UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO: CIÊNCIAS EM GASTROENTEROLOGIA E HEPATOLOGIA

Correlação inversa entre os níveisde HspB5 e a severidade da doença em modelo murino de colite ulcerativa

MICHELE ARAMBURU SERAFINI

DISSERTAÇÃO DE MESTRADO

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Somewhere, something incredible is waiting to be known.

Carl Sagan

SUMÁRIO

RESUMO

A colite ulcerativa (UC) é uma doença inflamatória intestinal caracterizada por inflamação recorrente e crônica do trato gastrointestinal. Seus sintomas incluem dor abdominal, cólicas, fadiga, diarréia persistente e perda de peso. A UC é caracterizada por inflamação da mucosa ao longo de todo o cólon e o reto. Durante o processo inflamatório, as moléculas de adesão VCAM-1 e E-selectina são expressas no endotélio vascular e ajudam na transmigração de células imunes do sangue para o tecido intestinal. Estudos recentes indicam que a proteínaHspB5, uma chaperona molecular membro da família de pequenas proteínas de choque térmico, pode estarenvolvida na expressão destas adesinas. Muito conservada na maioria das espécies, a HspB5 modula diversos processos celulares, tais como degradação proteica, apoptose, angiogênese, câncer e doenças inflamatórias. Assim, no presente trabalho buscamos avaliar os níveis deHspB5, TNF-α, E-selectina e VCAM-1 nas células endoteliais no tecido intestinal inflamado de animais com colite ulcerativa experimental. A colite ulcerativa aguda foi induzida em camundongos C57BL/6 por administração oral de 2% de *dextran sulfate sodium* (DSS) durante 7 dias na água de beber *ad libitum*. Foram usados como controles camundongos recebendo água pura ao invés de DSS. O índice de atividade da doença (IAD) foi avaliado diariamente, baseando-se nos critérios: perda de peso, consistência das fezes e presença de sangue nas fezes e no ânus. No dia 8, os cólons foram coletados e amostras de tecido foram processadas para avaliação histológica da colite e para avaliação dos níveis de HspB5, TNF-α, E-selectina e VCAM-1 por imuno-histoquímica. O grupo DSS apresentou um número maior de vasos em comparação ao grupo controle ($p< 0.05$), sugerindo que pode ter ocorrido

angiogênesedurante o período de indução da doença. Foi encontrada uma forte correlação negativa entre a severidade da doença e os níveis deHspB5*(r de Pearson*=-0.8912; *p*< 0.05) no grupo DSS. Animais com uma maior IAD apresentaram níveis reduzidos de HspB5, quando comparados com animais que apresentaram quadros menos severos dadoença. Ainda, os níveis de E-selectina (*p*<0,01) e TNF-α (*p*< 0.05) foram aumentados no grupo DSS em comparação ao grupo controle. Nossos resultados indicam que os níveis de HspB5são inversamente correlacionados à severidade da colite induzida por DSS, o que indica que esta proteína pode ter um papel protetor na indução da inflamação intestinal. Para o nosso conhecimento, este é o primeiro estudo a avaliar os níveisda proteína HspB5 nas doenças inflamatórias intestinais.

Palavras-chave:HspB5, doenças inflamatórias intestinais, colite ulcerativa, DSS, colite experimental

ABSTRACT

Ulcerative colitis (UC) is an inflammatory bowel disease characterized by chronic and recurrent inflammation of the gastrointestinal tract which includes symptoms of abdominal pain, cramps, persistent diarrhea, fatigue, and weight loss. UC is characterized by colonic mucosal inflammation along the entire colon and the rectum. During the inflammatory process, VCAM-1 and E-selectin adhesion molecules are expressed in the vascular endothelium and facilitate the transmigration of the leukocytes of the bloodstream into the intestinal tissue. Recent studies indicate that the HspB5 protein, a molecular chaperone and member of the small heat shock protein family, could be involved in the expression of these adhesion molecules. Highly conserved in most species, HspB5 modulates several cellular processes, such as protein degradation, apoptosis, angiogenesis, cancer and inflammatory diseases. We aimed to evaluate HspB5, TNF-α, E-selectin and VCAM-1 expression on endothelial cells in inflamed intestinal tissue of animals with experimental colitis. Acute colitis was induced in C57BL/6 mice by oral administration of 2% dextran sulfate sodium (DSS) from days 0 to 7 in drinking water *ad libitum*. Mice receiving pure water instead of DSS were used as controls. Disease activity index (DAI) was determined daily based on weight loss, stool consistency and presence of blood in the feces and anus. On day 8, colons were removed and tissue samples were processed for histological evaluation of colitis and immunohistochemical staining of HspB5, TNF-α, E-selectin and VCAM-1. DSS group demonstrated a greater number of vessels compared to control group ($P< 0.05$), suggesting that angiogenesis may occur during the period of induction of the disease. A strong negative correlation between disease severity and HspB5levels (Pearson's r=-

0.8912; $p< 0.05$) was found in DSS group. Animals with greater DAI presented reduced levels of HspB5, compared with animals with less severe disease. In addition, the levels of E-selectin ($p<0,01$) and TNF-α ($p< 0.05$) were higher in DSS group. Our results indicate HspB5 levels is inversely correlated to the severity of the DSS-induced colitis, indicating this protein may play a protective role in the induction of intestinal tissue inflammation. To the best of our knowledge, this is the first study to evaluate HspB5 levels in inflammatory bowel diseases.

Keywords: HspB5, inflammatory bowel disease, IBD, ulcerative colitis, UC, DSS-induced colitis, DSS model

LISTA DE ABREVIATURAS

CD – doença de Crohn / *Crohn'sDisease*

DAI – índice de Atividade da Doença / *DiseaseActivity Index*

DII – doenças Inflamatórias Intestinais

DSS –*dextran sulfate sodium*

HE – hematoxilina&eosina

Hsps–proteínas de choque-térmico / *Heat shock proteins*

NFκB –fator nuclear kappa B/*Nuclear Factor Kappa B*

PBS – solução salina fosfatada

ROS – espécies reativas de oxigênio / *Reactiveoxigenspecies*

TNF-α – fator de necrose tumoral α / *Tumor NecrosisFactor α*

TNBS – 2,4,6-*trinitrobenzene sulfonicacid*

UC – colite ulcerativa / *ulcerativecolitis*

VCAM-1 –*vascular celladhesinmolecule1*

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INTRODUÇÃO

ARTIGO ORIGINAL EM INGLÊS

1. INTRODUÇÃO E REVISÃO DA LITERATURA

1.1. Doençasinflamatóriasintestinais

As doenças inflamatórias intestinais (DII) são caracterizadas por uma inflamação no trato gastrointestinal, além de apresentar uma atividade aumentada anormal de linfócitos T em resposta à microbiota normal presente no órgão[1]. As DII incluem a doença de Crohn (CD) e a colite ulcerativa (UC) e são caracterizadas por fases alternadas de inflamação aguda e remissão, apresentando sintomas como dor abdominal, diarreia e perda de peso. Enquanto a CD pode causar inflamação transmural e afetar qualquer parte do trato gastrointestinal de forma descontínua, a UC é tipicamente uma inflamação na mucosa e é limitada ao cólon (Figura 1).

Embora a etiologia das DII ainda seja desconhecida, estudos recentes apontam que a suscetibilidade genética do indivíduo, o ambiente e a microbiota intestinal são fatores que estão envolvidos na patogênese desta doença[2]. Dentre os fatores ambientais que podem contribuir para o desenvolvimento da DII, podem ser citados o fumo, o estresse, a higiene, os hábitos alimentares, a atividade física e a qualidade do sono[3].

Atualmente, não há cura conhecida para as DII. Os tratamentos disponíveis consistem principalmente de aminosalicílicos, corticosteroides, imunossupressores e anticorpos monoclonais anti-fator de necrose tumoral α (anti-TNF-α). Entretanto, estes tratamentos visam apenas atenuar os sintomas clínicos das doenças, além de poderem

causar uma maior suscetibilidade a infecções intracelulares oportunistas, além de existir um risco potencial de linfoma e outras malignidades[4,5].

