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# EFEITO DO CINAMALDEÍDO SOBRE O METABOLISMO DE NUCLEOTÍDEOS E NUCLEOSÍDEO DE ADENINA EM ARTRITE POR ADJUVANTE

**DISSERTAÇÃO DE MESTRADO**

**Maria Luiza Prates Thorstenberg**

**Santa Maria, RS, Brasil**
**2014**

# EFEITOS DO CINAMALDEÍDO SOBRE O METABOLISMO DE NUCLEOTÍDEOS E NUCLEOSÍDEO DE ADENINA EM ARTRITE POR ADJUVANTE

**Maria Luiza Prates Thorstenberg**

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Ciências Biológicas, Área de Concentração em Bioquímica Toxicológica, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do grau de **Mestre em Ciências Biológicas: Bioquímica Toxicológica**

**Orientador: Daniela Bitencourt Rosa Leal**

**Santa Maria, RS, Brasil**
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# EFEITO DO CINAMALDEÍDO SOBRE O METABOLISMO DE NUCLEOTÍDEOS E NUCLEOSÍDEO DE ADENINA EM MODELO DE ARTRITE POR ADJUVANTE

elaborada por
**Maria Luiza Prates Thorstenberg**

Como requisito parcial para a obtenção do grau de
**Mestre em Ciências Biológicas: Bioquímica Toxicológica**

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**Suba o primeiro degrau com fé. Não é necessário que você veja toda a escada. Apenas dê o primeiro passo.**

***Martin Luther King***

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# RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica
Universidade Federal de Santa Maria, RS, Brasil

## EFEITO DO CINAMALDEÍDO SOBRE O METABOLISMO DE NUCLEOTÍDEOS E NUCLEOSÍDEO DA ADENINA E EM MODELO EXPERIMENTAL EM ARTRITE POR ADJUVANTE

AUTORA: MARIA LUIZA PRATES THORSTENBERG
ORIENTADORA: DANIELA BITENCOURT ROSA LEAL
Data e Local da Defesa: Santa Maria, 20 de dezembro de 2013

Artrite reumatoide (AR) é uma doença inflamatória crônica e multisistêmica, com características evidentes de autoimunidade, que expressa uma resposta celular do tipo $Th_1$. Caracteriza-se basicamente por sinovite crônica, simétrica e erosiva, preferencialmente de articulações periféricas, onde existe um intenso processo inflamatório. O sistema de sinalização purinérgica desenvolve um papel importante na modulação das respostas inflamatórias e imunes, através de biomoléculas extracelulares, como os nucleotídeos de adenina e seu derivado nuclesídeo adenosina, os quais são indispensáveis para a iniciação e manutenção de respostas inflamatórias. Os efeitos de tais moléculas são promovidos pela ação dos receptores purinérgicos específicos e controlados por ectoenzimas, na superfície das células. Com base nestes princípios esta pesquisa avaliou os efeitos do cinamaldeído no escore de artrite, edema de pata e hiperalgesia termal bem como em análises histológicas além da atividade de E-NTPDae e E-ADA em linfócitos de ratos com artritre induzida por adjuvante. Os ratos foram divididos em quatro grupos, dois grupos com artrite induzida por adjuvante e dois grupos controles. Estes animais receberam o composto na concentração de 2,1%, via oral por um período de 15 dias. Não se observou diferenças entre análises de escore de artrite e edema de pata, porém notou-se-se alterações em hiperalgesia termal em cerca de 60% no grupo com artrite induzida tratado com cinamaldeído. Em relação às análises histológicas foi notada uma leve redução do infiltrado inflamatório linfocitico em ratos com artrite induzida e tratados com cinamaldeído. Mostrou-se um aumento da hidrólise do ATP em 94,14% no grupo com artrite induzida quando comparado com o grupo controle e em 20,58% quando comparado com o grupo tratado com cinamaldeído. Contudo, a atividade da E-NTPDase quando utilizado ADP como substrato obteve um aumento 152,56% no grupo artrite induzida quando comparado com o grupo controle e 122,76% quando comparado com grupo tratado com cinamaldeído. Na atividade da E-ADA foi observado um aumento em cerca de 151,84% no grupo artrite induzida em relação ao grupo controle e em 69,7% quando comparado com a grupo artrite induzida e tratado com cinamaldeído. Em conclusão, os dados indicam que cinamadeído foi capaz de reduzir hiperalgesia termal e alterações histológicas, como também diminui a cascata das ectonucleotidases, uma vez que observamos uma queda gradativa das atividades nos linfócitos no grupo com artrite induzida por adjuvante tratados com cinamaldeído quando comparado com os demais grupos. Desta forma cinamaldeído foi capaz de exercer seus efeitos desfavorecendo alguns eventos inflamatórios característico da artrite induzida por adjuvante. Embora careça de maiores estudos, o cinamaldeído poderia ser utilizado como alvo terapêutico complementar para a artrite reumatoide.

**Palavras-chave:** Artrite reumatoide. Ecto- enzimas. Cinamaldeído. Nucleotídeos.

# ABSTRACT

Dissertation of Master's Degree
Post-Graduating Program in Biological Sciences (Toxicological Biochemistry) Federal University of Santa Maria, RS, Brazil

## EFFECT OF CINNAMALDEHYDE ON NUCLEOTIDE AND NUCLEOSISE OF ADENINA METABOLISM IN LYMPHOCYTES IN ARTHRITIS BY ADJUVANT

AUTHOR: MARIA LUIZA PRATES THORSTENBERG
ADVISOR: DANIELA BITENCOURT ROSA LEAL
Place and Date: Santa Maria, december 20th, 2013

Rheumatoid arthritis (RA) is a chronic, multisystem inflammatory disease with autoimmune features evident, expressing a cellular response of Th1. Characterized primarily by chronic synovitis, erosive and symmetrical, preferably peripheral joints, where there is an intense inflammatory process. The purinergic signaling system plays an important role in the modulation of inflammatory and immune responses through extracellular biomolecules such as nucleotides adenine and adenosine derivative nucleodise therefore, which are essential to providing the trigger and maintain the trigger inflammatory response. The effects of these molecules are promoted by the action of purinergic receptors specific and controlled by ectoenzymes, in cell surface. Based on these principles, this study investigated the effect of cinnamaldehyde in thermal hyperalgesia, , arthritis score, paw edema and thermal hyperalgesia as well histolgical parameters beyond the activity of the E-NTPDase and E-ADA in lymphocytes in rats adjuvant arthritis. The rats were divided in four groups, of wich two were adjuvant induced arthritis in the other two control groups. The animal received the compound at a concentration of 2,1% orally for a period of 15 days. Not differences were observed among analysis the arthritis score, paw edema it is however noted differences in thermal hyperalgesia in about 60% and in group induced arthritic and treated with cinnamaldehyde. Compared histological analysis it was noticed a slight reduction the lynphocitytic inflammatory infiltration in rats induced arthritis and treated with cinnamaldehyde. We found the increased the ATP hydrolysis in about 94,14% in arthritis induced when compared with control group and 20,58% when compared with groups treated with cinnmaldehyde . However, E-NTPDase activity when used ADP as substrate rised in 152,56% in relation the control group and 122,76% in relation the control group treated with cinnamaldehyde. In E-ADA activity was observed the increased in about 151,84%%in group arthritis induced when compared with control groups and rise 69,7% when compared with group arthritis induced treated with cinnamakdehyde. In conclusion, the data indicate that cinnamaldehyde was able to reduce thermal hyperalgesia and alterations histological in rats induced arthritis, as well as decreased the ectonucleotidase cascade, once we observed a activity gradual decrease in lymphocytes in group induced arthritis and treated with cynnamaldehyde in relation with all others groups. Therefore cinnamaldehyde was act to skewing some effects inflammatory of induced arthritis. Although requiring further study, cinnamaldehyde could be used as a complementary fot the benefit of people with rheumatoid arthritis.

**Keywords:** Rheumatoid arthritis. Ecto-enzymes. Cinnamaldehyde. Nucleotides.

# LISTA DE ABREVIATURAS

**ACRS:** Regiões conservadas da apirase

**ADP:** Adenosina difosfato

**AINHs:** Analgésicos e antiinflamatórios não esteroidais

**AMP:** Adenosine monofosfato

**AMPc:** Adenosine monofosfato cíclica

**AP-1:** Fator transcripcional 1

**AR:** Artrite reumatoide

**ATP:** Adenosine trifosfato

**CIN:** Cinamaldeído

**COX:** Cicloxigenase

**CTLA4:** Linfócito T citotóxico associado com proteína 4

**DMCD:** Drogas modificadoras do curso da doença

**E-ADA:** Adenosina desaminase

**E-NTPDase:** E-NTPDase

**GMP:** Guanosina monofosfato

**HLA II:** Antígeno leucocitário humano II

**HLA-DR:** Complexo de histocompatibilidade maior, classe 2, DR alfa.

**IgG:** Imunoglobulina do tipo G

**IL-1:** Interleucina 2

**IL-2:** Interleucina 2

**IL-17:** Interleucina 17

**IL-1β:** Interleucina 1- beta

**IL-6:** Interleucina 6

**IL-7:** Interleucina 7

**IL-12:** Interleucina 12

**IL-23:** Interleucina 23

**IκBK:** Kinase 2 IκB

**IFN-γ:** Interferon gama

**IMP:** Inosina 5’ monofosfato

**JAK-STAT:** Janus kinase- transudor de sinal e ativador de transcrição 3

**LPS:** Lipopolissacarídeo

**MAPK:** Proteína kinase ativadora de mitógeno

**MAPK P-38:** Proteína kinase ativadora de mitógeno 38

**MHC:** Complexo de histocompatibilidade maior

**MMP-1, MMP-9:** Metaloproteinases 1 e 9

**MHSP65:** Proteína de choque térmico 65

**NFκB:** Fator nuclear κB

**NK:** Célula Natural killer

**NKT:** Linfócito T natural killer

**NOD:** Domínio de ligação de nucleotídeo

**PADI4:** Arginase desaminase peptídica tipo 4

**PCC:** Peptídeo cíclico citrulinado

**PTPN22:**Proteína tirosina fosfatase tipo 22

**ROS:** Espécies reativas de oxigênio

**Th1:**Linfócito helper 1

**Th17:** Linfócito helper 17

**TLR2:** Receptors toll like 2

**TLR4:** Receptor toll like 4

**TLRs:** Receptor de linfócito T

**TNF-α:** Fator de necrose tumoral

**TRAF:** Peptidase conjugada de transferência de sinal

**TNF-α:** Fator de necrose tumoral alfa

**TGF-β:** Fator transformador de crescimento beta

**UMP:** Urudina monofosfato

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## INTRODUÇÃO

**MANUSCRITO**

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**MANUSCRITO**

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# APRESENTAÇÃO

Esta dissertação está organizada da seguinte forma: primeiramente é apresentada a introdução. A seguir, os resultados, discussão e a conclusão os quais se apresentam na forma de um manuscrito, no qual foi escrito, seguindo as normas do periódico ao qual o mesmo será submetido. As referências bibliográficas apresentadas no final da dissertação referem-se às citações que aparecem no item introdução.

Manuscrito será submetido para revista: **Food and Chemical Toxicology.**

# 1 INTRODUÇÃO

O papel primordial da resposta imune é proteger o indivíduo da invasão por patógenos infecciosos e promover a discriminação “próprio-não próprio” (MARTINEZ; ROSEN, 2005). Para realizar tais funções, o sistema imunológico precisa funcionar de forma harmônica para manter a tolerância imune, a qual pode ser quebrada, gerando distúrbios de imunorregulação com consequente emergência das doenças autoimunes (MILNER et al., 2005; SCHEINBERG, 2005).

O conceito de autoimunidade surgiu através da descoberta de auto anticorpos em condições de anemia hemolítica e artrite reumatoide (DONATH et al., 1904; WAALER, 1940). Sabe-se que os fatores que levam a autoimunidade não são bem definidos, uma vez que estão associados com variações genéticas em moléculas que regulam a ativação de células imunes, como complexo de histocompatibilidade maior (MHC) e componentes do complemento, citocinas, receptores de células T (TLRs), proteína domínio de ligação de nuclesídeo (NOD) e componentes do inflamassoma (ARNOTT, et al., 2004; KASTNER, 2005; SHIMIN, 2008).

Pesquisas evidenciam que infecções por microorganismos contribuem para a ativação de respostas autoimunes em indivíduos geneticamente pré-dispostos. A reação imune frente a estes agentes seria sustentada por uma reação cruzada devido ao mimetismo antigênico do hospedeiro, com consequente quebra da tolerância imunológica, levando a um processo crônico e destrutivo (ARNETT et al., 1987; KLINMAN, 2003).

Entre as patologias autoimunes, a Artrite Reumatoide (AR) é uma das mais estudadas sendo caracterizada por acometer principalmente a membrana sinovial, a cartilagem e o osso, podendo haver também envolvimento extrarticular (FIRESTEIN, 2003). É a artropatia crônica com uma prevalência mundial de 1% a 3% e uma incidência anual de 0,02% a 0,05%. Predomina mais no sexo feminino, surgindo entre a quarta e quinta década de vida, apresentando alta morbidade, declínio funcional, incapacidade permanente e aumento de mortalidade (PINCCUS; CALLAHAN, 1993; ALAMANOS; DROLOS, 2005).

Mudanças cruciais são encontradas na patogenia da AR, entre elas autoimunidade, inflamação crônica e degradação da articulação. A autoimunidade caracteriza-se pela produção de anticorpos específicos IgM e IgG (fator reumatoide) ou específico para peptídeo ciclíco citrulinado, os quais são os marcos iniciais para a

patogênese da AR (FIRESTEIN, 2003). Sabe-se que a AR é uma doença multifatorial resultando da interação entre fatores genéticos e ambientais, destacando-se como os principais fatores de risco: tabagismo, genética, idade, sexo, agente infeciosos, fatores hormonais e fatores mecânicos (Fig. 1) (ALAMANOS; DROSOS, 2005; KLARESKOG et al., 2009).

