UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

EFEITO DO EXTRATO DE *Uncaria tomentosa* (Wild.) D.C. NO METABOLISMO DE NUCLEOTÍDEOS DE ADENINA EM LINFÓCITOS DE RATOS SUBMETIDOS A MODELO EXPERIMENTAL DE ARTRITE REUMATÓIDE

DISSERTAÇÃO DE MESTRADO

Lívia Gelain Castilhos

Santa Maria, RS, Brasil
2012

EFEITO DO EXTRATO DE *Uncaria tomentosa* (Wild.) D.C. NO METABOLISMO DE NUCLEOTÍDEOS DE ADENINA EM LINFÓCITOS DE RATOS SUBMETIDOS A MODELO EXPERIMENTAL DE ARTRITE REUMATÓIDE

Lívia Gelain Castilhos

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

Orientadora: Prof. (Dra) Daniela Bitencourt Rosa Leal
Co-Orientadora: Prof. (Dra) Margareth Linde Athayde

Santa Maria, RS, Brasil
2012

Universidade Federal de Santa Maria
Centro de Ciências da Saúde
Programa de Pós-Graduação em Ciências Farmacêuticas

A Comissão Examinadora, abaixo assinada,
aprova a Dissertação de Mestrado

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elaborada por
Lívia Gelain Castilhos

como requisito parcial para obtenção do grau de
Mestre em Ciências Farmacêuticas

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Santa Maria, 18 de julho de 2012.

Dedico este trabalho...

... À minha mãe Angela e minha vó Didi, pelo incentivo, força e amor.

... À minha irmã Victória, pela presença, carinho e amizade.

... Ao meu namorado Ricardo, pelo amor e apoio.

...Sem eles nada disso seria possível.

"Cada pessoa que passa em nossa vida, passa sozinha,
é porque cada pessoa é única e nenhuma substitui a outra.
Cada pessoa que passa em nossa vida passa sozinha, e não nos deixa só,
porque deixa um pouco de si e leva um pouquinho de nós.
Essa é a mais bela responsabilidade da vida e a prova
de que as pessoas não se encontram por acaso."

Charles Chaplin

AGRADECIMENTOS

A DEUS, que todos os dias da minha vida me deu forças para nunca desistir.

À minha mãe Angela e a minha querida vó Didi, exemplos de força e dedicação, bases da minha educação, que semearam e cuidaram com atenção e carinho meu crescimento pessoal e profissional.

À minha irmã Victória, pela convivência, amor, preocupação e apoio. Obrigada pela companhia.

Ao meu amor Ricardo, por tantas alegrias, amor, compreensão, paciência e confiança. Obrigada por fazer parte da minha vida!

A toda minha família, pela união em todas as horas. Obrigada por acreditarem em mim.

A minha orientadora, Daniela Bitencourt Rosa Leal, por me mostrar o caminho da ciência e contribuir para o meu crescimento profissional. Por seu apoio e amizade, além de sua dedicação, competência e especial atenção nas revisões e sugestões, fatores fundamentais para a conclusão deste trabalho.

Aos amigos e colegas do laboratório 4229, João, Jeandre, Jader, Josiane, Carine, Karine Silveira, Karine Schlemmer, Maria Luiza, Bruna, Cristiano, Tatiana, Viviane, Francine e Pedro, pelo carinho, coleguismo, amizade e pela colaboração para a realização deste trabalho.

Aos professores e funcionários do Departamento de Microbiologia e Parasitologia e do Programa de Pós-Graduação em Ciências Farmacêuticas da UFSM pelo auxílio e apoio prestados.

A todos aqueles que colaboraram de alguma forma para a realização deste trabalho, meu muito obrigado!

"Insisto na caminhada.

O que não dá é pra ficar parado.

Se amanhã o que eu sonhei não for bem aquilo, eu tiro um arco-íris da cartola. E refaço. Colo. Pinto e bordo.

Porque a força de dentro é maior.

Maior que todo mal que existe no mundo.

Maior que todos os ventos contrários.

É maior porque é do bem. E nisso, sim, acredito até o fim.

O destino da felicidade me foi traçado no berço."

Caio Fernando de Abreu

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Ciências Farmacêuticas
Universidade Federal de Santa Maria

EFEITO DO EXTRATO DE *Uncaria tomentosa* (Wild.) D.C. NA HIDRÓLISE DE NUCLEOTÍDEOS DE ADENINA EM LINFÓCITOS DE RATOS SUBMETIDOS A MODELO EXPERIMENTAL DE ARTRITE REUMATÓIDE

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Data e local de Defesa: Santa Maria, 18 de Julho de 2012.

Os extratos do caule e da raiz da planta *Uncaria tomentosa* possuem diversas propriedades, dentre elas a propriedade anti-inflamatória, sendo bastante utilizados em casos de artrite reumatóide (AR). 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 AR, que por sua vez, é 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 essencialmente do tipo Th1. Dentre os mediadores capazes de modular as ações dos linfócitos, durante um processo inflamatório, destacam-se o ATP, o ADP, o AMP e o nucleosídeo adenosina, essenciais para o início e manutenção das respostas inflamatórias. Os efeitos destas moléculas são promovidos através da ativação de receptores purinérgicos específicos e controlados por um complexo enzimático localizado na superfície das células. O objetivo deste trabalho foi avaliar o efeito do extrato de *Uncaria tomentosa* no metabolismo de nucleotídeos da adenina através da atividade de ectoenzimas envolvidas na hidrólise do ATP em linfócitos de ratos submetidos a modelo experimental de AR. Os animais foram divididos em 4 grupos, controle (C), extrato (E), artrite (AR) e artrite associado ao extrato (AR+E). Quinze dias após a indução da AR por CFA, o extrato seco de *U. tomentosa* foi administrado por gavage 2 vezes ao dia na dose de 150mg/Kg durante 45 dias. Após o tratamento, o sangue foi coletado por punção cardíaca e os linfócitos foram separados para a realização da atividade da E-NTPDase e ADA, e o soro utilizado para a quantificação dos nucleotídeos e nucleosídeo. Os resultados demonstraram um aumento na atividade da E-NTPDase em ratos com AR induzida por CFA quando comparado ao controle. Já nos ratos tratados somente com o extrato de *Uncaria tomentosa* pode-se observar que o mesmo manteve a atividade da E-NTPDase e da E-ADA a níveis basais. Nos ratos com AR e tratados com o extrato pode-se observar que o mesmo foi capaz de prevenir o aumento da atividade da E-NTPDase, embora o nível de ATP e adenosina se encontram diminuídos e do ADP aumentado no meio extracelular. O aumento na atividade da E-NTPDase estaria relacionado com a tentativa de manter as concentrações basais de ATP e ADP no meio extracelular, uma vez que a indução da AR causa dano tecidual e consequentemente a liberação de grandes quantidades de ATP presentes no interior da célula. Dessa forma, o extrato de *Uncaria tomentosa* foi capaz de prevenir o aumento da atividade da E-NTPDase causado pela indução da AR.

Palavras-chave: *Uncaria tomentosa.* Artrite reumatóide. Linfócitos. E-NTPDase. Adenosina desaminase

ABSTRACT

Dissertation Master's Degree
Post-Graduating Program in Phamaceutical Sciences
Federal University of Santa Maria, RS, Brazil

EFEITO DO EXTRATO DE *Uncaria tomentosa* (Wild.) D.C. NA HIDRÓLISE DE NUCLEOTÍDEOS DE ADENINA EM LINFÓCITOS E PLAQUETAS EM MODELO EXPERIMENTAL DE ARTRITE REUMATÓIDE

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Place and Date: Santa Maria, July 18th, 2012.

The extracts of stem and root of the *Uncaria tomentosa* plant present several properties. Among them, the anti-inflammatory property is very important since it has been studied and widely observed in cases of rheumatoid arthritis (RA) treatment. The model of arthritis induced by complete Freund's adjuvant (CFA) in rats is a model widely used in searches for new therapies for chronic inflammatory arthropathies, such as RA, which in turn, is a chronic inflammatory disease, immune-mediated and with rather complex physiopathology. During the inflammatory process, a complex and hierarchical cytokines network rules this process triggering a Th1 type immune response. Among the mediators that modulate the action of lymphocytes during inflammatory process, we emphasize ATP, ADP, AMP and the nucleoside adenosine, which are essentials to the initiation and maintenance of inflammatory responses. The effects of these molecules are promoted by the action of specific purinergic receptors and controlled by an enzyme complex on the cell surface. The objective of this study was to evaluate the effect of the *Uncaria tomentosa* extract on the metabolism of adenine nucleotides through the activity of ectoenzymes involved in the ATP metabolism in lymphocytes of rats submitted to an experimental model of rheumatoid arthritis.The animals were divided into four groups, namely, control (C), extract (E), arthritis (AR) and arthritis associated with extract (AR+E). Fifteen days after AR induction by CFA, the *U. tomentosa* dry extract was administered two times a day at the dose of 150mg/kg for 45 days. After treatment, the blood was collected by cardiac puncture and the lymphocytes were isolated to E-NTPDase and ADA activity determination, and the serum used to purine level measurement. Results show an increase in the E-NTPDase activity in rats with CFA induced arthritis compared to control. Rats treated only with *Uncaria tomentosa* extract showed E-NTPDase and E-ADA activity maintained in basal levels. In rats with RA treated with *Uncaria tomentosa,* the extract was able to prevent the increase on the E-NTPDase activity, although the ATP and adenosine levels were decreased and ADP levels were increased in extracellular medium. The increase in E-NTPDase activity might be related to the attempt to maintain basal levels of ATP and ADP in basal levels in the extracellular medium, since the RA induction causes tissue damage and consequently large amounts of ATP in the cell. This way, the *Uncaria tomentosa* extract was able to prevent the increase in the E-NTPDase activity promoting RA induction.

Keywords: *Uncaria tomentosa*. Rheumatoid arthritis. Lymphocytes. E-NTPDase. Adenosine deaminase

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LISTA DE ABREVIATURAS

ADP: adenosina difosfato

AMP: adenosina monofosfato

AR: artrite reumatóide

ATP: adenosina trifosfato

Ca^{2+}: íon cálcio

CFA: adjuvante completo de Freund

DMCD: drogas modificadoras do curso da doença

E-NTPDase: ecto-nucleosídeo trifosfato difosfoidrolase

E-ADA: ecto-adenosina desaminase

eN: 5'-nucleotidase

IL-1: interleucina -1

IL-2: interleucina-2

IL-6: interleucina-6

IL-15: interleucina-15

IL-17: interleucina-17

INF-γ: interferon-gamma

NF-κB: fator nuclear kappa B

NK: *natural killer*

LDH: lactato desidrogenase

Mg^{2+}: íon magnésio

SEM: erro padrão da média

Th1: T *helper* 1

Th2: T *helper* 2

Th17: T *helper* 17

TNF-α: fator de necrose tumoral-alpha

Tregs: T reguladoras

U. tomentosa: *Uncaria tomentosa*

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SUMÁRIO

APRESENTAÇÃO

Esta dissertação está organizada na seguinte forma: primeiramente é apresentada a introdução. A seguir, os resultados, discussão e conclusões são apresentados na forma de manuscrito, o qual foi escrito, seguindo-se as normas do periódico ao qual o mesmo será submetido à publicação. As referências bibliográficas apresentadas no final da dissertação referem-se às citações que aparecem no item introdução. A formatação atende a MDT de 2012 da UFSM

1 INTRODUÇÃO

As plantas medicinais têm sido um importante recurso terapêutico desde os primórdios da antiguidade até os nossos dias. No passado, representavam a principal arma terapêutica conhecida, e hoje a busca por novos fitoterápicos vem estimulando pesquisas clínicas e assim fortalecendo a confiabilidade em sua eficácia e segurança. A utilização das plantas medicinais em pacientes acometidos por doenças reumáticas despertou o interesse da comunidade científica e tem sido tema de vários estudos no mundo todo (BRANDÃO, 2005).

A planta *Uncaria tomentosa*, tem sido muito utilizada para este fim. Há mais de 2000 anos, entre muitas tribos Peruanas, especialmente *Ashaninka*, acreditava-se que esta espécie possuía o poder da cura mágica, e hoje é extensivamente utilizada para o tratamento da artrite, doenças inflamatórias, reumatismo, asma, câncer, cirrose, gastrite, diabetes, gastroenterite, inflamação do trato urinário e muitas outras doenças (AKESSON et al., 2005; FALKIEWICZ et al., 2001; HEITZMAN et al., 2005; JURGENSEN et al., 2005; KEPLINGER et al., 1999; PILARSKI et al., 2006).

A *Uncaria tomentosa* (Willd) D.C. pertence à família Rubiaceae, gênero *Uncaria* e é uma planta medicinal originária da Amazônia brasileira e peruana (REINHARD, 1999). É caracterizada por um arbusto trepador, que cresce apoiando-se em outras árvores, podendo atingir até 20m de comprimento (CHANG, 1995). Seu caule tem textura fibroso-laminar, coloração mesclada de marrom avermelhado e creme e suas raízes são cilíndricas e tem segmentos papilosos (ALONSO, 1998). Apresenta folhas pecioladas, pareadas, ovais, de consistência membranosa, de coloração verde claro com presença de estípulas interpeciolares e espinhos axilares maciço-lenhosos levemente curvados. As inflorescências são esféricas, pedunculadas sozinhas ou em ramos. As flores são numerosas e pequenas, com ausência total de pelos e sem pecíolo. Os frutos são cápsulas unidas, bicarpeladas e as sementes são em forma de asa, bicaudadas e numerosas (Figura 1) (PEÑA et al., 1998).