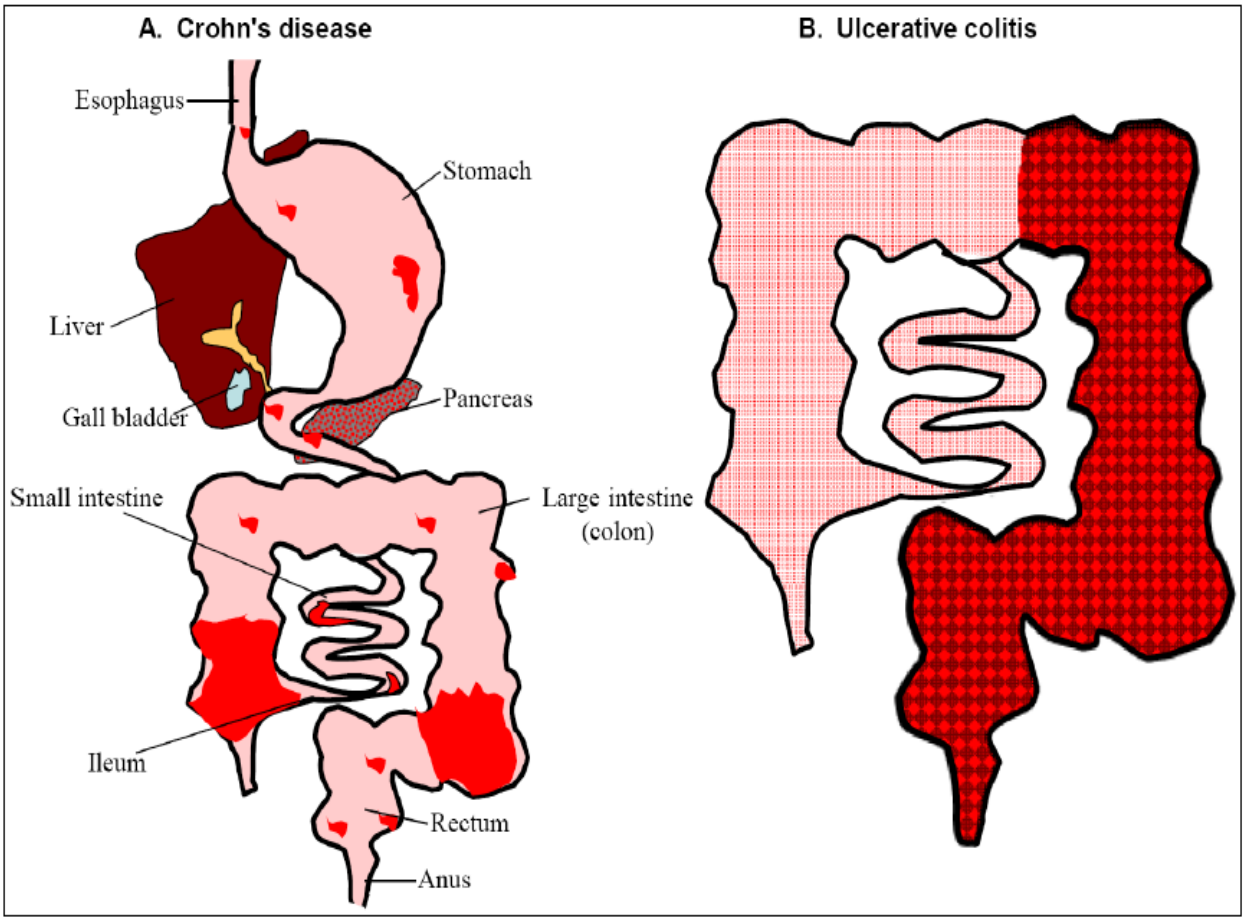

Figura 1. Representação esquemática das regiões afetadas (em vermelho) pelas Doenças Inflamatórias Intestinais. (A) Doença de Crohn: A inflamação pode afetar todo o trato digestivo, da boca ao ânus. (B) Colite Ulcerativa: A inflamação é limitada ao cólon. Fonte: Singh et al, 2011[1].

1.2. Epidemiologia das Doenças Inflamatórias Intestinais

Foram relatadas altas incidências das DII na América do Norte e na Europa, enquanto as incidências mais baixas foram observadas na África, na América do Sul e na Ásia[6]. Atualmente, a incidência anual de colite ulcerativa é ligeiramente mais alta na Europa (24.3 pessoas a cada 100.000 habitantes), enquanto na América do Norte a incidência média é de 19.2 pessoas a cada 100.000 habitantes. Já a incidência da doença de Crohn é mais alta na América do Norte (20.2 pessoas a cada 100.000 habitantes) do que na Europa (12.7 pessoas a cada 100.000 habitantes)[3]. Nas Américas Central e do Sul, os dados epidemiológicos ainda são escassos, o que reflete a baixa frequência ou uma possível falta de registros sobre essas doenças[7]. No Brasil, tem sido observado um aumento na incidência das DII na população nas últimas duas décadas[8,9,10].

As DII são mais comuns em países mais industrializados, sendo mais alta nas populações urbanas do que nas rurais, o que parece indicar que a urbanização é um fator de risco em potencial. A incidência destas doenças é mais elevada em pessoas mais jovens, sendo que o pico para a doença de Crohn é dos 20 aos 30 anos de idade, enquanto para a colite ulcerativa é dos 30 aos 40 anos de idade. A UC é ligeiramente mais frequente em homens (estes constituem 60% dos pacientes), enquanto a CD ocorre com uma frequência de 20% a 30% maior em mulheres[11,7].

1.3. Papel da ativação do endotélio vascular na inflamação intestinal

No início de um processo inflamatório, os macrófagos, células imunes residentes no tecido intestinal, produzem citocinaspró-inflamatórias que estimulam o recrutamento e a migração de outras células imunes. Dentre estes fatores, encontram-se o fator de necrose tumoral (TNF-α) e a interleucina1 (IL-1) que, ao se ligarem à membrana das células do endotélio vascular do tecido, promovem a ativação destas células através da via do fator nuclear kappa B *(*NFκB). Quando estes fatores se ligam na membrana da célula, são ativadas duas vias de sinalização que culminam na translocação do NFκB para o núcleo. Ao chegar ao núcleo, NFκB promove a transcrição de genes que codificam para proteínas inflamatórias, entre elas as moléculas de adesão E-selectina e a vascular celladhesionmolecule1 (VCAM-1) (Figura 2). Estas adesinas serão então expressas na membrana celular, o que favorecerá a migração dos linfócitos do sangue para o tecido[12, 13].

Enquanto a E-selectina promove uma interação fraca com os linfócitos que passam no fluxo sanguíneo, diminuindo a velocidade destes, VCAM-1 promove a adesão destes linfócitos ao vaso, sendo expressaem sítios de inflamação de pacientes com DII e pouco presente no tecido não-inflamado[13,14,15]. Foi demonstrado que o tratamento com anticorpos contra VCAM-1 e outras adesinas foi capaz de diminuir a inflamação intestinal[16]. Assim, estas adesinas promovem a migração de leucócitos presentes na corrente sanguínea para a mucosa do cólon, desempenhando, portanto,um papel fundamental no desenvolvimento da inflamação[17].

