BARBARA BRESCIANI COLOMBO

A VIMPOCETINA REDUZ A COLITE INDUZIDA POR ÁCIDO ACÉTICO EM CAMUNDONGOS

Londrina
2017

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Dissertação de mestrado apresentada ao Programa de Pós-Graduação em Ciências da Saúde da Universidade Estadual de Londrina, como requisito à obtenção do título de Mestre em Ciências da Saúde.

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Londrina, 20 de Fevereiro de 2017.

Dedico este trabalho aos meus pais e a Deus, por me formarem como ser humano.

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“O futuro pertence àqueles que acreditam na beleza de seus sonhos.”

Eleanor Roosevelt

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RESUMO

A Doença inflamatória intestinal (DII) inclui principalmente a Colite ulcerativa e a Doença de Crohn. Ela é caracterizada por uma inflamação crônica do trato gastrointestinal devido a uma resposta imune desregulada à microbiota intestinal em um individuo geneticamente suscetível. O recrutamento de neutrófilos e macrófagos para o tecido intestinal é um evento importante, no qual no foco inflamatório, essas células produzem EROs e citocinas pró-inflamatórias via NF-κB, contribuindo para o dano tecidual. A introdução do tratamento com anticorpo monoclonal anti-TNF-α em paciente com DII foi um avanço marcante. No entanto, essa terapia é muitas vezes limitada por uma perda de eficácia devido ao desenvolvimento de resposta adaptativa, dessa forma, acentuando a necessidade de novas terapias. A vimpocetina é uma droga nootrópica conhecida por suas propriedades anti-inflamatórias, em parte pela inibição do NF-κB e regulação de citocinas, além do seu efeito antioxidante. Portanto, o presente estudo avaliou o efeito da vimpocetina em modelo de colite induzida por ácido acético em camundongos. Os experimentos foram realizados em camundongos Swiss machos com aprovação do Comitê de Ética no Uso de Animais da Universidade Estadual de Londrina sob o número de processo 3307.2015.37. A colite foi induzida por injecção intracolônica de ácido acético a 7,5% (n = 6 camundongos por grupo). O tratamento com vimpocetina foi dividido em dois protocolos diferentes: (A) para análise do edema, estresse oxidativo e hiperalgesia mecânica visceral os camundongos foram tratados com vimpocetina 10 e 4h antes da injeção de ácido acético, e 2h após. Neste protocolo, a hiperalgesia mecânica visceral foi avaliada 3h após a indução da colite e o cólon distal foi coletado 4h após a indução da colite para avaliação do edema, da capacidade antioxidante total (ensaio ABTS) e níveis de GSH. (B) Para a produção de citocinas, ativação do NF-κB, recrutamento de neutrófilos, escore macroscópico e hiperalgesia mecânica visceral, os camundongos foram tratados com vimpocetina 2h antes da injeção de ácido acético e 4, 10 e 16h após. Neste protocolo, a hiperalgesia mecânica visceral foi avaliada 3 e 17h após a indução da colite e o cólon distal foi coletado 18h após indução da colite para análise de MPO (recrutamento de neutrófilos), escore de dano macroscópico, produção de citocinas e ativação do NF-κB .O tratamento com vimpocetina reduziu o edema, a atividade de MPO, o escore de dano macroscópico, a hiperalgesia mecânica visceral e os níveis das citocinas pró-inflamatórias IL-1β, TNF-α e IL-33 no cólon. A vimpocetina preveniu a redução dos níveis colônicos de GSH, da capacidade sequestradora do radical ABTS, da citocina anti-inflamatória IL-10 e inibiu a ativação do NF-κB. Desta forma, demonstramos pela primeira vez que a vimpocetina tem atividade anti-inflamatória, antioxidante e analgésica em modelo de colite induzida por ácido acético induzida em camundongos, portanto, é uma molécula promissora para o tratamento de DII.

Palavras-chave: Vimpocetina. Colite. Inflamação. Estresse oxidativo. Dor abdominal.

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ABSTRACT

Inflammatory Bowel Disease (IBD) includes mainly the Ulcerative Colitis and the Chron's Disease. It is characterized by a chronic inflammation of the gastrointestinal tract due to an immune response when the intestinal microbial was deregulated in a genetically sensitive individual. Neutrophils and macrophages recruitment towards intestinal tissues is an important event, which in the inflammatory foci, these cells produce ROS and NF-κB-dependent pro-inflammatory cytokines, contributing to tissue damage. A seminal advance was the introduction an anti-TNF-α monoclonal antibody as a treatment for IBD patients. However, this therapy is often limited by a loss of efficacy due to the development of adaptive response, underscoring the need for novel therapies. Vinpocetine is a nootropic drug known to have anti-inflammatory properties, partly by inhibition of NF-κB and downstream cytokines, in addition to its antioxidant effect. Therefore, the present study evaluated the effect of the vinpocetine in a model of acid acetic-induced colitis in mice. Experiments were conducted in male Swiss mice with Londrina State University Ethics Committee on Animal Research and Welfare approval under process number 3307.2015.37. Colitis was induced by intracolonic injection of acetic acid 7.5% (n = 6 mice per group). Vinpocetine treatment was divided in two different protocols: (A) for edema, oxidative stress analysis and visceral mechanical hyperalgesia mice were treated with vinpocetine 10 and 4h before acetic acid injection, and 2h after. In this protocol, mechanical hyperalgesia was evaluated 3h after colitis induction and distal colon was collected 4h after colitis induction for evaluation of edema, total antioxidant capacity (ABTS assay) and GSH levels. (B) For cytokine production, NF-κB activation, neutrophils recruitment, macroscopic score, and visceral mechanical hyperalgesia mice were treated with vinpocetine 2h before acetic acid injection, and 4, 10, and 16h after. In this protocol, mechanical hyperalgesia was evaluated 3 and 17h after colitis induction, distal colon was collected 18h after colitis induction for MPO analysis (neutrophils recruitment), cytokines production, and NF-κB activation. Treatment with vinpocetine reduced edema, MPO activity, macroscopic damage score, visceral mechanical hyperalgesia, and levels of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-33 in the colon. Vinpocetine prevented the reduction of colonic levels of GSH, ABTS radical scavenging ability, normalized levels of anti-inflammatory cytokine IL-10 and inhibits NF-κB activation. In this manner, we demonstrate for the first time that vinpocetine has anti-inflammatory, antioxidant, and analgesic activity in a model of acid acetic-induced colitis induced in mice and therefore is a promising molecule for the treatment of IBD.

Key words: Vinpocetine. Inflammation. Oxidative stress. Abdominal Pain

LISTA DE ABREVIATURAS E SIGLAS

5-ASA	Mesalazina
ABTS	2,2-azinobis (3-etilbenzotiazolina 6-sulfonato, sal de diamônio)
AINEs	Anti-inflamatórios não Esteroidais
CD14	*Cluster of Differentiation 14*
CU	Colite Ulcerativa
DC	Doença de Crohn
DII	Doenças Inflamatórias Intestinais
EDTA	*Ethylenediaminetetraacetic Acid*
	Ácido Etilenodiaminotetracético
ELISA	*Enzyme-linked Immunosorbent Assay*
	Ensaio de Imunoabsorção por Ligação Enzimática
EROs	Espécies Reativas de Oxigênio
ERNs	Espécies Reativas de Nitrogênio
FRAP	*Ferric Reducing Antioxidant Power*
	Capacidade Antioxidante Redutora do Íon Ferro
FDA	*Food and Drug Administration*
	Administração de alimentos e medicamentos
GSH	Glutationa Reduzida
GWS	*Genome Wide Association Studies*
	Estudos de Associação Ampla do Genoma
IASP	*International Association for the Study of Pain*
	Associação Internacional para o Estudo da Dor
IL-1β	Interleucina-1β
IL-33	Interleucina-33
IL-10	Interleucina-10
IL-2	Interleucina-2
IL-23	Interleucina-23
IL-23R	Receptor da Interleucina-23
IgG	Imunoglobulina G
iNOS	*Inducible Nitric Oxide Synthase*
	Óxido Nítrico Sintase Induzível
LPS	Lipopolissacarídeo
MCP-1	*Monocyte Chemoattractant Protein-1*
	Proteínas Quimiotáticas de Monócitos-1
MDP	Muramil Dipeptídeo

6-MMP	6-Metilmercaptopurina
6-MP	6-Mercaptopurina
MPO	Mieloperoxidase
NF-κB	*Nuclear Factor Kappa B*
	Fator Nuclear Kappa B
NGF	*Nerve Growth Fator*
	Fator de Crescimento Nervoso
NOD2	*Nucleotide-binding Oligomerization Domain Containing 2*
PCR-RT	*Reverse Transcription Polimerase Chain Reaction*
	Reação em Cadeia da Polimerase da Transcrição Reversa
6-TG	6-Tioguanina
Th17	*T Helper 17 Cells*
TNBS	*Trinitrobenzene Sulfonic Acid*
	Ácido Trinitrobenzeno Sulfônico
TNF-α	*Tumor Necrosis Factor- α*
	Fator de Necrose Tumoral-α
TPTZ	2,4,6-tripiridil-s-triazina
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-Carboxylic Acid
	Ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico
VCAM-1	*Vascular Cell Adhesion Protein-1*
	Molécula de Adesão Vascular Celular-1

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1 INTRODUÇÃO

1.1 Histórico e epidemologia da Doença Inflamatória Intestinal

A Doença Inflamatória Intestinal (DII) é caracterizada por inflamação crônica do trato gastrointestinal e inclui principalmente a Colite ulcerativa (CU) e a Doença de Crohn (DC) (SOUZA; FIOCCHI, 2015). Morgagni em 1761 descreveu pela primeira vez a DC em um paciente e denominou a doença como “ileal passion”. Outro caso foi descrito no início do século XIX por Combe e Saunders, um homem com queixas abdominais crônicas, no qual a autopsia revelou espessamento, inflamação e estenoses no íleo. Porém só no século XX Cronh e Col definiram a doença e a denominaram como DC (RAPOSO, 2008). A CU foi descrita por Samuel Wilks em 1959 como uma entidade individualizada, e o primeiro caso foi publicado no Times Gazette (MAGRO et al., 2007).

Estudos demonstram que a incidência da DII tem aumentado em muitas regiões do mundo, podendo tornar-se uma doença global (MOLODECKY et al., 2012). Ela ocorre com variações na incidência e prevalência de acordo com a região, mais comum em países industrializados, sendo a urbanização considerada um fator de risco (BURISCH et al., 2013; SOON et al., 2012). Afeta indivíduos de todas as faixas etárias, no entanto, apresenta picos de maior incidência dos 15 aos 30 anos e dos 50 aos 70 anos, sendo este último mais frequentemente associado a casos de DC (LOFTUS, 2004).

As maiores incidências da DII ocorrem no norte da Europa e na América do Norte. Na Europa a taxa de incidência da DC pode ser de até 11,5 casos em 100.000 pessoas por ano, e da CU pode ser de até 24 casos em 100.000 pessoas por ano; a prevalência da DC varia de 1,5 a 213 casos por 100.000 pessoas, e da CU varia de 2,4 a 294 casos em 100.00 pessoas. Na América do Norte, a taxa de incidência da DC pode ser de até 20,2 em 100.000 pessoas por ano, e da CU de até 24,0 em 100.000 pessoas por ano; a prevalência da DC varia 25,9 a 318,5 casos em 100.000 pessoas, e da CU varia de 37,5 a 248,6 casos em 100.000 pessoas (BURISCH, 2014; BURISCH et al., 2013; BURISCH; MUNKHOLM, 2015).

Nos Estados Unidos da América (EUA) a DII é uma das cinco condições intestinais crônicas mais comuns, com um custo total acima de 1,7 bilhões por ano (ABEGUNDE; MUHAMMAD; ALI, 2016). No Brasil, os dados ainda são escassos. Porém há alguns estudos, como o realizado por Victoria e colaboradores (2009) na região centro-oeste do Estado de São Paulo no período de 2001 a 2005, onde a incidência encontrada foi de 4,48 casos por 100.000 habitantes para CU e de 3,5 casos por 100.000 habitantes para DC, já as prevalências encontradas foram de 14,81 casos por 100.000 habitantes para a CU e de 5,65

casos por 100.000 habitantes para a DC. Em outro estudo realizado no Piauí, a prevalência em 2012 da DII foi de 12,8 casos por 100.000 por habitantes com um índice anual de 1,53 casos por 100.00 habitantes (PARENTE, 2015).

1.2 Histopatologia e manifestações clínicas da DII

A DII causa uma acentuada morbidade, e grande impacto na qualidade de vida e na capacidade de trabalho do indivíduo (NEURATH, 2014). Isso ocorre devido aos sintomas causados pela doença como diarreia (sangue e/ou muco), dor abdominal, vômitos, perda de peso, anemia, fadiga, febre, fístulas e manifestações extra-intestinais. Por se tratar de uma doença intermitente, os sintomas variam de leves a graves durante as recaídas e podem diminuir durante as remissões (BERNSTEIN et al., 2010; DE LANGE; BARRETT, 2015; NEURATH, 2014).

Embora a CU e a DC compartilhem algumas características, existem diferenças importantes a serem consideradas. Mais comum em mulheres do que em homens, a DC pode afetar todo o trato gastrointestinal, desde a boca até o ânus, de maneira descontínua, ou seja, áreas inflamadas intercaladas com áreas não inflamadas. O comprometimento intestinal é transmural, afetando todas as camadas do tecido (mucosa, submucosa, camada muscular e serosa). Pode haver complicações incluindo estenoses, abcessos ou fístulas. A presença de granulomas é característica da DC e pode ser útil para diferenciá-la da CU (BAUMGART; SANDBORN, 2007). Já a CU ocorre igualmente em ambos os sexos, afeta apenas o cólon, de maneira difusa, recidivante e não transmural, ou seja, afetando principalmente a mucosa. Conforme a extensão anatômica comprometida, ela pode ser classificada em proctite, quando atinge apenas o reto; colite do lado esquerdo, quando atinge o cólon sigmoide com ou sem envolvimento do cólon descendente; ou pancolite quando o cólon todo é comprometido. Apesar de raro, em casos de pancolite, há o acometimento de alguns centímetros do íleo terminal, evento denominado de ileíte de refluxo (BAUMGART; SANDBORN, 2007).

1.3 Fatores de risco e patogênese para DII

A DII é caracterizada por uma resposta imune desregulada à microbiota intestinal devido a gatilhos ambientais, em um indivíduo geneticamente susceptível (DE LANGE; BARRETT, 2015; MOLODECKY; KAPLAN, 2010). Têm sido propostas várias teorias para explicar os fatores de riscos ambientais envolvidos no desenvolvimento da DII (MOLODECKY; KAPLAN, 2010).

