UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE
CURSO DE GRADUAÇÃO EM BIOMEDICINA

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AVALIAÇÃO *IN VITRO* DO POTENCIAL TERAPÊUTICO DO EXTRATO DE *FASCIOLA HEPATICA* EM FIBROBLASTOS SINOVIAIS DE CAMUNDONGOS COM ARTRITE INDUZIDA POR COLÁGENO

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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 Bacharela em Biomedicina.

Orientador: Prof. Dr. Ricardo Machado Xavier

Coorientadora: Me. Mirian Farinon

Porto Alegre

2018

CIP - Catalogação na Publicação

Pedó, Renata Ternus
Avaliação in vitro do potencial terapêutico do extrato de Fasciola hepatica em fibroblastos sinoviais de camundongos com artrite induzida por colágeno / Renata Ternus Pedó. -- 2018.
58 f.
Orientador: Ricardo Machado Xavier.

Coorientadora: Mirian Farinon.

Trabalho de conclusão de curso (Graduação) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Curso de Biomedicina, Porto Alegre, BR-RS, 2018.

1. Artrite Reumatoide. 2. Fibroblastos Sinoviais . 3. Extrato de Fasciola hepatica. I. Xavier, Ricardo Machado, orient. II. Farinon, Mirian, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

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Aprovado em: 20 de dezembro de 2018.

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AGRADECIMENTOS

Aos meus pais, por todo o amor e carinho, por sempre terem me incentivado a estudar e lutar pelos meus sonhos. Sem o incentivo e apoio diários de vocês eu não teria chegado até aqui. Obrigada por sonharem comigo, terem me dado todo o suporte necessário e entenderem a ausência em muitos momentos desses últimos anos. Meu eterno obrigada!

As minhas irmãs, por terem dividido tantos momentos comigo ao longo desses anos, compartilhando as angústias e conquistas da faculdade. Muito obrigada!

Ao professor Xavier, pela orientação e ensinamentos nestes anos de iniciação científica.

A minha coorientadora, Mirian Farinon, a qual tornou possível a realização deste trabalho, com toda a sua orientação e ensinamentos. Obrigada por ter aceitado este convite. Ao teu lado aprendi muito, desde o meu primeiro dia como aluna de iniciação científica. Minha eterna gratidão!

A todos os meus colegas do LabDAI, por todos as sugestões, discussões sobre ciência e risadas compartilhadas.

Ao meu namorado Emerson, muito obrigada pela paciência nestes meses de TCC. Pelo apoio, compreensão, ajuda e carinho. Você tornou este período muito mais fácil.

RESUMO

A artrite reumatoide (AR) é uma doença autoimune, crônica e sistêmica, onde a inflamação da membrana sinovial articular leva à degradação da cartilagem e do osso, resultando na destruição articular, dor e incapacidade funcional. De etiologia ainda pouco esclarecida, sua prevalência é de cerca de 0,46% no Brasil e 1% no mundo, com ocorrência maior entre as mulheres. Diversos tipos celulares estão envolvidos na patogênese da AR, porém os fibroblastos sinoviais (FLS) se destacam por apresentarem um fenótipo agressivo que medeia a inflamação e a destruição articular. Apesar dos avanços no tratamento da AR, estes apresentam significativos efeitos adversos, altos custos e limitações, salientando a necessidade da busca por novas estratégias terapêuticas. A *Fasciola hepatica* (*F. hepatica*) é um helminto causador da doença fasciolose em ruminantes e humanos. Através de produtos excretores-secretores e antígenos do tegumento, a *F. hepatica* apresenta propriedades imunomoduladoras, as quais já foram estudadas em diferentes tipos celulares, mas não em FLS. Diante disso, avaliamos nesse trabalho o potencial terapêutico *in vitro* do extrato de *F. hepatica* em FLS isolados de camundongos com artrite induzida por colágeno. A viabilidade celular dos FLS foi determinada por ensaio de MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], em que os FLS foram expostos ao extrato de *F. hepatica* nas concentrações de 60µg/ml, 80µg/ml e 100µg/ml, por 24h, 48h e 72h. A capacidade de aderência dos FLS foi avaliada pela exposição das células ao extrato de *F. hepatica* na concentração de 100µg/ml por 24h. Os efeitos a longo prazo do tratamento com o extrato de F. hepatica na FLS foram avaliados utilizando o ensaio cumulativo de duplicação da população (CDP) após exposição dos FLS a 100µg/ml de extrato de *F. hepatica* durante 24h. As células foram contadas a cada 2 dias e replaqueadas na mesma concentração inicial por 8 dias. A liberação de interleucina-6 (IL-6) foi avaliada pelo ensaio de ELISA, para este ensaio os FLS também foram expostos ao extrato de *F. hepatica* na concentração de 100µg/ml por 24h e, após, foram estimulados com o fator de necrose tumoral α (TNF- α). O extrato de *F. hepatica* foi capaz de diminuir a viabilidade celular em 48h com a concentração de 100µg/ml quando comparado com o grupo DMEM ($p<0.05$). Em 72h, as doses de 60µg/ml ($p <0,001$), 80µg/ml ($p <0,001$) e 100µg/ml ($p <0,0001$) do extrato de *F. hepatica* diminuíram a viabilidade celular quando comparadas com o grupo DMEM. O tratamento com 100µg/ml do extrato por 24h apresentou uma tendência no aumento da liberação de IL-6 pelos FLS quando comparados com o grupo DMEM estimulado. No entanto, o extrato não alterou a capacidade de aderência e o crescimento a longo prazo dos FLS. A partir desses resultados, pode-se concluir que o extrato de *F. hepatica* não apresentou efeito sobre a aderência e o crescimento ao longo do tempo dos FLS, porém apresentou efeito na viabilidade dos FLS e foi capaz de aumentar a liberação de IL-6 pelos FLS. Portanto, embora esses resultados sejam preliminares, eles são importantes para entender a ação do extrato da *F. hepatica* nos FLS e indicam um efeito imunomodulatório do extrato sobre os FLS.

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

ABSTRACT

Rheumatoid arthritis (RA) is an autoimmune, chronic and systemic disease, where the inflammation of synovial joints leads to degradation of cartilage and bone, resulting in joint destruction, pain and functional disability. The prevalence of disease is around 0.46% in Brazil and 1% in the world, with a higher occurrence among women. Several cell types are involved in the pathogenesis of RA and fibroblast-like synoviocytes (FLS) have a central role mediating inflammation and joint destruction because of its aggressive phenotype. Despite advances in the treatment of RA, there are still significant adverse effects, high costs and limitations, emphasizing the need to search for new therapeutic options. *Fasciola hepatica (F. hepatica)* is a helminth that causes fasciolosis in ruminants and humans. Through excretory-secretory products and tegument antigens, *F. hepatica* presents immunomodulatory properties, which have already been studied in different cell types, but not in FLS. In this work, we evaluated the *in vitro* therapeutic potential of *F. hepatica* extract in FLS isolated from mice with collagen-induced arthritis. Cell viability of FLS was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, in which FLS were exposed to extract of *F. hepatica* at concentrations of 60µg/ml, 80µg/ml and 100µg/ml, for 24h, 48h and 72h. Adherence capacity of FLS was evaluated by exposing the cells to the extract of *F. hepatica* at a concentration of 100µg/ml for 24h. The long-term effects of treatment with *F. hepatica* extract on FLS were evaluated using the cumulative population doubling test (CDP) after exposure of FLS to 100µg/ml of *F. hepatica* extract for 24h. The cells were count every 2 days and were re-plated at the same initial density for 8 days. The release of interleukin-6 (IL-6) was evaluated by the ELISA assay. For this assay, FLS were exposed to 100µg/ml of extract of *F. hepatica* for 24h and then were stimulated with tumor necrosis factor (TNF-α). Extract of *F. hepatica* was able to decrease cell viability in 48h with the concentration of 100µg/ml when compared to DMEM group ($p < 0.05$). In 72h, doses of 60µg/ml ($p < 0.001$), 80µg/ml ($p < 0.001$) and 100µg/ml ($p < 0.0001$) of *F. hepatica* extract decreased cell viability when compared to DMEM group. Treatment with 100µg/ml of *F. hepatica* extract for 24h showed a tendency to increase IL-6 release by FLS when compared to stimulated DMEM. However, the extract did not alter adhesion capacity and long-term growth of FLS. In conclusion, extract of *F. hepatica* had no effect on adherence and growth over time of FLS, but showed an effect on the viability of FLS and was able to increase the release of IL-6 by FLS. Therefore, although these results are preliminary, they are important for understanding the action of *F. hepatica* extract on FLS and indicate an immunomodulatory effect of the extract on FLS.