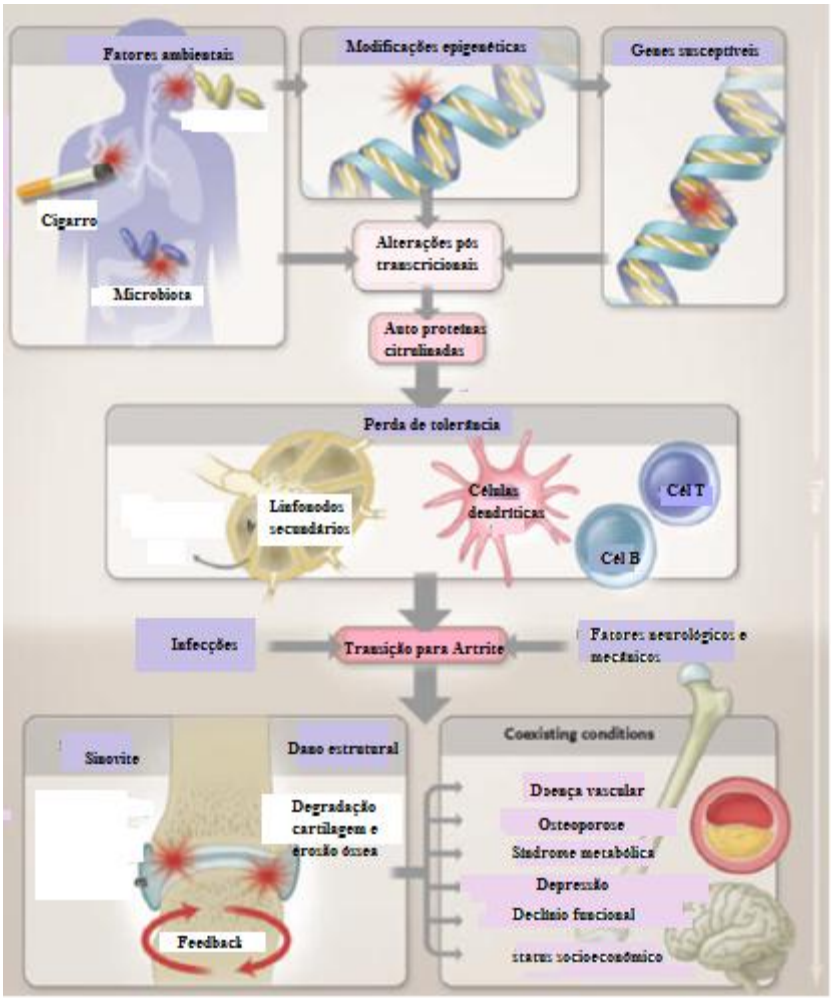


Figura 1: Patogênese da artrite reumatoide. (Adaptado de: MCIINES et al., 2011).

Os genes HLA-DR são associados com a gravidade da AR, bem como os genes que codificam IL-1 e TNF-α, PTPN22, PADI4, CTLA4 e as vias de sinalização da inflamação como TRAF, IκBK, NFκB e MAPK AP-1, JAK-STAT, MAPK P-38 codificando assim metaloproteinases, quimiocinas, moléculas de adesão e prostaglandinas, que perfazem a destruição da articulação e caracterizam mal prognóstico desta patologia (GREGERSEN et al., 1987; DELGADO; VAN DER HELM et al., 2005; ANAYA, 2007; DELGADO, 2007).

Devido às suas manifestações sistêmicas e crônicas, a artrite é marcada por uma hiperplasia das membranas sinoviais e estruturas articulares podendo levar à destruição óssea, edema e dor (VICENTI et al., 1994; FELDMAM et al., 1996; KINNE et al., 2000; FILLIPIN, 2008; ASQHIT et al., 2009; CARRILHO, 2009). Após ocorrer a perpetuação da doença, a membrana sinovial que é hipocelular, gera um ambiente hiperplástico, com a construção de uma camada de revestimento de células sinoviais e macrófagos que cobrem uma zona que possui um infiltrado celular com fibroblastos sinoviais, macrófagos, mastócitos, e ainda, células TCD4+, TCD8+, NK, NKT, B e plasmáticas (JIMENEZ, et al., 2005).

A patogênese da AR parece ser bastante complexa e está relacionada com a resposta imune inata e adaptativa bem como a resposta específica a antígenos mediado pelas células T e B. Citocinas como IL-1β, IL-6, IL-7, IL-12, IL-23 e TGF-β auxiliam na diferenciação e perpetuação de respostas pró-inflamatórias ocorrendo um equilíbrio entre respostas Th1 ou\e Th17 (Fig. 2) (BEREK; SCHRODER, 1997; PANAYI; CORRIGALL, 2001; BOISSER et al., 2008).

O padrão de resposta Th1 ou Th17 é caracterizado por apresentar linfócitos T autoreativos os quais interagem com os sinoviócitos, ocorrendo assim uma produção de mediadores inflamatórios como prostaglandina E2, agracanases, catepsinas, metaloproteínase (MMP-1, MMP-9) e IL-6 o que resulta na destruição da cartilagem e osso (CHABAUD et al., 1998;PANAY; CORRIGAL, 2001).

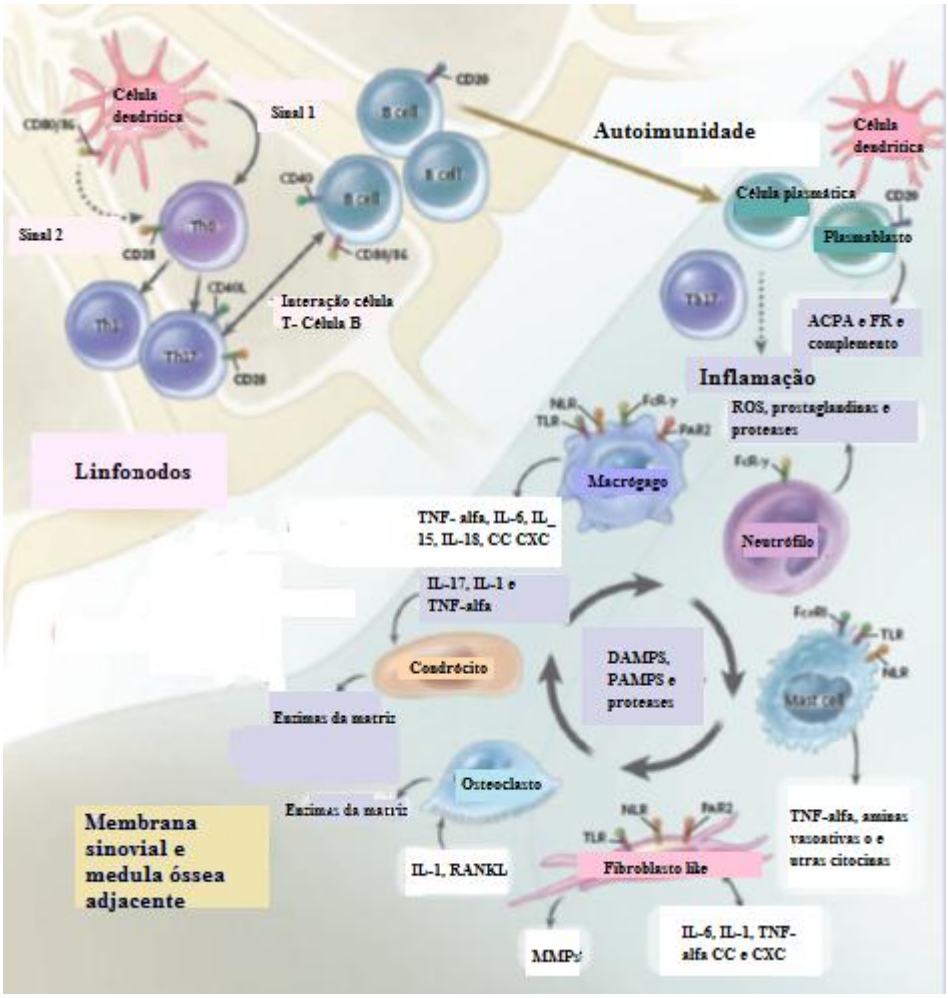


Figura 2: Ativação de células envolvidas na articulação na Artrite Reumatoide (Adaptado de: MCINNE et al., 2011).

É fato que a inflamação e desintegração óssea estão fortemente ligadas, uma vez que ocorre uma mudança de um perfil anabólico para catabólico, como também a inibição de síntese da matriz e indução da expressão de MMP (Fig. 3) (EBERHARDT et al., 2000; CATTERALL et al., 2001 GOLDRING, 2003; SCHETT et al., 2006).

Para manter a homeostase óssea há um equilíbrio entre reabsorção e formação, porém na fisiologia da artrite este equilíbrio é rompido, resultando em uma maior absorção que é dependente de osteoclastos e condrócitos, estas células induzem a reabsorção óssea as quais por sua vez permitem a invasão de células da membrana sinovial resultando na formação do *pannus* (TEITELBAUM, 2000; REDLICH et al., 2002). Como um ponto característico da AR, a *sinovite crônica* ou *pannus* é conhecido como o tecido neoformado, a partir da membrana sinovial de

uma articulação, este tecido cresce sobre a articulação se perpetuando sobre ela, também podendo chegar a revestir os ossos e tendões. Esta região é constituída por um infiltrado de células endoteliais, células TCD4+, macrófagos e células B (TEITELBAUM, 2000).

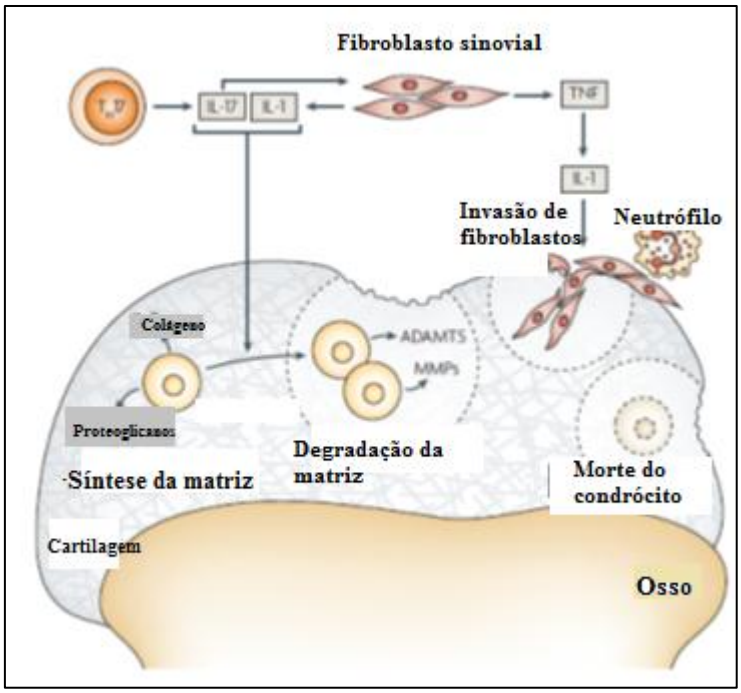


Figura 3: Ativacão de condrócitos e destruição da articulação (Adaptado de: MCINNES; SCHEET, 2007).

AR é a forma mais comum da poliartrite reumatoide a qual se apresenta por alterações articulares, estas ocorrem quando a doença é perpetuada, causando deformidades e incapacidade importantes (CORBACHO, 2001). Pode apresentar uma sintomatologia extra articular, envolvendo outros órgãos e tecidos. Dentre as feições não articulares temos o desenvolvimento de manifestações cardíacas (trombocitose e queda leve nos níveis de hemoglobina devido à ação de citocinas inflamatórias que agem nos precursores das hemácias), acometimento pulmonar (pleurite reumatoide), acometimento do fígado (anormalidade dos marcadores de função hepática), envolvimento cardíaco (pericardite levando a comprometimento cardíaco por artrite coronariana), envolvimento ocular (ceratoconjuntivite seca), e vasculite reumatoide (necrose fibrinoide da parede do vaso) (MONTENEGRO; ROCHA, 2009). O diagnóstico da AR é feito através da associação de

manifestações clínicas, radiológicas e laboratoriais. Baseado nos critérios de classificação do Colégio Americano de Reumatologia, revisados em 1987 (Figura 4), onde o paciente deve apresentar ao menos 4 dos 7 critérios estabelecidos (ARNETT et al., 1988)

**Quadro 1. 1987 Critérios para Classificação da AR (Colégio Americano de Reumatologia)**

| | |
|---|---|
| 1. Rigidez matinal | Rigidez matinal na e à volta das articulações, durando, pelo menos, uma hora até à melhoria máxima |
| 2. Artrite de 3 ou mais áreas articulares | Pelo menos 3 áreas articulares simultaneamente com edema dos tecidos moles ou fluido (não crescimento ósseo apenas) observado por um médico. As 14 áreas possíveis são as das seguintes articulações (à esquerda e à direita): interfalângicas proximais (IFP), metacarpofalângicas (MCP), punho, cotovelo, joelho, tornozelo e metatarsofalângicas (MTF) |
| 3. Artrite das articulações das mãos | Pelo menos uma área edemaciada (como descrito acima) das articulações seguintes: punho, MCP e IFP |
| 4. Artrite simétrica | Envolvimento simultâneo das mesmas áreas articulares (como definido em 2) em ambos os lados do corpo (o envolvimento bilateral das IFP, MCP ou MTF é aceitável sem simetria absoluta) |
| 5. Nódulos reumatóides | Nódulos subcutâneos, sobre proeminências ósseas, ou em superfícies extensoras, ou em regiões justa-articulares, observados por um médico |
| 6. Factor reumatóide sérico | Demonstração de quantidades anormais de factor reumatóide sérico por qualquer método para o qual o resultado foi positivo em <5% dos indivíduos do grupo controle normal |
| 7. Alterações radiográficas | Alterações radiográficas típicas de AR, na incidência póstero-anterior das radiografias da mão e do punho, que devem incluir erosões ou descalcificação óssea inequívoca, localizadas na ou mais marcadamente adjacentes às articulações envolvidas (apenas alterações de osteoartrite não qualificam) |

* Para efeitos de classificação, um paciente deve ser considerado como tendo AR se tiver satisfeito pelo menos 4 dos 7 critérios. Os critérios de 1 a 4 deverão estar presentes por, pelo menos, 6 meses. Pacientes com 2 diagnósticos clínicos não são excluídos.

**Fonte:** *Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315—24.*

Figura 4: Critérios de classificação do Colégio Americano de Reumatologia (Adaptado de: ARNETT et al., 1987).

