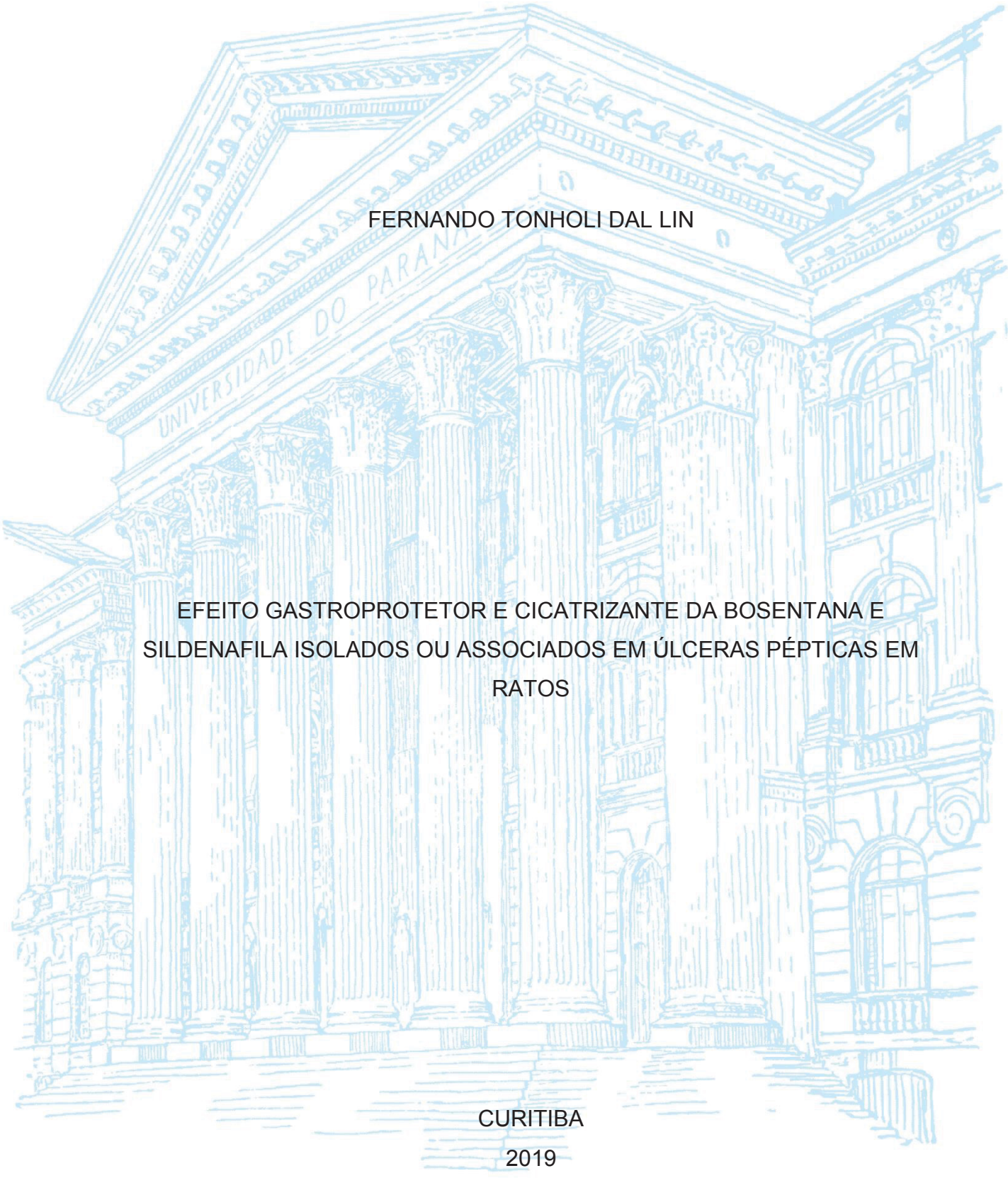
UNIVERSIDADE FEDERAL DO PARANÁ



FERNANDO TONHOLI DAL LIN

EFEITO GASTROPROTETOR E CICATRIZANTE DA BOSENTANA E SILDENAFILA ISOLADOS OU ASSOCIADOS EM ÚLCERAS PÉPTICAS EM RATOS

CURITIBA
2019

FERNANDO TONHOLI DAL LIN

EFEITO GASTROPROTETOR E CICATRIZANTE DA BOSENTANA E SILDENAFILA ISOLADOS OU ASSOCIADOS EM ÚLCERAS PÉPTICAS EM RATOS

Tese apresentada como requisito parcial à obtenção do grau de Doutor em Farmacologia, no Curso de Pós-Graduação em Farmacologia, no Setor Ciências Biológicas, na Universidade Federal Do Paraná.

Orientadora: Profa. Maria Fernanda de Paula Werner.

CURITIBA

2019

Universidade Federal do Paraná. Sistema de Bibliotecas.
Biblioteca de Ciências Biológicas.
(Rosilei Vilas Boas – CRB/9-939).

Dal Lin, Fernando Tonholi.
Efeito gastroprotetor e cicatrizante da bosentana e sildenafila isolados ou associados em úlceras pépticas em ratos. / Fernando Tonholi Dal Lin. – Curitiba, 2019.
106 f. : il.
Orientadora: Maria Fernanda de Paula Werner.
Tese (Doutorado) – Universidade Federal do Paraná, Setor de Ciências Biológicas. Programa de Pós-Graduação em Farmacologia.

1. Úlcera gástrica. 2. Citrato de Sildenafila. 3. Endotelinas. 4. Estômago – Inflamação. 5. Estresse oxidativo. 6. Bosentana. I. Título. II. Werner, Maria Fernanda de Paula. III. Universidade Federal do Paraná. Setor de Ciências Biológicas. Programa de Pós-Graduação em Farmacologia.

CDD (20. ed.) 616.343

MINISTÉRIO DA EDUCAÇÃO
SETOR DE CIENCIAS BIOLOGICAS
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO FARMACOLOGIA - 40001016038P0

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em FARMACOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da Tese de Doutorado de **FERNANDO TONHOLI DAL LIN**, intitulada: **EFEITO GASTROPROTETOR E CICATRIZANTE DA BOSENTANA E SILDENAFILA ISOLADOS OU ASSOCIADOS EM ÚLCERAS PEPTICAS EM RATOS**, sob orientação da Profa. Dra. MARIA FERNANDA DE PAULA WERNER, após terem inquirido o aluno e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.
A outorga do título de Doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 19 de Dezembro de 2019.

MARIA FERNANDA DE PAULA WERNER
Presidente da Banca Examinadora

ALEXANDRA ACCO
Avaliador Interno (UNIVERSIDADE FEDERAL DO PARANÁ)

ELIZABETH SOARES FERNANDES
Avaliador Externo (INSTITUTO PELÉ PEQUENO PRINCIPE)

FLAVIO HEUTA IVAND
Avaliador Externo (PONTIFÍCIA UNIVERSIDADE CATÓLICA DO PARANÁ)

DEDICATÓRIA

Dedico esse trabalho a meu falecido pai Paulo Fernando que até o seu último dia depositava em mim, uma fé incondicional como ninguém até hoje acreditou, acreditou em meus sonhos e planejou comigo como colocá-los em prática. Infelizmente ele não pode estar nesse plano para ver que estou fazendo de tudo para honrar tal fé inabalável. À minha mãe, que assim como meu pai, confiou em minhas escolhas e nunca duvidou de minha capacidade.

AGRADECIMENTOS

Agradeço imensamente à minha orientadora Maria Fernanda, com sua exímia competência e dedicação louvável, insistiu e me suportou como aluno. Agradeço por toda a paciência e empenho, que mesmo diante dos piores cenários, manteve-me como seu aluno até o fim dessa trajetória.

Agradeço a meus colegas de trabalho Bruna, Daniele, Jorge, Luiza, Paula e Beatriz. Sem eles esse trabalho nunca teria sido completado.

Agradeço a Coordenação de Aperfeiçoamento de Pessoal de Nível Superior pela concessão da bolsa de estudo. Assim como agradeço ao Conselho Nacional de Desenvolvimento Científico e Tecnológico pelo financiamento do projeto de pesquisa.

“O destino final nada importa,
a trajetória do viver é eterna.
Sente-se à janela ou fique em pé,
de qualquer modo, sempre,
sempre aproveite a viagem ao máximo.”

Fernando Tonholi Dal Lin, (2019).

NOTA EXPLICATIVA

Esta tese é apresentada em formato alternativo – artigos para publicação – de acordo com as normas do Programa de Pós-Graduação em Farmacologia da Universidade Federal do Paraná, constando de uma revisão de literatura, objetivos, dois artigos científicos e considerações finais. Os artigos foram formatados de acordo com as normas propostas por periódicos internacionais.

RESUMO

A úlcera gástrica é um problema de saúde pública mundial, caracterizada por extensas lesões na mucosa do estômago e/ou esôfago que podem se estender por todas as camadas do órgão, causando sérias complicações como sangramentos e perfurações que requerem hospitalização do paciente e intervenção cirúrgica. Como principais causas da úlcera gástrica temos o uso de anti-inflamatórios não esteroidais (AINEs), que diminuem a produção de muco-bicarbonato gastroprotetor, e a infecção pela bactéria *Helicobacter pylori*. Além da erradicação da *H. pylori*, o tratamento convencional visa a inibição da produção de ácido clorídrico pelo uso dos inibidores da bomba de prótons (e.g. omeprazol), ou de antagonistas de receptores H_2 da histamina (e.g. ranitidina). Contudo, estudos demonstram que a inibição da secreção ácida gástrica está associada ao aumento do risco de desenvolvimento de diversas doenças como osteoporose, infecções intestinais e até doença renal crônica. Além da secreção ácida gástrica, a ulcerogênese é um processo complexo que também envolve o fluxo sanguíneo. Os sistemas endotelinérgico e da via do óxido nítrico modulam a microcirculação bem como a inflamação e o estresse oxidativo na mucosa gástrica. Assim, o objetivo deste trabalho foi avaliar o efeito gastroprotetor e cicatrizante de drogas vasoativas, mais especificamente da bosentana e da sildenafila, em modelos de úlcera gástrica aguda e crônica. A administração oral de bosentana (antagonista dual de receptores ET_A e ET_B) e da sildenafila (inibidor da fosfodiesterase 5), isoladas ou em associação foram investigadas no modelo de úlcera aguda induzida por isquemia-reperfusão. O efeito cicatrizante da monoterapia com a bosentana foi investigada no modelo de úlcera crônica induzida por ácido acético. Por fim, o modelo de ligadura do piloro foi utilizado para investigar o efeito da bosentana na secreção ácida gástrica. Na úlcera aguda por IR, o pré-tratamento com bosentana, sildenafila ou a associação de baixas doses de ambos reduziram a área ulcerada, impediram a depleção de muco e das defesas antioxidantes no tecido gástrico. Complementarmente, esse pré-tratamento reduziu a infiltração de neutrófilos, importante marcador de inflamação tecidual. No modelo de úlcera crônica induzida por ácido acético foi constatado um aumento na expressão de receptores ET_A e ET_B na área ulcerada dos animais do grupo veículo comparado com animais *naïve*. O tratamento com a bosentana por cinco dias acelerou a cicatrização das úlceras crônicas, aumentou o conteúdo de mucina e a proliferação de células epiteliais da mucosa gástrica, reduziu a infiltração de neutrófilos e os níveis de citocinas inflamatórias, como fator de necrose tumoral-alfa e interleucina 1 beta. Os parâmetros plasmáticos da função renal e hepática não foram alterados pelo tratamento sub-crônico com a bosentana. A partir dos resultados do modelo agudo, conclui-se que o pré-tratamento com bosentana e sildenafila ou a associação de baixas doses dos fármacos exibe efeito gastroprotetor, e no modelo de úlcera crônica, o pós-tratamento com bosentana induziu a cicatrização de úlceras crônicas, sem modificar a secreção ácida gástrica. Nossos resultados reforçam a tendência mundial do reposicionamento de fármacos (uso *off label*), e de maneira especial este estudo demonstra que outras vias podem ser exploradas como adjuvantes no tratamento de úlceras gástricas, contornar os possíveis efeitos adversos do tratamento convencional.

Palavras chaves: Úlcera gástrica, sildenafila, bosentana, endotelina, inflamação, estresse oxidativo.

