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Thales Hein da Rosa

EFEITO DO TRATAMENTO COM EXTRATO DE *FASCIOLA HEPATICA* E CISTATINAS RECOMBINANTES DE *FASCIOLA HEPATICA* EM MODELO DE ARTRITE INDUZIDA POR ANTÍGENO

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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Bacharel em Biomedicina.

Orientador: Professor Doutor Ricardo Machado Xavier

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RESUMO

A artrite reumatoide (AR) é uma doença autoimune sistêmica com subsequente destruição articular devido à inflamação crônica sinovial, o que leva a dor e incapacidade funcional. Apesar dos recentes progressos no tratamento da AR, ainda há limitações na eficácia e efeitos adversos importantes, ressaltando a necessidade de encontrar novas estratégias terapêuticas que diminuam esses efeitos adversos e retardem a evolução da doença. A *Fasciola hepatica* é um verme do Filo Platyhelminthes e quando presente em um organismo hospedeiro apresenta produtos secretores-excretores (ESPs) e antígenos de tegumento, os quais podem atuar como moduladores da resposta imune e inflamatória. Dentre esses produtos estão as cistatinas, moléculas inibidoras de cisteíno-protease. Estudos prévios com cistatinas provenientes de *Schistosoma japonicum*, mostraram o potencial terapêutico em modelos animais de colite e artrite. Dentro desse contexto, esse estudo teve como objetivo avaliar o efeito das cistatinas recombinantes 1 e 3 e do extrato de *Fasciola hepatica* sobre parâmetros de inflamação e nocicepção em modelo agudo de artrite induzida por antígeno (AIA). Camundongos Balb/c foram imunizados através de injeção subcutânea de 200 μL de uma emulsão contendo adjuvante de Freund e albumina bovina sérica metilada (mBSA) no dia 0. O reforço foi feito nos dias 7 e 14 utilizando o adjuvante incompleto de Freund. No 21° dia, os camundongos receberam uma injeção intra-articular de mBSA no joelho direito. Os animais foram divididos em grupos: veículo (tratado com PBS), extrato de *Fasciola hepatica* (200 μg), cistatina 1 (100μg e 150 μg), cistatina 3 (100μg e 150 μg), e receberam tratamento via intraperitoneal 24 horas e 30 minutos antes da injeção intra-articular. Houve avaliação de nocicepção utilizando um analgesimetro digital 0, 3h, 6h e 24h após a injeção intra-articular. Após 24 horas da injeção, os animais foram eutanasiados e foi realizado lavado articular para avaliação da migração celular através da contagem de leucócitos totais por câmara de Neubauer. Os dados foram analisados com o programa GraphPad Prism 6.0 através de ANOVA seguida de testes post-hoc entre os grupos. A significância estatística foi considerada quando $p<0,05$. O tratamento com 200 μg de extrato de *F. hepatica* reduziu 44% da migração de leucócitos para o joelho inflamado em comparação com o grupo veículo e foi capaz de reduzir a nocicepção celular nos tempos 3h, 6h e 24h após a injeção de mBSA ($p<0,05$). Já no tratamento com a cistatina recombinante, 100 μg de ambas cistatinas 1 e 3 reduziu a migração de leucócitos para o joelho inflamado em 40% ($p<0,05$) e 37% ($p<0,001$), respectivamente; porém não foi capaz de reduzir a nocicepção celular. O tratamento com a dose mais alta de ambas cistatinas (150 μ g) não foi capaz de alterar os parâmetros avaliados. Portanto, esses resultados destacam o potencial anti-inflamatório e analgésico do extrato, bem como o potencial anti-inflamatório das cistatinas recombinantes em modelo de AIA. Como perspectivas, está a avaliação do tratamento em modelo crônico de artrite, bem como o estudo do mecanismo de ação, focando em células importantes na patofisiologia da artrite reumatoide, como linfócitos e fibroblastos sinoviais.

Palavras-chave: Artrite reumatoide, cistatina, extrato de *Fasciola hepatica*.

ABSTRACT

Rheumatoid Arthritis (RA) is a systemic autoimmune disease characterized by joint destruction due to synovial chronic inflammation which leads to pain and functional disability. Despite the recent progress in RA treatment, there are still limitations in efficacy and several adverse effects, emphasizing the need to search for novel strategies to decrease adverse effects and better control disease activity. *Fasciola hepatica* is a Trematode worm that presents excretion/secretion products (ESs) when infect its hosts. Besides, this parasite also have tegument antigens that, together with ESs, can act as immunomodulatory. Among these products are the cystatins, protease inhibitor molecules that have demonstrated some therapeutic potential in animal models of colitis and arthritis. In this context, this study aims to analyze the effects of recombinant cystatins and the extract of F. hepatica over inflammatory and antinociceptive parameters in an acute antigen-induced arthritis mice model. Balb/c mice were immunized through subcutaneous injection of an emulsion containing complete Freund's adjuvant plus methylated bovine serum albumin (mBSA) at day 0. Boosters injection were performed at day 7 and 14 using incomplete Freund's adjuvant. At day 21, mice received an intra-articular (ia) injection of mBSA in the right knee. Mice were divided into the following groups: vehicle, *F. hepatica* extract, cystatin 1 and cystatin 3 and received treatment via intraperitoneal 24h after ia injection and 30 minutes before ia injection. Nociception was measured 0h, 3h, 6h and 24h after ia injection of mBSA using a digital analgesimeter. 24h after ia, animals were euthanasiated and leukocyte migration to the knee was measured by counting the total number of leukocytes using a Neubauer chamber. Data was analyzed using GraphPad Prism 6.0 with ANOVA analysis followed by post-hoc test and results were considered significant when $p<0.05$. *F. hepatica* treatment reduced 44% of leukocyte migration when compared to vehicle group ($p<0.01$) and was able to reduce nociception ($p<0.05$) in mice subjected to AIA model 3h and 24h after ia injection. Cystatins 1 and 3 in 100µg dose were able to reduce leukocyte migration in 40% ($p<0.05$) and 37% ($p<0.01$), respectively. On the other hand, cystatins did not show significant effect in nociception. Therefore, our data indicate an anti-inflammatory and analgesic effect of the extract, and an anti-inflammatory effect of recombinant cystatins of F. hepatica in an acute arthritis model. As a perspective, we aim to study these treatments in a chronic model of arthritis, elucidating the mechanism of action, and to study the effect in key cells of the pathophysiology, as lymphocytes and synovial fibroblasts.

Keywords: Rheumatoid arthritis, cystatin, *F. hepatica* extract.