Keywords: Rheumatoid arthritis; fibroblast-like synoviocytes; *Fasciola hepatica* extract.

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LISTA DE ABREVIATURAS

ACPA	Anticorpos Anti-proteínas/peptídeos Citrulinados
AR	Artrite Reumatoide
CFA	Adjuvante Completo de Freund (Complete Freund's Adjuvante)
CIA	Artrite Induzida por Colágeno
CII	Colágeno Bovino do Tipo II
DMDC	Drogas Modificadoras do Curso da Doença
ESPs	Produtos Excretores-Secretores
F. hepatica	*Fasciola hepatica*
FLS	Fibroblastos Sinoviais
FR	Fator Reumatoide
IFN-γ	Interferon-γ
IL-1	Interleucina-1
IL-1β	Interleucina-1β
IL-17	Interleucina-17
IL-6	Interleucina-6
IL-8	Interleucina-8
MEC	Matriz Extracelular
MHC	Complexo Principal de Histocompatibilidade
MLS	Macrófago Sinovial
MMP	Metaloproteinases
PCR	Proteína C Reativa
RANKL	Ligante do Receptor Ativador do fator Nuclear κB
TGF-β	Fator de Crescimento β
TLRs	Receptor do Tipo Toll
VEGF	Fator de Crescimento Endotelial Vascular

TNF	Fator de Necrose Tumoral
VSG	Velocidade de Sedimentação Globular

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1 INTRODUÇÃO

1.1 ARTRITE REUMATOIDE

A artrite reumatoide (AR) é uma doença autoimune, inflamatória, sistêmica e de etiologia desconhecida. Ela é caracterizada por sinovite crônica, simétrica e erosiva, acometendo preferencialmente as articulações periféricas. Os sintomas clínicos da doença incluem edema, rigidez nas articulações e consequentemente redução dos movimentos (SCOTT; WOLFE; HUIZINGA, 2010). Apesar de envolver principalmente as articulações, a AR causa manifestações extra-articulares como nódulos reumatoides, fraqueza muscular, doenças de pele, manifestações oculares e vasculite, além de poder acometer rins, coração e sistema nervoso central e periférico (KHURANA; BERNEY, 2005). Essas manifestações agravam a doença, levando a um aumento da incapacidade funcional, diminuição da qualidade de vida e aumento da mortalidade (RADNER; SMOLEN; ALETAHA, 2010). Sua prevalência é de 0,46% no Brasil e 1% no mundo, sendo sua incidência mais comum em indivíduos entre os 40 e 70 anos de idade, com uma ocorrência maior em mulheres (SENNA et al., 2004) (LEE; WEINBLATT, 2001).

Apesar da etiologia da AR não ser completamente descrita, sabe-se que fatores ambientais e genéticos estão envolvidos com a suscetibilidade e severidade da doença (SMOLEN et al., 2018). Alguns fatores ambientais associados à AR são a exposição à sílica, o uso de cigarro, deficiência de vitamina D, obesidade e alterações na microbiota (TOBÓN; YOUINOU; SARAUX, 2010) (SMOLEN et al., 2018). Quanto aos fatores genéticos, sabe-se que 50% do risco de desenvolver AR é atribuído a eles. Atualmente, mais de 30 regiões gênicas associadas ao desenvolvimento da doença já foram identificadas, como os alelos do antígeno leucocitário humano (HLA)-DRB1, que contém um motivo comum, denominado de epítopo compartilhado e o gene PTPN22 (BARTON; WORTHINGTON, 2009).

Diversos mecanismos estão envolvidos na patogênese da AR. Um evento inicial para o desenvolvimento da doença são as modificações pós-traducionais, como a citrulinização de proteínas, a qual cria novos epítopos. Estes são reconhecidos pelo sistema imune adaptativo e apresentados pelas células apresentadoras de antígenos às células T, que por sua vez estimulam as células B a produzirem uma variedade de anticorpos (SMOLEN et al., 2018). Os anticorpos formados reconhecem auto-proteínas (proteínas do próprio indivíduo) sendo, portanto, denominados autoanticorpos. Os principais autoanticorpos presentes na AR são o fator reumatoide (FR), o qual reage contra a porção Fc da imunoglobulina G formando complexos imunes, e os anticorpos anti-proteínas/peptídeos citrulinados (ACPA) que possuem como alvo as proteínas/peptídeos citrulinados (HOLERS, 2013) (SMOLEN et al., 2018). A presença destes autoanticorpos circulantes, bem como de citocinas pró-inflamatórias e quimiocinas, pode ser detectada anos antes do aparecimento dos sinais clínicos da doença. Além disso, a presença de FR e de ACPA é associada com um pior prognóstico da AR (NIELEN et al., 2004).

A resposta autoimune gerada leva inicialmente a um processo inflamatório na membrana sinovial. Esta inflamação é caracterizada, primeiramente, pelo infiltrado e acúmulo de leucócitos, principalmente neutrófilos, os quais são atraídos por moléculas de adesão e quimiocinas expressas na sinóvia (MCINNES, 2011). Posteriormente, ocorre intensa proliferação de células T (principalmente $CD4^{+}$) e B, plasmócitos, mastócitos e células

dendríticas. Além disso, os macrófagos sinoviais (MLS) e fibroblastos sinoviais (FLS), que são uma importante fonte de citocinas e proteases, aumentam sua proliferação, levando à hiperplasia da membrana sinovial (MCINNES, 2011). Esta hiperplasia sinovial, juntamente com o infiltrado das células inflamatórias e a estimulação da angiogênese, leva à formação de um tecido invasivo denominado *pannus,* o qual invade as estruturas adjacentes, gerando danos à cartilagem e ao osso, destruindo progressivamente a articulação (MCINNES, 2011) (Figura 1).

A patologia da AR também envolve a produção e liberação de citocinas pró-inflamatórias e outros mediadores por diferentes tipos celulares. Os neutrófilos sintetizam prostaglandinas, proteases e espécies reativas de oxigênio e nitrogênio, além de secretarem fator de necrose tumoral (TNF) e metaloproteinases (MMPs) (CHOY, 2012). Os macrófagos produzem TNF-α, interleucina-1β (IL-1β), IL-6, IL-8, MMPs, quimiocinas e, também, realizam fagocitose e apresentação de antígenos. As células B, produzem IL-6, TNF-α e são precursores de plasmócitos secretores de autoanticorpos, além de processarem e apresentarem antígenos, promovendo a ativação das células T. Estas, por sua vez, sintetizam interferon-γ (IFN-γ), TNF e IL-17 (CHOY, 2012).

Embora ocorra a participação de várias citocinas na patogênese da AR, o TNF-α, a IL-6 e a IL-1 são mediadores cruciais na migração de células e no estabelecimento da inflamação (CHOY, 2012). O TNF-α desempenha um papel fundamental através da ativação da expressão de citocinas e quimiocinas, supressão de células T regulatórias, indução da angiogênese e da dor (MCINNES; SCHETT, 2007). A IL-6 promove a ativação local de leucócitos e a produção de autoanticorpos, já a IL-1, além de promover a ativação de leucócitos, também promove a ativação de células endoteliais, condrócitos e osteoclastos (MCINNES; SCHETT, 2007) (MCINNES, 2011).

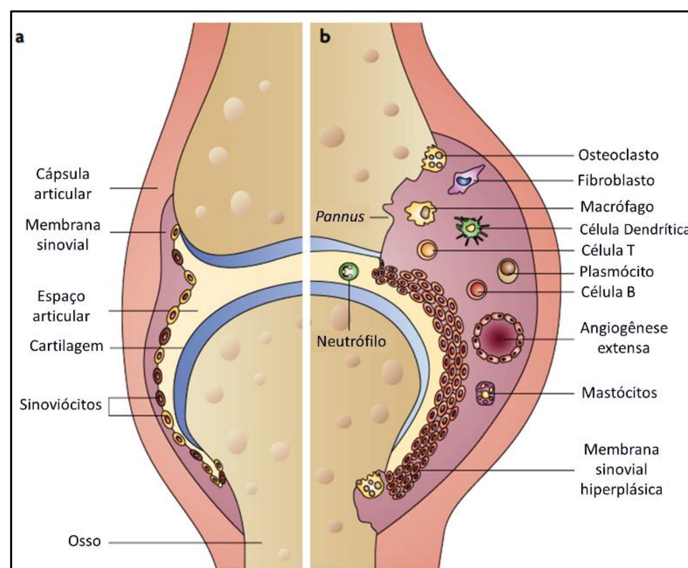

Figura1: Alterações articulares na artrite reumatoide.
a) Articulação saudável; b) Articulação doente (Adaptado de Smolen and Steiner, 2003).