Atualmente, as condutas para a terapêutica da artrite variam de acordo com o estágio da doença, sua atividade, responsividade e gravidade. Para o tratamento 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 (SHAVER et al., 2008). As drogas modificadoras do curso da doença

(DMCD), sozinhas ou em combinação com drogas biológicas, são indicadas para todo paciente a partir da definição do diagnóstico (BÉRTOLO, 2009).

A dificuldade da realização de estudos em pacientes por razões éticas ou técnicas leva à necessidade de modelos experimentais de artrite (ASQUITH et al., 2009). Modelos animais de doenças crônicas permitem uma melhor compreensão dos processos fisiopatológicos, bem como a avaliação do potencial de novas terapias. Para uma melhor compreensão da fisiopatologia da AR e desta forma uma melhor abordagem terapêutica, diversos modelos são empregados pela comunidade científica, destacando-se o modelo CIA (colágeno tipo II bovino) e o modelo por adjuvante completo de Freund (CFA) e por adjuvante incompleto de Freund (IFA), ambos com *Mycobacterium tuberculosis* inativado (KLEINAU et al., 1991; OLIVEIRA, 2007; DONG et al. 2010).O adjuvante completo de Freund (CFA), o qual contém o *Mycobacterium tuberculosis* inativado, foi convencionalmente utilizado para induzir artrite em ratos, no qual induz uma artrite crônica e progressiva conhecida como Artrite Induzida por Adjuvante (AIA). Após a indução, os animais desenvolvem uma inflamação poliarticular com uma consequente hiperplasia, desorganização da cartilagem e osso (BENDELE, 2001). Frente ao anteposto a Trealose dimicolato, Lipoarabinomana e proteína de choque térmico 65 (MHSP65) do *Mycobacterium*, vêm sendo relacionados na ativação de macrófagos e também produtoras de citocinas artritogênicas, incluindo TNF-α, IL-1β e IL-6, por TLR2 e TLR4 (BOWDISH et al., 2009; LEI et al., 2012). Evidências mostram que a ativação destes receptores está relacionada com AR, uma vez que em pacientes com a doença ativa, é encontrada uma regulação desta sinalização. A sinalização por TLR induzida por MHSP65 pode levar o aumento da IL-17 produzido pelas células Th17 auxiliando assim um processo pró-inflamatório característico desta patologia (SARKAR et al., 2010). Com o intuito de amenizar os diversos efeitos que doenças autoimunes ocasionam, a utilização de novas terapias alternativas vem sendo referenciadas, desta maneira a utilização de plantas medicinais, bem como de seus princípios ativos purificados vêm chamando a atenção de pesquisadores (SHCIPER, 1999; CHAO et al., 2005; LEE et al., 2009; LISHU et al., 2011).

O gênero *Cinnamomum* pertence à família Lauracea e compreende as espécies: *Cinnamomum osmophloeum, Cinnamomum zeylancum, Cinnamomum cassia,* sendo nativo do sudoeste da Ásia e Sri Lanka, popularmente conhecida como caneleira (NAGAI, 2003; MISHRA et al., 2009).

A caneleira é uma árvore de ciclo perene que atinge até 15 metros de altura, possui em seus caules cascas grosseiras e folhas simples opostas com nervuras longitudinais bem marcadas. Apresenta flores amarelo-esverdeadas, perfumadas e seu fruto é uma baga ovoide de cor escura (Figura 5). (BALME, 1978; SHCIPER, 1999). Na Ásia, *Cinnamom* foi popularmente usada na alimentação e na medicina tradicional. Seus extratos possuem uma composição variada incluindo polifenois, hidroxicalcanos e cinamaldeído (JARVILL, 2001; ANDERSON et al., 2004; PENG et al., 2008; ZHANG, 2008).

Figura 5: Caneleira. Adaptado de: (www.tradewindsfruit.com).

O cinamaldeído (CIN) (Figura 6) é um líquido amarelo pálido em temperatura ambiente, em suas propriedades sensoriais, apresenta-se com odor doce bem como paladar picante, sua concentração da caneleira é em torno de 60%. Têm sido amplamente utilizado na produção de produtos medicinais, bebidas, alimentos, perfumes, cosméticos, sabão, detergentes, cremes e loções, bem como para o tratamento de dispepsia, gastrite, distúrbios hematológicos e doenças alérgicas. Suas propriedades antifúngicas, antibacterianas e antiplaquetárias já foram descritas (N.T.P, 1993; LEUNG; FAUSTER, 1996; NAGAI et al., 2003; LEE et al., 2005; PASSOS et al., 2007; LEE et al., 2008). É quimicamente relacionado a uma ação toxicológica, podendo induzir fibrose renal e diminuir os níveis hematológicos, porém esses dados são controversos devido a esta toxicidade depender da frequência e da quantidade empregada (SIVAKUMAR; HALAGOWDER, 2008).

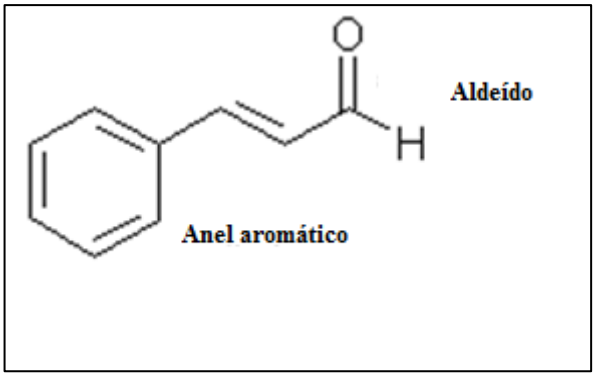


Figura 6: Estrutura molecular do cinamaldeído. (Adaptado de: CHAO et al., 2008).

Este componente possui uma gama diversa de propriedades biológicas já relatadas, como a capacidade de diminuir os níveis de glicose pós-prandial devido a uma sensibilização da insulina e recaptação da glicose, sendo uma abordagem para o tratamento de diabetes tipo 2 (WEIBEL; HANSEN, 1989; MACPHERSON et al., 2007; ZHANG et al., 2008; PLAISER, 2011). O CIN demonstrou ser um inibidor de cicloxigenase (COX-2) e um efetivo indutor de apoptose em células cancerígenas (WU et al., 2005). Alguns estudos destacaram os efeitos anti-inflamatórios do CIN, o qual interessantemente inibiu lipopolissacarídeo (LPS) induzido pela liberação de ROS. Também pode induzir a liberação de ROS de certas células tumorais (KA et al., 2003; CHAO et al., 2008). Este agente possui propriedades imunomodulatorias tais como indução da supressão da proliferação de células imunes e anti- LPS induzida pela atividade transcripcional do NF-κB, além de inibir a produção de citocinas tais como TNF-α, lL-6 e IL-1. (KOH et al., 1998; REDDY et al., 2001; LEE at al., 2002; LEE at al., 2005). Segundo Wen e col. (2002) o CIN pode ser um supressor de vários tipos de mediadores inflamatórios ambos em monócitos humanos THP-1, macrófagos J774A.1, bem como em modelos animas. Este estudo também encontrou que o CIN exerceu um melhor efeito inibitório na secreção de IL-1 do que na sua expressão (GUO et al., 2008). As propriedades anti-inflamatórias do CIN bem como sua ação antifúngica já são bem conhecidas. Assim, sabendo que AR requer uma complexa resposta pró-inflamatória para que ocorra a sua manutenção, as moléculas sinalizadoras, como os nucleotídeos extracelulares, estão envolvidos nestes eventos auxiliando na perpetuação da AR. Os nucleotídeos

de adenina, adenosina trifosfato (ATP), adenosina difosfato (ADP) e adenosina monofosfato (AMP) e seu derivado nucleosídeo adenosina, são secretados por leucócitos, plaquetas e células endoteliais danificadas e representam uma importante classe de moléculas extracelulares que desempenham um papel importante na modulação da resposta imune (ZIMMERMANN, 2000; BOURS et al., 2006). Estas moléculas interagem com receptores purinérgicos presentes na superfície celular e desencadeiam cascatas de eventos que modulam diversos efeitos biológicos (RALEVIC; BURNSTOCK, 2003).

O sistema purinérgico envolve três principais componentes: (1) nucleotídeos e nucleosídeos extracelulares, mediadores da sinalização; (2) receptores, através dos quais esses nucleotídeos e nucleosídeos exercem seus efeitos e, (3) as ectoenzimas, responsáveis pelo controle dos níveis extracelulares destas moléculas (Figura 7) (YEGUTKIN, 2008). Caracteriza-se 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; JUNGER, 2011).

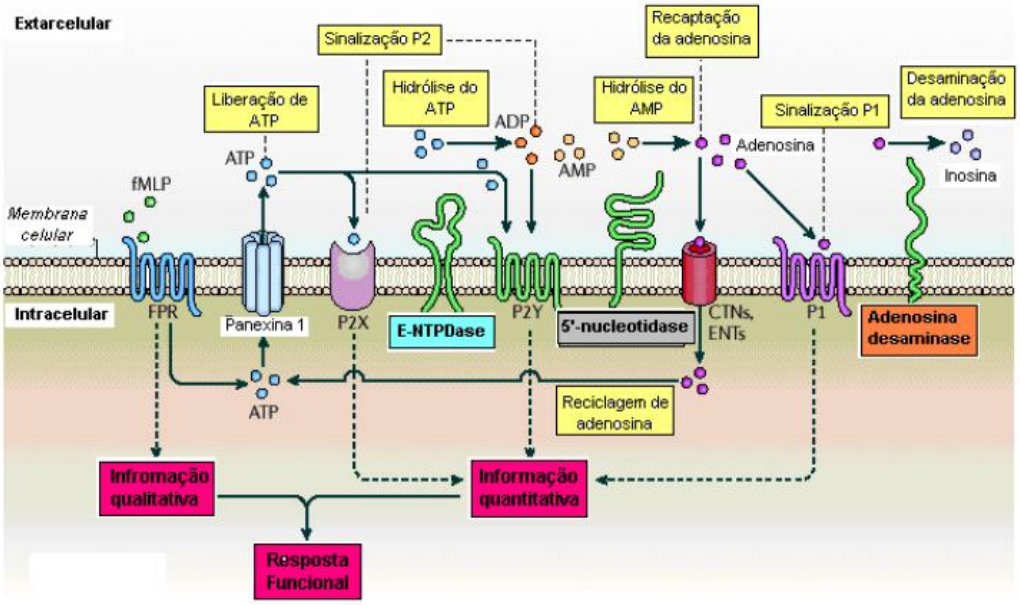


Figura 7: Componentes do sistema purinérgico (Adaptado de: Junger, 2011).

Os nucleotídeos da adenina tais como o ATP, ADP e AMP, e seu derivado nucleosídeo adenosina são liberados para o meio extracelular por células sanguíneas e vasculares, como eritrócitos, plaquetas, linfócitos e células endoteliais

(WOCHENSCHR, 1989; DUBYAK; EL-MOATASSIM, 1993), mas também podem ser liberados frente a um dano celular, nos sítios inflamatórios ou de estresse oxidativo, onde há um aumento da liberação de nucleotídeos. Já a adenosina pode ser liberada no meio extracelular como resultado da degradação do ATP e ADP por enzimas específicas (YEGUTKIN, 2008), ou através de transportadores na membrana das células que transportam a adenosina de dentro das células para o meio extracelular (BOROWIEC et al., 2008).

Em condições fisiológicas, os nucleotídeos são encontrados no meio extracelular em baixas concentrações (400-700nM) (DI VIRGILIO et al., 2001). Já em altas concentrações (3-10mM), podem atuar como uma molécula citotóxica e levar à morte celular, pela formação de poros na membrana plasmática (PODACK et al., 1985; YOUNG et al., 1986). Essas variações de concentração se dão devido a vários fatores como a quantidade de nucleotídeos liberada, os mecanismos de recaptação, situações de lise celular e a presença de enzimas como as ectonucleotidases (Figura 8) (BURNSTOCK, 2007).

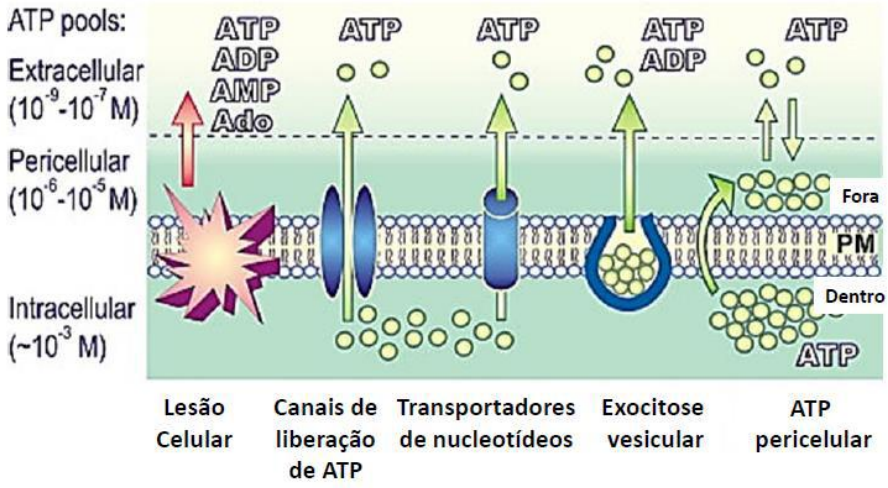


Figura 8: Caminhos da liberação de nucleotídeos por vias não líticas, incluindo movimento eletrodifusional através de canais de liberação de ATP, difusão facilitada por transportadores específicos de nucleotídeo e exocitose vesicular e ainda a segregação preferencial de concentração micromolar de ATP no espaço pericelular (Adaptado de: YEGUTKIN, 2008).

