the production of Th1 and Th2 cell/derived cytokines.^{6,16} Quercetin (3, 3', 4', 5, 7/pentahydroxy flavone) is a natural flavonoid that

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3 is widely distributed in hundreds of herbs (e.g., dill), vegetables (e.g., onions, broccoli,
4 peppers), fruits (e.g., apples, various berries, grapes), and some types of tea and wine.¹⁷
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6 Given that adjuvant arthritis is an experimental model of arthritis widely used for
7 preclinical testing of numerous anti/arthritis agents, it is of clinical interest to
8 investigate the therapeutic action of quercetin as an anti/inflammatory agent. However,
9 there is a paucity of information on the effect of quercetin on the purinergic system in
10 arthritis. The purpose of this study was to investigate the effect of quercetin on E/
11 NTPDase and E/ADA activities in rat lymphocytes, determine serum purine levels, and
12 evaluate the secretion of pro/and anti/inflammatory cytokines of complete Freund's
13 adjuvant (CFA)/induced arthritic rats.
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19 20 21 **2. Materials and methods**

22 23 24 **2.1. Chemicals**

25 Complete Freund's Adjuvant (CFA/0.6% suspension of heat/killed
26 *Mycobacterium tuberculosis* in liquid paraffin, ATP, ADP, adenosine, as well as bovine
27 serum albumin, Trizma base, and Coomassie Brilliant Blue G were obtained from
28 Sigma Chemical (St. Louis, MO, USA). All the other chemicals used in this experiment
29 were of the highest purity.
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34 35 36 **2.2 Animals**

37 Adult female heterogenic and conventional Wistar rats ($n = 40$; 70/90 days, 200/
38 300g) obtained from the Central Animal House of the Federal University of Santa Maria
39 (UFSM), Santa Maria, Brazil, were used for this experiment. The animals were kept on
40 a 12/h light/12/h dark cycle, at a temperature of $22 \pm 2^\circ\text{C}$, with free access to food and
41 water. The animals were handled according to the guidelines of the Committee on
42 Brazilian Society of Animal Science Laboratory, in accordance with international
43 guidelines. This project was approved by the Committee on the Use and Care of
44 Laboratory Animals, Federal University of Santa Maria (UFSM) and assigned the
45 project number (99700812140).
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52 53 54 **2.3 Complete Freund's adjuvant- induced arthritis**

55 To investigate the effects of quercetin in chronic inflammatory process, the
56 adjuvant/induced arthritis model was used. The animals were mildly anaesthetized with
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inhaled isoflurane, and intraplantar injection of 50 μL of CFA (0.6% suspension of heat/killed *Mycobacterium tuberculosis*, 1.0 mg/ μL , in liquid paraffin) or saline (used as a control negative) into the right paw was carried out according to the method described by Choi et al.¹⁸ to induce arthritis.

2.4 Treatment with Quercetin and Saline

The treatment of animals with quercetin was performed in doses of 5mg/kg, 25mg/kg and 50mg/kg during of 45 days. Quercetin was obtained from Sigma Chemical Co. (St. Louis, MO,USA) and freshly prepared in 25% ethanol and was administered orally, by gavage. According to the studies published in our research group, the dilution in 25% ethanol does not interfere with the activity of purinergic system ectoenzymes. The choice of 5, 25 and 50 mg/kg doses of quercetin was made based on previous works, where the therapeutic effects of this compound in rats were reported by Abdalla et al.¹⁹.

The rats were divided into eight groups and the groupings are as follow:

Group I	Normal rats administered saline (CS)
Group II	Normal rats administered quercetin 5 mg/kg (CQ5)
Group III	Normal rats administered quercetin 25 mg/kg (CQ25)
Group IV	Normal rats administered quercetin 50 mg/kg (CQ50);
Group V	Rats induced with CFA and administered saline (AS)
Group VI	Rats induced with CFA and administered quercetin 5 mg/kg (AQ5)
Group VII	Rats induced with CFA and administered quercetin 25 mg/kg (AQ25)
Group VIII	Rats induced with CFA and administered quercetin 50 mg/kg (AQ50)

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3 Rats were administered quercetin and saline for a period of 45 days prior to
4 euthanasia. After the treatment period, animals were anesthetized with isoflurane,
5 submitted to euthanasia and blood collected by cardiac puncture.
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8 9 **2.5 Evidences of arthritis induction or chronic inflammation evaluation**

10 To confirm the development of chronic inflammation and possible anti/
11 inflammatory effect of quercetin, the arthritis score, thermal hyperalgesia and paw
12 oedema were assessed. These tests were performed a day prior to induction of arthritis,
13 on the 15th day after the induction of arthritis, and the day before the euthanasia of the
14 animals.
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20 2.5.1 Arthritis score: To evaluate the progression of the arthritic response elicited by
21 intraplantar CFA injection. The following signs of inflammation were observed and
22 classified according to the scale: oedema formation (0—normal; 1—slight swelling at
23 the injection site; 2—swelling at the injection site and toes or ankle; 3—swelling at the
24 injection site, toes and ankle), redness (0—normal; 1—slightly red/purple; 2—
25 red/purple) and claw position (0—normal; 1—slightly curved; 2—almost closed).
26 Individual scores were added to give the total arthritis score.^{20,21}
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33 2.5.2 Thermal hyperalgesia: To evaluate the hypersensitivity to heat stimulation, the
34 paw immersion test was used of according with Dalmolin et al.²². Briefly, animals were
35 held, and the right hind paw was immersed in a water bath at 48°C. The time elapsed
36 between onset of the stimulus and manifestation of the paw withdrawal response was
37 measured automatically and was taken as an index of the thermal nociceptive threshold.
38 Significant decreases of paw withdrawal latency were interpreted as indicative of heat
39 hyperalgesia.
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46 2.5.3 Paw oedema: To observe the development of oedema, animals were held while
47 right hind paw thickness was measured using a digital caliper.²³ Fifteen days after the
48 induction of inflammation and one day after end treatment of quercetin and saline, new
49 measurements were taken and compared with basal values. An increase on these
50 measurement values compared with baseline was considered as representing oedema
51 values.
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2.6 Histopathological analysis

Sample joint tissues of the right hind paw were collected and fixed in 10% formalin solution and then dehydrated and embedded in paraffin, followed by sectioning and histological staining with haematoxylin and eosin. The slides were observed in optical microscope 100x to evaluate a possible damage.

2.7 Separation of blood plasm

Rats were anesthetized with isoflurane and blood was collected by cardiac puncture. The blood samples were collected in tubes containing dipotassium ethylenediamine tetra acetic acid (EDTA) as anticoagulant and centrifuged at 1,400 x g for 15 min at room temperature, as well as lymphocytes were isolated for the subsequent enzymatic assay and determination of MPO (myeloperoxidase) activity. Additionally, blood was collected without anticoagulant to serum separation.

2.8 Myeloperoxidase (MPO) activity in plasma of arthritis-induced rats

The MPO activity was analyzed spectrophotometrically by the peroxidase/ coupled assay system involving phenol, 4/ aminoantipyrine (AAP) and H_2O_2 .²⁴ Briefly, 390 μ L of 2.5 mM AAP and 20 mM phenol were placed in each tube, followed by 450 μ L of 1.7 mM H_2O_2 . In the presence of H_2O_2 as oxidizing agent, MPO catalyzed the oxidative coupling of phenol and AAP yielding a colored product, quinoneimine, with a maximum absorbance at 500 nm. The millimolar absorbance coefficient for the quinoneimine was determined to be $P = 14 \pm 0.1/\text{mM}/\text{cm}$, close to the previously reported values.²⁵ Results were expressed in micromolar of quinoneimine produced at 30 min.

2.9 Isolation of lymphocytes from blood

Blood was collected with 7.2 mg EDTA as anticoagulant and lymphocyte/ rich mononuclear cell were isolated from blood collected with EDTA and separated on Ficoll/Histopaque density by Boyum.²⁶ The percentage of lymphocytes was superior to 93% as previously outlined by Jaques et al.²⁷. The integrity of lymphocytes preparation was confirmed by determining the lactate dehydrogenase (LDH) activity in intact and disrupted lymphocytes using the kinetic method of the Labquest apparatus (Diagnostics Gold Analyzer). The procedure was repeated before and after the incubation period. The protocol was carried out according to the manufacturer's instructions. Triton X/100

(1%, final concentration) was used to disrupt the lymphocytes preparation. The enzymatic activity was expressed as units per liter, and one unit (1U) corresponds to 1] mol of NADH formed per minute per liter. The resultant lymphocytes samples were used immediately for enzymatic assays.

2.10 Protein determination

Protein was measured by the method previously described by Bradford ²⁸, using bovine serum albumin as standard.

2.11 E-NTPDase activity

E/NTPDase activity in lymphocytes was determined as previously described by Leal et al. ²⁹, in which the reaction medium contained 5 mM CaCl₂, 1,200 mM NaCl, 50 mM KCl, 600 mM glucose and 500 mM Tris-HCl buffer at pH 8.0, with a final volume of 200] L. Twenty microliters of the intact mononuclear cells suspended in saline solution was added to the reaction medium (0.1–0.2 mg/mL of protein) and pre/incubated for 10 min at 37°C; incubation proceeded for 70 min. The reaction was initiated by the addition of substrate (ATP or ADP) at a final concentration of 2.0 mM and stopped with 200] L of 10% trichloroacetic acid (TCA). The released inorganic phosphate (Pi) was assayed by a method previously described by Chan et al. ³⁰, using malachite green as colorimetric reagent and KH₂PO₄ as standard. Controls were carried out by adding the enzyme preparation after TCA addition to correct for non/enzymatic nucleotide hydrolysis. All samples were run in triplicate and the specific activity is reported as nmol of Pi released/min/mg of protein.

2.12 E-ADA activity

E/ADA activity in lymphocytes was measured by the method by Giusti and Galanti, ³¹ which is based on the direct measurement of ammonia produced when ADA acts in excess of adenosine. Briefly, 25] L of lymphocytes reacted with 21 mM of the substrate (adenosine), pH 6.5, and incubation was carried out for 1 h at 37°C. The reaction was stopped by adding 106.2 mM phenol and 167.8 nM sodium nitroprussiate and hypochlorite solution. Ammonium sulfate 75] M was used as ammonium standard. All the experiments were performed in triplicate and the values were expressed in nmolNH₃/min/mg protein.

2.13 Separation of blood serum

The blood samples were collected in tubes without anticoagulant and after the clot formation samples were centrifuged at 1,400 \times g for 15 min at room temperature. The resultant serum samples were aliquoted in microtubes and kept in the refrigerator until the purines and cytokines were quantified.

2.14 Serum purine levels

Purine compounds were analyzed by high/pressure liquid chromatography (HPLC) according to Voelter.³² The proteins were denatured by 0.6 mol/L perchloric acid. All samples were then centrifuged (16,000 \times g for 10 min at 4°C), supernatants were neutralized with 4.0N KOH and clarified with a second centrifugation (16,000 \times g for 30 min at 4°C). After the second centrifugation, the supernatants were collected and centrifuged again (16,000 \times g for 30 min at 4°C). Aliquots of 20 μ L were applied to a reversed/phase HPLC (LC/20AT model, Shimadzu, Kyoto, Japan) using a C₁₈ column (Ultra C18, 25 cm \times 4.6 mm \times 5 μ m, Restek – USA). The elution was carried out applying a linear gradient from 100% solvent A (60 mM KH₂PO₄ and 5 mM of tetrabutylammonium chloride, pH 6.0) to 100% of solvent B (solvent A plus 30% methanol) over a 40 min period (flow rate at 1.0 mL/min). Mobile phases were filtered through a 0.45 μ m Millipore filter prior to analysis, and all reagents utilized were of HPLC grade. The amounts of purines and metabolic residues were measured by absorption at 254 nm. The retention time of standards was used as parameter for identification and quantification by comparison of the peak area. Purine levels were expressed as nmol of different compounds per ml of serum.

2.15 Serum cytokine levels

Serum cytokines were simultaneously measured by Cytometric Bead Array (CBA). The Rat IL/4 and IFN/ γ Flex Set kit (BD Biosciences, San Jose, CA, USA) was applied following manufacturer's instructions. Quantitative results were generated using an Accuri flow cytometer and FCAP Array software. Results were expressed as picogram per milligram of serum.

2.16 Statistical analysis

Data were analyzed by two-way ANOVA. Post hoc analyses were carried out by the Tukey mean \pm standard error of the mean (SEM).

3. Results

3.1 Arthritis induction and effect of quercetin

To investigate the anti/inflammatory effects of quercetin treatment on chronic inflammation, the chronic inflammation induced by intraplantar injection of CFA was evaluated. Fifteen days after the injection of CFA, an increase in the arthritis score (Figure 1A) and paw thickness (Figure 1B) and a concomitant decrease in paw thermal latency (Figure 1C) was observed in all rat groups administered CFA. However, treatment with 50 mg/kg of quercetin caused a significant decrease in the arthritis score and paw thickness as well as an increase in paw thermal latency in rats.

3.2 Histological analysis

After 45 days, joint tissue from the paw of rats from the control saline (CS) group showed organized collagen, normal connective tissue, and no inflammatory infiltrate (Figure 2A). The other control groups (CQ5, CQ25, and CQ50) also showed normal structures (data not shown). The AS group (Figure 2B) showed that the joint space is distended by edema, filled and expanded by an intense inflammatory lymphocytic infiltrate, separating muscle fibers, adipose cells, and attachments such as nerves and vessels. The AQ5 group (Figure 2C) showed that the inflammatory infiltrate contains fewer lymphocytes than the AS group. Moreover, the AQ25 group (Figure 2D) showed less marked inflammatory changes than the AQ5 group. The AQ50 group (Figure 2E) demonstrated lower inflammatory infiltrate than group AQ25.

3.3 Myeloperoxidase (MPO) activity in plasma of arthritis-induced rats

Analysis of MPO activity is presented in Figure 2. MPO activity was significantly increased in the AS group (6.5 μmol quinoneimine, standard error of the mean [SEM] = 0.4, $n = 5$, $P < 0.001$) compared with the CS group (1.9 μmol quinoneimine, SEM = 0.2, $n = 5$, $P < 0.001$). However, treatment with 5, 25, and 50 mg/kg quercetin significantly reduced MPO activity (2.5 μmol quinoneimine, SEM = 0.7, $n = 5$, $P < 0.001$; 2.4 μmol quinoneimine, SEM = 0.4, $n = 5$, $P < 0.001$; 1.7 μmol quinoneimine, SEM = 0.3, $n = 5$, $P < 0.001$, respectively) compared with the AS group (6.5 μmol quinoneimine, SEM = 0.4, $n = 5$, $P < 0.001$).

3.4 Cellular integrity

Analysis of lactate dehydrogenase activity revealed that approximately 5% of lymphocytes of all the rat groups was disrupted, indicating that the preparation was predominantly intact after the isolation procedure (data not shown).

3.5 E-NTPDase activity

Analysis of lymphocyte E/NTPDase activity with ATP as a substrate are shown in Figure 4A. The hydrolysis of ATP was significantly increased in the AS group (43.5 nmol Pi/min/mg protein, SEM = 4.5, $n = 5$, $P < 0.01$) compared with the CS group (7.6 nmol of Pi/min/mg protein, SEM = 0.8, $n = 5$, $P < 0.01$). These results demonstrated that ATP hydrolysis in the AS group was increased approximately 5.7/fold compared with the CS group. However, groups that received quercetin (AQ25: 19.2 nmol of Pi/min/mg protein, SEM = 2.3, $n = 5$, $P < 0.05$; AQ50: 13.1 nmol of Pi/min/mg protein, SEM = 3.5, $n = 5$, $P < 0.01$) showed a decrease compared with the AS group, demonstrating that ATP hydrolysis was decreased approximately 2.2/ and 3.3/fold, respectively. Analysis of lymphocyte E/NTPDase activity with ADP as a substrate are shown in Figure 4B, where ADP hydrolysis was also higher in the AS group (77.5 nmol of Pi/min/mg protein, SEM = 4.5, $n = 5$, $P < 0.001$) compared with the CS group (21.2 nmol of Pi/min/mg protein, SEM = 1.2, $n = 5$, $P < 0.001$), representing an increase of 3.6/fold. On the other hand, treatment with 25 and 50 mg/kg quercetin resulted in a decrease compared with the AS group (AQ25: 31.7 nmol of Pi/min/mg protein, SEM = 9.9, $n = 5$, $P < 0.01$; AQ50: 18.6 nmol of Pi/min/mg protein, SEM = 3.8, $n = 5$, $P < 0.001$), indicating a decrease of 2.4/ and 4.2/fold, respectively.

3.6 E-ADA activity

Analysis of E/ADA activity is shown in Figure 5. The deamination of adenosine was lower in the AS group (5.1 nmol of Pi/min/mg protein, SEM = 1.1, $n = 5$, $P < 0.001$) compared with the CS group (42.6 nmol of Pi/min/mg protein, SEM = 5.9, $n = 5$, $P < 0.001$), indicating a decrease of 8.3/fold. Treatment with quercetin modulated the activity of E/ADA as revealed by the AQ5 (37.6 nmol of Pi/min/mg protein, SEM = 4.8, $n = 5$, $P < 0.001$), AQ25 (46.8 nmol of Pi/min/mg protein, SEM = 4.9, $n = 5$, $P < 0.001$), and AQ50 (54.4 nmol of Pi/min/mg protein, SEM = 2.8, $n = 5$, $P < 0.001$) groups, demonstrating increases of 7.4/, 9.2/, and 10.7/fold, respectively.

3.7 Serum purine levels

Purine levels in the serum were measured by high performance liquid chromatography (HPLC) (Table 1). There were no significant alterations in the levels of ATP, ADP, and AMP between the arthritis/saline and control/saline groups as well as the other groups. However, a higher level of adenosine was found in the AS group (8.12 nmol/mL of serum, SEM = 0.31, $n = 5$, $P < 0.05$) compared with the control/saline group (4.32 nmol/mL of serum, SEM = 0.97, $n = 5$, $P < 0.05$).

3.8 Serum cytokine levels

Serum IFN/ γ and IL/4 levels are shown in Figure 6. Results obtained for the proinflammatory cytokine IFN/ γ are shown in Figure 6A. Serum levels for IFN/ γ were significantly increased in the AS group (79.0 pg/mL, SEM = 4.3; $n = 5$; $P < 0.001$) compared with the CS group (17.7 pg/mL, SEM = 8.4; $n = 5$; $P < 0.001$). However, there was a significant decrease in IFN/ γ levels in the arthritis group that received 5 mg/kg (33.7 pg/mL, SEM = 6.9; $n = 5$; $P < 0.01$), 25 mg/kg (25.3 pg/mL, SEM = 2.9; $n = 5$; $P < 0.001$) and 50 mg/kg (17.9 pg/mL, SEM = 2.7; $n = 5$; $P < 0.001$) quercetin compared with the AS group (79.0 pg/mL \pm 4.3; $n = 5$; $P < 0.001$). As shown in Figure 6B, the average serum levels for the anti/inflammatory cytokine IL/4 were significantly increased in the AS group (29.6 pg/mL, SEM = 6.6; $n = 5$; $P < 0.001$) compared with the CS group (7.7 pg/mL, SEM = 3.8; $n = 5$; $P < 0.001$). Our results also revealed a significant decrease in IL/4 levels in the arthritis group treated with 5 mg/kg (8.2 pg/mL, SEM = 4.2; $n = 5$; $P < 0.01$), 25 mg/kg (1.1 pg/mL, SEM = 0.3; $n = 5$; $P < 0.001$), and 50 mg/kg (2.0 pg/mL, SEM = 0.7; $n = 5$; $P < 0.001$) quercetin compared with the AS group (29.6 pg/mL, SEM = 6.6; $n = 5$; $P < 0.001$).

4. Discussion

The involvement of cells such as lymphocytes, monocytes, neutrophils, and macrophages in the inflammatory response in RA has been well studied.^{33,34} Patients with RA require long/life therapy and there is a possibility of the development of resistance to synthetic drugs, leading to increased occurrence of adverse effects.¹⁴ Natural alternative and effective chemotherapeutic agents could provide promising anti/inflammatory effects.