A principal teoria é a hipótese da higiene, que propõe que o aumento da frequência de distúrbios imunológicos é devido à falta de exposição a patógenos entéricos na infância (BERNSTEIN; SHANAHAN, 2008; SHANAHAN; BERNSTEIN, 2009), isso pode levar a uma maior suscetibilidade ao desenvolvimento de respostas imunológicas inadequadas aos patógenos após a infância (GENT et al., 1994).

Além da hipótese da higiene, outros fatores ambientais têm sido propostos como fatores de risco. Uma meta-análise realizada em 2008 demonstrou uma associação positiva do uso de contraceptivos orais com o desenvolvimento da CU (CORNISH et al., 2008). Em uma revisão sistemática, o consumo elevado de gorduras totais, ácidos graxos poliinsaturadas, ácidos graxos ômega-6 e carne foram consistentemente associados com o aumento do risco do desenvolvimento da CU. Em contrapartida, o alto consumo de vegetais foi consistentemente associado à diminuição do risco de desenvolvimento da CU (HOU; ABRAHAM; EL-SERAG, 2011). Alguns estudos tem demonstrado o papel dos aditivos alimentares polissacarídeos como estabilizantes, espessantes, texturizantes, emulsificantes, edulcorantes e agentes de revestimento no risco do desenvolvimento da CU ao induzir alterações na barreira intestinal, mudanças na microbiota, supercrescimento de bactérias e prejuízos na resposta imune (DIXON et al., 2016). A amamentação parece ter um efeito protetor no desenvolvimento da doença (ACHESON; TRUE LOVE, 1961; KLEMENT et al., 2004), é através dela que se adquire a tolerância oral a microflora e antígenos alimentares e consequente efeito protetor (KLEMENT et al., 2004; KOLOSKI; BRET; RADFORD-SMITH, 2008). Por outro lado, os antibióticos na infância interferem no desenvolvimento da tolerância a bactérias entéricas, podendo levar ao desenvolvimento da DII (HILDEBRAND et al., 2008). O uso de anti-inflamatórios não esteroidais (AINEs), também tem sido associado com aumento do risco de desenvolvimento da DII (ANANTHAKRISHNAN et al., 2012). Foi demonstrado que os AINEs convencionais podem causar recidivas em 20% dos pacientes com DII em remissão (KVASNOVSKY; AUJLA; BJARNASON, 2014). Vários micro-organismos são propostos como causadores da CU, como o *Mycobacterium avium* subespécie *paratuberculosis* (MAP) e a estirpe de *Escherichia coli* aderente-invasiva (AIEC) (Molodecky, 2010). Algumas evidências sugerem que o estresse psicológico está envolvido na DII (MAWDSLEY; RAMPTON, 2005).

Estudos populacionais têm fornecido evidências consistentes de que fatores genéticos contribuem para a patogênese da DII, demonstrando de 8 a 10 vezes maior risco entre parentes (ORHOLM et al., 1991), e uma maior concordância genética para gêmeos, sendo a maior taxa de concordância entre gêmeos monozigóticos de 15,4% e em gêmeos dizigóticos de 3,9% para CU (BRANT, 2011). Recentes estudos de associação ampla do

genoma (GWAS) encontraram 163 loci gênicos associados a DII, 23 específicos da CU (JOSTINS et al., 2012).

Os fatores de risco ambientais (microorganismos, dieta, infecções, estresse, AINES, apendicectomia, antibióticos) e as alterações genéticas relacionadas a peptídeos antimicrobianos, autofagia, processamento microbiano, citocinas e quimiocinas podem iniciar alterações na barreira epitelial da mucosa intestinal. Dessa maneira, ocorre a translocação de bactérias comensais e produtos bacterianos do lúmen intestinal para dentro da parede intestinal causando a ativação de células imunes e produção de citocinas. Se a inflamação aguda da mucosa não for resolvida através de mecanismos anti-inflamatórios e da supressão da resposta imune inflamatória, essa ativação descontrolada de macrófagos, células T e células linfoides inatas gera uma inflamação crônica com elevada produção de citocinas. Por sua vez, essa inflamação crônica pode levar a complicações da doença, destruição de tecido, fibrose, abcessos, fístulas, câncer e manifestações extra-intestinais (NEURATH, 2014).

Nesse contexto, as citocinas têm um papel fundamental no desenvolvimento e perpetuação da doença. Como por exemplo, a IL-1β, uma citocina pró-inflamatória com uma ampla variedade de efeitos locais e sistêmicos, produzida por leucócitos do sistema imunológico e modula a função de células imunes e não imunes; promove ativação e a função efetora de células dendríticas, macrófagos e neutrófilos e ativação e sobrevivência de células T (BEN-SASSON et al., 2009; DINARELLO, 1996). A IL-1β foi encontrada em elevados níveis no cólon de pacientes com DII ativa (MCALINDON; HAWKEY; MAHIDA, 1998), os quais podem ser correlacionados com a atividade da doença e lesões ativas (LUDWICZEK et al., 2004). Outra citocina imporante, o TNF-α ligado à membrana ou solúvel, possui sua produção acentuada em pacientes com DII. As células mononucleares da lamina própria, como os macrófagos $CD14^{+}$, adipócitos, fibroblastos e células T têm demonstrado produzir grandes quantidades dessa citocina (ATREYA et al., 2011). Mais recentemente descoberta, a IL-33 é secretada por fibroblastos, adipócitos, células musculares, células endoteliais, macrófagos, células dendríticas, células respiratórias e células intestinais (NUNES; BERNARDAZZI; DE SOUZA, 2014). Foi demonstrado que os níveis de IL-33 na mucosa intestinal são maiores em pacientes com CU do que em pacientes com DC ou sem a doença, podendo seu nível ser relacionado com a atividade da doença (BELTRÁN et al., 2010). Por outro lado, a IL-10 uma citocina anti-inflamatória é secretada por células T reguladoras, macrófagos e células dendríticas participa na homeostasia da mucosa intestinal. É possível observar o desenvolvimento espontâneo de colite em camundongos *knockout* para a IL-10 apenas em presença de bactérias intestinais (SELLON et al., 1998). Além disso, pacientes com mutações que geraram perda de função

da IL-10 ou do seu receptor, desenvolveram DII grave e com início precoce (LEE et al., 2014).

Evidências sugerem que a inflamação intestinal crônica está associada com a produção aumentada de espécies reativas de oxigênio e nitrogênio (EROs/ERNs). Como resultado dessa produção elevada de EROs e ERNs, desenvolve-se o estresse oxidativo e modulação redox que possuem um importante papel na fisiopatologia da DII em humanos e animais (KARP; KOCH, 2006; PAVLICK et al., 2002). Uma característica da DII é o infiltrado celular de neutrófilos e macrófagos, essas células inflamatórias ativadas produzem grandes quantidades de superóxido e óxido nítrico a partir da NADPH oxidase (NOX2) e óxido nítrico sintase induzível (iNOS), respectivamente. A partir da reação do superóxido e do óxido nítrico ocorre formação de peroxinitrito, um composto com elevada capacidade oxidante envolvido na patogênese da DII (PACHER; BECKMAN; LIAUDET, 2007).

Outras enzimas também estão envolvidas na geração de EROs na DII, como a xantina oxidase, 5-lipoxigenase, enzimas do citrocomo P450 e a mieloperoxidase (MPO). A MPO é abundante principalmente em grânulos azurófilos de neutrófilos é secretada para dentro do compartimento fagolisossômico após a ativação do fagócito (NAITO; TAKAGI; YOSHIKAWA, 2007). Ela está envolvida na geração do ácido hipocloroso, um potente oxidante que gera dano tecidual (KLEBANOFF, 2005). A atividade aumentada da MPO é característica da inflamação intestinal e do estresse oxidativo em modelos de DII experimental. Dessa forma, ela é considerada um biomarcador na avaliação da fisiopatologia da doença e da intervenção com compostos antioxidantes (NAITO; TAKAGI; YOSHIKAWA, 2007). Por outro lado, na mucosa intestinal os níveis de antioxidantes como a glutationa reduzida (GSH), coenzima Q10, glutationa s-transferase, superóxido dismutase, catalase, paraoxonase 1 e metalotioneína estão reduzidos em pacientes com DII (CATARZI et al., 2011; REZAIE; PARKER; ABDOLLAHI, 2007).

A inflamação contribui para o aparecimento do estresse oxidativo, o qual por sua vez contribui para a perpetuação da inflamação intestinal na DII através da ativação do NF-κB, um fator de transcrição sensível ao estado redox. Consequentemente, ocorre o aumento de citocinas inflamatórias, moléculas de adesão e células inflamatórias (ZHU; LI, 2012).

Para a Associação Internacional para o Estudo da Dor (IASP), a dor é definida como sendo “uma experiência sensorial e emocional desagradável que é associada a lesões reais ou potenciais ou descrita em termos de tais lesões” (MERSKEY, 1980). A dor abdominal é um sintoma comum e uns dos primeiros apresentados em 50% a 70% dos pacientes que procuraram o auxílio médico para diagnóstico inicial ou em episódios de exacerbação da DII (AGHAZADEH et al., 2005; WAGTMANS et al., 1998). Embora alguns trabalhos ilustrem a etiologia multifatorial e sobreposição de fatores que contribuem para a percepção da dor na

DII, ela é frequentemente atribuída à inflamação (MORRISON et al., 2013; WAGTMANS et al., 1998). Neuromediadores inflamatórios como IL-1β, TNF-α e IL-33 e o fator de crescimento nervoso (NGF) liberados por macrófagos, mastócitos, células endoteliais ativam nociceptores induzindo a hiperalgesia (BINSHTOK; WANG; ZIMMERMANN, 2008; JIN, 2006; WRIGHT, 1999; ZARPELON et al., 2013). Além disso, ROS/ERS como o superóxido e o peroxinitrito também são capazes de induzir hiperalgesia (MA; ZHANG; WESTLUND, 2009; SALVEMINI et al., 2011; WANG; PORRECA; CUZZOCREA, 2004)

1.4 Modelos experimentais para estudo da DII

Há diversos modelos experimentais estabelecidos de DII, e são classificados em 5 grupos diferentes: indução química, células de transferência, espontâneo, congênito (mutação genética espontânea) e engenharia genética (MIZOGUCHI, 2012). Nos modelos de indução química, um agente exógeno é administrado para indução da DII, por exemplo, ácido acético, ácido trinitrobenzenosulfônico (TNBS), sulfato sódico de dextrana (DSS) (MORRIS et al., 1989). Nos modelos de células de transferência, populações celulares específicas são transferidas para um hospedeiro imunologicamente comprometido, um exemplo é o modelo CD45RB, em que há transferência adotiva de células T $CD4^+$ ($CD4^+CD45RB^{high}$) do baço de camundongos naíve para receptores imunodeficientes (POWRIE; MASON, 1990). Há um único modelo espontâneo, em que um macaco conhecido popularmente como Saguí-cabeça-de-algodão (*Saguinus oedipus*) nativo da Colômbia, desenvolve colite espontânea ao ser mantido nos EUA (CHALIFOUX; BRONSON, 1981). No modelo congênito, um exemplo é o modelo SAMP1/Yit, no qual uma estirpe de ratos SAMP1 derivada de 24 gerações de uma ninhada de irmãos AKR/J expressa um fenótipo de senescência acelerada e lesões inflamatórias no íleo terminal (MATSUMOTO et al., 1998). Nos modelos de engenharia genética os animais são manipulados geneticamente (*knockout* ou transgênicos) e desenvolvem DII, como exemplo, camundongos *knockout* para a IL-10 (KUHN et al., 1993).

Estes modelos são úteis não só para descobrir mecanismos envolvidos na doença, mas também para desenvolver novas estratégicas terapêuticas para a DII (MIZOGUCHI, 2012). Apesar desses modelos não mimetizarem exatamente a doença em humanos, eles têm sido úteis em muitos aspectos. Isso se deve ao fato de ser possível controlar o aparecimento da inflamação no laboratório, as falhas de tolerância imunológica, genes de susceptibilidade e vias pró-inflamatórias específicas envolvidas no desencadeamento da colite, possibilitando o entendimento de mecanismos de ação de fármacos bem como a fisiopatologia da doença (BRAMHALL et al., 2015).

Os modelos de indução com agente químico (por exemplo, acido acético) tem a vantagem de causar inflamação em linhagens puras de camundongos, com um sistema imune normal (NEURATH, 2012), baixo custo e fácil administração (LOW; NGUYEN; MIZOGUCHI, 2013). O ácido acético por via retal é capaz de reproduzir a colite de maneira difusa e dose-dependente, reprodutível na porção distal do cólon de camundongos (GUAZELLI et al., 2013; MACPHERSON; PFEIFFER, 1978), reproduz inflamação semelhante a CU em humanos, no que diz respeito mudanças moleculares, características histológicas e clínicas (GOYAL et al., 2014). A resposta inflamatória ocorre devido ao rompimento da barreira epitelial (KAWADA; ARIHIRO; MIZOGUCHI, 2007), com consequente liberação de prótons no espaço extracelular, promovendo acidificação intracelular e dano ao epitélio (RANDHAWA et al., 2014).

1.5 Tratamento convencional para CU

O protocolo terapêutico adotado depende do local acometido, severidade e complicações relacionadas à doença. Deve ser individualizado de acordo com a resposta sintomática e tolerância clínica. Seguindo o protocolo sequencial de tratamento dos sintomas, indução e manutenção da remissão (SOHRABPOUR; MALEKZADEH; KESHAVARZIAN, 2010). A terapia farmacológica utilizada consiste principalmente na utilização de aminossalicilatos, corticosteroides, imunossupressores não esteroidais, antibióticos e agente biológicos (FASANMADE et al., 2009; KINO et al., 1987; KORNBLUTH; SACHAR, 2004; MOWAT et al., 2011; NIELSEN, 1982).

Os aminossalicilatos (sulfassalazina e Mesalazina) são a primeira opção de tratamento para a indução e manutenção da remissão da CU leve a moderada (NIELSEN, 1982). Efeitos adversos da sulfassalazina foram encontrados em até 30% dos pacientes (NIELSEN, 1982), de maneira dose-dependente, devido a porção sulfapiridina (DAS et al., 1973). O efeito terapêutico do 5-ASA ocorre ao inibir a ciclooxigenase, a lipooxigenase, células B, citocinas inflamatórias (IL-1β e TNF-α), proliferação de células T, adesão de neutrófilos e macrófagos, ativação do fator nuclear NF-κB e sequestro de radicais livres (DESREUMAUX; GHOSH, 2006).