O ATP extracelular possui diversas funções fisiológicas, como: neurotransmissão, inibição da agregação plaquetária, contração do músculo liso,

inflamação e dor. Durante o desenvolvimento do processo inflamatório, este relacionado com a secreção de citocinas pró-inflamatórias (INF-γ, IL-12 e TNF-α) e a liberação de histaminas por mastócitos, provocando a produção de prostaglandinas (RALEVIC; BURNSTOCK, 1998; LANGSTON et al., 2003). O nucleotídeo ADP é o produto gerado na hidrólise do ATP e não possui um papel definido nos linfócitos (DI VIRGILIO et al., 2001), sendo conhecido por induzir a agregação plaquetária, alterar a forma das plaquetas, aumentar o cálcio citosólico e inibir a adenilato ciclase ativada (PARK; HOURANI, 1999). 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, também como substrato para a formação da adenosina (LATINI; PEDATA, 2001). Já a adenosina, a qual é formada a partir do precursor ATP nos espaços intra e extracelulares (BARSOTTI; IPATA, 2004) desempenha um papel importante como agente anti-inflamatório endógeno (CRONSTEIN, 1994), vasodilatadora, neuroprotetora (JACOBSON et al., 2006) e imunossupressora (SPYCHALA et al., 1997), através da inibição da liberação de citocinas, da adesão de células imune e do funcionamento de linfócitos citotóxicos (CRONSTEIN et al., 1983). A adenosina também atua como um potente inibidor da agregação plaquetária (BOROWIEC et al., 2006).

Os nucleotídeos da adenina e o nucleosídeo adenosina realizam suas ações biológicas através da ativação de receptores específicos presentes na superfície celular, denominados receptores purinérgicos (DI VIRGÍLIO et al., 2001). Os receptores purinérgicos se dividem em duas famílias, P1 e P2, presentes na superfície de diversas células cujos membros são ativados pela adenosina e por ATP e ADP respectivamente (BURNSTOCK, 2007).

Os purinoreceptores P2 podem ainda ser divididos em duas subclasses: acoplados à proteína G (metabotrópicos), chamados de P2Y e os ligados a canais iônicos, designados P2X, que são específicos para o ATP (DI VIRGÍLIO et al., 2001). Em mamíferos já foram identificados oito subtipos de receptores P2Y (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 e P2Y14), sete P2X (P2X1-7) e quatro subtipos de receptores P1 (A1, A2A, A2B e A3) que foram clonados e caracterizados farmacologicamente (RALEVIC; BURNSTOCK, 1998).

Os receptores P1 reconhecem a adenosina e também são metabotrópicos (BURNSTOCK, 2007). Os receptores subtipos A2A e A2B estão acoplados a proteínas estimulatórias G (Gs) e tipicamente suprimem as respostas celulares por

aumentar os níveis de AMPc intracelulares. Enquanto, os receptores subtipos A1 e A3 estão acoplados a proteínas Gi/0 ou Gq/11 e promovem a ativação celular (JUNGER, 2011).

O controle dos níveis extracelulares dos nucleotídeos da adenina e 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). Os nucleotídeos após desempenhar suas funções orgânicas, devem ser degradados de modo a manter seus níveis extracelulares em concentrações fisiológicas. Para isto, existe este sistema responsável pelo controle dos seus níveis extracelulares que é realizado por uma variedade de enzimas ancoradas à superfície celular ou localizadas no meio intersticial de forma solúvel, sendo conhecidas como ectonucleotidases (ZIMMERMANN et al., 2007).

As ectonucleotidases são ectoenzimas responsáveis pela hidrólise dos nucleotídeos da adenina (ATP, ADP e AMP) e incluem diversos membros das seguintes famílias: Ecto-nucleosídeo trifosfato difosfohidrolase (E-NTPDases), Ecto-nucleotídeo pirofosfatases/ fosfodiesterases (E-NPPs), Fosfatase Alcalina e Ecto-5'-nucleotidase (Figura 9). Outra ectoenzima também importante no metabolismo purinérgico é a adenosina desaminase (E-ADA), responsável pela desaminação do nucleosídeo adenosina (ZIMMERMANN, 2001; ZIMMERMANN et al., 2012). O conjunto de ações destas enzimas forma uma cadeia enzimática que tem início com a ação da E-NTPDase e da E-NPP, as quais catalisam a hidrólise do ATP e ADP formando AMP (ZIMMERMANN et al., 2007). A seguir a enzima E-5'-nucleotidase hidrolisa a molécula do AMP formando adenosina, a qual posteriormente é degradada pela ação da ADA gerando inosina (Figura 10) (YEGUTKIN, 2008).

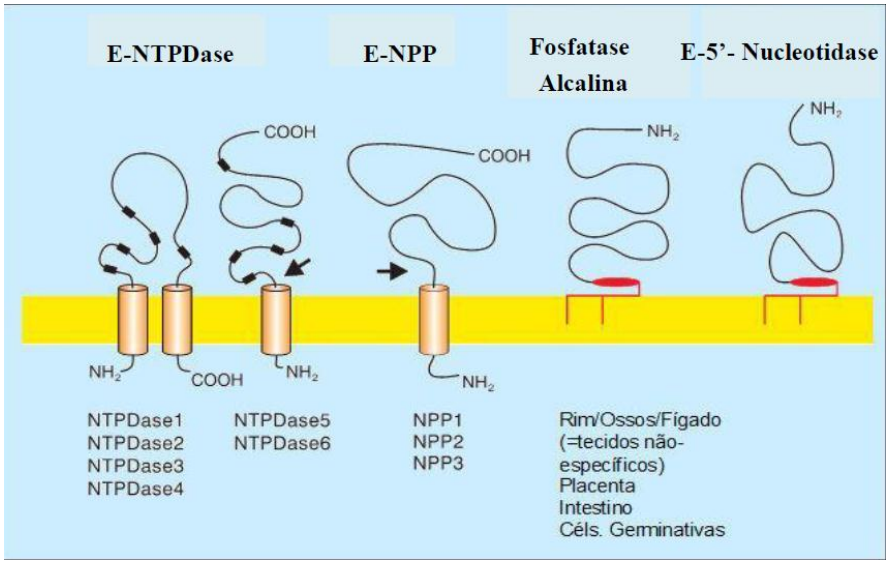


Figura 9: Estrutura das NTPDases (Adaptado de ZIMMERMANN, 2001).

As NTPDases (CD39; E.C 3.6.1.5) são uma família de enzimas responsáveis pela hidrólise de nucleotídeos di e trifosfatados a seus monofosfonucleotídeos correspondentes (ZIMMERMANN et al., 2007). As enzimas da família das NTPDases são expressas pelos genes Entpd, sendo que oito membros desta família já foram identificados e diferem quanto a especificidade de substratos, distribuição tecidual e localização celular (BIGONNESSE et al., 2004; SHI et al., 2001; ZIMMERMANN, 2001).

Quatro destes membros estão localizados na membrana celular com o sítio catalítico voltado para o meio extracelular (NTPDase 1, 2, 3, 8) e requerem $Ca^{2+}$ ou $Mg^{2+}$ para sua máxima atividade, sendo inativas na ausência destes cátions; e quatro exibem uma localização intracelular (NTPDase 4,5,6,7) (ZIMMERMANN, 2001; KUKULSKI et al., 2005; ROBSON et al., 2006).

A primeira NTPDase identificada foi a NTPDase-1, como proteína CD39, que está ancorada à membrana via dois domínios transmembrana e que hidrolisa os nucleotídeos ATP e ADP em proporções semelhantes (ZIMMERMANN, 2001). A NTPDase1 é um marcador de ativação de linfócitos, sendo também expressa em células natural killer, monócitos, células dendríticas e em um subconjunto de células T ativadas. Através da modulação da sinalização purinérgica a enzima desempenha um papel importante no controle da resposta imune celular (MIZUMOTO et al., 2002; ROBSON et al., 2006; DEAGLIO et al., 2007; DWYER et al., 2007). A NTPDase-2 é associada ao sistema nervoso central e periférico. A NTPDase-3 é associada com

estruturas neuronais, agindo na regulação dos níveis de ATP pré-sinápticos (YEGUTKIN, 2008). Já as NTPDases 4, 5, 6 e 7 estão localizadas no meio intracelular (ZIMMERMANN, 2001).

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 (SPANEVELLO et al., 2010), o infarto agudo do miocárdio (BAGATINI et al., 2008), e na síndrome da imunodeficiência adquirida (AIDS) (LEAL et al., 2005). A atividade da enzima também se encontra alterada em pacientes com hipercolesterolemia e processo inflamatório, onde a hidrólise do ATP e do ADP se encontra aumentada em plaquetas, assim como a expressão da CD39 na superfície da célula. Já em pacientes com AR, a atividade da E-NTPDase se encontra aumentada tanto em linfócitos como em plaquetas (BECKER et al., 2010; JAQUES et al., 2012).

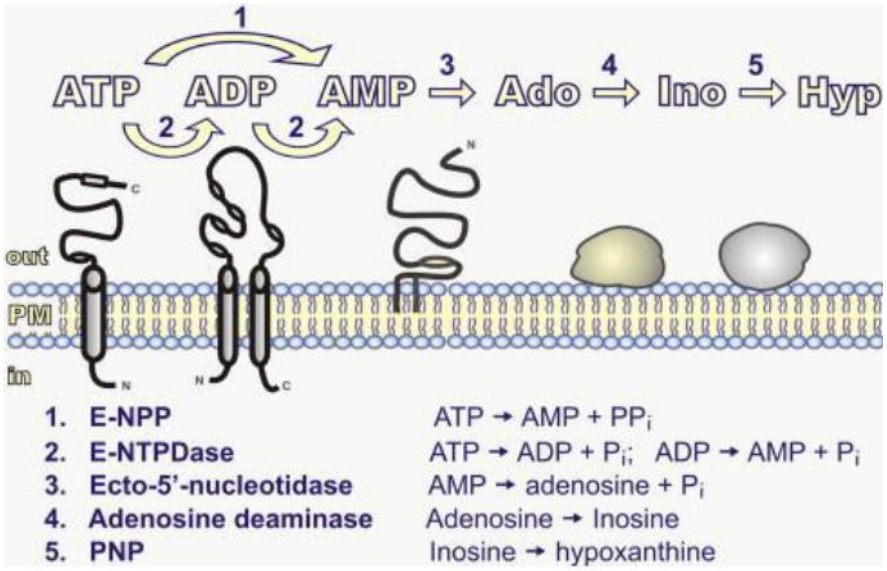


Figura 10: Ectoenzimas envolvidas na degradação de nucleotídeos e nucleosídeos (Adaptado YEGUTKIN, 2008).

Após a hidrólise do ATP e ADP pela E-NTPDase, a enzima ecto-5'-nucleotidase (E-5´-NT, CD73, E.C. 3.1.3.5) é responsável pela desfosforilação de ribo- e desoxiribonucleossídeos 5' monofosfatados como AMP, CMP, UMP, IMP e GMP, porém com uma maior afinidade pelo AMP, sendo por isto considerada a principal enzima responsável pela formação de adenosina (ZIMMERMANN et al.,

2012). A enzima adenosina desaminase (Figura 11) (ADA, E.C. 3.5.4.4) também faz parte do conjunto de enzimas responsáveis pela degradação sequencial dos nucleotídeos e nucleosídeos da adenina (YEGUTKIN, 2008). A E-ADA é responsável pela desaminação irreversível da adenosina e 2‘-deoxiadenosina em inosina e 2‘-deoxinosina, respectivamente (RESTA et al., 1998; ROBSON et al., 2006). A primeira proteína de superfície celular capaz de ancorar a ecto-ADA à membrana plasmática foi identificada como CD26 por Kameoka e cols. (1993), a qual se tornou conhecida como um marcador molecular de ativação de células T, pois quando estas células estão ativadas o nível de expressão da CD26 aumenta (FOX et al., 1984; FRANCO et al., 1997).

A E-ADA é uma enzima essencial para a proliferação e diferenciação dos linfócitos e monócito-macrófago no sistema imune, sendo usada para monitorar várias patologias imunológicas (HITOGLU et al., 2001; POURSHARIFI et al., 2008). Esta enzima é encontrada praticamente em todos os vertebrados. Em humanos existe na forma de duas isoenzimas classificadas como ADA1 e ADA2, cada uma com suas próprias características, como peso molecular, propriedades cinéticas e distribuição tecidual (SHAROYAN et al., 2006).

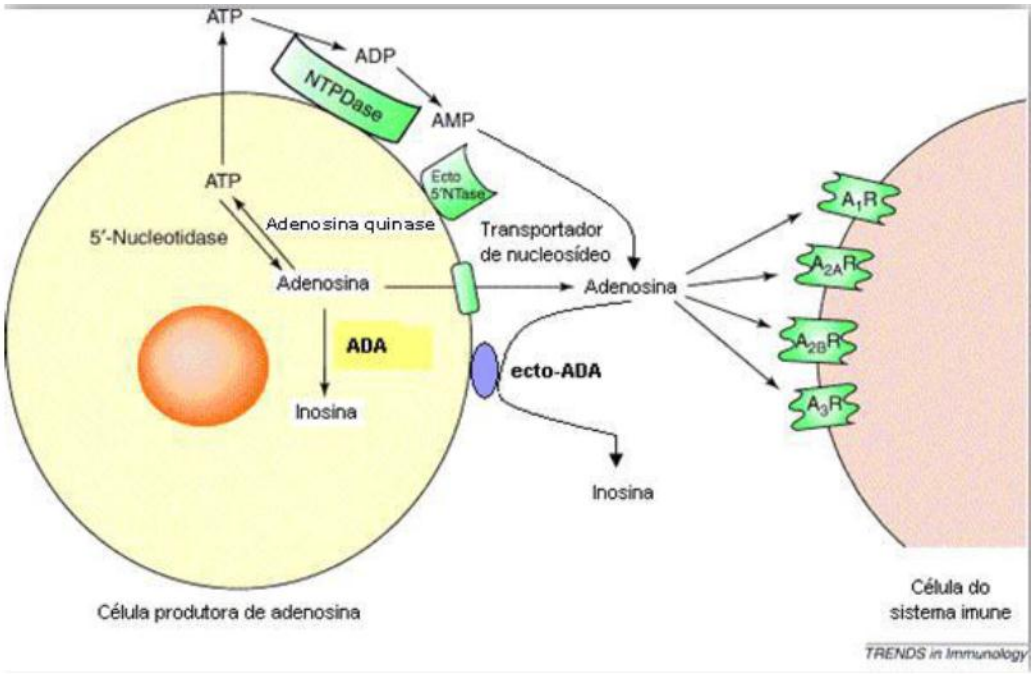


Figura 11: Vias envolvidas no metabolismo da Adenosina (Adaptado de HASKÓ; CROSTEIN, 2004).

A ADA1 está presente em todos os tecidos humanos, apresentando alta atividade em linfócitos e monócitos, e é responsável por grande parte do desaparecimento da adenosina circulante nesse meio (TSUBOI et al., 1995).