Seu nome provém da semelhança de seus espinhos com as unhas do gato, sendo conhecida em diversos países através dos nomes populares, tais como *cat's*

claw, uña de gato, paraguayo, garabato, garbato-casha, tambor-huasca, uña-huasca, uña-de-gavilán, hawk's claw, saventaro, cipó-de-gato, e arranha "gato" (KEPLINGER et al., 1999).

Figura 1 - *Uncaria tomentosa*: espinhos axilares maciço-lenhosos levemente curvados e inflorescências esféricas (Adaptado de REINHARD, 1999).

A *Uncaria tomentosa* contém diversos metabólitos secundários bioativos. A casca do caule e as raízes apresentam uma composição química variada, indicando predomínio de compostos polifenólicos, ácidos orgânicos, alcalóides oxindólicos tetracíclicos e pentacíclicos, triterpenos, taninos e esteróis (AQUINO et al., 1989; JONES, 1995). Entre os primeiros estudos farmacológicos da *Uncaria tomentosa* relata-se uma atividade anti-inflamatória moderada, associada à fração rica em esteróis (SENATORE et al., 1989). Posteriormente, essa atividade foi relacionada aos heterosídeos do ácido quinóvico e aos alcalóides. Porém seus dados farmacológicos disponíveis parecem indicar que essa atividade biológica é devida à ação sinérgica de diversos compostos presentes na mesma (AQUINO et al., 1991).

Vários extratos provenientes da planta *Uncaria tomentosa* com diferentes constituintes tem mostrado interessantes características em termos de atividades imunomodulatória e anti-inflamatória. Um extrato hidroalcoólico testado em camundongos BALBc durante 28 dias nas doses de 125, 500 e 1250 mg/kg apontou que o extrato não foi imunotóxico mas foi capaz de modular o sistema imune em dose dependente, pois uma polarização para citocinas tipo Th_2 foi observada. Já no

estudo in vitro, com 10-500 µg/mg do extrato por 48h, o mesmo promoveu um aumento no número de linfócitos T helper e linfócitos B. (DOMINGUES et al., 2011).

Em um estudo *in vitro* realizado com células sanguíneas, e com o intuito de verificar a ação dos alcalóides oxindólicos pentacíclicos e tetracíclicos da *U. tomentosa* sobre os linfócitos, demonstrou-se que os alcalóides pentacíclicos são capazes de induzir um fator de regulação e proliferação de linfócitos (WURM et al., 1998).

Mais tarde, Aguilar e col. (2002) demonstraram em um modelo *in vitro* que tanto o extrato hidroalcoólico como o extrato aquoso de *U. tomentosa* apresentaram uma atividade não significativa, mas moderada para cicloxigenase-1 e cicloxigenase-2. O mesmo também observou que ambos os extratos possuem uma atividade anti-inflamatória, sendo que o extrato hidroalcoólico mostrou um efeito anti-inflamatório similar ao anti-inflamatório não esteroidal indometacina com dose de 7mg/kg e o aquoso exibiu o mesmo efeito, porém em doses acima de 200mg/kg, quando testados em modelo de edema de pata induzido por carragenina.

Com relação aos processos inflamatórios, sabe-se que os radicais livres e o estresse oxidativo são fatores importantes no desenvolvimento da artrite e de outros processos inflamatórios do tipo crônico. Portando, substâncias que modificam a produção desses fatores e que, consequentemente reduzem o grau da lesão dos tecidos, serão efetivas no combate à inflamação (QUINTERA et al., 2003). Neste contexto, estudos *in vitro* revelaram que os extratos aquoso e hidroalcoólico das cascas do caule de *U. tomentosa* possuem propriedades anti-inflamatórias relacionadas com a supressão da síntese do fator de necrose tumoral (TNF-α), principal agente inflamatório envolvido em distúrbios digestivos e artríticos, (SANDOVAL et al., 2000).

Em outro estudo a fim de se investigar o mecanismo pelo qual a planta *U. tomentosa* emprega para regular a secreção de citocinas pro-inflamatórias, Allen-Hall e col. (2010), sugerem um possível mecanismo por meio da modulação do fator nuclear kappa B (NF-κB) mediando a regulação celular e a expressão de citocinas através da subunidade p52 do NF-κB. O papel da *U. tomentosa* como um inibidor do NF-kB é importante no tratamento da osteoartrite, pois duas classes de agentes anti-

inflamatórios que são amplamente utilizados, os salicilatos e glicocorticóides, são ambos inibidores do NF-κB (BEG; BALTIMORE, 1996).

Seguindo este mesmo contexto, pacientes com osteoartrite de joelho foram tratados com 100mg do extrato seco de *U. tomentosa* uma vez ao dia e durante 4 semanas, onde observou-se que mesma não desencadeou nenhum efeito deletério no sangue e na função hepática. A dor associada à atividade foi reduzida já na primeira semana de terapia. Estudos *in vitro* foram também realizados concluindo-se que suas propriedades anti-inflamatórias podem resultar de sua capacidade de inibir TNF-α e, em menor medida, a produção de PEG2 (PISCOYA et al., 2001).

Um estudo duplo-cego foi realizado em 70 pacientes com artrite reumatoide, os quais foram divididos em dois grupos que receberam “unha de gato” (2 cápsulas de 500mg, 3 vezes ao dia) ou placebo durante 6 meses. Os resultados concluem que a eficácia da unha de gato como anti-inflamatório em pacientes com artrite reumatóide está plenamente justificada, pois a mesma promoveu uma diminuição da rigidez matinal, a dor diurna e noturna foi sendo reduzida ao longo do tratamento, como também reduziu o número de articulações dolorosas e inchadas (CASTAÑEDA et al., 1998).

Mur e col. (2002) também realizaram um estudo duplo-cego onde avaliaram a ação do extrato seco de *U. tomentosa* (20mg – Immodal Pharmaka Áustria) em 40 pacientes com diagnóstico de artrite, mostrando uma evolução muito favorável do grupo que recebeu o extrato, com redução significativa da dor articular e da rigidez matinal.

A medicina complementar e alternativa está ganhando popularidade e seu uso nas sociedades ocidentais é responsável por significativos gastos em saúde pública e privada (RAMOS-EMUS et al., 1998). A artrite é uma das principais doenças para as quais os pacientes procuram a medicina complementar e alternativa (MCA) (BRUNE, 2004; SOEKEN, et al., 2003). A sua utilização em reumatologia tende a ser maior do que no geral da população, variando entre 28% e 90% (ASTIN, 1999), e ainda, relatórios nos mostram que mais de 70% dos pacientes em uso da MCA mencionam estes produtos para seus médicos.

A artrite reumatóide (AR) é uma doença crônica inflamatória e a mais comum das doenças reumáticas autoimunes, caracterizada por poliartrite periférica, simétrica, que leva à deformidade e à destruição das articulações por erosão do

osso e cartilagem (BIZZARO et al., 2001; LIPSKI, 1998). A AR afeta primariamente as articulações, mas pode incluir uma variedade de manifestações extra articulares, que vão desde febre e emagrecimento até o acometimento de órgãos como pulmões, coração, rins, entre outros (ENGEL et al., 1966; MIKKELSEN, 1967; O'BRIEN, 1967; WOLFE et al., 1968; WOOD, 1967).

As principais queixas dos pacientes com AR são a dor e a rigidez nas articulações. O enrijecimento das estruturas periarticulares e o edema sinovial impõem grave incapacidade às articulações envolvidas (BRANDÃO et al., 1997). Essa doença possui um curso crônico com períodos variáveis de remissão e exacerbação e sua evolução pode causar deformidades nas articulações e incapacidade funcional do indivíduo, com significativo impacto socioeconômico (HARRIS; JR., 1997; LAURINDO et al., 2002).

A AR é uma doença multifatorial, resultante da interação entre fatores genéticos e ambientais, os quais contribuem para sua ocorrência e expressão. Os fatores hormonais e o estilo de vida também podem influenciar o curso da doença (ALAMANOS; DROSOS, 2005). Sua prevalência é de, aproximadamente, 1% da população geral, com acometimento de ambos os sexos e predominância no sexo feminino, na proporção de 3-4:1(CARVALHO; XAVIER, 2001), sendo mais frequente na população adulta (40 a 60 anos), porém podendo acometer indivíduos de qualquer idade (BRANDÃO et al., 1997).

Como a AR é uma doença imunomediada, com fisiopatologia bastante complexa, acredita-se que o evento inicial seja provavelmente a ativação de células T dependentes de antígenos, desencadeando uma resposta imunológica essencialmente do tipo Th1. Essa ativação da reposta Th1 desencadearia múltiplos efeitos, incluindo ativação e proliferação de células endoteliais e sinoviais, recrutamento e ativação de células pró-inflamatórias, secreção de citocinas e proteases a partir de macrófagos e células sinoviais, e produção de auto-anticorpos (HARRIS, 1986).

Na sinovite, as células inflamatórias respondem com incremento na produção de prostaglandinas, citocinas e intensa formação de espécies reativas de oxigênio. Isso leva ao estresse oxidativo, que rapidamente amplifica o processo inflamatório, causando destruição articular, edema e dor (HENROTIN et al., 2005; VIGNAUD et al., 2005).

A destruição da cartilagem e do osso ocorre por erosão principalmente na junção da cartilagem, osso e membrana sinovial, uma região rica em macrófagos e conhecida como *pannus*. A patologia se estende por toda a articulação sinovial e as células mais abundantes da membrana sinovial são os macrófagos e linfócitos T, mas plasmócitos, células dendríticas e fibroblastos ativados também são encontrados (JANOSSY et al., 1981). As células do *pannus* migram sobre a cartilagem subjacente e no osso subcondral, fazendo com que ocorra a erosão subsequente destes tecidos (ALLARD et al., 1987). A destruição da cartilagem ocorre devido à atividade de metaloproteases de matriz (MMP), enzimas produzidas por macrófagos ativados e fibroblastos em resposta a citocinas como IL-1 e TNF-α (VINCENTI et al., 1994).

Os processos inflamatórios são regidos por uma rede complexa e hierarquizada de citocinas e quimiocinas. Durante a resposta inflamatória na AR um conjunto de citocinas tais como TNF-α, IL-1, IL-6 e IL-17 estão envolvidas no recrutamento, diferenciação e ativação dos osteoclastos, células multinucleadas que possuem capacidade de reabsorver tecido ósseo. Além disso, as citocinas podem afetar a diferenciação e função dos osteoclastos influenciando no equilíbrio entre a formação do osso e reabsorção óssea (HERMAN et al., 2008; McINNES; SCHETT, 2007). A IL-6 é uma citocina pró-inflamatória que desempenha um papel importante de condução de perda óssea (MAINI et al., 2006) (Figura 2).

O TNF-α, citocina importante na AR, é produzido por muitos tipos de células, incluindo monócitos e macrófagos ativados, neutrófilos, linfócitos T e B, mastócitos, basófilos, eosinófilos e células natural killer (NK). Seus efeitos incluem a ativação de monócitos/macrófagos e de outras células para produzir mediadores solúveis, como IL-1 e outras citocinas; estimulação da expressão de moléculas de adesão, permitindo a adesão e extravasamento subsequente de leucócitos (SHARON, 2000). O mediador pró-inflamatório IL-1β é induzido por TNF-α e é crucial para a indução da expressão de enzimas na membrana sinovial, bem como a formação dos osteoclastos (ZWERINA et al., 2007).

A IL-17 por sua vez, produzida pelas células T, é também uma das principais citocinas pró-inflamatórias derivada de uma população de células T (T helper 17 – Th17) e é considerada por desempenhar um papel chave na iniciação da doença inflamatória. A IL-17, produzida pelas células, é considerada o mais potente indutor

da produção de IL-6 pelos sinoviócitos (CHABAUD et al., 2001). Além do mais, a mesma induz a síntese de enzimas, como metaloproteases e participa da destruição da cartilagem e do osso na AR (SATO et al., 2006). Sabendo-se que a IL-17 é expressa na membrana sinovial de pacientes com AR e que seus níveis estão elevados nestes pacientes, seu controle poderia ser usado como estratégia no tratamento da mesma (MIOSSEC, 2003).