Os corticosteroides (prednisolona, prednisona, metilpredinisolona, hidrocortisona e budesonide) são utilizados no tratamento para pacientes com a forma moderada ou grave da doença e nos casos não responsivos aos aminossalicilatos (MOWAT et al., 2011; SOHRABPOUR; MALEKZADEH; KESHAVARZIAN, 2010). O seu efeito anti-inflamatório inclui inibição do recrutamento e proliferação de linfócitos, monócitos e macrófagos, migração de neutrófilos para os locais da inflamação, e diminuição da produção de

mediadores inflamatórios, incluíndo leucotrienos, citocinas e prostaglandinas (KATZ, 2004). Em longo prazo e/ou altas doses podem causar efeitos adversos graves, como perda óssea (LUKERT; RAISZ, 1990), tromboembolismo venoso (HIGGINS et al., 2015), má cicatrização (WANG; ARMSTRONG; ARMSTRONG, 2013), complicações cutâneas, oculares, músculo esqueléticas, gastrointestinais e infecciosas (ARDIZZONE; BIANCHI PORRO, 2002).

É recomendado o uso de imunossupressores não esteroidais, tais como azatioprina (AZA) e 6-mercaptopurina (6-MP) em pacientes com CU grave que necessitariam utilizar corticosteroides por mais de um ano, ou a pacientes com colite moderada não responsiva ao uso de corticosteroides (KORNBLUTH; SACHAR, 2004; LICHTENSTEIN et al., 2006). A AZA é pró-fármaco da 6-MP, e ambos são convertidos em 6-tioguanina (6-TG), metabólito com ação terapêutica. A 6-TG é convertida em um metabólito inativo e na 6-metilmercaptopurina (6-MMP), um metabólito hepatotóxico (REGUEIRO, 2000; STEIN; LICHTENSTEIN, 2001). A 6-TG inibe a proliferação de linfócitos através da sua incorporação em nucleotídeos celulares, dessa forma, ocorre a supressão da função das células T e atividade das células natural killer (REGUEIRO, 2000; SAHASRANAMAN; HOWARD; ROY, 2008). Alguns efeitos adversos estão relacionados às tiopurinas (ASA e 6-MP), incluindo náusea, artralgia, hepatotoxicidade, mielotoxicidade (leve e grave), prancreatite, elevação dos níveis de enzimas hepáticas, infecções oportunistas, linfoma, desordens mielóides e câncer de pele não melanoma (GOLDBERG; IRVING, 2015).

A Ciclosporina A é utilizada como segunda linha em casos agudos de CU grave e refratária aos corticosteroides. Ela é inibidor da calcineurina, sendo assim, atua diminuindo a resposta celular imunitária através do bloqueio da produção de IL-2 pelos linfócitos T auxiliares (LOFTUS; EGAN; SANDBORN, 2004). Porém, é considerada uma das terapias menos segura, devido ao risco de efeitos adversos graves, como anafilaxia, pneumonia por *Pneumocystis carinii* e nefrotoxicidade permanente (KATZ, 2005). Além disso, devido a sua janela terapêutica estreita é necessário acompanhamento dos níveis plasmáticos da droga durante o tratamento (COHEN; STEIN; HANAUER, 1999).

O Tacrolimus é um antibiótico macrolídeo obtido do *Streptomyces tsukubaenesis.* Ele também atua como inibidor da calcineurina, e embora seu modo de ação seja similar ao da Ciclosporina A, o seu efeito imunossupressor é de 30 a 100 vezes maior *in vitro* e de 10 a 20 vezes maior *in vivo*, com melhor absorção intestinal mesmo em presença de doença gastrointestinal (KINO et al., 1987). Os efeitos adversos mais comuns são neurotoxicidade, incluindo tremor e dor de cabeça, distúrbios gastrointestinais, nefrotoxicidade e distúrbios metabólicos (GONZALEZ-LAMA; GISBERT; MATE, 2006).

O Infliximab é um anticorpo monoclonal IgG que se liga com alta afinidade e especificidade ao TNF-α solúvel. Dessa forma, impede-o de ligar-se aos receptores

celulares e ativar a cascata inflamatória (RUTGEERTS; VAN ASSCHE; VERMEIRE, 2004). Além disso, liga-se ao TNF-α de membrana em células inflamatórias induzindo apoptose (WILHELM et al., 2008). Foi o primeiro anticorpo anti-TNF-α aprovado pela Administração de alimentos e medicamentos (FDA) para o tratamento de CU moderada a grave para reduzir os sinais e sintomas, induzir remissão, cicatrização da mucosa intestinal e eliminar o uso de corticosteroides em pacientes que não respondem adequadamente, são intolerantes ou possuem contra-indicações a corticosteroides ou a imunomoduladores (FASANMADE et al., 2009). Efeitos adversos graves como obstrução intestinal, insuficiência cardíaca, infecção, linfoma e neuropatia podem ocorrer devido ao uso do Infliximab (HANSEN et al., 2007).

O Adalimumab e o Golimumab são anticorpos monoclonais humanos que também são anti-TNF-α, como o Infliximab, aprovados pelo FDA em 2013 para pacientes com CU. Foram liberados como agentes de indução e manutenção da remissão em pacientes adultos com CU grave ou moderada que não respondem ou são intolerantes aos tratamentos convencionais ou necessitem do tratamento contínuo com corticosteroides (BLONSKI; BUCHNER; LICHTENSTEIN, 2014).

1.6 Vimpocetina

A vimpocetina ([3α,16α]-Eburnamenine-14-carboxylic acid ethyl ester) é um éster etílico sintético, derivada da Apovicamina, um alcaloide isolado a partir das folhas da *Vinca minor,* popularmente conhecida como Pervinca (LORINCZ C, SZASZ K, 1976). Nos EUA, mais de 300 marcas de suplementos alimentares regulamentadas como comida possuem em sua composição a vimpocetina e são vendidas diretamente ao consumidor (AVULA et al., 2016).

A vimpocetina é absorvida no estômago e intestino e é capaz de atravessar a barreira hematoencefálica. Sua metabolização em humanos e cachorros ocorre exclusivamente no fígado, porém a metabolização no plasma ocorre apenas em ratos (FANDY et al., 2016; SZAKÁCS, 2001). O ácido apovincamínico é o principal metabólito sem atividade farmacológica da hidrólise da vimpocetina e sua excreção ocorre através dos rins (GULYÁS et al., 2002). A vimpocentina é amplamente utilizada desde sua síntese, no final dos anos 60, para o tratamento de doenças cerebrovasculares, demonstrando um efeito neuroprotetor e aumento do fluxo sanguíneo cerebral (BERECZKI; FEKETE, 1999). O seu efeito clínico benéfico deve-se a sua ação antioxidante, anti-inflamatória, vasodilatadora e neuroprotetora (ZHAO et al., 2011).

Jeon e colaboradores (2010) demonstraram através de testes *in vitro* que a vimpocentina impede a ativação do fator de transcrição nuclear NF-κB induzido pelo TNF-α

em células lisas vasculares, em células endoteliais da veia umbilical humana, em células epiteliais respiratórias A549 e em uma linhagem celular de macrófagos. Além disso, a análise de PCR-RT demonstrou que a vimpocentina reduz a expressão de RNAm de moléculas pró-inflamatórias, tais como a interleucina-1β, proteínas quimiotáticas de monócitos-1 (MCP-1) e molécula de adesão vascular celular-1 (VCAM-1) induzida pelo TNF-α. *In vivo* a vimpocentina foi capaz de reduzir significativamente o recrutamento de neutrófilos induzido por TNF-α e lipopolissacarídeo (LPS) em tecidos do pulmão. Demonstrou-se que o mecanismo pelo qual a Vimpocentina é capaz de exercer seus efeitos anti-inflamatórios se dá através da inibição da IKK, prevenindo a degradação do IκB, a translocação do NF-κB para o núcleo, e consequentemente a inibição da produção de mediadores pró-inflamatórios, principalmente das citocinas IL-1β e TNF-α (JEON et al., 2010). Um outro possível mecanismo da vimpocetina é a inibição da fosforilação da enzima *upstream* Akt (ZHUANG et al., 2013). Corroborando esses dados, nosso laboratório demonstrou *in vivo* e *in vitro* que a vimpocetina inibe a ativação do NF-κB induzido por LPS e carragenina (RUIZ-MIYAZAWA et al., 2015a, 2015b). Além disso, outros estudos demonstraram a participação da vimpocetina na prevenção de danos à mucosa gástrica de animais (NOSÁLOVÁ; MACHOVÁ; BABULOVÁ, 1993), assim como propriedades antioxidantes em modelo *in vitro* (HORVATH et al., 2002). Dados sugerem o papel antinociceptivo da vimpocetina em modelo de dor visceral manifesta em ratos (ABDEL-SALAM, 2006).

Outro trabalho sugere que a vimpocetina é um potente bloqueador da atividade dos canais de sódio tetrodotoxina Na_v 1.8 presentes no gânglio da raiz dorsal de neurônios sensoriais. Esse tipo de canal é expresso de maneira seletiva em neurônios sensoriais, dessa maneira, demonstrando um mecanismo analgésico importante (ZHOU; DONG; CRONA, 2003). Foi demonstrado que a vimpocetina é capaz de inibir o transporte axoplasmático retrógrado do NGF periférico (CSILLIK et al., 2008; KNYIHAR-CSILLIK et al., 2007), evidências sugerem que o NGF tem um papel importante na geração e manutenção da dor em estudos experimentais e clínicos em uma ampla variedade de estados de dor (BANNWARTH; KOSTINE, 2014).

Tendo em vista que a vimpocentina possui efeito anti-inflamatório, antioxidante e analgésico ela apresenta-se como uma abordagem terapêutica promissora uma vez que há a participação de mediadores pró-inflamatórios, como citocinas e radicais livres na patogênese das DII, associado ao fato de que a mucosa colônica é relativamente pobre em antioxidantes endógenos. Uma vez que não existem dados na literatura sobre a utilização desta substância em modelos experimentais de colite, nós propomos avaliar o efeito

farmacológico da vimpocentina em modelo de colite experimental induzida por ácido acético em camundongos.

2 OBJETIVOS

2.1 Objetivo geral

Investigar o efeito anti-inflamatório, antioxidante e analgésico da vimpocetina em modelo de colite induzida por ácido acético em camundongos.

2.2 Objetivos específicos

- Avaliar o efeito do tratamento com vimpocetina sobre o edema colônico após a indução da colite com solução de ácido acético;
- Avaliar o efeito do tratamento com vimpocetina sobre o recrutamento de neutrófilos para o tecido colônico após a indução da colite com solução de ácido acético;
- Avaliar o efeito do tratamento com vimpocetina sobre as lesões macroscópicas no tecido colônico após a indução da colite com solução de ácido acético;
- Avaliar o efeito do tratamento com vimpocetina sobre a capacidade antioxidante no tecido colônico, através dos níveis de glutationa reduzida (GSH) e da capacidade sequestradora do radical ABTS, após a indução da colite com solução de ácido acético;
- Avaliar o efeito da vimpocetina sobre a hiperalgesia mecânica visceral após a indução da colite com solução de ácido Acético;
- Avaliar o efeito do tratamento com vimpocetina sobre os níveis das citocinas IL-1β, IL-10, TNF-α e IL-33 no tecido colônico após a indução da colite com solução de ácido acético;
- Avaliar o efeito do tratamento com a vimpocetina sobre a ativação do NF-κB após a indução da colite com solução de ácido acético;

3 MATERIAIS E MÉTODOS

3.1 Materiais

Vimpocetina foi adquirida da Tianjin Harmony Technology Development Co. (China), brometo de hexadecil trimetil-amonio (HTAB); dihidrocloreto de O-dianisidina; tween 80; glutationa reduzida (GSH); glutaraldeído; EDTA; cloreto de ferro hexahidratado; 2,4,6-tripiridil-s-triazina (TPTZ); 2,2-azinobis (3-etilbenzotiazolina-6-sulfonato, sal de diamônio; ABTS); Trolox (ácido 6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico); e persulfato de potássio foram adquiridos da Sigma Chemical Co. (St. Louis, USA). Kits para dosagem de IL-1β, IL-33 e IL-10 foram adquiridos da eBioscience (San Diego, USA). Para a determinação da ativação do NF-κB os anticorpos primários foram adquiridos da Santa Cruz Biotechnology (Santa Cruz, CA, USA), e os anticorpos secundários foram adquiridos da ©Jackson ImmunoResearch Inc. (IgG Affinity-Purified Antibodies, West Baltimore Pike, West Grove, PA, USA). Todos os reagentes utilizados foram de grau analítico.

3.2 Animais Experimentais

Os experimentos foram realizados em camundongos Swiss machos, pesando 20 ± 1g, provenientes do Biotério Central da Universidade Estadual de Londrina, PR, Brasil. Pelo menos 2 dias antes e durante os experimentos, os animais foram mantidos no Departamento de Ciências Patológicas da Universidade Estadual de Londrina, em caixas plásticas, forradas com maravalha, em ciclo de 12 horas claro/escuro e temperatura entre 21± 2°C. A água e ração foram administradas *ad libitum*, com exceção das 24 horas que antecediam os experimentos, período no qual os animais foram mantidos em jejum sólido. Os procedimentos de cuidado e manuseio dos camundongos foram realizados de acordo com as orientações da IASP e aprovado pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Estadual de Londrina (n° processo: 3307.2015.37). Todos os esforços foram feitos para minimizar o número de animais e seu sofrimento.

3.3 Indução da colite experimental

Os animais foram anestesiados com quetamina (80 mg/kg, im) e xilazina (10 mg/kg, im) após 24 horas de jejum sólido. Os camundongos receberam 100 µL (intracolônico) de solução salina estéril para lavagem do cólon dos animais. Uma hora após, os camundongos receberam 200 µL (intracolônico) de solução de ácido acético 7,5% (v/v em salina) ou salina

(intracolônico). Os camundongos foram mantidos de cabeça para baixo por 3 minutos para evitar o extravasamento do líquido. As injeções intracolônicas foram realizadas com uma cânula de polietileno de 3 cm de comprimento (GUAZELLI et al., 2013).

3.4 Protocolos experimentais e tratamento

Foram utilizados dois protocolos experimentais: (A) Os camundongos foram tratados 2 horas antes da indução da colite e 4, 10 e 16 horas após a indução. Nesse protocolo os camundongos foram eutanasiados 18 horas após e indução da colite. Após eutanásia, foi realizada a determinação da atividade da MPO, avaliação da lesão macroscópica, dosagem de citocinas (IL-1β, IL-10, IL-33 e TNF-α) de acordo com Guazelli et al. (2013) e ativação do NF-κB. A análise da hiperalgesia mecânica visceral foi realizada 3 e 17 horas após e indução da colite (figura 1A). No segundo protocolo (B), os camundongos foram tratados 10 e 4 horas antes da indução da colite, e 2 horas após a indução. Nesse protocolo, os animais foram eutanasiados 4 horas após e indução da colite. Após eutanásia, as amostras do cólon distal (1 cm) foram coletadas para os ensaios de GSH e ABTS de acordo com Guazelli et al. (2013), além disso, foi realizada a análise do edema. A hiperalgesia mecânica visceral foi realizada 3 horas após a indução da colite (figura 1B). Foram utilizados os seguintes grupos experimentais: (1) grupo controle, animais sem colite (animais receberam apenas salina intracolônico; (2) grupo colite, (animais receberam solução de ácido acético a 7,5% intracolônico, sem tratamento); (3) grupo vimpocetina, (animais receberam a solução de ácido acético a 7,5% intracolônico e tratados com vimpocetina [1, 3, 10 ou 30 mg/Kg solubilizada em 20% de tween 80 em salina] por via oral). A análise do edema, a determinação da atividade do MPO e a hiperalgesia mecânica visceral foram utilizadas para padronização de dose para os demais ensaios. Os protocolos de tratamento foram realizados conforme meia-vida da vimpocetina de aproximadamente 2h (POLGÁR; VERECZKEY; NYÁRY, 1985). A padronização do modelo e os tempos de coleta das amostras (4 e 18 horas após a indução da colite) foram determinados previamente por Guazelli et al. (2013).