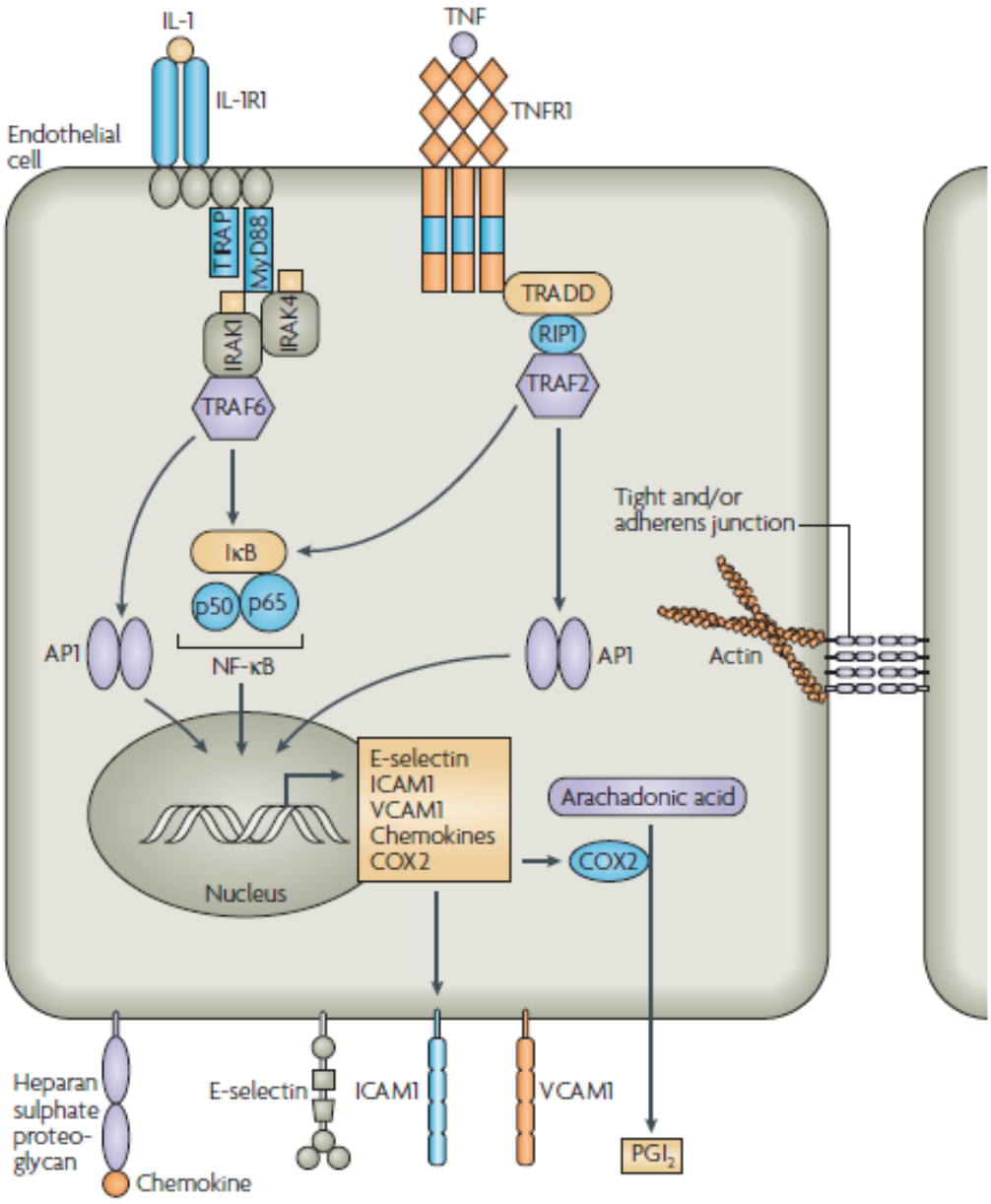

Figura 2. Ativação de células endoteliais. Os fatores TNF-α e IL-1 secretados pelos macrófagos ativam as células do endotélio vascular através da via do NFκB. Uma vez transportado ao núcleo, NFκB promove a transcrição de genes que codificam para proteínas inflamatórias como as moléculas de adesão VCAM-1 e E-selectina. Estas adesinas possibilitarão a migração das células inflamatórias presentes no sangue para o tecido. Fonte: Pober& Sessa, 2007[5].

1.4. Modelo murino de colite ulcerativa

O mecanismo pelo qual as doenças inflamatórias intestinais se estabelecem e se mantém de forma crônica ainda não está bem elucidado. Portanto, os modelos animais são ferramentas fundamentais para compreendermos estas doenças, o que pode contribuir para a identificação de alvos para novas alternativas terapêuticas e avaliação de novas drogas para o tratamento das DII.

O *2, 4, 6-trinitrobenzene sulfonicacid* (TNBS) induz a inflamação no cólon, caracterizada por infiltração leucocitária, edema e ulceração tecidual[18]. Desta forma, a indução da inflamação com TNBS se relaciona especificamente com a doença de Crohn em humanos[19,20]. Já a doença inflamatória induzida por *dextran sulfate sodium* (DSS) se assemelha morfologicamente e clinicamente à colite ulcerativa em humanos. Devido ao DSS possuir um efeito tóxico direto em células epiteliais do cólon, este polissacarídeo induz a inflamação causando erosões com completa perda de superfície epitelial na mucosa intestinal, o que deforma a integridade do tecido e aumenta a permeabilidade do tecido a microorganismos[21,22].

A colite ulcerativa induzida por DSS é um modelo robusto, amplamente utilizado e tem como principal característica níveis elevados de TNF-α tecidual[21]. Tipicamente, a colite aguda é induzida em camundongos C57BL/6 ou BALB/c, machos ou fêmeas, pela administração de 2 a 5% de DSS na água de beber *ad libitum* por um período de 5 a 8 dias[23,24,25]. Durante este período, são avaliados diariamente sintomas clínicos como perda de peso, consistência das fezes e presença de sangue nas fezes e no ânus, os

quais são utilizados para calcular o índice de atividade da doença (*disease activity index* - DAI)[26].

Após o período de indução da colite, os cólons são removidos e examinados histologicamente para avaliar a inflamação tecidual. O escore histológico é realizado conforme descrito porDieleman e colaboradores[27], e se baseia os parâmetros profundidade da inflamação (0-3), severidade da inflamação (0-3), dano às criptas (0-4) e regeneração tecidual (0-4) multiplicado pela porcentagem do tecido comprometido. Após a análise histológica, podem ser realizadas análises da expressão de proteínas no tecido. Além do TNF-α, também foram reportadas alterações nos níveis das citocinas IL-1β, IL-6, IL-10 e IL-17[28,29].

Os animais podem apresentar diferentes graus de severidade da doença conforme o peso molecular do DSS utilizado, bem como de sua concentração. No nosso grupo de pesquisa, a indução da colite ulcerativa por DSS foi padronizada em camundongos C57BL/6 machos. O DSS é administrado a 2% na água de beber *ad libitum* por 7 dias, e os animais são eutanasiados por superdosagem de isoflurano no oitavo dia[30,31,32].

1.5. HspB5 (αB-cristalina)

A proteína HspB5, também conhecida como αB-cristalina, pertence à família de pequenas proteínas de choque térmico (*smallheat-shockproteins*). Estas proteínas são ativadas durante o choque térmico ou outros insultos, e agem como chaperonas moleculares independentes de ATP, sendo capazes de regular a conformação

tridimensional de diversas outras proteínas. Além disso, também influenciam vias que podem modular o envelhecimento, o estresse oxidativo, os processos inflamatórios e a morte celular[33,34,35,36]. Muito conservada na maioria das espécies, a HspB5 modula diversos processos celulares como degradação proteica, apoptose, angiogênese e recuperação celular em situações de estresse[37,38].

A αB-cristalina está presente em uma ampla gama de tecidos, incluindo lentes oculares, coração, cérebro, músculo esquelético, cólon, pulmões, placenta, pele, esôfago, rins e tecido adiposo[39,40,41]. Na lente ocular, auxilia na manutenção da transparência do tecido; já no coração, atua na manutenção da viabilidade celular sob o estresse oxidativo. No cérebro, a HspB5 é mais presente na substânciabranca e acredita-se que esta proteína atue na estabilização e na proteção das bainhas de mielina das células nervosas[40,42,43,41].

A HspB5 é capaz de proteger astrócitos da apoptose sob diferentes estímulos tóxicos através da inibição da produção de espécies reativas de oxigênio (*reactiveoxygenspecies* - ROS) na mitocôndria[44]. Estudos *in vivo* e *in vitro* reportaram que a expressão desta proteína se correlaciona com níveis diminuídos de ROS, óxido nítrico e peroxidaçãolipídica[45,46]. Além disto, a atividade de chaperona molecular da HspB5 é necessária para estabilizar e evitar a agregação dos filamentos intermediários, o que torna esta proteína um importante modulador do citoesqueleto celular[47].

1.6. HspB5 e doenças inflamatórias

Embora a expressão aumentada de HspB5 tenha sido relatada em diversas doenças, tais como doenças neurodegenerativas[48], mal de Parkinson[49] e esclerose múltipla[50], incluindo doenças inflamatórias como a doença pulmonar obstrutiva crônica[51,52], doença celíaca[53] e na neuroinflamação[54], seu efeito no processo inflamatório ainda não está bem elucidado. Na doença pulmonar obstrutiva crônica, a HspB5 age como um mediador antiapoptótico nos pneumócitos alveolares, sendo vista como um mecanismo endógeno imunossupressor para controlar a inflamação excessiva[51,52]. Além disto, na esclerose múltipla, esta proteína é capaz de diminuir a inflamação através do bloqueio da ativação da via do NFκB nos neurônios e nas células da glia[50]. Em outro estudo, a administração intravenosa de HspB5 solúvel em modelo animal de esclerose múltipla teve um efeito anti-inflamatório, devido à capacidade desta proteína se ligar a proteínas pró-inflamatórias, impedindo sua ligação aos receptores celulares, o que resulta na diminuição da paralisia nos animais[55].

Entretanto, Dieterich e colaboradores[13] reportaram que a HspB5 pode ter um efeito pró-inflamatório. Este grupo de pesquisadores demonstrou que esta proteína é capaz de induzir a superexpressão da molécula de adesão E-selectina em resposta a TNF-α em células endoteliais *Human Umbilical VeinEndothelialCells* (HUVEC). Um resultado semelhante foi observado em uma linhagem de células endoteliaisnocaute para HspB5,*myocardial microvascular endotelial cells* (MyEnd), que apresentou a redução da expressão de E-selectina e VCAM-1 na presença de TNF-α, quando comparado ao grupo *wildtype*. Estes dados sugerem que um aumento nos níveis de

HspB5 poderia apresentar um efeito pró-inflamatório, devido ao aumento da expressão de adesinas e consequente transmigração de leucócitos.

Previamente, foi reportado que a proteína *heatshock* Hsp70 pode ter um efeito protetor contra a indução de colite por DSS, sendo que a inibiçãoda expressão desta proteína está associada a um aumento da morte celular induzida por ROS[56].Da mesma forma, Xue e colaboradores[57] demonstraram que uma expressão aumentada das *heatshocks* Hsp70 e Hsp25, induzida pela administração de glutamina, também é capaz de exercer um efeito protetor na colite induzida por DSS.

Entretanto, até o presente momento, não há estudos sobre a relação entre a expressão da*heatshock*HspB5 e as doenças inflamatórias intestinais. Devido aos resultados controversos reportados, torna-se necessária a investigação do papel desta proteína na inflamação, no desenvolvimento e na progressão das doenças inflamatórias intestinais, visto que esta compreensão pode auxiliar no desenvolvimento de novas alternativas terapêuticas para estas doenças.