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3 CFA/induced arthritis is a veritable model for assessing the series of
4 inflammatory processes that occur in RA.^{35,36} Reports have shown that approximately
5 90% of changes observed in this model are similar to those observed in human arthritis
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7³⁷. In this way, novel chemotherapeutic agents can be easily evaluated for the treatment
8 of chronic inflammatory conditions.^{38,39} Thus, this study was performed to assess the
9 effect of arthritis on purinergic enzyme activity, nucleotide and nucleoside levels, and
10 secretion of pro/ and anti/inflammatory cytokines, as well as the effect of three doses of
11 quercetin.
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15 To confirm the arthritis in rats, arthritis score, edema formation, thermal
16 hyperalgesia, histological analysis, and MPO activity were evaluated. The results of this
17 study showed that 50 mg/kg quercetin improved the physical features and ameliorated
18 the progression of arthritis induced by CFA. This agrees with the report of Mamani/
19 Matsuda et al.³⁸, where quercetin was shown to be effective in reducing signs of
20 arthritis.
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25 Histological evaluation revealed that all of the doses of quercetin tested in this
26 study reduced the inflammatory process. This is consistent with previous studies where
27 quercetin was shown to inhibit inflammatory aspects of synovial cell function and
28 neutrophil activation.^{40,41} It is believed that anti/inflammatory activity includes
29 inhibition of inflammatory agents and mediators such as enzymatic reactive oxygen
30 species and nitric oxide, regulation of the activity of cyclooxygenase enzymes and
31 inducible nitric oxide synthase, reduction of the levels and expression of cytokines, and
32 modulation of transcription factors such as factor nuclear kappa B (NF/ κ B)⁴².
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38 MPO activity is an important inflammatory marker and is present in the granules
39 of leukocytes, and primarily in neutrophils and macrophages. It is responsible for
40 secreting hypochlorite.⁴³ In this study, the AS group exhibited elevated MPO activity,
41 which indicates a progressive inflammatory process. This is consistent with the results
42 of Stamp et al.⁴³, where MPO activity was reported to be increased in arthritis patients,
43 indicating its role in the pathogenesis and severity of arthritis. Conversely, animals
44 treated with quercetin showed lower levels of MPO activity, indicating a decrease in
45 neutrophil infiltration and subsequent reduction in the inflammatory process, thereby
46 contributing to the decreased tissue damage observed in the histopathology of the paw.¹⁹
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53 Alterations in the activity of ectoenzymes, such E/NTPDase and E/ADA, have
54 been reported in several diseases, including ischemic heart disease⁴⁴, HIV⁴⁵, Chagas
55 disease⁴⁶, and multiple sclerosis⁴⁷, indicating that these enzymes could be involved in
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3 the pathogenesis of many diseases, including RA.^{36,48/50} However, this is the first study
4 where the role of quercetin on the purinergic system was determined in lymphocytes of
5 the CFA/induced arthritis rat model. The increase in E/NTPDase activity in arthritic rats
6 indicates that there is increased ATP and ADP hydrolysis in the extracellular milieu,
7 which would reduce their levels during the development of RA. This change is likely a
8 result of a dynamic response of lymphocytes in an attempt to maintain appropriate
9 levels.⁴⁹

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14 At high concentrations, extracellular ATP activates proinflammatory purinergic
15 P2X7 receptors, stimulates the Th1 immune response, and contributes to tissue damage
16 as well as inflammatory conditions.^{11,51} The increased E/NTPDase activity in
17 arthritis/saline rats compared with the control/saline rats is consistent with the results of
18 Castilhos et al.³⁶ and Silveira et al.⁵⁰ However, in groups treated with different doses
19 of quercetin, we observed that this flavonoid was able to reduce the activity of E/
20 NTPDase. At low concentrations, extracellular ATP possesses affinity for the P2Y
21 receptor subtype on the surface of lymphocytes. These purinergic receptors, when
22 stimulated, downregulate proinflammatory cytokines and stimulate the Th2 immune
23 response, leading to the production of anti/inflammatory cytokines.^{11,46} It has been
24 reported that quercetin exhibits anti/inflammatory properties and reduces cellular
25 damage⁵². The pro/inflammatory cytokine TNF/ α plays a central role in the
26 pathogenesis of RA⁵³. In RA, TNF/ α is expressed by macrophages, synovial lining
27 cells, and activated T cells within the inflamed joint⁵⁴. A study conducted by Nardi et
28 al.⁵⁵, suggests that quercetin has the ability to modulate the immune response and
29 exhibits anti/inflammatory activity by decreasing the endogenous production of TNF/ α .

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40 In addition to ATP and ADP, adenosine exhibits potent anti/inflammatory and
41 immunosuppressive actions through the inhibition of T cell proliferation, cytokine
42 secretion, and leukocyte migration through the endothelial barrier⁵⁶. Our results
43 demonstrated that E/ADA activity was decreased in lymphocytes of arthritis rats
44 compared with the control group. Consistent with our results, other studies have also
45 shown a decrease in E/ADA activity with arthritis.^{49,50} However, treatment with
46 quercetin (all doses) was able to reverse this effect in this study.

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51 In the study carried out by Abdalla et al.⁵⁷, the effect of quercetin (1, 5, 10, 25,
52 and 50 μ M) *in vitro* resulted in no significant differences in lymphocyte E/NTPDase
53 activity when ATP or ADP was used as a substrate. On the other hand, E/ADA activity
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3 *in vitro* showed a significant decrease proportional to the increase in quercetin
4 concentration compared with 0 μ M quercetin (control group).
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6 The products of nucleotide degradation were analyzed in the control and arthritis
7 group treated with saline and quercetin by HPLC. The results revealed that there were
8 no changes in the ATP, ADP, and AMP levels. However, adenosine levels were
9 increased in the AS rat group. Extracellular adenosine levels mediate an auto/regulatory
10 immunosuppressive loop to protect healthy tissues.⁵⁸
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13 The observed increase in adenosine levels may be attributed to the decreased E/
14 ADA activity in the AS group, as well as the attendant systemic changes caused by
15 inflammatory conditions. Adenosine plays a central and direct role in the regulation of
16 inflammatory responses by inhibiting lymphocyte activation and decreasing both Th1
17 and Th2 cytokine secretion through A2A receptor activation.⁵⁹ However, the observed
18 decrease in adenosine levels in rats treated with quercetin could suggest an anti/
19 inflammatory mechanism of quercetin. Previous studies have demonstrated that one of
20 mechanisms by which quercetin employs its beneficial effects in the central nervous
21 system is through modulation of E/ADA activity because this flavonoid inhibits activity
22 of this enzyme, thus contributing to the increased levels of adenosine.¹⁹
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30 Recent studies have shown that an imbalance in T cell subsets plays an important
31 role in RA. CD4⁺ T cells can be divided into Th1, Th2, Th17, and Treg subsets
32 according to differentiation and function.⁶⁰ Th1 cells secrete a large amount of IFN/ γ ,
33 which is responsible for promoting cell/mediated immunity.⁶⁰ IFN/ γ is synthesized in
34 the cellular immune response process and is a strong immune regulator, promoting
35 inflammation by inducing the expression of a variety of cytokines, including TNF/ α , IL/
36 2, and IL/10.⁶¹ Nair et al.¹⁶ showed that IL/4 and IFN/ γ play a significant role in the
37 regulation of immune responses by their mutually antagonistic mechanisms. Other
38 parameters evaluated in this study were the secretion of pro/ and anti/inflammatory
39 cytokines in rats with arthritis.
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46 Our results demonstrate higher levels of IFN/ γ in arthritis, indicating an
47 inflammatory state. Consistent with the results of our study, Niu et al.⁸ also found
48 significantly higher levels of serum IFN/ γ in an animal model of collagen/induced
49 arthritis. Pavlovic et al.⁶² also found significantly elevated IFN/ γ levels in the serum of
50 patients with early RA, indicating that this pro/inflammatory cytokine might play a key
51 role in maintaining immune homeostasis in patients with RA. Our data also
52 demonstrated decreased levels of IFN/ γ following treatment with 5, 25, and 50 mg/kg of
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3 quercetin. In addition, Yu et al.⁶³ observed that in supernatants from activated Th cells
4 cultured with either rutin or quercetin, only 20 and 40 μ M quercetin was able to reduce
5 IFN/ γ levels, demonstrating that quercetin exerts anti/inflammatory activities by
6 regulating inflammatory cytokine production mediated by macrophages and T
7 lymphocytes.
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11 Another cytokine evaluated in this study was IL/4. Our results demonstrated that
12 IL/4 levels were increased in the arthritis rats compared with the control group. Naciute
13 et al.⁶⁴ also found increased levels of IL/4 in the plasma of RA patients. However,
14 when arthritis rats were treated with 5, 25, and 50 mg/kg of quercetin, the levels of IL/4
15 were lower in relation to the arthritis group treated with saline. Our results are
16 consistent with the study of Braun et al.⁶⁵, which showed that pre/treatment with
17 quercetin reduced the levels of IL/4 in rats with hyperlipidemia, decreased eosinophil
18 recruitment, and inhibited NF/ κ B activation in the inflammatory process⁶⁶.
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24 Overall, our results demonstrate alterations in enzymes of the purinergic system
25 and in MPO activity of lymphocytes, as well in cytokine secretion in arthritic rats,
26 indicating that these alterations may be due to the inflammatory process of arthritis.
27 Interestingly, treatment with quercetin was able to prevent the alterations caused by
28 arthritis induced by CFA, thus we suggest that this flavonoid exhibits anti/inflammatory
29 effects and may be a promising natural compound to be used as an adjuvant in the
30 treatment of arthritis.
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46 **Compliance with ethical standards**

47 All procedures performed in studies involving animals were in accordance with the
48 ethical standards of the institution or practice at which the studies were conducted.
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52 **Conflict of interest**

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56 The authors declare that they have no conflict of interest.
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Legends to Figures

Figure 1. Effect of quercetin (5, 25 and 50 mg/kg) and saline on some inflammatory changes induced by intraplantar CFA on the arthritis score (A), oedema formation (B) and thermal hyperalgesia (C). *** $P < 0.001$ – basal values when compared to induction arthritis and # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ / arthritis saline treated when compared to other groups arthritis treated. Two-way analysis (ANOVA) followed by Tukey post test (n=5).

Figure 2. Histological image of joint tissues from treated or non/treated rats. Groups: CS (control/ saline, A), AS (arthritis/saline, B), AQ5 (arthritis/quercetin 5mg/kg, C), AQ25 (arthritis/quercetin 25mg/kg, D) and AQ50 (arthritis/quercetin 50mg/kg, E). Arrow demonstrates the lymphocytes and asterisks edema.

Figure 3. MPO activity in plasma of rats submitted to an experimental model of arthritis treated with saline and quercetin at doses 5, 25 and 50 mg/kg. Asterisks indicates significant differences from the control saline (*** $P < 0.001$). Ash indicates significant differences from the arthritis saline (### $P < 0.001$). Bars represent mean \pm SEM (n=5). Two-way ANOVA/Tukey test.

Figure 4. E/NTPDase activity (ATP and ADP hydrolysis, A and B respectively) in lymphocytes of rats submitted to an experimental model of arthritis treated with saline and quercetin at doses 5, 25 and 50 mg/kg. Asterisks indicates significant differences from the control saline ($P < 0.001$). Ash indicates significant differences from the arthritis saline (*** $P < 0.001$, ## $P < 0.01$ and ### $P < 0.001$). Bars represent mean \pm SEM (n=5). Two-way ANOVA/Tukey test.

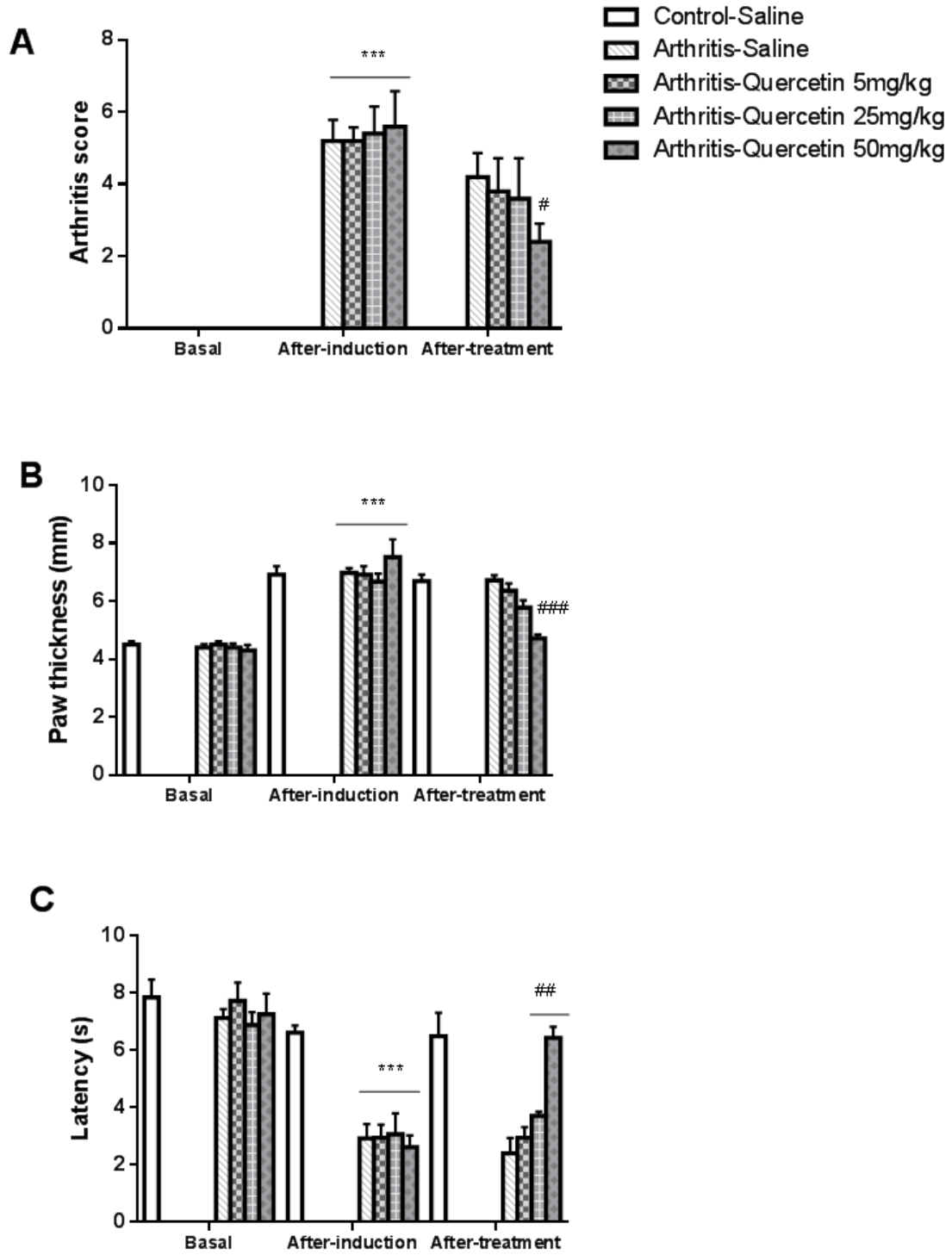
Figure 5. E/ADA activity (adenosine deamination) in lymphocytes of rats submitted to an experimental model of arthritis treated with saline, quercetin at doses 5, 25 and 50 mg/kg. Asterisks indicates significant differences from the control saline (*** $P < 0.001$). Ash indicates significant differences from the arthritis saline (### $P < 0.001$). Bars represent mean \pm SEM (n=5). Two-way ANOVA/Tukey test.

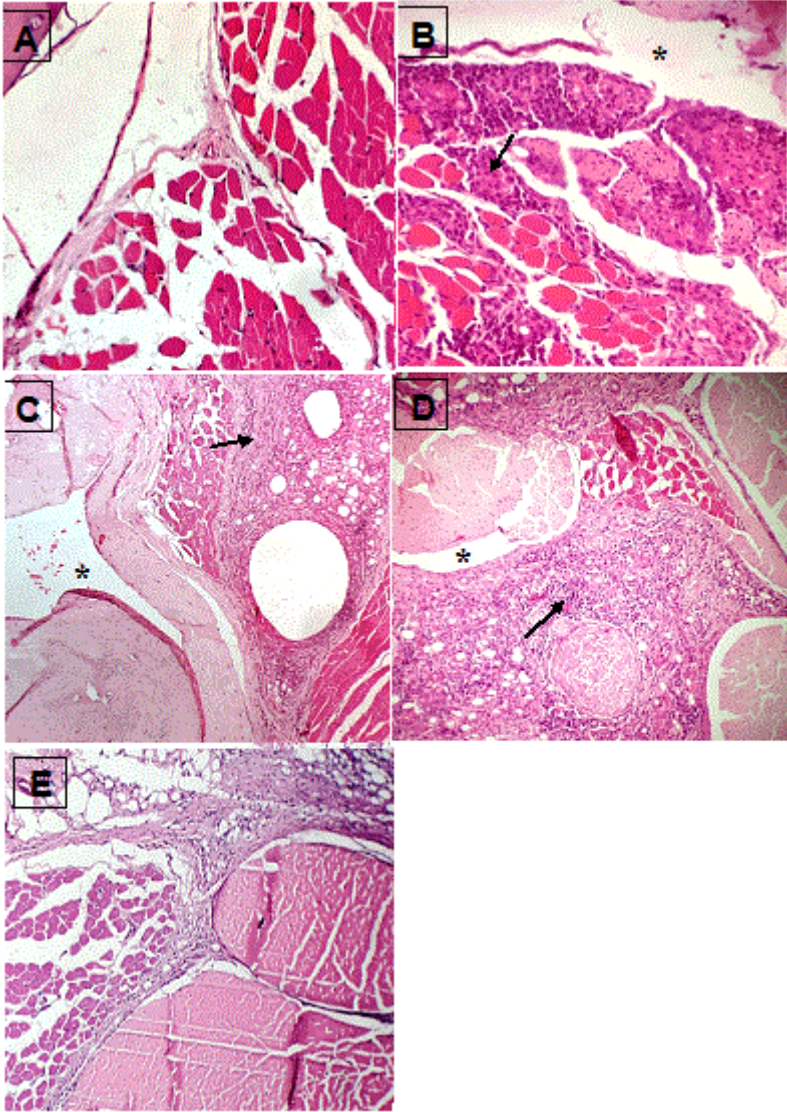
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2
3 Fig. 6 Serum levels of INF/ γ (A) and IL/4 (B) cytokines. Cytokines levels are reported
4 as of pg/mL. Groups: saline control (CS), saline+quercetin 5 mg/kg (CQ5),
5 saline+quercetin 25 mg/kg (CQ25), saline+quercetin 50 mg/kg (CQ50), arthritis +
6 saline (AS), arthritis +quercetin 5 mg/kg (AQ5), arthritis+quercetin 25 mg/kg (AQ25)
7 and arthritis+quercetin 50 mg/kg (AQ50). Bars represent mean \pm S.E.M. *Asteriks*
8 indicates significant differences from the control saline (** $P < 0.001$). *Ash* indicates
9 significant differences from the arthritis saline ($^{\#} P < 0.01$, $^{\#\#} P < 0.001$). Bars represent
10 mean \pm SEM (n=5). Two/way ANOVA/Tukey test
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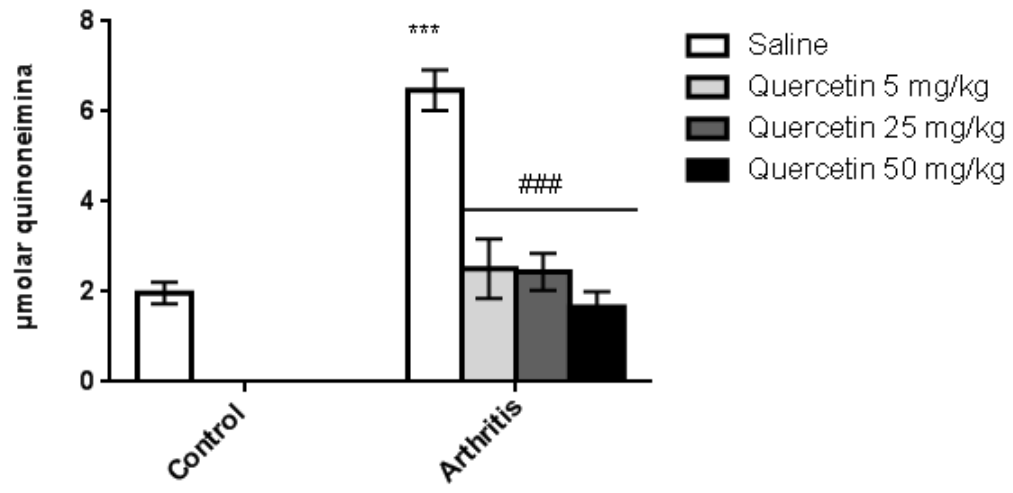
Table 1: Purine levels in rats with arthritis induced by CFA serum and treated with quercetin and saline

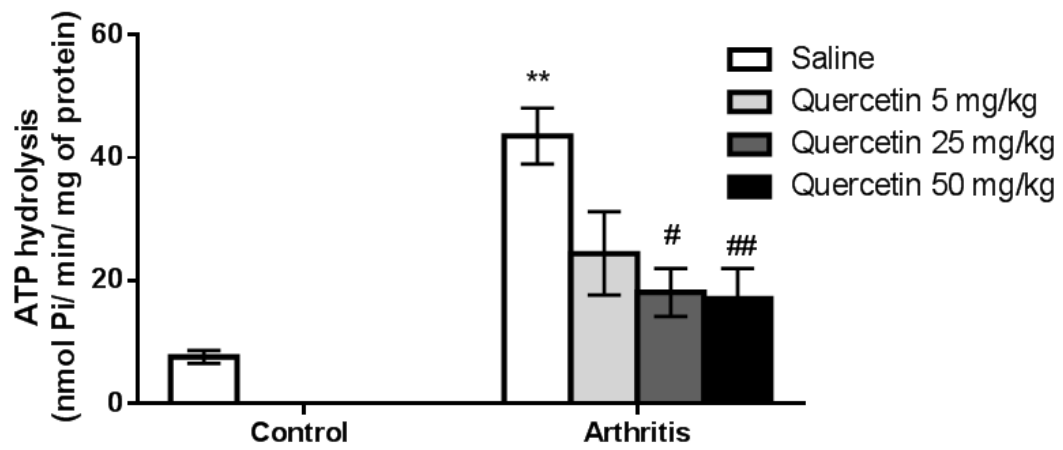
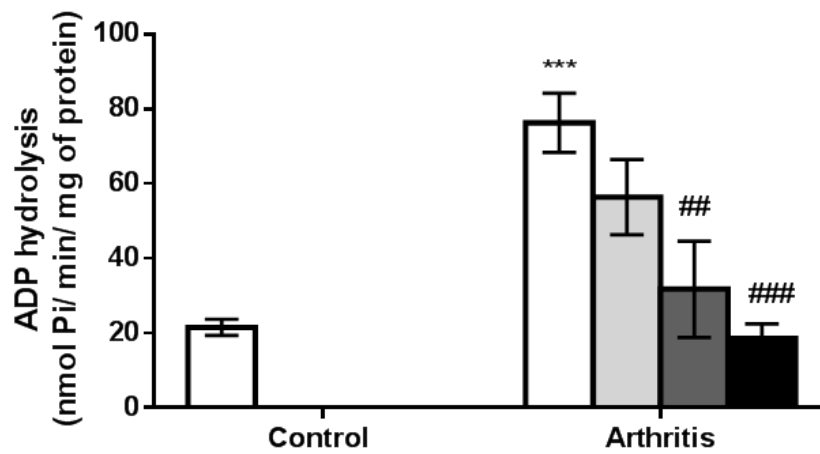
	ATP (nmol/mL of serum)	ADP (nmol/mL of serum)	AMP (nmol/mL of serum)	Adenosine (nmol/mL of serum)
CS	2.11±0.50 ^a	5.10±0.62 ^a	24.19±3.35 ^a	4.32±0.97 ^a
CQ5	1.62±0.10 ^a	6.61±1.49 ^a	31.09±4.20 ^a	5.87±0.34 ^a
CQ25	2.22±0.25 ^a	5.82±0.95 ^a	23.61±2.04 ^a	4.31±0.60 ^a
CQ50	2.25±0.62 ^a	5.22±2.29 ^a	29.84±1.79 ^a	4.00±1.14 ^a
AS	2.58±0.10 ^a	8.38±0.53 ^a	27.58±2.31 ^a	8.12±0.31 ^b
AQ5	2.06±0.41 ^a	7.83±2.12 ^a	32.64±1.79 ^a	6.89±0.64 ^a
AQ25	2.37±0.68 ^a	7.66±1.02 ^a	24.60±3.03 ^a	5.97±0.65 ^a
AQ50	1.84±0.30 ^a	8.40±0.46 ^a	27.22±1.22 ^a	6.15±0.63 ^a