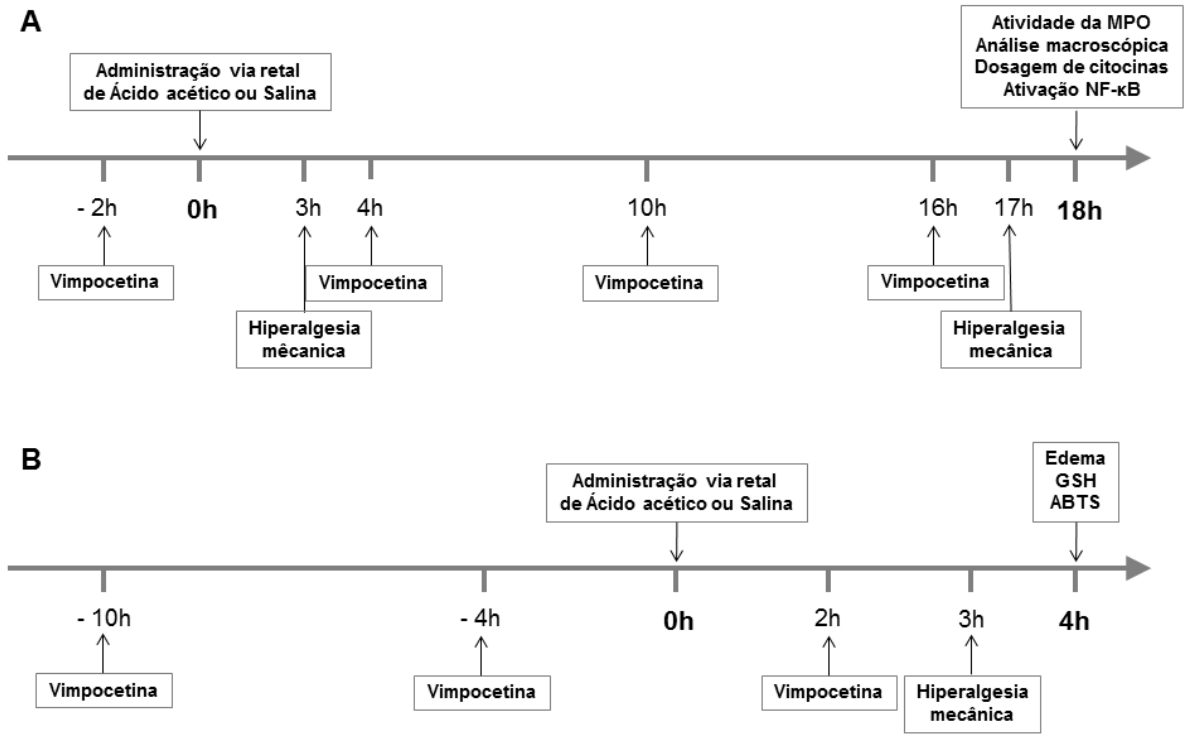

Figura 1 – Representação esquemática dos protocolos experimentais para indução da colite com ácido acético e tratamento com vimpocetina. (A) Os camundongos foram tratados com vimpocetina 2 horas antes; 4, 10 e 16 horas após a indução na colite. O teste do von Frey foi realizado 3 e 17 horas após a indução da colite e as amostras da porção distal do cólon (1 cm) foram coletadas 18 horas após a indução da colite para a determinação da atividade da MPO, análise macroscópica, dosagem de citocinas e ativação do NF-κB. (B) Os camundongos foram tratados com vimpocetina 10 e 4 horas antes; e 2 horas após a indução da colite. O teste do von Frey foi realizado 3 horas após a indução da colite e as amostras da porção distal do cólon (1 cm) foram coletadas 4 horas após a indução da colite para a avaliação do edema, GSH e ABTS.

3.5 Avaliação do Edema

Fragmentos da porção distal dos cólons dos camundongos, medindo 1 cm de comprimento, foram coletados e pesados para avaliação do edema no tecido. Após determinação do peso, em gramas, de 1 cm de tecido colônico, os resultados foram expressos em % de aumento [do peso (g) / comprimento do tecido colônico (cm)], em

relação ao grupo controle sem colite (BARBOSA, 2011; GUAZELLI et al., 2013; LEE et al., 2009).

3.6 Determinação da atividade da mieloperoxidase (MPO)

A atividade da MPO no cólon dos camundongos foi medida por método colorimétrico, descrito por Guazelli et al. (2013). Amostras de 1 cm da porção distal do cólon foram coletadas em tampão fosfato de potássio 50 mM (pH 6,0) contendo HTAB (brometo de hexadecil trimetil-amonio) 13,72 mM e armazenadas a -80°C. No dia do ensaio, as amostras foram homogeneizadas com auxílio do ultraturrax (ULTRA-TURRAX® – Ika), centrifugadas (16,1 *g*, 4°C, 2 minutos) e o sobrenadante foi utilizado para a reação colorimétrica em placa de 96 poços. A cada alíquota de 15 µL de amostra foi adicionado 200 µL da solução de reação contendo 52,64 mM de dihidrocloreto de O-dianisidina e 0,05% de H_2O_2 30% em tampão fosfato de potássio 50 mM (pH 6,0). As leituras foram realizadas a 450 nm (Multiskan GO Thermo Scientific), e o número de neutrófilos por mg de tecido foi determinado utilizando-se uma curva padrão de neutrófilos.

3.7 Hiperalgesia mecânica visceral

A hiperalgesia mecânica visceral foi avaliada pelo teste eletrônico do von Frey, de acordo com Pereira et al. (2013). Os camundongos foram alocados em gaiolas de acrílico (12 x 10 x 17 cm) com pavimentos de grades de arame em uma sala com temperatura controlada e silenciosa 30 minutos antes do início das medições. O teste consistiu em provocar uma resposta de retirada do animal com um transdutor de força portátil (analgesímetro eletrônico; Insight, Ribeirao Preto, SP, Brasil) adaptado a uma ponteira de polipropileno de 0.5 mm^2, que foi aplicada na região do abdômen inferior até o abdômen médio. O experimentador foi treinado e cuidados foram tomados para não estimular o mesmo ponto consecutivamente e evitada a estimulação da genitália externa. Após retirada, a intensidade da pressão foi automaticamente registrada, com valores da média de três medições. Foi considerada como resposta de retirada um dos seguintes comportamentos: retração acentuada do abdômen; lambida imediata ou coçar do local da aplicação da ponteira; salto; ou sacudida de pata (*flinches*) (LAIRD et al., 2001). Os resultados foram expressos como o delta (Δ) do limiar de retirada (em gramas), calculado subtraindo os valores médios obtidos 3 ou 17 horas após a indução da colite dos valores médios basais (antes do tratamento com vimpocetina e indução de colite).

3.8 Avaliação das lesões macroscópicas

Após a eutanásia dos camundongos, os cólons foram expostos, abertos longitudinalmente e os escores de lesão foram determinados de acordo com os achados macroscópicos (GUAZELLI et al., 2013), de acordo com a tabela 1.

Tabela 1 – Índices macroscópicos de colite segundo Guazelli *et al.*(GUAZELLI et al., 2013).

Escore	Achados macroscópicos
0	Sem danos
1	Hiperemia sem ulcerações
2	Ulcerações lineares sem inflamação significativa
3	Ulcerações lineares com inflamação em um local
4	Dois ou mais locais de inflamação e ulceração
5	Área da lesão > 1 cm ao longo do cólon
6-10	Área de lesão > 2 cm ao longo do comprimento do cólon. A quantificação é aumentando em 1 para cada centímetro adicional

3.9 Determinação da glutationa reduzida (GSH)

Os níveis de GSH nas amostras da porção distal do cólon dos camundongos foram determinados por método espectrofotométrico previamente descrito (GUAZELLI et al., 2013). Amostras de 1 cm da porção distal foram coletadas em solução de EDTA 0,02 M e homogeneizadas com auxílio do ultraturrax (ULTRA-TURRAX® – Ika). Os homogenatos foram tratados com ácido tricloroacético 30% e centrifugados (1,5 *g*, 4°C, 15 minutos). Em seguida, foram adicionados 200 µL de tampão Tris-HCl 0,4 M (pH 8,9) às alíquotas de 150 µL do sobrenadante de cada amostra. Após homogeneização, 10 µL de DTNB (ácido ditionitrobenzóico) 0,01 M em metanol foi adicionado. Após 5 minutos de reação, a leitura foi realizada a 412 nm (Multiskan GO Thermo Scientific). A curva padrão foi preparada com 0,5 µM de GSH, e os resultados expressos em nM de GSH por mg de tecido.

3.10 Determinação da capacidade sequestradora do radical ABTS

Amostras de 1 cm da porção distal do cólon foram coletadas em tampão KCl (500 µL, 1.15% peso/volume) e homogeneizadas com auxílio do ultraturrax (ULTRA-TURRAX® – Ika). Os homogenatos foram centrifugados (0,2 *g*, 4°C, 10 minutos) e os sobrenadantes foram utilizados no ensaio. À solução de ABTS diluída (200 µL) foi adicionada a 10 µL de amostra em cada poço. Após 6 minutos de incubação (25 °C) as absorvâncias foram determinadas a 730 nm (Multiskan GO Thermo Scientific). A capacidade sequestradora do radical nas amostras foi determinada a partir de uma curva padrão de Trolox (1,5 – 30 µmol/L) e os resultados foram expressos em mmol equivalente ao Trolox por mg de tecido, que representa a quantidade de Trolox (em mmol) com um potencial antioxidante equivalente a 1 mg do tecido (GUAZELLI et al., 2013).

3.11 Dosagem de citocinas

Amostras de 1 cm da porção distal do cólon foram coletadas em 500 µL de tampão apropriado contendo inibidores de protease e homogeneizadas com auxílio do ultraturrax (ULTRA-TURRAX® – Ika). Após homogeneização, as amostras foram centrifugadas (0,8 *g*, 4°C, 10 minutos) e o sobrenadante foi utilizado para avaliar os níveis de citocinas por ELISA (enzyme-linked immunosorbent assay) utilizando kits da eBioscience (Affymetrix, San Diego, CA, USA) em 450 nm (Multiskan GO Thermo Scientific), de acordo com instruções do fabricante. Os resultados foram obtidos comparando a densidade óptica com as densidades das curvas padrões, e expressos em pg por mg de tecido.

3.12 Ativação do NF-κB

Amostras de 1 cm da porção distal do cólon foram coletadas em 300 µL em tampão RIPA contendo inibidores de proteases e fosfatases e homogeneizadas com auxílio do ultraturrax (ULTRA-TURRAX® – Ika). Os homogenatos foram centrifugados (0.5 g, 4°C, 10 minutos) e os sobrenadantes utilizados para análise por western blot. Os extratos proteicos foram separados por SDS-PAGE (gel de 10%) e transferidos para uma membrana de nitrocelulose (GE Healthcare-Amersham, Pittsburgh, PA, USA). A membrana foi incubada em tampão de bloqueio [5% albumina de soro bovino (BSA) ou 5% de leite sem gordura em salina tamponada com Tris (TBS) com Tween 20] por 2 horas à temperatura ambiente, lavadas com tampão TBS com Tween 20 e incubadas a noite toda à 4°C na presença de anticorpo primário diluído em 5% BSA ou 5% de leite sem gordura em TBS com Tween 20.

Os anticorpos primários utilizados para o Western blot foram p-NFκB p65 (sc-166748, 1:100) e NFκB p65 (sc-372, 1:200). A massa molecular de proteína foi confirmada pelo PageRulerTM Prestained Protein Ladder (Thermo Scientific, Rockford, IL, USA). Após lavagem em TBS com Tween 20, a membrana foi incubada com anticorpo secundário por 2 horas à temperatura ambiente. A proteína foi visualizada por quimioluminescência com LuminataTM Forte Western HRP Substrate (Merck Millipore Corporation, Darmstadt, Alemanha). Todos os anticorpos primários foram adquiridos da Santa Cruz Biotechnology (Santa Cruz, CA, USA), e os anticorpos secundários foram adquiridos da ©Jackson ImmunoResearch Inc. (IgG Affinity-Purified Antibodies, West Baltimore Pike, West Grove, PA, USA). A membrana foi estripada e re-blotada com anticorpo contra a proteína total de interesse a ser utilizada como controle de carregamento. Os dados densitométricos foram analisados utilizando Sistemas de Imagem Científica (Image Lab 3.0 software; Bio-Rad Laboratories, Hercules, CA).

3.13 Análise estatística

Os dados foram expressos como média ± erro padrão da média de medidas feitas em 6 animais por grupo e são representativas de dois experimentos independentes, exceto para os experimentos envolvendo análise macroscópica (utilizados 17 animais por grupo e são representativos de dois experimentos independentes). Para dados em um único tempo as diferenças estatísticas significativas entre os grupos foram avaliadas através do teste paramétrico da análise de variância (ANOVA) de uma via seguida do pós-teste de Tukey. Para dados em múltiplos tempos (hiperalgesia), as diferenças estatísticas significativas entre os grupos foram avaliadas através do teste paramétrico da análise de variância (ANOVA) de duas vias medidas repetidas, seguida do pós-teste de Tukey. Para a análise dos escores macroscópicos de lesão colônica, foi utilizado o teste não paramétrico de Kruskall-Wallis seguido pelo teste de Dunn. As análises estatísticas foram realizadas usando-se o software GraphPad Prism 5 (GraphPad Software Inc., San Diego, EUA). As diferenças foram consideradas significativas para valores correspondentes a $p < 0,05$.

4 RESULTADOS

4.1 Artigo Científico

O presente trabalho foi realizado no Laboratório de Dor, Inflamação, Neuropatia e Câncer, da Universidade Estadual de Londrina e segue as normas da revista *European Journal of Pharmacology* (normas no ANEXO A). Os resultados parciais estão descritos no artigo intitulado “Vinpocetine ameliorates acetic acid-induced colitis by inhibiting neutrophils recruitment, oxidative stress, pro-inflammatory cytokines production, and NF-κB activation in mice”.