ABSTRACT

Gastric ulcer is a worldwide public health problem, characterized by extensive lesions in the mucosa of the stomach and / or esophagus that can extend through all layers of the organ, causing serious complications such as bleeding and perforations that require hospitalization and surgical intervention. The main causes of gastric ulcer are the use of non-steroidal anti-inflammatory drugs (NSAIDs), which decrease the production of gastroprotective mucus-bicarbonate, and infection by the bacterium Helicobacter pylori. In addition to the eradication of H. pylori, conventional treatment aims to inhibit the production of hydrochloric acid by the use of proton pump inhibitors (e.g. omeprazole), or histamine H2 receptor antagonists (e.g. ranitidine). However, studies show that inhibition of gastric acid secretion is associated with an increased risk of developing several diseases such as osteoporosis, intestinal infections and even chronic kidney disease. In addition to gastric acid secretion, ulcerogenesis is a complex process that also involves blood flow. The endothelinergic and nitric oxide pathway systems modulate microcirculation as well as inflammation and oxidative stress in the gastric mucosa. Thus, the objective of this work was to evaluate the gastroprotective and healing effect of vasoactive drugs, more specifically bosentan and sildenafil, in models of acute and chronic gastric ulcers. Oral administration of bosentan (dual antagonist of ETA and ETB receptors) and sildenafil (phosphodiesterase 5 inhibitor), either alone or in combination, were investigated in the ischemia-reperfusion-induced acute ulcer model. The healing effect of bosentan monotherapy was investigated in the acetic acid-induced chronic ulcer model. Finally, the pyloric ligation model was used to investigate the effect of bosentan on gastric acid secretion. In acute IR ulcers, pretreatment with bosentan, sildenafil or the combination of low doses of both reduced the ulcerated area, prevented mucus depletion and antioxidant defenses in gastric tissue. In addition, this pre-treatment reduced the infiltration of neutrophils, an important marker of tissue inflammation. In the acetic acid-induced chronic ulcer model, an increase in the expression of ETA and ETB receptors was found in the ulcerated area of animals in the vehicle group compared with naïve animals. Treatment with bosentan for five days accelerated the healing of chronic ulcers, increased mucin content and proliferation of gastric mucosal epithelial cells, reduced neutrophil infiltration and inflammatory cytokine levels, such as alpha-tumor necrosis factor and interleukin 1 beta. Plasma parameters of renal and liver function were not altered by sub-chronic treatment with bosentan. From the results of the acute model, it is concluded that pretreatment with bosentan and sildenafil or the association of low doses of the drugs exhibits a gastroprotective effect, and in the chronic ulcer model, post-treatment with bosentan induced the healing of ulcers. without changing gastric acid secretion. Our results reinforce the worldwide trend of drug repositioning (off label use), and in a special way this study demonstrates that other routes can be explored as adjuvants in the treatment of gastric ulcers, to circumvent the possible adverse effects of conventional treatment.

Keywords: Gastric ulcer, sildenafil, bosentan, endothelin, inflammation, oxidative stress.

LISTA DE ILUSTRAÇÕES

LISTA DE TABELAS

LISTA DE SIGLAS E ABREVIATURAS

AINEs - Anti-inflamatórios não esteroidais
ALT - Alanina aminotranferase
AST - Aspartato aminotranferase
ATP - Trifosfato de adenosina
CAT - Catalase
COX - Cicloxigenases
COX-1 - Cicloxigenase-1
COX-2 - Ciclooxigenase 2
EC - Célula Enterocromafin
EROs - Espécies reativas de oxigênio
ET-1 Endotelina 1
ET-2 Endotelina 2
ET-3 Endotelina 3
ETA - Receptor ETA de endotelina
ETB - Receptor ETB de endotelina
Ets - Endotelinas
GCs - Guanilato ciclase solúvel
GIP - Peptídeo inibidor gástrico
GLP-1 Peptídeo semelhante ao glucagon
GMPc - Monofosfato cíclico de guanosina
GSH - Glutationa Reduzida
GST - Glutationa tranferase
GTP - Trifosfato de guanosina
H+/K+-ATPase - Bomba de próton potássio ATPase
HCl - Ácido clorídrico
IBPs - Inibidores de bomba de prótons
IFN-y - Interferon Gama
IL-1 - Interleucina 1
IL-1β - Interleucina 1 Beta
IL-6 - Interleucina 6
IR - Isquemia-reperfusão
LPO - Lipoperóxidos

MAPK - Proteína quinase ativada por mitógeno

MPO - Mieloperoxidase

NADPH - Fosfato de dinucleotídeo de nicotinamida e adenina

NF-kB - Factor nuclear kappa B

NO - Óxido nítrico

O_2^- - Ânion superóxido

PAS - Ácido periódico de Schiff

PCNA - Antígeno nuclear de proliferação celular

PDE5 - Fosfodiesterase tipo 5

PGE1 - Prostagrandina E1

PGE-2 - Prostaglandina E2

PKG - Proteína quinase tipo dependente de GMPc

SOD - Superóxido dismutase

TGI- Trato gastrointestinal

TNF-α - Fator de Necrose Tumoral Alfa

VCAM-1 - Proteína 1 de adesão celular vascular

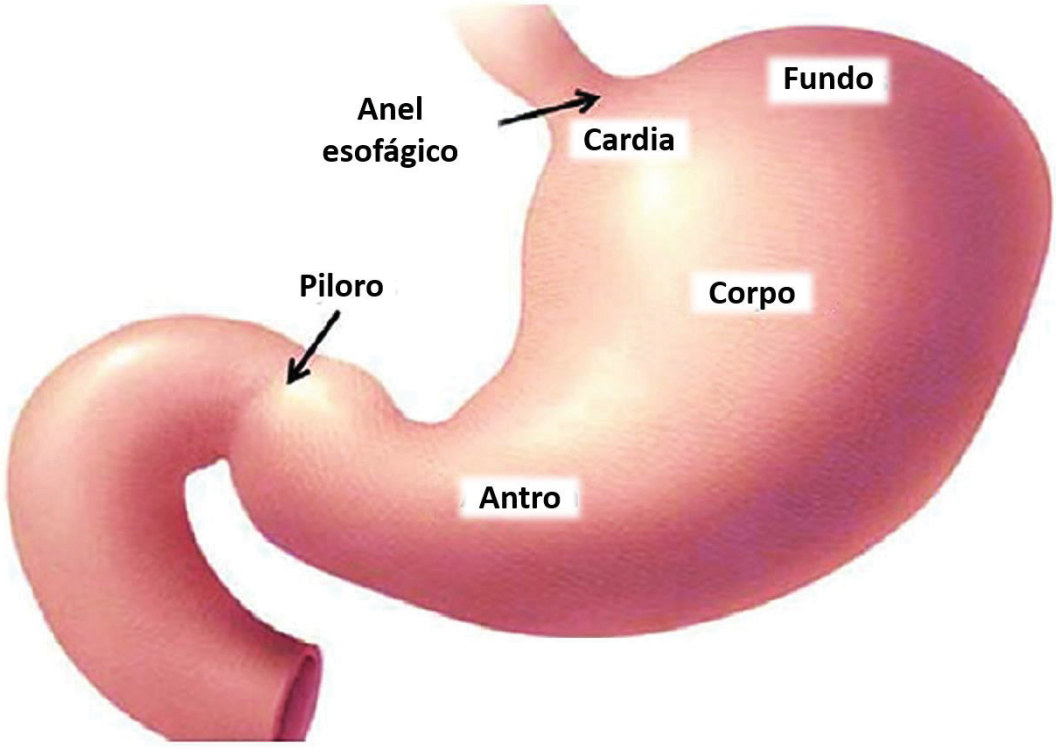
SUMÁRIO

1 INTRODUÇÃO

1.1 O ESTÔMAGO

O trato gastrointestinal (TGI) se estende da boca até o ânus, sendo composto por diversos órgãos com funções distintas e especializadas. Dentre esses órgãos temos a boca seguida pelo esôfago, o estômago, intestino delgado, intestino grosso e ânus. O estômago humano, um órgão muscular oco, é situado logo abaixo do diafragma entre a junção do esôfago e o anel pilórico ao lado esquerdo do abdómen. É formado por uma parte muscular (fundo e corpo) e uma parte secretora (antro) (FIGURA 1), essa possui glândulas gástricas que realizam a secreção do suco e muco gástricos, sendo que o suco gástrico realiza o processo digestório dos alimentos ingeridos, e o muco básico produzido pelas células epiteliais possui a função protetora impedindo a digestão do tecido gástrico pelo suco gástrico (HASLER; OWYANG, 2015).

FIGURA 1 - Ilustração da anatomia gástrica.

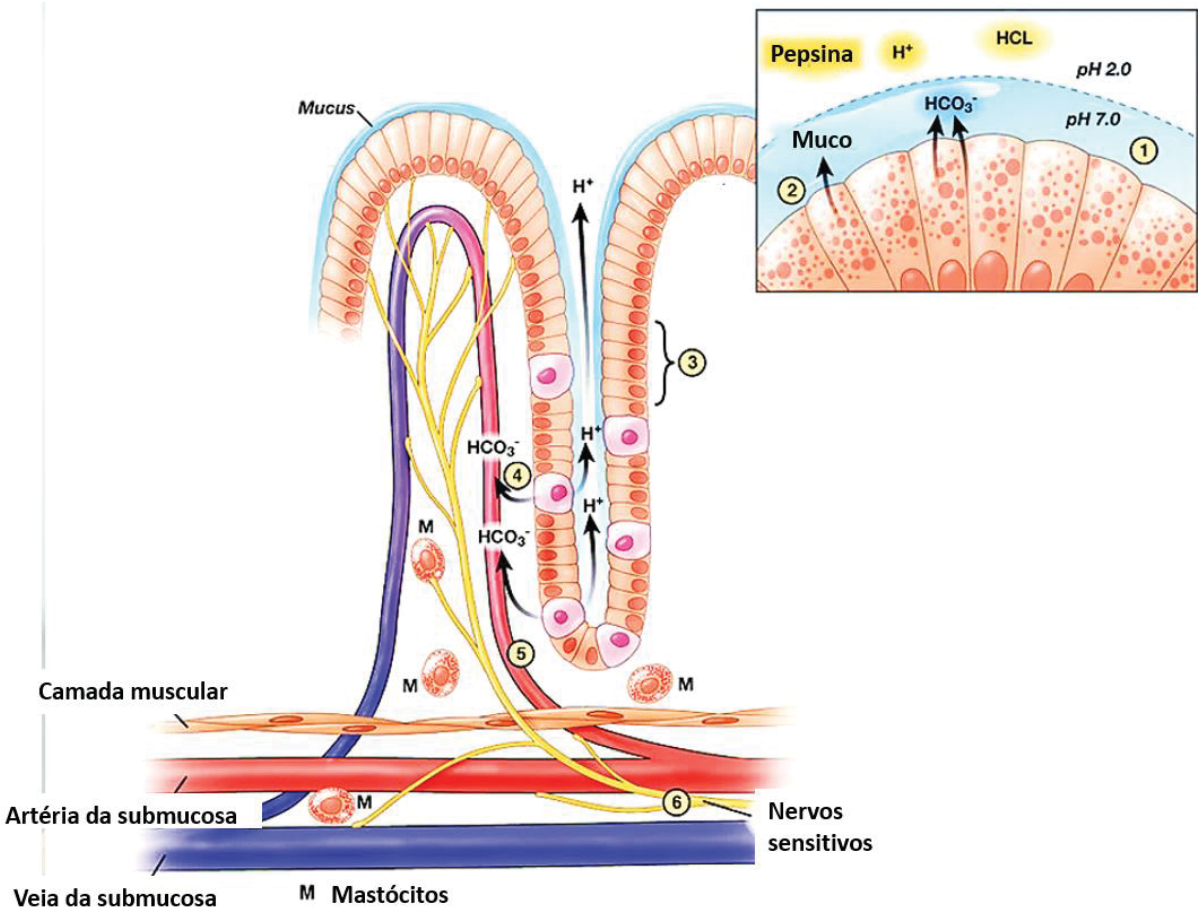


FONTE: Adaptado de(FORBES, 2019).

A porção funcional do estômago, a região da mucosa, é composta por células mucosas superficiais produtoras de mucina, células mucosas do colo responsáveis pela renovação das células superficiais e células parietais que produzem o ácido clorídrico (HCl) através das bomba de prótons (H^+/K^+-ATPase) e o fator intrínseco (YANDRAPU; SAROSIEK, 2015). Outros tipos celulares presentes no estômago são as células principais produtoras de pepsinogênio, células enterocromafins (EC), células semelhantes a EC que contêm histamina, células G que contêm grelina e obestatina e células D que secretam somatostatina (YANDRAPU; SAROSIEK, 2015). A produção do muco protetor é altamente dependente do fluxo sanguíneo e microcirculação (FIGURA 2), ambos mantidos pela ação vasodilatadora da prostaglandina E1 (PGE1) produzida constantemente pela enzima constitutiva cicloxigenase-1 (COX-1), e pela ação vasodilatadora do óxido nítrico pelas células endoteliais dos vasos sanguíneos do estômago (ARAKI et al., 2000; KOCHAR et al., 2011; MAIN; WHITTLE, 1973; WALLACE; MILLER, 2000; YANDRAPU; SAROSIEK, 2015).