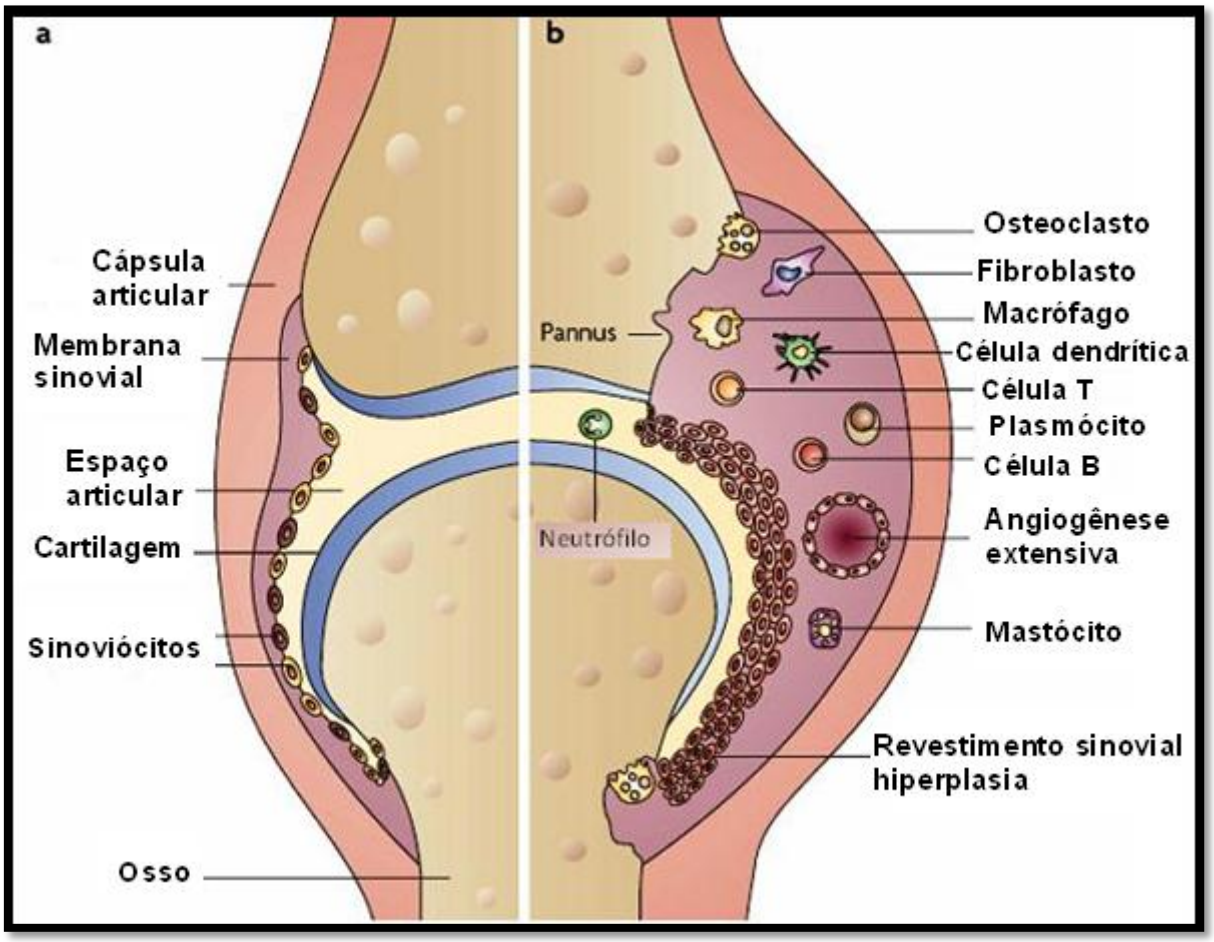

Figura 2 - Representação da articulação sinovial e células envolvidas no processo inflamatório na AR (Adaptado de HERMAN et al., 2008).

As células T reguladoras (Tregs) desempenham um papel central no controle da resposta autoimune e inflamação. Mudanças sutis no número, função e fenótipo das Tregs poderia levar ao desenvolvimento de doenças autoimunes como a AR, mas também pode ser uma consequência do processo inflamatório (CHAVELE; EHRENSTEIN, 2011). Em pacientes com AR, o número de Tregs presentes no fluido sinovial é maior do que no sangue periférico (MOTTONEN et al., 2005), e estas

Tregs, principalmente as presentes no fluido sinovial, são influenciadas por um perfil de citocinas. TNF-α, IL-6, IL-15 e IL-1 presentes na articulação inflamada agem para aumentar o número de Tregs no infiltrado inflamatório, mas ao mesmo tempo comprometem a sua função, talvez por um curto período a fim de se restaurar a tolerância imunológica (CHAVELE; EHRENSTEIN, 2011; PASARE; MEDZHITOV, 2003).

O diagnóstico da AR é realizado através de testes clínicos, pela evolução da doença, e laboratoriais, por marcadores como proteína C reativa e velocidade de eritrossedimentação, como também por estudos radiológicos (CAILLIET, 2001; LAURINDO et al., 2002). A orientação para diagnóstico é baseada nos critérios de classificação do Colégio Americano de Reumatologia revisado em 2002 (AMERICAN COLLEGE OF RHEUMATOLOGY SUBCOMMITTEE ON RHEUMATOID ARTHRITIS GUIDELINES, 2002).

As condutas para a terapêutica da artrite variam de acordo com o estágio da doença, sua atividade e gravidade. Para o tratamento da dor e do processo inflamatório articular faz-se o uso de analgésicos e anti-inflamatórios não hormonais (AINHs), associado ou não a doses baixas de glicocorticóiodes. As drogas modificadoras do curso da doença (DMCD) como hidroxicloroquina, metotrexato e sulfassalazina, as quais tem o potencial de reduzir ou prevenir o dano articular, são também indicadas para todo paciente a partir da definição do diagnóstico de artrite reumatoide (AMERICAN COLLEGE OF RHEUMATOLOGY SUBCOMMITTEE ON RHEUMATOID ARTHRITIS GUIDELINES, 2002).

Outro tipo de terapia também utilizada para pacientes com AR são os agentes biológicos como: o rituximabe que promove a depleção de células B e os agentes anti-TNF-α (infliximabe, golimumabe), anti-IL-1 (anakinra) e anti-IL-6 (tocilizumabe) os quais possuem sua atividade biológica diretamente voltada para uma citocina específica (VOLLENHOVEN, 2009) (Figura 3).

Frente a esses medicamentos de tecnologia avançada para o tratamento da AR, o Ministério da Saúde amplia ainda mais o espectro de possibilidades a ser oferecido aos pacientes quando incluiu o fitoterápico *Uncaria tomentosa* (unha-de-gato) na Relação Nacional de Plantas Medicinais de Interesse ao SUS (Renisus) disponibilizando-o para uso da população (MINISTÉRIO DA SAÚDE, 2009).

A dificuldade da realização de estudos em pacientes por razões éticas ou técnicas leva à necessidade de modelos experimentais de artrite. Os modelos animais de doenças crônicas permitem uma melhor compreensão dos processos fisiopatológicos, bem como a avaliação do potencial de novas terapias. Assim, 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 AR (JOE et al., 1999).

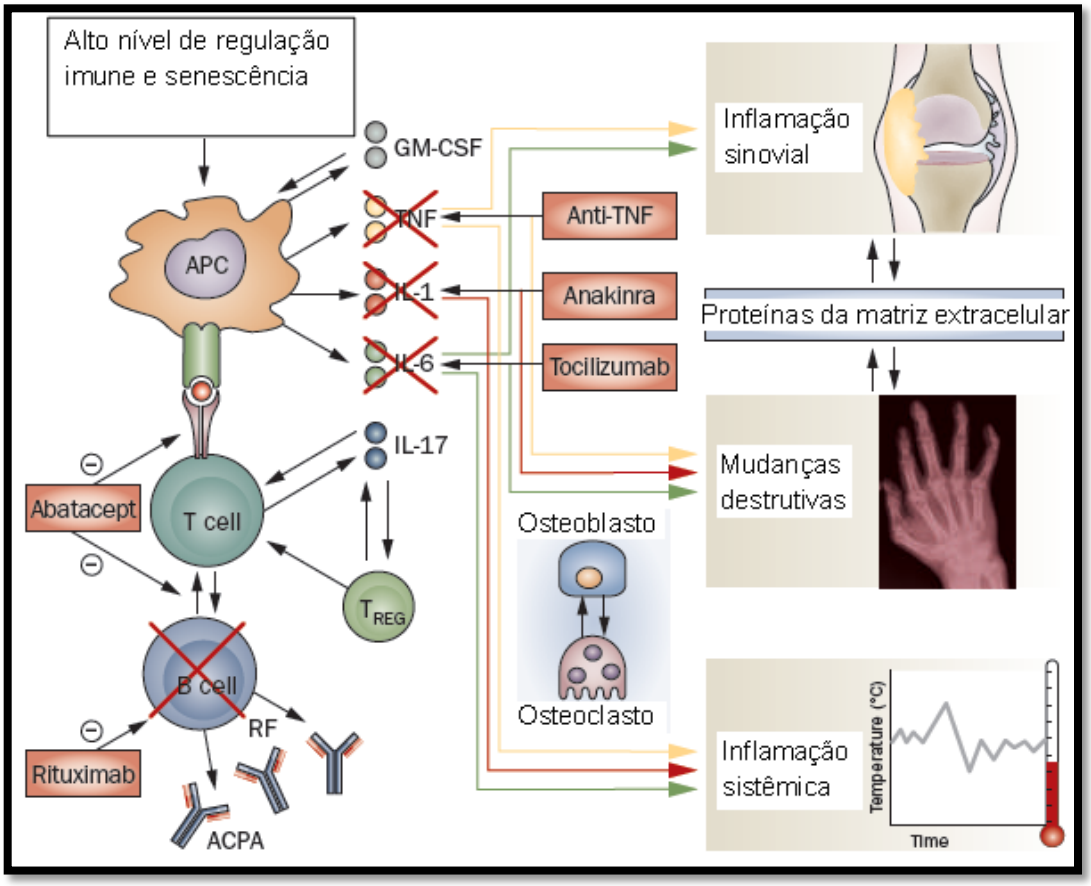

Figura 3 - Esquema simplificado dos mecanismos patofisiológicos da AR, e intervenção com agentes biológicos (Adaptado de VOLLENHOVEN, 2009).

A artrite induzida por adjuvante foi descrita primeiramente por Pearson em 1956, como uma doença induzida em ratos com susceptibilidade genética por uma inoculação simples de micobactéria (*Mycobacterium*) suspensa em óleo, substância conhecida como Adjuvante Completo de *Freund* (CFA) (WAUBEN et al., 1994). Durante a tentativa de induzir uma polimiosite através da injeção intramuscular de

CFA, Pearson percebeu o desenvolvimento de uma artrite crônica nos animais. Com o auxílio de Fae Wood, Pearson padronizou um modelo experimental de artrite induzida por CFA, o qual produzia sinais e sintomas semelhantes aos da artrite reumatóide em humanos (PEARSON, 1956; TAUROG et al., 1988).

Estudos realizados por Whitehouse e col. (1974) no laboratório de Pearson demonstraram que o desenvolvimento da artrite por adjuvante se deve tanto pela natureza do óleo utilizado para preparar o adjuvante quanto pela presença da peptidioglicana presente na parede celular da micobactéria, constituindo uma combinação que favorece o desenvolvimento da AR. Já outros estudos tem demonstrado que uma proteína (HSP) presente na parede bacteriana seria a responsável pela ativação das células T na patogênese da artrite (VAN EDEN et al., 1988; WAKSMAN, 2002). A característica autoimune da artrite induzida por CFA foi também primeiramente descrita por Pearson, quando este retirou clones de células T dos linfonodos dos animais que receberam injeção de CFA e transferiu para animais *naive*, os quais desenvolveram subsequentemente uma poliartrite (CARVALHO et al., 2002).

Segundo Oliveira e col. (2007), o CFA atua como um estímulo de resposta imunológico a antígenos desencadeando inicialmente um edema de pata 24h após sua indução, com características de inflamação aguda. Aproximadamente dez a quinze dias após sua indução, uma diminuição da inflamação aguda com subsequente desenvolvimento da inflamação crônica é observada, com a presença de fibrose, um infiltrado inflamatório com células gigantes, neutrófilos, linfócitos e plasmócitos e o consequente dano articular.

O edema característico pode ser avaliado por meio de um paquímetro digital, através da variação da espessura da pata após a administração do CFA, sendo que, aumentos nesta variação serão considerados como edema (CAO,1998). Em decorrência disto, o processamento sensorial será anormal; e os estímulos ambientais que normalmente são inócuos, como um leve toque, produzirá uma sensação de dor, ou seja, alodínia (LOESER; TREEDE, 2008). A alodínia mecânica pode ser avaliada usando o modelo de “up-and-down” por meio de filamentos de von Frey (DIXON, 1980). Cada pata é estimulada iniciando a série com um filamento de nylon de estímulo médio, caso o animal retire a pata do filamento outro próximo filamento com menor tensão será utilizado, porém caso ele não retire, utiliza-se um próximo

filamento de maior tensão. Assim, respostas aumentadas, definidas por diminuição do limiar de retração da pata ao estímulo mecânico, refletem as condições de alodínia (Figura 4) (LABUDA et al., 2001).

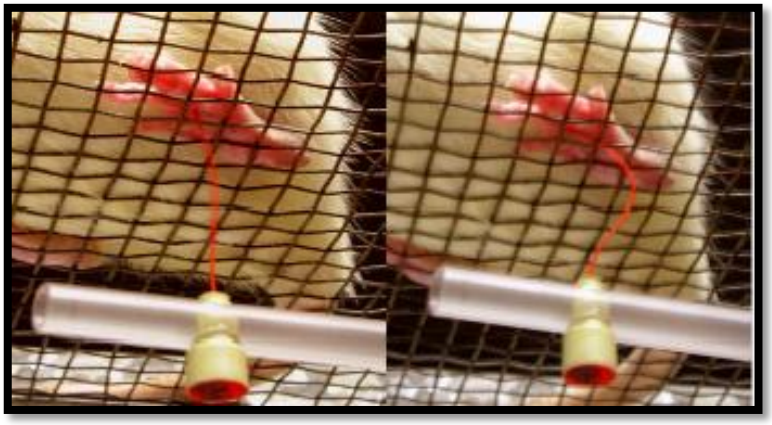

Figura 4 - Avaliação da alodínia mecânica por filamentos de nylon (COSTA, 2004).