Vinpocetine ameliorates acetic acid-induced colitis by inhibiting neutrophil recruitment, oxidative stress, pro-inflammatory cytokines production, and NF-κB activation in mice

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Abstract

The idiopathic inflammatory bowel diseases (IBD) comprise two types of chronic intestinal disorders: Crohn's disease and ulcerative colitis. Neutrophils and macrophages recruitment towards intestinal tissues is an important event given that in the inflammatory foci, these cells produce ROS and NF-κB-dependent pro-inflammatory cytokines, which contribute to tissue damage. A seminal advance was the introduction an anti-TNF-α monoclonal antibody as a treatment for patients with IBD. However, this therapy is often limited by a loss of efficacy due to the development of adaptive response, underscoring the need for novel therapies. Vinpocetine is a nootropic drug known to have anti-inflammatory properties, partly by inhibition of NF-κB and downstream cytokines, in addition to its antioxidant effect. Therefore, the present study evaluated the effect of the vinpocetine in a model of acid acetic-induced colitis in mice. Experiments were conducted in male Swiss. Treatment with vinpocetine reduced edema, MPO activity, macroscopic damage score, visceral mechanical hyperalgesia, NF-κB activation and thereby NF-κB-dependent pro-inflammatory cytokines IL-1β, TNF-α, and IL-33 in the colon. Vinpocetine prevented the reduction of colonic levels of GSH, ABTS radical scavenging ability, normalized levels of anti-inflammatory cytokine IL-10. Thus, we demonstrate for the first time that vinpocetine has anti-inflammatory, antioxidant, and analgesic activity in a model of acid acetic-induced colitis in mice and therefore is a promising molecule for the treatment of IBD.

Keywords: Vinpocetine. Inflammation. Oxidative stress. Abdominal Hyperalgesia.

1. Introduction

Inflammatory Bowel Disease (IBD) is characterized by chronic inflammation of the gastrointestinal tract and includes mainly the ulcerative Colitis (UC) and the Crohn Disease (CD) (Souza and Fiocchi, 2015). Its incidence has been increasing in several regions of the world, in this fashion, it can become a global disease (Molodecky et al., 2012). It takes place with variations in the incidence and predominance in accordance with the region, more commonly in industrialized countries, the urbanization being considered a risk factor (Burisch et al., 2013; Soon et al., 2012). In the United States of America (USA) the IBD is one of the five most common chronic intestinal conditions, with a total cost above 1.7 billion per year (Abegunde et al., 2016).

IBD has a great impact on the individuals' life quality and capacity of work (Neurath, 2014) due to the symptoms such as diarrhea, abdominal pain, vomiting, loss of weight, anemia, fatigue, fever, fistulas, and extra-intestinal manifestations. More specifically to UC, it occurs equally in both sexes, it affects only the colon, in a diffuse manner, recurrent and not transmural (Baumgart and Sandborn, 2007). IBD occurs due to an unregulated immune response triggered a response against intestinal microbiota in a genetically sensitive individual (de Lange and Barrett, 2015; Molodecky and Kaplan, 2010). Neutrophils and macrophages recruitment towards intestinal tissue is an important event. In the inflammatory foci, these cells produce reactive oxygen species (ROS) and NF-κB-dependent pro-inflammatory cytokines, contributing to tissue damage (Neurath, 2014). In this context, cytokines have a fundamental role in the development and perpetuation of the disease. Pro-inflammatory cytokines such as IL-1 β, TNF-α, and IL-33 are found in higher levels in patients with IBD (Atreya et al., 2011; Beltrán et al., 2010; McAlindon et al., 1998). In addition to those, patients with mutations in IL-10 production or its receptor have more severe disease with an early beginning (Lee et al., 2014). Chronic intestinal inflammation is associated with the increased production of ROS, which have an important role in the IBD physiopathology in both humans and animals (Karp and Koch, 2006; Pavlick et al., 2002).

Vinpocetine, a synthetic ethyl ester derived from vincamine, an alkaloid isolated from the leaves of the *Vinca minor* (Lorincz C, Szasz K, 1976) has been used for the treatment of cerebrovascular diseases, demonstrating a neuroprotective effect and an increase in the cerebral blood flow (Bereczki and Fekete, 1999). Vinpocetine has *in vitro* and *in vivo* antioxidant properties (Horvath et al., 2002; Ruiz-Miyazawa et al., 2015a, 2015b) in addition to analgesic effect in different experimental models (Abdel-Salam, 2006; Ruiz-Miyazawa et al., 2015a, 2015b). Jeon and colleagues demonstrated the anti-inflammatory property of vinpocetine through the inhibition of the IKK, preventing the degradation of the IκB, the

translocation of the NFκB to the nucleus, and consequently production of pro-inflammatory mediators, such as IL-1β and TNF-α *in vitro in* several cell types and *in vivo* mouse model of lung inflammation (Jeon et al., 2010). Vinpocetine reduces LPS- and carrageenan-induced hyperalgesia by reducing neutrophils recruitment, oxidative stress, NF-κB activation, and consequently pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-33 (Ruiz-Miyazawa et al., 2015a, 2015b).

Therefore, vinpocetine is a promising therapeutic approach once there is the participation of pro-inflammatory mediators, such as cytokines and ROS in the pathogenesis of the IBD. Considering that there are no studies demonstrating the role of vinpocetine in the model of colitis, to our knowledge, this is the first study evaluating the therapeutic effect of vinpocetine in an acid acetic-induced colitis in mice.

2. Materials and Methods

2.1 Materials

Vinpocetine was purchased from Tianjin Harmony Technology Development Co. (China), bromide of hexadecil trimethyl-ammonium (HTAB); dihydrochloride O-dianisidine; tween 80; reduced glutathione (GSH); glutaraldehyde; EDTA; iron chloride hexahydrate; 2,4,6-tripiridil-s-triazine (TPTZ); 2,2-azinobis (3-ethylbenzothiazoline-6-sulphonate, diammonium salt; ABTS); Trolox (acid 6-hydroxy-2,5,7,8-tetramethyl croman-2-carboxyl); and potassium persulfate were purchased from Sigma Chemical Co. (St. Louis, USA). Kits for the dosage of IL-1β, IL-33, and IL-10 were purchased from eBioscience (San Diego, USA). For determination of NF-κB activation, primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the secondary antibody purchased from Jackson© ImmunoResearch Inc. (IgG Affinity-Purified Antibodies, West Baltimore Pike, West Grove, PA, USA). All the used reagents were of analytical degree.

2.2 Experimental animals

The experiments were conducted in Swiss male mice, weighing 20 ± 1g, from the Central vivarium of the Londrina State University, PR, Brazil. At least 2 days before and during the experiments, the animals were maintained in the Department of Pathology of the Londrina State University, in plastic boxes, covered with shavings, in clear/dark cycle of 12 hours and temperature between 21 ± 2°C. The water and ration were administered *ad libitum*, with the exception of 24 hours that preceded the experiments, a period in which the animals were maintained in solid fast. The proceedings of care and handling of the mice

were carried out in accordance with the directions of the International Association for the Study of the Pain (IASP) and approved by the Londrina State University Ethics Committee on Animal Research and Welfare (process number: 3307.2015.37). All the efforts were made to minimize the number of animals and their suffering.

2.3 Induction of experimental colitis

Mice were anaesthetised with ketamine (80 mg/kg, im) and xylazine (10 mg/kg, im) after 24 hours of solid fast. Mice received 100 µL (intracolonic) of sterile saline solution for washing the colon of animals. One hour later, mice received 200 µL (intracolonic) of acetic acid 7.5 % (v/v, in saline) or saline (intracolonic). Mice were maintained upside down for 3 minutes in order to avoid liquid extravasation. Intracolonic injection was performed with a polyethylene cannula of 3 cm length (Guazelli et al., 2013).

2.4 Experimental protocols and treatment

Two experimental protocols were used: (A) mice were treated 2 hours before colitis induction; and 4, 10, and 16 hours after colitis induction. In this protocol, mice were euthanized 18 hours after colitis induction. After the euthanasia, the samples of the distal colon (1 cm) for the determination of MPO activity, dosage of cytokines (IL-1 β, IL-10, IL-33, and TNF-α) (Guazelli et al., 2013) and NF-κB activation. Macroscopic score was evaluated using the whole colon (Guazelli et al., 2013). The visceral mechanical hyperalgesia was evaluated 3 and 17 hours after colitis induction (figure 1A). The second protocol (B), mice were treated 10 and 4 hours before colitis induction; and 2 hours after the induction. In this protocol, mice were euthanized 4 hours after colitis induction. After the euthanasia, the samples of the distal colon (1 cm) were collected for GSH and ABTS assays (Guazelli et al., 2013), and edema. The visceral mechanical hyperalgesia was evaluated 3 hours after colitis induction (figure 1B). For both protocols, the following experimental groups were used: (1) control groups, animals without colitis (animals that received only saline, intracolonic); (2) colitis group, (animals received intracolonic solution of acetic acid to 7.5%, without treatment); (3) vinpocetine group, (animals received intracolonic solution of 7.5% acetic acid and treated with vinpocetine [1, 3, 10, or 30 mg/kg solubilized in 20% of Tween 80 in saline] orally). The vinpocetine dose was determined after analysis of edema, MPO activity, and visceral mechanical hyperalgesia. The treatment protocols were performed considering the vinpocetine half-life (approximately 2 hours) (Polgár et al., 1985). The standardization of the model and sample collection times (4 and 18 hours after colitis induction) were previously determined by Guazelli et al. (2013).

2.5 Edema

Fragment measuring 1 cm of the distal portion of the colon, was collected and weighed for evaluation of the edema in the tissue. After determination of the weight, in grams, of 1 cm of colon tissue, the results were expressed in % of increase (of the weight [g] / length of the colon tissue [cm]), regarding the control group without colitis (Barbosa, 2011; Guazelli et al., 2013; Lee et al., 2009).

2.6 Myeloperoxidase activity assay

MPO activity in the colon of the mice was measured by the colorimetric method, described by Guazelli et al. (2013). Samples of 1 cm of the distal portion of the colon were collected in ice-cold 50 mM K_2HPO_4 buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HTAB) and kept at -80°C until use. Samples were homogenized, centrifuged (16,100 g × 2 minutes × 4°C), and the resulting supernatant was assayed for MPO activity spectrophotometrically at 450 nm (Multiskan GO Microplate Spectrophotometer, Thermo Scientific, Vantaa, Finland), with three readings in 1 min. The MPO activity of samples was compared to a standard curve of neutrophils. Briefly, 10 µL of sample was mixed with 200 µL of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/mL o-dianisidine dihydrochloride and 0.015% hydrogen peroxide. The results are presented as MPO activity.

2.7 Visceral mechanical hyperalgesia

The visceral mechanical hyperalgesia was valued by the electronic test of von Frey, in accordance with Pereira et al. (2013). The mice were allocated in acrylic cages (12 x 10 x 17 cm) with road surfaces of wire grills in a room with controlled temperature and silent 30 minutes before the beginning of the measurements. The test consisted in provoking a withdrawal response from the animal with a transducer of portable force (electronic analgesimeter; Insight, Ribeirao Preto, SP, Brazil) adapted to a tip of polypropylene of 0.5 mm^2, which was applied in the lower abdomen up to the middle abdomen area. The experimenter was trained and care was taken not to stimulate the same point consecutively and the stimulation of the external genitalia was avoided. After withdrawal, the intensity of the pressure was automatically registered, with values of the average of three measurements. One of the following behaviors was considered as a withdrawal response: sharp retraction of the abdomen; immediate licking or scratching of the tip application spot; jump; flinches (Laird et al., 2001; Pereira et al. 2013). The results were expressed as the delta (Δ) of the withdrawal threshold (in grams), calculated subtracting the mean of the values at 3 and 17

hours after the colitis induction from the basal values (before vinpocetine treatment and colitis induction).

2.8 Macroscopic damage score

After the euthanasia, colon was exposed, opened longitudinally and the damage scores were determined in accordance with the macroscopic findings using the following criteria outlined (Guazelli et al., 2013): no damage (score 0); localized hyperemia but no ulcers (score 1); linear ulcers with no significant inflammation (score 2); linear ulcers with inflammation at one site (score 3); two or more sites of ulceration and inflammation (score 4); one site of inflammation >1 cm along the length of the colon (score 5); site of inflammation >2 cm along the length of the colon, with quantification increased by 1 for each additional centimeter (score 6−10).

2.9 GSH assay

The levels of GSH in the samples of the mice's distal portion of the colon were determined by the spectrophotometric method previously described (Guazelli et al. 2013). Samples of 1 cm of the distal portion were collected and homogenized in a solution of EDTA 0.02 M using an ultraturrax (ULTRA-TURRAX® – Ika). The homogenates were treated with acid trichloroacetic at 30% and centrifuged (1.5 *g* × 15 minutes × 4°C). Next, 200 µL of Tris-HCl 0.4 M (pH 8.9) buffer was added to the brackets of 150 µL of the supernatant of each sample. After the homogenization, 10 µL of DTNB (dytionitro benzoic acid) 0.01 M in methanol was added. After 5 minutes of reaction, the reading was carried out at 412 nm (Multiskan GO Thermo Scientific). The standard curve was prepared with 0.5 µM of GSH, and the results expressed in nM of GSH per mg of tissue.

2.10 ABTS assay

Samples of 1 cm of the distal colon were homogenized immediately in ice-cold KCl buffer (500 µL, 1.15% weight/volume). The homogenates were centrifuged (0,2 *g* × 10 minutes × 4°C), and the supernatants were used in the assay. Diluted ABTS solution (200 µL) was mixed with 10 µL of sample in each well. After 6 min of incubation (25 °C), the absorbance was measured at 730 nm (Multiskan GO, Thermo Scientific). The ABTS radical scavenging ability in the samples was compared with a standard curve of Trolox (1.5 – 30 µmol/L) and the results are expressed in mmol of Trolox equivalent per mg of tissue (Guazelli et al. 2013).

2.11 Cytokines measurements

Samples of 1 cm of the distal colon were homogenized in 500 µL of the appropriate buffer containing protease inhibitors. Cytokine levels were determined as described previously by an enzyme-linked immunosorbent assay (ELISA) using eBioscience kits (eBioscience, San Diego, CA, USA) accordingly with manufacturer instructions. The results were expressed as picograms (pg) of cytokine per mg of tissue.