JUSTIFICATIVA

Os métodos terapêuticos atualmente disponíveis para a colite ulcerativa visam principalmente manter o estado de remissão e aliviar seus sintomas clínicos, não havendo nenhum tratamento disponível que possa reverter o quadro da doença até o momento. Além disso, as drogas convencionais muitas vezes causam efeitos colaterais indesejados e não são efetivas em todos os pacientes.

Tendo em vista a necessidade de novos métodos de tratamento, torna-se necessário o estudo de novas estratégias terapêuticas. Neste contexto encontram-se as Hsps (*Heatshockproteins*), que podem proporcionar um maior entendimento da colite ulcerativa devido a sua relação com a inflamação e situações de estresse. Do nosso conhecimento, nenhum grupo estudou os níveis da proteína HspB5 na colite ulcerativa.

QUESTÃO DE PESQUISA

Os níveis da proteína HspB5 são alterados durante o processo inflamatório na colite ulcerativa experimental induzida por DSS?

HIPÓTESE

Os níveis da proteína HspB5 são aumentados durante o processo inflamatório na colite ulcerativa experimental induzida por DSS.

OBJETIVOS

Objetivo Geral

Avaliar os níveis da proteína HspB5 no cólon de camundongos submetidos ao modelo murino de colite ulcerativa e sua correlação com marcadores envolvidos no processo de migração de células inflamatórias.

Objetivos Específicos

Avaliar os níveis imuno-histoquímicos nas células endoteliais do cólon de animais doentes e controle com anticorpos contra os marcadores:

- HspB5

- VCAM-1

- TNF-α

- E-selectina

ARTIGO ORIGINAL

Inverse correlation between HspB5 expression and disease severity in DSS-induced colitis

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Abstract

Ulcerative colitis (UC) is an inflammatory bowel disease characterized by chronic and recurrent inflammation of the gastrointestinal tract which includes symptoms of abdominal pain, cramps, persistent diarrhea, fatigue, and weight loss. UC is characterized by colonic mucosal inflammation along the entire colon and the rectum. During the inflammatory process, VCAM-1 and E-selectin adhesion molecules are expressed in the vascular endothelium and facilitate the transmigration of the leukocytes of the bloodstream into the intestinal tissue. Recent studies indicate that the HspB5 protein, a molecular chaperone and member of the small heat shock protein family, is involved in the expression of these adhesion molecules. Highly conserved in most species, HspB5 modulates several cellular processes, such as protein degradation, apoptosis, angiogenesis, cancer and inflammatory diseases. We aimed to evaluate HspB5, TNF-α, E-selectin and VCAM-1 expression on endothelial cells in inflamed intestinal tissue of animals with experimental colitis. Acute colitis was induced in C57BL/6 mice by oral administration of 2% dextran sulfate sodium (DSS) from days 0 to 7 in drinking water ad libitum. Mice receiving pure water instead of DSS were used as controls. Disease activity index (DAI) was determined daily based on weight loss, stool consistency and presence of blood in the feces and anus. On day 8, colons were removed and tissue samples were processed for histological evaluation of colitis and immunohistochemical staining of HspB5, TNF-α, E-selectin and VCAM-1. DSS group demonstrated a greater number of vessels compared to control group ($P < 0.05$), suggesting that angiogenesis may occur during the period of induction of the disease. A strong negative correlation between disease severity and HspB5 expression (Pearson's $r=-0.8912$; $p < 0.05$) was found in DSS group. Animals with greater DAI presented reduced expression of HspB5, compared with animals with less severe disease. In addition, the expression of E-selectin ($p<0,01$) and TNF-α ($p < 0.05$) was higher in DSS group. Our results indicate HspB5 expression is inversely correlated to the severity of the DSS-induced colitis, indicating this protein may play a protective role in the induction of intestinal tissue inflammation. To the best of our knowledge, this is the first study to evaluate HspB5 expression in inflammatory bowel diseases.

Keywords	HspB5, inflammatory bowel disease, IBD, ulcerative colitis, UC, DSS-induced colitis, DSS model
Taxonomy	Experimental Pathology, Gastrointestinal Pathology
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FIGURE 1 TIFF.tif [Figure]

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Inverse correlation between HspB5 expression and disease severity in DSS-induced colitis

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Summary

Ulcerative colitis (UC) is an inflammatory bowel disease characterized by chronic and recurrent inflammation of the gastrointestinal tract which includes symptoms of abdominal pain, cramps, persistent diarrhea, fatigue, and weight loss. UC is characterized by colonic mucosal inflammation along the entire colon and the rectum. During the inflammatory process, VCAM-1 and E-selectin adhesion molecules are expressed in the vascular endothelium and facilitate the transmigration of the leukocytes of the bloodstream into the intestinal tissue. Recent studies indicate that the HspB5 protein, a molecular chaperone and member of the small heat shock protein family, is involved in the expression of these adhesion molecules. Highly conserved in most species, HspB5 modulates several cellular processes, such as protein degradation, apoptosis, angiogenesis, cancer and inflammatory diseases. We aimed to evaluate HspB5, TNF-α, E-selectin and VCAM-1 expression on endothelial cells in inflamed intestinal tissue of animals with experimental colitis. Acute colitis was induced in C57BL/6 mice by oral administration of 2% dextran sulfate sodium (DSS) from days 0 to 7 in drinking water *ad libitum*. Mice receiving pure water instead of DSS were used as controls. Disease activity index (DAI) was determined daily based on weight loss, stool consistency and presence of blood in the feces and anus. On day 8, colons were removed and tissue samples were processed for histological evaluation of colitis and immunohistochemical staining of HspB5, TNF-α, E-selectin and VCAM-1. DSS group demonstrated a greater number of vessels compared to control group ($P< 0.05$), suggesting that angiogenesis may occur during the period of induction of the disease. A strong negative correlation between disease severity and HspB5 expression (Pearson's r=-0.8912; $p< 0.05$) was found in DSS group. Animals with greater DAI presented reduced expression of HspB5, compared with animals with less severe disease. In addition, the expression of E-selectin ($p<0,01$) and TNF-α ($p< 0.05$) was higher in DSS group. Our results indicate HspB5 expression is inversely correlated to the severity of the DSS-induced colitis, indicating this protein may play a protective role in the induction of intestinal tissue inflammation. To the best of our knowledge, this is the first study to evaluate HspB5 expression in inflammatory bowel diseases.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder which includes Crohn's disease (CD) and ulcerative colitis (UC), and is characterized by alternating phases of clinical relapse and remission. Symptoms include abdominal pain, visceral hypersensitivity and diarrhea. UC is characterized by colonic mucosal inflammation along the entire colon and the rectum, and presents symptoms such as rectal bleeding, diarrhea and abdominal pain[1,2,3]. There are evidences that IBD is a result of the interaction between environmental and microbial factors in the context of a genetically susceptible individual, which contributes to an imbalanced mucosal immune response to the normal intestinal flora[1,4].

Although inflammation research has focused mainly on the functions and identities of immune cells, recent reports indicate vascular endothelial cells also have a key role in this process. In an inflammatory response, tissue-resident macrophages secrete proinflammatory cytokines including tumor-necrosis factor (TNF-α) and interleukin-1 (IL-1). These molecules bind to colonic submucosa endothelial cells and activate a pathway that leads to nuclear factor-kB (NFkB) gene transcription. NFkB promotes the expression of adhesion molecules such as E‑selectin and vascular cell adhesion molecule 1 (VCAM1), that bind to circulating leukocytes by the endothelium[5,6]. E-selectin contributes with a weak interaction with leukocytes, which decreases the blood flow velocity of these cells. Moreover, VCAM-1 promotes leukocyte firm adhesion to the vessel. Therefore, these adhesion molecules facilitate the migration of leukocytes present in the bloodstream to the colon mucosa. It has been demonstrated that TNF-a, E-selectin and VCAM-1 molecules are expressed in patients with IBD and present low levelsin non-inflamed tissues[7,8,9].

HspB5 protein, also known as αB-crystalline, is part of the small heat-shock protein family and assists cell recovery in stressful situations[10]. Expressed in the majority of species, from prokaryotes to humans, HspB5 modulates several cellular processes such as protein degradation, apoptosis, angiogenesis, cancer and inflammatory diseases[11]. This protein is transiently induced as a result of intense oxidative metabolism in distinct organsincluding kidneys, heart and muscle tissue[12]. In the brain, HspB5 protects astrocytes from cell death under different toxic stimuli by inhibiting ROS production from cerebral mitochondria[13]. It has been reported both *in vivo* and in *vitro* that αB-crystalline expression correlates to decreased levels of reactive oxygen species, nitric oxide, and lipid peroxidation[14,15].