Aparentemente não existem diferenças, tanto catalíticas quanto moleculares, entre a enzima presente no citosol e a ecto-ADA (FRANCO et al., 1997). A ADA2 é a isoenzima predominante no soro e representa a menor parte da atividade da ADA total em tecidos (ZUKKERMAN et al., 1980). Diferentemente da ADA1, a ADA2 apresenta diferenças tanto estruturais quanto cinéticas e é encontrada predominantemente no soro de indivíduos normais (UNGERER et al., 1992). Dados recentes têm sugerido que ADA2 no plasma humano pode ser secretada por monócitos ativados em processos inflamatórios, tendo a habilidade de regular a proliferação celular (IWAKI-EGAWA et al., 2006). A adenosina é liberada pelas células dependendo da sua concentração intracelular ou pode ser proveniente da degradação do ATP extracelular devido à ação das ectonucleotidases. O controle da sinalização adenosinérgica também pode ser exercido através da via de recuperação de adenosina por transportadores de nucleosídeos, seguida por fosforilação à AMP pela adenosina quinase ou desaminação à inosina pela ADA (HASKÓ; CRONSTEIN, 2004).

Além de possuírem importante atividade na regulação dos níveis de nucleotídeos e nucleosídeos da adenina, as ectoenzimas possuem ações extremamente importantes no sistema imunológico (SALAZAR-GONZALEZ et al., 1985; BENREZZAK et al., 1999). Enzimas como a E-NTPDase e a E-ADA, estão presentes na membrana dos linfócitos desempenhando um importante papel na resposta inflamatória. As respostas imunes pró-inflamatórias desencadeadas pela AR são moduladas por nucleotídeos e nucleosídeos, que se correlacionam diretamente com a atividade das ecto-nucleotidases. Uma vez que o CIN por atuar como um potente modulador do sistema imune com propriedades anti-inflamatórias, torna-se relevante e de interesse científico a investigação do seu efeito na atividade da E-NTPDase e da E-ADA em linfócitos de ratos com artrite induzida por adjuvante. Desta forma, espera-se contribuir para a busca de novas terapias complementares que possam beneficiar pacientes com AR.

# 2 OBJETIVOS

## 2.1 Objetivo geral

Avaliar o efeito do cinamaldeído no metabolismo de nucleotídeos e nucleosídeo de adenina em modelo de artrite por adjuvante.

## 2.2 Objetivos específicos

Em ratos com artrite induzida por adjuvante tratados com cinamaldeído:

- Avaliar score de artrite, edema de pata e hiperalgesia termal;
- Avaliar perfil histológico nas patas;
- Avaliar a atividade das enzimas de dano hepático alanina aminotransferase (ALT), a aspartato aminotransferase (AST) em soro e índices hematológicos;
- Avaliar a atividade das enzimas E-NTPDase em linfócitos;
- Avaliar a atividade da enzima E-ADA em linfócitos.

# 3 MANUSCRITO

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de um manuscrito. Os itens Materiais e métodos, Resultados e Discussão e Referências bibliográficas encontram-se compondo o próprio manuscrito e representam a íntegra deste estudo.

O manuscrito será submetido à revista **Food and Chemical toxicology**.

**MANUSCRITO**

# E-NTPDase AND E-ADA ACTIVITIES IN LYMPHOCYTES FROM ADJUVANT-INDUCED ARTHRITIC RATS AND TREATED WITH CINNAMALDEHYDE

**Maria Luiza Thorstenberg[a], João F. P. Rezer[a], Lívia G. Castilhos[a], Karine L. Silveira[a], Cláudia M. Bertoncheli[a,b], Pedro Henrique Doleski[a], Nara Maria B. Martins[b], Mateus F. Rossato[c], Kássia Bagolin[d], Jamile F. Gonçalves[a], Juliano Ferreira[c], Sônia Terezinha A. Lopes[d], Daniela B. R. Leal[a]**

[a] Centro de Ciências da Saúde, Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900, Santa Maria, RS, Brasil.

[b] Centro de Ciências da Saúde, Departamento de Patologia, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900, Santa Maria, RS, Brasil.

[c] Centro de Ciências Naturais e Exatas, Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Santa Maria, Campus Universtário, Camobi, 97105-900, Santa Maria, RS, Brasil.

[d] Centro de Ciências Rurais, Departamento de Pequenos Animais, Universidade Federal de Santa Maria, Campus Universitário, Camobi, 97105-900, Santa Maria, RS, Brasil.

thorstenbergml@gmail.com
joaofeliperezer@gmail.com
liviagelain@gmail.com
kah_ls@yahoo.com.br
c_bertoncheli@yahoo.com.br
ph_doleski@hotmail.com
nbmartins@via-rs.net
mateusrossato@gmail.com
cassiabagolin@hotmail.com
jamilefabbrin@yahoo.com.br
ferreiraj99@gmail.com
soniatldosanjos@hotmail.com

*** Corresponding author:**

Daniela Bitencourt Rosa Leal
Fax: + 55-55322-08242
Phone: + 55 55 3220 9581
Departamento de Microbiologia e Parasitologia, Centro de Ciências da Saúde,
Universidade Federal de Santa Maria, Av. Roraima, 1000, prédio 20, 97105-900.
Santa Maria, RS, Brazil
e-mail: dbitencourtrosaleal@gmail.com

**Abstract**

Cinnamaldehyde (CIN) occurs naturally in the genus *Cinnamomum* and has long been used as an anti-mutagenic, anti-oxidative and anti-microbial agent in tradicional medicine. Furthermore, CIN has been shown to inhibit the production of pro-inflammatory cytokines making it a potent drug against inflammatory and autoimmune diseases. The present study was undertaken to assess the plausible effect of CIN against chronic arthritis in Wistar rats by evaluation the E-NTPDases and E-ADA activities in lymphocytes. For this, chronic arthritis was stimulated by a subcutaneous injection of Complete Freund's Adjuvant (CFA) into the hind paw of rats and CIN was administered to the rats by gavage at concentration of 2,1%. The paw volume was measured using the digital caliper and, hyperalgesia was evaluated by ankle flexion. The ankle joints were isolated and examined by histological analysis. Hematological parameters and markers of liver injury were measured among the groups. Moreover, the lymphocytes were isolated from whole blood to assay the E-NTPDase and E-ADA activities. The results of the present study showed that CIN did not change the arthritis score and paw edema but reduced the thermal hyperalgesia. Also, this compound was able in slight reduced cell infiltration in CFA-induced arthritis compared to the control. The hematological parameters as well as the markers of liver injury were not statistically significant between both groups. The E-NTPDase activities (both ATP and ADP as substrate) and E-ADA in lymphocytes were increased in adjuvante-induced arthritic rats as compared to the control. In arthritic rats treated with CIN it was observed a decreased E-NTPDase and E- ADA in relation to control Moreover, CIN did not alter neither E-NTPDase nor E-ADA activity in healthy animals. This study thus provided evidence that CIN might be an effective and promising agent to supplement the treatment of chronic arthritis. However, detailed molecular mechanisms of CIN action need to be estudied in order to confirm these protective functions.

*Keywords:* Cinnamaldehyde; CFA, E-ADA; E-NTPDase; Lymphocytes; Inflammation

## 1. Introduction

Rheumatoid arthritis (RA) is a progressive, disabling and chronic multisystem disease that is characterized by pain, swelling and stiffness of the synovial joints. The exact etiology of this debilitating disease is not known, but it is believed to be the result of an autoimmune response of the body which can be triggered by a variety of genetic and enviromental factors, including microbial infection (McInnes & Schett, 2011).

In recent years, animal models of arthritis contributed to the better understanding of the RA etiology and pathogenesis (Vicent et al., 2012). Numerous animal models for arthritis have been studied. Many of these have been used to identify therapeutics targets for the development of new therapies, like model adjuvant-induced arthritis (Kollias et al., 2011). This model is induced by a single intradermal injection of complete Freund's adjuvant (CFA). It is characterized by a rapid progression of a polyarticular inflammation, with marked bone resorption and periosteal bone proliferation. Histological analysis allows the identification of infiltrating cells, particularly neutrophils, and joint destruction. Adjuvant-induced arthritis is a T cell-dependent disease, which shares some characteristics with human RA, including swelling of the extremities, cartilage degradation, loss of joint function and lymphocyte infiltration the joints (Hegen et al., 2008).

Due to non-responsiveness and to toxicity of some therapies, currently there is a resurgence of interest in safe herbal medicines in all the countries as alternative sources of drugs for incurable diseases, such as RA (Kang et al., 2006, Lee et al., 2008). Cinnamaldehyde (CIN) is a constituent of the essential oil obtained from the bark of *Cinnamomum* tree. CIN is the main bioactive compound isolated from the leaves of *Cinnamomum* and it is widely used as flavoring agent in beverages, ice-creams, sweets and condiments (NTP, 1993). Furthermore, CIN is an α,β-unsaturated carbonyl derivative with a mono-substituted benzene ring (Reddy et al., 2004) thus previous biological studies have demonstrated that CIN has shown anti-bacterial activities,

anti-tumourigenic effects (Ka et al., 2003), immunomodulatory (Chao et al., 2005) and anti-fungal effects (Subash et al., 2007) as well as effects on hyperglycemia, angiogenisis, tumourignenisis (Kwon et al., 1997.; Lee et al., 1999).

In RA, the synovium becomes infiltrated with chronic inflammatory cells, the residente fibroblastos secretion of cytokines, chemokines and enzymes that reinforce the inflammation and catalyse joint destruction. The resulting pannus acquires the ability to invade and destroy adjacente articular cartilage (Chang et al., 2010). Inflammation is a key player in the pathophisology of arthritis and the purinergic signalling system plays an important role in modulating the inflammatory and immune response through adenosine nucleotides (ATP, ADP and AMP) and their derived adenosine. These molecules are important in the mediation of many biological and pathological events (Bours et al., 2006) and are dynamically controlled during inflammation by ecto-nucleotidase thiphosphate diphosphohydrolase (E-NTPDase; CD39; EC 3.6.1.5) and ecto-adenosine deaminase (E-ADA; EC 3.5.4.4), which are anchored in the cellular surface of immune cells (Bours et al., 2006).

Considering that RA is characterized by pro-inflammatory disease and the involvement of purinergic system as such the ectonucletidases, that have fundamental biological in the proliferation and modulation of immunes cells and these events, the purpose of this study was to investigate the activity of E-NTPDase and E-ADA in lymphocytes from CFA-induced arthritis treated with CIN, to achieve better comprehension of their immune status.

## 2. Materials and methods

### *2.1 Chemicals*

Cinnamaldehyde (CIN), adenosine 5’- triphosphate disodium salt (ATP), adenosine 5’-diphosphate sodium salt (ADP), adenosine, bovine serum albumin, Trizma base, and Coomassie Brilliant Blue G were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ficoll-

Hypaque (Lymphoprep) was obtained from Nycomed Pharma (Oslo, Norway). All the chemicals used in this experiment were of analytical grade and of the highest purity.

*2.2 Animals*

Twenty adult female Wistar rats (200-300g) from the Central Animal House of the Universidade Federal de Santa Maria (UFSM) were used in this experiment. Animals were kept on a 12-h light/12-h dark cycle, at a temperature of 22±2$^{o}$C, with free access to food and water. The animals were used according to the guidelines of the Committee on Brazilian Society of Animal Science Lab (SBCAL, 2009) in accordance with international guidelines and were approved by the Committee on the Use and Care of Laboratory Animals of our University (n. 042.2013).

*2.3 Chronic immunological CFA-induced arthritis in rats*

To investigate of CIN anti-inflammatory effect, a CFA-induced arthritis model was used. Animals were slightly anesthetized with isoflurane and 100 μL of Complete Freund's Adjuvant (CFA - 0.6% suspension of heat-killed *Mycobacterium tuberculosis* in liquid paraffin) or saline (used as control) was injected into the right hind paw (Sauzem et al., 2009). After fourteen days later, the measurement of inflammatory and nociceptive parameters (arthritis score, see bellow) was assessed to confirm de development of inflammatory process.

*2.4 Experimental procedure*

The treatment of animals with CIN began 15 days after induction of arthritis by CFA. The animals were randomly divided into four groups (n=4 per group): control (C); cinnamaldehyde (CIN); arthritis (AR); and arthritis associated with CIN (AR+CIN). CIN was administered at 2.1% by gavage daily during 15 days (Yoon- Young et al, 2011). CIN was freshly prepared in

corn oil and administered (1mL/Kg) between 9 and 11 a.m. After the treatment period, the animals were anesthetized and submitted to euthanasia. The total blood was collected by cardiac puncture to separate the lymphocytes. The ankle joint tissues were separated for histopathological analysis.

*2.5 Evidences of arthritis induction*

*2.5.1 Arthritis score*

To evaluate the progression of the arthritic response elicited by intraplantar CFA injection, animals were observed daily, before administration. The following sings of inflammation were observed and classified according the scale: edema formation (0 – normal; 1 – slightly slowed in the point of injection; 2 – slowed in the point of the injection and toe or ankle; 3 – slowed in the point of injection, toes and ankle), redness (0 – normal; 1 – slightly red/purple; 2 – red/purple) and claw position (0 – normal; 1 – slightly curved; 2 – almost closed). The sum of the different score were made and considered as total arthritis score (Simjee et al., 2007; Gemeinhardt et al., 2011).

*2.5.2 Paw edema*

To observe the development of edema, animals were held and the right hind paw thickness was measured using a digital caliper (Cao et al., 1998). Fourteen days later after the induction of inflammation, and before each CIN administration, new measurements were taken and compared to basal values.

*2.5.3 Thermal hyperalgesia*

To evaluate the hypersensitivity to heat stimulation, we used the paw immersion test, according to Dalmolin et al. (2007). Briefly, animals were held and the right hind paw was immersed in a

water bath at 48°C. The time elapsed between onset of the stimulus and manifestation of the paw withdrawal response was measured automatically and was taken as an index of the thermal nociceptive threshold. Significant decreases of paw withdrawal latency were interpreted as indicative of heat hyperalgesia.

*2.6 Histopathological observation*

Samples of ankle joints right were collected and fixed in 10% formalin solution and then dehydrated and embedded in paraffin, followed by sectioning and histological staining with hematoxylin and eosin (H&E). The slides were observed in optical microscope (400x) to evaluate a possible damage.