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ACPA	Anticorcos Contra Proteína Citrulinada
ACR	American college of rheumatism
AINE	Anti-inflamatório não-esteroidal
APC	Célula apresentadora de antígeno
AR	Artrite Reumatoide
CD	Célula dendrítica
CIA	Artrite induzida por colágeno
COX	Ciclo-oxigenase
DAMP	Padrão molecular associado à dano
DAS-28	Escore da Atividade da Doença em 28 articulações
DMARD	Droga antireumática modificadora da doença
EULAR	Liga européia contra o reumatismo
FhHDM	Molécula de defesa do helminto da F*asciola hepatica*
Fhteg	Antígenos do tegumento da *Fasciola hepatica*
FR	Fator Reumatoide
HLA	Antígeno Leucocitário Humano
HpARI	Inibidor de liberação de alarmina de *H. polygyrus*
IFN- gama	Interferon gama
IgG	Imunoglobulina G
IL-1	Interleucina 1
Il-10	Interleucina 10
IL-11	Interleucina 11
IL-12	Interleucina 12
IL-17	Interleucina 17
IL-33	Interleucina 33
IL-6	Interleucina 6
LPS	Lipopolissacarídeo
MHC	Complexo Maior de Histocompatibilidade
MTX	Metotrexato
PAMP	Padrão molecular associado à patógeno
PCR	Proteína C-reativa
rSJcystatin	Cistatina recombinante do *Schistossoma japonicum*
TGF-beta	Fator de transformação de crescimento
Th	Célula T auxiliar
TIMP	Inibidor tecidual de metaloproteinases
TLR	Receptor tipo "toll"

TNF-alfa	Fator de Necrose Tumoral Alfa
Treg	Célula T regulatória
Treg	Célula t regulatória
VSG	Velocidade de sedimentação globular

SUMÁRIO

1 -INTRODUÇÃO

1.1- Artrite Reumatoide

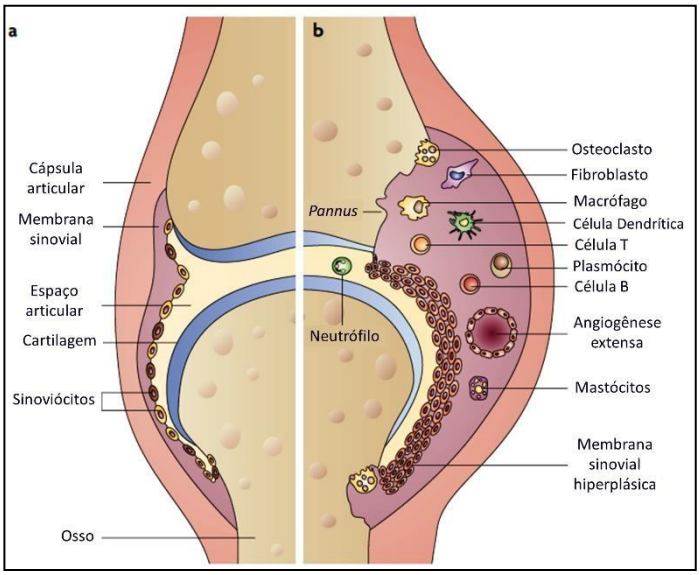
A artrite reumatoide (AR) é uma doença autoimune sistêmica e de caráter inflamatório que envolve primariamente o acometimento das articulações sinoviais. Tem como característica a inflamação crônica da membrana sinovial, de forma erosiva e simétrica, envolvendo principalmente as articulações periféricas (SMOLEN e colab., 2016). Os principais sinais clínicos articulares da doença incluem rigidez nas articulações, redução e perda dos movimentos. Além do dano articular, sabe-se que a AR causa diversas manifestações extra-articulares, como fraqueza muscular, uveíte, vasculite, doenças cutâneas e acometimento de órgãos como rins, coração e sistema nervoso (DOUGADOS, 2016). Essas condições podem vir a provocar comorbidades severas que, junto ao acometimento articular, fazem da AR uma importante causa de incapacidade e mortalidade (SCOTT e colab., 2010).

A AR aparece com uma incidência mundial que varia entre 0,5% e 1% e uma prevalência de 0,46% no Brasil (ALAMANOS e colab., 2006; SENNA e colab., 2004). A despeito das diferenças de prevalência entre regiões, idade e sexo indicarem o envolvimento de fatores genéticos e ambientais para o desenvolvimento da doença, a etiologia da AR não é totalmente conhecida. Dentre os fatores genéticos estudados destacam-se, além da heritabilidade (JIANG e colab., 2015), o envolvimento de mais de cem loci gênicos associados ao risco de desenvolver AR, sendo a maioria relacionados à expressão de componentes do sistema imune como os loci do antígeno leucocitário humano (HLA)(SILMAN, Alan J e PEARSON, 2002). A maioria dos genes presentes nessa região codificam moléculas do complexo maior de histocompatibilidade (MHC) de classe II, molécula responsável por apresentar antígenos para células da resposta imune. Dessa forma, mutações nos genes dessa região (principalmente HLADR01/04) podem implicar em perda da tolerância e consequente apresentação de autoantígenos às células T (GREGERSEN e colab., 1987). Junto a isso, fatores ambientais como tabagismo, agentes infecciosos e perfil alterado de microbiota estão relacionados com aumento no risco de AR (SCHER e colab., 2016; SILMAN, A J e colab., 1996; WILSON e EBRINGER, 2000).

A patofisiologia da AR envolve mecanismos celulares, moleculares e epigenéticos alterados que resultam no estabelecimento de autoimunidade e inflamação. Um dos mecanismos epigenéticos mais associado com a produção de autoanticorpos é a citrulinação aberrante de proteínas próprias (ZHAO e colab., 2008). Os anticorpos contra proteínas citrulinadas (ACPA) são capazes de interagir com os antígenos citrulinados e formar complexos que ativam o sistema complemento de forma exagerada (SABHARWAL e colab., 1982). Outro autoanticorpo importante é o fator reumatoide (FR), que é capaz de ligar-se à porção Fc das imunoglobulinas e formar imunocomplexos que causam ativação de macrófagos e expressão de citocinas (ANQUETIL e colab., 2015). A presença desses autoanticorpos pode servir como ferramenta no diagnóstico e na caracterização da severidade da doença (ALETAHA e SMOLEN, 2018).

A inflamação na membrana sinovial é gerada como consequência da ativação da resposta imune. A sinovite na AR é caracterizada pela infiltração e proliferação leucocitária na membrana, sendo inicialmente composta por células da imunidade inata (neutrófilos, monócitos, macrófagos, células dendríticas (CD) e mastócitos) que posteriormente irão recrutar células da imunidade adaptativa (células T, células B e plasmócitos). Na presença de citocinas como o fator de necrose tumoral alfa (TNF-α), interleucina 1 (IL-1) e interleucina 6 (IL-6), o perfil dos fibroblastos e macrófagos sinoviais se altera e culmina na secreção de moléculas como metaloproteinases e colagenases, que estimulam a invasão dos fibroblastos sinoviais (CHOY, 2012). Junto a isso, a presença de espécies pró-inflamatórias também induz angiogênese e limita a regeneração dos condrócitos . Com isso, ocorre a formação de um tecido invasivo e agressivo, denominado pannus, que acaba por degradar e invadir os tecidos adjacentes como o tecido cartilaginoso e ósseo (MCINNES e SCHETT, 2011).