O uso do modelo de artrite induzida por adjuvante em animais reside na semelhança que este modelo apresenta com a doença reumatóide em humanos (CAI et al., 2006; NAGAKURA et al., 2003; PEARSON, 1956; TAUROG et al., 1988; WHITEHOUSE, 2007). Tais semelhanças incluem mudanças histopatológicas, a infiltração celular, a hipersensibilidade e o edema da articulação afetada (DONALDSON et al., 1993; WILSON et al., 2006). Este modelo de artrite induzida por adjuvante permite o estudo da etiopatogenia da artrite autoimune, o prognóstico da doença e possibilita o estudo da efetividade do tratamento (WHITEHOUSE, 2007).

Sabendo-se que o processo inflamatório decorrente da AR promove a ativação das respostas imune e inflamatória, os nucleotídeos extracelulares são moléculas essenciais para o início e para a manutenção das reações inflamatórias, visto que são importantes moléculas sinalizadoras. Os nucleotídeos de adenina (ATP e ADP) 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; RALEVIC; BURNSTOCK, 2003). Estas moléculas interagem com receptores purinérgicos presentes na superfície celular e desencadeiam cascatas de eventos que modulam diversos efeitos biológicos, dentre

eles, a resposta imune, a agregação plaquetária, a inflamação, e a dor (RALEVIC; BURNSTOCK, 1998).

O sistema purinérgico envolve três principais componentes: nucleotídeos e nucleosídeos extracelulares, mediadores da sinalização; receptores, através dos quais estes nucleotídeos e nucleosídeos exercem seus efeitos e, as ectoenzimas, responsáveis pelo controle dos níveis extracelulares destas moléculas (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).

Diferentes tipos celulares, como plaquetas, linfócitos, células endoteliais entre outros, expressam distintos conjuntos de componentes de sinalização purinérgica descritos acima, permitindo a formação de complexos personalizados de sinalização purinérgica (Figura 5) (JUNGER, 2011).

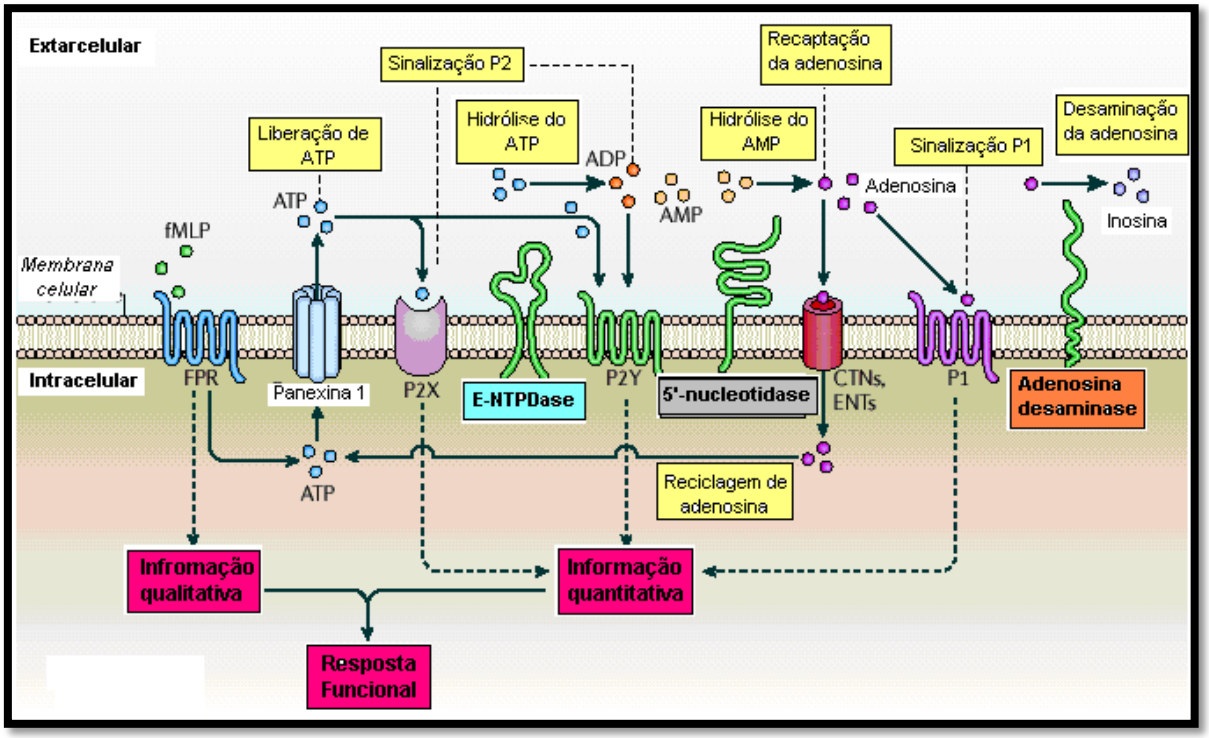

Figura 5 - Representação dos componentes do sistema purinérgico (adaptado de JUNGER, 2011).

Os nucleosídeos são moléculas resultantes da união de uma base nitrogenada (púrica ou pirimídica) a uma pentose. Exemplos destas moléculas

incluem a citidina, a uridina, a guanosina, a timina, a inosina e a adenosina. Quando estes nucleosídeos são fosforilados por quinases específicas formam moléculas denominadas de nucleotídeos (ATKINSON et al., 2008).

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 e células endoteliais danificadas (DUBYAK; EL-MOATASSIM, 1993; WOCHENSCHR, 1989), mas também podem ser liberados frente à injúria 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 (HUNSUCKER et al., 2005; 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., 2006).

Em condições fisiológicas, os nucleotídeos são encontrados no meio extracelular em baixas concentrações (DI VIRGILIO et al., 2001). Já em altas concentrações, podem atuar como uma molécula citotóxica e levar à morte celular, pela formação de grandes poros na membrana plasmática (PODACK et al., 1985; YOUNG et al., 1986).

O ATP possui diversas funções fisiológicas, como a neurotransmissão, a inibição da agregação plaquetária, e induz a secreção de importantes mediadores por parte dos linfócitos T como interferon-γ (INF-γ) e interleucina-2 (IL-2) que estão envolvidos na indução da resposta imune. Também apresenta outros efeitos como contração do músculo liso, inflamação e dor (RALEVIC; BURNSTOCK, 1998). O ATP pode funcionar como uma molécula sinalizadora no controle da inflamação e da resposta imune (DI VIRGILIO et al., 2001). A modulação do processo inflamatório e da resposta imune pelo ATP extracelular é complexa e resulta de efeitos específicos sobre uma grande variedade de células imunes e não imunes. O ATP é capaz de desencadear funções pró-inflamatórias nos neutrófilos, estimular a produção de citocinas inflamatórias como IL-1 e TNF-α e ainda estimular a proliferação de linfócitos (BOURS et al., 2006).

O ADP não possui um papel definido nos linfócitos (DI VIRGILIO et al., 2001). Porém, nas plaquetas, ele age como um importante mediador da agregação

plaquetária e da tromboregulação, podendo ser liberado na circulação sanguínea após danos teciduais (ZIMMERMANN, 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, servindo também como substrato para a formação da adenosina (CUNHA, 2001; 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) e imunossupressor, através da inibição da liberação de citocinas, da adesão de células imune e do funcionamento de linfócitos citotóxicos (CRONSTEIN et al., 1983).

Os nucleotídeos da adenina como ATP e ADP, e o nucleosídeo adenosina não atravessam a membrana celular, mas podem realizar suas ações biológicas através de receptores específicos presentes na superfície celular, denominados receptores purinérgicos (DI VIRGÍLIO et al., 2001).

O ATP extracelular e seus metabólitos são reconhecidos por duas famílias de receptores purinérgicos, 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 (DI VIRGÍLIO et al., 2001). Em mamíferos já foram identificados oito subtipos de receptores P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ e $P2Y_{14}$), sete P2X ($P2X_{1-7}$) e quatro subtipos de receptores P1 (A_1, A_{2A}, A_{2B} e A_3) que foram clonados e caracterizados farmacologicamente (RALEVIC; BURNSTOCK, 1998).

Os receptores P1 reconhecem a adenosina e também são metabotrópicos (BURNSTOCK, 2007). Os receptores subtipos A_{2A} e A_{2B} estão acoplados à 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).

Portanto, o ATP liberado no meio extracelular exercerá seus efeitos ao se ligar a receptores P2X ou a P2Y, e consequentemente será metabolizado à

adenosina por ectoenzimas localizadas na superfície da membrana celular, que controlam seus níveis extracelulares. A adenosina formada, por sua vez, exercerá seus efeitos biológicos através da ativação de receptores P1 (Figura 6) (DI VIRGILIO et al., 2001).

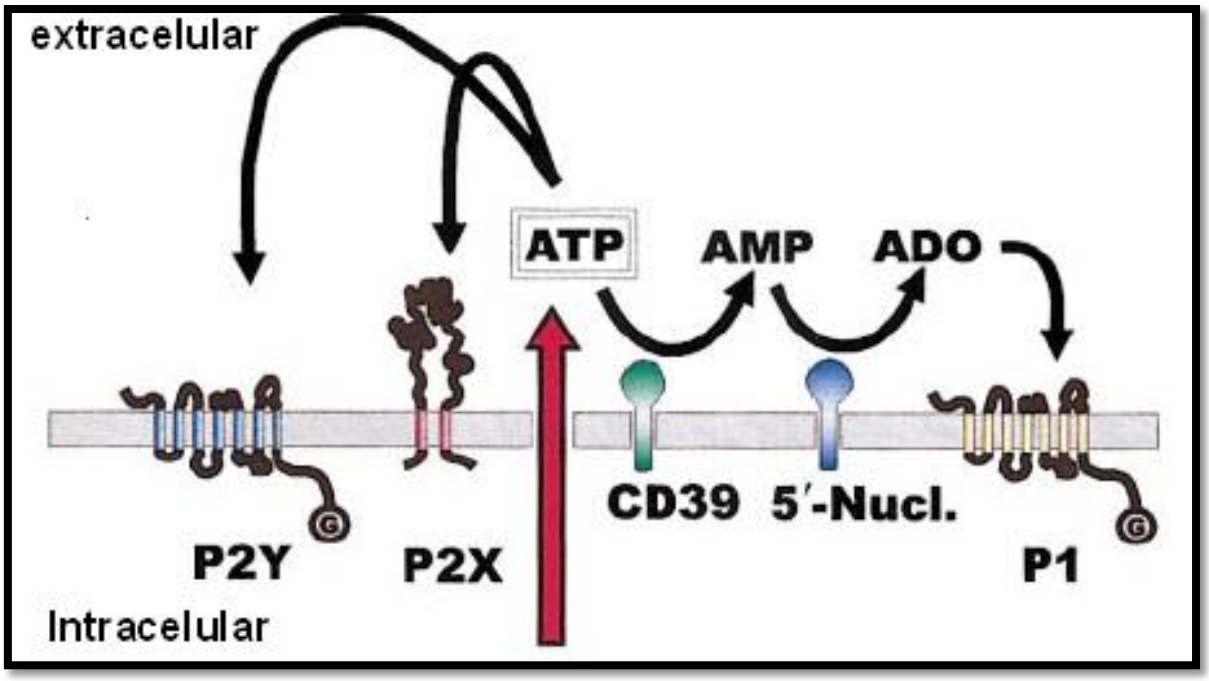

Figura 6 - Liberação de ATP no meio extracelular (DI VIRGILIO et al., 2001).

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). Este controle 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 (eN) (Figura 7). Outra ectoenzima também importante no metabolismo

purinérgico é a adenosina deaminase (E-ADA), responsável pela desaminação do nucleosídeo adenosina (ZIMMERMANN, 2001; ZIMMERMANN et al., 2012).

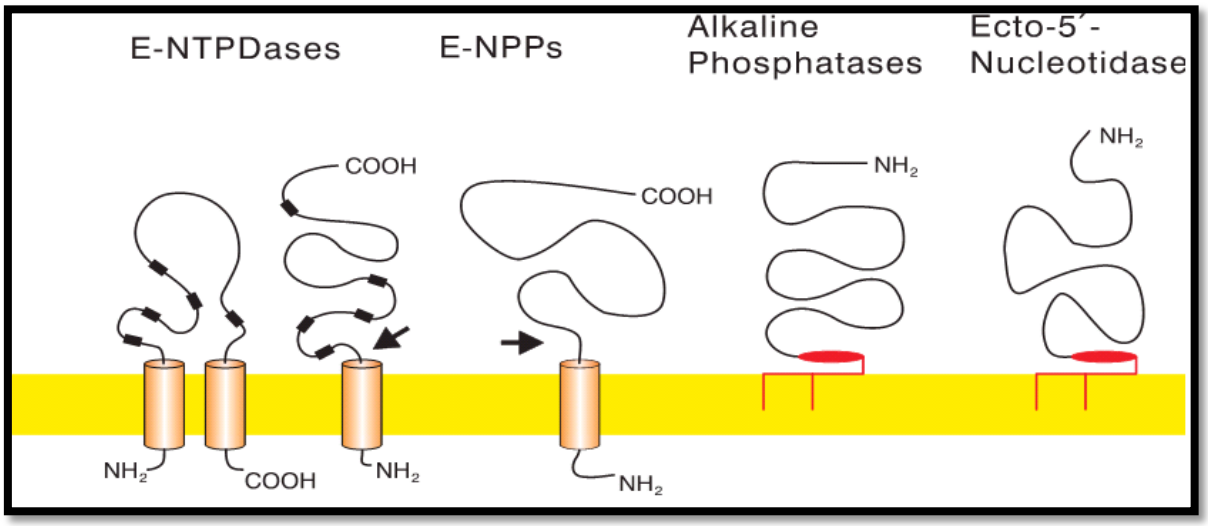

Figura 7 - Topografia das ectonucleotidases (adaptado de Zimmermann, 2001).