*2.7 Liver damage markers*

Aspartate transaminase (AST), alanine transaminase (ALT), were evaluated in a semi-automatic analyzer (TP Analyzer Plus®, Thermoplate) using commercial kits (Labtest® Diagnóstica S.A.). Tests were carried out in duplicate.

*2.8 Hematological parameters*

A complete hemogram was performed in the blood samples collected in the tubes containing 7.2 mg dipotassium EDTA as an anticoagulant and the quantitative determination the hematological parameters was performed by automated haematology analyzer (SYSMEX XT-1800i, Roche Diagnostic, USA).

*2.9 Isolation of lymphocytes from blood*

Lymphocytes-rich mononuclear cells were isolated from peripheral blood collected with 7.2 mg dipotassium EDTA as anticoagulant and separated on Ficoll-Histopaque density gradients as

described by Böyum (1968). The percentage of lymphocytes was superior to 93% as previously described by our research group Jacques et al. (2011). The integrity of the lymphocytes preparation was confirmed by determining the lactate desydrogenase (LDH) activity in intact and disrupted lymphocytes using the Kinetic method of the Labquest apparatus (Diagnostics Gold Analyzer). The procedure was performed before and after the incubation period. Samples with more than 10% of disrupted cells were excluded.

*2.10 Protein Determination*

Protein was measured by the Comassie Blue method according to Bradford (1976) using serum albumin as standard.

*2.11 E-NTPDase enzyme assays*

NTPDase activity in lymphocytes was determined as described by Leal et al. (2005a). This method is based on the measurement of inorganic phosphate (Pi) released by colorimetric assay. The reaction medium contained 0.5 mM $CaCl_2$, 120 mM NaCl, 5 mM KCl, 60 mM glucose and 50 mM Tris-HCl buffer at pH 8.0, in final volume of 200 µL. Twenty microliters of intact lymphocytes suspended in saline solution were added to the reaction medium (2-4 µg protein) and pre-incubated for 10 min a 37 °C. The reaction was started by the addition of substrate (ATP or ADP) at a final concentration of 2 mM and stopped with 200 µL 10% trichloroacetic acid (TCA) to provide a final concentration of 5%. The incubation proceeded for 70 min and the released Pi was assayed by the method of Chan et.al. (1986) using malachite green as colorimetric reagent and $H_2PO_4$ 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 triplicate and specific activity reported as nmol Pi released/mim/mg of protein.

*2.12 E-ADA enzyme assay*

ADA activity was measured in lymphocytes by the method of Giusti and Galanti (1984), which is based on the direct measurement of the formation of ammonia produced, when ADA acts in excess of adenosine. Briefly, 25 µL of lymphocytes reacted with 21 mM of the substrate (adenosine), pH 6.5, and incubation was carried out for 1h at 37 °C. The reaction was stopped by adding 106.2 mM phenol and 167.8 nM sodium nitroprissiate and hypochlorite solution. Ammonium sulfate (75 µM) was used as ammonium standard. All the experiments were performed in triplicate and the values were expressed in U/Lfor ADA activity. One unit (1U) of ADA is defined as the amount of enzymes required to release 1 mmol of ammonia per minute from adenosine at standard assay conditions.

*2.13 Statistical analyses*

Data from enzyme assays, hematological parameters and markers of liver injury were submitted to analysis of variance one-way (ANOVA) followed by the Tukey's test. The results of evidences of arthritis induction were evaluated by analysis of variance two-way (ANOVA) followed by the Bonferoni's test. $P<0.05$ was considered to represent a significant difference among the analyses used. All data were expressed as mean ± standard error of the mean (SEM).

## 3. Results

*3.1 Evidences of arthritis induction and effects of CIN*

To investigate the possible anti-inflammatory effect of repeated CIN administration for 14 days, we evaluated the development of arthritis score, paw edema and thermal hyperalgesia (Figure 1). We observed that CFA injection was capable to induce an increase in arthritis score and paw edema, and develop thermal hyperalgesia. While it, CIN treatment for 14 days in CFA-induced arthritis animals no change in both arthritis score (Figure 1A) and paw edema (Figure 1B), but

induced an inhibition of 60.7 ± 5.4% in the thermal hyperalgesia (Figure 1C). CIN treatment in saline injected animals induced no behavioral alteration.

*3.2 Hystological analysis*

Ankle joint section of control group and control group treated with CIN for 14 days showed organized collagen and absence of inflammatory infiltrate (Figure A e B). The CFA- induced arthritis group presented organized collagen with in the presence the inflammatory lymphocytic infiltrate and neutrophil with some giant cells (Figure C), while CFA- induced arthritis group treated with CIN for 14 days showed separation of collagen fibers in some fields but organized together with lymphocytic inflammatory infiltrate (Figure D).

*3.3 Liver damage markers*

The activities of the hepatic enzymes AST and ALT in rat serum did not differ among the different groups (Table 1).

*3.4 Hematologial parameters*

The hematological parameters as well RBC, hemoglobin, MCV, MCHC, WBC, lymphocytes, neutrophils, monocytes, eosinophils and platelets showed no difference among the groups (Table 2).

*3.5 Cellular integrity*

LDH activity measurement showed that approximately 5% of the lymphocytes of both groups was disrupted, indicating that the preparation was predominantly intact after the isolation procedure (data not showed).

*3.6 E-NTPDase and E-ADA activity in lymphocytes before and after (15 days) of arthritis induction*

Figure 3 shows the E-NTPDase and E-ADA activities in lymphocytes of rats before and after 15 days of arthritis induction. As can be observed, E-NTPDase activity with ATP as substrate was altered in CFA- induced arthritis rats (126.24 nmol of Pi/min/mg of protein; SEM=15.41, n=4; $P<0.05$), demonstrating that ATP hydrolysis was increased in 231% when compared to the control group (38.09 nmol of Pi/min/mg of protein; SEM=10.52, n=4; $P<0.05$) (Figure 3A). The same behaviour was observed in ADP hydrolysis for CFA- induced arthritis group (122.48 nmol of Pi/min/mg of protein; SEM=18.73, n=4; $P<0.05$) when compared to control group (60.66 nmol of Pi/min/mg of protein; SEM=8.17; n=4; $P<0.05$) showing an increase of 101% (Figure 3B). The groups showed no significant alterations in the E-ADA activity when adenosine was used as substrate (Figure 3C).

*3.7 E-NTPDase and E-ADA activity in lymphocytes with 14 days of treatment with cinnamaldehyde*

The results obtained for ATP and ADP hydrolysis in lymphocytes of CFA- induced arthritis rats and treated with cinnamaldeyde 2.1% for 14 days are shown in Figure 4. ATP hydrolysis (Figure 4A) was increased in 94.14% in the AR group (111.687 nmol of Pi/min/mg of protein; SEM=16.41, n=4; $P<0.05$ ) when compared to control group (57.53 nmol pf Pi/mim/mg of protein; SEM=10.44, n=4; $P<0.05$), in 79.74% when compared to CIN group (72.137 nmol of Pi/min/mg of protein; SEM=2.96, n=4; $P<0.05$) and in 20.59% when compared to AR+CIN (69.38 nmol of Pi/min/mg of protein; SEM=4.81, n=4; $P<0.05$). The results showed enhanced ADP hydrolysis (Figure 4B) of 152.56% in the AR group (138.00 nmol of Pi/min/mg of protein; SEM=18.76, n=4; $P<0.05$) when compared to control group (54.64 nmol of Pi/min/mg

of protein; SEM=10.10, n=4; $P<0.05$), in 122.76 % when compared to CIN group (61.95 nmol of Pi/min/mg of protein; SEM=6.01, n=4; $P<0.05$), however the AR group showed no significant alterations in the ADP hydrolysis when compared to AR+CIN group (159.5 nmol of Pi/min/mg of protein; SEM=22.96, n=4; $P<0.05$). Results obtained for E-ADA activity in lymphocytes is show in (Figure 4C). We observed a increase of 151.84% in the AR group (98.83 U/L of ADA/mg of protein; SEM=2.61; n=4; $P<0.05$) when compared to control group (39.24 U/L of ADA/mg of protein; SEM=8.90; n=4; $P<0.05$), in 296.1% when compared to CIN group (24.95 U/L of ADA/mg of protein; SEM=1.64; n=4; $P<0.05$) and in 69.7% when compared to AR+CIN group (58.23 U/L of ADA/mg of protein; SEM=8.74; n=4; $P<0.05$).

## 3. Discussion

RA is a chronic, inflammatory and systemic autoimmune disease which is characterized by synovial inflammation and hyperalgesia (McInnes et al., 2011). Chemokine and enzymes reinforce the inflammation and catalyse joint destruction giving deformity, autoantibody production and systemic features, including cardiovascular, pulmonary and skeletal disordens (Walsh et al., 2010). It is a disease of global scope with a prevalence in women and being mediated by Th1, Th17 and Treg cells (Chabaud et al., 1998; Miossec et al., 2009). The exact etiology of this debilitating disease is not known, but it has been reported as a complex interaction between genotype and environmental factors (MacGregor et al., 2000).

Several animal models have been employed to research anti-arthritogenic agents. The Complete Freund's Adjuvant (CFA)-induced arthritis is a widely used model (Yang et al., 2010). In this model mycobacterial heat-shock protein 65 (MHPS65) of *Mycobacterium tuberculosis* triggers signals through receptors toll like 3 and 4 (TLR-3 and TLR-4) with production the pro-inflammatory cytokynes such as IL-1β, IL-6 and TNF-α, that are implicated in the pathogenesis of the arthritis induced by CFA (Bowdish et al., 2009). CFA has been

utilized to induce an arthritic immunopathological disease that displays many of the pathological features of human (Hegen et al., 2008).

In the immune synapse, the recognition of antigens by T lympchocytes happens through their T cell receptors (TCRs) which are associated with peptides presented on major histocompatibility complex (MHC) molecules by antigens-presenting cells (APCs) (Valitutii et al., 1995). It is believed that purinergic signalling in the immune synapse could serve as an amplification response for antigen recognition. A number of studies have shown that T lymphocytes are capable to release ATP in response to various extracellular stimuli as infections. Thus, purinergic signalling with ATP may be involved in migration and activation of T lymphocytes (Canady et al., 2002; Into et al., 2002). Moreover, T lymphocytes present ectoenzymes as E-NTPDase and E-ADA and also express many members receptor families such as P2X, P2Y and P1 on their surface (Di Virgilio et al.,2001; Wang et al.,2004).

In the present study, it was induced arthritis in rats using CFA and the inflammatory process was confirmed through the measurement of increased arthritis score, paw edema and thermal hyperalgesia which characterizes an arthritis process. Here, we verified the ability of cinnamaldehyde (CIN) to reverse this process. It was remarkable that CIN reversed the thermal hyperlgesia but did not change the arthritis score and paw edema. However, Jung-Chun et al. (2012) showed that cinamic aldehyde, a CIN metabolic, was effective to inhibit the development of paw edema induced by carrageenan (5 and 10 mg/Kg). Moreover, the histological parameters showed that CIN was able to reverse mild histological changes due to arthritis. Adjuvant-induced arthritis model has been showed to size areas of necrosis with the accumulation of inflammatory exudate on cartilage surfaces (Richard et al., 1985).

Here, AST and ALT activities in rat serum and the hematological parameters showed no difference among the groups. However, according to Sivakumar et al. (2008) CIN (500mg/Kg) changed ALT and AST when used for 90 days nevertheless we not found alterations in hepatic

parameters thus suggesting that CIN was safer when used in this time and concentration. Besides, Banji et al. (2011) reported decreased hemoglobin concentration and increased number of platelets in arthritic rats.

In addition, it is well known that pro-inflammatory cytokines such as tumour necrosis fator (TNF-α), interleukin-1 (IL-1), interleukin-6 (IL-6) and auto reative T lymphocytes have significant involvement in the RA pathogenisis (Kokkonen et al., 2010). Furthermore, the synovial fluid are enriched for imune cells such as neutrophil, macrophages, dendritic cells and T lymphocytes, which contribute to immunostimulatory profile of this pathology (Feldmann et al, 1996). Levels of purine and pyrimidine nucleotides are significant increased on the involved sites contributing to the amplification of the inflammatory reactions and favoring the reactives process (Miyara and Sakaguchi, 2007). Extracellular ATP can act as a damage-associated molecular patterns (DAMPs), when released at high local levels in response to damage, infection or other inflammatory stimuli interacting with specific receptors on cellular surface (La Sala et al., 2003., Elliot et al., 2009)

The results of this study showed an increase in the E-NTPDase lymphocyte activity (ATP and ADP as substrate) after 14 days of CFA-induced arthritis as well as after 14 days of treatment period when compared to control. The possible association between NTPDase activity and immune diseases has been evaluated considering that this enzyme activity could be used as an activation marker of lymphocytes during the immune response (Leal et al., 2005b; Jaques et al., 2012). In fact, recently, our research group reported an increased E-NTPDase activity in lymphocytes of patients with arthritis (Jaques et al., 2012). Once released to the extracellular medium, ATP activates the pro-inflammatory purinergic receptors P2X1, P2X4 and P2X7 that contributes to inflammation and migration of immune cells to the inflammatory foci and an increased production of pro-inflammatory mediators (Bours et al., 2006; Woehrle et al., 2010). Thus, the increased activity of E-NTPDase indicates that hydrolysis the ATP and

ADP are highest as a dynamics response of lymphocytes in an attempt to the maintenance their appropriate levels (Jaques et al., 2012).

In addition, here we demonstrated that the groups showed no significant alterations in the E-ADA activity after 14 days of CFA-induced arthritis. However, ADA activity was increased in arthritic rats after 14 days of treatment period when compared to control. Thus, the increase in ADA activity could be responsible for the possible decreased adenosine concentration in extracellular medium. It is important to note that adenosine, ADA substrate, exhibits potent anti-inflammatory and immunosuppressive action by inhibiting the proliferation of T cells, the secretion of cytokines and the migration of leukocytes across endothelial barriers (Kobie et al., 2006; Thompson et al., 2008).