Figura 1- Alterações articulares na artrite reumatoide



1.2- Diagnóstico e Tratamento da AR

Entre as manifestações clínicas mais observadas em pacientes com AR estão a dor e rigidez nas articulações, sendo afetadas principalmente as articulações interfalângicas e metacarpofalângicas. Da mesma forma, sintomas extra-articulares como fadiga e perda de peso também estão presentes nas formas mais comuns de AR. Ademais, o paciente com AR também pode apresentar perfil sorológico distinto, com frequente presença de FR (não tão específico para AR) e ACPA (mais específico para AR). Por fim, outros sinais clínicos são avaliados, como presença de proteína C-reativa (PCR) e sedimentação eritrocitária (VSG). Tendo em vista esses aspectos, foram criados critérios de classificação para AR visando um diagnóstico precoce da doença. O critério mais utilizado foi elaborado em 2010 pela ACR e pela Liga Europeia Contra Reumatismo (EULAR) e estabelece uma pontuação levando em conta os aspectos clínico-laboratoriais apresentado pelos pacientes (Tabela 1) (ALETAHA e colab., 2010).

Tabela 1. Critérios de classificação para AR segundo ACR 2010 ((Aletaha et al., 2010).

Envolvimento Articular	**0-5**
1 articulação média a grande	0
2-10 articulações médias a grandes	1
1-3 articulações pequenas (não contando articulações grandes)	2
4-10 articulações pequenas (não contando articulações grandes)	3
>10 articulações (pelo menos uma articulação grande)	5
Sorologia	**0-3**
fator reumatoide e anticorpo contra antígenos citrulinados negativos	0
FR e ACPA fracamente positivos	2
FR e ACPA fortemente positivos	3
Reagentes de Fase Aguda	**0-1**
proteína C-reatica e taxa de sedimentação eritrocitária normal	0
proteína C-reatica e/ou taxa de sedimentação eritrocitária anormal	1
Duração dos Sintomas	**0-1**
<6 semanas	0
6 semanas ou mais	1
Ponto de corte para AR: 6 ou mais	

O tratamento para AR deve levar em conta a severidade da doença e tem como objetivo diminuir sua atividade e retardar a destruição das articulações. Nesse contexto, as drogas mais utilizadas para controle de AR são as drogas antirreumáticas modificadoras de doença (DMARDs), anti-inflamatórios não esteroidais (AINEs), e glicocorticoides (ALETAHA e SMOLEN, 2018); outras intervenções como prática de exercícios físicos e dietas também podem ser importantes para a melhora do quadro da doença (BRODIN e colab., 2008; HAGEN e colab., 2009). Do ponto de vista farmacológico, as drogas de primeira escolha geralmente são os DMARDs, tanto como monoterapia ou em combinação com outro fármaco (associação de DMARDs ou com corticoesteroides (ALETAHA e SMOLEN, 2018).

A categoria dos DMARDs compreende compostos químicos sintéticos e biológicos que, por diferentes mecanismos de ação, visam a imunossupressão do paciente. Como exemplos de DMARDs sintéticos destacam-se o metotrexato (MTX) e a sulfassalazina, e em contrapartida o adalimumabe e etanercepte são exemplos de DMARDs biológicos que atuam neutralizando o TNF-α. Para a escolha da droga a ser administrada, não existe DMARD superior para início de

tratamento de AR, no entanto o MTX tem menor custo e é capaz de diminuir a progressão da doença assim como os outros fármacos (SMOLEN e colab., 2017).

Sobre a farmacologia, o MTX age como análogo do ácido fólico, e é capaz de inibir as enzimas dihidrofolato redutase e a timidilato sintetase, responsáveis por manter o folato intracelular viável para a síntese de DNA. Em última análise, o MTX irá provocar a diminuição da proliferação celular e da secreção de citocinas inflamatórias. A despeito de sua ação desejada, essas drogas também apresentam efeitos adversos importantes, sendo o risco aumentado de infecções a principal preocupação nesse aspecto. Além disso, o MTX aumenta o risco de desenvolvimento de fibrose e cirrose hepática, doença pulmonar intersticial, linfomas malignos e supressão da medula óssea (SEGAL e colab., 1990). Por outro lado, os DMARDs biológicos são fármacos que têm como objetivo neutralizar citocinas e inibir componentes da resposta imune como inibidores de TNF-α, IL-1, IL-6, células T e células B. Devido à tecnologia empregada para a produção dessas drogas, elas apresentam custo de tratamento elevado. Além dos efeitos indesejados relacionados à imunossupressão, uma parcela dos pacientes não respondem ao tratamento com DMARDs, sendo necessário a mudança do plano de tratamento (SMOLEN e colab., 2017).

Além dos DMARDs, o tratamento com AINEs para AR visa a diminuição da inflamação no paciente. Geralmente, estes fármacos agem pela inibição da ciclo-oxigenase (COX), enzima responsável pela transformação de ácido araquidônico em moléculas que sinalizam inflamação como prostaglandinas, leucotrienos e tromboxanos (SMITH, William L. e colab., 2000) . As prostaglandinas por sua vez, se ligam em receptores de células inflamatórias (célula T e B, macrófagos e CD) e ativam a expressão e liberação de IL-10, TNF-α, IL-4, juntamente com a supressão da liberação de IL-12 e IFN-ɣ (HARRIS e colab., 2002). Por outro lado, o uso de AINEs pode causar efeitos adversos importantes como problemas gastrointestinais, risco cardiovascular aumentado, toxicidade renal e hepatotoxicidade (JONES, 2001).

Oss glicocorticoides, outra classe de fármacos utilizados no tratamento da AR, também são capazes de diminuir a atividade de células do sistema imune e reduzir a inflamação. É descrito que essas moléculas, assim como seus análogos endógenos, são capazes de sinalizar a expressão de genes que regulam a resposta imune de forma anti-inflamatória. Sabe-se que os glicocorticoides agem inibindo a secreção de muitas citocinas pró-inflamatórias como IL-1, IL-17, IL-11 e TNF-α. Da mesma forma, atuam inibindo a apresentação de antígenos, a co-

estimulação, a expressão de receptor de célula T e a produção de anticorpos. Assim como os outros fármacos, o uso de glicocorticoides pode causar efeitos indesejáveis como a síndrome de Cushing, a qual o paciente pode apresentar sintomas como ulceração gastrointestinal, retenção de líquidos, hipertensão, imunossupressão, complicações glicêmicas, distúrbios de humor, entre outros (WEISS, 1989).