O suco gástrico é secretado pelas células parietais da mucosa em um volume de cerca de 1,5L por dia, tendo em sua composição água, sais, HCl, fator intrínseco e a enzima proteolítica pepsina (YANDRAPU; SAROSIEK, 2015). O baixo pH (1–3 pH) conferido pelo HCl possui importantes funções como diminuição da carga microbiana ingerida, auxilio na conversão do pepsinogênio em pepsina, assim como auxilio no aumento da biodisponibilidade de vitaminas como a B_{12} e minerais como o ferro e magnésio (EVENEPOEL, 2001).

FIGURA 2 - Ilustração da produção de mucina e HCl gástricos.



FONTE: Adaptado de (YANDRAPU; SAROSIEK, 2015).

1.1.1 Regulação da secreção ácida gástrica

O controle da secreção ácida gástrica envolve sinalizações distintas como a neuronal (acetilcolina), hormonal (gastrina) e parácrina (histamina), e é divido em fases cefálica, gástrica e intestinal. Mediada pelo nervo vago, a fase cefálica ocorre pela estimulação colinérgica e liberação de acetilcolina, que atua sobre os receptores muscarínicos M3 nas células parietais; liberação na submucosa de gastrina pelas células G e histamina pelas células ECL, que por sua vez ligam-se nos receptores H_2 para a histamina e CCK_2 para a gastrina presentes na célula parietal, induzindo a inserção de bombas K^+/H^+ ATPase na membrana apical das células parietais, aumentando a secreção de HCl para o lúmen gástrico.

A fase gástrica é o resultado da presença de alimentos que distendem a parede gástrica que por reflexos vagais estimulam a liberação de acetilcolina, assim como pela presença de aminoácidos, peptídeos e etanol que estimulam a liberação de

gastrina. Sendo a inibição da gastrina é conferida pelo baixo pH do suco gástrico resultante da secreção de HCl. Quando o alimento alcança o duodeno é iniciado a fase intestinal, em que ocorre a inibição da secreção gástrica por meio da liberação do peptídeo inibidor gástrico (GIP), colecistocinina, secretina e do peptídeo semelhante ao glucagon 1 (GLP-1), que através da mediação pela somastatina inibem a secreção de ácido gástrico (RICHARDSON et al., 1976; YANDRAPU; SAROSIEK, 2015).

1.2 A ÚLCERA GÁSTRICA

Assim como os outros órgãos do TGI o estômago é acometido por diversas doenças e desordens intestinais como úlceras gástricas, síndrome de Zollinger-Ellison, pólipos e neoplasias (HUNT et al., 2015). A úlcera gástrica ou úlcera péptica constitui uma das mais importantes doenças que acometem o trato gastrointestinal, caracterizada por uma ruptura da integridade da mucosa gástrica com formação de lesões necróticas, isoladas e recorrentes localizadas comumente no estômago ou duodeno proximal (DEL VALLE, 2015). Tais lesões no tecido gástrico penetram a mucosa, submucosa até a camada muscular, podendo romper a membrana serosa formando uma perfuração no estômago. (FASHNER; GITU, 2015).

Usualmente, a úlcera é resultado de um desequilíbrio entre os fatores ditos agressores e os protetores da mucosa gástrica, sendo os agressores classificados como endógenos (ácido clorídrico e pepsina) e exógenos (*Helicobacter pylori*, anti-inflamatórios não esteroidais (AINEs) e consumo de álcool). Por outro lado, dentre os principais fatores protetores temos a produção de muco, sistema antioxidante endógeno, fluxo sanguíneo tecidual e microcirculação da mucosa (DEL VALLE, 2015; LANAS et al., 2017). Tais fatores protetores têm uma íntima relação com o óxido nítrico, pois esse exerce uma gama de funções no estômago, como a de aumentar a secreção de muco e o fluxo sanguíneo, diminuir a adesão vascular de leucócitos, inibir a apoptose e modular a função da barreira epitelial de modo a manter a integridade da mucosa gástrica (CALATAYUD et al., 1999; FIORUCCI et al., 1999; KIM; KIM, 1996; MURAKAMI et al., 1996; PIQUE; WHITTLE; ESPLUGUES, 1989; PRICE; HANSON; WHITTLE, 1994).

1.2.1 Sintomas e complicações

Entre os sintomas apresentados por pacientes com úlcera gástrica são comumente citados: dor epigástrica, que é exacerbada pelo jejum e aliviada logo após as refeições, refluxo gástrico, náusea, dispepsia e pirose (NAJM, 2011). Entretanto a úlcera gástrica pode ser assintomática, principalmente em pacientes idosos nos quais a prevalência pode chegar a 35% (HILTON et al., 2001). Com isso, a úlcera pode evoluir silenciosamente levando a sérias complicações como hemorragia digestiva alta, podendo ser acompanhada de dor epigástrica, tontura e síncope pela hipotensão e perda de sangue (LU et al., 2004). A hemorragia digestiva alta é a complicação mais comum dentre as citadas, com incidência entre 19 a 57 casos em cada 100.000 pessoas (KEMPENICH; SIRINEK, 2018). Diversas drogas foram associadas ao aumento de risco de hemorragia gástrica, como AINEs, inibidores seletivos da recaptação de serotonina, corticosteroides, antagonistas de angiotensina e anticoagulantes (MASCLEE et al., 2014). A segunda complicação mais comum e mais grave da úlcera gástrica é a perfuração, em que a cirurgia de emergência e tratamento agressivo contra sepse são os pilares da terapia para essa complicação (SØREIDE et al., 2015). Nos casos de perfuração, mesmo com cirurgia e antibioticoterapia combinada, a sepse é responsável por metade da mortalidade associada a esta complicação (KROBOT et al., 2004; SØREIDE et al., 2015). Sua incidência é de 4 a 14 por 100.000 pessoas (KEMPENICH; SIRINEK, 2018).

Embora a incidência geral de complicações esteja diminuindo nas últimas décadas, complicações como sangramento, perfuração e obstrução são responsáveis por quase 150.000 hospitalizações anualmente nos Estados Unidos, com mortalidade de cerca de 30% (SØREIDE et al., 2015).

1.2.2 Epidemiologia

A prevalência da úlcera gástrica na população mundial é estimada entre 5 a 10%, e sua incidência entre 0,1 a 0,3% (ARO et al., 2006; KURATA et al., 1992; LAU et al., 2011; MALMI et al., 2014; NAJM, 2011; ROSENSTOCK; JØRGENSEN, 1995). Contudo, os últimos 20 a 30 anos mostraram uma diminuição na incidência, complicações, taxas de hospitalização e mortalidade associadas às úlceras gástricas (LANAS et al., 2011; MALMI et al., 2014; SONNENBERG, 2007a, 2007b, 2013). Esses

declínios podem ser associados às novas terapias aplicadas na década de 1990, como a erradicação da bactéria *H. pylori*, uso generalizado de drogas anti-secretoras como os inibidores de bomba de prótons, que reduzem drasticamente a acidez gástrico e, ao uso mais racional dos AINES (LANAS, 2016; LANAS et al., 2011; MALMI et al., 2014).

1.2.3 Etiologia

Infecções pela bactéria *Helicobacter pylori* e uso de anti-inflamatórios não-esteroides (AINEs) são identificadas como principais causas de úlcera péptica (KEMPENICH; SIRINEK, 2018). A infecção por *H. pylori,* costuma ser oral já na primeira infância, ocorre em todo o mundo com uma alta variação na taxa de prevalência entre diferentes países, principalmente por fatores socioeconômicos (SUERBAUM; MICHETTI, 2002). Em países em desenvolvimento a prevalência da *H. pylori* em adultos é superior a 80%, já em países desenvolvidos a prevalência é de 20 a 50% (MALATY; GRAHAM, 1994).

A idade avançada é um importante fator que aumenta o risco de desenvolvimento de úlcera crônica, vários estudos epidemiológicos na literatura demonstram que 60% dos pacientes com úlcera duodenal apresentam mais de 60 anos (LAU et al., 2011). Outros fatores que são associados ao desenvolvimento de úlceras gástricas são o tabagismo e o consumo de álcool (KNOLL et al., 1998; KO; CHO, 2000).

Além disso, também há possíveis fatores genéticos que podem aumentar a susceptibilidade ao desenvolvimento de úlcera gástrica (TANIKAWA et al., 2012). Estudos mostraram uma associação do uso do ácido acetilsalicílico, um AINE, com risco aumentado de desenvolvimento de úlcera gástrica de pacientes asiáticos que apresentam polimorfismos de nucleotídeo único do gene da cicloxigenase-1 (A-842G e C50T), contudo a relevância clínica desses estudos não foi determinada devido a fraqueza da associação encontrada dos estudos (SHIOTANI et al., 2014; SHIOTANI; FUJITA; NISHIO, 2015; WANG et al., 2014).

1.2.4 Tratamento farmacológico

Diante da elucidação da fisiopatologia da úlcera gástrica, particularmente o descobrimento da fisiologia da secreção ácida gástrica, há inúmeras estratégias

terapêuticas disponíveis, como descrito na Tabela 1 (adaptado de CAVALLINI et al., 2006).

Tabela 1 – Classificação das drogas para tratamento de úlceras gástricas crônicas

ANTIÁCIDOS	Bicarbonato de sódio, carbonato de cálcio, hidróxidos de alumínio/magnésio
AGENTES CITOPROTETORES DE BARREIRA	Sucralfato, bismuto coloidal
ANTAGONISTAS DE RECEPTOR H_2	Cimetidina, ranitidina, famotidina, nizatidina
ANÁLOGO DE PGE1	Misoprostrol
INIBIDORES DE H^+K^+ ATPASE	Omeprazol, pantoprazol, lansoprazol, rabeprazol, esomeprazol, dexlansoprazol vonoprazan

FONTE: adaptado de CAVALLINI et al., 2006.

Após a década de 90 o tratamento cirúrgico para úlcera gástrica se tornou muito menos necessário, visto que com o tratamento associado de antibióticos com inibidores de bomba de prótons (IBPs), como o omeprazol, tem alcançado alta taxa de cura e foi amplamente difundido. Os IBPs agem reduzindo a produção de ácido gástrico em até 95% através da ligação irreversível com a enzima H^+/K^+ ATPase nas células parietais gástricas (LARSON; SULLIVAN, 1984). Ainda assim, estão entre as duas classes de fármacos predominantemente prescritas devido a melhor eficácia e segurança comparada com as outras classes (NEHRA et al., 2018; SCHWESINGER, 2001). O omeprazol foi a primeira droga da classe dos IBPs e introduzida no mercado em 1989 com grande aceitação e boa segurança para o uso. Nos anos seguintes diversas outras drogas semelhantes, foram lançadas, como o lansoprazol, rabeprazol, pantoprazol, esomeprazol e dexlansoprazol (VAEZI; YANG; HOWDEN, 2017). Desde então, há uma preocupação crescente com o uso de IBPs, pois em muitos países como EUA e Brasil é possível a compra sem receita médica, o que resulta em um fácil acesso para o público. Além disso, os IBPs também são receitados para dispepsia, azia frequente, dor abdominal, inchaço, eructação excessiva e esôfago de Barrett (VAEZI; YANG; HOWDEN, 2017).

Os antibióticos empregados tem a finalidade de eliminar à bactéria *H. pylori* do ambiente gástrico, sendo usada a combinação de dois antibióticos como a amoxicilina, claritromicina, tetraciclina ou metronidazol (SUERBAUM; MICHETTI, 2002). A

resistência de microrganismos devido ao uso disseminado de antibióticos é uma tendência mundial, por isso a *H. pylori* já apresenta uma taxa de resistência que varia de 20% na Suécia até 83% na China (HUNT et al., 2011; ZHANG, 2014).