Adicionalmente à inflamação sinovial, ocorre o dano à cartilagem e ao osso subcondral (subjacente à cartilagem articular). A hiperplasia sinovial é a principal causa do dano à

cartilagem, uma vez que os MLS e FLS são ativados por TNF-α, IL-1 e IL-6 e, consequentemente, passam a sintetizar substâncias nocivas à cartilagem, como as MMPs (CHOY, 2012). Citocinas também ativam os condrócitos, levando à liberação direta de MMPs na cartilagem. Somado a isto, o potencial de regeneração do tecido cartilaginoso fica limitado, devido às citocinas e espécies reativas de nitrogênio presentes neste ambiente, que promovem a apoptose dos condrócitos (CHOY, 2012) (MCINNES, 2011). O dano ao tecido ósseo ocorre devido a ativação e diferenciação de osteoclastos pelo ligante do receptor ativador do fator nuclear κB (RANKL), o qual é expresso principalmente por osteoblastos, mas também por células T e FLS. Esta ativação e diferenciação dos osteoclastos também é estimulada, em menor proporção, por citocinas como TNFα, IL-1, IL-6 e IL-17 (JUNG et al., 2014). Os osteoclastos, então ativados, produzem proteases que degradam a matriz óssea mineralizada e realizam a reabsorção óssea (SCHETT; GRAVALLESE, 2012).

1.2 FIBROBLASTOS SINOVIAIS

A membrana sinovial saudável é um tecido que reveste internamente as articulações e é composta por uma camada fina de células, normalmente dividida em dois compartimentos denominados camada íntima e camada sub-íntima. A íntima possui de 1 a 3 camadas de células e é composta por dois tipos celulares em proporções relativamente iguais: MLS e FLS. A sub-íntima é composta de tecido conjuntivo frouxo, irregular, com poucos fibroblastos, macrófagos e células adiposas (BARTOK; FIRESTEIN, 2010). No entanto, na AR a membrana sinovial sofre modificações se tornando hiperplásica. A camada íntima pode atingir de 10 a 15 camadas de células e a sub-íntima também sofre expansão com um infiltrado de células inflamatórias (células dendríticas, mastócitos, células T e B) e aumento da deposição de matriz extracelular (MEC). Além disso, na camada sub-íntima ocorre a neoangiogênese, em resposta ao aumento da demanda metabólica do tecido inflamado e hipóxico (TURNER; FILER, 2015).

Apesar de diversos tipos celulares estarem envolvidos nas modificações que ocorrem na membrana sinovial, os FLS se destacam por possuírem um importante papel tanto no indivíduo saudável quanto na patogenia da AR (BARTOK; FIRESTEIN, 2010). Nas articulações saudáveis, eles controlam a composição do líquido sinovial, produzindo lubricina e ácido hialurônico que auxiliam na lubrificação articular. Eles também são responsáveis pelo remodelamento da MEC, através da secreção de vários dos seus componentes, como colágenos, fibronectina e laminina, além de secretarem enzimas que degradam a matriz, como as MMPs, e seus inibidores (BARTOK; FIRESTEIN, 2010). Contudo, na AR o comportamento dos FLS se modifica pois, após sua ativação, estas células adquirem um fenótipo agressivo e medeiam a inflamação e a destruição articular (BARTOK; FIRESTEIN, 2010).

No ambiente de inflamação articular encontrado na AR, citocinas pró-inflamatórias, quimiocinas e fatores de crescimento produzidos pelos MLS ativam os FLS, os quais aumentam a secreção de uma variedade de citocinas, quimiocinas e fatores pró-angiogênicos, como o fator de crescimento endotelial vascular (VEGF). Dessa forma, os FLS exercem importantes funções imunorreguladoras, através da secreção de citocinas e interações diretas com células imunes. Eles secretam IL-6 e IL-8, que estimulam a ativação de células do sistema imune inato e adaptativo na articulação, bem como IL-1 e fator de crescimento beta (TGF-β) (BOTTINI; FIRESTEIN, 2013) (CROW, 2010). Ainda, os FLS na AR também apresentam expressão

aumentada de moléculas de adesão, como caderinas e integrinas, as quais auxiliam na fixação da sinóvia hiperplásica à superfície da cartilagem. Com esse processo, é estabelecida uma rede parácrina/autócrina que perpetua a inflamação, recruta novas células inflamatórias para a articulação e contribui para a degradação da MEC (BARTOK; FIRESTEIN, 2010). Em adição à produção e secreção de fatores inflamatórios, a capacidade proliferativa dos FLS aumenta, por conta de um desequilíbrio entre proliferação e apoptose, devido a mudanças nas vias que promovem a invasividade e a secreção de citocinas, aumentando assim, a expressão de moléculas antiapoptóticas (BOTTINI; FIRESTEIN, 2013).

Além do mais, os FLS apresentam características semelhantes a um tumor, como o aumento da migração, redução da inibição por contato, expressão de proto-oncogenes e a capacidade de invadir os tecidos adjacentes (BOTTINI; FIRESTEIN, 2013). O fenótipo de invasão se deve à capacidade dos FLS de produzirem componentes de degradação da MEC, como colagenases, MMPs e catepsinas, que orientam o equilíbrio entre as proteases e seus inibidores para a destruição tecidual. Essa invasividade também é estimulada por citocinas como IL-1 e TNF-α e espécies reativas de oxigênio e nitrogênio que têm sua produção favorecida pela hipóxia local (MÜLLER-LADNER et al., 2007). Somado a este potencial invasivo, responsável pelo dano à cartilagem, os FLS também podem migrar de uma articulação para a outra, propagando a doença (LEFÈVRE et al., 2009). Por conta disto, os FLS secretam RANKL que promove a diferenciação de osteoclastos, os quais geram a erosão óssea, levando ao dano articular (BOTTINI; FIRESTEIN, 2013).

Devido ao fato dos FLS serem considerados um dos principais contribuintes para a inflamação e destruição articular, essas células constituem um importante alvo para novas abordagens terapêuticas na AR. Uma característica dos FLS que propicia seu estudo, é que eles podem ser isolados do tecido sinovial e cultivados *in vitro* (BARTOK; FIRESTEIN, 2010). No cultivo *in vitro*, seu fenótipo agressivo e invasivo é mantido, inclusive apresentando correlação do grau de capacidade de invasão com o dano articular. Estudos já demonstraram que seu fenótipo in vitro está associado com a taxa de destruição articular de pacientes com AR (TOLBOOM et al., 2005), bem com o dano histológico da articulação em ratos (LARAGIONE et al., 2008).

1.3 DIAGNÓSTICO E TRATAMENTARO DA AR

O diagnóstico da AR é um processo individualizado, baseado em critérios de classificação que incluem manifestações clínicas e testes sorológicos (SMOLEN et al., 2018). Atualmente, os critérios de classificação mais utilizados são os propostos pelo Colégio Americano de Reumatologia (American College of Rheumatology - ACR) e pela Liga Europeia Contra o Reumatismo (European League Against Rheumatism - EULAR) em 2010 (SMOLEN et al., 2018). Estes critérios avaliam o envolvimento e distribuição das articulações acometidas, a duração dos sintomas, a sorologia (FR e ACPA) e marcadores de fase aguda (níveis da proteína C reativa [PCR] e velocidade de sedimentação globular [VSG]), atribuindo uma pontuação para cada critério (NEOGI et al., 2010).

Quanto às pontuações dos critérios de classificação, são atribuídos 0 a 5 pontos para o envolvimento e distribuição das articulações (1 grande articulação = 0 pontos; 2 a 10 grandes

articulações = 1 ponto; 1 a 3 pequenas articulações = 2 pontos; 4 a 10 pequenas articulações = 3 pontos e mais que 10 articulações = 5 pontos); 0 a 1 ponto para a duração dos sintomas (por menos de 6 semanas = 0 pontos; igual ou por mais de 6 semanas = 1 ponto); 0 a 6 pontos para a sorologia (negativo para FR e ACPA = 0 pontos; fracamente positivo para FR ou para ACPA = 2 pontos e fortemente positivo para FR ou para ACPA = 3 pontos) e 0 a 1 ponto para os marcadores de fase aguda (níveis normais para PCR e VSG = 0 pontos e níveis anormais de PCR ou de VSG = 1 ponto) (Tabela 1). No entanto, estes critérios de classificação são restritos a indivíduos que tenham pelo menos uma articulação inchada e é necessária uma pontuação de 6 ou mais pontos para diagnóstico definitivo de AR (NEOGI et al., 2010). O diagnóstico precoce previne que o paciente tenha perdas funcionais e diminuição na sua qualidade de vida (ALETAHA et al., 2005).