Tendo em vista o custo elevado, a ocorrência de diversos efeitos adversos e a ineficácia dos tratamentos atuais para doenças autoimunes, é necessária a prospecção de novos alvos, avaliação de fármacos e moléculas promissoras, bem como a elaboração de novas hipóteses que proponham novas estratégias terapêuticas.

1.3- Hipótese dos Velhos Amigos e autoimunidade

A hipótese dos velhos amigos (ou hipótese da higiene) sugere, através de uma perspectiva evolutiva, que organismos que coevoluíram com os mamíferos são capazes de modular a resposta imune do hospedeiro para facilitar sua evasão, e a falta de exposição a esses parasitas nos dias atuais está associada à crescente incidência de doenças autoimunes e inflamatórias em alguns lugares do mundo. Ainda, segundo a hipótese, os organismos que acabam modulando o sistema imune do hospedeiro são aqueles que precisam ser tolerados no organismo humano, como bactérias simbiontes da microbiota intestinal normal e helmintos (OKADA e colab., 2010). A diminuição da exposição aos agentes infecciosos está relacionada com a mudança no estilo de vida e hábitos dos seres humanos modernos. Duas transições epidemiológicas foram responsáveis pela depleção do contato dos seres humanos com alguns de seus antigos parasitas. A primeira transição se refere ao momento da história em que seres humanos começaram a se organizar em grupos maiores, domesticar animais, viver em fazendas e ambientes rurais. Isso resulta em um perfil infeccioso distinto, com aumento de infecções orofecais e virais e diminuição da carga de helmintos. Já a segunda transição se refere ao estabelecimento do ser humano em grandes cidades, tratamento de água, menor contato com animais e utilização de antibióticos. Isso corresponde à mudança no perfil de microbiota e ainda menos contato com parasitas helmínticos e, segundo, a hipótese a consequência é um empobrecimento na quantidade de estímulos que o sistema imune de seres humanos atuais recebe, facilitando o aparecimento de desordens inflamatórias e autoimunes, como diabetes tipo 1, doenças inflamatórias intestinais, esclerose múltipla e AR (ROOK, 2012).

Os microrganismos que fazem parte da microbiota intestinal normal são importantes imunomoduladores. Em ensaios com animais, verificou-se a capacidade de bactérias de diminuir a incidência de artrite em modelo induzido por antígeno (KOHASHI e colab., 1985). Ainda, sabe-se da capacidade da colonização com bactéria de indução de células Tregs (GEUKING e colab., 2011) e diminuição da resposta Th17 (ROUND e colab., 2011). Corroborando com a hipótese dos velhos amigos, muitos estudos demonstram a diversidade de perfis de microbiota nas diferentes regiões do mundo, bem como a ocorrência de microrganismos característicos em cada região (DE FILIPPO e colab., 2010).

Através de estudos de associação, observou-se que a esclerose múltipla ocorre de forma mais rara em regiões de alta prevalência de *Trichuris trichiura* (FLEMING e COOK, 2006). Da mesma forma, foi vista a relação entre aumento de casos de diabetes tipo 1 e erradicação de infecção por *Enterobios vermicularis* (GALE, 2002) e ainda, a correlação entre a falta de exposição à helmintos e ocorrência de doenças inflamatórias intestinais (WEINSTOCK e ELLIOTT, 2009).

Como já citado, helmintos são eficientes moduladores da resposta imune do hospedeiro e o fazem por diversos mecanismos. Dentre os mecanismos estudados, sabe-se que esses organismos são capazes de estimular o aumento de população de células Tregs (CORREALE e FAREZ, 2007), aumento de população de células B secretoras de IL-10, modulação de células dendríticas (HANG e colab., 2010), regulação da microbiota intestinal (WALK e colab., 2010) e ativação de macrófagos secretores de IL-10 (SCHNOELLER e colab., 2008).

1.4- Helmintos e moléculas de imunomodulação

Como visto acima, existe um grande número de evidências que mostram que os helmintos são capazes de regular o sistema imune do hospedeiro, e que o fazem por diversos mecanismos. Podemos destacar, ainda, a habilidade desses parasitas de regular as diferentes etapas da resposta imunológica pela secreção ou expressão de moléculas, que vão participar inibindo a iniciação da resposta, o reconhecimento de antígenos, modulação da resposta adaptativa, regulação da resposta efetora e controle do reparo tecidual (MAIZELS e colab., 2018).

Helmintos possuem a capacidade de neutralizar sinais de alarminas, citocinas liberadas após dano epitelial responsáveis, junto aos padrões moleculares associados à patógenos (PAMPs)

e aos padrões moleculares associados à dano (DAMPs), pela iniciação da resposta imune com a ativação de macrófagos e células dendríticas. O verme *Heligmosomoides polygyrus* é capaz de inibir a sinalização de alarminas, bloqueando o receptor de IL-33 através de seu produto excretor inibidor de liberação de alarmina (HpARI) (OSBOURN e colab., 2017). Esse helminto também é capaz da secreção de apirases que degradam ATP extracelular indutor de sinal inflamatório (CEKIC e LINDEN, 2016).

Quanto à capacidade de regular o processamento e apresentação de antígenos, os helmintos podem fazê-lo através da secreção de bloqueadores de TLR de macrófagos e CD, inibição da expressão de TLR, estimulação de células apresentadoras de antígenos (APCs) e indução de resposta Th2, secreção de fatores de crescimento análogos, inibição da inflamação por bloqueio de moléculas inflamatórias e indução de liberação de citocinas anti-inflamatórias. Por exemplo, a molécula secretada pelo helminto *Acanthocheilonema viteae* ES-62 induz a supressão de TLR e IL-33, através do sequestramento da molécula sinalizadora MyD88. Ainda, a molécula ES-62 já demonstrou ter ação protetora em modelo murino de AR (PINEDA e colab., 2015).

A secreção de glicanos por helmintos do gênero *Schistosoma* é capaz de polarizar macrófagos e CD para uma resposta anti-inflamatória e induzir a liberação de IL-10 e TGF-β (OKANO e colab., 2001). Os parasitas *Brugia malayi* e *Trichinella spirallis* secretam análogos de moléculas de inibição de migração (MIFs) e induzem a liberação de IL-8 por monócitos e a ativação de macrófagos (CHO e colab., 2015; TAN e colab., 2001) .