Além dos IBPs, os antagonistas de receptor H_2 da histamina também são capazes de reduzir a secreção ácida gástrico, porém, no caso da cimetidina, com efeitos colaterais mais proeminentes e maior interação medicamentosa com outros fármacos (RICHTER, 1986). Os efeitos colaterais dessa classe incluem bradicardia, hipotensão, confusão mental e podem até induzir ginecomastia por interação com andrógenos, como é o caso da cimetidina (SPENCE; CELESTIN, 1979). O uso intenso dessa classe foi de 1970 até 1990, caindo em desuso após a entrada os IBPs no mercado farmacêutico (ERIKSSON et al., 1995). Os IBPs estão entre as 10 drogas que mais representaram custos com a saúde nos EUA em 2015, sendo as mais prescritas para tratamento de úlceras gástricas e doença do refluxo gastresofágico (KANTOR et al., 2015; SCHUMOCK et al., 2016). Contudo, o uso dos antagonistas de receptores H_2 tem aumentado gradativamente, devido ao bloqueio menos intenso da secreção ácida gástrica tem sido visto na clínica menos efeitos colaterais dos antagonistas como a ranitidina (PANULA et al., 2015)

Após o ano 2000, com a quebra de patente e comercialização de genéricos e similares dos inibidores de bomba de prótons, houve um aumento considerável do uso dos IBPs principalmente na Europa, em que o omeprazol foi a droga mais comercializada em 2010 na Espanha (LANAS, 2016). Consequentemente, aumentou-se significativamente o número de relatos de prescrições inapropriadas, em que são passadas doses acima do recomendado e por um longo período de tratamento, tanto em hospitais e ambulatórios quanto em centros de saúde (VAN VLIET et al., 2008).
Em 2015 um novo fármaco inibidor da secreção ácida foi aprovado para uso no Japão, o vonoprazan, da classe dos bloqueadores de ácido competitivo com o potássio que inibe reversivelmente a atividade da H^+K^+ ATPase (GARNOCK-JONES, 2015). Seu perfil farmacológico de início de ação mais rápido, exibe uma maior segurança e eficácia na inibição da secreção ácida gástrica comparado com os IBPs clássicos, demonstrando assim um grande potencial para sua aprovação e uso no âmbito mundial para o tratamento de úlceras gástricas e doenças relacionadas (HUNT; SCARPIGNATO, 2015). Contudo, mesmo com a superioridade farmacológica do mais recente fármaco aprovado (vonoprazan), esses tratamentos ainda se limitam a apenas uma via fisiológica que é a inibição da secreção ácida.

A úlcera gástrica impacta substancialmente no cenário econômico dos países. Na Alemanha um estudo chegou ao valor de 3,5 bilhões de doses diárias definidas para os IBPs no tratamento de úlceras gástricas e doenças relacionadas (MÖSSNER, 2016). Na década de 90 o custo estimado nos EUA tanto direto, quanto indireto pela perda da produtividade, foi de U$ 5,65 bilhões anuais, chegando a U$ 11 bilhões com as complicações da úlcera gástrica contribuindo consideravelmente para tal custo (FORGACS; LOGANAYAGAM, 2008; NEHRA et al., 2018; SONNENBERG; EVERHART, 1997). O custo estimado nos Países Baixos para a úlcera hemorrágica está foi estimado em €12000,00, já a úlcera perfurada chega ao valor de €19000,00 e esse valor sobe para €26000,00 quando ambas ocorrem simultaneamente, considerando que são estudos de décadas passadas, esses valores de custos tendem a ser consideravelmente mais elevados (DE LEEST et al., 2004). Contudo dados mais recentes sobre o impacto econômico de úlceras gástricas são escassos.

1.2.5 Efeitos adversos do tratamento farmacológico

Como todo medicamento os IBPs não estão isentos de efeitos adversos pelo seu uso. A difusão global do uso de IBPs levou a estudos que relataram o uso inapropriado, sendo receitado muitas vezes erroneamente sem a real necessidade pelo paciente (FORGACS; LOGANAYAGAM, 2008; VAN VLIET et al., 2008). O uso dos IBPs continua a crescer a cada ano no mundo, sendo que esse fenômeno evidencia que há necessidade de reavaliação das indicações corretas dos IBPs, com melhores diretrizes baseadas em evidências (BHATIA et al., 2019; SAVARINO et al., 2018). Essa prescrição imprópria ocorre principalmente em ambiente ambulatorial, em que foi constatado um desperdício de U$ 1,5 milhões em um centro de saúde na cidade de Ann Arbor, MI, EUA (HEIDELBAUGH; GOLDBERG; INADOMI, 2010).

Embora os IBPs sejam considerados muito seguros para uso terapêutico, estudos recentes sobre seu uso prolongado têm observado potenciais efeitos adversos como risco de fraturas ósseas, pneumonia, infecção intestinal por *Clostridium difficile,* hipomagnesemia, deficiência de vitamina B_{12}, doença renal crônica (NEHRA et al., 2018; O'CONNELL et al., 2019). Contudo, a maioria das evidências publicadas mostram uma fraca associação entre o uso de IBP e o risco de desenvolvimento de efeitos adversos sérios. Assim, quando clinicamente indicado, é

altamente recomendável a prescrição dos IBPs na menor dose efetiva para o controle dos sintomas, reduzindo o potencial risco de tais eventos adversos.

Dentro os efeitos adversos do uso dos IBPs, em que as evidências sugerem uma possível associação causal, estão a hipomagnesemia, a deficiência de vitamina B_{12} e um supercrescimento bacteriano no intestino delgado. A hipomagnesemia associada ao uso dos IBPs foi descrita primeiramente em 2006 em pacientes que faziam uso de IBPs por mais de 1 ano e apresentavam espasmos musculares dos pés e mãos (carpopedais) (EPSTEIN; MCGRATH; LAW, 2006). Posteriormente, os níveis séricos de magnésio nesses pacientes retornaram ao valor normal com a interrupção do uso de IBPs. Uma meta análise de 9 estudos observacionais com 109798 pacientes reportou-se um risco de 43% para o desenvolvimento de hipomagnesemia em pacientes recebendo IBPs (CHEUNGPASITPORN et al., 2015).

O ácido gástrico é necessário para liberação da vitamina B_{12} de proteínas dos alimentos e facilitar a absorção dessa vitamina no íleo intestinal. Com base nisso, um estudo revelou uma associação tipo causativa de um risco de 65% no desenvolvimento de deficiência de vitamina B_{12} com o IBPs (MILLER, 2018). Outro efeito adverso com evidências de associação causal com IBPs é o supercrescimento bacteriano intestinal, condição a qual resulta em fermentação excessiva com inchaço abdominal, diarreia, inflamação intestinal, perda de peso, má absorção nutricional, e lesão celular pela produção de enterotoxinas e adesão bacteriana (HOFFMANN; ZEITZ, 2002; HUSEBYE et al., 1992; KIRSCH, 1990; RANA; BHARDWAJ, 2008; WILLIAMS, 2001). Segundo metanálise de 11 estudos há risco de aumento de supercrescimento bacteriano intestinal com o uso de IBPs, contudo a importância clínica desse evento adverso para os pacientes estudados ainda não foi avaliada (LO; CHAN, 2013).

Além dos efeitos adversos, estudos relataram associação entre uso de IBPs com maior risco de fraturas ósseas, de infecção intestinal por *Clostridium difficile* e com doença renal crônica (NEHRA et al., 2018). Em relação às fraturas ósseas devido à osteoporose, essas exibem uma alta taxa de mortalidade, entre 13% a 22% para homens e 40% a 50% para mulheres (JOHNELL; KANIS, 2005). Com base no estudo clínico duplo cego, placebo controlado, em que foi contatado uma menor absorção de cálcio em mulheres idosas após uma semana de uso do omeprazol, foi proposto que o risco de fraturas aumentado poderia estar associado à redução da absorção nutricional do cálcio e redução da densidade mineral óssea (O'CONNELL et al., 2005).

Em estudo coorte retrospectivo com 851631 crianças foi constatada associação do uso de IBPs com maior risco de fratura, que se mostrou amplificado pela maior duração do tratamento (MALCHODI et al., 2019). Em uma metanálise de 18 estudos observacionais foi relatada uma associação de risco 33% maior de fraturas ósseas pelo uso de IBPs (ZHOU et al., 2016). Contudo, há estudos que não encontraram diferenças significativas na densidade e estrutura óssea pelo uso de IBPs por 5 anos, o que denota a fraqueza da possível associação (TARGOWNIK et al., 2017). Estudos sugerem que há uma ligação entre infecção por *Clostridium difficile* e uso de IBPs, o que guiou o FDA (*Food and Drug Administration*) a alertar sobre o risco de infecção intestinal pela terapia com IBPs e indicar sempre a menor dose efetiva (NEHRA et al., 2018). Tal recomendação é baseada na metanálise de estudos observacionais que constataram aumento de risco da incidência e recorrência de infecções intestinais pelo uso de IBPs, além de que outro estudo relacionou a alteração da microbiota intestinal pelo isso de IBPs também com maior risco de infecção pela *C. difficile* (FREEDBERG et al., 2015; JANARTHANAN et al., 2012; KWOK et al., 2012; PARK et al., 2019; TARIQ et al., 2017).

Relatos levantaram preocupações em relação à associação de lesão renal crônica e aguda e uso de IBPs. A primeira hipótese foi levantada a partir do estudo prospectivo de comunidades com risco de desenvolvimento de aterosclerose com função renal normal, com duração de 13,9 anos (LAZARUS et al., 2016). Após uma análise ajustada os resultados revelaram que o uso de IBPs aumentava em 50% o risco de desenvolvimento de doença renal crônica. Outro estudo de base populacional de 290.592 pacientes com mais de 65 anos encontrou um aumento de 2,5 vezes no risco de desenvolvimento de lesão renal aguda e um risco três vezes maior de nefrite intersticial aguda entre pacientes idosos que recentemente iniciaram a terapia com IBP (ANTONIOU et al., 2015).

Foi observado em camundongos que IBPs podem interagir com enzimas cerebrais como a gama secretase 1 (BACE1), o que leva a uma menor degradação proteica lisossomal na micróglia pela inibição da H^+/ATPase vacuolar, impedindo a depuração de peptídeos beta amiloides e levando à demência da doença de Alzheimer (BADIOLA et al., 2013). Estudos observacionais com 3076 pacientes idosos sem histórico de demência foi relataram um risco maior de 38% de desenvolvimento de demência e 44% para doença de Alzheimer com uso de IBPs (HAENISCH et al., 2015). Um achado semelhante foi obtido em um estudo de coorte com 73679 indivíduos idosos,

em que se encontrou um risco 44% maior de desenvolvimento de demência associado com IBPs (GOMM et al., 2016). Contudo esses achados contrastam com o estudo posteriores, com o estudo prospectivo de 13864 mulheres de meia idade e idosas, em que não foi contatada a associação do uso de IBPs com queda na função cognitiva (LOCHHEAD et al., 2017). Em uma metanálise com 642000 pacientes não foi constatada relação entre o uso de IBPs com desenvolvimento de demência na doença de Alzheimer (SONG et al., 2019).

Visto os efeitos adversos mais comuns com o uso, há também efeitos adversos incomuns como a síndrome da hipersecreção ácida gástrica de rebote, que ocorre com a interrupção do tratamento com IBPs (LEROTIĆ et al., 2011). Por mecanismo de inibição por retroalimentação, a secreção de ácido gástrico inibe a secreção endócrina de gastrina pelas células G. Na redução da secreção de HCl, as células G aumentam a secreção de gastrina, levando ao quadro de hipergastrinemia. Como a gastrina é um hormônio que estimula a proliferação de várias células estomacais, como das próprias células parietais secretoras de HCl. Então, com a interrupção do tratamento com IBPs a produção ácida gástrica é intensificada, levando ao quadro de hipercloridria. Visto que HCl é um agente agressor da mucosa, o excesso de sua produção juntamente com diminuição dos fatores protetores da mucosa pode resultar em lesão gástrica que pode evoluir para úlcera gástrica (LEROTIĆ et al., 2011; TEIXEIRA, 2011; WALDUM et al., 2010).

1.3 ÚLCERA GÁSTRICA: ALÉM DA INIBIÇÃO DA SECREÇÃO ÁCIDA

A supressão ácida gástrica tem sido o foco do tratamento farmacológico desde a década de 1990. Contudo, estudos com animais e com humanos têm observado que além da secreção ácida gástrica, outros fatores como a redução do fluxo sanguíneo também contribuem para o processo de ulcerogênese. Um importante sistema endógeno, representado pela sinalização endotelinérgica, merece ser melhor explorado (KREJCI et al., 2003; LAZARATOS et al., 1995), uma vez que existem poucos estudos demonstrando o envolvimento do sistema endotelinérgico na ulcerogênese, particularmente tratando-se de úlceras gástricas crônicas.