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

1. Envolvimento articular (0-5)

- 1 articulação média a grande (0)
- 2-10 articulações médias a grande (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 pequena) (5)

2. Sorologia (0-3)

- Fator reumatoide (FR) e Anticorpo contra antígenos citrulinados (ACPA) negativo (0)
- RF e ACPA fracamente positivos (2)
- RF e ACPA fortemente positivos (3)

3. Reagentes de fase aguda (0-1)

- Proteína C reativa e taxa de sedimentação eritrocitária normal (0)
- Proteína C reativa e/ou taxa de sedimentação eritrocitária anormal (1)

4. **Duração dos sintomas (0-1)**

- < 6 semanas (0)
- 6 semanas ou mais (1)

Ponto de corte para artrite reumatoide: 6 ou mais

O tratamento da doença atualmente envolve uma abordagem de tratamento baseado em metas (treat-to-target), através do monitoramento rigoroso da atividade da doença e na mudança do manejo, caso a meta de tratamento não seja atingida (SMOLEN et al., 2016). O principal objetivo do tratamento não é a cura, mas a remissão ou pelo menos uma baixa taxa de atividade da doença, com a finalidade de restaurar a função física na doença precoce e maximizar a função física na doença estabelecida, bem como a eliminação da inflamação articular ativa (SMOLEN et al., 2018). Outros objetivos do tratamento incluem a redução da dor, controle das comorbidades e preservação de atividades recreativas e de trabalho (SCOTT; WOLFE; HUIZINGA, 2010).

A base do tratamento da AR são as drogas modificadoras do curso da doença (DMCDs). Embora seus diversos mecanismos de ação não sejam completamente conhecidos, seus efeitos são a redução do inchaço e da dor nas articulações, diminuição dos marcadores de fase aguda (PCR e VSG), limitação dos danos progressivos nas articulações e melhora da função. O DMCD padrão é o metotrexato, cujos efeitos adversos, bem como dos demais DMCDs (leflunomida, sulfasalazina e hidroxicloroquina), incluem desde náuseas até hepatotoxicidade e doenças pulmonares intersticiais. O tempo de início da ação dos DMCDs é lento, assim até que seu efeito farmacológico se inicie, são utilizados glicocorticoides por períodos curtos de tempo (SMOLEN et al., 2018). Os glicocorticoides utilizados por períodos curtos de tempo reduzem a inflamação sinovial e seu uso por tempo prolongado diminui o dano nas articulações, porém seus efeitos adversos a longo prazo incluem infecções e osteoporose, assim, a relação custo/benefício é considerada desfavorável. Ainda, podem ser utilizados de forma localizada, através de infiltração nas articulações, sendo um tratamento altamente eficaz (SMOLEN et al., 2018).

Outra classe de medicamentos utilizada é a de agentes biológicos (SCOTT; WOLFE; HUIZINGA, 2010), desenvolvidos devido a avanços na biologia molecular e bioquímica nos últimos anos, que propiciaram a compreensão detalhada da estrutura e função de vários receptores chave nas células do sistema imune, possibilitando a produção de citocinas recombinantes e receptores de citocinas. Dentro dessa classe, os inibidores de TNF foram os primeiros licenciados para uso (O'SHEA; LAURENCE; MCINNES, 2013). Dentre eles, o infliximabe e o adalimumabe são anticorpos anti-TNF, e o etanercepte é uma proteína de fusão do receptor II do TNF (SMOLEN; STEINER, 2003). Outros agentes biológicos são o rituximabe, um anticorpo monoclonal anti-CD20 (molécula de superfície encontrada na célula B) e o abatacepte, um inibidor de sinais coestimulatórios de células T. Atualmente, os agentes biológicos são usados combinados com metotrexato ou algum outro DMCD (que também podem ser combinados entre si) com o objetivo de reduzir a formação de anticorpos contra os tratamentos. Os efeitos adversos incluem reações e infecções nos locais de infusão e injeção, além do aumento do risco de tuberculose com o uso dos inibidores do TNF (SMOLEN; ALETAHA; MCINNES, 2016).

Conforme citado, os medicamentos empregados no tratamento da AR são utilizadas de maneira combinada, de acordo com o estado do paciente e o estágio da doença. O tratamento da AR inicial utiliza o metotrexato como primeira opção de DMCD. Em pacientes com respostas incompletas aos DMCDs, a adição precoce de agentes biológicos parece altamente eficaz, no entanto, quando os pacientes atingem a remissão deve ser utilizado um único DMCD. Os anti-inflamatórios não-esteroidais (AINEs), também possuem indicação de uso na AR inicial, com o objetivo de aliviar os sintomas antes que o tratamento antirreumático específico seja iniciado. Quando a AR já está estabelecida, se almeja a diminuição da atividade da doença pelo uso em conjunto de DMCDs e agentes biológicos, com ou sem glicocorticoides. Neste estágio da AR, os agentes biológicos, geralmente são continuados, a menos que se tornem ineficazes ou surja um efeito adverso relevante. As recidivas e a doença ativa persistente são tratados com a troca ou combinação de DMCDs, adição de glicocorticoides e início ou troca de agentes biológicos. Caso a doença continue ativa, é realizada a troca ou combinação de

DMCDs, adição de glicocorticoides e início ou troca de agentes biológicos. Ainda, pacientes que não responderem aos inibidores de TNF, geralmente recebem um agente biológico alternativo (SCOTT; WOLFE; HUIZINGA, 2010; SMOLEN et al., 2018).

Um outro aspecto importante no tratamento da AR, é o manejo das comorbidades associadas, como doenças cardíacas, renais e depressão, pois estas refletem tanto o processo da doença como o seu tratamento (SCOTT; WOLFE; HUIZINGA, 2010). Apesar dos avanços no tratamento da AR e das diversas classes disponíveis para o tratamento, a presença dos efeitos adversos e a necessidade do uso de diversos fármacos ao mesmo tempo, em alguns casos, leva muitos pacientes a desistirem ou não seguirem o tratamento corretamente. Além disso, as opções atuais falham na recidiva ou reativação da doença e muitos fármacos possuem custos elevados, como os agentes biológicos. Portanto, se faz necessária a busca por novas terapias, com o intuito de solucionar os problemas relacionados ao tratamento e, assim, melhorar a qualidade de vida dos pacientes (O'SHEA; LAURENCE; MCINNES, 2013; SMOLEN et al., 2018).

1.4 MODELO ANIMAL DE ARTRITE INDUZIDA POR COLÁGENO (CIA)

Modelos animais que mimetizam doenças humanas são importantes para o estudo da patogenia das doenças, das vias e mecanismos biológicos envolvidos. Dessa forma, eles possibilitam o estudo de novos alvos terapêuticos, bem como o desenvolvimento de agentes terapêuticos e de prevenção. Para a escolha de um modelo, alguns critérios para a seleção são importantes, como a capacidade de prever a eficácia em humanos dos agentes estudados, a preferência por um protocolo experimental de fácil execução e reprodutibilidade, bem como um período de execução relativamente curto, além de apresentar uma patologia e/ou patogenia semelhante à da doença em humanos (BENDELE, 2001).

Dentro do estudo experimental da artrite, diversos modelos animais são utilizados, os quais mimetizam diversas características da artrite em humanos (KOLLIAS et al., 2011). Quase todos os modelos apresentam as características gerais da AR, como a degradação das articulações, a produção de citocinas e a proliferação e infiltração celular (WEBB, 2014). Apesar das limitações de cada modelo, eles viabilizaram o entendimento de diversos mecanismos da AR e contribuíram para vários avanços em seu tratamento (ASQUITH et al., 2009).