Outro mecanismo utilizado por helmintos é a interferência na resposta adaptativa do hospedeiro. Com essa interferência o parasita pode direcionar a resposta para um perfil menos inflamatório, de modo que a memória imunológica os perceba como próprios. Para isso, esses organismos desenvolveram diversos métodos que resultam preferencialmente em uma resposta regulada por Tregs ou por células B. Quanto à modulação de Tregs, uma molécula que apresenta essa finalidade é a análoga de inibidor tecidual de metaloproteinases (TIMP) de ancilostomídeos, capaz de modular a resposta de CD e estimular uma resposta de célula T mais tolerante (CUÉLLAR e colab., 2009). Além disso, estudos mostram que essas moléculas são capazes de serem efetivas na inibição de modelos de alergia e de colite em camundongos (FERREIRA e colab., 2017; NAVARRO e colab., 2016). Outra forma de induzir a resposta de Tregs é a secreção da molécula PAS-1 pelo helminto *Ascaris suum*, que atua estimulando a proliferação de

populações de Tregs CD25+ supressoras de respostas alérgicas e pró-inflamatórias (ARAÚJO e colab., 2008). Ainda, os parasitas podem se aproveitar da secreção de homólogos de moléculas do hospedeiro que estimulam o estabelecimento e estabilidade da população de Tregs. Um exemplo disso é a produção de molécula homóloga da galectina-9 pelo *Toxocaris leonina* que é capaz de induzir a produção de IL-10 e TGF-β da mesma forma e juntamente com a galectina do hospedeiro (KIM e colab., 2010).

Quando o alvo é a resposta de células B, os helmintos procuram diminuir a efetividade da resposta, interferindo na migração endotelial de células B (TRIBOLET e colab., 2015), modificando a expressão de receptores com consequente sinalização para resposta ineficiente (DEEHAN e colab., 2001), e induzindo fenótipo regulatório desse tipo celular (HUSSAARTS e colab., 2011) através de inúmeras moléculas excretadas/secretadas.

A resposta imunológica efetora está relacionada com a neutralização do patógeno ou toxina potencialmente prejudicial. Isso ocorre preferencialmente através da atração e migração de granulócitos para o local da infecção e consequente liberação dos grânulos para controle da infecção. Sendo assim, os helmintos desenvolveram métodos para evadir a ação da resposta efetora contra eles, como o bloqueio de quimiocinas (atração de células efetoras) e inibição de integrinas e moléculas de adesão. Estudos mostram a capacidade de ancilostomídeos de inibir a respostas de quimiocinas responsáveis pela atração de eosinófilos através da secreção de metalopreoteinases que clivam essas quimiocinas (CULLEY e colab., 2000). Da mesma forma, o *S. mansoni* secreta uma proteína de ligação a quimiocinas (SmCKBP) que bloqueia a migração de neutrófilos (SMITH, Philip e colab., 2005). Junto aos mecanismos de supressão de quimiocinas, ancilostomídeos conseguem inibir a adesão de neutrófilos através da secreção da proteína NIF, que liga as integrinas ao fibrinogênio e evita a ligação com o endotélio vascular (MOYLE e colab., 1994).

1.5- *Fasciola hepatica*

A *Fasciola hepatica* é um helminto trematódeo que habita preferencialmente ductos biliares de ruminantes e seres humanos, e sua infecção pode causar a fasciolose. Apesar do potencial estabelecimento de uma doença parasitária, sua infecção apresenta, por um outro lado,

aspectos importantes na modulação do sistema imune do hospedeiro (BRADY e colab., 1999). Estudos mostram que a infecção com esse parasita pode atenuar a manifestação de modelo animal de esclerose múltipla inibindo respostas de perfil Th1 e Th17 através da liberação de fator de transformação do crescimento beta (TGF-β) e IL-10 (WALSH e colab., 2009). O parasita se aproveita de um amplo espectro de moléculas excretadas e secretadas para realizar essa imunomodulação (ROBINSON e colab., 2013).

Dentre as moléculas secretadas e excretadas pelo verme, podemos destacar espécies que irão atuar no processamento de antígeno e antioxidantes. De fato, a molécula de defesa do helminto do tipo 1 da *F. hepatica* (FhHDM-1) é capaz de acidificar o meio endolisossomal e prejudicar o processamento de antígenos em macrófagos (ROBINSON e colab., 2012) e de se ligar em lipopolissacarídeo (LPS), inibindo a resposta contra LPS e ativação de macrófagos (ROBINSON e colab., 2011). De forma parecida, os produtos Fh12 e Fh15 suprimem a ativação de macrófagos pela ligação dessas moléculas em TLR2, TLR4, TLR5 e TLR8 e inibição da liberação de citocinas (MARTIN e colab., 2015; RAMOS-BENÍTEZ e colab., 2017). Ainda, a *F. hepatica* secreta cisteíno-proteases capazes de degradar o TLR intracelular e suprimir sua expressão em células mieloides (DONNELLY e colab., 2010). Ademais, as peroxirredoxinas da *F. hepatica*, além de ação antioxidante protetora ao helminto, mostrou ser efetiva em dirigir o perfil de macrófagos para um estado M2/regulatório (DONNELLY e colab., 2008).

Além do potencial modulatório das moléculas secretadas e excretadas pela *F. hepatica*, os antígenos presentes em seu próprio tegumento também apresentam efeito regulador (RAVIDÀ e colab., 2016). Os antígenos do tegumento da *F. hepatica* (FhTeg) quando administrados em modelo murino de choque séptico, mostrou potencial supressor de células Th1, reduzindo os níveis de IFN-ɣ (HAMILTON e colab., 2009). Além disso, quando estimuladas por FhTeg, CD foram capazes de induzir anergia em células T (ALDRIDGE e O'NEILL, 2016). Além disso, sabe-se que glicanos expressos no tegumento da *F. hepatica* são capazes de interferir nas moléculas de adesão de células dendríticas e provocar anergia de células T, indução de liberação de IL-10 e aumento da população de Tregs (RODRÍGUEZ e colab., 2017).

1.6- Cistatinas

As cistatinas fazem parte da família de moléculas chamada de inibidores de cisteíno-proteases e estão entre as mais importantes moléculas secretadas por helmintos utilizadas como imunomoduladoras (MAIZELS e colab., 2018). Como se sabe, as cistatinas são capazes de interferir em diversos processos relacionados com a resposta imune como a sinalização de citocinas e processamento e apresentação de antígenos (HARTMANN e colab., 1997).

Inicialmente, foi verificado a capacidade *in vitro* da cistatina de modular processos do sistema imune. De fato, estudos mostraram que a cistatina foi capaz de atuar sobre macrófagos e regular a produção de citocinas (KLOTZ e colab., 2011). Quando testada em estudos *in vivo* as cistatinas também se mostraram eficazes na regulação do sistema imune. Em modelo experimental de colite, a cistatina recombinante de *Schistosoma japonicum* (rSjcystatin) foi capaz de aliviar a patogenia causada pela resposta Th1 e reduzir a inflamação no cólon, apresentando potencial terapêutico. Esse efeito foi relacionado com a capacidade de regulação de Tregs e Th2 induzida por rSjcystatin nos tecidos estudados (LIANG e colab., 2018).