1.3.1 Sistema endotelinérgico

As endotelinas são peptídeos endógenos que desde sua descoberta em 1988, são os vasoconstritores mais potentes conhecidos, e sua denominação foi devido à primeira fonte descrita, o endotélio (YANAGISAWA et al., 1988). Nos mamíferos há diferentes isoformas, como a endotelina-1 (ET-1), que é mais abundante e de maior relevância biológica, endotelina-2 (ET-2) e endotelina-3 (ET-3) (INOUE et al., 1989; SAIDA; MITSUI; ISHIDA, 1989). Tais peptídeos ligam-se em duas subclasses de receptores acoplados à proteína G, o receptor ET_A e o receptor ET_B, que através de uma sinalização por Ca^{2+} culmina na ativação da fosfolipase C (DAVENPORT, 2002). Predominantemente, ambos receptores ET_A e ET_B são encontrados em todo sistema circulatório, como na musculatura lisa vascular, enquanto o receptor ET_B é encontrado apenas no endotélio vascular (MAGUIRE; DAVENPORT, 2015).

Todas as isoformas de ETs ligam-se com a mesma afinidade ao receptor ET_B, enquanto apenas a ET-3 possui baixa afinidade ao receptor ET_A (RUBANYI; POLOKOFF, 1994). Os receptores ET_A e ET_B compartilham sinalização por proteína $G_{q/11,}$ com uma sustentada liberação de Ca^{2+} dos estoques intracelulares seguida da entrada extracelular de Ca^{2+}. (RUBANYI; POLOKOFF, 1994).

Além do endotélio diversos outros tipos de células sintetizam as endotelinas, como células musculares lisas, epiteliais renais, neurônios, fibroblastos, miofibroblastos, mastócitos, monócitos, macrófagos, leucócitos e células dendríticas (FINSNES et al., 2001; HOUDE; DESBIENS; D'ORLÉANS-JUSTE, 2016; IWATA et al., 2009; JUERGENS et al., 2008; SHI-WEN et al., 2006; SPRAGUE; KHALIL, 2009; SUTCLIFFE et al., 2009). A expressão de ET-1 pode sofrer influência de diversos fatores, como os que aumentam sua expressão, como a trombina, bradicinina, angiotensina II, fator β de transformação de crescimento, hipóxia e miRNAs (FRIES, 2019; RUBANYI; POLOKOFF, 1994; VON BRANDENSTEIN; RICHTER; FRIES, 2012).

Há também fatores que podem diminuir a expressão de ET-1, como o óxido nítrico e estresse por força de cisalhamento (GRATTON et al., 1997; RAPOPORT, 2014). A degradação de ET-1 livre é dependente dos mastócito dependente por atividade intracelular da carboxipeptidase A3 (SCHNEIDER et al., 2007). Já a ET-1 ligada ao receptor ET_B é degradada pela internalização do complexo seguida de digestão lisossomal (BREMNES et al., 2000).

A ativação dos receptores de ET, que induz um aumento sustentado no Ca^{2+} citosólico, influencia em uma variedade de segundos mensageiros, como a via da proteína quinase ativada por mitógeno (MAPK), via da rho-quinase e adenilato ciclase (IVEY; OSMAN; LITTLE, 2008). Como exemplos específicos, através da MAPK, fatores de transcrição c-fos e c-myc a ET-1 atua como mitógeno nas células musculares lisas, assim como inibidores de MAPK, c-Src, PI-3K e Janus tirosina quinase anulam o efeito vasoconstritor de ET-1 (BOBIK et al., 1990; FUJITANI et al., 1995). Isso denota que as ETs podem exercer muitos outros efeitos além da manutenção da pressão sanguínea, como por exemplo em condições de doenças cardíacas e pulmonares, insuficiência renal e tumores (DAVENPORT et al., 2016).

1.3.2 Endotelina-1 e estresse oxidativo

A ativação de receptores ET_A/ET_B por ET-1 é capaz de induzir estresse oxidativo, por via de fosfato de dinucleotídeo de nicotinamida e adenina (NADPH) dependente de Ca^{2+}, o qual resulta na produção de ânion superóxido ($O_2^{\cdot-}$) seguido de dano oxidativo, demonstrado tanto *in vitro* quanto *in vivo* (DUERRSCHMIDT et al., 2000; MONTEZANO et al., 2010; WEDGWOOD et al., 2001). Essa relação é fortificada pela demonstração que inibindo a atividade da NADPH oxidase diminui-se a vasoconstrição mediada por ET-1 (LOOMIS, 2005; MEYER; BARTON; PROSSNITZ, 2014).

Similarmente a relação ET-1 com estresse oxidativo foi demonstrada por sua injeção intravenosa, que induziu intensa e persistente vasoconstrição, levando à isquemia de órgãos e geração de espécies reativas (DONG et al., 2005; LOOMIS, 2005; LÓPEZ-SEPÚLVEDA et al., 2011; THAKALI et al., 2005). Tal estresse induzido por ET-1 resulta na redução da glutationa intracelular (GSH) e aumento da peroxidação lipídica pelo aumento de geração de lipoperóxidos (SCALERA et al., 2002; VISWANATHA SWAMY et al., 2011). Ambos receptores ET_A/ET_B estão envolvidos com a geração de espécies reativas, e o bloqueio desses receptores com antagonista diminui os danos oxidativos (BRIYAL; PHILIP; GULATI, 2011; DONG et al., 2005; ELMARAKBY et al., 2005; XU; LIN; YUAN, 2003).

Em pacientes com doença arterial coronariana, o antagonista peptídico do receptor ET_A, o BQ123, que apresenta meia vida curta, foi capaz de reduzir a produção de $O_2^{\cdot-}$, com provável envolvimento da NADPH oxidase, fato relacionado

com preservação da vasodilatação dependente do endotélio (CERRATO et al., 2012). Em relação ao antagonista peptídico do receptor ET_B BQ788, também foi mostrado uma redução da produção de espécies reativas de oxigênio (EROs) em gânglios do sistema nervoso simpático de ratos hipertensos (DAI et al., 2004). No mesmo estudo foi constatado um aumento na expressão do receptor ET_B devido ao quadro hipertensivo, o que explica em parte o efeito do antagonista ET_B. Outro estudo demonstrou o efeito do antagonista peptídico seletivo para o receptor ET_B, o BQ788, que reverteu o quadro hipotensivo e reduziu o estresse oxidativo induzidos por lipopolissacarídeo (LPS) em ratos (PIECHOTA-POLAŃCZYK; GORĄCA, 2012).

1.3.3 Endotelina-1 e o processo inflamatório

Muitos estudos têm demonstrado a participação da ET-1 em processos inflamatórios, nos quais o peptídeo é responsável pela ativação de fatores de transcrição como o NF-kB e expressão de citocinas pró inflamatórias como TNF-α, IL-6 e IL-1 (YEAGER et al., 2012). Adicionalmente, foi demonstrado que esses fatores pró-inflamatórios, como NF-kB, também podem estimular a produção de ET-1 (VIRDIS; SCHIFFRIN, 2003). Essa relação ET-1 com citocinas inflamatórias é evidenciada por diversos estudos, como no que avaliou o antagonista seletivo ET_A BQ123, que foi capaz de bloquear o aumento de TNF-α e IL-1β em modelo de enfisema pulmonar em ratos. (CHEN et al., 2010).

Em pacientes com esclerose sistêmica foi constatado um aumento sérico de ET-1 e citocinas inflamatórias como IL-2, IL-6, IL-8 e IFN-y, as quais tiveram seus níveis reduzidos pelo tratamento com a bosentana, o antagonista de receptores ET_A/ET_B (BELLISAI et al., 2011). Em estudo com pacientes pós-cirúrgicos de ponte de safena, o antagonista BQ123 foi capaz de reduzir os níveis de TNF-α, efeito o qual foi associado a melhor desfecho cirúrgico para o paciente (FORD et al., 2008). A produção de EROs foi aumentada em diferentes tipos celulares por vias dependentes de NF-kB, COX e NADPH oxidase (DONATE et al., 2012; KLENIEWSKA et al., 2013; PIECHOTA; GORACA, 2011).

O inibidor de NF-kB, o BAY 11-7082, preveniu o edema e estresse oxidativo induzidos por ET-1 exógena nos tecidos pulmonar e hepático, demonstrando a relação de ET-1 com mediadores inflamatórios (KLENIEWSKA et al., 2013; PIECHOTA; GORACA, 2011). O tratamento com bosentana em um modelo de artrite

reumatoide mostrou efeito anti-inflamatório pela redução de infiltração de leucócitos, redução das citocinas pró-inflamatórias IL-1β, TNF-α e IL-17, conjuntamente com melhora do quadro geral da doença (DONATE et al., 2012). Tanto o BQ123 quanto BQ788 foram capazes de melhorar a hemodinâmica e diminuir a peroxidação lipídica plasmática induzidas pelo LPS em ratos, mas apenas BQ123 reduziu o aumento do marcador inflamatório TNF-α no plasma (PIECHOTA-POLAŃCZYK; GORĄCA, 2012).

Com isso, o aumento dos níveis de citocinas inflamatórias ativam a produção de prostaglandinas durante o processo inflamatório em diferentes tipos celulares, como células endoteliais vasculares e musculatura lisa. Através da atividade da cicloxigenase (COX) ocorre a produção das prostaglandinas, por processo que se inicia na catálise do ácido araquidônico pela fosfolipase A2, esse por sua vez serve de substrato para COX na produção de prostaglandinas. A COX-1 é a isoforma constitutiva expressa em condições fisiológicas normais na maioria dos tecidos, já a isoforma COX-2 é expressa apenas quando há um processo inflamatório. Estudos recentes mostraram que ET-1 é capaz de induzir a expressão de COX-2 e elevar a produção de PGE_2 por via MAPK e NF-κB (LIN et al., 2013).

ET-1 é capaz de aumentar a expressão de proteína de adesão celular VCAM-1 no endotélio vascular e estimular a infiltração de neutrófilos polimorfonucleares, contribuindo para inflamação e disfunção endotelial (LI et al., 2003). A infiltração de neutrófilos no processo inflamatório contribui para a geração de EROs, proteases e metabólitos do ácido araquidônico, como demonstrado em dano por isquemia-reperfusão em miocárdio de modelos experimentais (HANSEN, 1995). Estudos indicam que ET-1 causa acúmulo de neutrófilos, seguido da geração de EROs, produção de proteases e disfunção da mucosa intestinal em ratos (OKTAR et al., 2000). Com isso, foi relatado que o bloqueio dos receptores endotelinérgicos ou knock-out de ET-1 atenuou a infiltração de neutrófilos após isquemia no miocárdio (ANGGRAHINI et al., 2009; GONON et al., 2001). A própria geração de EROs contribui para a disfunção endotelial na aterosclerose, como mostrado em modelo experimental e em humanos, em que o aumento da produção de ET-1 foi relacionado ao desenvolvimento da placa aterosclerótica (BARTON; TRAUPE; HAUDENSCHILD, 2003; DASHWOOD; TSUI, 2011; HAUG et al., 1996). Achados como esse foram corroborados por estudos posteriores, como em que se verificou estresse oxidativo e infiltração de monócitos e macrófagos em camundongos com superexpressão de ET-1 endotelial. Contudo, deve-se atentar neste estudo a maior importância da ET-1

tecidual em relação à plasmática, como mostrado no estudo em que a quantidade de ET-1 tecidual aumentada é capaz de predizer a formação de placa aterosclerótica em pacientes com doença renal crônica (NOSHAD et al., 2009). EROs induzidos por ET-1 são um importante fator no desenvolvimento de doenças, como a aterosclerose. Foi proposto que antagonistas de receptores de endotelinas podem ser úteis na prevenção de doenças vasculares, visto que a superexpressão de ET-1 pelo endotélio vascular é acompanhada de aumento na sensibilidade da ativação de canais de potássio voltagem dependente, prejudicando a vasodilatação dependente do endotélio (KITADA et al., 2009; KITADA; OHKITA; MATSUMURA, 2012; MIAN et al., 2013).

Vale notar que em determinadas condições como a sepse, estratégias anti-inflamatórias com corticosteroides, anti-TNF-α, terapias baseadas em IL-1 e ativadores de proteína C não se mostraram promissoras para essa doença (XIE et al., 2014). Devido a isso, a modulação da via endotelinérgica tem sido proposta como uma alternativa viável para melhorar quadros inflamatórios.