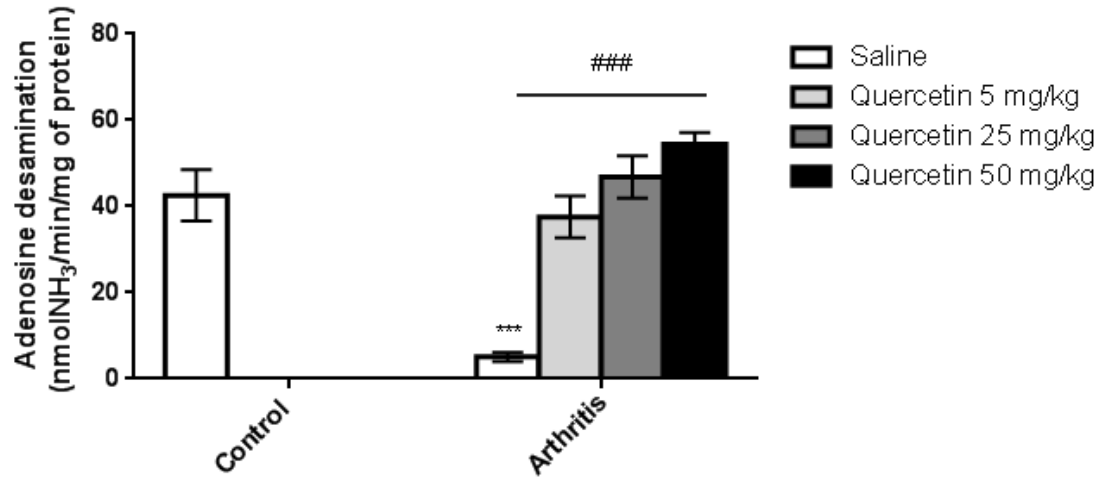
Adenine nucleotides and adenosine levels measurement in serum of control and arthritis rats and treated for 45 days with quercetin (5, 25 and 50 mg/kg) and treated with saline. Purine levels measurement are reported as of nmol/ml of serum. Groups: saline control (CS), saline+quercetin 5 mg/kg (CQ5), saline+quercetin 25 mg/kg (CQ25), saline+quercetin 50 mg/kg (CQ50), arthritis + saline (AS), arthritis +quercetin 5 mg/kg (AQ5), arthritis+quercetin 25 mg/kg (AQ25) and arthritis+quercetin 50 mg/kg (AQ50). Bars represent mean ± S.E.M. Groups with different letters are statistically different. ($P < 0.05$; $n = 5$) (Two-way ANOVA/Tukey Multiple Comparison Test).

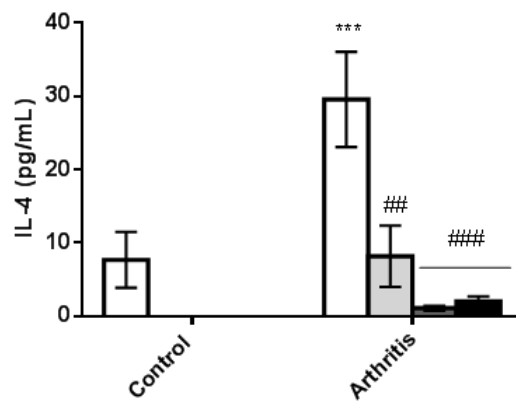
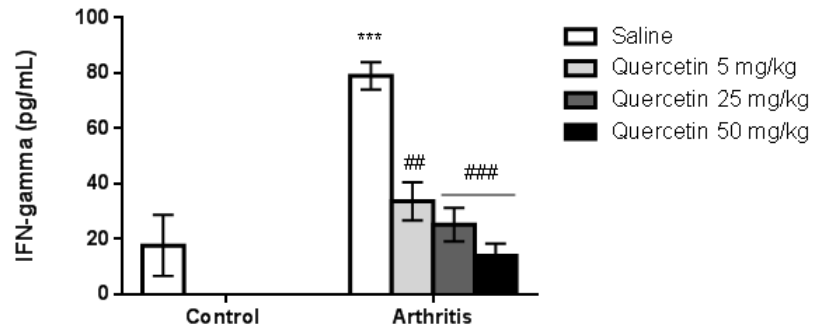


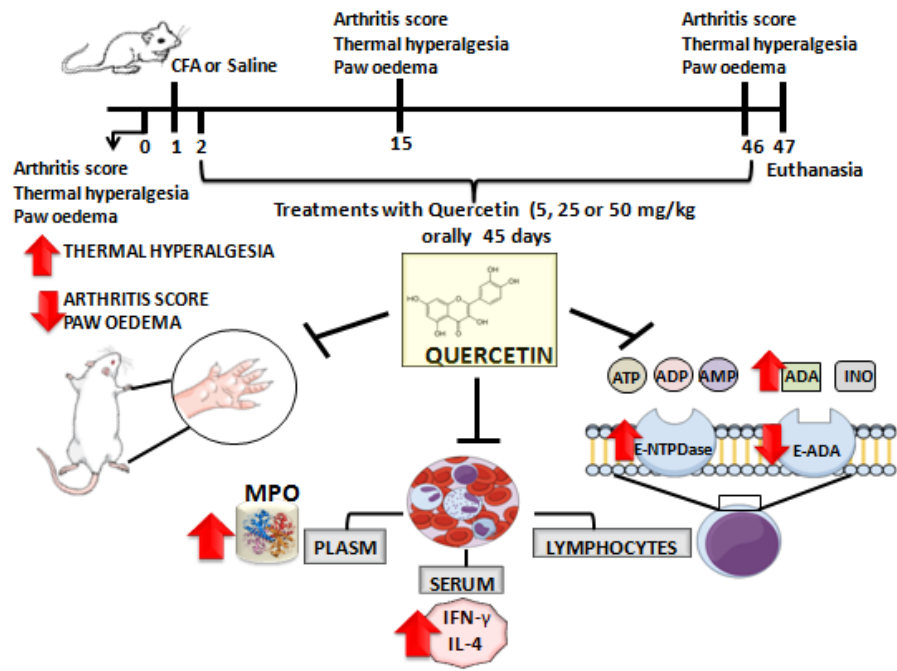




A**B**







4.2 MANUSCRITO II

Submetido ao periódico Journal of Cellular Biochemistry



Antioxidant, hepatoprotective, genoprotective and cytoprotective effects of quercetin in a murine model of arthritis

Journal:	<i>Journal of Cellular Biochemistry</i>
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Keywords:	Rheumatoid arthritis, Oxidative stress, genotoxicity, cytotoxicity, quercetin

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Manuscripts

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3 **Antioxidant, hepatoprotective, genoprotective and cytoprotective effects**
4 **of quercetin in a murine model of arthritis**
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6 **Running title:** Quercetin for treatment of arthritis
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For Peer Review

ABSTRACT

Rheumatoid arthritis is a highly debilitating, inflammatory autoimmune disease which is characterised by joint destruction. The present study sought to investigate the effect of quercetin in rats with complete Freund's adjuvant-induced arthritis. Animals were divided into control/saline, control/quercetin 5 mg/kg, control/quercetin 25 mg/kg, control/quercetin 50 mg/kg, arthritis/saline, arthritis/quercetin 5 mg/kg, arthritis/quercetin 25 mg/kg or arthritis/saline 50 mg/kg; and the saline or quercetin treatments were administered for 45 days. Biochemical parameters, oxidative stress, genotoxicity and cytotoxicity were evaluated. The results showed that treatment with all doses of quercetin reduced the levels of aspartate aminotransferase, reactive oxygen species and thiobarbituric acid reactive substances; although only treatment with 25 or 50 mg/kg increased catalase activity levels. Total thiol and reduced glutathione levels were not significantly affected. Genotoxicity, assessed by DNA damage and cytotoxicity through picogreen assay, decreased following treatment with quercetin. Our results present evidence of the antioxidant, hepatoprotective, genoprotective and cytoprotective effects of quercetin, indicating its potential as a candidate for adjuvant therapy.

Keywords: Rheumatoid arthritis, Oxidative stress, genotoxicity, cytotoxicity, quercetin.

1. Introduction

Rheumatoid Arthritis (RA) is a chronic, autoimmune and inflammatory arthropathy that mainly affects the synovial joints and is characterised by the presence of erosion, degradation and destruction of cartilage and bone (Ma et al., 2018; Sokka et al., 2009). The aetiology of RA is multifactorial and as yet incompletely elucidated; however, several studies have reported that inflammation, lipid peroxidation, oxidative stress and increased production of reactive oxygen species (ROS) affect the progression and severity of the pathology (Shahmohamadnejad et al., 2015; Mackness & Mackness, 2004; Li, Liu, & Liang, 2003). The imbalance between free radical production and the reduction of antioxidant defence status is defined as oxidative stress (Valko et al., 2007).

Production of ROS occurs in every cell type, and these molecules can affect various cellular functions including cell signalling, angiogenesis, apoptosis and cell proliferation (Dornelles et al., 2017; Fernandez, Fornari, & Barbosa, 2007; Giorgio, Trinei, Migliaccio, & Pelicci, 2007). They may damage biological molecules, such as lipids, resulting in highly reactive and unstable lipid hydroperoxides that then decompose to form thiobarbituric acid reactive substances (TBARS) (Ghani, Barril, Bedgood, & Prenzler, 2017; Montes-Nieto et al., 2017; Tsikas, 2017).

In addition, DNA damage caused by ROS can include genome instability, increased mutation, apoptosis and associated tissue regeneration and cell proliferation (Altindag, Karakoc, Kocyigit, Celik, & Soran, 2007; Sawa & Ohshima, 2006). In chronic inflammatory diseases such as RA and systemic lupus erythematosus, ROS are released by phagocytic cells at the lesion site, and pass through cell membranes to react with nuclear DNA (Bashir, Harris, Denman, Blake, & Winyard, 1993). The inflammatory nature of RA, particularly during periods of exacerbation, implies that a state of oxidative stress may exist in this disease (Kocyigit et al., 2005). Damage caused by oxidative stress can be attenuated by antioxidant enzymatic systems, represented by the enzymes superoxide dismutase, catalase and glutathione peroxidase (GSH-Px) and non-

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3 enzymatic systems such as vitamin C, vitamin E, carotenoids and flavonoids
4 (Gutteridge & Halliwell, 2010; Vasiljevic et al., 2016).
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7 Quercetin is an important exogenous flavonoid with anti-inflammatory
8 and antioxidant properties (Borghi et al., 2018; Guazelli et al., 2018; Salem
9 Alrawaiq & Abdullah, 2014; Valerio et al., 2009), as well as anticarcinogenic,
10 analgesic, antihypertensive and antiulcer effects (Anjaneyulu & Chopra, 2003;
11 Borghi et al., 2018). Clinical and experimental data have shown the cardio- and
12 bowel-protective functions of this flavonoid (Borghi et al., 2018; Guazelli et al.,
13 2018; Salem Alrawaiq & Abdullah, 2014; Valerio et al., 2009). Quercetin is
14 found in several fruits and vegetables including capers, dill, cilantro, broccoli,
15 onions and various berries (such as chokeberries, cranberries and
16 lingonberries) (Darband et al., 2018; Sak, 2014).
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25 The antioxidant action of quercetin is effected through the elimination of
26 free radicals and inhibition of oxidation of many molecules (Aviram & Fuhrman,
27 2002; Chen, 2010; Williams, Spencer, & Rice-Evans, 2004). Furthermore, the
28 flavonoid effectively blocks the proliferation of synoviocytes and development of
29 angiogenesis in inflammatory processes associated with arthritis. These
30 mechanisms demonstrate the potential of quercetin as an antirheumatic drug
31 (Guazelli et al., 2018; Jackson, Higo, Hunter, & Burt, 2006).
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38 Taking the above-mentioned evidence into account, the aim of this study
39 was to investigate whether treatment with quercetin provides protection against
40 complete Freund's adjuvant (CFA)-induced arthritis using a murine model. To
41 this end; biochemical parameters, oxidative stress, genotoxicity and cytotoxicity
42 were evaluated.
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50 **2. Materials and methods**

51 **2.1. Chemicals**

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53 Complete Freund's Adjuvant (CFA-0.6% suspension of heat-killed
54 *Mycobacterium tuberculosis* in liquid paraffin, bovine serum albumin, Trizma
55 base, and Coomassie Brilliant Blue G were obtained from Sigma Chemical (St.
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3 Louis, MO, USA). All the other chemicals used in this experiment were of the
4 highest purity. Commercial kits for the measurement of donated from Bioclin
5 Quibasa (Belo Horizonte-MG, Brazil). The Quant-iT™ PicogreenH dsDNA
6 Assay Kit was obtained from Life-Technologies (Carlsbad, CA, USA).
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10 **2.2 Animals**

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13 Forty Adult female, heterogenic, and conventional Wistar rats (200–300
14 g) were used in this experiment. The animals were obtained from the Central
15 Animal House of the Federal University of Santa Maria (UFSM), Brazil. They
16 were maintained at constant temperature (23±1°C) on a 12 h light/dark cycle
17 with free access to food and water. All animals were maintained in accordance
18 with the guidelines on the use of animals, approved by the Ethics committee of
19 the UFSM (protocol number: 99700812140).
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29 **2.3 Experimental design and Complete Freund's adjuvant-induced 30 arthritis**

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33 The animals were mildly anesthetized with inhaled isoflurane, and
34 intraplantar injection of 50 µL of CFA (0.6% suspension of heat-killed
35 *Mycobacterium tuberculosis*, 1.0 mg/µL, in liquid paraffin) or saline (used as a
36 control negative) into the right paw was carried out according to the method
37 described by Oliveira et al. (2007) (Oliveira et al., 2007) to induce arthritis
38 (Figure 1). The rats were divided into eight groups of five animals each,
39 according to the treatment administered are follow: Group I: saline solution only
40 (NaCl 0.9%) (CS); Group II: 5 mg/kg of quercetin only (CQ5); Group III 25
41 mg/kg of quercetin only (CQ25); Group IV: 50 mg/kg of quercetin only (CQ50);
42 Group V: saline solution only (NaCl 0.9%) (AS), 14 days post-induction with
43 CFA; Group VI 5 mg/kg of quercetin only (AQ5), 14 days post-induction with
44 CFA; Group VII: 25 mg/kg of quercetin only (AQ25), 14 days post-induction with
45 CFA; Group VIII: 50 mg/kg quercetin only (AQ50), 14 days post-induction with
46 CFA. The animals were treated with either sterile saline (NaCl 0.9%) or
47 quercetin (5, 25 and 50 mg/kg) by oral gavage for 45 days. In this study, the
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3 quercetin dose was chosen based on an earlier report by Abdalla et al. (2014)
4 (Abdalla et al., 2014).
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10 **2.4 Sample collection**

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12 After 45 days of treatment (day 59), the rats were anesthetized with
13 isoflurane and blood samples were collected by cardiac puncture. The blood
14 samples were collected in tubes without anticoagulant, with
15 ethylenediaminetetraacetic acid (EDTA) and with citrate. Specimens were
16 centrifuged at 2,500×g for 15 min at 4 °C. Serum was used to determine alanine
17 aminotransferase (ALT), aspartate aminotransferase (AST), alkaline
18 phosphatase (ALP), reactive oxygen species (ROS), thiobarbituric acid reactive
19 substances (TBARS) and picogreen. Plasma was used to total thiols (T-SH)
20 and reduced glutathione (GSH). Lymphocytes were separated from whole blood
21 with EDTA for DNA damage. Whole blood collected with citrate was used to
22 assess catalase.
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32 **2.5 Biochemical parameters**

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34 Commercial Kits (Bioclin®) were used for ALT, AST and ALP
35 measurements, in semi-automatic equipment (Bioclin 100®).
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42 **2.6 Oxidative stress determinations**

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44 Oxidative stress markers were determined in serum or whole blood, and
45 assays were run in triplicate.
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51 **2.6.1 Measurement of reactive oxygen species (ROS) production**

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53 ROS production by cells was determined by the levels of 2'-7'-
54 dichlorofluorescein (DCF) (Myhre, Andersen, Aarnes, & Fonnum, 2003). Fifty µL
55 aliquots of serum supernatant were added to the medium comprising distilled
56 water and 2'-7'-dichlorofluorescein diacetate (DCFHDA) (1 mM) and incubated
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3 in the dark for 1 h. Then, the fluorescence was evaluated (excitation at 488 nm
4 and emission at 525 nm). A standard curve was used to determine DCF levels
5 and results were corrected for protein levels and were expressed as DCFCH-
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7 DA fluorescence.
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10 11 12 13 **2.6.2 Thiobarbituric acid-reactive substance (TBARS) measurement**

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16 The method described by Jentzsch et al. (1996) (Jentzsch, Bachmann,
17 Furst, & Biesalski, 1996) was used to measure TBARS levels. Lipid
18 peroxidation index, by the reaction with thiobarbituric acid, was measured
19 through the concentration of malondialdehyde (MDA). Two hundred μL of serum
20 or standard (0.03 mM MDA), 1 mL of 0.2 M orthophosphoric acid and 250 μL of
21 0.1M thiobarbituric acid (TBA) composed the reaction mixture, which was
22 incubated at 95°C for 120 min and read in a spectrophotometer at 532 nm.
23 Seric levels of TBARS were expressed as nmol MDA/mg of protein.
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33 **2.6.3 Catalase activity (CAT)**

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36 Nelson and Kiesow (1972) (Nelson & Kiesow, 1972) method was used to
37 quantify catalase (CAT) activity. Twenty μL of citrate whole blood was added to a
38 mixture of 50 mM potassium phosphate buffer (pH 7), 10 mM H_2O_2 . The
39 absorbance was observed for 2 min (every 10 s) at 240 nm to determine the
40 rate of H_2O_2 reaction. The results were expressed in nmol/mg protein/minute.
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48 **2.6.4 Total thiols determination (T-SH)**

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51 Ellman (1959) (Ellman, 1959) modified method was used for
52 determination of total thiols (T-SH) levels. After the addition of 50 μL of 10 mM
53 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), 100 μL of plasma was used in a
54 final volume of 1000 μL solution. The reaction product was measured at 412
55 nm. The thiols groups content was calculated using a cysteine standard curve
56 and expressed as nmol T-SH/g protein.
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2.6.5 Reduced glutathione measurement (GSH)

GSH was measured spectrophotometrically with Ellman's reagent (Ellman, 1959). Firstly, the plasma proteins were precipitated using TCA 10%. An aliquot of 100 μ l of the supernatant of plasma in a final volume of 1000 μ l of solution was used for the reaction. The reaction product was measured at 412 nm after the addition of 10 mM 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB)(0.05 ml). A standard curve using reduced glutathione was added to calculate the content of thiol groups in samples and was expressed as nmol GSH/g protein.

2.7 Protein determination

The method of Bradford (Bradford, 1976) was used for protein content, bovine serum albumin was used as a standard.

2.8 Genotoxicity

2.8.1 Lymphocyte separation

Lymphocyte separation was performed by gradient density with Histopaque-1077 (Sigma–Aldrich Co.) (St Louis, USA), according to Böyum, 1968 (Boyum, 1968).