Adicionalmente, a rSjcystatin foi capaz de aliviar os efeitos deletérios da artrite induzida por colágeno (CIA) quando administrada de forma profilática. Esse efeito está relacionado com o aumento de citocinas Th2, IL-4 e IL-10, IgG1 colágeno-específico, e à redução de IFN-γ, IgG2a colágeno-específico e citocinas pró inflamatórias IL-6, IL-17e TNF-α (LIU e colab., 2016). Em estudo com cistatina não recombinante de *Schistosoma japonicum* (SjCystatin) foi demostrado o efeito regulatório em cultura de macrófagos através da inibição da produção de óxido nítrico, TNF-α e IL-6 e estimulação da produção de IL-10, demonstrando que a SjCystatin pode induzir polarização de macrófagos M2 (LI e colab., 2017).

Juntamente às evidências históricas e epidemiológicas que relacionam as incidências de doenças infecciosas e doenças autoimunes, o grande repertório de moléculas imunomodulatórias que helmintos apresentam fazem que experimentos avaliando o seus potenciais terapêuticos como imunomoduladores sejam de grande relevância. Nesse contexto, a busca por moléculas secretada por helmintos pode ser útil, como vimos, no controle da doença em vários momentos da resposta autoimune presente em doenças como a AR.

1.7- Justificativa

Em conjunto, os estudos epidemiológicos sobre infecções por helmintos e os

experimentos envolvendo as moléculas secretadas por esses parasitas, demonstram um potencial imunomodulador que pode ser explorado no tratamento de doenças autoimunes. Considerando também as limitações que envolvem as atuais formas de tratamento de artrite reumatoide como o alto custo, efeitos adversos e ineficácia para uma parcela dos pacientes, destaca-se a necessidade de buscar alternativas aos tratamentos padrões utilizados. Com base nisso, tanto o extrato da *F. hepatica* quanto as cistatinas mostram-se como tratamentos em potencial para serem utilizadas no controle da AR.

1.8- Objetivos

1.8.1- Objetivo Geral

Avaliar o efeito e a eficácia do extrato de *F. hepatica* e das suas cistatinas como terapia anti-inflamatória e imunomoduladora *in vivo* em modelo de artrite induzida antígeno (AIA) em camundongos Balb/c.

1.8.2- Objetivos específicos

Avaliar a ação das cistatinas recombinantes e do extrato de *F. hepatica* sobre a inflamação em camundongos submetidos ao modelo de AIA:

1. Através da migração de leucócitos totais para a cavidade articular;
2. Através da nocicepção das articulações.

2- ARTIGO CIENTÍFICO

Therapeutic effect of *Fasciola hepatica* extract and recombinant cystatins in antigen-induced arthritis.

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Abstract

Fasciola hepatica is a fluke worm that causes fasciolitis. Its infection and secretory/excretory products present the ability to modulate the immune system. Here, we evaluate the potential of *F. hepatica* extract and its recombinant cystatins to regulate immunity in antigen-induced arthritis (AIA). AIA was performed using Balb/C mice sensitized with an intra articular (ia) injection of methylated bovine serum albumin (mBSA) and treatment was made in times 24h and 30 minutes before ia injection. Nociception was measured in times 0, 3, 6 and 24h after ia injection and leukocyte migration was evaluated after mice eutanasia. Results showed that *F. hepatica* extract reduced significantly nociception ($p<0.001$) while recombinant cystatins were not able to reduce pain ($p> 0.05$). Extract (200µg) ($p<0.01$), cystatin 1(100µg) ($p<0.05$) and cystatin 3 (100µg) ($p<0.01$) showed effect in reducing leukocyte migration. Our findings suggest that both *Fasciola hepatica* extract and recombinant cystatins can be further explored in order to reach novel approaches for autoimmune diseases including rheumatoid arthritis.

Introduction

Rheumatoid Arthritis (RA) is a chronic inflammatory disease that has a global incidence of 0.5 to 1.0% and impacts directly in public health issues [1]. RA clinical manifestation involves bone erosion, synovial hyperplasia and systemic inflammation that lead to joint destruction. The disease is associated with autoantibody production, lymphocyte migration and activation, synovial fibroblast proliferation and invasion, and osteoclast activation. RA etiology is not totally clear, but there are several environmental factors that seems to be involved in the development of the condition [2]. RA treatment basically consist in control the inflammation

through use of anti-inflammatory drugs, such as glucocorticoids and non-steroidal anti-inflammatory drug,s and with disease modifying anti-rheumatic drugs (DMARDs). However, current treatments can lead to several adverse effects, being immunosuppression the most common, with a consequent liability to opportunistic infections [3]. Hence, it is important to search for novel treatments that have lower impacts in patient's quality of life.

Epidemiological studies correlate the increased incidence of allergic and inflammatory disorders in developed countries with a decreased occurrence of infectious diseases. On the other hand, in underdeveloped countries the opposite occurs: high indexes of infectious diseases in contrast of lower indicators of inflammatory disorders [4]. Based on this, the *Old Friends* hypothesis suggests that the lack of contact with immune modulators such as helminths and natural bacterias could result in dysregulation of the host immune response and trigger the establishment of autoimmunity [5].

Additionally, an anti-inflammatory potential of helminth infections has been shown, with the use of helminths-derived excretory/secretory molecules to control autoimmune diseases [6]. In this context, the infection with *Fasciola hepatica* was able to attenuate autoimmunity in encephalomyelitis mice model by inhibition of Th17 and Th1 responses [7]. In the same way, the immune modulation potential of the secretome of this parasite was demonstrated [8], associating relevant products secreted by the worm with the potential to regulate the host immune system, such as cystatins. These molecules are part of the cysteine-protease inhibitors and are vital to many survival processes of the parasite. The main interaction between cystatins and host immune system are in the antigen processing and presentation, when cystatins can inhibit proteases involved in antigenic protein cleavage [9]. In addition, cystatin induced IL-10 release by macrophages and monocytes and suppressed T cell activation [10]. Furthermore, studies have shown that cystatins from several helminths were effective to control disease in animal models of colitis and allergic lung inflammation [11, 12].

In the present study we aimed to evaluate the anti-inflammatory and analgesic potential of *F. hepatica* extract and its recombinant cystatins in antigen-induced arthritis (AIA) mice model.

Materials and Methods

Fasciola hepatica extract and recombinant cystatins preparation

F. hepatica extract was prepared following *Cancela et al. 2004* [13]. Briefly, living flukes were homogenized in phosphate buffer saline (PBS) following centrifugation at 20.000g per 1h. Recombinants cystatins (rFhcystatins) were obtained by cloning and expressing the *F. hepatica* cystatin genes in *E. coli*. For this, the coding DNA sequences of cystatins were amplified by PCR, then amplicons were attached into plasmidial vectors and were inserted in bacterial genome using a restriction enzyme for its expression. Proteins were expressed with glutathione-S-transferase tags, and purification was made by chromatography in glutathione-sepharose 4B following cleavage of tags with thrombin.

Animals

For this study, 59 male Balb/C with 8-12 weeks were used according with the guidelines for the use of animals. The study was approved by the Committee on the Ethics of Animals Experimentation of the Hospital de Clínicas de Porto Alegre (number 170310).