Although increased expression of HspB5 has been associated with inflammatory diseases[16,17,11,18,19,20], its effect in inflammatory processes has not been elucidated. In chronic obstructive pulmonary disease (COPD), HspB5 generally acts as an antiapoptotic mediator in alveolar pneumocytesand can be seen as an endogenous immunosuppressive attempt to control excessive inflammation[21,19]. Moreover, αB-crystallin was reported to decrease the activation of NF-κB, which inhibits NF-κB-mediated transcription of proinflammatory cytokines in brain cells[22]. Nevertheless, Dieterichet al[23] reported HspB5 induces overexpression of E-selectin in response to TNF-α in the endothelial cell lineage HUVEC (Human Umbilical Vein Endothelial Cells). The same group observed that another endothelial cell lineage, myocardial microvascular endothelial cells (MyEnd), knocked out for HspB5, shows reduced expression of E-selectin and VCAM-1 in the presence of TNF-α.These data suggest an increase in HspB5 levels could result in a proinflammatory effect, due to an enhanced expression of adhesins in endothelial cells.

Thus, we aimed to evaluate HspB5 and adhesins expression in inflammation of intestinal tissue in an experimental model of colitis. To our knowledge, this is the first study to evaluate HspB5 expression in inflammatory bowel diseases.

2. Material andMethods

2.1. Mouse DSS-inducedcolitis

Male C57BL/6 micewereobtainedfrom*Unidade de Experimentação Animal* (UEA) of*Hospital de Clínicas de Porto Alegre* (HCPA). Animals were maintained in a 12h light-dark cycle at humidity and temperature controlled at house facilities. All procedures were accomplished in accordance to Brazilian Federal Law 11.794/08, which regulates the registration of experimentation centers and establishes rules for scientific use of animals.

Animals received oral administration of 2% dextran sulfate sodium (DSS; MP Biomedicals, United States) in drinking water *ad libitum* from days 0 to 7 for inducing acute colitis according to our previous studies[24,25,26]. Animals receiving pure water were used as controls (n = 6 mice/group). Weight loss, stool consistency and presence of blood in feces and anus were observed daily. Score from 0 to 4 was assigned for each parameter, resulting in the total disease activity index (DAI) score ranging from 0 (unaffected) to 12 (severe colitis). The DAI score was assessed by and investigator blinded to the protocol. After 8 days of DSS administration, animals were euthanized and colons were removed from the cecum to the rectum.

2.2. Histological evaluation of colitis

Colons were fixed in 10% formalin, processed and paraffin-embedded. Colon sections (4 µm) were stained with hematoxylin-eosin (HE) and analyzed in a halogen light microscope by a blinded investigator as described by Dieleman[27] et al. Parameters of the histological score, such as depth of inflammation (0-3), severity of inflammation (0-3), crypt damage (0-4) and regeneration (0-4) were multiplied by the percentage of compromised tissue (1 point for 25%, 2 points for 26%-50%, 3 points for 51%-75%, and 4 points for 76%-100%). Accordingly, inflammation and extent have a range from 0 to 12, and regeneration and crypt damage have a range from 0 to 16.

2.3. Immunohistochemistry Reaction

Colon samples from C57BL/6 mice with DSS-induced colitis (n=6) were evaluated by indirect immunohistochemistry method with secondary antibody conjugated to streptavidin-coupled peroxidase. Healthy mice were used as control (n=6). 4µm thick longitudinal sections of the colon were obtained to perform the immunohistochemical reactions. Sections were deparaffinized in xylol and hydrated through ethanol.

For antigen retrieval, sections were immersed in citrate-EDTA buffer (10mM Citric Acid, 2mM EDTA, 0.05% Tween 2) for 20 minutes at 94°C. Endogenous peroxidase activity was blocked using 10% hydrogen peroxide-methanol buffer for 20 min at room temperature. Nonspecific reactions were blocked with casein for 20 min at room temperature. Next, sections were washed with PBS and incubated with primary antibodies overnight at 4 °C accordingly to the desired protein, as demonstrated in **Table 1**.

After incubation with primary antibody, slides were washed twice with PBS and incubated with respective biotinylated secondary antibodies for 30 min in the dark, at room temperature. Sections were then rinsed and incubated with streptavidin-coupled peroxidase for 30 min and visualized by a 1 min incubation with liquid 303-diaminobezidin in buffered substrate. Finally, hematoxicillin was used as counterstaining.

2.4. Evaluation of immunohistochemical staining

The evaluation of the slides was performed by a blinded investigator, who analyzed approximately 1 cm length of the distal portion of the colon. Samples were evaluated manually under an optical microscope at 400 x magnification, and the number of positive and negative blood vessels were counted. Vessels that presented lumen and, at least, one endothelial cell nucleus were included in the analysis.

2.5. Statistical analysis

Results were shown as the mean ± SE. Statistical analysis was performed using Graph Pad Prism 5 software. Generalized Estimated Equations (GEE) was used for DAI and weight loss analysis. Data of immunohistochemical were analyzed for statistical significance either by Student's t-test or one-way analysis of variance (ANOVA). Correlation between the DAI and HSpB5 expression was evaluated by Pearson's linear correlation analysis. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. DSS administration promoted significant DAI increase, weight loss and altered histological score

Mice exposed to oral administration of 2% DSS presented a significant DAI increase, characterized by bloody diarrhea, rectal bleeding and sustained weight loss. In DSS group, DAI score have shown a significant increase on day 5 of disease induction compared to control group (5.00 ± 1.31 DSS group and 1.00 ± 0.36 control group, $P < 0.01$). The disease severity peak was on day 8 (9.00 ± 0.68 DSS on day 8 and 5.00 ± 1.31 DSS on day 5; $P < 0.001$) (**Figure 1A**). Additionally, DSS administration was associated with significant changes in mice body weight compared to control group. The baseline of the weight change was the mean weight of first day (day 0). A significant weight loss of 11.99 ± 2.63% ($P< 0.01$), 18.48 ± 2.48% ($P< 0.001$) and 20.00 ± 2.77% ($P < 0.001$) was observed on days 6, 7 and 8 in DSS group, respectively. (**Figure 1B**).

As expected, intense colonic inflammation was observed in DSS group, characterized by extensive mucosal ulceration, loss of globlet cells and crypt damage. DSS mice intensity of inflammation score was 7.50 ± 0.67 (P< 0.01), crypt damage 10.00 ± 1.26 (P< 0.01) and regeneration 8.0±0.63 (P< 0.01), while extension score was 6.66 ± 0.80 (P< 0.01) compared with control group. Histological score of DSS mice was on average 17.50 ± 1.80 (P< 0.001), which was consistent with the clinical score. (**Figure 2**).

3.2. DSS treated animals presented a greater number of vessels

First, we compared the number of colon vessels between control group and DSS group. DSS group demonstrated a greater number of vessels compared to control group (38.87 ± 5.8 number of vessels in DSS group and 19.5 ±2.00 number of vessels in control group, P< 0.05). (**Figure 3**).

3.3. Gut vessels from DSS treated animals presented higher expression of TNF-α and E-selectin

The expression of the cell signaling protein TNF-α and thecell adhesion moleculesE-selectin and VCAM 1was analyzed in colon inflamed tissue by immunohistochemistry. In DSS group, 88.41 ± 3.95% of the vessels were positive for TNF-α, whereas only 67.30 ± 8.89% were positive in control group ($P < 0.05$). Percentage of vessel expressing E-selectin was 95.45 ± 1.37% in DSS group, whereas only 73.72 ± 5.74% were positive in control group ($P < 0.01$). Interestingly no statistical difference were observed on VCAM 1 expression (80.54 ± 5.63% in DSS group and 60.00 ± 7.43% in control group) (**Figure 4**).

3.4. HspB5 expression is inversely related to the severity of inflammatory bowel disease

No differences were observed regarding the expression of HspB5 from sick and healthy animals (34.95 ± 9.00% in DSS group and 38.09 ± 10.60% in control group). Indeed very interestingly a strong negative correlation between the percentage of vessels positive for HspB5 and DAI score in DSS group was found (P< 0.05; Pearson's r = -0.8912). The animals that

presented a greater disease severity also demonstrated a lower percentage of vessels positive for HspB5 when compared with animals with a milder disease (**Figure 5**).

4. Discussion

HspB5 protein is part of the small heat-shock protein family and assists cell recovery in stressful situations. This protein modulates several cellular processes such as protein degradation, apoptosis, angiogenesis, cancer and inflammatory diseases[11]. Although increased expression of HspB5 has been associated with inflammatory diseases, its expression in inflammatory bowel disease has not been evaluated.

In the present study, we observed a higher number of vessels after DSS-induced colitis period. This data suggests inflammation probably stimulated angiogenesis in colon tissue within colitis induction period. Same results were observed by Jerkicet al[28] in DSS-induced colitis.