2.8.2 Comet assay

Comet assay was performed using Singh et al. (1995) (Singh, McCoy, Tice, & Schneider, 1988), in agreement with the comet assay general guidelines (Hartmann et al., 2003; Tice et al., 2000). Slides were made and immersed in a lysis solution at 4 °C for 1 h, followed by electrophoresis at 25 V and 300 mA for 40 min at a steady temperature. The slides were then silver-stained, as described by Nadin et al. (2001) (Nadin, Vargas-Roig, & Ciocca, 2001). A total of 100 cells from each rat were carefully chosen and evaluated. The tail length

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3 of the cells was visually scored from 0 (no migration) to 4 (maximal migration).
4 The comet parameters analyzed were the percentage of DNA in the comet tail
5 and tail moment.
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11 **2.9 Cytotoxicity**

12 **2.9.1 DNA Picogreen**

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17 The PicoGreen fluorescence assay was performed according to the
18 manufacturer's protocol (Quant ITTM, Invitrogen). The Picogreen reagent will
19 be diluted 1: 200 with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and
20 incubated with 10 μ l of plasma in the dark at room temperature for 5 min. To
21 minimize photobleaching effects, the time for fluorescence measurement will be
22 kept constant for all samples. A standard curve will be generated using double-
23 stranded calf thymus DNA supplied by the manufacturer. All fluorescence
24 measurements will be recorded on a fluorescence plate reader (Multi-mode M2
25 /M2e SpectraMax Reader Plate, Molecular Devices Corporation, Sunnyvale,
26 CA, USA) at the emission wavelength of 528 nm and excitation 485 nm at room
27 temperature (25°C). Results were expressed as a percentage of double-strand
28 DNA (dsDNA).
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44 **2.10 Statistical analysis**

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46 Data were analyzed by two-way ANOVA. Post hoc analyses were carried
47 out by the Tukey mean \pm standard error of the mean (SEM).
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53 **3. Results**

54 **3.1 Biochemical parameters**

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57 The results of the biochemical analyses are shown in Table 1. Regarding
58 markers of liver damage, an increase in serum AST was observed in the AS
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3 group (251.5 U/L, SEM = 2.8, $P < 0.001$) when compared with the CS group
4 (120.6 U/L, SEM = 9.7). Treatment with quercetin confirmed that the flavonoid
5 reduced the levels of AST in the AQ5 (133.7 U/L, SEM = 9.3, $P < 0.001$), AQ25
6 (123.6 U/L, SEM = 12.0, $P < 0.001$) and AQ50 groups (111.7 U/L, SEM = 9.3, P
7 < 0.001) when compared with the AS group. The levels of ALT and ALP were
8 no significantly different between the groups.
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17 **3.2 Oxidative stress analysis**

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19 Figure 2 illustrates the measured levels of ROS levels in serum.
20 Significantly increased ROS levels were observed in the AS group (0.0036
21 DCFH-DA Fluorescence, SEM = 0.001, $P < 0.001$) compared with the CS group
22 (0.0011 DCFH-DA Fluorescence, SEM = 0.001). However, treatment with 5, 25
23 or 50 mg/kg quercetin significantly reduced ROS levels in the AQ5 (0.0018
24 DCFH-DA Fluorescence, SEM = 0.001, $P < 0.001$), AQ25 (0.0015 DCFH-DA
25 Fluorescence, SEM = 0.001, $P < 0.001$) and AQ50 groups (0.0014 DCFH-DA
26 Fluorescence, SEM = 0.001, $P < 0.001$) compared with the AS group.
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37 **3.3 Thiobarbituric acid reactive substances measurement**

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39 The serum TBARS levels are presented in Figure 3. In the AS group, an
40 increase in TBARS level was observed (0.20 nmol MDA/mg of protein, SEM =
41 0.04, $P < 0.001$) compared with the CS group (0.04 nmol MDA/mg of protein,
42 SEM = 0.01). Treatment with 5, 25 or 50 mg/kg of quercetin prevented this
43 increase, with lower levels of TBARS recorded in the AQ5 (0.06 nmol MDA/mg
44 of protein, SEM = 0.01, $P < 0.001$), AQ25 (0.06 nmol MDA/mg of protein, SEM
45 = 0.01, $P < 0.001$) and AQ50 groups (0.05 nmol MDA/mg of protein, SEM =
46 0.01, $P < 0.001$) compared to AS group.
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3.4 Total thiol determination and reduced glutathione measurement

The results of total thiol determination and reduced glutathione measurement in plasma are shown in Table 2. No statistical difference was observed between the thiol levels of the AS (1326.4 nmol T-SH/g protein, SEM = 91.5, $P > 0.05$) and CS groups (1598.8 nmol T-SH/g protein, SEM = 164.9). Similarly, no statistical difference was observed between the reduced glutathione levels of the AS (61.36 nmol G-SH/g protein, SEM = 3.04, $P > 0.05$) and CS groups (70.47 nmol G-SH/g protein, SEM = 2.36).

3.5 Catalase activity

Results of the catalase activity assays are presented in Figure 4. Activity levels were significantly higher in the AQ25 (8.55 nmol/mg protein/minute, SEM = 1.35, $P < 0.05$) and AQ50 groups (9.31 nmol/mg protein/minute, SEM = 1.54, $P < 0.01$) compared with the AS group (3.66 nmol/mg protein/minute, SEM = 0.48).

3.6 Genotoxicity and Cytotoxicity

The potential genotoxicity in lymphocytes and the cytotoxic effects in serum are shown in Table 3. The results show a statistically significant difference in terms of DNA damage in the AS group (117.8 DNA damage index, SEM = 3.0, $P < 0.001$) compared with the CS group (88.8 DNA damage index, SEM = 1.6). In contrast, the level of DNA damage was significantly reduced in groups treated with quercetin; AQ5 (95.4 DNA damage index, SEM = 2.6, $P < 0.001$), AQ25 (81.6 DNA damage index, SEM = 1.3, $P < 0.001$) and AQ50 (73.0 DNA damage index, SEM = 1.6, $P < 0.001$).

The dsDNA assay indicated that the percentage of dsDNA was increased in the AS group (122.7 dsDNA, SEM = 3.9, $P < 0.05$) compared with the CS group (120.2 dsDNA, SEM = 5.1). However, after treatment with quercetin, dsDNA levels were significantly decreased in the AQ5 (87.0 dsDNA, SEM = 5.5, $P < 0.001$), AQ25 (68.7 dsDNA, SEM = 5.2, $P < 0.001$) and A50 groups (65.4

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3 dsDNA, SEM = 2.5, $P < 0.001$).

4 5 6 7 8 **4. Discussion**

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10 In the present study, we evaluated the protective effects of quercetin in
11 CFA-induced arthritis in rats. The successful induction of arthritis and
12 subsequent inflammatory process were confirmed by histological analysis,
13 arthritis scores, paw oedema and thermal hyperalgesia; as well as the serum
14 cytokine profile (data not shown). Arthritic animals exhibited increased hepatic
15 damage, oxidative stress, and genotoxicity and cytotoxicity parameters; and
16 were observed to have a reduced capacity of antioxidant defence. Quercetin
17 treatment led to an improvement in all these parameters.

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19 Quercetin is a flavonoid that has been identified as having potential
20 therapeutic effects in many models of disease (Valerio et al., 2009; Casagrande
21 et al., 2006; Jackson et al., 2006). The CFA model is a scientifically-justified
22 experimental method in which immunopathological arthritis is induced in
23 animals with similar cellular immune responses and pathological mechanisms to
24 humans (Mahdi, Khan, Asmawi, Mahmud, & Murugaiyah, 2017; Nair, Singh, &
25 Gupta, 2012). The model presents several advantages, including prediction of
26 efficacy of novel compounds, superior clinical manifestation and progression of
27 disease compared with other models and low inter- and intra-variability among
28 tested animals. These features allow the use of fewer laboratory animals than
29 other arthritis models require (Mahdi et al., 2017; Tuncel et al., 2016).

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31 One of the consequences of arthritis is hepatic damage associated with
32 the systemic chronic inflammation that is characteristic of this condition (De,
33 Ravishankar, & Bhavsar, 1994). Liver dysfunction is assessed by the level of
34 aminotransferases released by damaged cells (Raj Kapoor et al., 2009). The
35 only change in biochemical parameters indicative of liver dysfunction that was
36 observed in our study was an increase in AST activity in the AS group.
37 Corroborating our results, da Silveira et al. (2016) (da Silveira et al., 2016) also
38 reported increased AST levels in rats with induced arthritis, which may be a
39 result of the arthritic inflammatory process.

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3 Treatment with quercetin at any dosage caused the serum AST levels of
4 arthritic rats to revert to normal levels, demonstrating the hepatoprotective
5 potential effect of this flavonoid. This effect has been verified in other studies
6 (Afifi, Ibrahim, & Galal, 2018; Wei et al., 2017; Miltersteiner et al., 2003), and
7 has been suggested to be due to the antioxidant capacity of quercetin; effected
8 through its ability to scavenge reactive oxygen species (Tzankova et al., 2017;
9 Filipe et al., 2001), inhibit lipoxygenase and cyclooxygenase and stabilise cell
10 membranes (Tzankova et al., 2017; Kumarappan et al., 2011; Cos et al., 1998).

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13 In the present study, we observed increased ROS and TBARS levels in
14 the untreated arthritic group, which can be the production of ROS as a result of
15 inflammation, leading to the destruction of cartilage and bone, neutrophil
16 degranulation and the release of various potentially harmful enzymes (Vasiljevic
17 et al., 2016; Stamp et al., 2012). Oxidative stress can induce the peroxidation of
18 membrane lipids, which can lead to inactivation of membrane-bound receptors
19 or enzymes with a consequential increase in tissue permeability and damage to
20 normal cellular function (Dalle-Donne, Rossi, Colombo, Giustarini, & Milzani,
21 2006). One of the markers used to evaluate lipid peroxidation is the level of
22 TBARS (Bauerova et al., 2015) which is elevated in patients with RA due to
23 excessive lipid peroxidation in cells and plasma, or as a result of excessive
24 generation and diffusion of lipid peroxides in inflamed joints (Vijayakumar,
25 Suresh, & Manoharan, 2006).

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28 Besides that, it was found that quercetin treatment reversed the
29 increases in ROS and TBARS levels in arthritic subjects. This corroborates with
30 other studies which have demonstrated that quercetin could decrease levels of
31 ROS and TBARS (Baldissareli et al., 2016; Abdalla et al., 2014), possibly
32 through the inactivation of highly reactive species by hydroxyl groups present in
33 the flavonoid (Jeyadevi et al., 2013). Previous studies have also shown that
34 quercetin prevents lipid peroxidation and eliminates superoxide radicals
35 (Johnson & Loo, 2000; Kumar, Sharma, Khanna, & Raj, 2003) by scavenging
36 free radicals and thus delaying oxidative pathway-mediated cell injury, ensuring
37 protection against lipid peroxidation and chelating metal ions (dos Santos et al.,
38 2018; Kumar et al., 2003; Johnson & Loo, 2000).

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3 The antioxidant defence system includes antioxidant enzymes, catalase,
4 glutathione S-transferase, superoxide dismutase and glutathione peroxidase as
5 well as non-enzymatic antioxidants such as flavonoids, carotenoids, vitamins
6 (A, C and E), glutathione and minerals (Jeyadevi et al., 2013). We observed
7 increased catalase activity following treatment with quercetin at doses of 25 and
8 50 mg/kg compared with the untreated arthritic group, although no effect was
9 observed in total thiol or reduced glutathione levels. The effect of quercetin on
10 catalase activity has been demonstrated previously by Sherif (2018) (Sherif,
11 2018) in a model of hepatotoxicity, where quercetin treatment led to increased
12 hepatic catalase levels. This suggests that quercetin is able to protect the liver
13 from the deleterious effects of melphalan by scavenging free radicals and
14 enhancing the activities of the hepatic antioxidant enzymes superoxide
15 dismutase and catalase.
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26 Another important result of this study was the reduction in DNA damage.
27 It is known that chronic inflammatory diseases such as RA and systemic lupus
28 erythematosus are characterised by ROS release by phagocytic cells at the
29 lesion site. These ROS pass through cell membranes and react with nuclear
30 DNA (Johnson & Loo, 2000), which can contribute to increased mutation rates,
31 genome instability, apoptosis and associated tissue regeneration and cell
32 proliferation (dos Santos et al., 2018; Ansar, Siddiqi, Zargar, Ganaie, &
33 Abudawood, 2016). In the present study, DNA damage was higher in the
34 untreated arthritic group, which corroborates the findings of Altindag et al.
35 (2007) (Altindag et al., 2007), who reported significantly higher DNA damage in
36 RA patients than in healthy subjects. However, we observed a significant
37 reduction in DNA damage in groups treated with quercetin. This result is
38 consistent with results presented by Marcolin et al. (2013) (Marcolin et al.,
39 2013), which demonstrated the protective role of quercetin in an experimental
40 model of non-alcoholic steatohepatitis. Taken together, these results indicate
41 that quercetin is able to scavenge ROS, resulting in decreased levels of DNA
42 strand breaks and oxidised bases.
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56 In a study conducted by Ansar et al. (2016) (Ansar et al., 2016),
57 quercetin was found to decrease DNA damage in rats with acrylamide-induced
58 toxicity, suggesting that the antioxidant effects of this flavonoid are due to high
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3 membrane diffusion which aids the removal of oxyradicals from the lipid bilayer.
4 Furthermore, the pentahydroxyl structure of quercetin allows the chelation of
5 metal ions, thereby eliminating lipid alkoxyl and peroxy radicals (Lien, Ren, Bui,
6 & Wang, 1999). This flavonoid may also be indirectly involved in the induction of
7 detoxifying genes (Kahraman, Sabuncuoğlu, & Yilmaz, 2006; Ha et al., 2003;
8 Molina, Sanchez-Reus, Iglesias, & Benedi, 2003; Polat, Tokyol; Youdim,
9 Spencer, Schroeter, & Rice-evans, 2002).

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16 Double strand DNA fragmentation was found to be increased in arthritic
17 rats, indicating cytotoxicity which was likely due to oxidative damage and
18 apoptosis. This observation was reversed by treatment with quercetin, in line
19 with the results of Abdalla et al. (2014) and Lovato et al. (2016) (Lovato et al.,
20 2016; Abdalla et al., 2014) from studies on murine models of chemical
21 cytotoxicity. This effect may be associated with the antioxidant action of
22 quercetin, due to the presence of the O-dihydroxyl structure at the 3' and
23 4' positions in the B-ring—referred to as the catechol group—which has a high
24 scavenging capacity due to its configuration (Lovato et al., 2016; D'Andrea,
25 2015).

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34 In summary, the present study demonstrates that quercetin can provide
35 protection against tissue damage by reversing the deleterious effects of CFA-
36 induced arthritis. This protective effect was demonstrated by reductions in AST,
37 ROS, TBARS levels and an increase in CAT activity following administration of
38 quercetin, as well as by reductions in DNA damage and fragmentation of double
39 strand DNA. Cellular damage is associated with clinical signs of AR such as
40 pain, stiffness and joint deformity, which make this condition highly debilitating.
41 The systemic effects of AR also result in damage to tissues other than joints
42 and bones. Our findings illustrate the potential antioxidant effects of quercetin,
43 highlighting possible therapeutic applications of this flavonoid. Thus, quercetin
44 is a potential candidate for adjuvant treatment of RA, worthy of further
45 investigation to establish its use in clinical practice.
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Compliance with ethical standards

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Conflict of interest

The authors declare that they have no conflict of interest.

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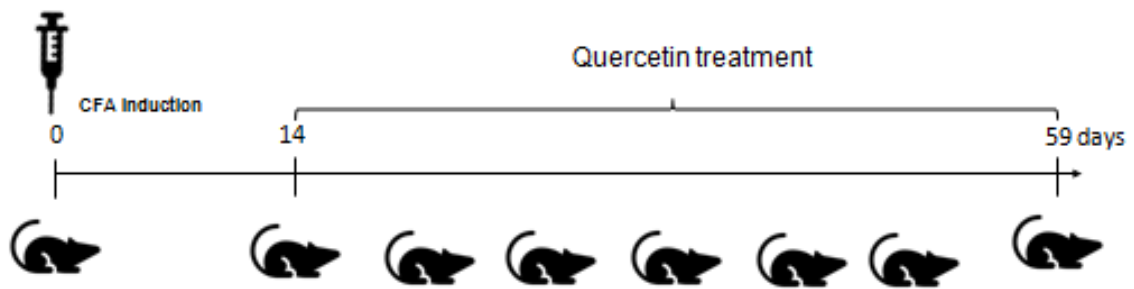


Figure 1. Experimental design of the study detailing animal grouping

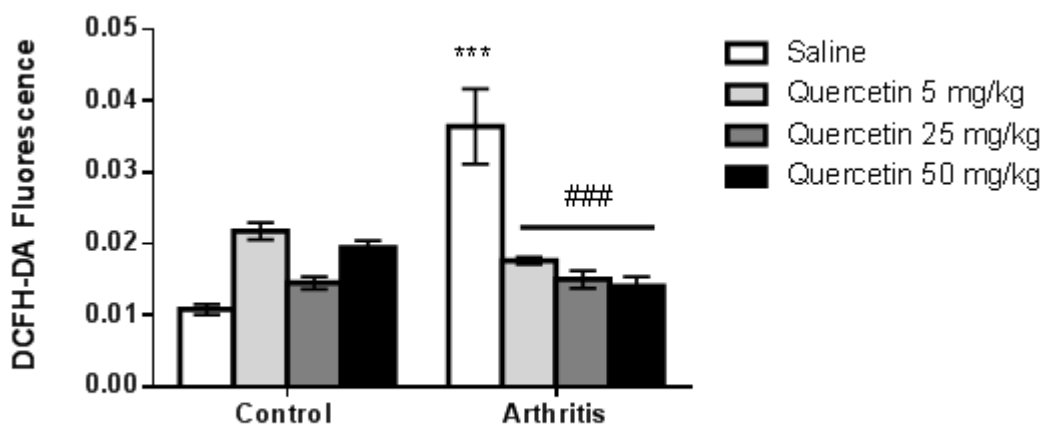


Figure 2. Measurement of reactive oxygen species (ROS) in serum of rats submitted to an experimental model of arthritis treated with saline, quercetin at doses 5, 25 and 50 mg/kg. Asterisks indicates significant differences from the control saline (***) $P < 0.001$). Ash indicates significant differences from the arthritis saline (###) $P < 0.001$. Bars represent mean \pm SEM ($n=5$). Two-way ANOVA-Tukey test.

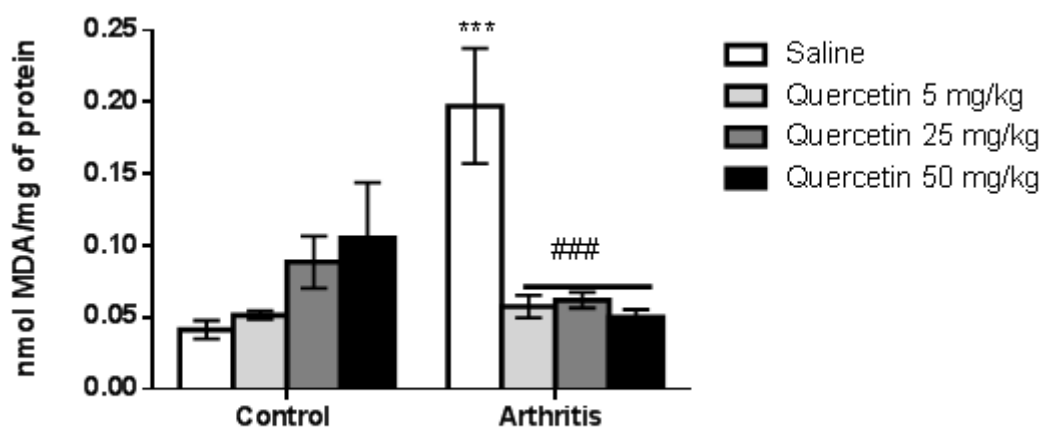


Figure 3. Measurement of thioarbituric acid reactive substances (TBARS) in serum of rats submitted to an experimental model of arthritis treated with saline, quercetin at doses 5, 25 and 50 mg/kg. Asterisks indicates significant differences from the control saline (***) $P < 0.001$). Ash indicates significant differences from the arthritis saline (###) $P < 0.001$. Bars represent mean \pm SEM (n=5). Two-way ANOVA-Tukey test.

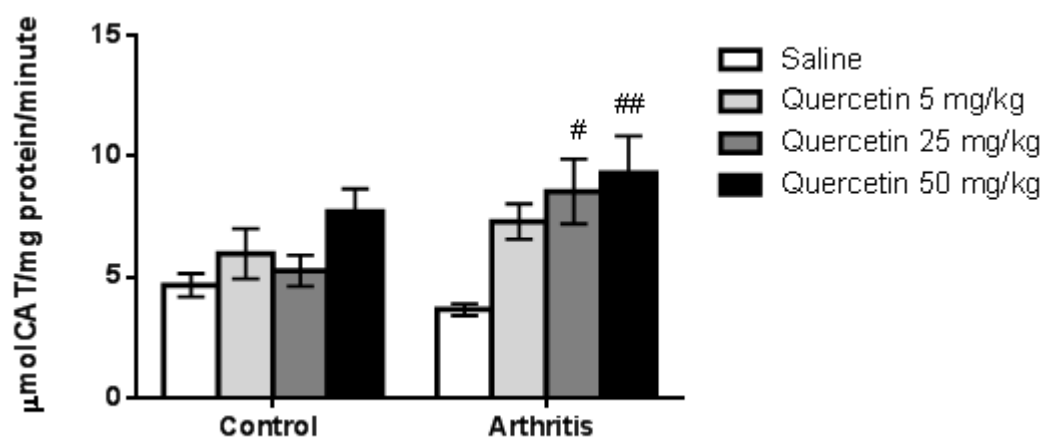


Figure 4. Catalase activity in total blood of rats submitted to an experimental model of arthritis treated with saline, quercetin at doses 5, 25 and 50 mg/kg. Ash indicates significant differences from the arthritis saline (# $P < 0.05$, ## $P < 0.01$). Bars represent mean \pm SEM (n=5). Two-way ANOVA-Tukey test.

Figure Legend

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Table 1: Seric levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) of rats submitted to an experimental model of arthritis treated with saline, quercetin at doses 5, 25 and 50 mg/kg

	ALT	AST	ALP
	(U/L)	(U/L)	(U/L)
CS	57.5±5.3 ^a	120.6±9.7 ^a	395.5±58.8 ^a
CQ5	50.5±3.8 ^a	123.9±5.9 ^a	504.0±32.1 ^a
CQ25	55.0±6.7 ^a	129.7±15.4 ^a	422.3±81.6 ^a
CQ50	57.6±3.0 ^a	122.4±6.4 ^a	480.3±58.1 ^a
AS	65.00±6.3 ^a	251.5±2.8 ^b	457.2±54.0 ^a
AQ5	54.5±6.1 ^a	133.7±9.3 ^c	391.2±72.9 ^a
AQ25	54.6±2.4 ^a	123.6±12.0 ^c	498.5±53.7 ^a
AQ50	46.8±4.0 ^a	111.7±9.3 ^c	361.7±59.0 ^a

Groups: saline control (CS), saline+quercetin 5 mg/kg (CQ5), saline+quercetin 25 mg/kg (CQ25), saline+quercetin 50 mg/kg (CQ50), arthritis + saline (AS), arthritis +quercetin 5 mg/kg (AQ5), arthritis+quercetin 25 mg/kg (AQ25) and arthritis+quercetin 50 mg/kg (AQ50). Bars represent mean ± S.E.M. Groups with different letters are statistically different. ($P < 0.001$; $n = 5$) (Two-way ANOVA-Tukey Multiple Comparison Test).