Furthermore, other studies reported that the E-NTPDase and E-ADA have important implications in immune response once alteration in their activities have been observed in cancer lung (Zanini et al., 2012), HIV (Leal, 2005b) and lupus (Loza et al., 2011). Knowing that these enzymes act in a cascade, the present data suggest that increased E-NTPDases and E-ADA activity in CFA-induced arthritis rats could cause co-stimulatory signalling in the immune synapse, resulting in increased proliferation of T helper 1 (TH1)- type cytokines (Franco et al., 2007; Zavialov et al., 2010; Gessi et al., 2011). The E-ADA activity represent the real status of RA since adenosine low levels are due to increased of its activity being related to a decreased IL-10 and TGF-β levels, both necessary to sustain the expansion of $T_{reg}$ cells, thus contributing to the pathogenesis of RA (McInnes et al., 2007).

Some studies have demonstrated the anti-inflammatory effects of CIN (Lee et al., 2008). Here, we evaluated the effect of CIN on the metabolism of adenine and adenosine nucleotides in healthy and arthritic animals. In normal rats that received the treatment with CIN, the activities of E-NTPDase and E-ADA were maintained at basal levels. Taking into account the

CFA-induced arthritis group that receveid CIN, we observed that this compound was able to partially prevent the increase on the E-NTPDase and E-ADA activity. At low levels, ATP can associate with P2Y receptors in surface the lymphocytes thus decreasing the pro-inflammatory cytokines. Furthermore, we suggest that enzymatic cascade was able to form extracellular adenosine, this possible adenosine exerts its immunosuppressive effects, once E-ADA activity is elevated in humans and contributes to the pathogenesis of RA. It is known that high adenosine levels may counterbalance inflammatory stimuli by inhibiting the production of Th1-like response (i.e., TNF-α, IFN-γ, IL-1, IL-12) (Forrest et al., 2005), impairing migration and emerging to a Th2-like response (Bours et al., 2006). Adenosine is released from a variety of immune cells as T lymphocytes in response to inflammatory process and may be considered a potent immunosuppressive which can interact with P1 cell surface receptors subtypes: $A_1$,$A_2A$, $A_{2B}$ and $A_3A$ ARs. Adenosine receptors are presented in lymphocytes, platelets, macrophages, neutrophils where they mediate pro- and anti- inflammatory effects. The $A_1$ and $A_3$ARs receptors exert an inhibitory effect on cAMP production while $A_{2A}$ and $A_{2B}$ ARs mediate an increase of cAMP accumulation (Gessi et al., 2011).

It is known that high levels of pro-inflammatory cytokines act via an upregulation of NF-κB which is relevent in the arthritis pathogenesis and also regulates ARs receptors (Bar-yehuda et al., 2007). It has been found the overexpression of $A_3$ARs in cell extracts derived from paw and mononuclear cells from arthritic rats in comparison to healthy animals (Rath- Wolfson et al., 2006) and also in peripherial blood mononuclear cells of patients with RA, psoriasis and Crohn's (Ochaion et al., 2009).

Currently studies have shown that CIN inhibits NF-κB activity transcriptional through inhibits of DNA binding (Reddy et al., 2004, Hyung et al., 2008). NF-κB was also revealed to bloked mRNA expression of iNOS as well as other proinflammatory cytokines such as TNF-α and IL-1β (Hyung et al., 2008). and to be an α,β-unsaturated carbonyl exerting suppressive

effect on TLR-4 and TLR-2 oligomerization suggesting that thiolation is the major chemical mechanism of CIN inhibition (Heiss et al., 2001; Reddy et al., 2004). Thus, we suggest that there are a relationship between CIN and expression the adenosine receptors, once CIN has been showed to inhibited NF-κB in which this transcriptional factor regulate $A_3A$ expression levels, thus decreasing E-ADA activity. When $A_2A$ and $A_3A$ receptors are stimulated there is a decrease in the levels of TNF-α found in RA. Recently, has been reported an relationship between disease activity and serum E-ADA levels which in turn may predict actual disease activity as well as treatment responsivity in RA (Varani et al., 2011).

## 4. Conclusions

In conclusion, our data demonstrate that the cinnamaldehyde was able to partially reduce the thermal hyperalgesia and histological injuries. In addition, the compound was able to partially prevent the increased E-NTPDase and E-ADA activities in lymphocytes of rats submitted to an experimental adjuvante arthritis model. Furthermore, we showed a CIN modulation in E-ADA activity, once decreased their activities, in turn this mediator possibly inhibits pro-inflammatory response. Thus, this study suggests that CIN might be an effective and promising agent to supplement the treatment of chronic arthritis. However, detailed molecular mechanisms of CIN action need to be estudied in order to confirm these protective functions.

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**Table 1.** ALT and AST activities in serum of rats with CFA- induced arthritis and treated for 14 days with cinnamaldehyde 2.1%.

| **Groups** | **C** | **CIN** | **AR** | **AR + CIN** |
|---|---|---|---|---|
| ALT (UI/L) | 67.0 ± 6.99 | 70.00 ± 3.02 | 63.50 ± 7.08 | 63.50 ± 3.13 |
| AST (UI/L) | 192.2 ±70.80 | 288.3 ± 66.30 | 363.1 ± 41.63 | 363.1 ± 41.63 |

ALT and AST activities were expressed as (UI/L). Groups: C (control), CIN (cinnamaldehyde), AR (CFA- induced arthritis) and AR+CIN (CFA- induced arthritis treated with CIN). Results are presented as means ± S.E.M. (n=4 for group). $P<0.005$ was considered statistically significant according to analysis of variance one-way (ANOVA) followed by the Tukey's test. ALT: alanine-L-transaminase, AST: alanina-S-transaminase.

**Table 2.** Hematological determination of rats with CFA- induced arthritis and treated for 14 days with cinnamaldehyde 2.1%.

| **Groups** | **C** | **CIN** | **AR** | **AR + CIN** |
|---|---|---|---|---|
| RBC ($x10^6$/ µl)[a] | 7.33 ± 0.11 | 7.72 ± 0.14 | 7.75 ± 0.04 | 7.89 ± 0.12 |
| Hemoglobin (g/dL)[b] | 13.18 ± 0.22 | 13.28 ± 0.26 | 13.60 ± 0.20 | 13.63 ± 0.18 |
| HCT (%)[c] | 44.5. ± 2.76 | 46.48 ± 3.61 | 48.80.68 ± 1.98 | 48.40 ± 0.68 |
| MCV (pg)[d] | 60.69 ± 1.17 | 60.14 ± 0.53 | 62.93. ± 1.6671 | 61.40 ± 1,24 |
| MCHC (g/dL)[e] | 29.62 ± 0.46 | 28.57 ± 0.13 | 27.88 ± 0.35 | 28.16 ± 0.49 |
| WBC ($x10^3$/µl)[f] | 7.36 ± 0.46 | 7.37 ± 0.46 | 10.15 ± 0.33 | 10.17 ± 0.43 |
| Lymphocytes ($x10^3$/µl) | 81. ±3.13 | 84.24 ± 2.75 | 81 ± 5.50 | 76.25 ± 1.87 |
| Neutrophils ($x10^3$/µl) | 17.75± 3.49 | 14.0 ± 2.97 | 17.33 ± 4.84 | 20 ± 1.85 |
| Monocytes ($x10^3$/µl) | 0.2 ±0.0 | 1.0 ± 0.0 | 0.60 ± 0.53 | 1.50 ± 0.57 |
| Eosinophils (x $10^3$/µl) | 0.75 ± 0.95 | 2.00 ± 2.70 | 1.62 ± 0.74 | 1.00 ± 1.15 |
| Platelets ($x10^3$/µl) | 55033 ± 3.30 | 43250 ± 3.75 | 59633 ± 2.42 | 63300± 2.42 |

Groups: C (control), CIN (cinnamaldehyde), AR (CFA- induced arthritis) and AR+CIN (CFA- induced arthritis treated with CIN). B. Barsrs represent mean ± S.E.M. n=4 for group (one-way ANOVA following TUKEY Comparison Test). RBC[a]:Eryhtrocytes ; HCT: Hematocrit; MCV:mean corpuscuçar volum; MCHC: Concentration HB mean corpuscular; WBC: Leukocytes

**Figure captions**

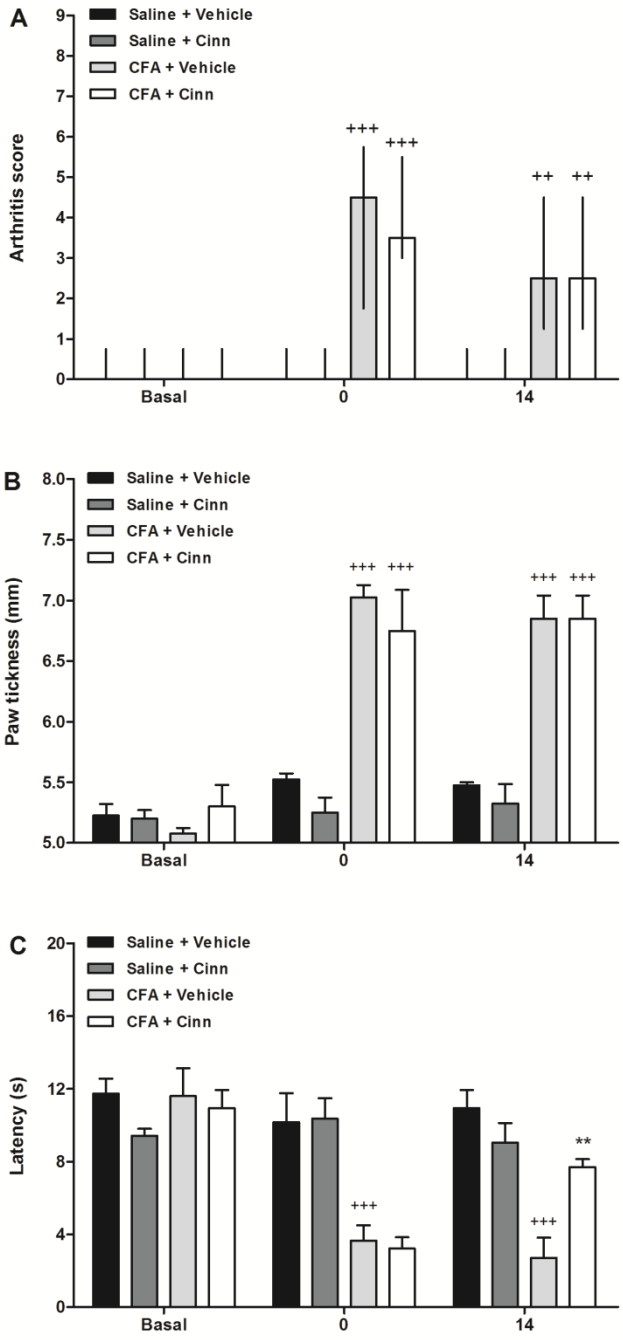
**Figure 1.** Evaluation of behavioral changes induced by CFA-injection. Evaluation of arthritis score (A), paw edema (B) and thermal hyperalgesia (C) induced by CFA intraplantar injection, and CIN (2.1%) effects on these parameters after 14 days of treatment (n = 4 for group). $^{++}$ $P<0.005$ and $^{+++}$ $P<0.001$ in comparison to correspondent saline-injected group and ** $P<0.005$ in comparison to CFA- induced arthritis group, according to two-way analysis of variance (ANOVA), followed by Bonferroni post-test.

**Figure 2.** Histological image of joint tissues from treated or non-treated rats. The control (A); cinnamaldehyde (B); CFA- induced arthritis (C); and CFA- induced arthritis treated with CIN (D).

**Figure 3.** E-NTPDase and E-ADA activities in lymphocytes of rats before and after (15 days) of arthritis induction, using ATP (A), A DP (B) and ADA as substrate (C). Groups: C (control) and AR (CFA- induced arthritis). Bars represent means ± SEM. Groups with different letters are statistically differences (P <0.05; n=4 for group). Unpaired test t.

**Figure 4.** E-NTPDase and E-ADA activity in lymphocytes of CFA-induced arthritis rats and treated for 14 days with cynnamaldehyde 2,1% using ATP (A) and ADP (B) and ADA (C) as substrate. Groups: Controle (C), CIN (cinnamaldehyde), AR (CFA- induced arthritis) and AR+CIN (CFA- induced arthritis treated with CIN). Groups with different letters are statistically different (P<0.05; n=4 for group). One-way ANOVA- Tukey Test.