Previous studies have focused mainly on the functions and identities of immune cells in inflammatory diseases. It was only recently several researchers reported endothelial cells also have a key role in the inflammatory process[5,29,30,31,32,33]. TNF-α secreted by resident immune cells activates NFkB, which promotes the transcription of adhesion molecules in endothelial cells such as E-selectin and VCAM-1[5]. These adhesins then facilitate the migration of leukocytes to the tissue. In the present work, we evaluated TNF-α, E-selectin and VCAM-1 expression in DSS-induced colitis. TNF-α is an important pro-inflammatory molecule with diverse mechanisms of action in intestinal inflammation. In intestinal epithelium cells, TNF-α is able to reduce the production of intestinal mucus, to influence in tight-junction permeability, and to induce cell death, compromising the integrity of the mucosal barrier that separates the host from its environment[29,30]. Moreover, E-selectin is an important transmembrane glycoprotein that mediates the initial endothelial cell adhesion to leukocytes in an inflammatory process[31]. E-selectin knockout mice were remarkably protected from the leukocyte infiltration in an adipose tissue inflammation model[32]. Additionaly, E-selectin blockade significantly inhibited 25–60% of the migration of CD4 T cells to TNF-a sites in dermal inflammation[33].

In colitis, E-selectin expression in endothelial cells is thought to be an initial sign of inflammation relapse from remissive disease[34]. In a study with biopsies of UC patients, it was observed E-selectin-positive endothelial cells were significantly more frequent in all patients

compared to specimens in disease remission[35]. Endothelial cell expression of TNF-α can increase reactive oxygen species production and decrease bioavailability of nitrogen oxide, thus resulting in endothelial dysfunction[36]. Significant higher TNF-α levels have been found in serum concentrations[37], in mucosal cell secretion in the colon[38,39] and in stool[40] of patients with ulcerative colitis. Accordingly, our results demonstrated a greater expression of inflammation markers E-selectin and TNF-α in the colon vessels of animals from DSS group. Therefore, our data indicate these molecules have an important role in the development of inflammation in DSS-induced colitis model.

AlthoughDieterichet al[23] reported HspB5 induces overexpression of E-selectin in response to TNF-α, suggesting that an increase in HspB5 levels could result in a proinflammatory effect, in the present work we found no difference in HspB5 expression between DSS group and control group. Our data indicates this protein expression is not altered with inflammation induced by DSS. Interestingly, we have found a strong negative correlation between the percentage of vessels positive for HspB5 and DAI score of DSS group. In other words, mice with lowest disease activity index presented an increased percentage of vessels positive for HspB5. This enhanced expression indicates HspB5 may play a protective role against colonic inflammation induced by DSS. Recent research established a correlation between the molecular chaperone activity of HspB5 and its therapeutic function. It has been proposed the therapeutic benefit of this protein is related to its capacity to bind proinflammatory proteins temperature-dependent within inflammatory foci[41,11]. Moreover, HspB5 has been shown to have anti-inflammatory properties by inhibition of NF-κB and p38 MAP kinase[18].

Masilamoniet al[42] reported the therapeutic activity of HspB5 in an inflammation-induced by silver nitrate model in mice. They demonstrated intraperitoneal injection of HspB5 into mice decreases lipid peroxidation, increases antioxidant enzyme activities and reduces glutathione levels. In an experimental autoimmune encephalomyelitis, HspB5 was able to reduce serological levels of IL-6 and attenuate paralysis[19,43]. IL-6 is a pro-inflammatory cytokine also highly expressed in both acute and chronic multiple sclerosis (MS) lesions, in which HspB5 has also been shown to be an effective therapeutic anti-inflammatory protein in animal models[18,43]. Also, in chronic obstructive pulmonary disease, it was shown HspB5 acts as an antiapoptotic mediator in alveolar pneumocytes and can be seen as an endogenous immunosuppressive attempt to control excessive inflammation[21].

Accordingly, our data suggest HspB5 may play an important role in the suppression of inflammation in DSS-induced colitis. HspB5 protein most likely offers a protective effect to the induction of the inflammation, being able to lower disease severity in mice that express it in a greater amount, possibly by aiding intestinal epithelial cell survival.

5. Conclusions

Our results demonstrate HspB5 expression is inversely related to the severity of the disease in experimental model of colitis, which indicates this protein may play a protective role in the inflammation of the intestinal tissue. In the DSS model, expression of TNF-α and E-selectin inflammation markers is increased, as observed in several inflammatory diseases, which indicates these molecules have an important role in the development of inflammation in DSS-induced colitis model.

To the best of our knowledge, this is the first study to evaluate HspB5 in inflamed tissue with colitis. However, more studies are necessary to understand the mechanisms underlying inflammation remission in DSS-induced colitis by HspB5.

6. Conflicts of Interest

The authors report no conflicts of interest.

7. Funding

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Tables

Antibody	Dilution in PBS	Description
HspB5	1:100	Anti-CRYAB, aB-crystallin, Sigma Aldrich, SAB4500485, polyclonal, produced in rabbit
E-selectin	1:50	Anti-CD62E, AbCAM, ab18981, polyclonal, produced in rabbit
TNF-α	1:100	Anti-TNF-α, Invitrogen, AMC3012, polyclonal, produced in rabbit
VCAM-1	1:100	Anti-VCAM-1, AbCAM, ab134047, monoclonal, produced in rabbit

Table 1: Dilution of primary antibodies for each protein evaluated.

Figures

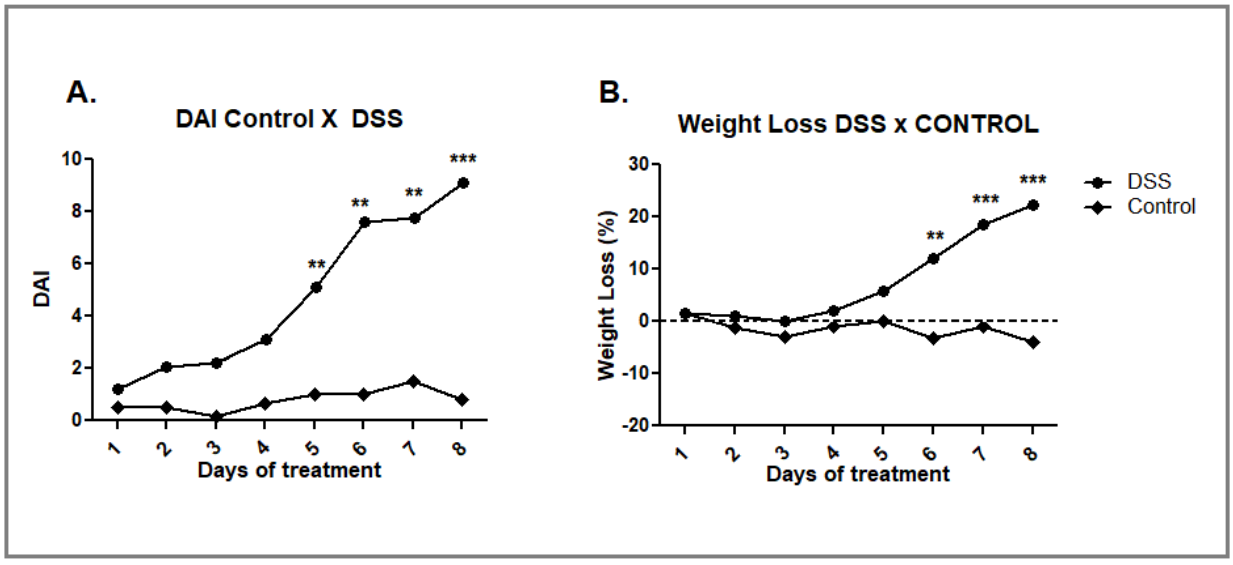

Figure 1. Clinical analysis of DSS-induced colitis.**A**. Disease activity index (DAI) from days 0 to 8. In DSS group, DAI score have shown a significant increase on day 5 of disease induction compared to control group (5.00 ± 1.31 DSS group and 1.00 ± 0.36 control group, $P < 0.01$). The disease severity peak was on day 8 (9.00 ± 0.68 DSS on day 8 and 5.00 ± 1.31 DSS on day 5; $P < 0.001$**. B.** Percentage of weight loss from total body weight from days 0 to 8. A significant weight loss of 11.99 ± 2.63% ($P< 0.01$), 18.48 ± 2.48% ($P< 0.001$) and 20.00± 2.77% ($P < 0.001$) was observed on days 6, 7 and 8 in DSS group, respectively. n = 6 mice/group.