Table 2: Total thiols determination and reduced glutathione measurement in plasma of rats submitted to an experimental model of arthritis treated with saline, quercetin at doses 5, 25 and 50 mg/kg

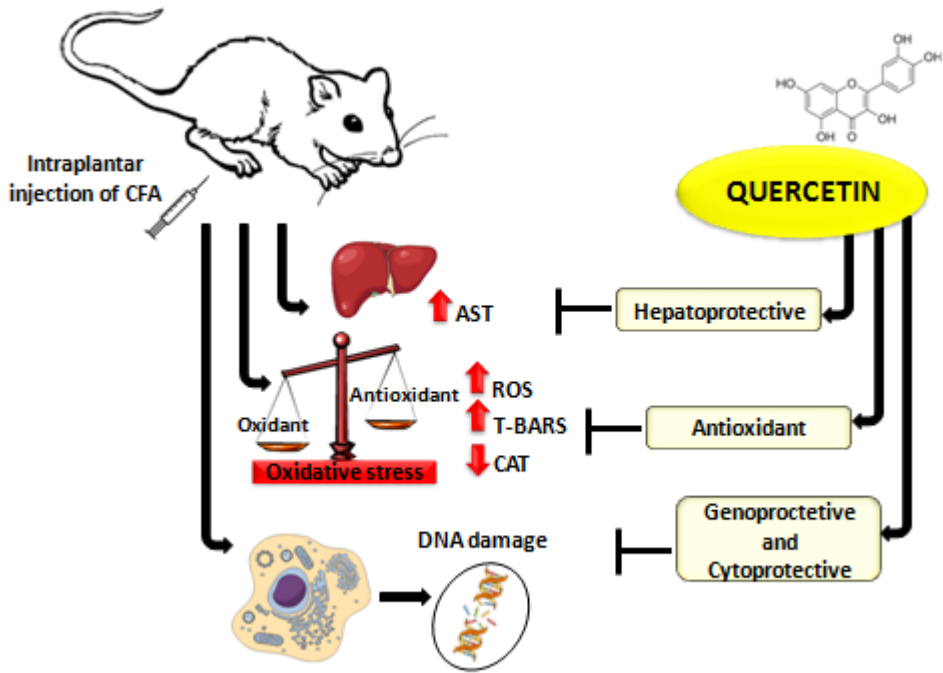
	T-SH	G-SH
CS	1598.8±164.9 ^a	70.5±2.4 ^a
CQ5	1150.7±53.1 ^a	73.9±2.0 ^a
CQ25	1278.7±79.2 ^a	72.7±1.1 ^a
CQ50	1634.5±158.0 ^a	72.1±2.0 ^a
AS	1326.4±91.5 ^a	61.4±3.0 ^a
AQ5	1328.5±209.7 ^a	71.7±3.3 ^a
AQ25	1267.8±28.7 ^a	68.8±5.0 ^a
AQ50	1291.7±76.0 ^a	69.5±3.6 ^a

Total thiols and reduced glutathione. Groups: saline control (CS), saline+quercetin 5 mg/kg (CQ5), saline+quercetin 25 mg/kg (CQ25), saline+quercetin 50 mg/kg (CQ50), arthritis + saline (AS), arthritis +quercetin 5 mg/kg (AQ5), arthritis+quercetin 25 mg/kg (AQ25) and arthritis+quercetin 50 mg/kg (AQ50). Bars represent mean ± S.E.M (Two-way ANOVA-Tukey Multiple Comparison Test).

Table 3: Genotoxicity and cytotoxicity of rats submitted to an experimental model of arthritis treated with saline, quercetin at doses 5, 25 and 50 mg/kg

	DNA damage index	dsDNA
CS	88.8±1.6 ^a	120.2±5.1 ^a
CQ5	117.8±1.4 ^a	95.5±5.0 ^a
CQ25	72.8±1.9 ^a	90.9±2.1 ^a
CQ50	61.4±2.0 ^a	79.8±4.2 ^a
AS	117.8±3.0 ^b	122.7±3.9 ^b
AQ5	95.4±2.6 ^c	87.0±5.5 ^a
AQ25	81.6±1.3 ^a	68.7±5.2 ^a
AQ50	73.0±1.6 ^a	65.4±2.5 ^a

DNA damage and dsDNA. Groups: saline control (CS), saline+quercetin 5 mg/kg (CQ5), saline+quercetin 25 mg/kg (CQ25), saline+quercetin 50 mg/kg (CQ50), arthritis + saline (AS), arthritis +quercetin 5 mg/kg (AQ5), arthritis+quercetin 25 mg/kg (AQ25) and arthritis+quercetin 50 mg/kg (AQ50). Bars represent mean ± S.E.M. Groups with different letters are statistically different. ($P < 0.001$; $n = 5$) (Two-way ANOVA-Tukey Multiple Comparison Test).



Research highlights

► The effect of quercetin on biochemical, stress oxidative, genotoxicity and cytotoxicity were evaluated in serum, plasma and lymphocytes of rats with arthritis induced. ► An increase in AST, ROS, T-BARS, genotoxicity and citotoxicity and decreased CAT levels was found in the arthritic group. ► Quercetin demonstrated an anti-inflammatory effect and might be a promising natural compound to be used an adjuvant in the treatment of arthritis.

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01 November 2018

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Antioxidant, hepatoprotective, genoprotective and cytoprotective effects of quercetin in a murine model of arthritis

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4.3 MANUSCRITO III

Submetido ao periódico Nutrition Research

1 **Antioxidant effect of quercetin on the liver and kidney of rats submitted to**
2 **CFA-induced arthritis**

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ABSTRACT

Rheumatoid arthritis (RA) is an autoimmune and inflammatory disease that affects articular and extra-articular systems. Quercetin is flavonoid found in many fruits and vegetables and has antioxidant action, so we hypothesized that quercetin might have a protective effect against oxidative stress in liver and kidney tissue of arthritic rats. Herein, we investigated the effects of quercetin in oxidative stress parameters in liver and kidney in rats with complete Freund's adjuvant (CFA)-induced arthritis. Wistar rats were divided into control/saline (CS), control/quercetin 5 mg/kg (CQ5), control/quercetin 25 mg/kg (CQ25), control/quercetin 50 mg/kg (CQ50), arthritis/saline (AS), arthritis/quercetin 5 mg/kg (AQ5), arthritis/quercetin 25 mg/kg (AQ25) and arthritis/saline 50 mg/kg (AQ50) and treated with saline and quercetin for 45 days. Results showed that both liver and kidney tissue of arthritic rats present a pronounced oxidative stress, suggesting this model was able to cause extra-articular manifestations. However, treatments with quercetin were effective preventing the damage caused by arthritis induction. Thereby, our results present evidence of an antioxidant effect of quercetin showing it is a promising candidate for adjuvant therapy of RA.

Keywords: CFA-induced arthritis, oxidative stress, liver, kidney, quercetin.

57 **1. Introduction**

58

59 Rheumatoid arthritis (RA) is an autoimmune, inflammatory, chronic and
60 progressive disease which may progress to severe disability with direct negative
61 impact on life quality and increase in mortality rate [1]. This inflammatory
62 disease is known for affecting the synovial joints damaging the synovial lining
63 and leading to the formation of pannus tissue [2]. RA may often manifest in
64 tissues and systems other than the musculoskeletal, including hematological,
65 gastrointestinal, dermatological, cardiovascular, respiratory, ocular and renal,
66 known as extra-articular manifestations [3].

67 Extra-articular manifestations are clinically important due to its association
68 with morbidity and increased risk of premature mortality [4]. The extra-articular
69 manifestations include amyloidosis, rheumatoid vasculitis, Felty Syndrome,
70 rheumatoid nodules, lung and neurologic commitment, as well as, cardiac,
71 muscle, renal and liver abnormalities [5]. Previous studies have shown that
72 extra-articular manifestations play an important role as predictors of early
73 mortality in patients with RA [6,7].

74 The etiology and pathogenesis of RA remain unknown; however, it is known
75 that interactions between several factors, such as genetic and environmental
76 factors lead to an inappropriate immunomodulation resulting in an inflammatory
77 process. Independently of the exact trigger, the reactive oxygen species (ROS)
78 has an important role in this disease [8]. The imbalance between pro-oxidants
79 and antioxidants biomarkers leads to oxidative stress. It can harm the cell
80 structures such as proteins, membranes, lipids and nucleic acids resulting in
81 kidneys in rat model with CFA-induced arthritis cell death or altered cell function

82 [9]. Besides, oxidative stress is a critical factor in inflammation, endothelial
83 dysfunction and multiple organ damage [10].

84 Antioxidants are compounds which neutralize the harmful effects of free
85 radicals generated by chemical stressors such as the inflammatory process.
86 Quercetin is a widely known flavonoid found in many fruits, vegetables, and
87 leaves, which has antioxidant, anti-inflammatory, antiviral, and antiallergic
88 activities [11][12]. Its average consumption is of approximately 3 to 38 mg per
89 day [13]. High levels of quercetin are found in cherries, onions, apples, berries,
90 asparagus and red leaf lettuce [14]. The absorption of this flavonoid occurs in
91 the gastrointestinal tract and it is metabolized by phase II enzymes in both the
92 stomach and intestines, whereas the resultant metabolites are processed
93 further in the liver and kidney [15].

94 Considering that rheumatoid arthritis is a disease which affects other
95 organs, beyond the joints, and the oxidative stress is implicated in this
96 pathology, we evaluated oxidative stress parameters in the liver and kidney of
97 rats submitted to a model of adjuvant-induced arthritis. Due to the previously
98 demonstrated antioxidant and anti-inflammatory properties of quercetin in the
99 literature, we hypothesized that quercetin might have a protective action against
100 oxidative stress in the liver and kidney tissue of arthritic rats. Thus, we have
101 designed this study to evaluate the effects of quercetin on oxidative stress
102 parameters in the liver and kidneys in a rat model of CFA-induced arthritis.

103

104

105 **2. Materials and methods**

106

107 **2.1. Chemicals**

108 Complete Freund's Adjuvant (CFA-0.6% suspension of heat-killed
109 *Mycobacterium tuberculosis* in liquid paraffin, bovine serum albumin, Trizma
110 base, and Coomassie Brilliant Blue G were obtained from Sigma Chemical (St.
111 Louis, MO, USA). All the other chemicals used in this experiment were of the
112 highest purity.

113

114 **2.2 Animals**

115 In this study were used 40 adult female, heterogenic, and conventional
116 Wistar rats (200–300 g) with approximately 200-300 g of body weight from the
117 Central Animal House of the Universidade Federal de Santa Maria (UFSM),
118 Brazil. The animals were maintained on an experimental room under constant
119 temperature ($23\pm 1^{\circ}\text{C}$) on a 12 h light/dark cycle with free access to food and
120 water. All animals were maintained in accordance with the guidelines on the use
121 of animals, approved by the Ethics committee of the UFSM (protocol number:
122 99700812140).

123

124 **2.3 Complete Freund's adjuvant-induced arthritis**

125 To induce of arthritis, the animals were mildly anaesthetized with inhaled
126 isoflurane, and intraplantar injection of 50 μL of CFA (0.6% suspension of heat-
127 killed *Mycobacterium tuberculosis*, 1.0 mg/ μL , in liquid paraffin) or saline (used
128 as a control negative) into the right paw was carried out according to the
129 method described by Oliveira et al., 2007 [16] to induce arthritis.

130 The rats were divided into eight groups of five animals each, according to the
131 treatment administered, namely;

132 Group I: saline solution only (NaCl 0.9%) (CS);

133 Group II: 5 mg/kg of quercetin only (CQ5)
134 Group III 25 mg/kg of quercetin only (CQ25);
135 Group IV: 50 mg/kg of quercetin only (CQ50);
136 Group V: saline solution only (NaCl 0.9%) (AS), 14 days post-induction with
137 CFA;
138 Group VI 5 mg/kg of quercetin only (AQ5), 14 days post-induction with CFA;
139 Group VII: 25 mg/kg of quercetin only (AQ25), 14 days post-induction with CFA;
140 Group VIII: 50 mg/kg quercetin only (AQ50), 14 days post-induction with CFA.
141 The animals were treated with either sterile saline (NaCl 0.9%) or quercetin (5,
142 25 and 50 mg/kg) by oral gavage for 45 days. In this study, the quercetin dose
143 was chosen based on an earlier report by Abdalla et al., 2014 [17].

144

145 **2.4 Liver and kidney preparation**

146 After 45 days of treatment, the rats were anesthetized with isoflurane
147 then submitted to euthanasia. A fragment of liver and kidney was collected,
148 excised, and homogenized 1:10 (weight/volume) in 10 mM solution of Tris-HCl,
149 pH 7.4. These procedures were carried out at 0-4°C. The homogenate was
150 centrifuged at 1800 rpm for 10 min in a refrigerated centrifuge. Aliquots of the
151 supernatants were stored at -20°C until utilization. The supernatants were used
152 subsequently for the determination of reactive oxygen species (ROS), total
153 thiols (T-SH), catalase (CAT) and superoxide dismutase (SOD).

154

155 **2.5 Oxidative stress determinations**

156 The evaluation of oxidative stress markers was performed in triplicates for each
157 sample. The samples used were liver and kidney tissues.

158 **2.5.1 Reactive oxygen species (ROS) quantification**

159 The determination of ROS production was performed through the measurement
160 of the levels of 2'-7'-dichlorofluorescein (DCF)[18]. Aliquots of tissue
161 homogenate (50 µL) supernatant were added to a distilled water and 2'-7'-
162 dichlorofluorescein diacetate (DCFHDA) (1mM) medium followed by an 1h dark
163 incubation. Next, to determine DCF levels the fluorescence was read (excitation
164 at 488 nm and emission at 525 nm) using a standard curve and results were
165 corrected according to protein levels.

166

167 **2.5.2 Total thiols determination (T-SH)**

168 T-SH levels were determined according to Ellman modified method [19].
169 Following the addition of 50 µL of 10 Mm 5,5'-dithio-bis(2-nitrobenzoic acid)
170 (DTNB), tissue homogenate (100 µL) was added to the solution to a final
171 volume of 1000 µL. Readings were made at 412 nm. A cysteine standard curve
172 was used to determine the thiols groups content in nmol T-SH/g tissue.

173

174 **2.5.3 Reduced glutathione measurement (GSH)**

175 A spectrophotometric method with Ellman's reagent was performed to
176 measured GSH. Precipitation of proteins with TCA 10% to a 100 µL of the
177 supernatant of tissue homogenate to a 1000 µL of 10 mM 5,5'-dithio-bis (2-
178 nitrobenzoic acid) (DTNB) (0.05 mL) solution. Readings were made at 412 nm.
179 A glutathione curve was used for the calculation and expressed in nmol GSH/g
180 tissue.

181

182

183 **2.5.4 Catalase activity (CAT)**

184 CAT activity was measured by means of Nelson and Kiesow (1972) method
185 [20]. Tissue homogenate (20 μ L) was mixed to 50 mM potassium phosphate
186 buffer (pH 7), 10 Mm H₂O₂. Absorbance measurements were made at 240 nm
187 for 2 min (every 10 s) in order to access the rate of the H₂O₂ reaction. CAT was
188 expressed in nmol/mg/protein/minute.

189

190 **2.5.5 Superoxide dismutase activity (SOD)**

191 SOD activity was accessed using the method developed by Misra and
192 Fridovich, (1972) [21], based on the inhibition of the radical superoxide (O₂⁻)
193 reaction with epinephrine, which suffers auto-oxidation (Ph 10.5) producing
194 adrenochrome, detected at 480 nm. The auto-oxidation of epinephrine was
195 stopped by the addition of tissue homogenate samples (20, 40, 60 μ L)
196 containing SOD inhibits. The rate of inhibition was observed for 2 min. SOD
197 activity was expressed in units of SOD/mg of protein.

198

199 **2.6 Protein determination**

200 Protein was determined by means of Bradford method (1976) [22], bovine
201 serum albumin was used as a standard.

202

203 **2.7 Statistical analysis**

204 For data were analyzed two-way ANOVA was used followed by the Tukey test
205 and expressed as the mean \pm standard error of the mean (SEM).

206

207

208 **3. Results**

209

210 **3.1 Oxidative stress determination**

211 The ROS levels in the liver and kidney are demonstrated in Figure 1. In the
212 hepatic tissue (figure 1A), an increased in AS was observed (1.34 DCFH-DA
213 Fluorescence, SEM= 0.12 n=5, $P < 0.05$) when compared to CS (0.90 DCFH-DA
214 Fluorescence, SEM= 0.09, n=5), however treatment with quercetin was able to
215 decrease the ROS levels, as following, AQ5 (0.94 DCFH-DA Fluorescence,
216 SEM= 0.11, n=5, $P < 0.05$), AQ25 (0.88 DCFH-DA Fluorescence, SEM= 0.05,
217 n=5, $P < 0.05$) and AQ50 (0.68 DCFH-DA Fluorescence, SEM= 0.13, n=5, $P <$
218 0.001) when compared to AS (1.34 DCFH-DA Fluorescence, SEM= 0.12 n=5).
219 In the kidney tissue (Figure 1B), we verified an increased in AS (22.76 DCFH-
220 DA Fluorescence, SEM= 2.49, n=5, $P < 0.01$) compared to CS (13.42 DCFH-DA
221 Fluorescence, SEM= 0.85, n=5). Treatment with quercetin decreased the ROS
222 levels, AQ5 (10.49 DCFH-DA Fluorescence, SEM= 0.86, n=5, $P < 0.001$), AQ25
223 (8.88 DCFH-DA Fluorescence, SEM= 0.97, n=5, $P < 0.001$) and AQ50 (10.01
224 DCFH-DA Fluorescence, SEM= 1.15, n=5, $P < 0.001$) when compared to AS
225 (22.76 DCFH-DA Fluorescence, SEM= 2.49, n=5).

226

227 **3.2 Total thiols determination (T-SH)**

228 The total thiols determinations in the liver and kidney are shown in Figure 2. In
229 the liver (Figure 2A), no statistical difference was observed between AS (692.00
230 nmol T-SH/g plasma, SEM= 51.08, n=5, $P > 0.05$) and CS (766.77 nmol T-SH/g
231 plasma, SEM= 37.58, n=5) and the kidney (Figure 2B), no statistical difference

232 was verified between AS (893.17 nmol T-SH/g plasma, SEM= 111.97, n=5, $P >$
233 0.05) and CS (856.81 nmol T-SH/g plasma, SEM= 119.98, n=5).

234

235 **3.3 Reduced glutathione measurement (GSH)**

236 In Figure 3, the reduced glutathione measurements in the liver (Figure 3A) and
237 kidney (Figure 3B) can be seen. No statistical difference was observed in liver
238 between AS (72.36 nmol G-SH/g plasma, SEM= 3.00, n=5, $P > 0.05$) and CS
239 (63.54 nmol G-SH/g plasma, SEM= 2.12, n=5). In the kidney, too no statistical
240 difference was observed between AS (27.65 nmol G-SH/g plasma, SEM= 2.95,
241 n=5, $P > 0.05$) and CS (32.71 nmol G-SH/g plasma, SEM= 1.00, n=5).

242

243 **3.4 Catalase activity (CAT)**

244 Results obtained for catalase in liver (Figure 4A) and kidneys (Figure 4B) are
245 presented in Figure 4. In the liver, catalase activity was significantly decreased
246 in AS (45.89 μ mol CAT/mg protein/minute, SEM=2.53, n=5, $P < 0.01$) when
247 compared to CS (82.75 μ mol CAT/mg protein/minute, SEM=9.43, n=5).
248 Meanwhile, only treatment with quercetin 50 mg was able to increased the
249 catalase activity, as observed AQ50 (83.55 μ mol CAT/mg protein/minute,
250 SEM=1.74, n=5, $P < 0.01$) compared to AS (45.89 μ mol CAT/mg
251 protein/minute, SEM=2.53, n=5).

252 A similar result was observed in the kidney, the catalase activity was
253 significantly decreased in AS (3.79 μ mol CAT/mg protein/minute, SEM=0.23,
254 n=5, $P < 0.05$) when compared to CS (15.51 μ mol CAT/mg protein/minute,
255 SEM=0.99, n=5), while treatment with 50 mg/kg of quercetin increased catalase
256 activity, respectively AQ50 (23.55 μ mol CAT/mg protein/minute, SEM=1.55,

257 n=5, $P < 0.001$) compared to AS (3.79 $\mu\text{mol CAT/mg protein/minute}$,
258 SEM=0.23, n=5).

259 **3.5 Superoxide dismutase activity (SOD)**

260 In Figure 5, the superoxide dismutase activity in the liver (5A) and kidney (5B)
261 can be seen. In the figure 5A, superoxide dismutase activity was significantly
262 decreased in AS (1.13 U SOD/ mg of protein, SEM=0.12, n=5, $P < 0.01$) when
263 compared to CS (3.98 U SOD/ mg of protein, SEM=0.15, n=5). However,
264 treatment with the 50 mg dose of quercetin was able to increase the activity of
265 this enzyme, as follows AQ50 (5.40 U SOD/ mg of protein, SEM=0.38, n=5, $P <$
266 0.001) compared to AS (1.13 U SOD/ mg of protein, SEM=0.12, n=5).