2.12 NF-κB activation

Samples of 1 cm of the distal colon were homogenized in RIPA buffer containing protease and phosphatase inhibitors. The lysates were centrifuged (0.5 *g* × 10 min × 4°C) and the supernatants used in western blot analysis. The protein extracts were separated by SDS-PAGE and transferred to a nitrocellulose membrane (GE Healthcare-Amersham, Pittsburgh, PA, USA). Membranes were then incubated in blocking buffer [5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) with Tween 20 or 5% nonfat milk] for 2 h at room temperature and incubated overnight at 4°C in the presence of primary antibody diluted in 5% BSA in TBS with Tween 20 or 5% nonfat milk. The antibodies and Western blot conditions were p-NFκB p65 (sc-166748, 1:100) and NFκB p65 (sc-372, 1:200) on 10% gel. The molecular protein mass was confirmed by PageRuler^{TM} Prestained Protein Ladder (Thermo Scientific, Rockford, IL, USA). After washing in PBS with Tween 20, the membrane was incubated with secondary antibody for 2 h at room temperature. Protein was visualized by chemiluminescence with Luminata^{TM} Forte Western HRP Substrate (Merck Millipore Corporation, Darmstadt, Alemanha). The membranes were reprobed with antibody against the total protein of interest to be used as loading control in addition to loading the same amount of protein. Densitometric data were measured using Scientific Imaging Systems (Image Lab 3.0 software; Bio-Rad Laboratories, Hercules, CA).

2.13 Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM) of measures made in 6 mice per group and are representative of two independent experiments, except for those in the macroscopic score (which were used 17 animals per group and are representative of two independent experiments). Single time point analysis was evaluated using one-way analysis of variance (ANOVA), followed by Tukey's test. Multiple time points analysis (hyperalgesia) was evaluated using the two-way analysis of variance (ANOVA) repeated measures, followed by Tukey's test. For categorical variables (analysis of the macroscopic damage score of the colon), was evaluated using the nonparametric test of Kruskal-Wallis was used, followed by Dunn's test. The statistical analyses were performed in

the GraphPad Prism 5 software (GraphPad Software Inc., San Diego, USA). The differences were considered significant for corresponding values at $p < 0,05$.

3. Results

3.1 Vinpocetine reduces acetic acid-induced edema and MPO activity

In the first series of experiments, it was evaluated edema and MPO activity, 4h and 18h after the colitis induction, respectively. Mice were treated with vinpocetine (1, 3, 10, or 30 mg/kg, p.o.), following protocol A (edema evaluation) or B (MPO activity), respectively. Sample of the colon (1 cm) was collected 4h (edema evaluation) or 18h (MPO activity) after colitis induction. All the doses of vinpocetine reduced edema significantly (Figure 2A). On the other hand, only the dose of 30 mg/kg of vinpocetine reduced significantly MPO activity (Figure 2B).

3.2 Vinpocetine reduces acetic acid-induced visceral mechanical hyperalgesia

Mice were treated with vinpocetine (1, 3, 10, or 30 mg/kg, p.o.) as per protocol A or with vinpocetine (30 mg/kg, p.o.) as per protocol B. Mechanical hyperalgesia was evaluated 3h after colitis induction, following protocol A; or 3 and 17h after colitis induction following protocol B. Intracolonic injection of acetic acid induced visceral mechanical hyperalgesia (as observed with an increase of the delta threshold), which was reduced with vinpocetine (30 mg/kg) treatment in both protocols (Figure 3A and 3B). Given that, we choose the dose of 30 mg/kg of vinpocetine for the next experiments.

3.3 Vinpocetine reduces acetic acid-induced macroscopic damage

Mice were treated with vinpocetine (30 mg/kg, p.o.) following protocol B. The whole colon was collected 18h after colitis induction. Intracolonic injection of acetic acid induced severe tissue damage as observed at the site of inflammation higher than 2cm along the length of the colon (Figure 4B and 4D). On the other hand, mice treated with vinpocetine (30 mg/kg) presented focal sites of milder inflammatory response (Figure 4C and 4D).

3.4 Vinpocetine inhibits acetic acid-induced oxidative stress

Next, the antioxidant effect of vinpocetine was evaluated. For this, mice were treated with vinpocetine (30 mg/kg, p.o.), as per protocol B. Sample of the colon (1 cm) was collected 4h after colitis induction. Acetic acid reduced the levels of GSH and the treatment with vinpocetine (30 mg/kg, p.o.) normalized it (Figure 5A). Also, acetic acid depleted total

antioxidant capacity (ABTS radical scavenging ability assay) and the treatment with vinpocetine (30 mg/kg) restored total antioxidant capacity (Figure 5B).

3.5 Vinpocetine reduces acid acetic-induced production of pro-inflammatory cytokines (IL-1 β, TNF-α, and IL-33) and normalizes the anti-inflammatory cytokine IL-10

Mice were treated with vinpocetine (30 mg/kg, p.o.), as per protocol B. Sample of the colon (1 cm) was collected 18h after colitis induction. Acetic acid induced the production of pro-inflammatory cytokines (IL-1β, TNF-α, and IL-33) and the treatment with vinpocetine (30 mg/kg) reduced significantly the production of IL-1β, TNF-α, and IL-33 induced by acetic acid (Figure 6A-C). In addition, intracolonic injection of acetic acid reduced the levels of IL-10. Treatment with vinpocetine (30 mg/kg) normalized IL-10 levels (Figure 6D).

3.6 Vinpocetine inhibits acetic acid-induced NF-κB activation.

Mice were treated with vinpocetine (30 mg/kg, p.o.), as per protocol B. Sample of the colon (1 cm) was collected 18h after colitis induction. The concentration of phosphorylated and total NF-κB p65 subunit was determined by western blot by measuring the ratio of phosphorylated and total NF-κB. Therefore, the elevation in the ratio points the increase in NF-κB activation. Acetic acid induced the NF-κB activation and the treatment with vinpocetine (30 mg/kg) inhibits NF-κB activation (Figure 7A and 7B).

4. Discussion

In the present work, we observed that vinpocetine presents anti-inflammatory, antioxidant, and analgesic effect in a model of acetic acid-induced colitis in mice. This effect was demonstrated through the reduction of edema; MPO activity; macroscopic damage; visceral mechanical hyperalgesia; oxidative stress (normalization of GSH levels and ABTS radical scavenging ability); NF-κB activation; production of pro-inflammatory cytokines such as IL-1 β, TNF-α, and IL-33, and normalization of the anti-inflammatory cytokine IL-10 levels. To our knowledge, this is the first demonstration of vinpocetine effect on experimental colitis.

IBD has a great impact on the patients' quality of life, and the most common symptoms are diarrhea (blood and/or mucus), abdominal pain, vomiting, loss of weight, anemia, fatigue, fever, fistulas, and extra-intestinal manifestations (Neurath, 2014). It is an intermittent disease switching from light to severe during the acute phase, and lessening during the remission phase (Bernstein et al., 2010; de Lange and Barrett, 2015; Neurath, 2014). The current treatment of the UC consists mainly in the use of mesalazine, corticosteroids, immunosuppressant, and anti-TNF-α monoclonal antibodies (Kane, 2006).

However, these therapies present several side effects and monoclonal antibodies efficacy are often limited by a loss of efficacy due to the development of adaptive response or present several side effects, underscoring the need for novel therapies (Goldberg and Irving, 2015; Hansen et al., 2007; Higgins et al., 2015; Lukert and Raisz, 1990). In this sense, some clinical studies demonstrated that vinpocetine is a safe drug in the long term, without significant side effects (Balestreri et al., 1987; Thal et al., 1989; Zhang et al., 2016). Vinpocetine is absorbed in the stomach and intestine, being able to cross the blood-brain barrier. Its metabolizing process, in humans and dogs, occurs exclusively in the liver, but plasma metabolism occurs only in rats (Fandy et al., 2016; Szakácz et al., 2001). In spite of being known as a fosfodieterase-1 (PDE-1) inhibitor, recent data suggest that vinpocetine also has anti-inflammatory action independent on targeting PDE-1. In fact, vinpocetine reduces LPS- and carrageenan-induced NF-κB activation *in vivo* (Ruiz-Miyazawa et al., 2015a, 2015b). This mechanism accounts for the inhibition of IKK (Jeon et al., 2010) or the inhibition of the NF-κB upstream enzyme Akt (Zhuang et al., 2013), which prevents the degradation of IκB, translocation of NF-κB to the nucleus, and consequently inhibition of NF-κB-dependent cytokines, such as IL-1β and TNF-α (Jeon et al., 2010).

Neutrophils infiltration in the inflamed mucosal membrane is an important histological finding in patients with IBD, specifically in UC. In the inflammatory foci, neutrophils contribute to tissue damage and dysfunction of the mucosal membrane (Higa et al., 1997). In fact, neutrophil is an important source of ROS and pro-inflammatory cytokines, which can further contribute to tissue damage. The disease activity correlates with the neutrophils influx in the mucosal membrane, resulting in the formation of abscesses crypts (Blake et al., 2004; Carrigan et al., 2005). MPO is an abundant enzyme mainly in azurophils granules of neutrophils (Naito et al., 2007). It is involved in the generation of hypochlorous acid, a powerful oxidant that possesses bactericidal activity, but also produces tissue damage (Klebanoff, 2005). MPO can be used as a quantitative indicator of the inflammation due to its activity being directly proportional to a number of neutrophils (Krawisz et al., 1984). Our results show that vinpocetine reduces edema, macroscopic score, and MPO activity in a model of colitis induced by acetic acid. In lung tissue, vinpocetine was able to reduce TNF-α- and lipopolysaccharide (LPS)-induced neutrophils recruitment (Jeon et al., 2010). Recent data demonstrated what vinpocetine reduces MPO activity and leukocytes recruitment (Ruiz-Miyazawa et al., 2015a, 2015b). Therefore, the reduction of neutrophils recruitment is an important finding.

Reactive species of oxygen (ROS) play an important role in the pathophysiology of IBD (Karp and Koch, 2006; Pavlick et al., 2002; Pravda, 2005). In fact, patients with IBD have lower antioxidant capacity (even in the asymptomatic phase of the disease) (Achitei et

al., 2013) and present higher levels oxidative DNA damage (Pereira et al., 2016) than healthy individuals. Inflammatory cells activation such as neutrophils and macrophages causes the elevated production of superoxide anion, nitric oxide, and peroxynitrite, resulting in the establishment of oxidative stress in the IBD (Pacher et al., 2007). Importantly, ROS also modulates the NF-κB activation and consequently, increase the expression of cytokines pro-inflammatory, adhesion molecules, and the perpetuation of the inflammation (Chiurchiu and Maccarrone, 2011; Christman and Blackwell, 2000; Maloy and Powrie, 2011). In fact, treatment with antioxidants such as the flavonoid quercetin (Guazelli et al., 2013) or the GSH precursor N-acetylcysteine (NAC) (Amrouche-Mekkioui and Djerdjouri, 2012) reduces colitis-induced oxidative stress. *In vitro* data, demonstrated that vinpocetine is a powerful antioxidant molecule (Horvath et al., 2002). Vinpocetine reduces *in vivo* lipid peroxidation, nitrite production, and restores GSH and total antioxidant defense in different models, such as carrageenan- and LPS-induced inflammation (Ruiz-Miyazawa et al., 2015a, 2015b). This mechanism is related to the ability of scavenges hydroxyl radicals and other ROS (Santos et al., 2000; Zaki and Abdelsalam, 2013). Corroborating, our results show that vinpocetine inhibits the oxidative stress by normalizing total antioxidant capacity and GSH levels and prevented the reduction of ABTS radical scavenging ability in acetic acid-induced colitis.

Abdominal pain is a common symptom reaching up to 70% of the patients who seek medical assistance, either in cases of initial diagnosis or in episodes of relapses (Aghazadeh et al., 2005; Wagtmans et al., 1998). As matter of fact, depression and anxiety are common symptoms of patients with IBD due to the chronic abdominal pain (Regueiro et al., 2017). Inflammatory mediators such IL-1β, TNF-α, IL-33, and the nerve growth factor (NGF) released by neutrophils, macrophages, and mast cells can activate nociceptors and induce pain (Binshtok et al., 2008; Jin, 2006; Wright, 1999; Zarpelon et al., 2013). In fact, mice lacking ST2 (IL-33 receptor) or TNFR1 (TNF-α receptor) present decreased abdominal pain (Magro et al., 2013; Yamacita-Borin et al., 2015). In addition to that, ROS such as superoxide and peroxynitrite are also able to induce hyperalgesia (Ma et al., 2009; Maioli et al., 2015; Salvemini et al., 2011; Wang et al., 2004). In fact, intraperitoneal injection of a superoxide anion donor causes abdominal contortions in mice (Maioli et al., 2015). Vinpocetine has been shown to reduce pain in different experimental models (Abdel-Salam, 2006; Ruiz-Miyazawa et al., 2015a, 2015b). Vinpocetine reduces LPS- and carrageenan-induced hyperalgesia by reducing neutrophils recruitment, oxidative stress, NF-κB activation, and consequently pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-33 (Ruiz-Miyazawa et al., 2015a, 2015b). Whole-Cell voltage-clamp recording demonstrated that vinpocetine blocks Nav1.8 sodium current inward (Zhou et al., 2003), and is another possible mechanism by which vinpocetine exerts its analgesic effect. This is important given that Nav1.8 are channels

present in DRG sensory neuron and its activity is related to pain (Zhou et al., 2003). Vinpocetine also blocks the axoplasmic transport of NGF in the peripheral sensory neurons and reduces the release of the pain-related neuropeptides substance P and CGRP in the spinal cord (Knyihar-Csillik et al., 2007). Herein, we observed that vinpocetine reduced colonic neutrophils recruitment and production of hyperalgesic cytokines, such as IL-1β, TNF-α, and IL-33, which may account for its analgesic effect. Therefore, the inhibition of acetic acid-induced visceral mechanical hyperalgesia is an important finding, given that abdominal pain is a symptom inherent of patients with IBD (Akbar et al., 2008). Of note, vinpocetine also presented antidepressant-like activity in the forced swimming test (Abdel-Salam, 2006). Given the prevalence of depression on IBD, vinpocetine could not only modulates pain but also modulates mood.

Cytokines have a central role in the development and perpetuation of IBD (Neurath, 2014). In fact, higher levels of IL-1β was found in the colon of patients with active IBD (McAlindon et al., 1998). This finding can be correlated with disease activity and colon damage (Ludwiczek et al., 2004). Another important cytokine, TNF-α attached to the membrane or soluble, is increased in patients with IBD (Atreya et al., 2011). IL-33 is another important cytokine, given that its level is correlated with disease activity and is found in higher levels in patients with UC when compared with patients with CD or healthy individuals (Beltrán et al., 2010). On the other side, the anti-inflammatory cytokine IL-10 has important role homeostatic regulation of the intestinal inflammatory response. In fact, mice lacking IL-10 develops spontaneous gut inflammation, which is reverted by treatment with recombinant IL-10 (Steidler et al., 2000). Patients with mutations in IL-10 production or its receptor have more severe disease with an early beginning (Lee et al., 2014). Our results show that vinpocetine inhibits the NF-κB activation, which explains the reduced levels of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-33, and normalized levels of anti-inflammatory cytokine IL-10. Corroborating, other studies demonstrated that vinpocetine reduces the production of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-33 by inhibiting NF-κB activation (Jeon et al., 2010; Ruiz-Miyazawa et al., 2015a, 2015b) demonstrating that this signaling pathway is one of the targets of vinpocetine. Importantly, *in vitro* evidence demonstrate that NF-κB p65 antisense oligonucleotides reduces the expression of NF-κB p65 subunit and the NF-κB-dependent cytokines IL-1β and IL-8 in the lamina propria mononuclear cells from patients with ulcerative colitis (Li et al., 2008), demonstrating the clinical relevance of inhibiting this signaling pathway.