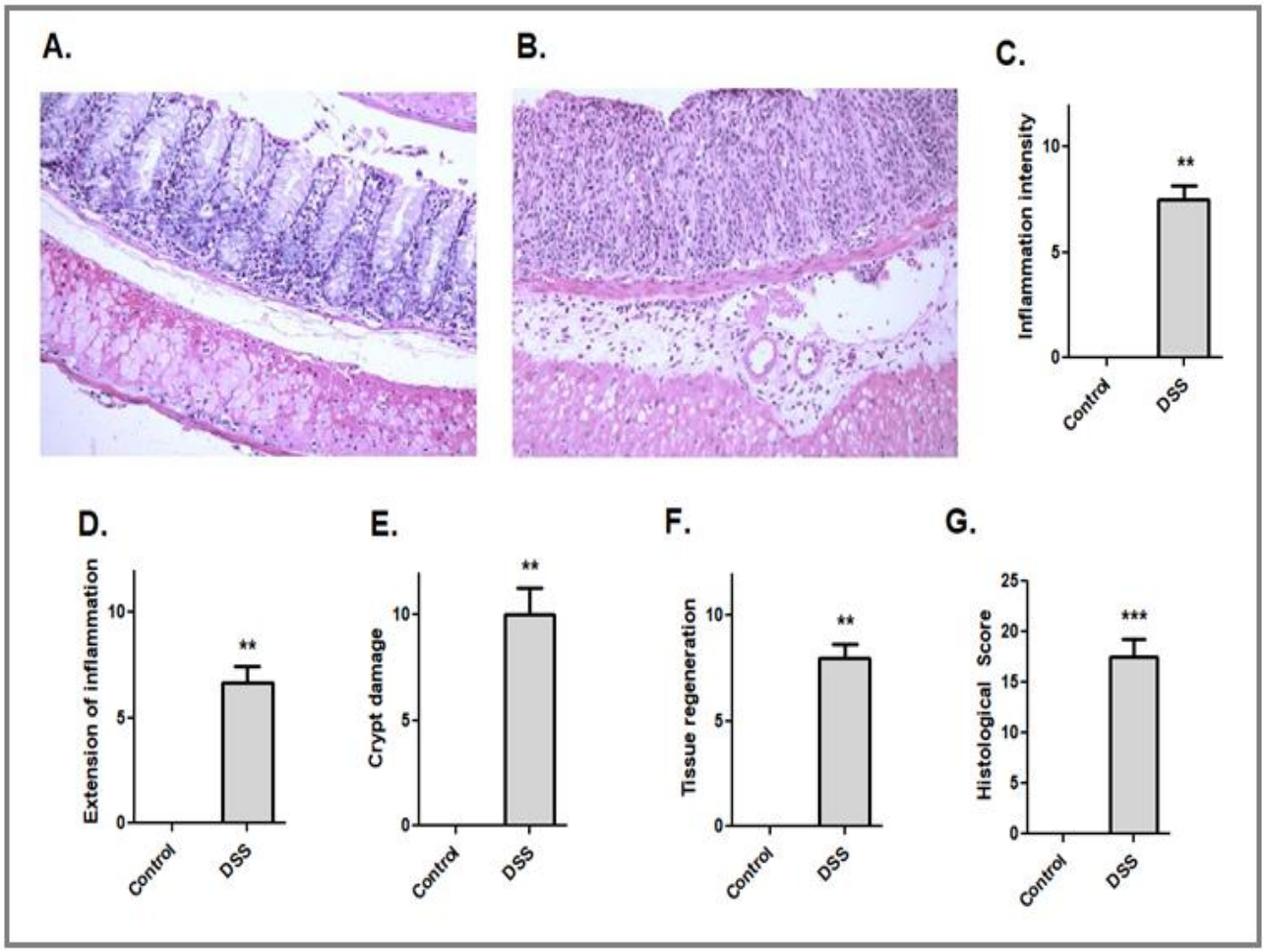

Figure 2: Histological analysis of DSS-induced colitis versus control group. **A**. Hematoxylin-Eosin (HE) slide of a control specimen showing preserved colon mucosa. **B.** HE slide of DSS specimen presenting damage to structure of the glands of the colon mucosa. Optical microscopy, 200 x magnification. **C–G.** Graphs demonstrating inflammation intensity (7.50 ± 0.67 in DSS, *P*< 0.01), extension of inflammation (6.66 ± 0.80 in DSS, *P*< 0.01), crypt damage (10.00 ± 1.26 in DSS, *P*< 0.01), tissue regeneration (8.0 ± 0.63 in DSS, *P*< 0.01) and histological score (17.50 ± 1.80 in DSS, *P*< 0.001).

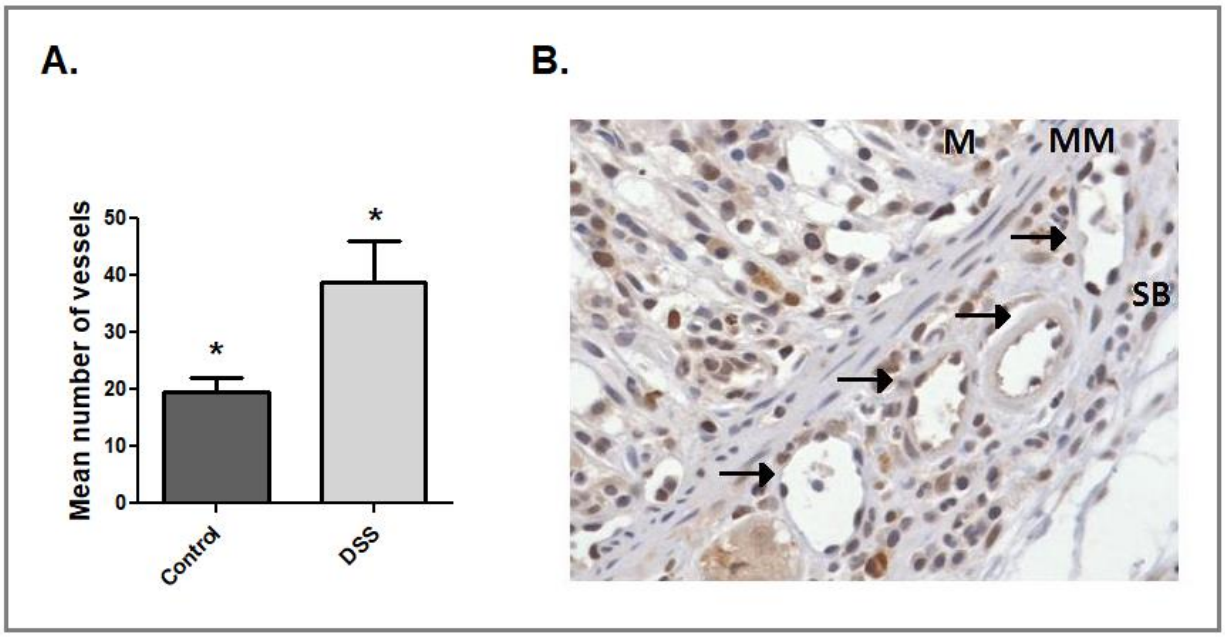

Figure 3. Total number of vessel in DSS and control group.**A.** The total number of vessels per group (38.87 ± 5.80 number of vessels in DSS group and 19.5 ±2.00 number of vessels in control group; *P*< 0.05). **B.**Immunohistochemical image of the colon with HspB5 expression from DSS group displaying four vessels on the same area. Label: M = mucosal layer, MM = muscularis mucosae layer, SB = submucosal layer. Optical microscopy, 400 x magnification.