1.3.4 Sistema endotelinérgico e úlceras gástricas

As primeiras pesquisas a explorar a relação do sistema endotelinérgico com a ulcerogênese demonstraram que ET-1 exógena é capaz de induzir hipóxia no estômago e comprometer a integridade da mucosa gástrica, efeito esse impedido pelo pré-tratamento oral com a bosentana em modelos animais (KREJCI et al., 2003; LAZARATOS et al., 1995). Com isso, deu-se início da pesquisa com antagonistas de endotelinas, como a bosentana, em modelos experimentais de úlceras gástricas induzidas por AINEs, estresse e a lesão por isquemia reperfusão. Nesses modelos, o efeito do pré-tratamento com a bosentana reduziu significativamente a área ulcerosa e diminuiu a atividade da mieloperoxidase, um marcador de infiltração de neutrófilos (DU et al., 2013; HASSAN; KASHIMURA; MATSUMARU, 1997; MATSUMARU, 1997; PADOL; HUANG; HUNT, 1999). Assim como relatado em estudos com modelos animais, constatou-se efeito gastroprotetor do pré-tratamento com a bosentana também em humanos, tanto contra danos por ingestão de álcool como pelo AINE ácido acetilsalicílico (DUGGAN et al., 1999).

1.3.5 Uso *off-label* de fármacos vasoativos

Cerca de 30% até 50% dos pacientes com esclerose sistêmica são afligidos por ulcerações dolorosas nas extremidades das mãos conhecidas como úlceras digitais (AMBACH et al., 2009; CHUNG, 2007; HACHULLA et al., 2007). Essas ulcerações são desencadeadas pelo fenômeno de Raynaud, em que devido à vasculopatia de pequenos vasos sanguíneos ocorre uma intensa e prolongada vasoconstrição, causando hipoperfusão e hipóxia tecidual, levando à necrose das extremidades afetadas e podendo levar até a amputação do membro afetado (ROSS; NIKPOUR, 2019). Outros eventos ocorrem em paralelo, como vasoespasmo secundário ao fenômeno de Raynaud, fibrose da túnica íntima vascular e trombose arterial, de modo que quando iniciado, o processo de ulceração tende a progredir pelo membro ou local com ausência de qualquer processo cicatricial (CHUNG; FIORENTINO, 2006).

A fisiopatologia das úlceras digitais na esclerose sistêmica é complexa e não está completamente elucidada. Contudo, um fator que foi intimamente relacionado com a severidade das úlceras digitais foi o elevado nível de ET-1 nos pacientes (YAMANE et al., 1992). Durante um estudo clínico com a bosentana 125 mg/dia para hipertensão arterial pulmonar em pacientes com esclerose sistêmica constatou-se que, como efeito secundário ocorreu a cicatrização de úlceras digitais nesses pacientes de modo jamais visto em estudos anteriores (HUMBERT, 2003). Estudos de caso posteriores reforçaram tal evidência, em que demonstram que além de induzir a cicatrização das ulcerações a bosentana foi capaz de prevenir a formação de novas lesões em pacientes com histórico de úlceras digitais (KORN et al., 2004). Com base nesses estudos de caso, estudos clínicos foram realizados atestando a efetividade da bosentana na cicatrização e prevenção de úlceras digitais na esclerose sistêmica, sendo recomendado seu uso *off-label* com segurança e efetividade (KORN et al., 2004; MATUCCI-CERINIC et al., 2011). Em paralelo, outras drogas vasoativas foram testadas em estudos clínicos e observacionais partindo-se do uso *off-label* com bloqueadores de canais de cálcio, iloprosta e dentre eles a sildenafila isoladamente e a associação da bosentana com sildenafila. Esses pacientes apresentavam adicionalmente uma menor produção de óxido nítrico, o qual foi relacionado intimamente com o surgimento e progressão das úlceras digitais, denotando assim a relevância da manutenção do fluxo sanguíneo tanto para prevenção quanto para

cicatrização dessas graves lesões (AMBACH et al., 2009; FREEDMAN; GIRGIS; MAYES, 1999; KOWAL-BIELECKA et al., 2017). Em virtude disso, estudos clínicos partiram do pressuposto que aumentando a disponibilidade de óxido nítrico nesses pacientes poderia se obter uma melhora do quadro de úlceras digitais, como foi constado por um estudo clínico piloto com a sildenafila nos pacientes com esclerose sistêmica (BRUECKNER et al., 2010; HACHULLA et al., 2016).

1.3.6 Bosentana: Antagonista duplo de receptores de endotelina

Em 2001, a bosentana (Tracleer®) foi o primeiro antagonista duplo de receptor ET_A/ET_B registrado nos EUA como tratamento oral para pacientes com hipertensão pulmonar funcional de classe III e IV (DUPUIS; HOEPER, 2008; MOTTE; MCENTEE; NAEIJE, 2006). Diversos estudos clínicos demonstraram a efetividade da bosentana
na melhora significativa da capacidade de exercício e na hemodinâmica por análise de Doppler e ecocardiograma (MONTANI et al., 2013; MOTTE; MCENTEE; NAEIJE, 2006). Outros importantes parâmetros também foram melhorados pela bosentana, como aumento da microcirculação de órgãos internos e tecidos periféricos, diminuição da superexpressão de ET-1, iNOS e COX-2 (ISKIT et al., 2004; KELLER et al., 2006; KREJCI et al., 2003). Além da bosentana outros antagonistas foram lançados posteriormente, como antagonista duplo tezosentana, antagonistas seletivos para o receptor ET_A como a macitentana, ambrisentana, atrasentana, sitaxsentana são alguns exemplos (BURNIER et al., 2010; MONTANI et al., 2009; SIDHARTA; VAN GIERSBERGEN; DINGEMANSE, 2013).

1.3.7 Sildenafila: Inibidor da fosfodiesterase tipo 5

Em 1998 o citrato de sildenafila foi aprovado pela *Food and Drug Administration* dos EUA e pela Agência Europeia de Medicamentos para o tratamento sob demanda de disfunção eréctil, sendo o primeiro inibidor da fosfodiesterase tipo 5 (PDE5) licenciado para tal indicação (GIULIANO et al., 2009). A eficácia e segurança da sildenafila no tratamento da disfunção erétil foram estabelecidas em mais de 120 ensaios clínicos pelo fabricante (Pfizer Limitada, Sandwich, Kent, Reino Unido), atingindo uma exposição cumulada de mais de 14 mil pacientes, somando outros

estudos independentes (LATIES; SHARLIP, 2006; SALONIA; RIGATTI; MONTORSI, 2003). Contudo, efeitos adversos de intensidade leve a moderada foram relatados, como cefaleia, congestão nasal e rubor facial, sendo considerada uma droga com alta segurança para o uso (BROCK et al., 2002; HELLSTROM et al., 2002, 2003). Tais efeitos adversos foram relacionados tanto com o tratamento com sildenafila quanto com outros inibidores de PDE5, como vardenafila (Levitra ® , da Bayer AG, Leverkusen, Alemanha) e a tadalafila (Cialis ® , Eli Lilly Nederland BV, Houten, Países Baixos) com diferença não significativa entre as drogas descritas (CAMPBELL, 2005; GRESSER; GLEITER, 2002).

A sildenafila e outros inibidores da PDE5 agem na via do óxido nítrico que é gerado pelas células epiteliais a partir do aminoácido L-arginina e oxigênio molecular. O óxido nítrico então se difunde até o citoplasma das células musculares lisas vasculares, local onde ativa a enzima guanilato ciclase solúvel (GCs), que por sua vez a GCs converte o trifosfato de guanosina (GTP) em monofosfato de guanosina cíclico (GMPc) ativando a proteína quinase dependente de GMPc (PKG). Em seguida a PKG fosforila múltiplas proteínas, como a fosfatase da cadeia leve da miosina, a RhoA, o receptor IP_3, a fosfolipase C entre outras, culminando na diminuição do Ca^{2+} citosólico e abrindo os canais de potássio ATP dependente, que hiperpolarizam as células musculares lisas vasculares, induzindo assim o relaxamento da musculatura lisa vascular (vasodilatação) e aumento do fluxo sanguíneo (FRANCIS; BUSCH; CORBIN, 2010; FRANCIS; CORBIN, 2005).

Em virtude do seu efeito vasodilatador, foram realizados estudos clínicos com a sildenafila na cicatrização de úlceras digitais. Um estudo unicêntrico com 19 pacientes com úlceras digitais decorrentes de esclerose sistêmica, em que foram tratados com sildenafila na máxima dose tolerada (até 150 mg) por 6 meses, mostrou um efeito cicatrizante nas ulcerações existentes e reduziu formação de novas úlceras (BRUECKNER et al., 2010). Igualmente em outro estudo clínico com 83 pacientes, o tratamento com 60 mg/dia de sildenafila induziu cicatrização significativa das úlceras digitais quando comparada com placebo (HACHULLA et al., 2016).

Contudo, o efeito da sildenafila assim como da bosentana vai muito além do aumento da vasodilatação e aumento do fluxo sanguíneo. Estudos mostraram que a sildenafila é capaz de diminuir a geração de EROs e diminuir o dano tecidual isquêmico em diversos órgãos como intestino, fígado, ovário, rim, pulmão e coração. (GUERRA-MORA et al., 2017; INAN et al., 2013; INCEBIYIK et al., 2015; MOHEY et

al., 2016; WANG et al., 2015). De forma semelhante, parâmetros inflamatórios foram melhorados com o tratamento com sildenafila em modelos *in vitro*, *in vivo* e em humanos (LAXMI et al., 2019; TAIBI et al., 2010; VLACHOPOULOS et al., 2015; ZAHRAN et al., 2015). Tratando-se do estômago, o pré-tratamento com sildenafila exibiu de forma similar proteção em modelos de lesão gástrica induzidas por AINEs como a indometacina, etanol e ácido acético, em que alguns autores consideram tal efeito intimamente associada à via do NO (AYDINLI et al., 2007; DUFFIN; SHAW; ROSSI, 2008; MEDEIROS et al., 2008; MOUSTAFA et al., 2013; SANTOS et al., 2005; SARAC et al., 2017). Contudo, há uma carência de estudos com modelo de úlcera gástrica aguda por isquemia reperfusão com a sildenafila, visto os inúmeros estudos com tal modelo para outros órgãos.

No caso de úlceras digitais, tanto bosentana quanto a sildenafila exibem efeitos vasodilatador e cicatrizante por vias farmacodinâmicas distintas. Estudos levantaram a hipótese de que se poderia obter um sinergismo farmacológico com combinação de ambas as drogas, como foi visto no estudo de caso em que a monoterapia com a bosentana (125 mg/dia) não exibiu efeito clínico satisfatório para cicatrização de úlceras digitais, contudo o efeito cicatrizante foi visto adicionando de modo sustentado com baixa dose de sildenafila (12,5 mg/dia) (AMBACH et al., 2009). Outro estudo de caso semelhante constatou o mesmo efeito sinérgico do tratamento combinado da bosentana com a sildenafila para cicatrização de úlceras digitais (OMARJEE et al., 2017). Enquanto ainda há poucos estudos com a combinação de ambas as drogas (bosentana e sildenafila) para úlceras digitais, para hipertensão arterial pulmonar há muito mais evidências de eficácia na combinação. Como mostrado no estudo retrospectivo com pacientes com hipertensão arterial pulmonar, em que a bosentana foi combinada com diferentes inibidores da PDE5, inclusive a sildenafila, foi constatado o benefício clínico dessa combinação (SITBON et al., 2016). Interessantemente, em estudo anterior, pacientes com hipertensão arterial pulmonar em tratamento com 250 mg/dia com bosentana, tiveram essa dose diminuída para 125 mg/dia e combinada com 50 mg/dia sildenafila, obtendo-se um resultado clínico superior à monoterapia (AMIN et al., 2015).

A partir desses achados elaborou-se a hipótese de que tanto a monoterapia quanto a combinação da bosentana e sildenafila poderiam exibir um efeito gastroprotetor contra úlceras gástricas agudas, assim como acelerar a cicatrização de úlceras gástricas crônicas.

2 MANUSCRITO 1

Author's Manuscript to be submitted

Journal: Digestive Diseases and Sciences

Title: NON-CLINICAL STUDY REVEAL THE GASTROPROTECTIVE EFFECTS OF BOSENTAN AND SILDENAFIL IN ACUTE GASTRIC ULCERS INDUCED BY ISCHEMIA-REPERFUSION

Corresponding Author: Maria Fernanda Werner

Co-Authors: Fernando T Dal Lin, master; Daniele Maria-Ferreira, PhD; Bruna B da Luz, master; Paula G. Ciapparini, graduated.

NON-CLINICAL STUDY REVEAL THE GASTROPROTECTIVE EFFECTS OF BOSENTAN AND SILDENAFIL IN ACUTE GASTRIC ULCERS INDUCED BY ISCHEMIA-REPERFUSION

Fernando Tonholi Dal Lin[a], Daniele Maria Ferreira[a], Bruna Barbosa da Luz[a], Paula Giovana Ciapparini[a], Maria Fernanda de Paula Werner[a].