267 In the figure 5B, we observed that the superoxide dismutase activity was
268 decreased in AS (1.47 U SOD/ mg of protein, SEM=0.27, n=5, $P < 0.05$) when
269 compared to CS (5.18 U SOD/ mg of protein, SEM=0.45, n=5), but only
270 treatment with 50 mg/kg of quercetin increased superoxide dismutase activity,
271 AQ50 (8.70 U SOD/ mg of protein, SEM=0.27, n=5, $P < 0.001$) compared to AS
272 (1.47 U SOD/ mg of protein, SEM=0.27, n=5).

273

274 **4. Discussion**

275

276 We investigated the effect of quercetin on oxidative stress parameters in the
277 liver and kidney of rats with arthritis induced by adjuvant. The induction of
278 arthritis promoted an increase of reactive species of oxygen (ROS) as well as a
279 decrease in the enzymatic antioxidant defense. Treatments with all doses of
280 quercetin reduced ROS levels while the dose of 50 mg increased the
281 antioxidant defense.

282 The increased production of reactive oxygen species ROS and
283 decreased of antioxidant associated with the inflammatory process are common
284 features among all autoimmune diseases [23]. CFA-induced arthritis is an
285 experimental model which replicate most of the pathological and clinical
286 features of this disease, thus being the most frequently used model for
287 detection of new compounds with anti-arthritic activity [24].

288 Oxidative stress occurs due to the disruption of the balance between the
289 body's oxidants load and antioxidants reservoir. Numerous studies have shown
290 that ROS are implicated in the pathophysiology of RA [25–27]. Also, the
291 pathogenesis of liver and kidney diseases involve an imbalance between free
292 radical production and the antioxidant defense [28,29].

293 In this study, an increase was in the ROS levels in the liver and kidney of
294 rats with arthritis induced by CFA. Increased ROS levels in the liver were
295 observed in a study conducted by Gonçalves et al., 2015 [30] with liver and
296 brain of arthritic rats. This may be owing to an increased oxidative metabolism,
297 generating a more oxidizing environment resulting in augmented production of
298 ROS [30,31].

299 In addition, liver cells need energy to perform their numerous functions
300 and the high metabolic rate of the liver is directly associated with a high electron
301 flow in the mitochondrial respiratory chain. Meantime, some of these electrons
302 are diverted, producing new ROS, leaving the body susceptible to the process
303 of oxidative stress [32,33].

304 The same result was observed in the kidney, however, to our knowledge
305 this is the first study that determined the levels of ROS in the kidney in model of
306 induced arthritis. In a model of diabetes and in another model of kidney and

307 liver damage by anabolic steroids, an increase in ROS levels was observed in
308 the kidney, which can be explained by the depletion of the antioxidant defense
309 and the compromised the ability to combat ROS, with consequent oxidative
310 damage [32].

311 In contrast, in the quercetin-treated groups, ROS levels were decreased
312 when compared to the arthritic group. The antioxidant role of quercetin is well-
313 established and given by its ability to eliminate free radicals and bind transition
314 metal ions [34,35] and within the flavonoid family. Among all flavonoids,
315 quercetin is considered the most potent scavenger of ROS [34].

316 The harmful effects of free radicals are neutralized by the action of
317 antioxidants, which are compounds or substance with the ability to eliminate
318 free radicals or inhibit the oxidation process in cells[8]. Catalase (CAT),
319 glutathione peroxidase (GPx) and superoxide dismutase (SOD) are
320 representative of the enzymatic antioxidants, while the non-enzymatic ones are
321 vitamin E and C, thiols antioxidants (glutathione, thioredoxin, and lipoic acid),
322 melatonin, carotenoids, natural flavonoids, and other compounds [36].

323 Thiols are the very important molecules in the process of antioxidation
324 containing thiols groups (-SH). The groups -SH can reduce the electrons so that
325 the organism can be protected from the oxidative damages caused by ROS
326 [37]. CAT is an enzyme responsible for the conversion of hydrogen peroxide
327 into water and oxygen, protecting cells from the harmful effects of accumulated
328 hydrogen peroxide [25]. SOD is responsible for detoxification of ROS by
329 dismutating the superoxide radical anion to oxygen and hydrogen peroxide [38].

330 In the present study, no significant differences were found in total thiols
331 and reduced glutathione (GSH), however, CAT and SOD activities in the liver

332 and kidney were found to be decreased in group arthritic induced. Our results
333 are in agreement with Ramprasatha, Shanthib and Sachdanandama, 2005 [39].
334 High levels of ROS are capable of inhibiting the activity of antioxidants; hence
335 the activities of these enzymes appear to be reduced. However, treatment with
336 quercetin 50 mg/kg was able to increase the CAT and SOD activities, which we
337 can suggest by the antioxidant action of this flavonoid. Quercetin has a strong
338 electron-donating capability that stabilizes the free radicals [40]. The prevention
339 of oxidative damage by quercetin is attributed to its free -OH substituent, which
340 is believed to increase the stability of the flavonoid radical and the catechol
341 group, which are responsible by the chelating action of quercetin [41].

342 In the present study, we found that CFA caused a significant increase in
343 ROS and a decrease in SOD and CAT activity in the liver and kidney,
344 suggesting that CFA-induced arthritis (AIA) was able to cause extra-articular
345 manifestations in the animals. Moreover, our results clearly demonstrated a
346 protective antioxidant role of quercetin in the liver and kidney of animals with
347 AIA. In conclusion, our findings demonstrated that quercetin represents a
348 promising therapeutic approach to extra-articular manifestations arising from
349 rheumatoid arthritis, evidencing the advantages of quercetin and encouraging
350 its use in further preclinical and clinical studies as an antioxidant agent.

351

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357

358 **Compliance with ethical standards**

359 All procedures performed in studies involving animals were in accordance with
360 the ethical standards of the institution or practice at which the studies were
361 conducted.

362

363 **Conflict of interest**

364 The authors declare that they have no conflict of interest.

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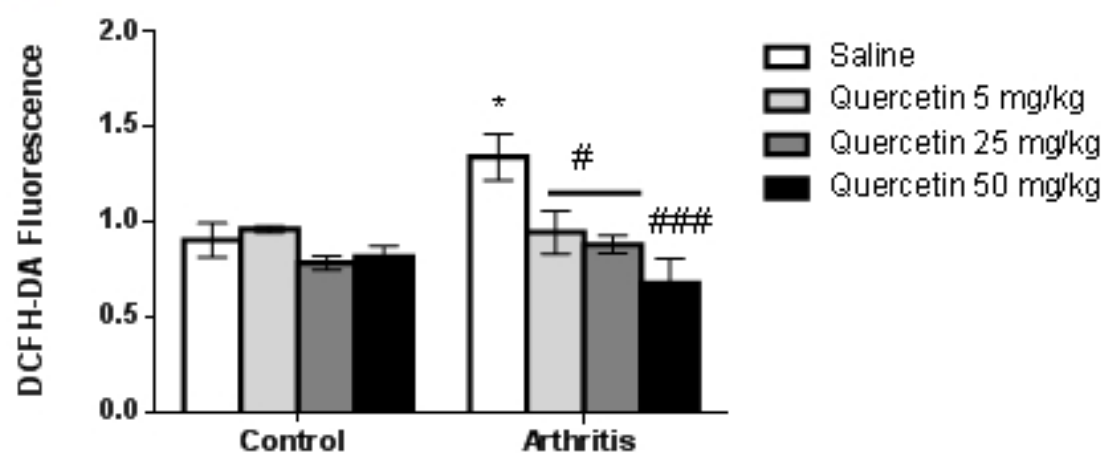
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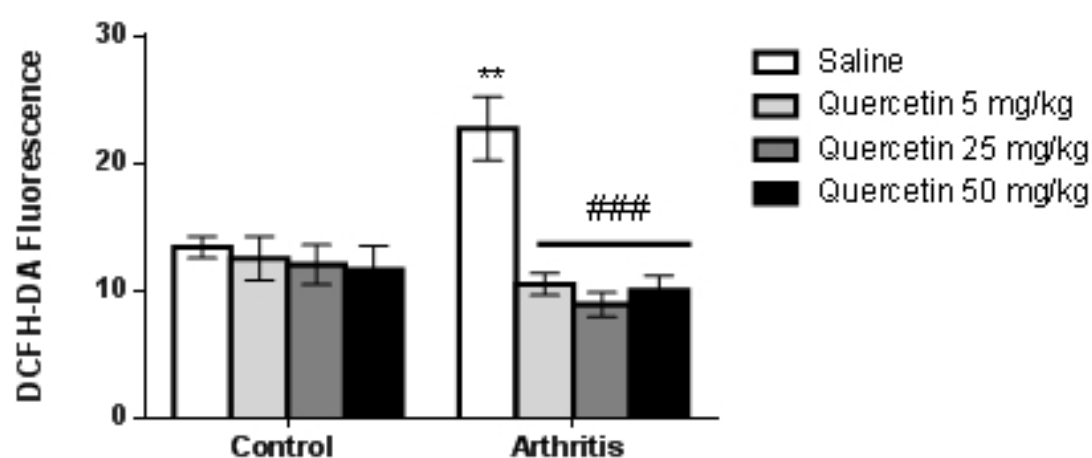
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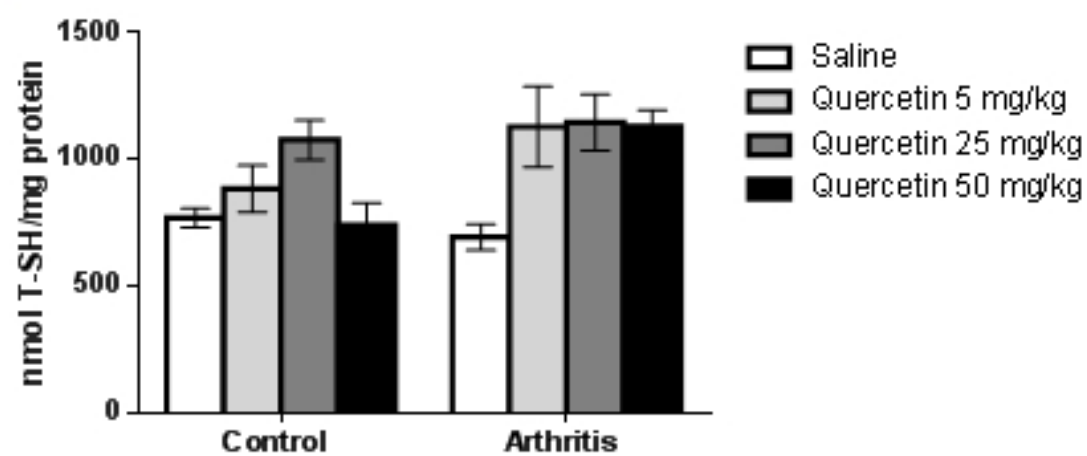
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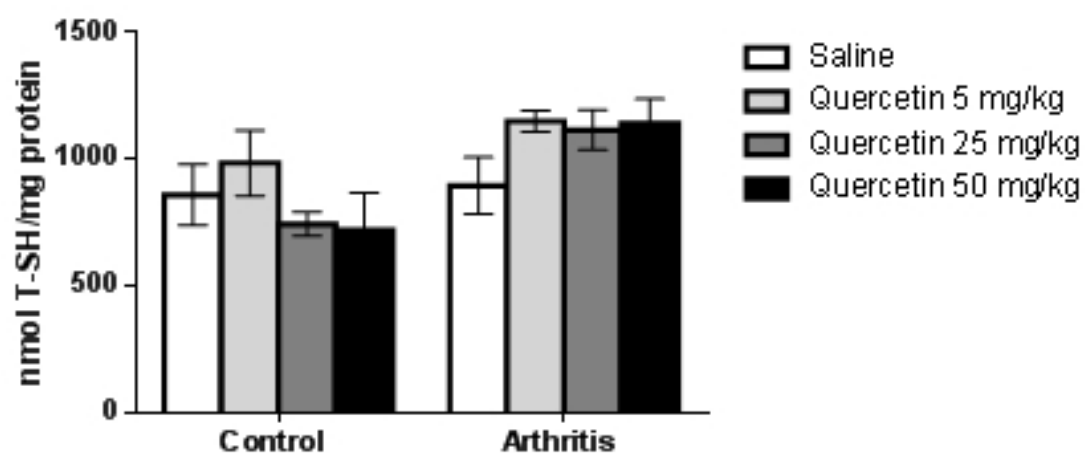
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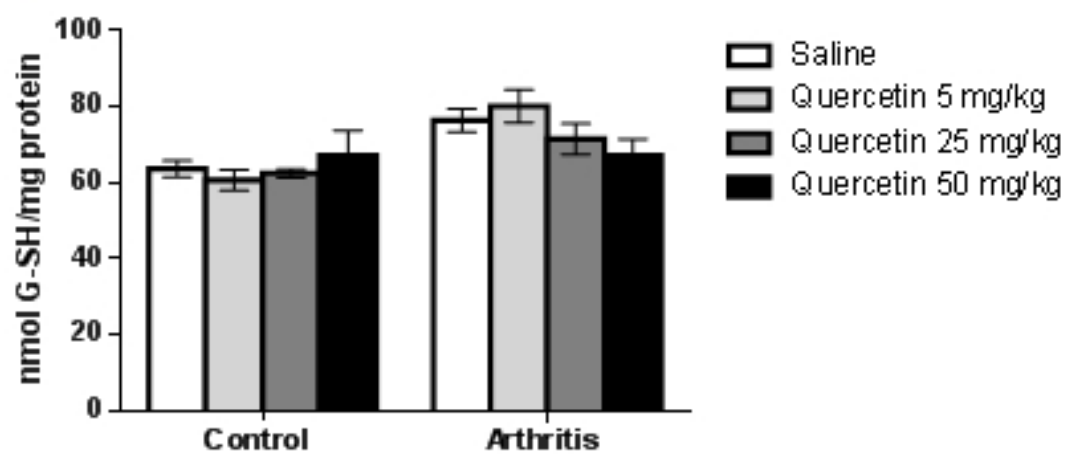
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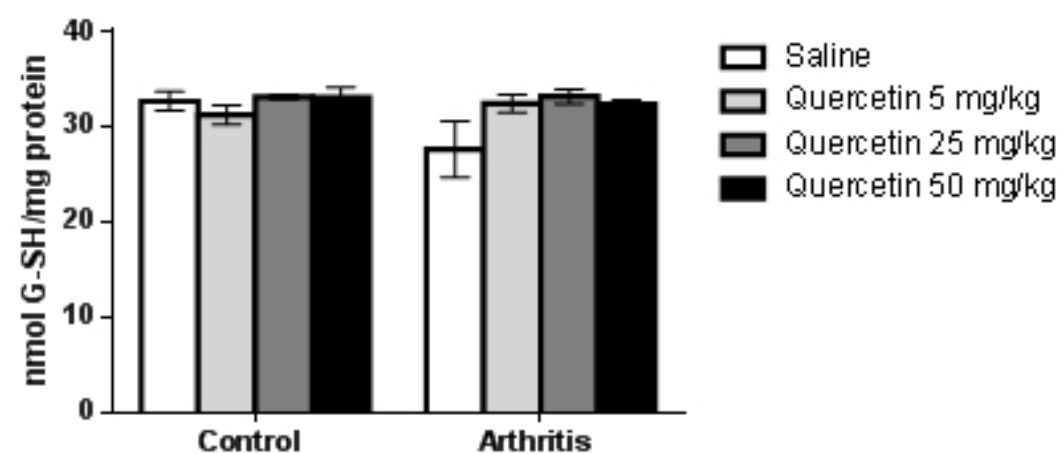
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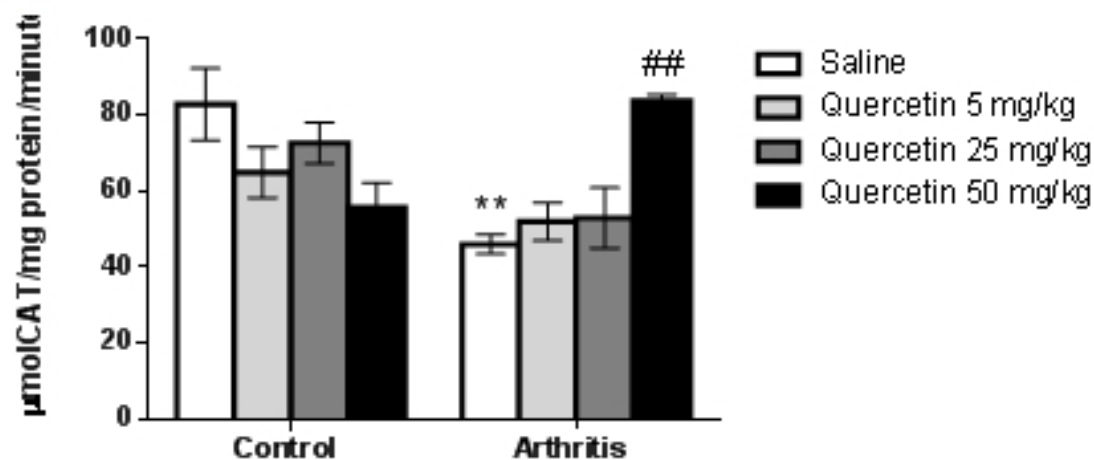
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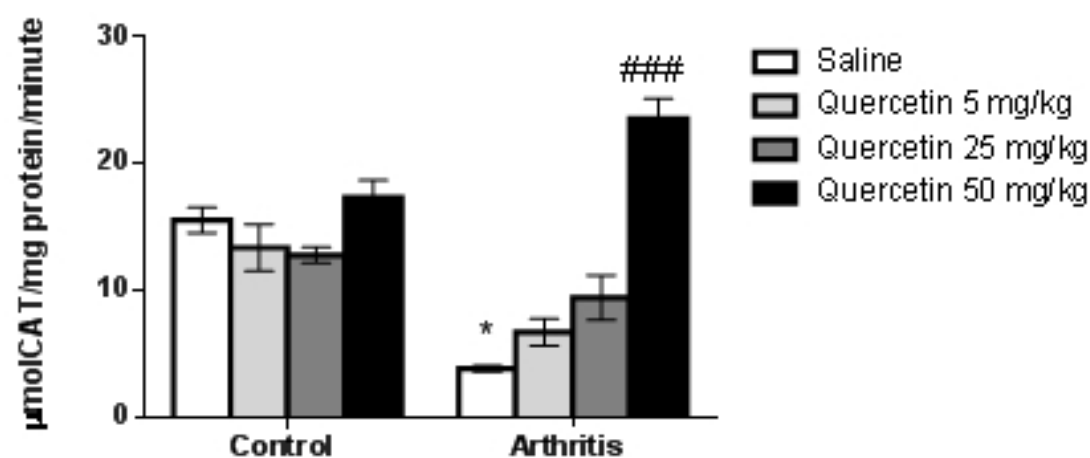
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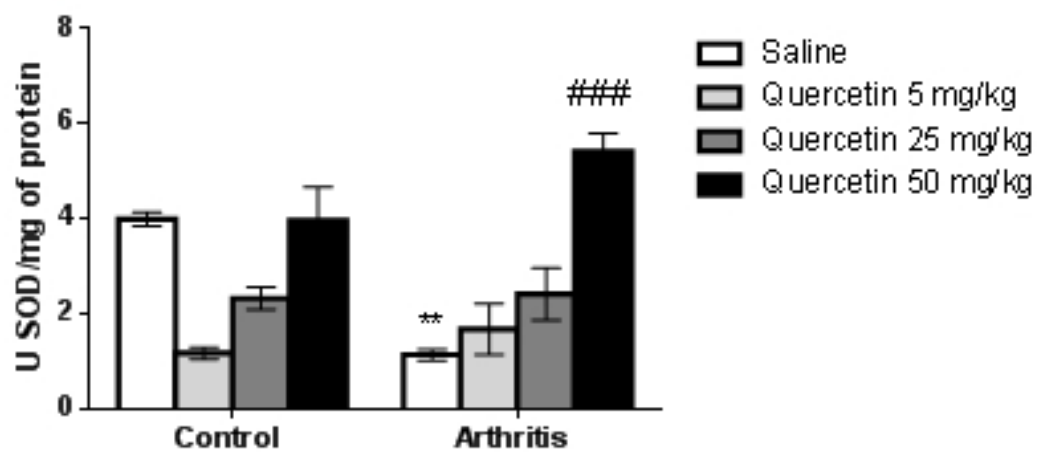
4A



4B



5A



5B

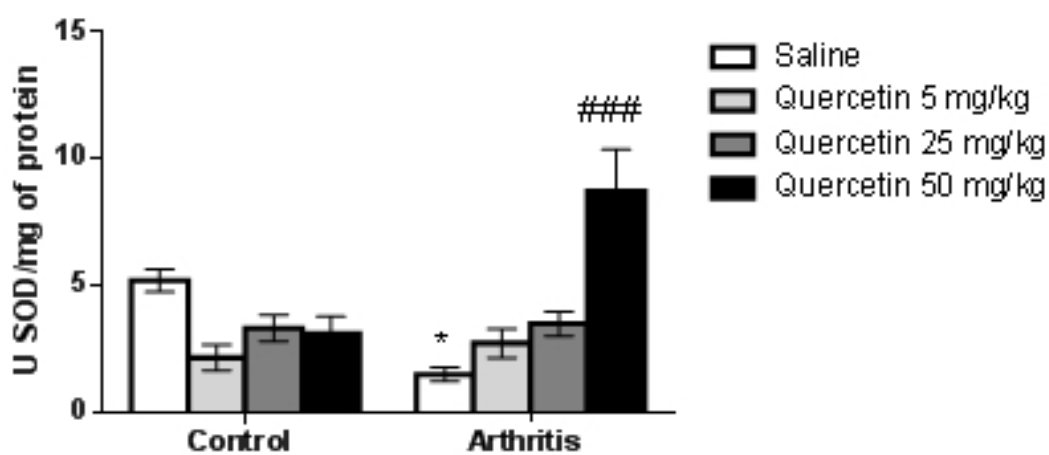


Figure Legend

Figure 1. Measurement of reactive oxygen species (ROS) in liver (1A) and kidney (1B) of rats submitted to an experimental model of arthritis treated with saline, quercetin at doses 5, 25 and 50 mg/kg. *Asterisks* indicates significant differences from the control saline (* $P<0.05$, ** $P<0.01$). *Ash* indicates significant differences from the arthritis saline (# $P<0.05$, ## $P<0.01$, ### $P<0.001$). Bars represent mean \pm SEM (n=5). Two-way ANOVA-Tukey test.