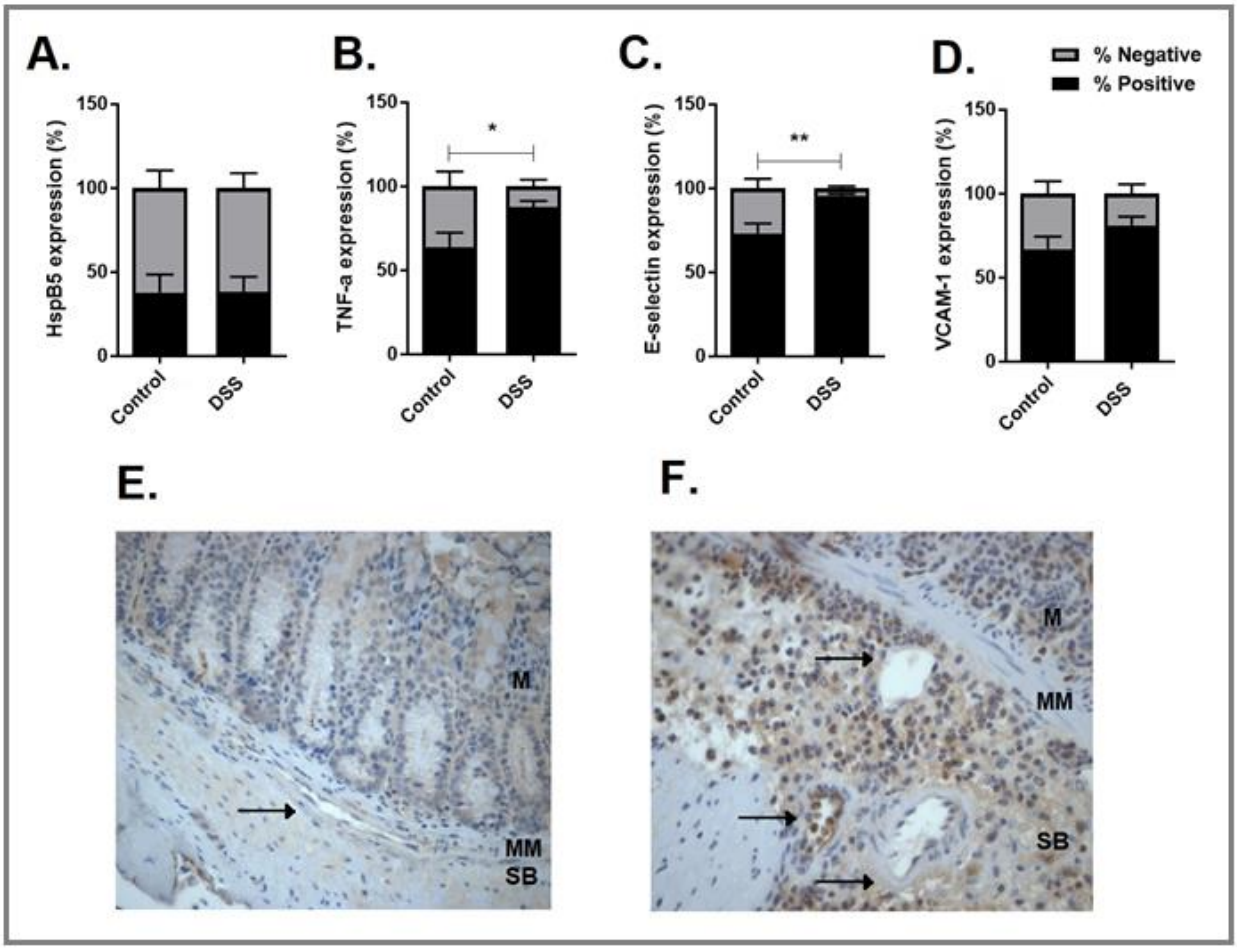

Figure 4: Immunohistochemistry analysis of HspB5 and inflammation markers. **A.** Percentage of vessels expressing HspB5. **B**. Percentage of vessels expressing TNF-α (88.41 ± 3.95% in DSS group and 67.30 ± 8.89% in control group; *P*< 0.05). **C.** Percentage of vessel expressing E-selectin (95.45 ± 1.37% in DSS group and 73.72 ± 5.74% in control group; *P*< 0.01). . **D.** Percentage of vessels expressing VCAM-1. **E.**Immunohistochemical staining for E-selectin in control mice tissue displaying one negative vessel. Optical microscopy, 400 x magnification **F.** Immuno staining for E-selectin in DSS mice tissue displaying one negative and two positive vessels. Optical microscopy, 400 x magnification. Label: M = mucosal layer, MM = muscularis mucosae layer, SB = submucosal layer.

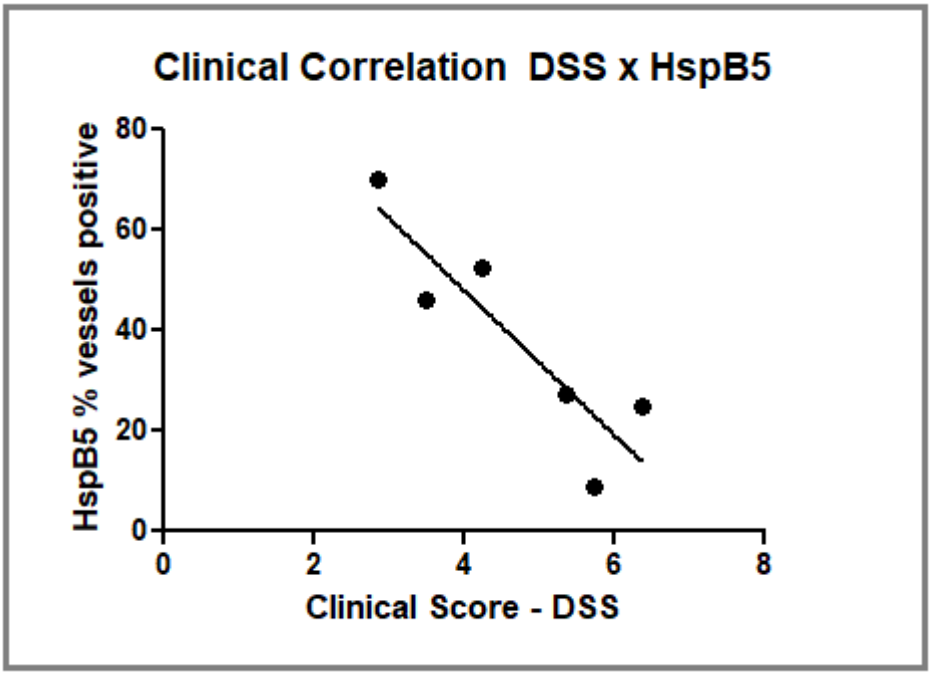

Figure 5: Strongnegative correlation between the percentage of vessels positive for HspB5 and DAI score in DSS group (*P*< 0.05, Pearson's r = -0.8912).

CONCLUSÕES

Os resultados obtidos neste trabalho nos permitem concluir que:

1.Os níveisde TNF-α e da adesina E-selectina estão aumentadosneste modelomurino de colite ulcerativa, o que aponta que estas moléculas podem possuir um papel importante no desenvolvimento da inflamação no cólon induzida por DSS.

2. Os níveisda proteínaHspB5 se correlacionam de forma inversa à severidade da doença no modelo animal de colite ulcerativa com DSS, isto é, animais com uma DAI maior apresentaram menores níveis desta proteína, enquanto que animais com uma doença mais leve apresentaram níveis aumentados de HspB5.

3. Os níveis aumentados de HspB5 em animais com severidade da doença mais baixa pode indicar que esta proteína possui um papel protetor na indução da doença, sendo capaz de diminuir a inflamação nos animais que a apresentaram em uma maior quantidade.

PERSPECTIVAS

Para o nosso conhecimento, o presentetrabalho é o primeiro a avaliar a expressão da HspB5 em doenças inflamatórias intestinais. Sendo assim, pouca informação é conhecida a respeito dos efeitos desta proteína nestas doenças, sendo necessários mais estudos para melhor compreender quais mecanismos levam à remissão da inflamação induzida por DSS com HspB5. A partir destes estudos futuros, poderia ser considerada a avaliação da administração sistêmica de HspB5 solúvel como alternativa terapêutica para atenuar a inflamação induzida por DSS. Caso sejam comprovados efeitos clínicos benéficos e eficazes neste modelo, este pode vir a ser um tratamento promissor para reduzir a inflamação e aumentar a qualidade de vida dos pacientes de doenças inflamatórias intestinais.

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APÊNDICE

Resumos produzidos durante o mestrado:

Resumo 1

"Effect of Hepatitis C drugs sofosbuvir and daclatasvir treatment on mesenchymal stem cells viability, autophagy and migration capacity"

Michele Aramburu Serafini[1], Diórlon Nunes Machado[1], Raquel Ayres[1], Ana Carolina Henzel Raymundo[1], Eduardo CremoneseFilippi Chiela[1], Anelise Bergmann Araújo[2], Themis Reverbel da Silveira[1], Mário Reis Álvares-da-Silva[1], Fabiany da Costa Gonçalves[1] and Ana Helena Paz[12].

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Porto Alegre/RS, May 2018

ACCEPTANCE LETTER

MICHELE ARAMBURU SERAFINI,

We would like to inform that you abstract was APPROVED for presentation at **1ST TERMIS-AM WORKSHOP – 4th International Meeting on Tissue Engineering and Regenerative Medicine** that took place from June 29 to July 1 at the Barra Shopping Sul Event Center. The day of its presentation will be announced on the website www.termisamerica2018.com.br on 06/15/2018. Check the poster's standards on the event website. In addition, you may only present your poster at the event and appear in the annals if your entry is removed until June 10, 2018.

Title: Effect of Hepatitis C drugs sofosbuvir and daclatasvir treatment on mesenchymal stem cells viability, autophagy and migration capacity

Form of presentation: Pôster

Resumo 2

"GRX, a stem cell in the liver tissue, is not affected by Hepatitis C sofosbuvir and daclatasvir drug treatment in vitro"

Michele Aramburu Serafini[1], Raquel Ayres[1], Ana Carolina Henzel Raymundo[1], Eduardo CremoneseFilippi Chiela[1], Themis Reverbel da Silveira[1], Mário Reis Álvares-da-Silva[1], Fabiany da Costa Gonçalves[1] and Ana Helena Paz[12].

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Title: GRX, a liver stem cell line, is not affected by Hepatitis C sofosbuvir and daclatasvir drug treatment in vitro

Form of presentation: Pôster

Co-autoria de trabalhos realizados durante o mestrado:

Artigo 1 – status: em revisão (Cytotherapy)

“Bioactive factors secreted from mesenchymal stromal cellsprotect the intestines from experimental colitis in a three-dimensional culture MSC-secreted factors protect the intestine from colitis”

Fabiany da Costa Gonçalves[1,2], Michele Aramburu Serafini[1,2], Bianca Pfaffenseller[2], Anelise Bergmann Araújo[3], Fernanda Visioli[4] and Ana Helena Paz [1,2]

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