Essas enzimas atuam em conjunto formando 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 5'-nucleotidase hidrolisa a molécula do AMP formando adenosina, a qual posteriormente é degradada pela ação da ADA gerando inosina (Figura 8) (YEGUTKIN, 2008).

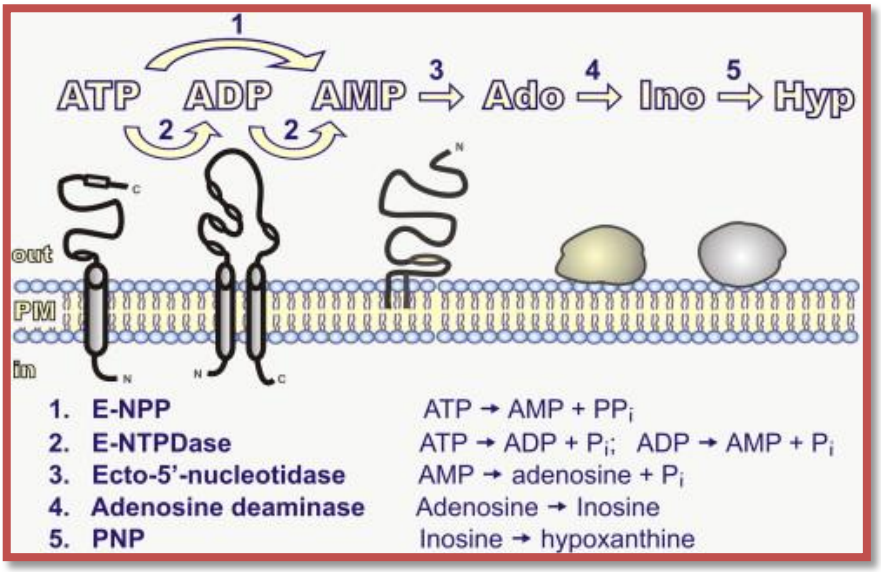

Figura 8 - Enzimas envolvidas na degradação extracelular de nucleotídeos e nucleosídeo da adenina (Adaptado de YEGUTKIN, 2008).

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 (Figura 9) 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) (KUKULSKI et al., 2005; ROBSON et al., 2006; ZIMMERMANN, 2001).

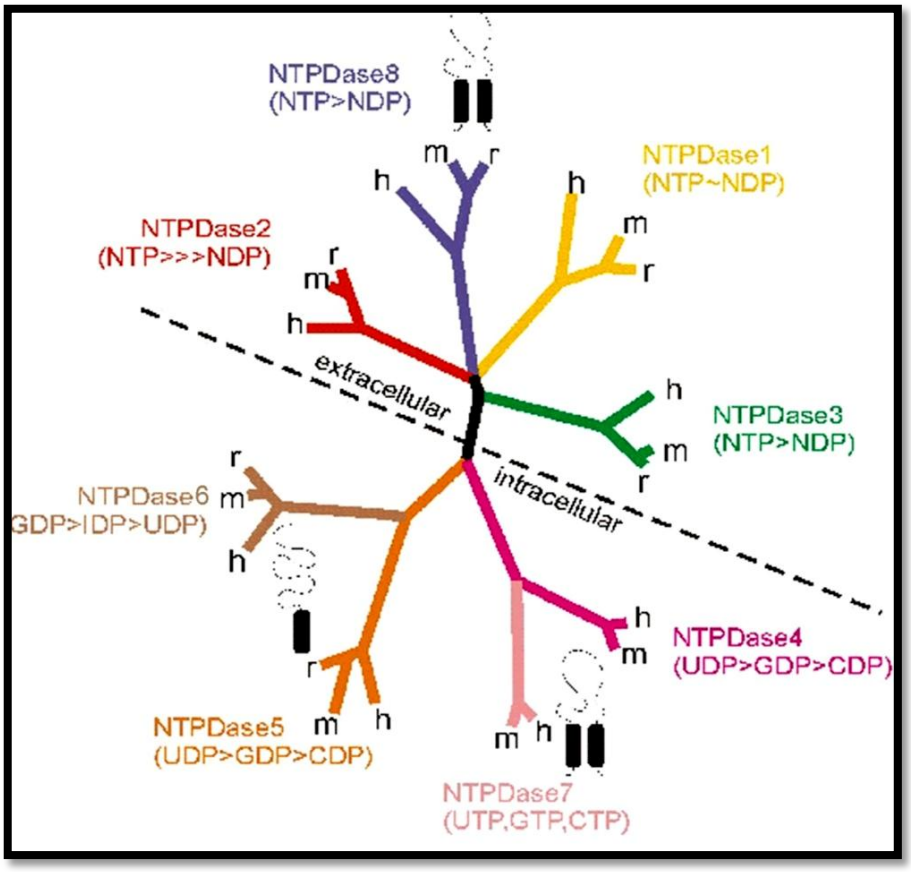

Figura 9 - Membros da família das NTPDases (1 - 8) (Adaptado de ROBSON et al., 2006).

As NTPDases possuem 5 regiões denominadas regiões conservadas da apirase (ACRs), que são locais que apresentam grande similaridade na sequência de aminoácidos (ZIMMERMANN, 2001). Tais regiões estão envolvidas no reconhecimento do substrato, ligação e hidrólise (KIRLEY et al., 2006). As NTPDases são proteínas oligoméricas e apresentam dois domínios transmembrana (domínio I e domínio II), de tamanhos similares, com segmentos NH2 e COOH terminais citoplasmáticos e um grande domínio extracelular com a atividade enzimática formando uma grande fenda que compõe o sítio catalítico (VORHOFF et al., 2005).

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 (DEAGLIO et al., 2007; ROBSON et al., 2006; DWYER et al., 2007; MIZUMOTO et al., 2002).

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). Além disso, Becker e col. (2010) demonstraram um aumento na atividade da E-NTPDase em plaquetas de pacientes com artrite reumatóide, o que reflete no aumento da degradação dos nucleotídeos, possivelmente funcionando como um mecanismo orgânico compensatório.

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).

Após a hidrólise do ATP e ADP pela E-NTPDase, a enzima ecto-5'-nucleotidase (eN, 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 eN é uma proteína homodimericamente ancorada à membrana plasmática via um glicosil fosfatidilinositol (GPI) com seu sítio catalítico voltado para o meio extracelular (Figura 10) (HUNSUCKER et al., 2005; ZIMMERMANN, 2001).

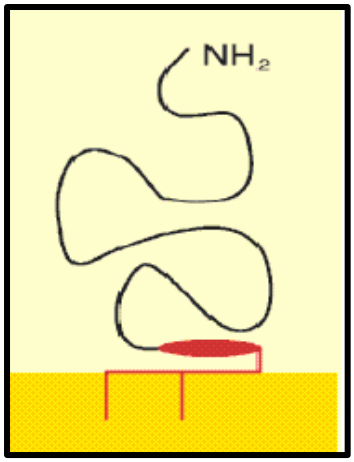

Figura 10 - Estrutura da ecto-5'-nucleotidase ancorada à membrana (Adaptado de ZIMMERMANN, 2001).

Esta enzima possui uma ampla distribuição tecidual, sendo expressa por subpopulações de linfócitos T e B, sendo que sua atividade catalítica varia consideravelmente entre os tecidos (JIN et al., 2010). Como outras enzimas localizadas na superfície celular, a eN, tem sido implicada em funções não-enzimáticas como ativação de células T, adesão célula-célula (RESTA et al., 1998; STRATER, 2006; ZIMMERMANN, 2001) e sinalizações transmembrana (KAWASHIMA et al., 2000).

A enzima adenosina desaminase (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 col. (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

consideravelmente (FOX et al., 1984; FRANCO et al., 1997). Martin e col. (1995) demonstram que a ecto-ADA poderia atuar como uma molécula co-estimulatória, uma vez que, a proliferação celular é acelerada quando as células T periféricas são ativadas na presença da ecto-ADA.

A E-ADA é uma enzima essencial para a proliferação e diferenciação dos linfócitos e monócito-macrófago no sistema imune, e alterações em sua atividade têm sido considerada um indicador de distúrbios imunológicos (HITOGLU et al., 2001; POURSHARIFI et al., 2009). 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 propriedades e particularidades bioquímicas (SHAROYAN et al., 2006). A ADA1 está presente em todos os tecidos humanos, apresentando alta atividade em linfócitos e monócitos, e representa a maior parte da atividade da ADA total, enquanto que a ADA2 é a isoenzima predominante no soro e representa a menor parte da atividade da ADA total em tecidos (ZUKKERMAN et al., 1980).

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 citosólica (Figura 11) (HASKÓ; CRONSTEIN, 2004).

Os receptores de adenosina A1 e A_{2B} também são proteínas responsáveis pelo ancoramento da ecto-ADA à membrana (HERRERA et al., 2001; SAURA et al., 1996). Sugere-se que esta interação aumenta a afinidade da adenosina ao seu receptor específico, e consequentemente permite eficiência nos seus processos de sinalização (FRANCO et al., 1997; ROMANOWSKA et al., 2007). Segundo VARANI e col. (2011), um aumento na densidade dos receptores A_{2A} e A_3AR foi observado e associado com respostas celulares inflamatórias.

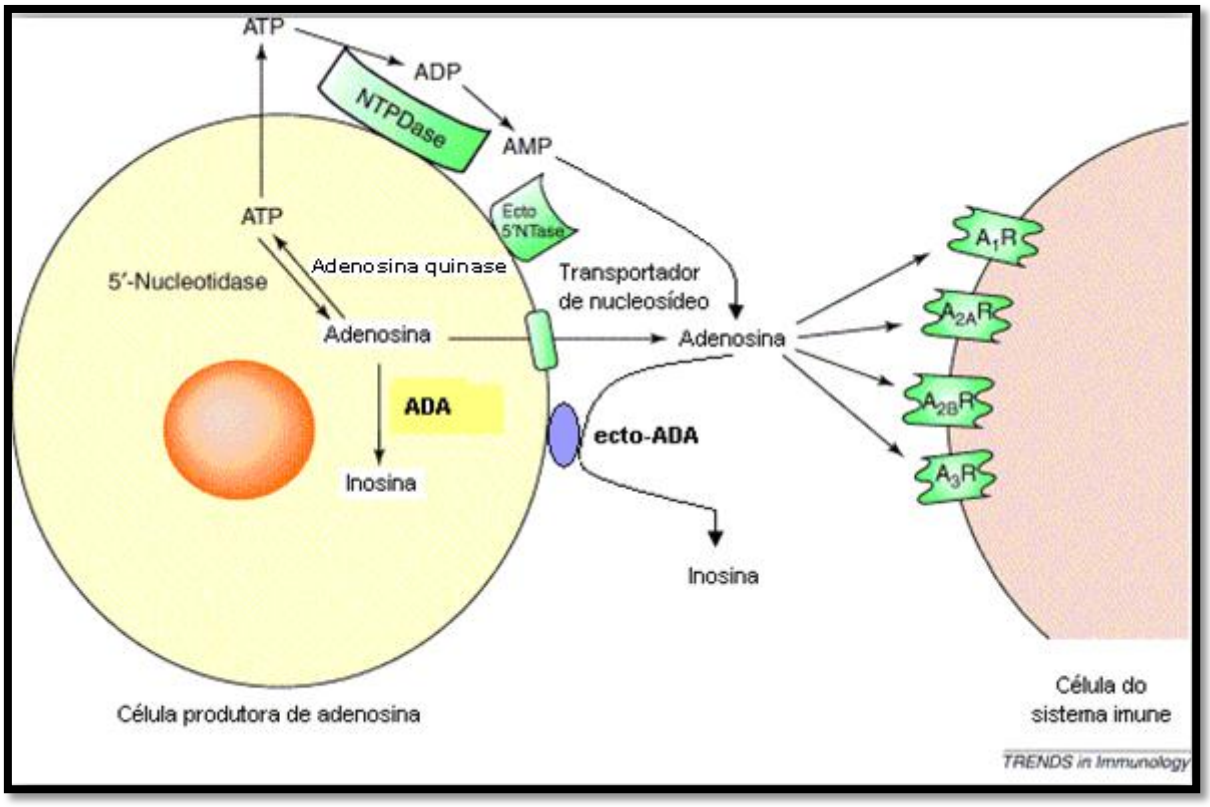

Figura 11 – Vias envolvidas no metabolismo da adenosina (Adaptado de HASKÓ; CRONSTEIN, 2004).

Enzimas do sistema purinérgico, como a E-NTPDase e a E-ADA, estão presentes na membrana dos linfócitos desempenhando um importante papel na resposta inflamatória. Considerando que a AR é uma doença caracterizada por sinovite crônica com consequente amplificação do processo inflamatório e destruição articular, é de interesse clínico investigar a ação terapêutica dos compostos com propriedade anti-inflamatória, como os da *U. tomentosa*. Até o momento existem poucos estudos demonstrando os efeitos da *U. tomentosa* no metabolismo de nucleotídeos da adenina. Portanto, é relevante investigar o efeito do extrato na atividade da E-NTPDase e a E-ADA em linfócitos de ratos submetidos a modelo experimental de AR.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar o efeito do extrato de *Uncaria tomentosa* (Wild.) D.C no metabolismo de nucleotídeos de adenina em linfócitos de ratos submetidos a um modelo experimental de artrite reumatóide.