Induction of AIA

AIA was performed according to *Grespan et al., 2008* [14]. Mice were sensitized on day 0 with a subcutaneous (sc) injection of 200 µl of a solution containing equal ratio of methylated bovine serum albumin (mBSA) (Sigma-Aldrich, Saint Louis, MO, USA) plus saline and complete Freund's adjuvant (CFA) (Sigma-Aldrich). Then, on days 7 and 14 mice were subjected to booster sc injections of mBSA with incomplete Freund's adjuvant (IFA) (Sigma-Aldrich). Arthritis was induced on day 21, with an intra-articular (ia) injection of 30µg of mBSA into the left knee joint. The right knee joint was injected with saline solution as a negative control of arthritis (saline group).Treatment was performed intraperitoneally in 100 µl 24 hours and 30 min before ia injection. *F. hepatica* extract treatment was performed with a 200µg dose of extract while rFhcystatins treatment was performed using cystatins 1 and 3, each one in two doses: 100µg dose and 150µg dose.. Vehicle group received saline 0.9%. Nociception was evaluated before ia injection 3h, 6h and 24h after ia injection. Euthanasia and leukocyte migration was performed 24h after ia injection.

Nociception and Leukocyte migration assay

Nociception was measured at 0, 3h, 6h and 24h after ia injection using a digital analgesimeter (Insight Instruments, Ribeirão Preto, SP, Brazil) following the method described by *Oliveira et al.* 2011 [15]. In this test, a force was applied to the hind paws of the animal, then the equipment measured the mechanical threshold to paw withdrawal. Leukocyte migration was evaluated 24h after ia injection. The knee cavity of mice was washed three times with PBS-EDTA and the recovered liquid was mixed in Turk's reagent (1:1). Total number of cells was counted in optical microscope using a Neubauer Chamber (HBG, Giessen-Lützellinden, Germany).

Statistical analysis

Statistical data is present as mean ± standard error of the mean (S.E.M.). Groups were compared by one or two-way analysis of variance (ANOVA) with Bonferroni's adjustment for multiple comparisons and by Student's t-test using GraphPad Prism 6.0 (San Diego, USA). Statistical differences were considered significant with a p value <0.05.

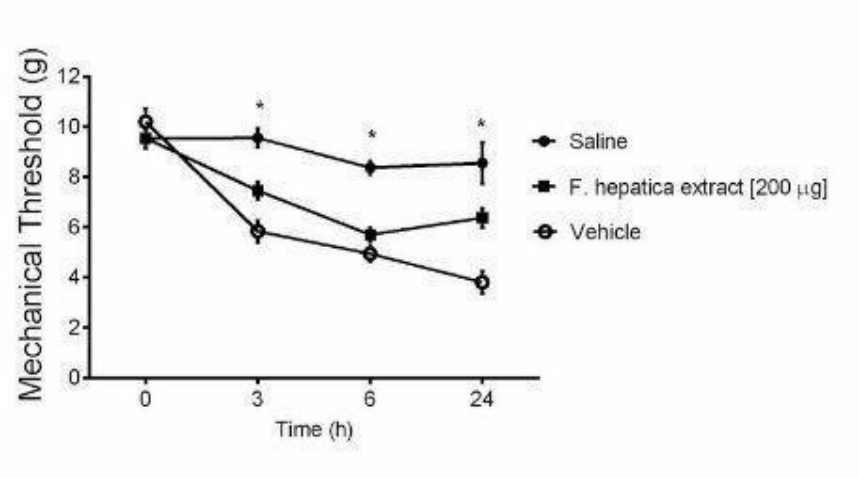
Results

F. hepatica extract, but not cystatin, reduce nociception in AIA model

First, we evaluated the analgesic potential of the treatments, considering an increase of pain in response to mBSA injection and the consequent inflammatory process.

Mice treated with vehicle had an increased nociception 3, 6 and 24 h after ia injection in comparison to Saline group (P<0,001). Animals treated with 200µg of extract showed increased values of mechanical threshold (40,00±19,00) when compared to vehicle-treated mice (90,90±28,72) at the end of the experimentation (p<0.001) (fig. 1a). In contrast, recombinant cystatins in both doses were not able to reduce the nociception, showing the same progression as the vehicle-treated group (p>0.05) (fig. 1b).

A



B

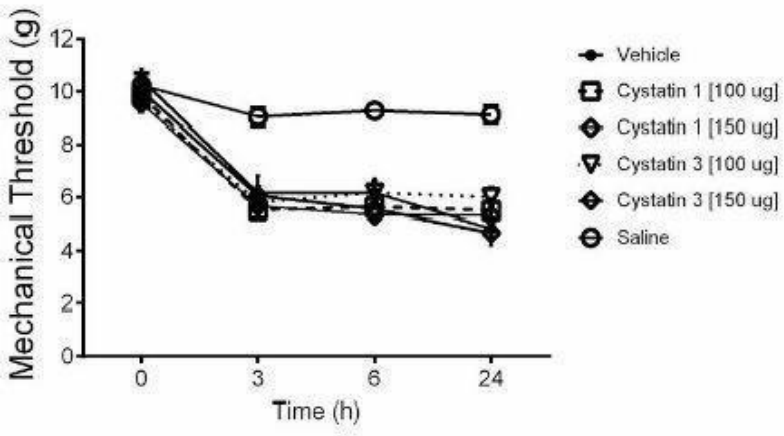


Fig. 1- Effect of F. hepatica extract and recombinant cystatins on nociception in antigen-induced arthritis. Treatment with *F. hepatica* extract reduced nociception 3h, 6h and 24h after intraarticular injection of mBSA (A). Treatment with both recombinant cystatins did not reduce mechanical response at any dose (B). Results are expressed in mean ± SEM and data was analyzed by two-way ANOVA following Bonferroni's post test. * $p<0.05$ vehicle versus extract.

F. hepatica extract and cystatins reduce leukocyte migration in AIA model

Leukocyte migration to the local of ia injection represent an inflammatory response to mBSA antigen. Therefore, this parameter can be used as a predictive for an anti-inflammatory effect of the tested treatments.

Animals treated with *F. hepatica* extract presented 44% of reduction in leukocyte migration in comparison to vehicle treated group ($p<0.01$) (fig. 2a). In the same way, cystatin 3 ($p<0.01$) and cystatin 1 ($p<0.05$) in 100µg decreased leukocytes migration in 37% and 44% respectively (fig. 2b).