Entre os modelos experimentais utilizados, podem ser citados o de artrite induzida por zymosan (ZIA), artrite induzida por adjuvante (AA), artrite induzida por antígeno (AIA) e de artrite induzida por colágeno (CIA). O modelo de CIA compartilha várias similaridades com a AR humana, como a hiperplasia sinovial, o infiltrado celular, a degradação da cartilagem e a suscetibilidade ligada à expressão de genes específicos da classe II do complexo principal de histocompatibilidade (MHC). Além das características já mencionadas, a quebra da auto tolerância e a produção de auto anticorpos contra o próprio colágeno, o tornam o modelo padrão ouro para estudos de AR (ASQUITH et al., 2009; BRAND; LATHAM; ROSLONIEC, 2007). No entanto, apesar das similaridades entre este modelo e a AR, a patologia articular na AR é crônica e simétrica, enquanto no modelo de CIA ela é transitória e não-simétrica; além disso,

em CIA o FR não está presente, há pouco ou nenhum viés entre os sexos e a doença experimental é geralmente monofásica (BILLIAU; MATTHYS, 2011).

O modelo de CIA é induzido em cepas de camundongos geneticamente suscetíveis, sendo a mais utilizada a de camundongos DBA/1J. A indução é realizada através de uma emulsão de adjuvante completo de Freund (CFA) e colágeno bovino do tipo II (CII). De acordo com o protocolo experimental, a artrite é induzida nos camundongos por uma imunização intradérmica de 100µl na base da cauda, contendo CII dissolvido em ácido acético 0,1M (2mg/ml) e emulsificado em um volume igual de CFA (2mg/ml de *Mycobacterium tuberculosis*). Após 18 dias, é realizada um reforço em outro ponto da cauda com 100µl de emulsão contendo CII e adjuvante incompleto de Freund (sem o *Mycobacterium tuberculosis*). Os primeiros sinais da doença aparecem entre 21 a 28 dias após a primeira imunização, sendo identificados através de edema e eritema nas patas dos animais (BRAND; LATHAM; ROSLONIEC, 2007).

A principal causa do desenvolvimento do CIA é a superestimulação da reatividade imunológica inata pelo adjuvante, levando a uma resposta inflamatória sistêmica. Devido à presença de partículas de *M. tuberculosis*, o CFA também gera a imunidade adaptativa contra antígenos micobacterianos, com envolvimento das células T auxiliar do tipo 1 (Th1) e T auxiliar do tipo 17 (Th17) (BILLIAU; MATTHYS, 2011). Além disso, seu desenvolvimento está associado com a resposta de linfócitos B e T, através da produção de anticorpos anti-colágeno do tipo II e células T específicas para o colágeno (ASQUITH et al., 2009).

Os FLS neste modelo também passam pelas alterações que ocorrem com os FLS na doença humana. Após serem isolados e cultivados *in vitro*, são capazes de manter suas características, como o fenótipo agressivo, invasivo e a resistência à apoptose, mesmo sem um estímulo exógeno, e podem ser cultivados *in vitro* por longos períodos (BOTTINI; FIRESTEIN, 2013). Além disso, produzem espontaneamente citocinas, fatores de crescimento, MMPs e moléculas de adesão da mesma maneira que os FLS em pacientes com AR (BARTOK; FIRESTEIN, 2010). Dessa maneira, os FLS isolados de camundongos com CIA, são uma importante ferramenta para o entendimento da patogênese da AR e para a avaliação de novos alvos terapêuticos.

1.5 *FASCIOLA HEPATICA*

A erradicação do contato humano com agentes parasitas pela melhora da higiene ao longo dos anos está relacionada com o aumento de doenças autoimunes e alérgicas, sendo essa ideia centrada na "hipótese da higiene". Segundo essa hipótese, essa relação se baseia na ausência de um papel imunomodulatório que esses vermes têm quando parasitam o organismo (JACKSON et al., 2009). Assim, doenças crônicas causadas por helmintos, geram uma resposta imune Th2, com potencial de imunomodulação. Além disso, a literatura evidencia o papel regulatório do sistema imune por parasitas em diversas doenças autoimunes, como na esclerose múltipla (CORREALE; FAREZ, 2007), na doença de Crohn (SUMMERS et al., 2005a), no diabetes tipo 1 (SAUNDERS et al., 2007), na colite (SUMMERS et al., 2005b) e na AR (OLIVEIRA et al., 2017; PAULA MONTEIRO GOMIDES et al., 2017).

Outros estudos evidenciam que produtos secretados por helmintos possuem moléculas com propriedades anti-inflamatórias (HAMILTON et al., 2009; WANG et al., 2016) e, dentre estes helmintos, a *Fasciola hepatica (F. hepatica)* é um parasita que vem sendo estudado com essa finalidade. Este parasita é um helminto trematódeo causador da doença fasciolose em ruminantes e humanos. A infecção ocorre pela ingestão de água e vegetais contaminados por metacercárias do parasita, afetando em torno de 17 milhões de pessoas no mundo (MORALES; ESPINO, 2012; NYINDO; LUKAMBAGIRE, 2015). A infecção é crônica e pode persistir por vários anos no hospedeiro, resultando em uma grande liberação de ovos do parasita nas fezes, o que contribui para a transmissão da infecção (MAS-COMA; BARGUES; VALERO, 2005).

Na infecção, a *F. hepatica* utiliza estratégias para regular a resposta imune de seus hospedeiros, através de produtos excretores-secretores (ESPs), os quais têm os mesmos efeitos imunomodulatórios presentes na infecção com o verme e são capazes de suprimir a resposta imune via Th1 (DONNELLY et al., 2005), podendo desta maneira reduzir a inflamação. Além disso, os antígenos do tegumento da *F. hepatica* apresentam um mecanismo de regulação imune. Tanto os ESPs quanto os antígenos do tegumento também apresentaram capacidade de suprimir a ativação de células dendríticas em reposta aos receptores do tipo Toll 3, 4 e 9 (Toll-like receptors, TLRs) em estudos *in vitro* e em modelo de fasciolose em camundongos (FALCÓN et al., 2010, 2012).

A preparação de antígenos presentes no tegumento da *F. hepatica* (FhTeg) e administrada em modelo de choque séptico em camundongos, exibiu potentes propriedades supressoras de células Th1, reduzindo os níveis séricos de IFN-γ. Além disso, células dendríticas e mastócitos ativados por FhTeg, são hipo-responsivos à ativação de TLRs e, com isso, suprimem a produção de citocinas inflamatórias como TNF-α, IL-6, IFN-γ e IL-10, importantes na condução de respostas imunes adaptativas (RAVIDÀ et al., 2016).

A *F. hepatica* também é capaz de induzir apoptose de duas células importantes da resposta imune inata, os eosinófilos e os macrófagos, demonstrado em estudo *in vitro* (GUASCONI; SERRADELL; MASIH, 2012). Estas células possuem papéis importantes na resposta imune desenvolvida durante as infecções por helmintos. A indução de apoptose dessas células pode ser importante na modulação do sistema imune, se apresentando como mais uma estratégia para evadir a resposta imune do hospedeiro (GUASCONI; SERRADELL; MASIH, 2012).

Durante a infecção, a *F. hepatica* secreta moléculas como as cistatinas, as quais são inibidoras de cisteíno-proteases e seu papel tem sido estudado na supressão da resposta imune. As cisteínos-proteases, especialmente as catepsinas, desempenham um importante papel no desenvolvimento e progressão da destruição articular na AR. Já foi demonstrado que a catepsina B é muito expressa em FLS, sendo capaz de regular sua capacidade de migração e invasão (TONG et al., 2014). Dessa forma, a ação das cistatinas sobre os FLS poderia diminuir seu fenótipo agressivo. As cistatinas também são capazes de regular citocinas inflamatórias e apresentar antígenos (VRAY; HARTMANN; HOEBEKE, 2002) (KLOTZ et al., 2011). Estudo com a cistatina recombinante de *Schistosoma japonicum* em modelo de CIA em camundongos, demonstrou que ela foi capaz de aliviar os efeitos deletérios no modelo quando administrada de

forma profilática. Esta administração preveniu a destruição da cartilagem e a inflamação das articulações, com diminuição das citocinas pró-inflamatórias, IL-6, IL-17 e TNF-α (LIU et al., 2016). Diante disso, estudos com a *F. hepatica* são importantes para um maior conhecimento sobre os seus componentes e produtos e, possivelmente, a aplicação de suas moléculas imunossupressoras como agentes terapêuticos para doenças como a AR.

1.6 JUSTIFICATIVA

Apesar dos avanços e das diversas classes de medicamentos disponíveis para o tratamento da AR, a presença de efeitos adversos e a necessidade do uso de diversos fármacos ao mesmo tempo, em alguns casos, leva os pacientes a desistirem ou não seguirem o tratamento corretamente. Além disso, as opções atuais por vezes falham em manter a remissão da doença e muitos fármacos possuem custos elevados. Portanto, se faz necessária a busca por novas terapias, com o intuito de solucionar os problemas relacionados ao tratamento e, assim, melhorar a qualidade de vida dos pacientes.