Figure 2. Total thiols determination in liver (2A) and kidney (2B) of rats submitted to an experimental model of arthritis treated with saline, quercetin at doses 5, 25 and 50 mg/kg. Bars represent mean \pm SEM (n=5). Two-way ANOVA-Tukey test.

Figure 3. Reduced glutathione measurement in liver (3A) and kidney (3B) of rats submitted to an experimental model of arthritis treated with saline, quercetin at doses 5, 25 and 50 mg/kg. Bars represent mean \pm SEM (n=5). Two-way ANOVA-Tukey test.

Figure 4. Catalase activity in liver (4A) and kidney (4B) of rats submitted to an experimental model of arthritis treated with saline, quercetin at doses 5, 25 and 50 mg/kg. *Asterisks* indicates significant differences from the control saline (* $P<0.05$). *Ash* indicates significant differences from the arthritis saline ## $P<0.01$, ### $P<0.001$). Bars represent mean \pm SEM (n=5). Two-way ANOVA-Tukey test.

Figure 5. Superoxide dismutase activity in liver (5A) and kidney (5B) of rats submitted to an experimental model of arthritis treated with saline, quercetin at doses 5, 25 and 50 mg/kg. *Asterisks* indicates significant differences from the control saline (* $P<0.05$, ** $P<0.01$). *Ash* indicates significant differences from the arthritis saline ## $P<0.01$, ### $P<0.001$). Bars represent mean \pm SEM (n=5). Two-way ANOVA-Tukey test.

5 DISCUSSÃO

A artrite reumatoide é uma doença que expressa uma resposta predominantemente do tipo Th1 e Th17, e as citocinas desenvolvem um papel importante na patogênese da mesma, sendo a citocina pró-inflamatória TNF- α é a principal responsável pelo infiltrado inflamatório (BRYANT; MOORE, 2006), pela ativação de linfócitos, pela estimulação à liberação de enzimas proteolíticas pelos macrófagos e pela produção de outras citocinas inflamatórias, tais como a IL-6 e IL-13 (FALEIRO et al., 2011). A artrite induzida por CFA é amplamente utilizada para a avaliação farmacológica de agentes antiartríticos devido ao elevado grau de semelhança que tem com a doença humana (NEWBOULD, 1963; KUMAR et al., 2016).

Os resultados deste trabalho foram divididos em 3 manuscritos. O objetivo do primeiro manuscrito foi avaliar o efeito da quercetina nas atividades da E-NTPDase e E-ADA em linfócitos, e nos níveis séricos de purinas, bem como, avaliar a secreção de citocinas pró e anti-inflamatórias em ratos com artrite induzida por adjuvante. No segundo manuscrito foi investigado se o tratamento com quercetina era capaz de fornecer proteção contra a artrite induzida por CFA em um modelo animal, através da avaliação de parâmetros bioquímicos, de estresse oxidativo, de genotoxicidade e de citotoxicidade. Por fim, o terceiro manuscrito objetivou verificar os efeitos protetivos da quercetina em parâmetros de estresse oxidativo no fígado e rins no mesmo modelo.

No primeiro manuscrito, para confirmar o desenvolvimento do processo inflamatório crônico no modelo experimental, testes comportamentais de artrite foram avaliados. Os animais com artrite induzida por CFA demonstraram um aumento no edema de pata, do escore de artrite e uma diminuição da hiperalgesia termal. Entretanto, nos animais com artrite induzida e tratados com quercetina, foi possível observar que as doses de 25 e 50 mg/kg foram capazes de reduzir o edema de pata e o escore da artrite, bem como aumentar o tempo da hiperalgesia termal. Esses resultados corroboram com um estudo realizado por Mamani-Matsuda et al. (2006), que demonstrou que as doses mais altas de quercetina foram mais eficientes na redução dos sinais artríticos. Ainda, Kumar et al. (2016) relataram que a *Paederia foetida* Linn, na qual o principal componente é a quercetina foi capaz de reduzir o edema de pata e o escore da artrite.

Estes resultados foram reforçados com a avaliação histológica, na qual podemos observar que no grupo artrítico, o espaço articular apresentou-se distendido por edema, preenchido e expandido por um intenso infiltrado inflamatório linfocitário. Entretanto, nos grupos tratados com quercetina, todas as doses de quercetina utilizadas foram capazes de

reduzir o processo inflamatório, o que consiste com estudos anteriores, em que a quercetina demonstrou inibir os aspectos inflamatórios da função das células sinoviais e ativação de neutrófilos (JACKSON et al., 2006; TUNON et al., 2009). A atividade anti-inflamatória da quercetina se deve a sua capacidade de inibir a resposta Th1, regular as atividades das enzimas cicloxigenase e óxido nítrico sintase induzível (iNOS), bem como modulação de fatores de transcrição, como o fator nuclear kappa B (NF- κ B) (BLANK et al., 2000).

Ainda para comprovar a indução da artrite, a atividade da mieloperoxidase foi avaliada. A mieloperoxidase é uma heme enzima presente nos grânulos de leucócitos, principalmente neutrófilos e macrófagos, responsável pela conversão da H₂O₂ em HOCl, sendo considerada também como um clássico marcador do processo inflamatório (BLANK et al., 2000; CHAGAS et al., 2017). Neste estudo, o grupo artrítico exibiu uma atividade elevada de mieloperoxidase, indicando um processo inflamatório progressivo, o que está de acordo com Stamp et al. (2012) que demonstrou que a atividade da mieloperoxidase foi elevada em pacientes com artrite, indicando seu papel na patogênese e gravidade desta patologia. Por outro lado, os animais tratados com quercetina apresentaram menores níveis de atividade da mieloperoxidase, indicando uma diminuição da infiltração de neutrófilos e consequente redução do processo inflamatório, contribuindo para a diminuição do dano tecidual observado na histologia das patas.

Atualmente, há um crescente número de estudos sobre os papéis fisiopatológicos da sinalização purinérgica e o seu potencial terapêutico para uma variedade de doenças, incluindo as doenças autoimunes (BURNSTOCK; DI VIRGILIO, 2013). As atividades das ectoenzimas E-NTPDase e E-ADA estão alteradas em diversas doenças inflamatórias (BAGATINI et al., 2011; SPANEVELLO et al., 2010; BECKER et al., 2010; JAQUES et al., 2013) e infecciosas (LEAL et al., 2011; SOUZA et al., 2012).

A AR é caracterizada pela proliferação predominante sinovial, destruição óssea e da cartilagem articular (KAMANLI et al., 2004). O fluido sinovial é rico por células imunes, tais como neutrófilos, macrófagos, células dendríticas e linfócitos T, que contribuem para a cronicidade da doença (FELDMAN; BRENNAN; MAINI, 1996). Os linfócitos estão intimamente envolvidos em processos inflamatórios e uma das maneiras pelas quais a função dos linfócitos é regulada é através de uma família de enzimas conhecidas como ectonucleotidases (BARANKIEWICZ; DOSCH; COHEN, 1988).

Diante do exposto, em nosso estudo avaliamos a atividade de ectoenzimas em linfócitos, tais como: E-NTPDase e E-ADA. Podemos observar que houve um aumento na atividade da E-NTPDase no grupo artrítico quando comparado ao grupo controle/salina,

indicando que houve um aumento da hidrólise de ATP e ADP no meio extracelular, reduzindo os seus níveis durante o desenvolvimento da AR, provavelmente como um mecanismo regulatório para manter a homeostase (JAQUES et al., 2013; SILVEIRA et al., 2016). Ainda, a diminuição dos níveis de ATP e ADP, decorrente, do aumento da atividade da E-NTPDase, favorecendo a geração de uma quantidade aumentada de adenosina, uma molécula com propriedades anti-inflamatórias e analgésicas (FREDHLOM et al., 1994; SILVEIRA et al., 2016).

Níveis altos de ATP extracelular ativam os receptores P2X7, estimulando uma resposta pró-inflamatória, o que contribui para o dano tecidual e manutenção de um processo inflamatório crônico (BOURS et al., 2006; RUCHEL et al., 2016). Estudos prévios com ratos artríticos mostraram um aumento na atividade da E-NTPDase (CASTILHOS et al., 2015; SILVEIRA et al., 2016). No presente estudo, o tratamento com quercetina reduziu a atividade desta enzima, reduzindo portanto os níveis de ATP extracelular. Em baixas concentrações, o ATP extracelular possui afinidade pelos receptores P2Y na superfície dos linfócitos, que quando estimulados, promovem uma mudança da resposta Th1 para Th2 (BOURS et al., 2006; SOUZA et al., 2012).

É bem relatado na literatura que a quercetina apresenta propriedades anti-inflamatórias e reduz o dano celular (CHEN et al., 2005). A citocina pró-inflamatória TNF- α desempenha um papel central na patogênese da AR (KAYSER et al., 2007). Na AR, o TNF- α é expresso por macrófagos, células sinoviais e células T ativadas dentro da articulação inflamada (ALVES et al., 2016). Um estudo conduzido por Nardi et al. (2016), sugere que a quercetina tem a capacidade de modular a resposta imune e exibe atividade anti-inflamatória, diminuindo a produção endógena de TNF- α .

Em relação à atividade da E-ADA, nossos resultados demonstraram que a atividade da E-ADA foi diminuída no grupo artrítico quando comparado ao grupo controle/salina, o que pode ser corroborado por um estudo conduzido por Jaques et al. (2013) e Silveira et al. (2016), sugerindo que a diminuição da atividade da E-ADA seria uma resposta dinâmica dos linfócitos numa tentativa de aumentar as concentrações de adenosina na tentativa de reduzir o processo inflamatório decorrente da artrite (JAQUES et al., 2013). Os níveis de adenosina apresentaram um aumento no grupo artrítico, que pode ser atribuído à diminuição da atividade da E-ADA neste grupo, agindo como um mecanismo regulatório para o controle do processo inflamatório (CSOKA et al., 2008). Entretanto, o tratamento com quercetina foi capaz de modular a atividade da E-ADA reverter este efeito neste estudo, aumentando a atividade da E-ADA, evidenciando que os compostos naturais são capazes de modular o sistema purinérgico

(BALDISSARELLI et al., 2016), porém os mecanismos pelos quais, esta modulação ocorre ainda não estão bem estabelecidos.

O equilíbrio entre as citocinas pró-inflamatórias e anti-inflamatórias na AR determina o grau de extensão da inflamação, e conduz diferentes desfechos clínicos (SIVALINGAM et al., 2007). Recentes estudos mostraram que há um desequilíbrio nos subconjuntos das células T desempenham um importante papel na AR. As células TCD4⁺ podem ser subdivididas em subconjuntos Th1, Th2, Th17 e Treg, de acordo com a sua diferenciação e sua função. As células do tipo Th1 secretam uma grande quantidade de IFN- γ , que é responsável por promover a imunidade mediada por células (YU et al., 2008). O IFN- γ é sintetizado no processo de resposta imune celular e é um forte regulador imunológico, promovendo a inflamação através da indução da expressão de uma variedade de citocinas, incluindo TNF- α , IL-2 e IL-10 (BI et al., 2017).

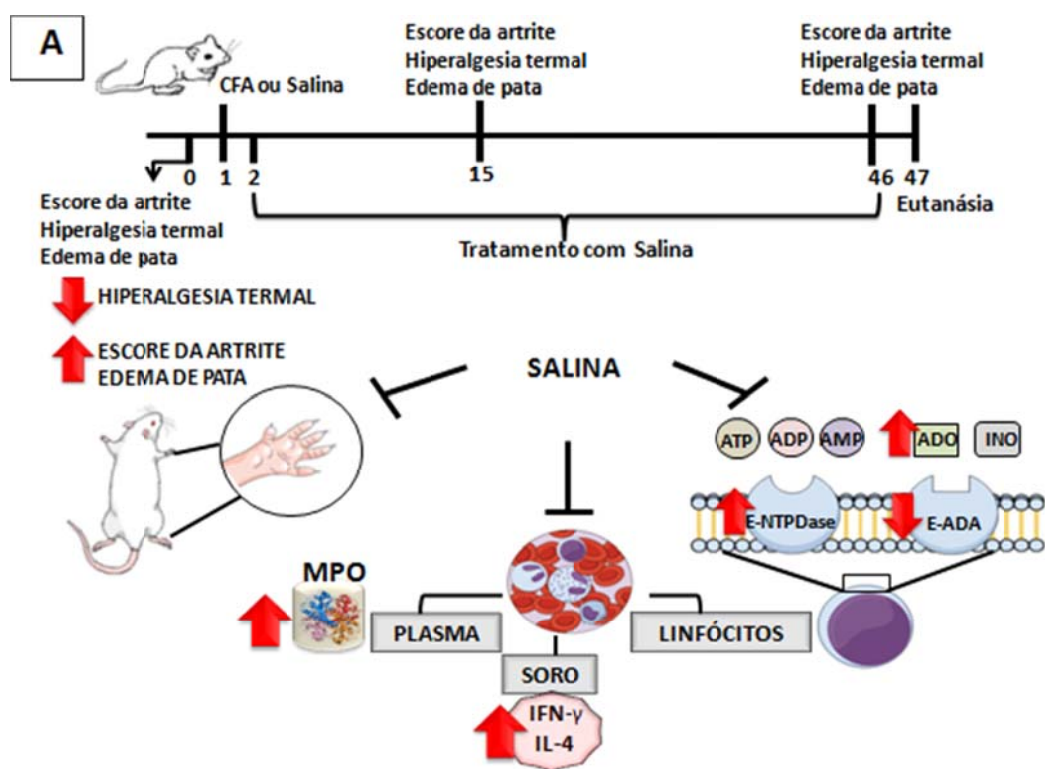
Outros parâmetros avaliados neste estudo foram à secreção de citocinas pró-inflamatórias e anti-inflamatórias. Podemos observar níveis mais elevados de IFN- γ no grupo artrítico quando comparado ao grupo controle/salina, o que pode ser corroborado com Niu et al. (2017), que também encontrou níveis significativamente mais altos de IFN- γ sérico em um modelo animal de artrite induzida por colágeno e Pavlovic et al., 2014 também observaram níveis mais elevados em pacientes com AR inicial, indicando que esta citocina pró-inflamatória têm um papel fundamental na manutenção da homeostase imune em pacientes com AR. Nossos resultados também demonstraram níveis reduzidos de IFN- γ após o tratamento com 5, 25 e 50 mg/kg de quercetina. Em um estudo realizado por Yu et al. (2008) foi verificado que em sobrenadantes de células Th ativadas cultivadas com quercetina, apenas as doses de 20 e 40 μ M de quercetina foram capaz de reduzir os níveis de IFN- γ , demonstrando que a quercetina exerce atividades anti-inflamatórias regulando a produção de citocinas inflamatórias mediadas por macrófagos e linfócitos T .

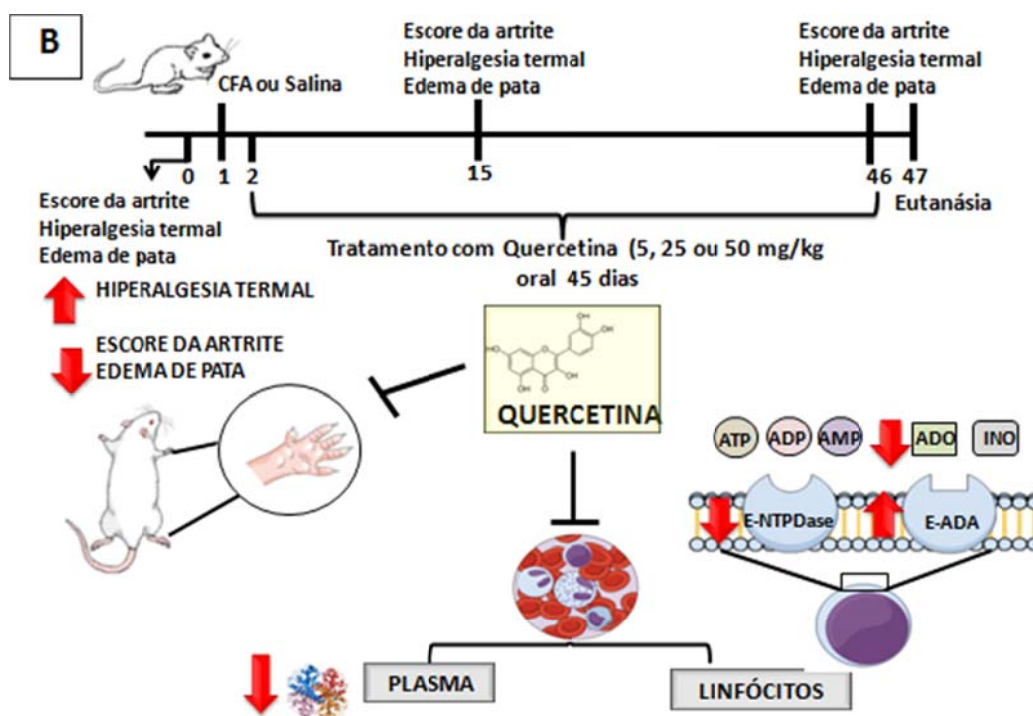
A IL-4 é produzida por mastócitos e linfócitos Th2 e tem a função de inibir a produção das citocinas pró-inflamatórias, como IFN- γ , TNF- α e IL-1, além disso, é capaz de bloquear a reabsorção óssea por inibição da atividade dos osteoclastos (BRZUSTEWICZ; BRYL, 2015). A citocina anti-inflamatória avaliada em nosso estudo foi a IL-4 e os nossos resultados demonstraram que os níveis de IL-4 foram aumentados no grupo artrítico quando comparados ao grupo controle/salina. Naciute et al. (2017) também encontraram níveis de IL-4 aumentados em pacientes com AR. Apesar disso, nos grupos tratados com quercetina houve uma diminuição dos níveis desta citocina, sendo consistente com o estudo de Braun et al., 2017 que mostrou que o pré-tratamento com a quercetina reduziu os níveis de IL-4 em ratos

com hiperlipidemia, promovendo uma diminuição do recrutamento dos eosinófilos recrutamento e inibição da ativação do NF- κ B no processo inflamatório (ROGERIO et al., 2010).

Na figura 10, estão ilustrados os resultados do manuscrito 1, sendo que a letra A demonstra o grupo dos animais que receberam salina como tratamento e na letra B, os grupos de animais que foram tratados com quercetina.

Figura 10 - Resumo gráfico dos resultados do manuscrito I





Podemos concluir, que os nossos resultados demonstraram alterações nas enzimas do sistema purinérgico em linfócitos, atividade da MPO, bem como na secreção de citocinas no grupo de ratos artríticos, indicando que essas alterações podem ser decorrentes do processo inflamatório decorrente da artrite. Por outro lado, o tratamento com quercetina foi capaz de prevenir essas alterações causadas pela artrite induzida por CFA, sugerindo que este flavonoide possui efeitos anti-inflamatórios e pode ser um composto natural promissor para o uso como um adjuvante no tratamento da artrite.

No segundo manuscrito, foi investigado se o tratamento com quercetina é capaz de fornecer proteção contra o dano oxidativo causado pela artrite induzida por CFA em um modelo animal. Os parâmetros avaliados foram bioquímicos, de estresse oxidativo, genotoxicidade e citotoxicidade.

Uma das consequências da artrite é o dano hepático associado à inflamação crônica sistêmica que é característica dessa condição (RAVISHANKAR; BHAVSAR, 1994). A disfunção hepática é avaliada pelo nível de aminotransferases liberadas pelas células danificadas (RAJKAPOOR et al., 2009). A única alteração nos parâmetros bioquímicos indicativos de disfunção hepática que foi observada em nosso estudo foi um aumento na atividade da AST no grupo artrítico. Corroborando com os nossos resultados, da Silveira et al. (2016) também relataram um aumento dos níveis de AST em ratos com artrite induzida, o que pode ser resultado do processo inflamatório artrítico.

O tratamento com quercetina em qualquer dosagem fez com que os níveis séricos de AST de ratos artríticos voltassem aos níveis normais, demonstrando o efeito hepatoprotetor deste flavonoide. Este efeito foi verificado em outros estudos (MILTERSTEINER et al., 2003; WEI et al., 2017; AFIFI; IBRAHIM; GALAL, 2018), e tem sido sugerido que seja devido à capacidade antioxidante da quercetina, realizada através de sua capacidade de sequestrar espécies reativas de oxigênio (FILIPE et al., 2001; TZANKOVA et al., 2017), além da inibição da lipoxigenase e ciclooxigenase (COS et al., 1998; KUMARAPPAN et al., 2011; TZANKOVA et al., 2017).

O estresse oxidativo é definido como o desequilíbrio entre a produção de radicais livres e o status de defesa antioxidante (VALKO et al., 2007) e está envolvido na patogênese da sinovite inflamatória. As células presentes na articulação inflamada (macrófagos, neutrófilos, linfócitos e células endoteliais), uma vez isoladas e estimuladas, são capazes de produzir radicais livres. Estes radicais, na presença de moléculas de lipídeos, DNA, proteínas ou carboidratos provocam lesão oxidativa (SEVANIAN; HOCHSTEIN, 1985; RAMOS; RAMOS; DOMINGUEZ, 2000).