5. Conclusions

The present study demonstrated for the first time that vinpocetine ameliorates acetic acid-induced colitis due to inhibition of neutrophils recruitment, abdominal hyperalgesia, oxidative stress, pro-inflammatory cytokines production, and NF-κB activation. To the best of our knowledge, this is the first report demonstrating the effect of vinpocetine on experimental colitis. Given the therapeutic effects herein observed, the fact that vinpocetine is a safe drug because it presents no side effects and is widely used in the clinic for cerebrovascular diseases, our data suggest that vinpocetine is a promising molecule for the treatment of IBD such as ulcerative colitis.

Conflict of interest

The authors declare no conflict of interest.

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Figures

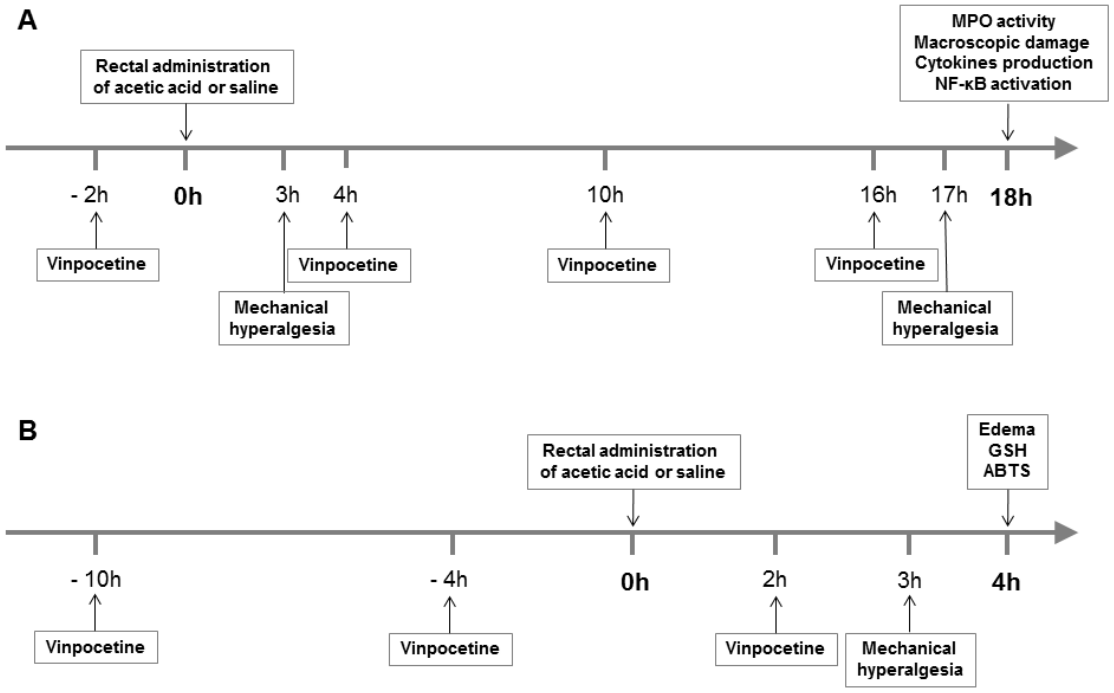

Figure 1. Schematic representation of colitis experimental protocols and treatment with vinpocetine. In the first protocol, mice were treated with vinpocetine 2 hours before; 4, 10, and 16 hours after colitis induction. Visceral mechanical hyperalgesia was assessed 3 and 17 hours after colitis induction. Samples from the distal portion of the colon (1 cm) were collected 18 hours after colitis induction for determination of MPO activity, macroscopic damage, cytokine production, and NF-κB activation (Panel A). In the second protocol, mice were treated with vinpocetine 10 and 4 hours before; and 2 hours after colitis induction. Visceral mechanical hyperalgesia was performed 3 hours after colitis induction. Samples from the distal portion of the colon (1 cm) were collected 4 hours after colitis induction for evaluation of edema, GSH and ABTS (Panel B).

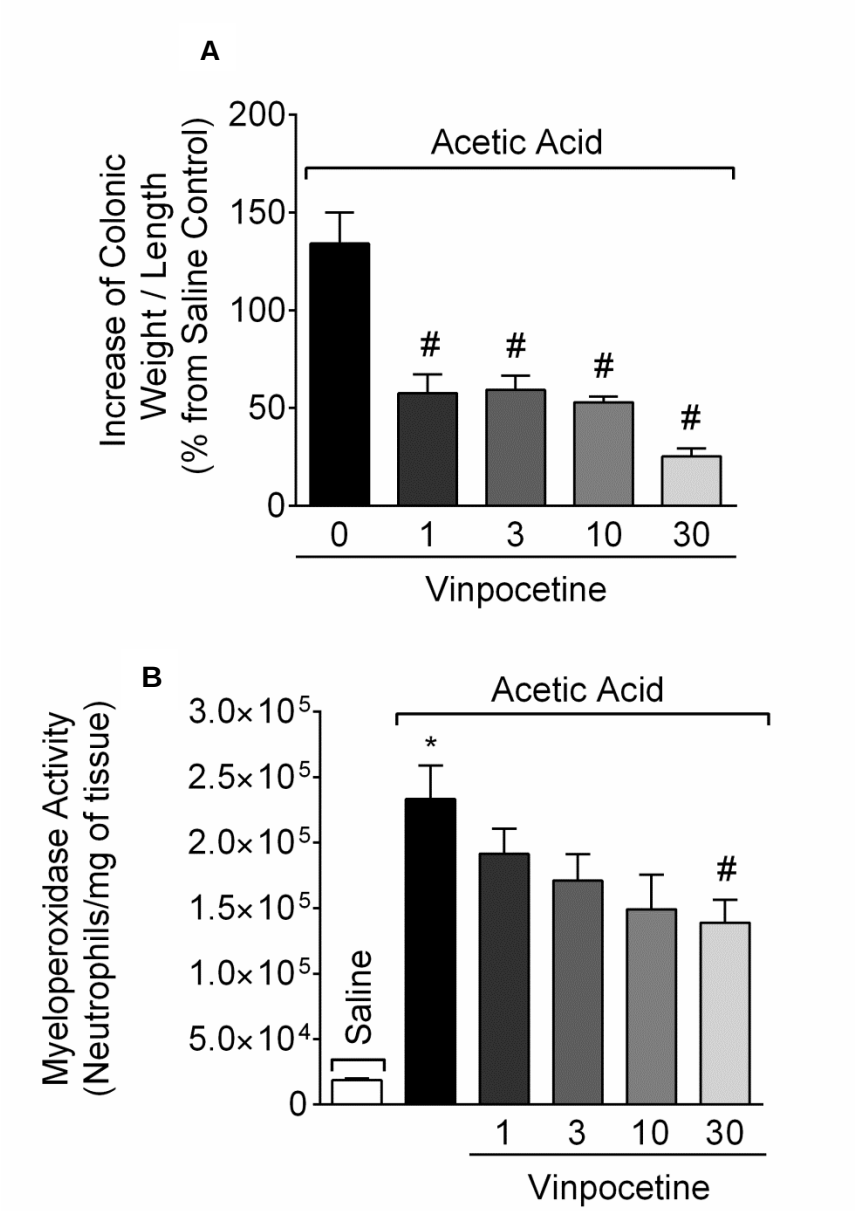

Figure 2. Vinpocetine reduces acetic acid-induced edema and MPO activity. Mice were treated with vinpocetine (1, 3, 10, or 30 mg/kg, p.o.) or vehicle (20% tween 80 in saline, p.o.) 10 and 4 hours before injection of acetic acid (7.5%, 200 µL) or saline (200 µL), respectively; and 2 hours after (Panel A), as per protocol B. Mice were treated with vinpocetine (1, 3, 10, or 30 mg/kg, p.o.) or vehicle 2 hours before acetic acid (7.5%, 200 µL) or saline (200 µL) injection, respectively; and 4, 10, and 16 hours after (Panel B), as per protocol A. Samples from distal colon were collected 4 hours after colitis induction (Panel A) and 18 hours after colitis induction (Panel B). Results are presented as means ± SEM, *n* = 6 mice per group per experiment, two independent experiments [*p < 0.05 vs. saline control; #p < 0.05 vs. 0 mg/kg (vehicle), (ANOVA followed by Tukey's test)].

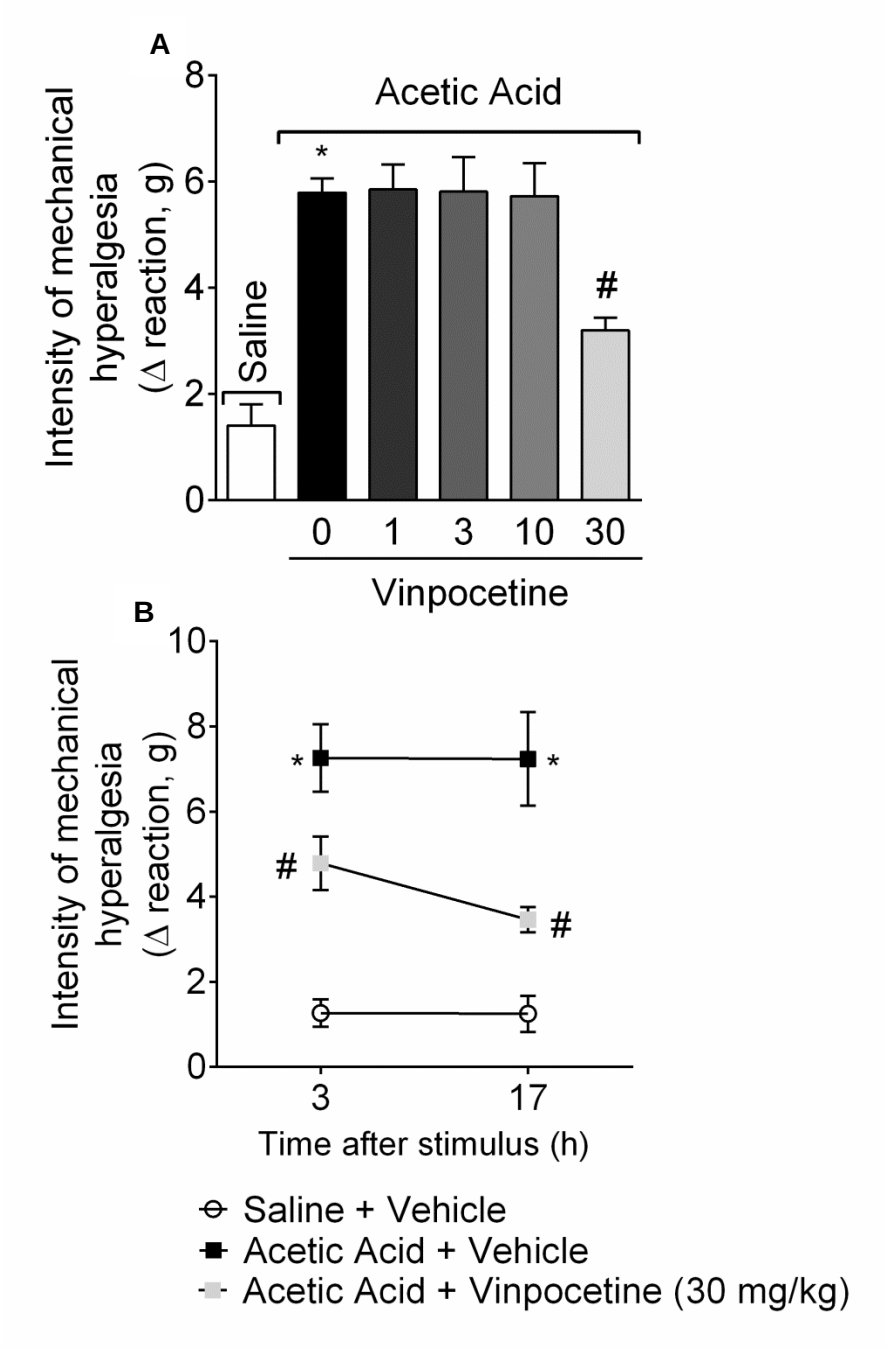

Figure 3. Vinpocetine reduces acetic acid-induced visceral mechanical hyperalgesia. Mice were treated with vinpocetine (1, 3, 10, or 30 mg/kg, p.o.) or vehicle (20% tween 80 in saline, p.o.) 10 and 4 hours before injection of acetic acid (7.5%, 200 µL) or saline (200 µL), respectively; and 2 hours after (Panel A), as per protocol B. Mice were treated with vinpocetine (1, 3, 10, or 30 mg/kg, p.o.) or vehicle 2 hours before injection of acetic acid (7.5%, 200 µL) or saline (200 µL), respectively; and 4, 10, and 16 hours after (Panel B), as per protocol A. Visceral mechanical hyperalgesia was assessed 3 hours after colitis induction (Panel A); or 3 and 17 hours after colitis induction (Panel B). Results are means ± SEM, *n* = 6 mice per group per experiment, two independent experiments {*p < 0.05 vs. saline control; #p < 0.05 vs. 0 mg/kg [vehicle], [one-way ANOVA followed by Tukey's test (Panel A)] [two-way ANOVA followed by Tukey's test (Panel B)]}.