2.2 Objetivos específicos

- Em ratos submetidos a modelo experimental de artrite e tratados com extrato de *Uncaria tomentosa:*

- Determinar a atividade das enzimas NTPDase e ADA em linfócitos;
- Quantificar nucleotídeos e nucleosídeo da adenina no soro através de Cromatografia Líquida de Alta Eficiência (HPLC).

3 MANUSCRITO

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

O manuscrito será submetido ao Journal of Ethnopharmacology.

Effect of *Uncaria tomentosa* extract in the activity of E-NTPDase and ADA in lymphocytes of rats submitted to experimental model of rheumatoid arthritis

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Abstract

Ethnopharmacological relevance: There are few studies demonstrating the effect of *Uncaria tomentosa* on the metabolism of adenine nucleotides. During the inflammatory process the nucleotides and nucleoside are regulated by an enzyme complex located on cell surface, modulating the lymphocytes action. The extract of *U. tomentosa* has anti-inflammatory properties and it has been widely used in cases of rheumatoid arthritis (RA).

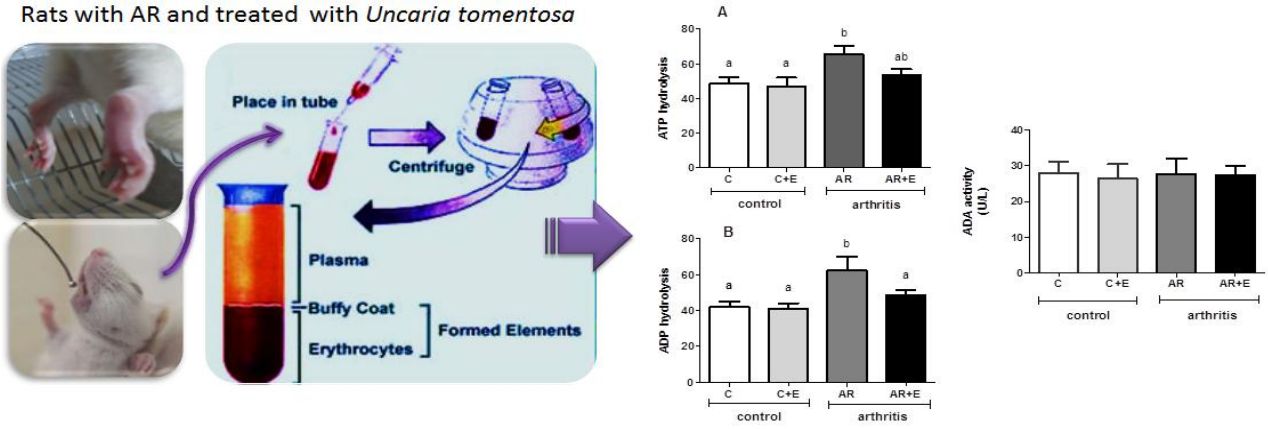
Aim of the study: To evaluate the effect of the *Uncaria tomentosa* extract on the activity of E-NTPDase and ADA in lymphocytes of rats submitted to experimental model of RA.

Material and methods: Rats had RA-induced and were treated with the extract. Peripheral lymphocytes were isolated and E-NTPDase and E-ADA activities were determined.

Results: E-NTPDase activity was increased in rats with RA-induced. In rats with RA-induced that received the *U. tomentosa* extract, the results were similar to control. The *U. tomentosa* extract did not alter neither E-NTPDase nor E-ADA activity in healthy animals.

Conclusions: The increase in E-NTPDase activity might be related to the attempt to maintain basal levels of ATP and ADP in basal levels in the extracellular medium, since the RA induction causes tissue damage and consequently large amounts of ATP in the cell. This way, the *Uncaria tomentosa* extract was able to prevent the increase in the E-NTPDase activity promoting RA induction.

Graphical abstract:



Keywords: Rheumatoid arthritis; *Uncaria tomentosa*; lymphocytes; E-NTPDase; adenosina desaminase

1. INTRODUCTION

Uncaria tomentosa (Willd.) DC. is a giant vine of the Rubiaceae family that grows in the Amazon rainforest and because of its curved thorns it is commonly known as ‘cat’s claw’ or ‘uña de gato’. This species among several Peruvian tribes has been extensively used for the treatment of many ailments, such as arthritis and other inflammatory disorders (Reinhard, 1999). In most of the latest studies, its anti-inflammatory activity has been uniquely attributed to tetracyclic and pentacyclic oxindole alkaloids. However, currently available pharmacological data have indicated that this biological activity is due to the synergistic action of several compounds present in this species (Wagner et al, 1985; Aquino et al, 1989, 1991; Laus et al, 1997; Falkiewicz and Lukasiak, 2001).

Rheumatoid arthritis (RA) is a human autoimmune disease that affects the synovial membranes of the peripheral joints. RA characteristically involves the infiltration of leukocytes into the synovium, which undergo inflammation and swelling (Feldmann, 1996). During the inflammatory process, the immune and inflammatory responses are active and it is well known that an imbalance between pro- and anti-inflammatory cytokine activities favors the induction of autoimmunity, chronic inflammation and thereby joint damage (Iain and Georg, 2007).

Extracellular nucleotides are essential molecules for the onset and maintenance of inflammatory reactions, whereas they are important signaling molecules (Luttikhuizen, 2004). The purinergic signaling system plays an important role in modulating the inflammatory and immune responses by extracellular biomolecules such as adenine nucleotides (ATP, ADP and AMP) and their derived nucleoside adenosine (Ralevic and Burnstock, 2003). Evidence indicates that high extracellular ATP level acts through specific cell surface receptors as a pro-inflammatory agent that potentiates the release of pro-inflammatory cytokines (Bours et al, 2006) from activated lymphocytes (Langston et al, 2003).

Extracellular ATP and adenosine levels as well as the ensuing purinergic signaling can be dynamically controlled during inflammation by the action of enzymes expressed in immune cells (Bours et al, 2006). E-NTPDase (CD39) is the membrane-bound enzyme involved in the breakdown of ATP and ADP to AMP, which is

sequentially hydrolyzed by 5'-nucleotidase to adenosine (Robson et al, 2006; Zimmermann et al, 2007; Yegutkin, 2008).

E-ADA is another important enzyme that catalyzes the irreversible deamination of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine respectively, therefore contributing to the removal of adenosine from the extracellular compartment (Franco et al, 1997). This enzyme has fundamental biological role in the proliferation and differentiation of lymphoid cells, particularly T lymphocytes, and maturation of monocytes (Bota et al, 2001), performing an important function in the immune system and inflammatory processes (Antonioli et al, 2008).

Considering that RA is a disease characterized by chronic synovitis and therefore amplification of inflammation and joint destruction, it is of clinical interest to investigate the therapeutic action of compounds with anti-inflammatory properties, such as *Uncaria tomentosa*. Until the moment there are few studies demonstrating the effect of *Uncaria tomentosa* on the metabolism of adenine nucleotides. Thus, it is relevant to investigate its effect on the activity of E-NTPDase and E-ADA in lymphocytes of rats submitted to an experimental model of RA.

2. MATERIAL AND METHODS

2.1 Chemicals

Complete Freund Adjuvant (CFA - 0.6% suspension of heat-killed *Mycobacterium tuberculosis* in liquid paraffin), 5-(N,N-diethylamino) pentyl-3,4,5-trimethoxybenzoate (TMB), hexadecyltrimethylammonium bromide (HTAB), the substrates ATP, ADP, adenosine, as well as Trizma base, Coomassie Brilliant Blue G and bovine serum albumin were obtained from Sigma Chemical Co (St. Louis, MO, USA) and K_2HPO_4, from Reagen. All the other chemicals used in this experiment were of the highest purity.

2.2 Animals

Twenty eight adult female Wistar rats (200-300g) were used in this experiment. Animals were divided into four groups, namely, control (C); extract (E); arthritis (AR); and arthritis associated with extract (AR+E). Animals were kept on a 12-h light/12-h

dark cycle, at a temperature of 22±2°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), in accordance with international guidelines and were approved by the Committee on the Use and Care of Laboratory Animals of our university (n. 125/2010(2)).

2.3 Induction of arthritis

To investigate the effect of the extract of *Uncaria tomentosa* over the inflammatory process, the adjuvant-induced arthritis model was used and described by Sauzem et al., 2009. Animals were slightly anesthetized with halotane and 100 µL of Complete Freund Adjuvant (CFA - 0.6% suspension of heat-killed *Mycobacterium tuberculosis* in liquid paraffin) was injected into the right hind paw to induce arthritis.

2.4 Treatment with *Uncaria tomentosa extract*

The treatment of animals with extract began 15 days after induction of arthritis by CFA. The *Uncaria tomentosa* root dry extract was a donation from Herbarium Botanical Laboratory, PR-Brazil, lot number 991260. The extract was prepared daily with water as vehicle and administered into groups E and AR+E by gavage two times a day at the dose of 150mg/kg for 45 days, mimicking the Unha de Gato® phytotherapeutic from Herbarium Botanical Laboratory, indicated for treatment of patients with RA. The C and AR groups were treated with water in the same condition.

2.4 Evidences of induction

Evidences of induction as mechanical sensitivity and paw thickness of each rat were evaluated briefly before induction of arthritis by CFA and then 15 days after induction whereas an increase in the mechanical sensitivity and paw thickness were considered as markers of the inflammatory process. Moreover, these measurements were made 45 days after *U. tomentosa* treatment to observe the effect of *U. tomentosa* over the inflammatory process. To observe the development of edema, animals were held and the right hind paw thickness was measured using a digital calipter (Cao, 1998). An increase in the thickness was considered as formation of

edema. Mechanical allodynia was evaluated using the up-and-down method, described by Dixon (1980), using von Frey filaments. Briefly, rats were placed in cages with a wire mesh bottom which allowed full access to the paws. The paw was touched with 1 of a series of 7 von Frey hairs with logarithmic increments (6, 8, 10, 15, 26, 60 and 100). Von Frey hairs were applied perpendicularly to the plantar surface with sufficient force to cause slight buckling against the paw, and held for approximately 2–4 s. Stimuli were presented at intervals of several seconds, allowing for apparent resolution of any behavioral responses to previous stimuli. To evaluate neutrophil infiltration, mieloperoxidase activity (MPO) was evaluated in paw skin sample, as described by Suzuki et al. (1983). Briefly, sample was homogenized in acetate buffer (80 mM, pH 5.5) containing 0.5% HTAB and centrifuged at 16.000 xg during 20 minutes at 4°C. After, 10 µL of supernatant were added to 200 µL of acetate buffer and 20 µL of TMB (18.4 mM) and incubated at 37ºC for 3 minutes. To stop the reaction, microplate were taken to the ice bath and 30 µL of acetic acid were added. The color formed was assessed at 630 nm and the results were expresses as optical density per mg of tissue (OD / mg tissue).

2.5 Isolation of lymphocytes from blood

Rats were anesthetized with isoflurane and blood was collected by cardiac puncture. Blood was collected with 7.2 mg dipotassium EDTA as anticoagulant and lymphocyte-rich mononuclear cell were isolated from blood collected with Ethylenediamine tetraacetic acid (EDTA) and separated on Ficoll-Histopaque density (Böyum, 1968) as previously described. The percentage of lymphocytes was superior to 93% as previously described (Jaques et al., 2011). The integrity of the 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 instructions. Triton X-100 (1%, final concentration) was used to disrupt the lymphocytes preparation. The enzymatic activity is expressed as units per liter, and one unit (1U) corresponds to 1 µmol of NADH formed per minute per liter.

2.6 Protein determination

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

2.7 E-NTPDase activity determination

After lymphocytes isolation, the E-NTPDase activity was determined as previously described by Leal et al (2005), in which 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, with a final volume of 200 µL. Twenty microliters of the intact mononuclear cells suspended in saline solution was added to the reaction medium (2-4 µg of protein), and pre-incubated for 10 min at 37^{o}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 µL of 10% trichloracetic acid (TCA). The released inorganic phosphate (Pi) was assayed by a method previously described by Chan et al (1986) using malachite green as colorimetric reagent and KH_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 in triplicate and the specific activity is reported as nmol of Pi released/min/mg of protein.

2.8 Adenosine deaminase activity determination (ADA)

ADA activity was measured spectrophotometrically 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 1 h at 37ºC. The reaction was stopped by adding 106 mM and 167.8 mM sodium nitroprussiate and hypochlorite solution. Ammonium sulfate of 75 µM was used as ammonium standard. All the experiments were performed in triplicate and the values were expressed in U/L for ADA activity. One unit (1U) of ADA is defined as the amount of enzyme required to release 1 mmol of ammonia per minute from adenosine at standard assay conditions.

2.9 Separation of blood serum

Rats were anesthetized with isoflurano and blood was collected by cardiac puncture. The blood samples were colleted in tubes without anticoagulant and after the clot formation were centrifuged at 1400 g for 15 min at room temperature. The resultant serum samples were aliquotted in microtubes and kept on ice until the purines quantification.