[a] *Department of Pharmacology, Federal University of Parana, Curitiba, PR, Brazil.*

[a] Corresponding author at: Federal University of Parana, (UFPR), Biological Science Sector, Department of Pharmacology, PO Box 19031, Curitiba, PR 81531-980, Brazil. Tel.: +55 41 3361-1721, e-mail address: mfernanda.werner@ufpr.br (M.F. P. Werner).

Abstract

Introduction: Several factors are known to induce gastric ulcer in the postoperative period, due to gastric ischemia-reperfusion injury (IR), which can cause serious complications. Conventional treatment for gastric ulcers caused by IR is based on inhibition of acid secretion with proton pump inhibitors (PPIs) due to omeprazole. Recent studies have shown correlations between the use of PPIs and complications such as intestinal infections in a hospital setting. Vasoactive drugs, such as bosentan and sildenafil, have a gastroprotective effects without change gastric acid secretion, thus avoiding risks associated with PPIs. Therefore, this study aims to analyze the gastroprotective effect of bosentan and sildenafil isolated or in association against gastric ulcers induced by IR. **Methods**: In male Wistar rats, gastric ulcers were induced by 1h of ischemia by celiac artery occlusion and one hour of reperfusion. The rats were orally

pretreated with vehicle (1 ml/kg), omeprazole (40 mg/kg), bosentan (3, 10 or 30 mg/kg), sildenafil 1 or 5 mg/kg) or the combination of bosentan (3 mg/kg) and sildenafil (1 mg/kg) at the lowest ineffective doses. The efficacy of pretreatments was measured by ulcer area and of gastric mucus. The neutrophil infiltration level, an inflammatory parameter, was measured by myeloperoxidase activity. The gastric oxidative stress was measured by quantification of reduced glutathione, lipid peroxides, and antioxidant enzymes superoxide dismutase, glutathione S-transferase and catalase activities. **Results**: Pretreatment with bosentan, sildenafil or low-doses combination decreased the injury area by IR and neutrophil infiltration in gastric tissue, as well as preserving gastric defenses such as mucus and endogenous antioxidant and decreased the oxidative stress. **Conclusion**: Bosentan and sildenafil were effective in promoting gastroprotection against IR gastric injury. Both of these drugs have effects of preventing protective gastric mucus depletion, decreasing the infiltration of inflammatory neutrophils and preventing depletion of enzymatic and non-enzymatic endogenous antioxidants. Therefore, this research shows that other pathways besides gastric acid secretion are involved with gastroprotection, such as the endothelergic system and the nitric oxide signaling, pathways that do not interfere with gastric acid secretion.

1. INTRODUCTION

Protective factors of the gastric mucosa such as gastric mucus, blood flow, microcirculation, and endogenous antioxidant defenses are essential for tissue defense against aggressive factors of the stomach such as ethanol, non-steroidal anti-inflammatory drugs and ischemia-reperfusion (IR) [1,2]. Gastric IR injury is a clinical problem caused by multiplus events such as shock, acute gastric dilation, vasculitis, atherosclerosis, esophageal hernia, disseminated intravascular coagulation, and as a postoperative complication [3–8]. Despite the

low rate of occurrence, IR gastric injury may require a long period of hospitalization and is associated with an increased risk of death [9]. The pathogenesis of IR injury involves leukocyte adhesion, neutrophil infiltration, and intense oxidative stress. [10–13].

Conventional pharmacological therapy for acute gastric lesions is based on the inhibition of gastric acid secretion using predominantly proton pump inhibitors (PPIs) [14]. PPIs are available for sale without a prescription in several countries such as Brazil and the USA [15,16]. The widespread use of PPIs has led to several observational studies bringing evidence of the association of PPI use with increased risk for osteoporotic fracture development, intestinal bacterial overgrowth, intestinal *Clostridium difficile* infection, vitamin B12 deficiency and even increased risk of death. [14,17–20].

Seeing that the potential outcomes harmful to health due to gastric acid inhibition, it is remarkable the importance of exploring other pharmacological targets for gastroprotection of acute injuries to avoid such adverse possible effects associated with the use of PPIs. The maintenance of blood flow and microcirculation are essential for gastroprotection, vasoactive drugs such as endothelin receptor antagonists and phosphodiesterase 5 inhibitors, such as bosentan and sildenafil respectively, have demonstrated gastroprotective effects in animal models of acute gastric ulcer caused by multiple factors, such as non-steroidal anti-inflammatory drugs, ethanol, and stress [21–27]. These studies denote the involvement of the endothelergic system and the nitric oxide pathway on gastric injury by these ulcer models, but not for the IR model yet. Therefore, this study aims to explore the gastroprotective effect of bosentan and sildenafil alone or in low-doses combination for treatment to IR ulcers.

2. METHODS

2.1. Animals

Male Wistar rats supplied by the BSU of Federal University of Paraná, Curitiba, Paraná, were used in all the *in vivo* experiments. The rats were housed in cages at 22 ± 2 °C, light/dark 12h/12h cycles and free access to water and food. All *in vivo* experimental protocols were approved by the Animal Ethics Committee of Biological Science Sector of Federal University of Paraná (CEUA/BIO-UFPR) on number 1054.

2.2. Acute gastric ulcer by ischemia-reperfusion injury

As described by Wada (1997), the acute ulcer was induced with some modifications. Rats kept fasted for 18 h before gastric ulcer induction. The rats were orally pretreatment with vehicle (water, 1 mL/kg), omeprazole (40 mg/kg), bosentan (3, 10 or 30 mg/kg - Actelion Pharmaceuticals Ltd. Allschwil, Switzerland - PubChem CID: 104865), sildenafil (1 or 5 mg/kg – PubChem CID: 135398744) or low-dose combination of bosentan (3 mg/kg) and sildenafil (1 mg/kg), prior 30 minutes before the gastric ischemia. The rats were underwent general anesthesia by ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.) and an incision was made in the abdomen. The celiac trunk was identified and clamped with a small clamp on holding force 145g for 1 h. After this period, the reperfusion was initiated by clamp removal. After 60 min of reperfusion, the animals were euthanized by a lethal dose of ketamine (100 mg/kg) and xylazine (100 mg/kg), then the stomachs were removed and opened along the lesser curvature to determine the lesion area.

2.3. Preparation of gastric samples

IR induced gastric ulcers were homogenized in 200 mM phosphate potassium buffer pH 6.5 and centrifuged at 9.000g for 20 min at 4ºC. The homogenates were used for lipoperoxides (LPO) quantification. The supernatants were used for quantification of reduced glutathione (GSH), glutathione S-transferase activity (GST), catalase (CAT) activity and superoxide dismutase (SOD) activity. The pellets were used for myeloperoxidase (MPO) activity measurement.

2.4. Determination of gastric mucus quantification

The gastric mucus was measured according to Corne et al (1974). The gastric mucus of the glandular segment of the stomach was complexed with a dye solution of 0.1% Alcian Blue for 2 h. After, the tissue was washed with 250 mM sucrose twice for 15 and 45 min respectively, and then the complex mucus-dye was extracted adding 500 mM magnesium chloride and stirred intermittently for 2 h. The solution extracted was mixed with the same ether volume and centrifuged for 10 min at 3600g. The aqueous layer was separated to measure the absorbance at 580 nm and the results were expressed in µg Alcian blue/g of glandular tissue.

2.5. Determination of myeloperoxidase actividy

The myeloperoxidase (MPO) activity, an enzyme present in neutrophils, was measured by the method of Bradley and Priebat, 1984, modified by De Young et al., 1989 [28,29]. The ulcer samples were homogenized in 200 mM phosphate potassium buffer pH 6,5 and centrifuged at 9000 g for 20 min at 4 ºC. The pellets were resuspended with 1 mL of phosphate potassium buffer in the presence of 0.5% hexadecyltrimethylammonium (HTAB). The samples were sonicated for 1 min and centrifuged at 11.000 g for 20 min at 4 °C. In 96 well plates, 30 µL of the supernatant was added in 220 µL of a solution containing: 100 µL of 80 mM potassium

phosphate buffer, 85 µL of 22 mM sodium phosphate buffer and 15 µL 0.017% of H_2O_2. The reaction was started with the addition of 20 µL of tetramethylbenzidine (TMB) and incubated for 3 min at 37 °C. The reaction was interrupted by 30 µL of 1.46 M sodium acetate (pH = 3.0) and the enzyme levels were determined in a spectrophotometer at 620 nm. The results were expressed as a unit of optical density (O.D.)/mg protein.

2.6. Determination of reduced glutathione content

The method of Sedlak and Lindsay (1968) was used for gastric GSH content. In 50 µL of homogenates was added 40 µL of 12,5% trichloroacetic acid, vortexed briefly and centrifuged at 9000 g for 15 minutes. Then, in a 96-well plate, 40 µL of the supernatants were added in 290 µL of Tris-HCl buffer (0.4 M, pH 8.9) and 5 µL of DTNB (0.01 M, 5,5′-dithiobis (2-nitrobenzoic acid)). The procedures were performed at 4ºC. Subsequently, the absorbance was measured by spectrophotometry at 415nm. The values obtained were interpolated with a standard curve of GSH and results were expressed as µg/g of tissue.

2.7. Determination of glutathione S-transferase activity

The determination of glutathione-S-transferase activity (GST) was performed based on the method of Habig et al. (1974). The specific activity of GST was determined by the conjugation of dichloro-nitrobenzene with GSH, forming a thioether which can be monitored by increasing absorbance. Thus, 100 µl of the supernatant was added to 200 µl of reaction solution containing 3 mM dichloro-nitrobenzene (diluted in ethanol) and 3 mM GSH (diluted in phosphate buffer). The activity of the GST was quantified in intervals of 10 s for 1 min, in a spectrophotometer at 340 nm and expressed in µmoles/mg of protein/min.

2.8. Determination of superoxide dismutase activity

According to the method of Marklund and Marklund (1974) and Gao et al (1998), the superoxide dismutase activity was determined. The method is based on the inhibition of pyrogallol autoxidation by superoxide dismutase. Thus, 20 µl of supernatant was mixed with 25µl of 1 mM of pyrogallol and 300uL of 200 mM Tris HCl-EDTA buffer solution at pH 8.5, at room temperature for 20 min. The reaction was interrupted by the addition of 12.5 µl of 1M HCl and centrifuged for 4 min at 14000 g. The absorbance was measured at 405 nm using a microplate reader. The amount of superoxide dismutase that inhibited the oxidation of pyrogallol by 50%, relative to the control, was defined as one unit of SOD activity. The enzymatic activity was expressed as U/mg of protein.

2.9. Determination of catalase activity

The catalase activity was determined by measuring the rate of H_2O_2 conversion to O_2 at room temperature (Aebi, 1984). In a 96-well UV, the microplate was added 5 µl of supernatant in 295 µl of phosphate buffer pH 7 and 30 mM H_2O_2. The absorbance was measured for 1 minute at 240 nm in a microplate reader. The molar extinction coefficient of H_2O_2 43.6 cm^{-1} was used to determine the catalase activity, expressed as U/mg of protein.

2.10. Determination of lipoperoxides content

Ulcer tissue homogenates were diluted in 90% methanol (1:1) pH 6.5, sonicated and centrifuged at 14000 g for 15 min at 4°C. The number of lipoperoxides was determined by the FOX method described by Jiang et al. (1992). The FOX method is based on the oxidation of Fe^{2+} (ferrous ammonium sulfate) to Fe^{3+} by lipid hydroperoxide, in acidic medium, in the presence of a complex of Fe^{3+} (III) pigments of xylenol orange. Aliquots of the sample (30 µl) will be incubated for 30 min at room temperature with 270 µl of the reaction media containing

1 ml xylene orange, 250 μM $FeSO_4$, 25 mM H_2SO_4 and 4 mM BHT (Butylated Hydroxytoluene) in 90% methanol. The absorbances were measured at 560 nm in a spectrophotometer. The results were expressed in mmol of hydroperoxide/mg of tissue.

2.11. Statistical analysis

Results are expressed as mean ± standard error of the means (S.E.M). Therefore, statistical comparisons were performed by one-way analysis of variance (ANOVA), followed by Bonferroni's multi-comparison *pos-hoc* test for comparison of more than two groups. Unpaired *t*-test was used for comparison between two groups. The analyses were performed using GraphPad Prism® version 6.0 (GraphPad Software, San Diego, USA). Differences were considered as statistically significant when $*P \leq 0.05$.