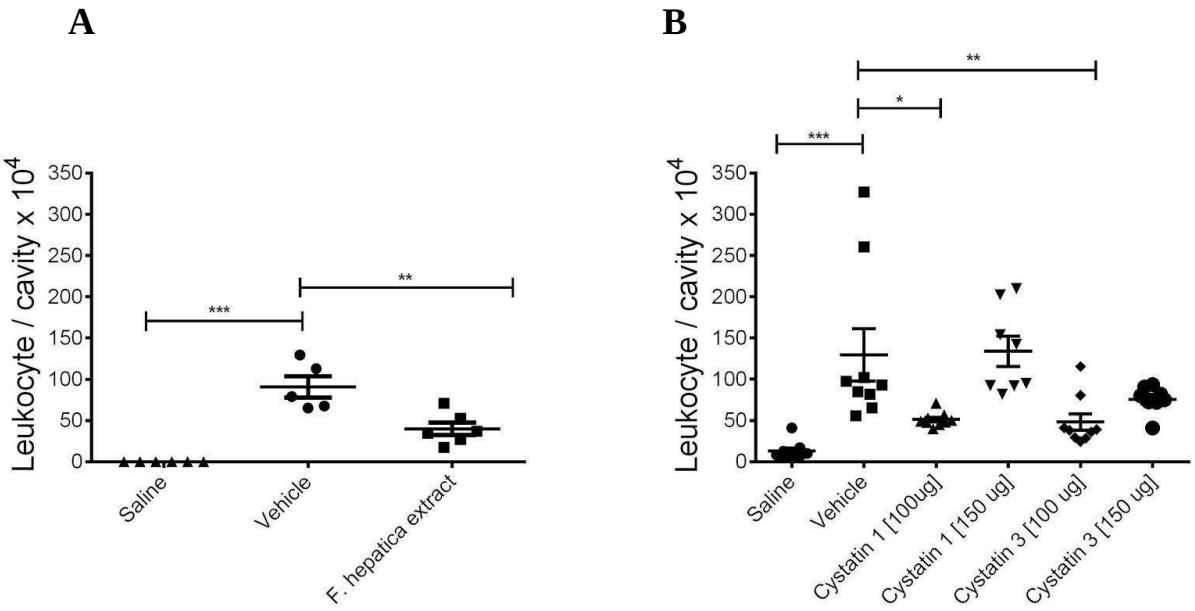


Fig. 2- Effect of F. hepatica extract and recombinant cystatins on leukocyte migration in antigen-induced arthritis. Treatment with F. hepatica extract reduced leukocyte migration (fig. 2a). Treatment with recombinant cystatin 1 and cystatin 3 reduced leukocyte migration in 100µg dose (p) (fig. 2b). Results are expressed in mean ± SEM and data was analyzed by one-way ANOVA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Discussion

In AIA model, there is an acute establishment of arthritis in mice, simulating symptoms that are also present in RA patients, such as pain and migration of inflammatory cells to the affected joint [14]. Then, in order to study novel treatments for RA patients, the proposed intervention must be effective in neutralize these clinical manifestations to proceed to later pre-clinical trials. Moreover, helminths present the ability to modulate the host immune system in order to evasion. Based on this, in the present study we evaluated two different treatments in AIA model: *F. hepatica* extract and recombinant cystatins. Our study demonstrated the potential of *F. hepatica* to reduce nociception and the effect of both extract and cystatins 1 and 3 to reduce leukocyte migration to the joint of AIA mice.

Elevated pain in joint is reported by patients as a frequent symptom in RA and, since inflammation is closely related to pain induced by mediators such as TNF-α, IL-6, IL-1β and IFNγ [16], it is important that treatments present an effect in reducing pain parameters and pain sensation. To assess the analgesic potential of treatments, we analyzed mice nociception using the mechanical threshold parameter with Von Frey. *F. hepatica* extract was able to reduce pain in mice knee while cystatins had no effect. The effect of extract treatment could be explained by the worm secretome which have a variable repertory of molecules [17] that seem to modulate the inflammatory and pain pathways, such cathelicidin-like helminth defence molecule, peroxiredoxins and other excretory products [8]. In addition, it has been described the presence of proteases, enolases, paramyosins and other immunomodulatory molecules in *F. hepatica* tegument that could potentially modulate nociception [18]. Due to its preparation, we speculate that *F. hepatica* extract contain both excretory/secretory products and tegument proteins that are involved in pain reduction.

Leukocyte migration in RA characterizes an important pathological feature of the disease. This lead to hypercellularity, edema and establishment of an inflammatory environment in the joint that contributes to subsequent synovial fibroblast activation, *pannus* formation and joint destruction [2]. Some mechanisms are highlighted as fundamental in this process, as autoantigen presentation, cytokine signaling and leukocytes activation [20]. In our findings, both *F. hepatica* extract and cystatins were able to reduce leukocyte migration in AIA model. The extract contains a repertory of molecules that could interfere in migration of cells. Indeed, *F. hepatica* glycans can modulate dendritic cells adhesion and reduce T cell activation [21].

A previous study has shown that cystatins are protease inhibitors of antigen processing and presenting mechanisms [9] and our results corroborates the link of inhibition of autoantigen presenting with a reduction in the initiation of response and consequent recruitment of cells. It is interesting that both cystatins did not affect nociception, indicating that while the treatment seems to affect the inflammatory process it is not able to modulate nociception, at least not at the early phase of AIA. Nevertheless, more studies are needed for a better understanding of these effects.

In conclusion, our study shows that *F. hepatica* extract and recombinants cystatins were effective in controlling cell migration in an acute model of arthritis. In addition, *F. hepatica* extract ameliorated nociception, suggesting further investigation to elucidate compounds present

in this extract that could be important in pain control. Taken together, our results suggest that the extract and recombinant cystatins of *F. hepatica* present a potential anti-inflammatory and anti-arthritic action and serve as a proof-of-concept for further studies in chronic arthritis models, as well as in its direct effect in different cells types involved in RA pathology.

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Disclosures

None**.**

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3- CONCLUSÕES E PERSPECTIVAS

Os resultados obtidos no estudo mostram um potencial terapêutico tanto do extrato de *Fasciola hepatica* quanto das cistatinas recombinantes. Segundo os dados, o extrato foi efetivo em controlar a dor e a migração leucocitária em modelo de artrite induzida por antígeno. As cistatinas recombinantes se mostraram capazes de controlar a migração leucocitária na dose de 100µg no modelo testado. Com base nisso, e levando em consideração as dificuldades presentes nas terapias atuais, as intervenções propostas no presente estudo se candidatam como potenciais alvos de estudos posteriores, visando a elaboração de novas estratégias terapêuticas.

As perspectivas do grupo em relação aos resultados obtidos são de testar os tratamentos em modelo crônico de artrite (artrite-induzida por colágeno (CIA)), bem como o teste *in vitro* em células-chave do desenvolvimento e manutenção da AR.

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ANEXO 1 - GUIDELINES PARA SUBMISSÃO DE ARTIGO NA REVISTA *CLINICAL RHEUMATOLOGY*

Clinical Rheumatology
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ISSN: 0770-3198 (print version) ISSN: 1434-9949 (electronic version) Journal no. 10067

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