Em nosso estudo, encontramos um aumento nos níveis de EROs e TBARS no grupo artrítico, o que pode ser explicado pelo fato de as ERO possam ser produzidas como resultado de inflamação, levando à destruição de cartilagens e ossos, degranulação de neutrófilos e liberação de uma variedade de enzimas potencialmente prejudiciais (STAMP et al., 2012; VASILJEVIC et al., 2016). A peroxidação de lipídios de membrana induzida pelo estresse oxidativo é prejudicial, levando à inativação de receptores ou enzimas ligados à membrana, e como consequência pode aumentar a permeabilidade do tecido e danificar a função celular normal (DALLE-DONE et al., 2006). Um dos marcadores utilizados para avaliar a peroxidação lipídica é o TBARS (BAUEROVA et al., 2015), que foi encontrado em níveis elevados em pacientes com AR devido à excessiva peroxidação lipídica observada nas células e no plasma, ou como resultado da geração e difusão excessivas de peróxidos lipídicos nas articulações inflamadas (VIJAYAKUMAR et al., 2006).

O tratamento com quercetina reverteu às alterações nos níveis de EROS e TBARS. Estes resultados corroboram com outros estudos que demonstraram que a quercetina diminuiu os níveis de ROS e TBARS (ABDALLA et al., 2014; BALDISSARELLI et al., 2016), provavelmente devido as EROs serem inativadas por grupos hidroxila altamente reativos presentes neste flavonoide (JEYADEVI et al., 2013). Estudos anteriores revelaram que a quercetina previne a peroxidação lipídica e elimina os radicais superóxidos (JOHNSON; LOO, 2000; KUMAR et al., 2003), retardando a lesão celular mediada pela via oxidativa ao

eliminar os radicais livres, garantindo proteção contra a peroxidação lipídica e os íons metálicos quelantes (KUMAR et al., 2003; DOS SANTOS et al., 2018).

O mecanismo de defesa que previne danos por estresse oxidativo inclui enzimas antioxidantes, tais como: catalase (CAT), glutathione S-transferase (GST), superóxido dismutase (SOD) e glutathione peroxidase (GPx) e antioxidantes não enzimáticos como os flavonoides, carotenoides, vitaminas (A, C e E), glutathione e minerais (JEYADEVI et al., 2013). Verificamos um aumento nos níveis de CAT em animais tratados com quercetina nas doses de 25 e 50 mg/kg quando comparado ao grupo artrítico, entretanto a quercetina não apresentou efeito sobre a dosagem dos tióis totais e da glutathione reduzida. Este efeito da quercetina já havia sido demonstrado por Sherif, 2018, em um modelo de hepatotoxicidade, onde o tratamento com quercetina aumentou os níveis hepáticos de CAT, sugerindo que a quercetina protegeu o fígado dos efeitos deletérios do melfalano, eliminando os radicais livres e estimulando as enzimas antioxidantes hepáticas SOD e CAT, usando os mesmos mecanismos descritos acima.

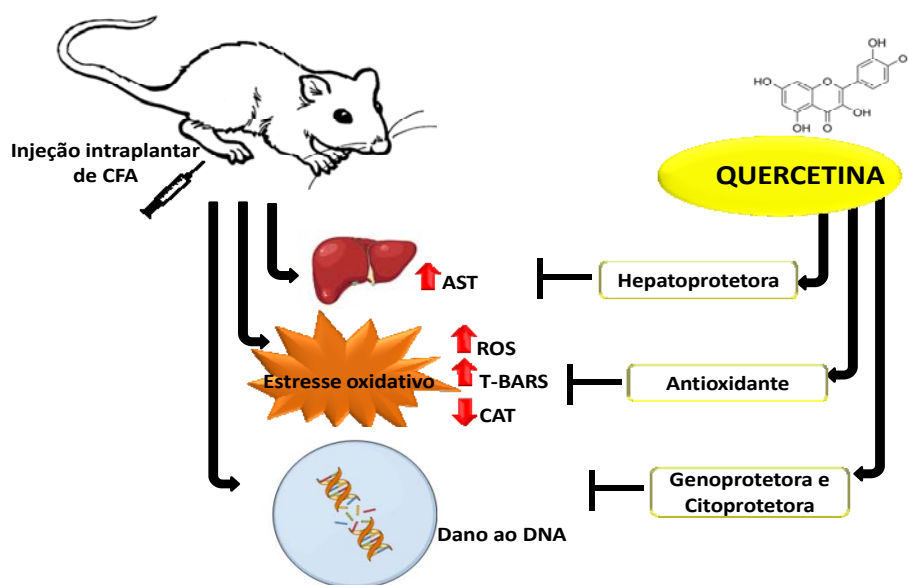
Outro resultado importante a ser discutido é o dano ao DNA. Sabe-se que nas doenças inflamatórias crônicas, como AR e LES, as EROs são liberadas pelas células fagocitárias no local da lesão e passam pelas membranas celulares que reagem com o DNA nuclear (JOHNSON; LOO, 2000), o que pode contribuir para o aumento das taxas de mutação, instabilidade do genoma, apoptose, regeneração tecidual associada e proliferação celular (ANSAR et al., 2016; DOS SANTOS et al., 2018). No presente estudo, o dano ao DNA foi aumentado no grupo artrítico, corroborando com os achados de Altindag et al. (2007), em que o dano ao DNA foi significativamente maior em pacientes com AR do que nos controles. Nos grupos tratados com quercetina, podemos verificar que houve uma redução significativa nos níveis de dano ao DNA. Esse resultado é consistente com o de Marcolin et al. (2013), que demonstrou que o papel protetor da quercetina em um modelo experimental de esteato-hepatite não alcoólica (NASH), propondo que a quercetina é capaz de eliminar as EROs e consequentemente, diminuir as quebras das cadeias de DNA e das bases oxidadas.

Além disso, em um estudo realizado por Ansar et al. (2016), a quercetina foi capaz de diminuir o dano ao DNA em ratos com toxicidade induzida por acrilamida (ACR), sugerindo que os efeitos antioxidantes desse flavonoide são devidos à sua alta difusão nas membranas, auxiliando na remoção de oxirradicais ao longo da bicamada lipídica. Alternativamente, a estrutura da quercetina penta-hidroxila permite a quelação de íons metálicos, eliminando assim os radicais alcóxila e peróxila lipídicos (LIEN et al., 1999). Este flavonoide também

pode estar envolvido na indução indireta de genes desintoxicantes (YOUDIM et al., 2002; MOLINA et al., 2003; HA et al., 2003; POLAT et al., 2006).

Em relação aos níveis de fragmentação do DNA de cadeia dupla, avaliado através do método do Picogreen®, constatamos um aumento no grupo artrítico, indicando citotoxicidade, que provavelmente é devido ao dano oxidativo e apoptose e este aumento foi revertido pelo tratamento com a quercetina. Estes resultados estão de acordo com Abdalla et al., 2014 e Lovato et al., 2016 em modelos murinos de citotoxicidade química. Este efeito pode estar associado à ação antioxidante da quercetina, devido à presença da estrutura O-dihidroxi nas posições 3'e 4'no anel B, conhecido como grupo catecol, que possui alta capacidade de varredura devido à sua configuração (D'ANDREA et al., 2015; LOVATO et al., 2016). Na figura 11, estão ilustrados os resultados do manuscrito 2.

Figura 11 - Resumo gráfico dos resultados do manuscrito II



Em resumo, neste manuscrito podemos demonstrar que a quercetina forneceu proteção contra o estresse oxidativo proveniente da AR. Nossos achados ilustram os potenciais efeitos antioxidantes da quercetina, destacando possíveis aplicações terapêuticas desse flavonoide. Assim, a quercetina é um potencial candidato para o tratamento adjuvante da AR, merecedor de uma investigação mais aprofundada para estabelecer seu uso na prática clínica.

Na AR, além do característico acometimento poliarticular, pode envolver, diretamente ou indiretamente, diversos órgãos e tecidos, incluindo os aspectos hematológicos,

gastrointestinais, dermatológicos, cardiovasculares, respiratórios, oculares e renais. Essas manifestações clínicas são conhecidas como manifestações extra-articulares (CHANDRASHEKARA et al., 2017). Essas manifestações têm importância clínica devido à sua associação com a morbidade e aumento do risco de mortalidade prematura (NYHALL-WAHLIN et al., 2009).

Deste modo, avaliamos marcadores de estresse oxidativo em tecido hepático e renal neste modelo animal e podemos verificar que tanto no tecido hepático como no renal, nos animais com artrite induzida, podemos constatar um acentuado estresse oxidativo, confirmando que este modelo foi capaz de gerar manifestações extra-articulares significativas.

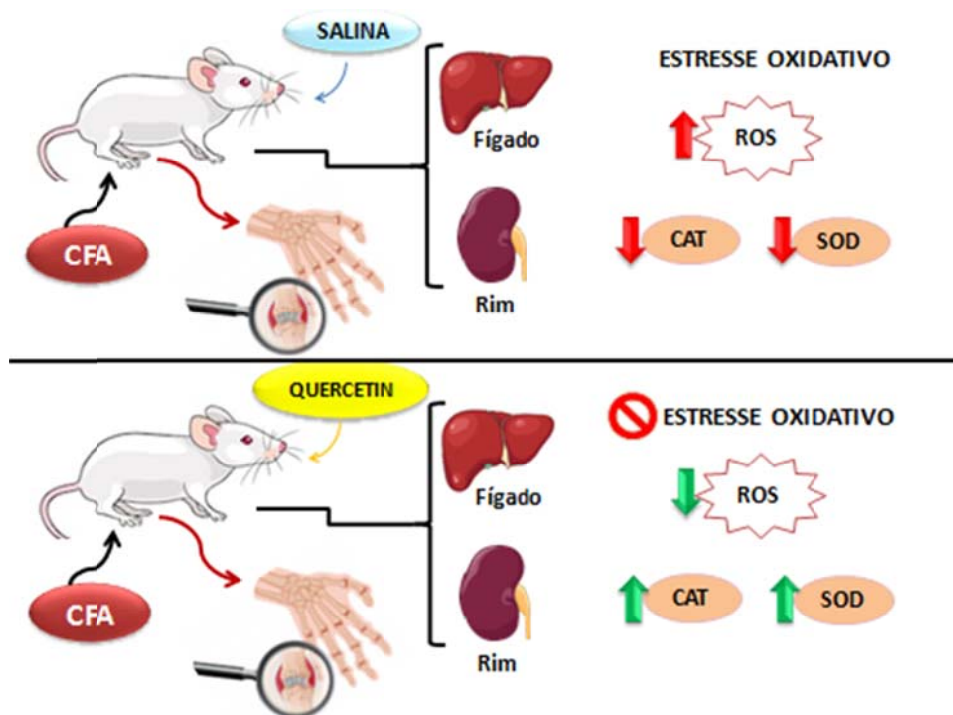
Em relação aos níveis das EROS, observamos um aumento no tecido hepático e tecido renal no grupo artrítico. Os níveis de EROs aumentados no fígado também foram observados em um estudo realizado por Gonçalves et al. (2015), o que pode ser explicado pelo metabolismo oxidativo mais intenso, gerando um ambiente mais oxidante e uma produção mais intensa de EROs (COMAR et al., 2013; GONÇALVES et al., 2015). Além disso, as células do fígado precisam de energia para realizar muitas funções e a alta taxa metabólica do fígado está diretamente associada a um alto fluxo de elétrons na cadeia respiratória mitocondrial. Enquanto isso, alguns desses elétrons são desviados, produzindo novas EROs, deixando o corpo suscetível ao processo de estresse oxidativo (OGONOVSKY et al., 2005; DORNELLES et al., 2017).

O mesmo resultado foi observado em tecido renal, no entanto, em nosso conhecimento este foi o primeiro estudo que determinou os níveis de EROS em tecido renal em um modelo de artrite induzida. Em um modelo de diabetes e em outro modelo de dano renal e hepático causado por esteroides anabolizantes, também se verificou um aumento dos níveis de EROs nos rins, o que pode ser explicado pela depleção das defesas antioxidantes, comprometendo a capacidade de combater as EROs e com consequente dano oxidativo (OLIVEIRA et al., 2015; DORNELLES et al., 2017). Em contraste, neste estudo os grupos tratados com quercetina, tiveram os níveis de EROs reduzidos quando comparados ao grupo artrítico. O papel antioxidante da quercetina é bem estabelecido pela sua capacidade de eliminar os radicais livres e vincular a transição de íons metálicos (DE SOUZA; DE GIOVANI, 2004; D'ANDREA, 2015), e dentro da família dos flavonoides; a quercetina é a mais potente depuradora de EROS (D'ANDREA, 2015).

Dentre os parâmetros antioxidantes avaliados, neste presente estudo, não foram encontradas diferenças significativas em tióis totais e glutathiona reduzida, mas a atividade de CAT e SOD no fígado e rim foi diminuída no grupo artrítico. Nossos resultados estão de

acordo com Ramprasatha, Shanthib e Sachdanandama, 2005. Os altos níveis de EROS são capazes de inibir a atividade de antioxidantes e, portanto, as atividades dessas enzimas parecem ser reduzidas. No entanto, apenas o tratamento com quercetina na dose de 50 mg/kg foi capaz de aumentar as atividades de CAT e SOD, o que pode ser sugerido pela ação antioxidante desse flavonoide. A quercetina tem uma forte capacidade de doação de elétrons que estabiliza os radicais livres (ZHENG et al., 2017). A prevenção do dano oxidativo pela quercetina é atribuída ao seu substituinte livre -OH, que, acredita-se que possa aumentar a estabilidade do radical flavonoide e do grupo catecol, sendo responsável pela ação quelante da quercetina (ALRAWAIQ; ABDULLAH, 2014). Na figura 12 estão ilustrados, os resultados encontrados no manuscrito III.

Figura 12 - Resumo gráfico dos resultados do manuscrito III



Assim, neste manuscrito podemos observar que o modelo induzido por CFA causou um aumento significativo de EROS e diminuição da atividade da SOD e CAT no fígado e rim, sugerindo que a artrite induzida por CFA (AIA) foi capaz de causar manifestações extra-articulares nos animais. No entanto, nossos resultados demonstraram claramente que um papel antioxidante protetora do tratamento com quercetina no fígado e rim de animais com AIA. Além disso, nossos achados demonstraram que a quercetina representa uma abordagem terapêutica promissora para complicações decorrentes da artrite reumatoide, evidenciando as

vantagens da quercetina e incentivando seu uso em estudos pré-clínicos e clínicos adicionais como um agente antioxidante. Assim, a quercetina pode ser um candidato ideal para o tratamento adjuvante da AR e suas manifestações extra-articulares.

Em um modelo de artrite avaliamos marcadores de estresse oxidativo no soro e parâmetros do sistema purinérgico em linfócitos. Em ratos artríticos observamos que a atividade das enzimas do sistema purinérgico foram alteradas na superfície dos linfócitos, bem como um aumento na atividade da mieloperoxidase, no soro verificamos, ainda um aumento dos níveis de adenosina e de marcadores de estresse oxidativo e dano ao DNA. Assim demonstramos a presença de um processo inflamatório sistêmico capaz de causar danos em outros tecidos do organismo, que pode ser constatado com o aumento dos níveis da AST. A seguir, investigamos a produção de EROs e a defesa antioxidante. Nestes órgãos foi observado um desequilíbrio que favorece a produção de EROs em detrimento da defesa antioxidante. A quercetina foi capaz de reverter todas estas mudanças descritas acima, sendo capaz de prevenir as alterações causadas pela artrite e podendo ser um composto natural promissor sendo usado como adjuvante no tratamento da artrite.

6 CONCLUSÃO

- O CFA foi capaz de induzir um processo inflamatório nos animais com artrite observados através do escore de artrite, edema de pata, hiperalgesia termal, avaliação histológica, bem como níveis séricos de IFN- γ e o tratamento com a quercetina foi capaz de reverter estes parâmetros.
- Acompanhando o processo o inflamatório foi demonstrado pela avaliação da atividade da mieloperoxidase e dos níveis de EROs. A quercetina também foi capaz de reverter o estresse oxidativo.
- A quercetina foi capaz de reverter às alterações causadas pela artrite nas atividades da E-NTPDase e da E-ADA e nos níveis de adenosina, sugerindo que a quercetina exerce efeitos sob o sistema purinérgico.
- Ratos artríticos apresentaram dano ao DNA evidenciado pela técnica de Cometa e Picogreen®, que foi revertido com o tratamento com a quercetina, demonstrando que a quercetina possui um efeito genoprotetor.
- O processo inflamatório sistêmico observado pelos parâmetros descritos acima foi capaz de alterar a AST (marcador de dano hepático), indicando manifestações extra-articulares e a quercetina reverteu este dano.
- O desequilíbrio entre a produção de EROs e a defesa antioxidante foi observado nos tecidos hepático e renal, indicando dano oxidativo nestes órgãos. A quercetina exerceu o seu efeito antioxidante, reduzindo estes níveis.
- A quercetina apresentou efeito anti-inflamatório, antioxidante, hepatoprotetora, genoprotetora e citoprotetora, podendo ser uma alternativa promissora no tratamento da AR, incluindo as suas manifestações extra-articulares.

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CERTIFICADO

Certificamos que a proposta intitulada "QUERCETINA E SEUS MECANISMOS CAUSAIS: ESTUDOS IN VITRO E EX VIVO DOS SEUS POSSÍVEIS EFEITOS NA SINALIZAÇÃO PURINÉRGICA E NO METABOLISMO OXIDATIVO-INFLAMATÓRIO", protocolada sob o CEUA nº 9970081214, sob a responsabilidade de **Daniela Bitencourt Rosa Leal** - 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 - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal de Santa Maria (CEUA/UFSM) na reunião de 16/01/2015.

We certify that the proposal "QUERCETIN AND ITS MECHANISMS CAUSAL: IN VITRO STUDIES AND EX VIVO OF ITS POSSIBLE EFFECTS ON SIGNAL PURINERGIC AND OXIDATIVE-INFLAMMATORY METABOLISM", utilizing 50 Heterogenic rats (50 females), protocol number CEUA 9970081214, under the responsibility of **Daniela Bitencourt Rosa Leal** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University of Santa Maria (CEUA/UFSM) in the meeting of 01/16/2015.

Finalidade da Proposta: [Pesquisa \(Acadêmica\)](#)

Vigência da Proposta: de [03/2015](#) a [12/2017](#)

Área: [Microbiologia E Parasitologia](#)

Origem: [Biotério Central UFSM](#)

Espécie: [Ratos heterogênicos](#)

sexo: [Fêmeas](#)

idade: [8 a 8 semanas](#)

N: [50](#)

Linhagem: [Wistar](#)

Peso: [250 a 300 g](#)

Resumo: A quercetina, um flavonóide presente em vários alimentos exerce diversas funções terapêuticas no organismo como atividade antioxidante antiviral e antiinflamatória. Os flavonoides presentes nas frutas e nos vegetais têm recebido considerável atenção por causa de seu baixo custo e ampla margem de segurança. Avaliar os possíveis efeitos citotóxicos e genotóxicos faz parte dos processos da exploração de flavonóides, e a partir deles, podemos estabelecer a potencialidade de aplicação clínica de uma droga, minimizando os possíveis riscos à saúde humana e conseqüentemente acrescentando sua eficácia e segurança. Os flavonóides têm se destacado por seus excelentes potenciais terapêuticos em diversos ensaios experimentais. O modelo de artrite induzida por adjuvante completo de Freund (CFA) em ratos é um modelo bastante empregado na investigação de novas terapias para artropatias inflamatórias crônicas, como a artrite reumatóide. A artrite reumatóide é caracterizada como uma doença crônica inflamatória, imunomediada e com fisiopatologia bastante complexa. Durante o processo inflamatório, uma rede complexa e hierarquizada de citocinas rege este processo, desencadeando uma resposta imunológica. O processo inflamatório possui importantes mediadores, entre eles os componentes que fazem parte da sinalização purinérgica: nucleotídeos e nucleosídeos da adenina, ectoenzimas e receptores purinérgicos. Dessa forma, este projeto tem como objetivo avaliar os possíveis efeitos da quercetina sobre o sistema purinérgico e no metabolismo oxidativo-inflamatório, auxiliando na avaliação toxicológica dos constituintes deste flavonóide e propiciando subsídios para elaboração de estudos pré-clínicos.

Local do experimento: LABORATÓRIO DE IMUNOBIOLOGIA EXPERIMENTAL E APLICADA DO DEPARTAMENTO DE MICROBIOLOGIA E PARASITOLOGIA DA UFSM.

Santa Maria, 27 de novembro de 2018

Prof. Dr. Denis Broock Rosemberg
Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

Prof. Dr. Saulo Tadeu Lemos Pinto Filho
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal de Santa Maria

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Subject: Cell Biochemistry & Function - Manuscript number CBF-18-0060

Body: 04-Apr-2018

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Your manuscript entitled "Role of quercetin in activity of E-NTPDase/E-ADA and cytokine secretion of complete Freund's adjuvant-induced arthritic rats" has been successfully submitted online and is presently being given full consideration for publication in Cell Biochemistry & Function.

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We are pleased to receive your manuscript entitled "Antioxidant, hepatoprotective, genoprotective and cytoprotective effects of quercetin in a murine model of arthritis" by Saccol, Renata; Silveira, Karine; Manzoni, Alessandra; Abdalla, Fatima; Oliveira, Juliana ; Dornelles, Guilherme; Barbisan, Fernanda; Passos, Daniela; Casali, Emerson; Andrade, Cinthia ; da Cruz, Ivana; Leal, Daniela.

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Dear Dr Pereira,

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Corresponding author: Dr Daniela Leal

Listed co-author(s): Ms Juliana Oliveira, Mrs Daniela Ferreira Passos, Dr Renata Pereira, Dr Karine Lanes Silveira, Miss Alessandra Guedes Manzoni, Dr Cinthia Melazzo de Andrade

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