**Figure 1**


A
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CFA + Vehicle
CFA + Cinn
Paw tickness (mm)
8.0
7.5
7.0
6.5
6.0
5.5
5.0
+++
+++
+++
+++
Basal
0
14
C
Saline + Vehicle
Saline + Cinn
CFA + Vehicle
CFA + Cinn
Latency (s)
20
16
12
8
4
0
+++
+++
**
Basal
0
14

**Figure 2**

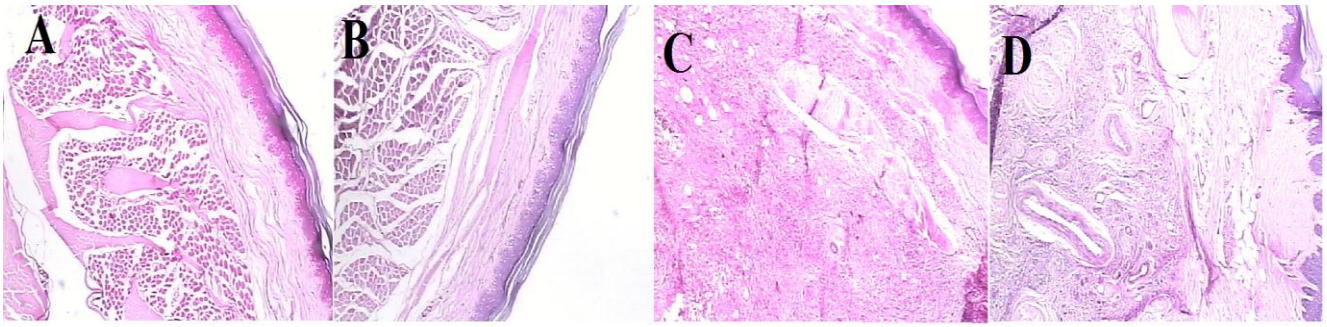


**Figure 3**

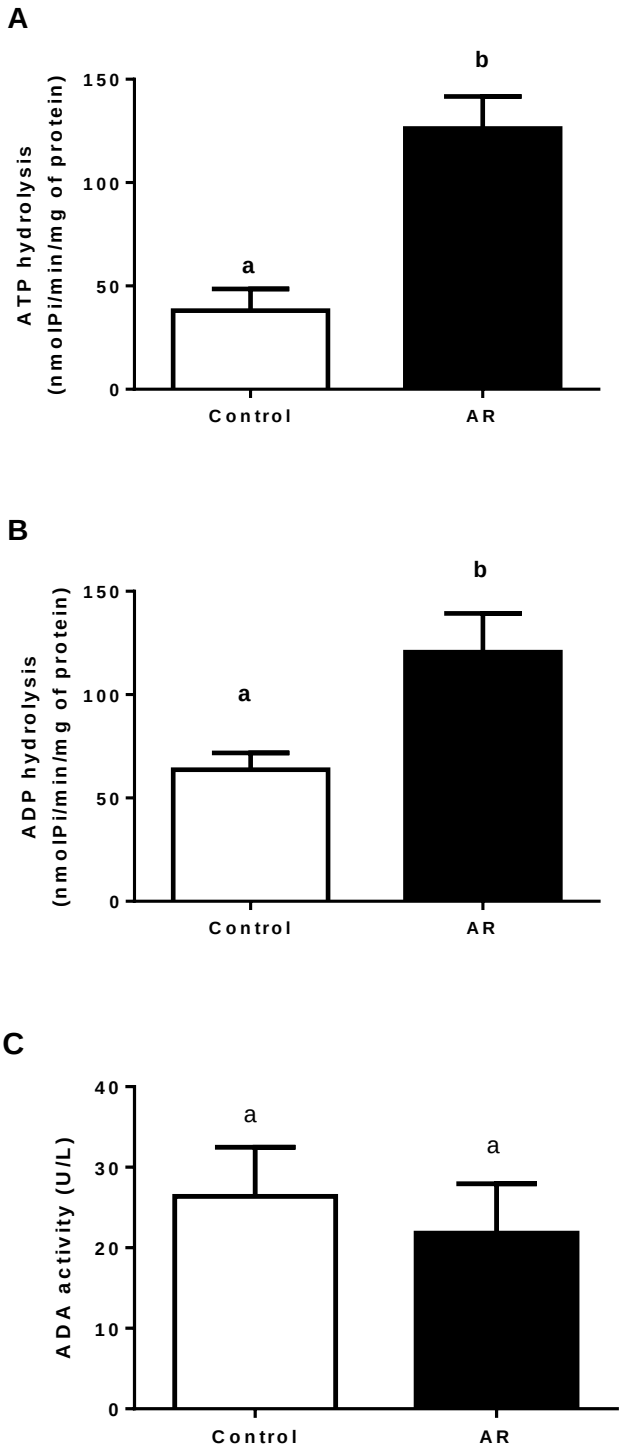
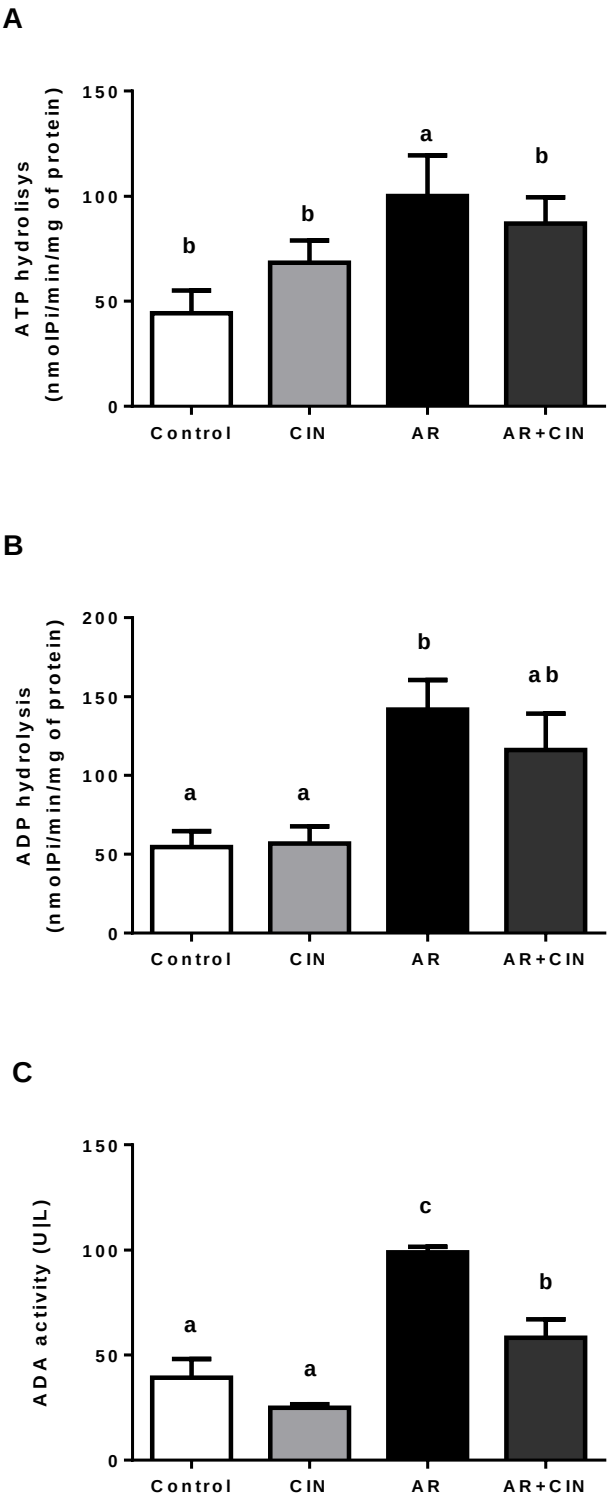

A
ATP hydrolysis (nmolPi/min/mg of protein)
150
100
50
0
a
b
Control
AR
B
ADP hydrolysis (nmolPi/min/mg of protein)
150
100
50
0
a
b
Control
AR
C
ADA activity (U/L)
40
30
20
10
0
a
a
Control
AR

**Figure 4**


A
ATP hydrolisys (nmolPi/min/mg of protein)
150
100
50
0
b
b
a
b
Control
CIN
AR
AR+CIN
B
ADP hydrolysis (nmolPi/min/mg of protein)
200
150
100
50
0
a
a
b
a b
Control
CIN
AR
AR+CIN
C
ADA activity (U|L)
150
100
50
0
a
a
c
b
Control
CIN
AR
AR+CIN

# 4 CONCLUSÃO

- Evidências de indução de artrite foram bem estabelecidas, uma vez que score de artrite, edema de pata e hiperalgesia termal estavam alterados em ratos com artrite induzida por adjuvante em relação a ratos controles. O cinamaldeído induziu a inibição hiperalgesia termal em ratos com artrite induzida por adjuvante em relação ao grupo controle, porém score de artrite e edema de pata não se alteraram na dose de 2,1% e no tempo de tratamento de 14 dias.

- Observou-se pelas análises histológicas a presença de infiltrado inflamatório linfocitico e neutrofilico com a presença de algumas células gigantes no grupo artrite induzida por adjuvante. No grupo artrite induzida por adjuvante tratado com cinamaldeido pode-se visualizar uma redução do infiltrado inflamatório linfocitico.

- Cinamaldeído foi seguro na dose de 2,1% tempo de tratamento de 14 dias, uma vez que não foi observado alterações nas dosagens de enzimas hepáticas e nos índices hematológicos.

- Como não foi observada influência do CIN sobre a atividade das enzimas E-NTPDase e E-ADA em linfócitos de ratos controle, os resultados obtidos foram em decorrência da artrite induzida por adjuvante e da associação da artrite induzida por adjuvante com o cinamaldeído. A atividade da E-NTPDase em linfócitos foi diminuída em ratos com artrite induzida por adjuvante e tratados com cinamaldeído, sugerindo desta maneira que cinamaldeído está alterando o processo pró-inflamatório gerado por este modelo.

- E-ADA em linfócitos também mostrou alteração em ratos com artrite induzida por adjuvante e tratados com cinamaldeído, demostrando influência deste agente no sistema purinérgico. A diminuição da atividade da E-ADA aumenta

as concentrações de adenosina o qual exerce seus efeitos imunossupressores, protegendo o organismo de possíveis danos causados pela artrite induzida.

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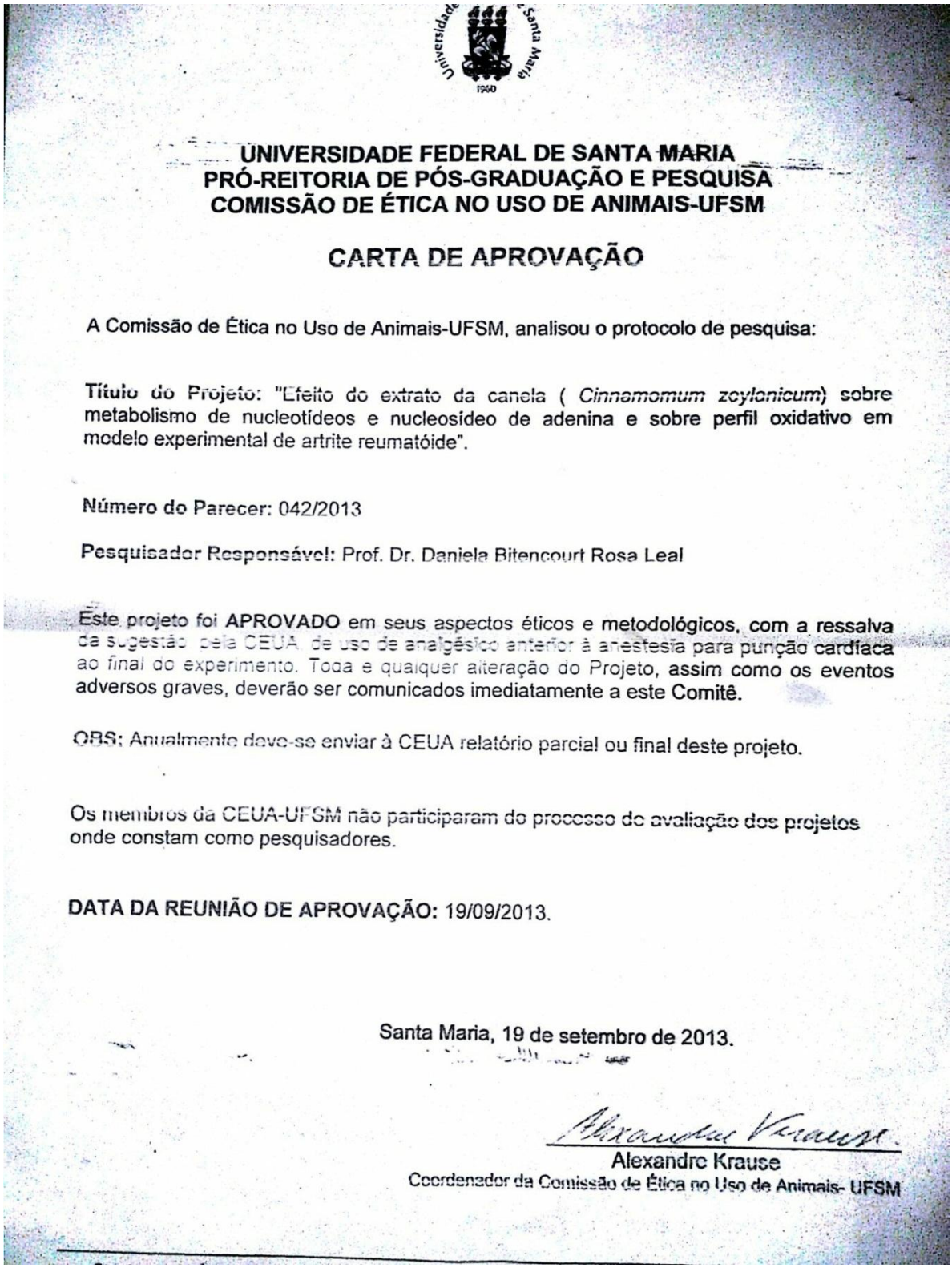
# ANEXO 1



**UNIVERSIDADE FEDERAL DE SANTA MARIA**
**PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA**
**COMISSÃO DE ÉTICA NO USO DE ANIMAIS-UFSM**

## CARTA DE APROVAÇÃO

A Comissão de Ética no Uso de Animais-UFSM, analisou o protocolo de pesquisa:

**Título do Projeto:** "Efeito do extrato da canela ( *Cinnamomum zeylanicum*) sobre metabolismo de nucleotídeos e nucleosídeo de adenina e sobre perfil oxidativo em modelo experimental de artrite reumatóide".

**Número do Parecer:** 042/2013

**Pesquisador Responsável:** Prof. Dr. Daniela Bitencourt Rosa Leal

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos, com a ressalva da sugestão pela CEUA de uso de analgésico anterior à anestesia para punção cardíaca ao final do experimento. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

**OBS:** Anualmente deve-se enviar à CEUA relatório parcial ou final deste projeto.

Os membros da CEUA-UFSM não participaram do processo de avaliação dos projetos onde constam como pesquisadores.

**DATA DA REUNIÃO DE APROVAÇÃO:** 19/09/2013.

Santa Maria, 19 de setembro de 2013.

Alexandre Krause
Coordenador da Comissão de Ética no Uso de Animais- UFSM

## ANEXO 2

# FOOD AND CHEMICAL TOXICOLOGY

**AUTHOR INFORMATION PACK**

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**ISSN:** 0278-6915

## DESCRIPTION

For a journal statement regarding "Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize", published in *Food and Chemical Toxicology*, Volume 50, Issue 11, November 2012, Pages 4221-4231, please click here.

Food and Chemical Toxicology (FCT), an internationally renowned journal, aspires to publish original research articles and reviews on **toxic effects**, in animals or humans, of natural or synthetic chemicals occurring in the human environment with particular emphasis on **food, drugs, and chemicals, including agricultural and industrial safety**, and **consumer product safety.** Areas such as safety evaluation of **novel foods and ingredients, biotechnologically-derived** products, and **nanomaterials** are included in the scope of the journal. FCT also encourages submission of papers on **inter-relationships between nutrition and toxicology** and on *in vitro* techniques, particularly those fostering the **3 Rs.**

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