1.7 OBJETIVOS

1.7.1 Objetivo geral

Avaliar os efeitos *in vitro* do extrato de *F. hepatica* em FLS obtidos de camundongos com CIA.

1.7.2 Objetivos específicos

Avaliação *in vitro* dos efeitos do extrato de *F. hepatica* em culturas de FLS através dos ensaios de:

- Viabilidade celular (MTT);
- Aderência celular;
- Ensaio cumulativo de duplicação da população;
- Dosagem de IL-6 por ELISA.

2 ARTIGO CIENTÍFICO

In vitro evaluation of the therapeutic potential of _Fasciola hepatica_ extract in fibroblast-like synoviocytes from mice with collagen-induced arthritis

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Abstract

Rheumatoid arthritis (RA) is an autoimmune disease that leads to joint destruction and fibroblast-like synoviocytes (FLS) has a central role on the disease pathophysiology. *Fasciola hepatica (F. hepatica)* is a parasite that is able to regulate the immune responses of its hosts, through excretory-secretory products (ESPs) and tegument antigens. The present study aimed to evaluate the *in vitro* effects of *F. hepatica* extract in fibroblast-like synoviocytes from mice with collagen-induced arthritis (CIA). FLS viability was determined by MTT assay and after FLS were exposed to 60µg/ml, 80µg/ml and 100µg/ml of *F. hepatica* extract for 24h, 48h and 72h. FLS adherence efficiency was evaluated after exposure to 100µg/ml of *F. hepatica* extract for 24h. The long-term effects of treatment with *F. hepatica* extract on FLS was assessed by using the cumulative population doubling assay after FLS exposure to 100µg/ml of *F. hepatica* extract for 24h. The cells were count every 2 days and were re-plated at the same initial density for 8 days. Finally, TNF-α-induced IL-6 release by FLS was accessed by ELISA after exposure to 100µg/ml of *F. hepatica* extract for 24h. *F. hepatica* decreased FLS viability in 48h of treatment with 100µg/ml when compared with DMEM group ($p<0.05$) and in 72h with 60µg/ml ($p<0.001$), 80µg/ml ($p<0.001$) and 100µg/ml ($p<0.0001$) doses when compared with DMEM group. *F. hepatica* extract also showed a tendency to increased IL-6 release by FLS, however, did not affected adherence and long-term proliferation. Taken all together, this results are important to understand the *F. hepatica* extract action in FLS and support new studies to elucidate *F. hepatica* extract effects in other FLS parameters, as invasion and migration behavior.

Keywords: Rheumatoid arthritis; fibroblast-like synoviocytes; *Fasciola hepatica* extract.

1 Introduction

Rheumatoid arthritis (RA) is an autoimmune, inflammatory, systemic and chronic disease that affects 0.5-1% of world population. RA is characterized by chronic, symmetrical and erosive synovitis, preferably in peripheral joints [1]. Early events in RA pathogenesis include the development of synovial hyperplasia, neoangiogenesis and synovial tissue infiltration with mononuclear cells such as macrophages, T and B lymphocytes. Synovial hyperplasia leads the formation of an invasive tissue called *pannus* that invades adjacent structures and causes damage to cartilage and bone, progressively destroying the joint [2]. Despite advances in the treatment of RA, many patients are not able to attain remission and current options sometimes fail to maintain the disease in remission [3,4], emphasizing the need in developing new therapeutic strategies.

Fibroblast-like synoviocytes (FLS) have a central role in synovial hyperplasia and joint damage. In the arthritic joint environment, these cells are altered and present a tumor-like behavior such as resistance to apoptosis, increased proliferation, migration and invasion capacity [5]. Therefore, these cells are an important target for new therapeutic approaches in RA [6].

Fasciola hepatica (F. hepatica) is a helminth trematode that causes fasciolosis disease in ruminants and humans [7]. In infection, *F. hepatica* uses strategies to regulate the immune response of its hosts through excretory-secretory products (ESPs) which are proteases and antioxidants secreted by the shedding of tegument [8]. These ESPs are able to reduce the Th1 response, contributing to the reduction of inflammation through activation of macrophages that increase the differentiation of Th2 cells and participate in the suppression of Th1 inflammatory responses [9,10]. The tegument of *F. hepatica* is released every 2 to 3h during the course of infection, representing a constant source of antigen in direct contact with the host's immune cells. By releasing tegument, immune complexes that have formed on the surface can also be released and regulate immune system of host organism [11]. Moreover, both ESPs and tegument antigens have the ability to suppress the activation of dendritic cells in response to Toll-like receptor 3, 4 e 9 [8,12].

During infection, *F. hepatica* also secretes cystatins, inhibitors of cysteine proteases that are able to regulate inflammatory cytokines and to present antigens [13]. Cysteine proteases, especially cathepsins, play an important role in the development and progression of joint destruction in RA and it has been shown that cathepsin B is highly expressed in FLS, being able to upregulate its migration and invasion capacity [14]. In addition, *F. hepatica* cystatins was able to inhibit cathepsin B [15]. Based on these observations, the aim of this study was to evaluate the *in vitro* effects of *F. hepatic* extract in fibroblast-like synoviocytes from mice with collagen-induced arthritis (CIA).

2 Materials and methods

2.1 Isolation and culture of FLS from mice with CIA

CIA was performed according to Brand et al. [16]. DBA/1J mice were immunized by intradermal injection in the base of the tail on day 0 with 50 µl of an emulsion consisting in equal parts of Complete Freund's Adjuvant (CFA) with 2 mg/ml of heat inactivated *Mycobacterium tuberculosis* (Strain H37 RA) (Difco Laboratories, Lawrence, KS, USA) and bovine type II collagen (CII) (2 mg/ml) (Chondrex, Redmond, WA, USA). After eighteen days, a booster injection was performed in another site of the tail with 50 µl of an emulsion with Incomplete Freund's Adjuvant and CII. After booster, animals were monitored daily for clinical signs of arthritis and when reached the highest scores, animals were euthanized. Then joints were collected and incubated with collagenase I (1 mg/ml) (Sigma-Aldrich) for 2h at 37 °C. A pool of joints from 2 to 3 animals was made to obtained each FLS culture. The supernatant was collected and centrifuged at 1100 rpm for 10 min and the pellet was re-suspended in Dulbecco's modified Eagle's medium high glucose (DMEM-HG), supplemented with 15% fetal bovine serum (FBS), 1% penicillin-streptomycin and 0.125% gentamicin (Gibco). Afterwards, FLS cells were transferred to a plate for culture at 37 °C and 5% CO_2 atmosphere. The initial culture was monitored daily until 70–90% of confluence and then cells were detached with trypsin-EDTA (Gibco) and transferred to a culture flask. FLS were used for experiments after five passages and six different cultures were used for all experiments.

2.2 Preparation of *Fasciola hepatica* extract

Mature *F. hepatica* were extracted from the bile ducts of bovine livers obtained at local abattoirs to obtain the extract. Liver bundles were washed for 2h at 37°C in 10 mM phosphate-buffered sal[15]ine (PBS) pH 7.3 and the extract was prepared according to Cancela et al. [15]. Briefly, 10 mL of cold PBS per gram (wet weight) of flukes was added and the parasites were homogenized in a glass tissue grinder and sonicated in ice bath five times with 60s bursts at 20% power followed by 30s pauses. After centrifugation at 20,000 g for 30min at 4 °C, supernatant was stored at −80 °C until use.

2.3 FLS viability assay

FLS viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay [17] and performed in three separated and independent experiments. Semiconfluent (70–80%) cultures of FLS were harvested with trypsin-EDTA digestion and re-suspended in DMEM-HG supplemented with 10% FBS. Cells were added in 96-well plates at a density of 1×10^3 cells/well. After 24h, FLS were exposed to different doses (60µg/ml, 80µg/ml and 100µg/ml) of *F. hepatica* extract for 24h, 48h and 72h at 37 °C in 5% CO_2. Parallel control was carried out with DMEM supplemented with 10% FBS (cell viability control) in the absence of the test compound. Culture medium was replaced with 100µl of DMEM-HG with MTT (0.5 mg/ml) and after 4h of incubation the supernatants were removed, 100µl of dimethyl sulphoxide (DMSO, Sigma Aldrich, St. Louis/USA) was added to dissolve the MTT formazan crystals and the absorbance of each well was measured at 570 nm and 690 nm in SpectraMax M3 spectrophotometer.