2.10 Purine level measurement

The quantitative determination of adenine nucleotides and adenosine levels were performed in serum blood by HPLC. At first, proteins were denatured by the addition of 0.6 mol/L of perchloric acid. Then, all samples were centrifuged (14000 x g for 10 min). The obtained supernatants were neutralized with 4 N KOH and clarified with a second centrifugation (14000 x g for 15 minutes). Aliquots of 40 µL were applied to a reversed-phase HPLC system using a 25 cm C18 Shimadzu column (Shimadzu, Japan) at 260 nm with a mobile phase containing 60 mM KH_2PO_4, 5 mM tetrabutylammonium chloride, pH 6.0, in 30% methanol according to a method previously described by Voelter (1980). The peaks of purines (ATP, ADP, AMP and adenosine) were identified by their retention times and quantified by comparison with standards. Results are expressed as nmoles of the different compounds per mL of serum.

2.11 Statistical analysis

Data were analyzed by a one- or two-way ANOVA. Post hoc analyses were carried out by the Student's Newman-Keuls (SNK) Multiple Comparison Test. Purine level were log-transformed to achieve normal distribution of data. A probability of $P<0.05$ was considered significant. All data are ex*p*ressed as mean ± Standard Error of the Mean (SEM).

3. Results

3.1 Evidences of AR induction and effect of *Uncaria tomentosa* extract

As shown in Figure 1, CFA injection was capable of increase mechanical sensitivity (mechanical allodynia) (Figure 1A), paw thickness (Figure 1B) and MPO activity (Figure 1C), in 72.4%, 120.1% and 35.3% respectively when compared basal" to basal characterizing an arthritic process. After 45 days of treatment, *Uncaria tomentosa* extract was able to partially reverse the mechanical allodynia (23.1 ± 2.7%, $P<0.01$), edema (21.6 ± 5.7%, $P<0.001$) and MPO activity (35.5 ± 5.2%, $P<0.05$).

3.2 E-NTPDase activity determination

Figure 3 shows the effect of oral administration of *Uncaria tomentosa* extract on ATP and ADP hydrolysis by E-NTPDase in lymphocytes of rats submitted to an experimental model of RA. Results of lymphocytes E-NTPDase activity with ATP as substrate are shown in Figure 3A. As can be observed, the hydrolysis of ATP was altered in rats with AR (65.5 nmol of Pi/min/mg of protein; SEM=4.8; n=7; $P<0.05$), demonstrating that ATP hydrolysis was increased in 25.7% when compared to the control group (C) (48.7 nmol of Pi/min/mg of protein; SEM=3.2; n=7; $P<0.05$) and in 28.2% when compared to the extract group (E) (47 nmol of Pi/min/mg of protein; SEM=4.9; n=7; $P<0.05$). However, two-way ANOVA showed no significant interaction [$F(1,15)=1.458$; $p=0.246$; n=7] among the variables. In addition, results obtained for the lymphocytes E-NTPDase activity with ADP as substrate are shown in figure 3B, where the ADP hydrolysis was also increased in 32.5% in the AR group (62.4 nmol Pi/min/mg; SEM=7.6; n=7; $P<0.05$) when compared to C (42.1nmol of Pi/min/mg of protein; SEM=3.0; n=7; $P<0.05$), in 34.3% when compared to E group (41.0 nmol of Pi/min/mg of protein; SEM=2.9; n=7; $P<0.05$) and in 22% when compared to AR+E group (48.7 nmol of Pi/min/mg of protein; SEM=2.7; n=7; $P<0.05$). Two-way ANOVA showed no significant interaction [$F(1,13)=2.606$; $p=0.130$; n=7]. The results of lymphocytes E-NTPDase activity in the group E with both ATP substrate (47.0 nmol of Pi/min/mg of protein; SEM=4.9; n=7; $P<0.05$) and ADP (41.0 nmol of Pi/min/mg of protein; SEM=2.9; n=7; $P<0.05$) as substrate were similar to ATP (48.7 nmol of Pi/min/mg of protein; SEM=3.2; n=7; $P<0.05$) and ADP control (42.1nmol of Pi/min/mg of protein; SEM=3.0; n=7; $P<0.05$), showing that in healthy rats the extract did not alter the E-NTPDase activity.

3.3 Adenosine deaminase activity determination (ADA)

Results obtained for adenosine hydrolysis by E-ADA are shown in Figure 4. As can be seen, adenosine hydrolysis was not altered. The groups showed no significant alterations in the E-ADA activity when adenosine was used as substrate. Two-way ANOVA showed no significant interaction [F(1,15)=1.572, p=0.229, n=7].

3.4 Purine level measurement

Purine levels in serum were measured by HPLC. Levels of ATP, ADP, AMP and adenosine showed no significant alterations in the AR group when compared to control. The levels of these nucleotides and nucleoside were also normal in the E group when compared to control, showing that the extract did not interfere in the purine level. However, in the AR+E group, the level of ATP and adenosine was decreased, whereas ADP levels were increased (Table 1).

4. Discussion and Conclusion

Experimental animal models of chronic diseases allow better understanding of the physiopathologic processes, and the evaluation of potential new therapies. *Uncaria tomentosa,* because of its well known anti-inflammatory effects, has been widely used for the treatment of arthritis, rheumatism, inflammatory diseases, among others (Akesson et al, 2005, Jurgensen et al, 2005; Pilarski et al, 2006).

Based on that, the model of arthritis induced by Complete Freund's Adjuvant (CFA) in rats is a model widely used in the research of new therapies for chronic inflammatory arthropathies, such as RA (Joe et al, 1999). In this study, we induced arthritis in animals by the use of CFA and the inflammatory process was confirmed through the measurement of increased paw thickness, mechanical thresholds and MPO activity (neutrophil marker), what characterizes an arthritic process. After that, we analyzed the ability of *U. tomentosa* extract to reverse this process. We observed that *U. tomentosa* was able to partially reverse this process. Similar results are also observed with carrageenan induced paw edema demonstrating that both hydro-alcoholic and aqueous extract have an anti-inflammatory activity by decreasing the carrageenan-induced increase in paw volume when compared with control rats (Keplinger et al, 1999; Aquino et al, 1991; Aguilar et al, 2002). Human tests with *U.*

tomentosa were also carried out in patients with osteoarthritis and RA demonstrating that it was able to reduce pain, morning stiffness and swelling joints (Castañeda et al, 1998; Piscoya et al, 2001; Mur et al, 2002).

Extensive tissue damage in inflammatory processes may lead to a significant increase in the levels of purine and pyrimidine nucleotides on the sites involved, probably contributing to the amplification of the inflammatory reaction (Miyara and Sakaguchi, 2007). Extracellular ATP can act as a damage-associated molecular patterns, given that it is normally confined to intracellular sites but can be released at high local levels following cell lysis, infection, or via regulated efflux. ATP released into the extracellular space can modulate the immune response through its capacity to bind and activate multiple nucleotide receptor family members (la Sala et al, 2003; Gordon, 1986).

While the enzymes E-NTPDase and E-ADA constitute a multiple system for extracellular nucleotide hydrolysis, the increase of these activities reflects an increased degradation of nucleotides as a compensatory organic response. The results of the present study show an increase in the E-NTPDase activity in rats with induced-RA when compared to control. Corroborating our results, Becker et al (2010) found increased E-NTPDase activity in platelets of patients with RA.

Also, many other studies have shown that the E-NTPDase and E-ADA have significant roles in immune response. Alterations in their activities have been observed in some autoimmune diseases such as multiple sclerosis, lupus and diabetes (Loza et al, 2011; Schmatz et al, 2009; Spanevello et al, 2010). The increased activity of E-NTPDase lead the hydrolysis of the nucleotides ATP and ADP, as a compensatory mechanism, leading to the maintenance of their appropriate levels since that, the ATP released to the extracellular medium at high concentrations activates the pro-inflammatory purinergic P2X7 receptors and contributes to tissue damage and inflammation (Di Virgilio, 1995).

Knowing that these enzymes act in a cascade, in this data we can suggest that the 5’-nucleotidase activity in rats with induced-RA could be also increased resulting in a greater amount of adenosine in the extracellular medium to offset the pro-inflammatory effects of ATP. However, in this same group the levels of ATP, ADP, AMP and adenosine are normal in the extracellular medium as well as E-ADA activity. It is supposed that the adenosine is being produced in excess by the increased activity

of 5'-nucleotidase supposed. This adenosine could be binding to specific receptors expressed on the cell surface exercising its anti-inflammatory function and maintaining the levels of adenosine normal on extracellular medium. Thus, adenosine acts as a negative feedback signal to counteract ATP-mediated immune stimulation, preventing uncontrolled inflammation and lessening the collateral damage to healthy tissues (Gessi et al, 2007).

Knowing that *U. tomentosa* has an anti-inflammatory property, we evaluated the effect of the *U. tomentosa* dry extract on the metabolism of adenine nucleotides. In healthy animals that received the treatment with extract, the activities of E-NTPDase and E-ADA were maintained at basal levels, what was also confirmed by purine levels on the serum measured by HPLC showing that these levels are in normal concentration in the extracellular medium.

Taking into account the group of arthritic rats that received the *U. tomentosa* dry extract, we can note that the extract was able to prevent the increase on the E-NTPDase activity although, the purine levels on the serum showed that ATP levels are decreased and ADP levels are increased. It seems that low-level purinergic signaling induced by nucleotides at decreased concentrations, modulates ongoing inflammatory and immune responses by P2 receptors (Di Virgilio et al, 2003). At low concentration, extracellular ATP possesses affinity for P2Y receptor subtype on the surfaces of lymphocytes. These purinergic receptors, when stimulated, develop a down-modulation of pro-inflammatory cytokines and stimulate the Th2 immune response, leading to the production of anti-inflammatory cytokines, protection from oxidative damage and down-production of oxygen radicals in whole blood (Bours et al, 2006). P2Y receptor signaling may therefore be an important stop signal to prevent excessive stimulation of inflammation and avoid conditions that might favor autoimmunity (Di Virgilio et al, 2009).

No changes were observed in the ADA activity, but a decrease in adenosine levels was observed in serum of the arthritic rats that received *U. tomentosa* extract. Corroborating our results, a study previously published by our research group (Becker et al, 2010) has showed that the level of adenosine in serum of RA patients was also decreased. In addition, in this same group the ADP levels showed to be increased. What may be happening is that the adenylate kinase (EC 2.7.4.3) could be activated in an attempt to reconstitute the pool of ADP. As proposed by YEGUTKIN et al (2012), an

opposite via could lead to the recovery of adenine nucleotides, since that adenylate kinase was identified as another key player in the metabolism of circulating ADP. This extracellular ADP could be linking to P2Y receptor, the adenine-nucleotide-preferring receptors mainly responding to ADP, and leading the anti-inflammatory response.

In conclusion, our data demonstrate that the *U. tomentosa* extract was able to reduce partially the paw thickness, mechanical thresholds and MPO activity in a model of RA-induced. In addition, the extract was able to prevent the increase on the E-NTPDase activity in lymphocytes of rats submitted to an experimental model of RA.

Acknowledgments

The authors wish to thank the HERBARIUM Laboratório Botânico LTDA to supply the extract used to perform the experiment. This study was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Rio Grande do Sul (FAPERGS), Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and PRONEX, Brazil.

Conflicts of Interest statement

There are no actual or potential conflicts of interest.

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Figure Legends

Figure 1- Evidences of AR induction and the effect of *U. tomentosa* extract treatment over the inflammatory process induced by CFA. A) Mechanical sensitivity, B) paw edema and C) MPO activity before (C) and after CFA injection (AR), as well as the effect of *Uncaria tomentosa* treatment (150 mg/kg, 2 times a day, v.o.) for 45 days over these parameters. * $P<0.05$, with n=7 in comparison to AR group, analyzed by One Way ANOVA followed by Student Newman Keuls (SNK).

Figure 2- (A) ATP hydrolysis in lymphocytes of rats with CFA induced arthritis and treated for 45 days with *Uncaria tomentosa* extract in the dose of 150mg/kg 2 times a day. (B) ADP hydrolysis in lymphocytes of rats with CFA induced arthritis and treated for 45 days with *Uncaria tomentosa* extract in the dose of 150mg/kg 2 times a day. Enzyme specific activities are reported as nmol of Pi released/min/mg of protein. Groups: C (control), E (extract), AR (arthritis) and AR+E (arthritis + extract). Bars represent mean S.E.M. (^{a,b}) Indicates a significant $P<0.05$, with n=7 (one-way ANOVA-Newman-Keuls Multiple Comparison Test).

Figure 3- Adenosine hydrolysis in lymphocytes of rats with CFA induced arthritis and treated for 45 days with *Uncaria tomentosa* extract in the dose of 150mg/kg 2 times a day. Enzyme activities are reported as U/mg of protein. Groups: C (control), E (extract), AR (arthritis) and AR+E (arthritis + extract). Bars represent mean S.E.M. (^{a,b}) Indicates a significant $P<0.05$, with n=7 (one-way ANOVA-Newman-Keuls Multiple Comparison Test).