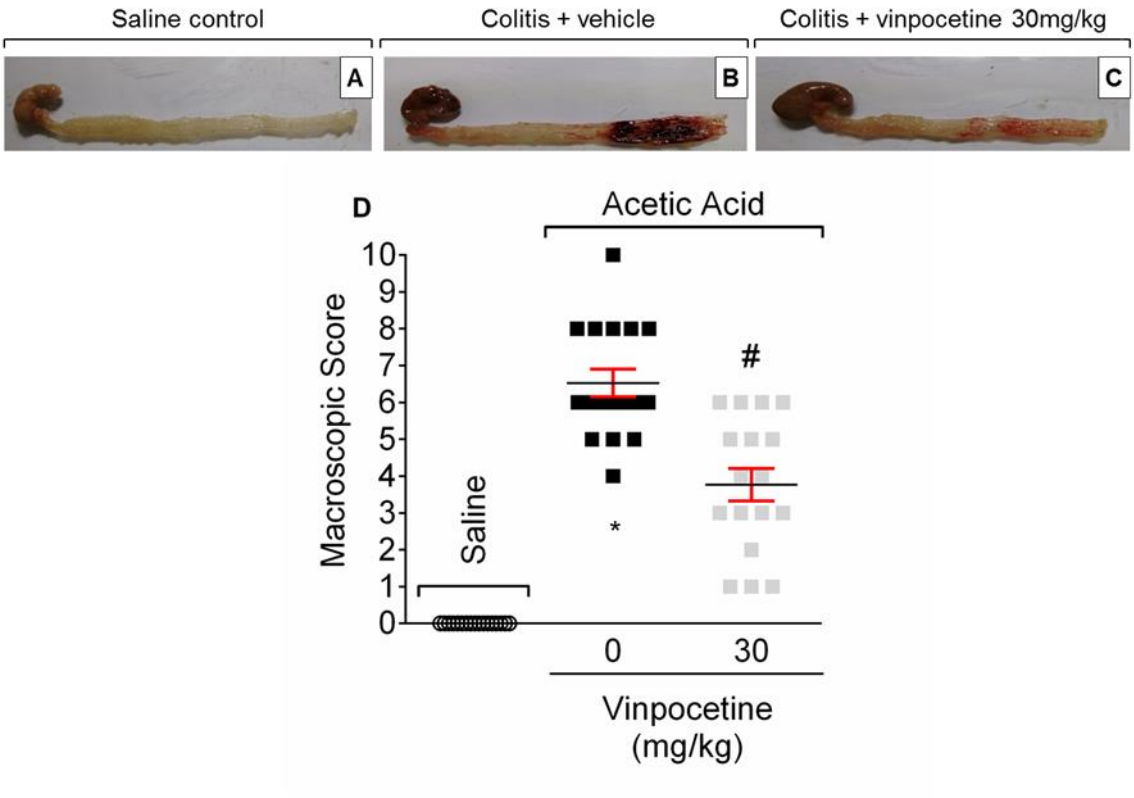

Figure 4. Vinpocetine decreases acetic acid-induced macroscopic damage. Mice were treated with vinpocetine (30 mg/kg, p.o.) or vehicle (20% tween 80 in saline, p.o.) 2 hours before injection of acetic acid (7.5%, 200 µL) or saline (200 µL), respectively; and 4, 10, and 16 hours after (Panel B), as per protocol A. Samples from distal colon were collected 18 hours after colitis induction and the macroscopic damage score was determined. Saline control (Panel A), Colitis + vehicle (Panel B), Colitis + vinpocetine 30 mg/kg (Panel C), and macroscopic score (Panel D). Results are presented as means ± SEM, *n* = 17 mice per group per experiment, two independent experiments [*p < 0.05 vs. saline control; #p < 0.05 vs. 0 mg/kg (vehicle), (Kruskal-Wallis non-parametric test followed by Dunn's test)].

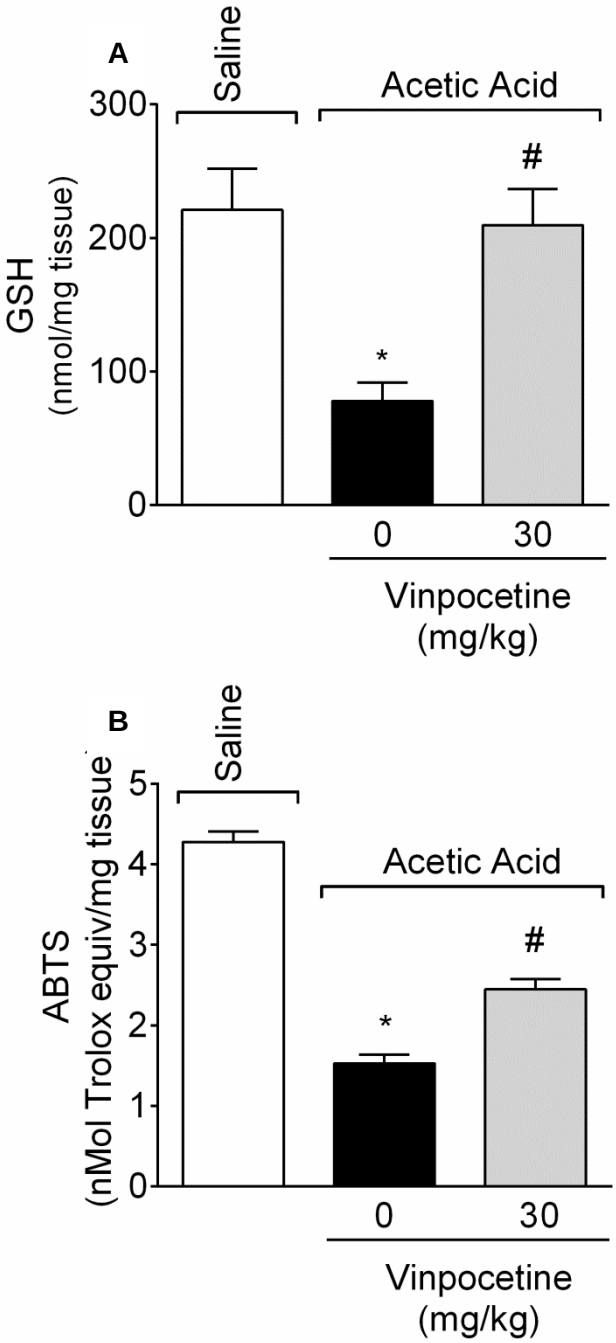

Figure 5. Vinpocetine inhibits acetic acid-induced oxidative stress. Mice were treated with vinpocetine (30 mg/kg, p.o.) or vehicle (20% tween 80 in saline, p.o.) 10 and 4 hours before injection of acetic acid (7.5%, 200 µL) or saline (200 µL), respectively; and 2 hours after (Panel A and B), as per protocol A. Samples from distal colon were collected 4 hours after colitis induction. Results are presented as means ± SEM, *n* = 6 mice per group per experiment, two independent experiments [*p < 0.05 vs. saline control; #p < 0.05 vs. 0 mg/kg (vehicle), (ANOVA followed by Tukey's test)].

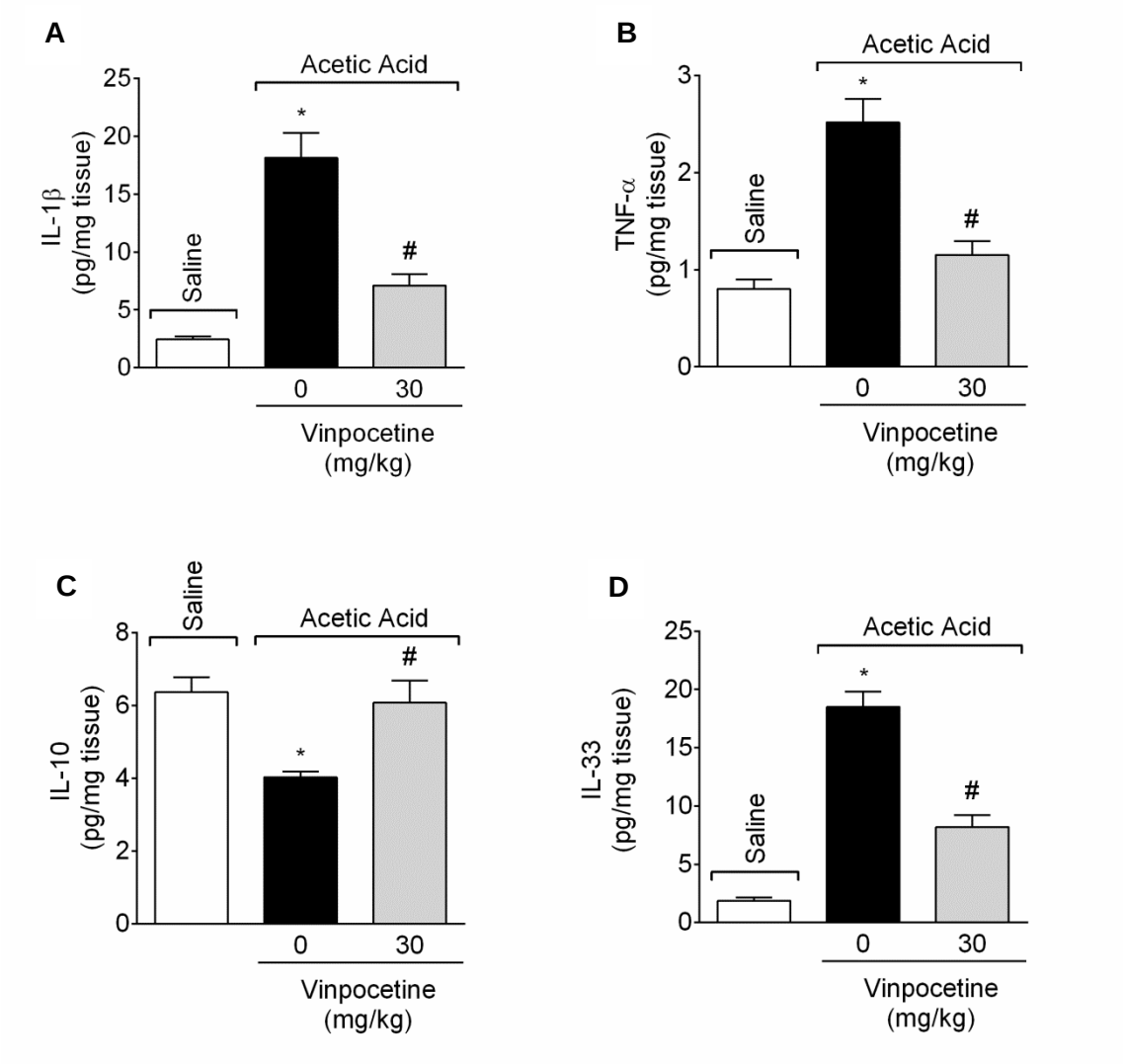

Figure 6. Vinpocetine reduces acetic acid-induced pro-inflammatory cytokines (IL-1β, TNF-α, and IL-33) and prevents the depletion of the anti-inflammatory cytokine IL-10. Mice were treated with vinpocetine (30 mg/kg, p.o.) or vehicle (20% tween 80 in saline, p.o.) 2 hours before injection of acetic acid (7.5%, 200 µL) or saline (200 µL), respectively; and 4, 10, and, 16 hours after, as per protocol A. Samples from distal colon were collected 18 hours after colitis induction for the determination of IL-1β (Panel A), TNF-α (Panel B), IL-10 (Panel C), and IL-33 (Panel D) production. Results are presented as means ± SEM, *n* = 6 mice per group per experiment, two independent experiments [*$p < 0.05$ vs. saline control; #$p < 0.05$ vs. 0 mg/kg (vehicle), (ANOVA followed by Tukey's test)].

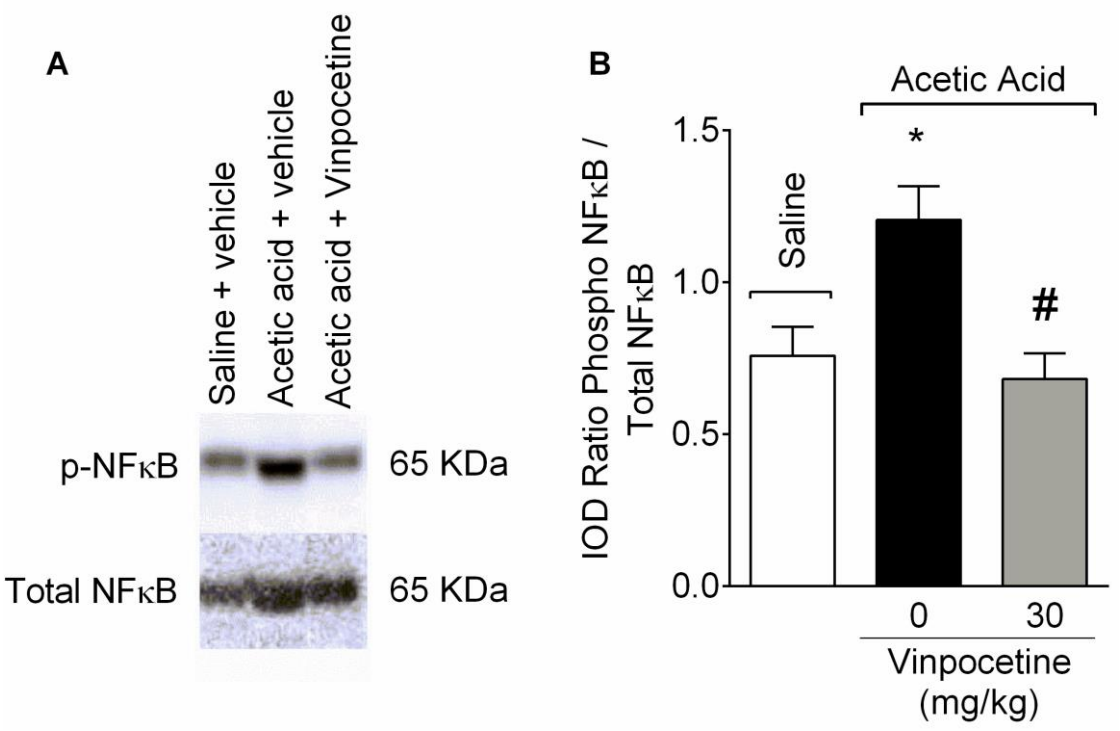

Figure 7. Vinpocetine inhibits acetic acid-induced NF-κB activation. Mice were treated with vinpocetine (30 mg/kg, p.o.) or vehicle (20% tween 80 in saline, p.o.) 2 hours before injection of acetic acid (7.5%, 200 µL) or saline (200 µL), respectively; and 4, 10, and, 16 hours after, as per protocol A. Samples from distal colon were collected 18 hours after colitis induction for the determination of NF-κB activation (Panel A and B). Results are presented as means ± SEM, *n* = 6 mice per group per experiment, two independent experiments [*p < 0.05 vs. saline control; #p < 0.05 vs. 0 mg/kg (vehicle), (ANOVA followed by Tukey's test)].

5 CONCLUSÕES

No presente estudo, verificamos que a vimpocetina possui efeito anti-inflamatório, antioxidante e analgésico em modelo de colite induzida por ácido acético. Esse efeito foi demonstrado através da redução de parâmetros inflamatórios no cólon dos animais, como edema; da atividade da MPO; do dano macroscópico; da hiperalgesia mecânica visceral; da inibição do estresse oxidativo observado pela normalização dos níveis do GSH e da capacidade antioxidante (ensaio ABTS); da redução da produção de citocinas pró-inflamatórias como a IL-1β, o TNF-α e a IL-33, normalização dos níveis da citocina anti-inflamatória IL-10 e inibição da ativação do NF-κB. Considerando que a terapia convencional pode levar ao aparecimento de efeitos adversos graves, e que em contrapartida, a vimpocetina é segura por apresentar poucos efeitos adversos e ser amplamente utilizada na clínica para doenças cerebrovasculares, em adição aos dados do presente estudo, podemos concluir que essa molécula se mostra como uma alternativa viável para tratamento da DII, como a colite ulcerativa.

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ANEXO A

Normas técnicas para a publicação na revista científica *European Journal of Pharmacology*.

ANEXO A

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