2.4 Adherence assay

Adherence assay was performed in three separated and independent experiments. Semiconfluent (70–80%) cultures of FLS were harvested with trypsin-EDTA digestion and re-suspended in DMEM plus 10% FBS. Cells were seeded in 24-well plate at a density of 5×10^4 cells/well. After 24h, FLS were exposed or not to 100µg/ml of *F.hepatica* extract for 24h and then re-plated in triplicate at a density of 100 cells/well in 96-well plate. After 24h, cells were fixed with 4% paraformaldehyde and stained with crystal violet. Adhered cells were counted in an optical microscopy and the adhesion efficiency was calculated by the ratio between adhered cells and plated cells.

2.5 Cumulative population doubling

The long-term effects of treatment with *F. hepatica* extract was assessed by using the cumulative population doubling (CPD) assay. Briefly, FLS cells were seeded in a 24-well plate, at the density of 5×10^4 cells/well. After 24h, the cells were exposed or not to 100 µg/ml of *F.hepatica* extract. After 24h, cells were re-plated in duplicate at a density of $2x10^4$ cells/well. The cells were count every 2 days and were re-plated at the same initial density for 8 days. The CPD was calculated using the equation PD = [log N(t) * logN(to)]/log 2, where N(t) is the number of cells/well at the time of the count, and N(to) is the initial number of cells. The sum of the PDs was plot versus the time of the culture.

2.6 Enzyme-linked immunosorbent assays (ELISA)

Cells were re-suspended in DMEM supplemented with 2% FBS and $4x10^4$ cells/well were seeded in a 24-well plate. When wells reached the confluence of 80%, FLS were exposed in duplicates to DMEM supplemented with 0.1% FBS or 100µg/ml of *F.hepatica* extract. After 23h, cells were re-treated and 1h later, were stimulated with 10ng/ml of TNF-α. After 24h, the supernatant was collected and stored at -80°C until use. The concentration of IL-6 in supernatants was measured by Mouse IL-6 ELISA MAX Standard Sets (BioLegend, San Diego, CA, USA). The absorbance of each well was measured at 450 nm and 570 nm in SpectraMax M3 spectrophotometer. Data analyses were performed in My Assays software ("Four Parameter Logistic Curve" online data analysis tool, MyAssays Ltd, Sussex, UK).

2.7 Statistical analyses

Data are presented as mean ± standard error of the mean (SEM). 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. Statistical differences were considered significant with a p value <0.05.

3 Results

3.1 *F. hepatica* extract altered the cellular viability in 48h and 72h of treatment

Treatment with *F. hepatica* extract did not affect cell viability on doses used in 24h. In 48h of treatment, the 100µg/ml dose of *F. hepatica* extract decreased cell viability when

compared with DMEM group ($p<0.05$). In 72h of treatment, the 60µg/ml ($p<0.001$), 80µg/ml ($p<0.001$) and 100µg/ml ($p<0.0001$) doses of *F. hepatica* extract, decreased cell viability when compared with DMEM group. Based on these results, the dose of 100µg/ml and the time of 24h were chosen as the treatment protocol for the following analyses. This dose and time were chosen so we could observe a therapeutic effect of *F. hepatica* extract, independent of cytotoxicity. (Figure 1).

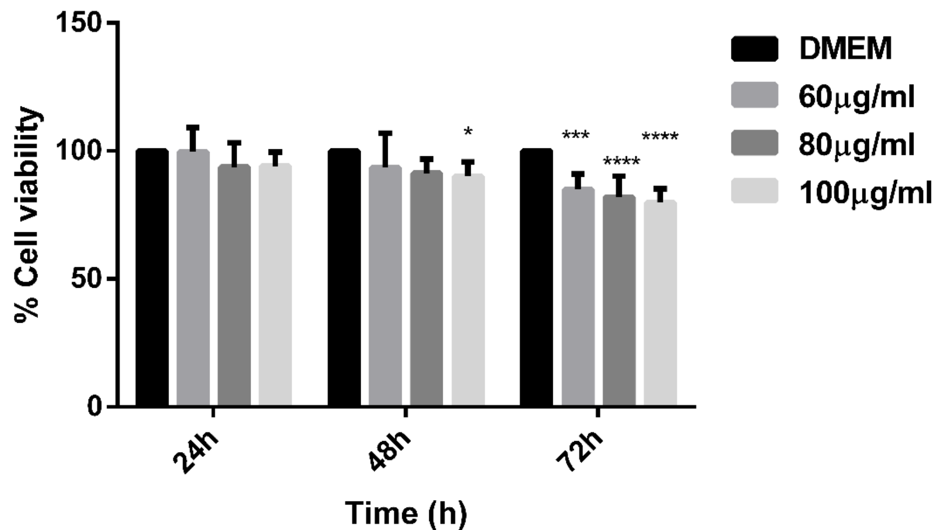

Figure 1: Effect of *F. hepatica* extract in FLS viability. Treatment with *F. hepatica* extract did not alter FLS viability in 24h. *F. hepatica* extract decreased FLS viability with 100µg/ml when compared with DMEM in 48h. *F. hepatica* extract decreased FLS viability with 60, 80 and 100µg/ml when compared with DMEM in 72h. Data were analyzed by two-way ANOVA, followed by Bonferroni's post-test. Significantly different from DMEM group (*$p < 0.05$); (***$p < 0.001$); (****$p<0.0001$).

3.2 *F. hepatica* extract did not alter the FLS adhesion efficiency

To analyze the FLS adhesion efficiency after treatment with *F. hepatica* extract, FLS were treated for 24h with 100µg/ml of *F. hepatica* extract. After this period, the cells adhered were count. FLS treated with 100µg/ml of *F. hepatica* extract did not have their adhesion capacity altered when compared to DMEM group (Figure 2).

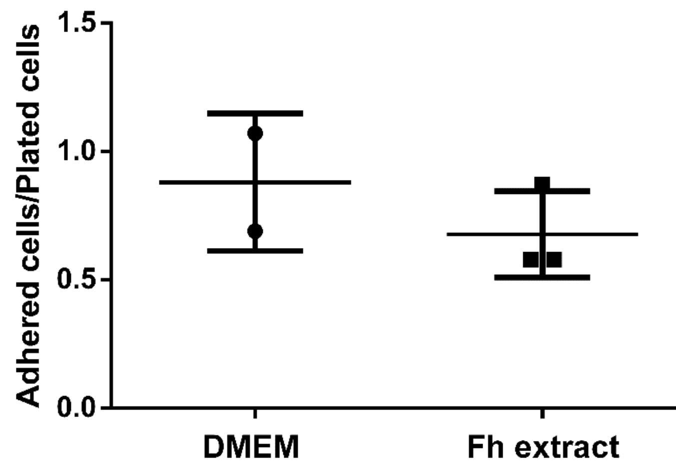

Figure 2: Effect of *F. hepatica* extract in FLS adherence. Treatment with 100µg/ml of *F. hepatica* extract did not alter FLS adhesion capacity when compared to DMEM group. Data were analyzed by Student t test.

3.3 *F. hepatica* extract did not alter FLS cumulative population doubling

The long-term effects of treatment with *F. hepatica* extract was analyzed for 8 days. Treatment with 100µg/ml of *F. hepatica* extract did not affect cell growth on days 0, 2, 4, 6 and 8 when compared to DMEM group (Figure 3).

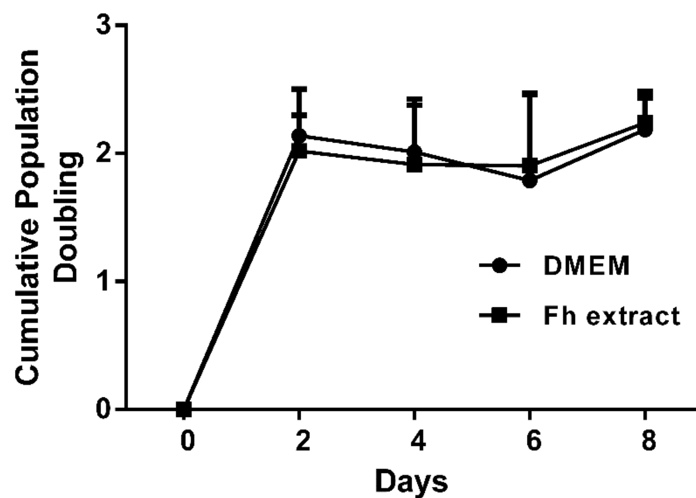

Figure 3: Long-term effects of treatment with *F. hepatica* extract in FLS. Treatment with 100µg/ml of *F. hepatica* extract did not alter FLS growth when compared to DMEM group. Data were analyzed by two-way ANOVA, followed by Bonferroni's post-test.