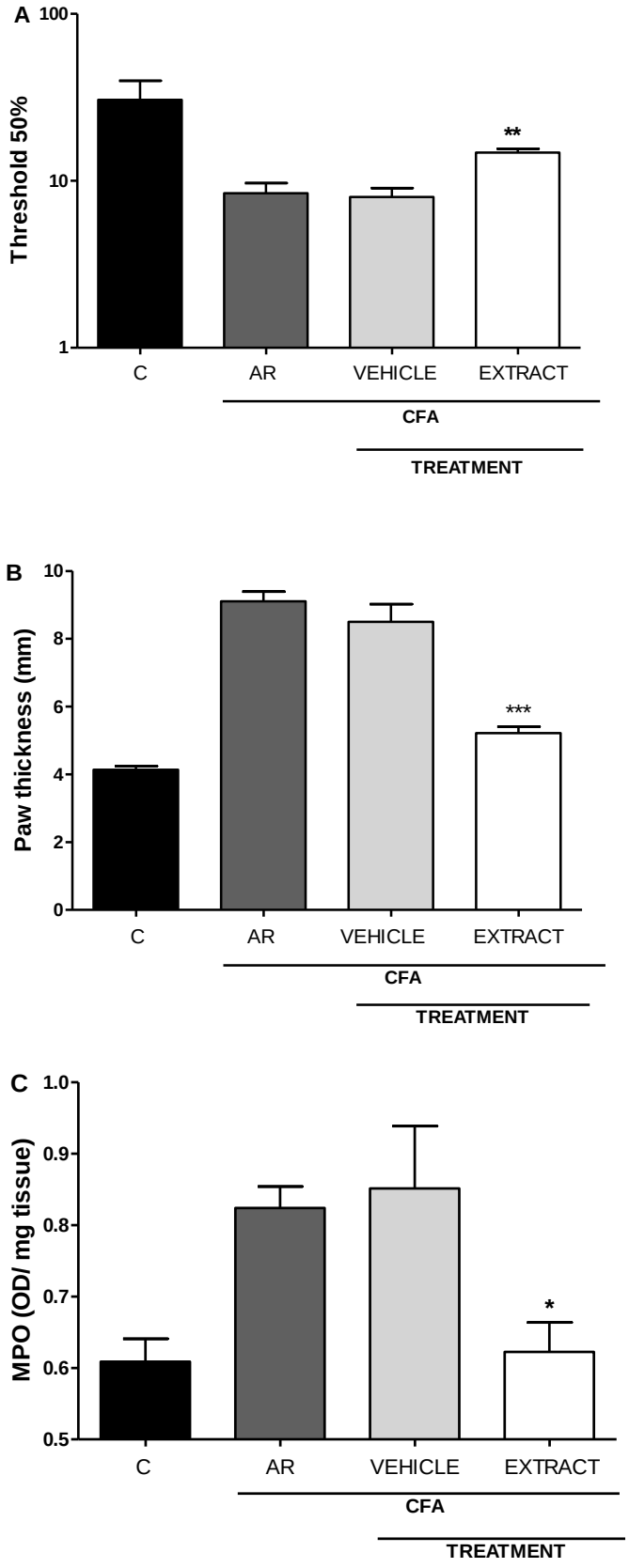
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Figure 1

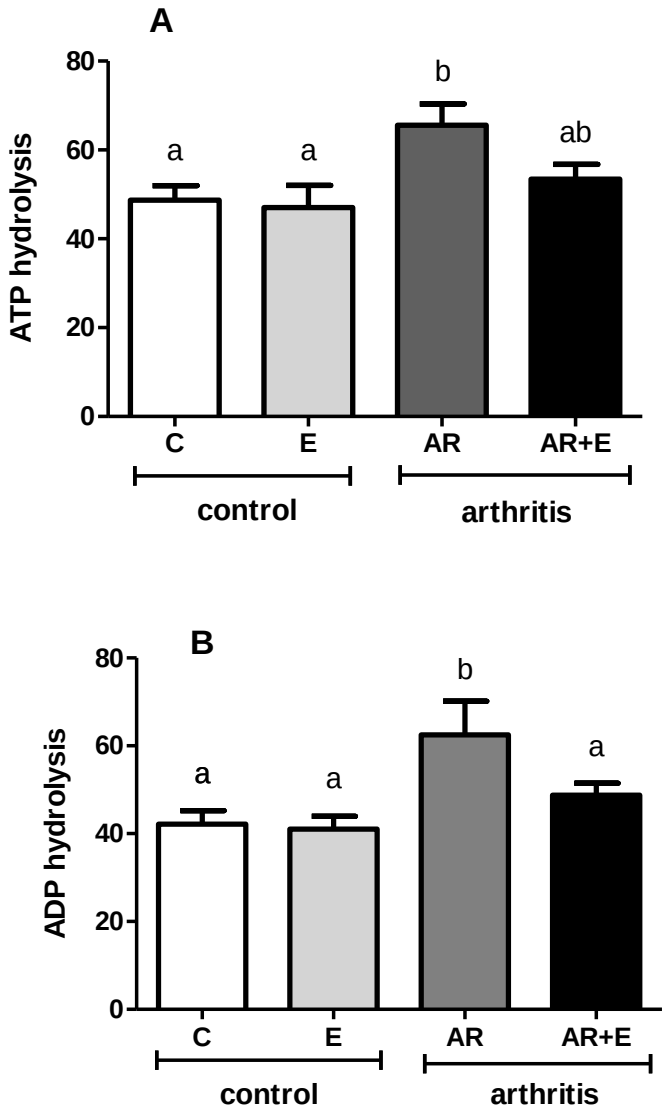
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Figure 2

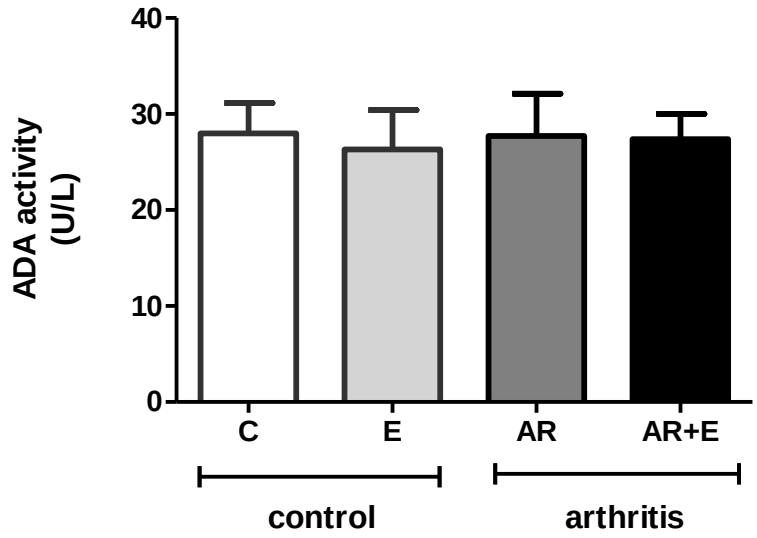

Figure 3

Table 1:

Purine level measurement: adenine nucleotides and adenosine levels measurement in rats with RA-induced and treated with *Uncaria tomentosa* extract.

	C (log of nmol/ml)	**E** (log of nmol/ml)	**AR** (log of nmol/ml)	**AR+E** (log of nmol/ml)
ATP	1.04±0.003[a]	1.02±0.01[a]	1.04±0.02[a]	0.89±0.01[b]
ADP	1.25±0.002[a]	1.28±0.01[a]	1.25±0.01[a]	1.34±0.001[b]
AMP	1.00±0.005	1.05±0.008	1.04±0.008	1.02±0.02
Adenosine	1.35±0.001[a]	1.29±0.02[ab]	1.33±0.01[ab]	1.11±0.08[b]

The measurement of purine levels from serum of rats with CFA induced arthritis and treated for 45 days with *Uncaria tomentosa* extract in the dose of 150mg/kg 2 times a day was addressed using HPLC methodology. Purine levels measurement were log-transformed and are reported as log of nmol/ml. Groups: C (control), E (extract), AR (arthritis) and AR+E (arthritis + extract). Bars represent mean S.E.M. ([a,b]) Indicates difference among the groups, $P<0.05$ with n=7 (one-way ANOVA-Newman-Keuls Multiple Comparison Test).

3 CONCLUSÕES

- O modelo de indução da artrite por adjuvante completo de Freund mostrou-se capaz de mimetizar um processo artrítico, o qual foi comprovado pelo desenvolvimento do edema, aumento da alodínia mecânica, atividade da mieloperoxidase e pela histologia. O extrato de *Uncaria tomentosa* mostrou ser capaz de reverter parcialmente estes processos.

- O modelo experimental de AR não causou alterações na atividade da ADA, assim como o tratamento com o extrato também não causou efeito na atividade dessa enzima.

- O aumento da atividade da NTPDase nos ratos com AR induzida está relacionado com a tentativa de manter as concentrações basais de ATP e ADP no meio extracelular uma vez que a indução da AR causa dano tecidual e, consequentemente a liberação de grandes quantidades de ATP presentes no interior da célula.

- O extrato de *U. tomentosa* por sua vez foi capaz de prevenir o aumento na atividade da E-NTPDase causado pela indução da AR, e níveis diminuídos de ATP e adenosina, assim como níveis aumentados de ADP foram observados no meio extracelular.

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Anexos

Anexo A - Carta de Aprovação pelo Comitê de Ética

UNIVERSIDADE FEDERAL DE SANTA MARIA
PRÓ-REITORIA DE PÓS-GRADUAÇÃO E PESQUISA
COMITÊ INTERNO DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL-UFSM

CARTA DE APROVAÇÃO

O Comitê Interno de Ética em Experimentação Animal-UFSM, analisou o protocolo de pesquisa:

Título do Projeto: "Efeito dos extratos de uncaria tomentosa (wild) d.c. na hidrólise de nucelotideos de adenina em linfócitos"

Numero do Parecer: 125/2010(2)

Pesquisador Responsável: Daniela Bitencourt Rosa Leal

Este projeto foi **APROVADO** em seus aspectos éticos e metodológicos. Toda e qualquer alteração do Projeto, assim como os eventos adversos graves, deverão ser comunicados imediatamente a este Comitê.

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

DATA DA REUNIÃO DE APROVAÇÃO:

S .nta Maria, 14 de Março de 2011.

Marta Lizandra do Rêgo Leal
Coordenador do Comitê Interno de Ética em Experimentação
Animal-UFSM

Comitê Interno de Ética em Experimentação Animal - UFSM - Av. Roraima, 1000 – Prédio da Reitoria - 2º andar - Campus Universitário 97105-900 – Santa Maria – RS - - Tel: 0 xx 55 3220 9362

Anexo B - Normas Journal of Ethnopharmacology

Journal of Ethnopharmacology

An Interdisciplinary Journal Devoted to Indigenous Drugs

Guide for Authors

The *Journal of Ethnopharmacology* is dedicated to the exchange of information and understandings about people's use of plants, fungi, animals, microorganisms and minerals and their biological and pharmacological effects based on the principles established through international conventions. Early people, confronted with illness and disease, discovered a wealth of useful therapeutic agents in the plant and animal kingdoms. The empirical knowledge of these medicinal substances and their toxic potential was passed on by oral tradition and sometimes recorded in herbals and other texts on *materia medica*. Many valuable drugs of today (e.g., atropine, ephedrine, tubocurarine, digoxin, reserpine) came into use through the study of indigenous remedies. Chemists continue to use plant-derived drugs (e.g., morphine, taxol, physostigmine, quinidine, emetine) as prototypes in their attempts to develop more effective and less toxic medicinals.

Please note that figures and tables should be embedded in the text as close as possible to where they are initially cited. It is also mandatory to upload separate graphic and table files as these will be required if your manuscript is accepted for publication.

Classification of your paper
Please note that upon submitting your article you will have to select **at least one classification** and **at least three of the given keywords.** You can preview the list of classifications and keywords (here). This information is needed by the Editors to more quickly process your article. In addition to this, you can submit free keywords as described below under "Keywords".

The "rules of 5"
The Editors and Editorial Board have developed the "Rules of 5" for publishing in JEP. We have produced five clear criteria that each author needs to think about before submitting a manuscript and setting the whole process of editing and reviewing at work. Click here.
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Authors are encouraged to submit video material or animation sequences to support and enhance your scientific research. For more information please see the paragraph on video data below.

Types of paper

The *Journal of Ethnopharmacology* will accept the following contributions:

1. Original research articles - whose length is not limited and should include Title, Abstract, Methods and Materials, Results, Discussion, Conclusions, Acknowledgements and References. As a guideline, a full length paper normally occupies no more than 10 printed pages of the journal, including tables and illustrations.
2. Ethnopharmacological communications (formerly Short Communications) - whose average length is not more than 4 pages in print (approx. 2000-2300 words, including abstract and references). A maximum of 2 illustrations (figures or tables) is allowed. See paragraph below for description and format.
3. Letters to the Editors.
4. Reviews - Authors intending to write review articles should consult and send an outline to the Reviews Editor (see inside front cover for contact information) before preparing their manuscripts. The organization and subdivision of review articles can be arranged at the author's discretion. Authors should keep in mind that a good review sets the trend and direction of future research on the subject matter being reviewed. Tables, figures and references are to be arranged in the same way as research articles in the journal. Reviews on topics that address cutting-edge problems are particularly welcome.

Outlines for potential reviews need to include:

A detailed abstract using the structure provided in the guidelines

An annotated table of contents

A short CV of the lead author

Book reviews - Books for review should be sent to the Reviews Editor.

6. Commentaries - *invited*, peer-reviewed, critical discussion about crucial aspects of the field but most importantly methodological and conceptual-theoretical developments in the field and should also provide a standard, for example, for pharmacological methods to be used in papers in the *Journal of Ethnopharmacology*. The scientific dialogue differs greatly in the social / cultural and natural sciences, the discussions about the common foundations of the field are ongoing and the papers published should contribute to a transdisciplinary and multidisciplinary discussion. The length should be a maximum of 2-3 printed pages or 2500 words. Please contact the Reviews Editor j.ethnopharmacol@pharmacy.ac.uk with an outline.
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