3.4 *F. hepatica* extract affect the release of IL-6 by FLS

Stimulation with TNF-α showed a tendency to increased IL-6 release by FLS. In addition, treatment with 100µg/ml of *F. hepatica* extract for 24h showed a tendency to increase

approximately 3 times the IL-6 release by FLS when compared to stimulated DMEM (Figure 4).

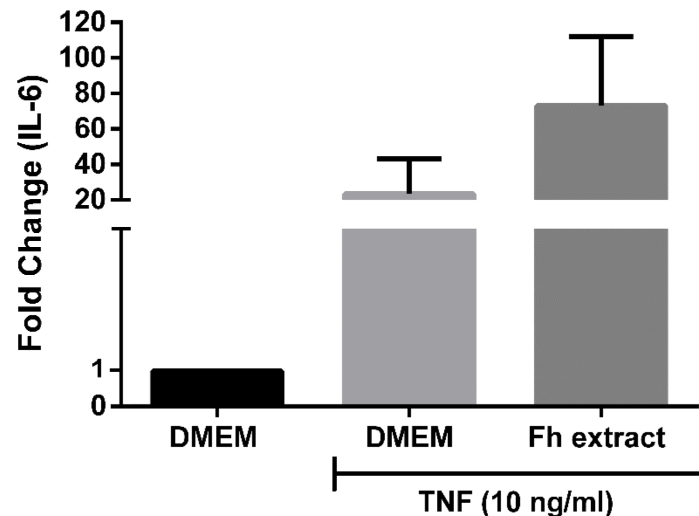

Figure 4: Effect of *F. hepatica* extract in IL-6 release by FLS. Treatment with 100µg/ml of *F. hepatica* extract increased IL-6 release by FLS when compared to stimulated DMEM group. Data were analyzed by one-way ANOVA, followed by Bonferroni's post-test.

4 Discussion

Due to the fact that FLS are the major component of *pannus* and present a key role in the joint damage observed in RA, we evaluated the *in vitro* effects of *F. hepatica* extract on FLS obtained from mice with CIA. Our findings demonstrated that *F. hepatica* extract decreased FLS viability in MTT assay and showed a tendency to increases IL-6 release by FLS, but had no effect in adherence and long-term proliferation.

Initially, we performed a MTT assay to analyze FLS viability and determine a *F. hepatica* extract dose and time for subsequent analyses. The fact that *F. hepatica* extract altered cell viability in 48h and 72h of treatment is consistent with other findings. Wędrychowicz et al. [18] demonstrated that *F. hepatica* EPSs decreased survival of human hepatocyte cell line *in vitro* with 72h of treatment. Similar results have also been obtained when rat hepatocytes were treated with 100µg/ml of *F. hepatica* somatic proteins for 72h [19]. Based on this, the dose of 100µg/ml and 24h of treatment were chosen because our objective was to verify therapeutic effect of *F. hepatica* extract on FLS independently of cytotoxic.

A striking feature of arthritic FLS is its reduced contact inhibition, which promotes the expansion of FLS population and leads to hyperplasia of synovial membrane [5]. Thus, we evaluated the cumulative population doubling of cells. Treatment with *F. hepatica* extract had no effect over FLS cumulative population doubling. Cervi et al. [20] demonstrated that the antigen gluthatione S transferase (GST) present in the *F. hepatica* ESPs, inhibited the proliferation of healthy rat spleen cells in response to concanavalin A (ConA) stimulation *in vitro*. In contrast, in our study FLS duplication was analyzed without an exogenous stimulation,

characterizing a basal proliferation. Thus, if FLS were stimulated before treatment with *F. hepatica* extract, likely its duplication could be affected.

The activation of FLS to become aggressive cells is associated to several stimuli, like cytokines as TNF-α secreted by infiltrating inflammatory cells in the synovium [21] which stimulate FLS to produce and secrete proinflammatory factors such as IL-6 [22]. Therefore, we evaluated the IL-6 release by FLS treated with *F. hepatica* extract after TNF-α stimulation. Our result demonstrated that *F. hepatica* extract showed a tendency to increased IL-6 release by FLS when compared to stimulated DMEM. This increase in IL-6 release can be associated with the balance between IL-6 and TNF-α. Yimin et al. [23] demonstrated *in vitro* that macrophages and dendritics cells from TNF-α gene-deficient mice infected by Rhodococcus aurantiacus enhanced production of IL-6. They also treated wild-type mouse cells with anti-TNF-α or anti-IL-6 and treatment increased IL-6 or TNF- α production. Thus, they suggested that the production of TNF- α and IL-6 could be negatively regulated by each other. Additionally, Gonzalez-Amaro et al. [24] demonstrated the negative relationship between TNF-α and IL-6 in the acute stage of the liver abscess induced by *Entamoeba histolytica* infection. IL-6 is a metabolism regulator and is classified as both a pro and anti-inflammatory cytokine [25], moreover the TNF-α/IL-6 balance may be a key factor in regulating immune responses [23]. In view of this, *F. hepatica* extract could have stimulated IL-6 release by FLS as an attempt to balance the inflammation by reducing TNF-α levels.

In an antigen-induced arthritis mice model performed by our research group, treatment with *F. hepatica* extract reduced nociception and inhibited leukocyte migration to the knee joint, a marker of acute inflammation (unpublished data), indicating that components from *F. hepatica* could have an interesting effect in RA. Moreover, treatment with recombinant cystatin of *Schistosoma japonicum* prevented the destruction of cartilage and joint inflammation in mice with CIA, with reduction of the pro-inflammatory cytokines, IL-6, IL-17 and TNF-α [26]. It is interesting that recombinant cystatins from *F. hepatica* are able to inhibit Cathepsin B, that is highly expressed in FLS and upregulate their migration and invasion capacity, in an enzymatic inhibition assay [15] [14].

As far as we know, this was the first study that evaluated the therapeutic effect of any *F. hepatica* product in FLS. Previous work have demonstrated anti-inflammatory effects of components of *F. hepatica* in different cells type. *F. hepatica* tegument is highly glycosylated, with the presence of oligosaccharide motifs and glycoproteins, which have immune modulatory properties [27] GST present in the *F. hepatica* ESPs downregulated nitric oxide production by healthy peritoneal macrophages [28]. Moreover, *in vitro* and *in vivo* studies have shown that antioxidant peroxiredoxin from *F. hepatica* (FhPrx) is responsible for activating M2 phenotype macrophages, being able to promote differentiation of T cells into a Th2 phenotype [29].

As mentioned, *F. hepatica* extract has many different molecules with potential pharmacological effects, such as cystatins, glycoproteins, GST and FhPrx [15,27–29]. Therefore, the concentration of these components in the extract are not known. It raises the possibility that they are in under therapeutic concentrations in the dosages we used here in order

to evaluate the effects of *F. hepatica* extract in adhesion and cumulative population doubling of FLS.

In this way, treatment with *F. hepatica* did not affect the adherence and cumulative population doubling properties of FLS. However, *F. hepatica* extract decreased FLS viability in MTT assay and showed a tendency to induce an increase in IL-6 release, indicating an immunomodulatory effect in FLS. Therefore, although these results are preliminary, they are important to understand the *F. hepatica* extract action in FLS and support new studies to elucidate *F. hepatica* extract effects in other FLS parameters, as invasion and migration behavior.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by Fundação de Incentivo à Pesquisa e Eventos do Hospital de Clínicas de Porto Alegre (FIPE-HCPA).

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3 CONCLUSÕES E PERSPECTIVAS

Nossos resultados demonstraram que o extrato de *F. hepatica* não afetou a capacidade de aderência celular e não alterou o crescimento dos FLS ao longo do tempo. No entanto, foi capaz de diminuir a viabilidade dos FLS em 48h e 72h e também apresentou uma tendência em aumentar a liberação de IL-6 pelos FLS. Portanto, embora esses resultados sejam preliminares, eles são importantes para entender a ação do extrato da *F. hepatica* nos FLS e indicam um efeito imunomodulatório do extrato sobre os FLS.

Este trabalho tem como perspectivas avaliar a ação do extrato de *F. hepatica* sobre os macrófagos que também estão envolvidos na patogênese da AR, bem como, avaliar a ação de cistatina recombinante sobre os FLS.

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