3. RESULTS

3.1. *Bosentan, sildenafil or low-doses combination exhibited a gastroprotective effect against gastric ischemia-reperfusion injury*

Oral pretreatment of the animals with bosentan significantly reduced the lesion area in 51.1% and 61.3% at doses of 10 and 30 mg/kg respectively when compared to the vehicle group (190.7 ± 24.9 mm^2) (Fig. 1A). Sildenafil treatment at dose 5 mg/kg significantly reduced in 66.2% when compared to the vehicle group (204.0 ± 11.0 mm^2) (Fig. 1B). Low-doses combination of bosentan 3 mg/kg and sildenafil 1 mg/kg significantly reduced in 51.0% when compared to the vehicle group (182.1 ± 20.2 mm^2) (Fig. 1C).

Representative photographs of gastric tissues reveal extensive tissue injury by IR in the vehicle group (Fig. 2B), bosentan 3 mg/kg (Fig. 2C), sildenafil 1 mg/kg (Fig. 2D) or low-dose combination (Fig. 2E).

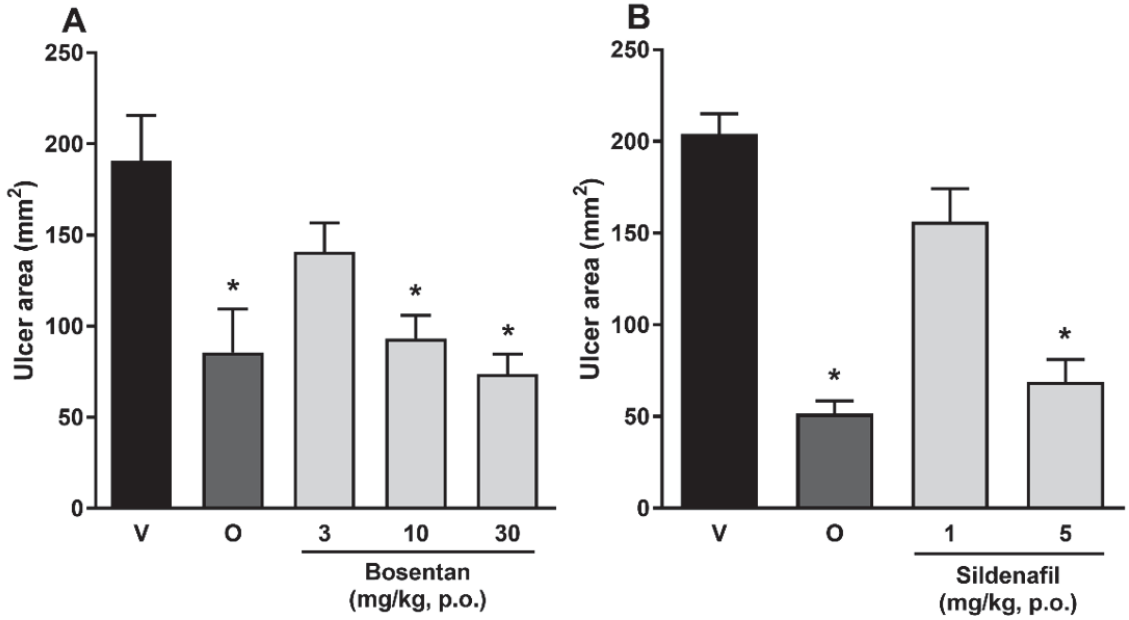


Fig. 1 Effect of bosentan and sildenafil on ulcer area on ischemic-reperfusion acute gastric ulcer in rats. Ulcer area (mm^2). **A-B:** Animals were orally pre-treated with vehicle (V), omeprazole (O: 40 mg/kg), bosentan (3, 10 or 30 mg/kg), sildenafil (1 or 5 mg/kg) 1h prior to ischemia induction. The results are expressed as mean ± S.E.M. (n=7). One-way ANOVA followed by Bonferroni's test. *P<0.05 when compared to the vehicle group (V).

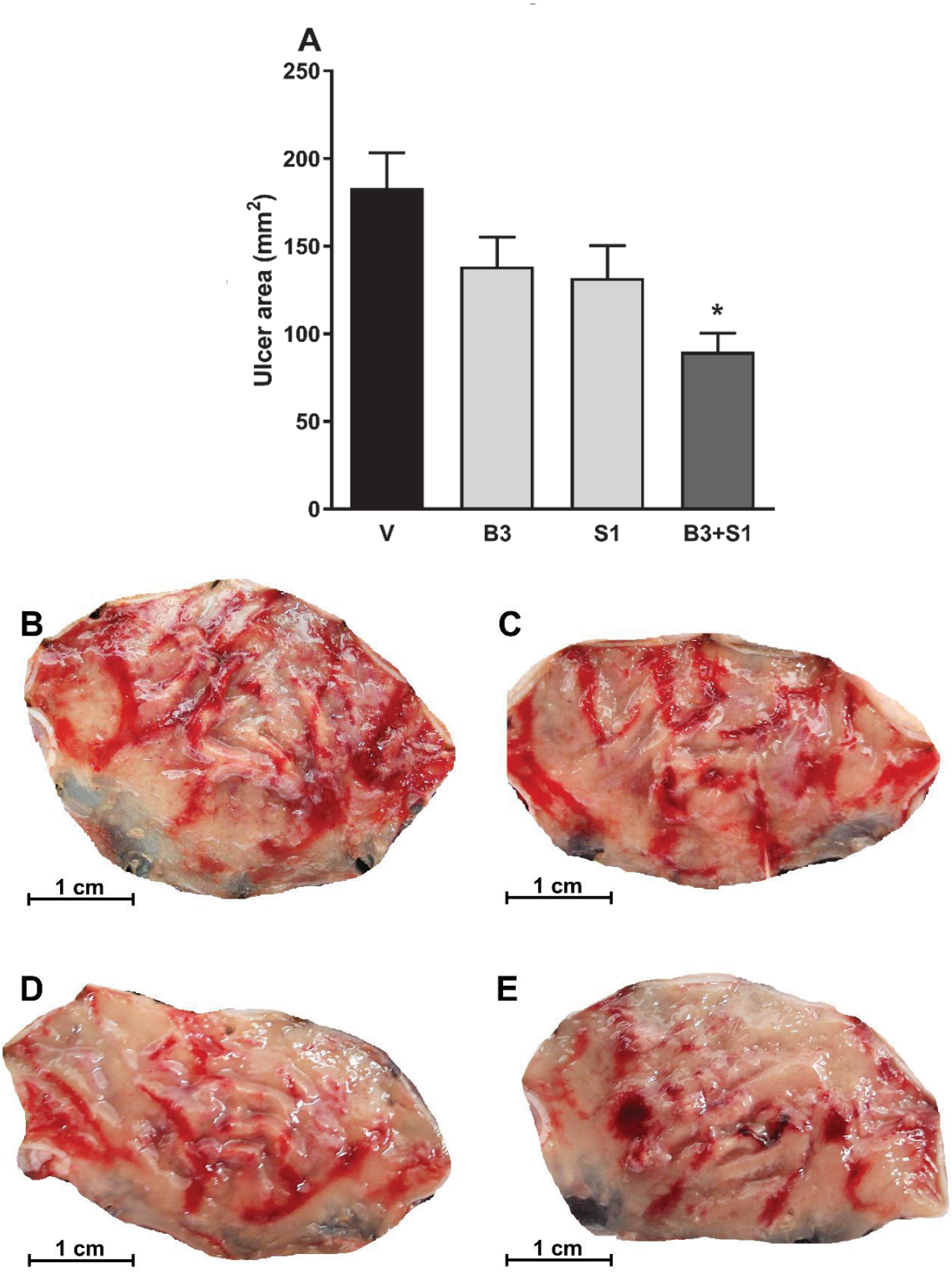


Fig. 2 A: Effect of bosentan, sildenafil or low dose combination on ulcer area on ischemic-reperfusion acute gastric ulcer in rats. Ulcer area (mm2). Animals were orally pretreated with vehicle (V), bosentan (B3: 3 mg/kg), sildenafil (S1: 1 mg/kg) or low-doses combination bosentan (B3: 3 mg/kg) and sildenafil (S1: 1 mg/kg) 1h prior to ischemia induction. The results are expressed as mean ± S.E.M. (n=7). One-way ANOVA followed by Bonferroni's test.

*P<0.05 when compared to the vehicle group (V). **B-E:** Representative macroscopic photograph of ulcer area on ischemic-reperfusion acute gastric ulcer in rats. Animals were orally treated with vehicle (Panel B), sildenafil (1 mg/kg; Panel C), bosentan (3 mg/kg; Panel D) or low-doses combination (bosentan 3 mg/kg and sildenafil 1 mg/kg) (Panel E), 1h prior to ischemia induction.

3.1. *Bosentan, sildenafil or low-doses combination preserves gastric mucus barrier against gastric ischemia-reperfusion injury*

The quantification of gastric mucus after gastric IR revealed that pretreatment with bosentan at doses of 10 or 30 mg/kg significantly preserved at 70.6% and 54.9%, respectively, when compared with the vehicle group (893.7 ± 80.0 µg of Alcian Blue/g of tissue) (Fig. 3A). Sildenafil at dose 5 mg/kg significantly preserved 74.6% when compared to the vehicle group (966.4 ± 34.5 µg of Alcian Blue/g of tissue) (Fig. 3B). The low-doses combination (bosentan 3 mg/kg and sildenafil 1 mg/kg) significantly preserved mucus in 56.4% when compared to the vehicle group (758.1 ± 42.8 µg of Alcian Blue/g of tissue) (Fig. 3C).

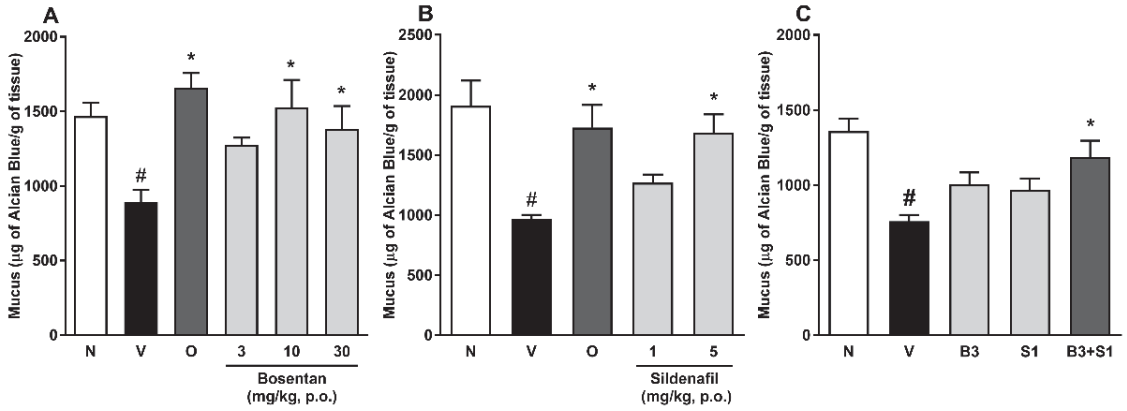


Fig. 3. Effect of bosentan, sildenafil or low dose combination on mucus content on ischemic-reperfusion acute gastric ulcer in rats. **A-C:** Mucus content (µg of Alcian Blue/g of tissue). Animals were orally pre-treated with vehicle (V), omeprazole (O: 40 mg/kg), bosentan (3, 10 or 30 mg/kg), sildenafil (1 or 5 mg/kg) or low-doses combination bosentan (B3: 3 mg/kg) and sildenafil (S1: 1 mg/kg) 1h prior to ischemia induction. The results are expressed as mean ± S.E.M. (n=5-8). One-way ANOVA followed by Bonferroni's test. *P<0.05 when compared to the vehicle group (V), #P<0.05 when compared to the naïve group (N).

3.2. Bosentan, sildenafil or low-doses combination prevent neutrophil infiltration to ulcerated gastric tissue

The myeloperoxidase content, an enzyme present in neutrophils infiltrated into the gastric mucosa due to IR injury, was significantly reduced by treatment with bosentan at doses 10 and 30 mg/kg in 42.3% and 38.8% respectively, compared to vehicle group (4.3 ± 0.3 mO.D/mg of protein) (Fig. 4A), while sildenafil at a dose of 5 mg/kg significantly reduced by 29.9% when compared to the vehicle group (4.5 ± 0.2 mO.D/mg of protein) (Fig. 4B), and the combination of bosentan and sildenafil at doses 3 mg/kg and 1 mg/kg respectively, significantly reduced in 42.2% when compared to the vehicle group (4.5 ± 0.4 mO.D/mg of protein) (Fig. 4C).

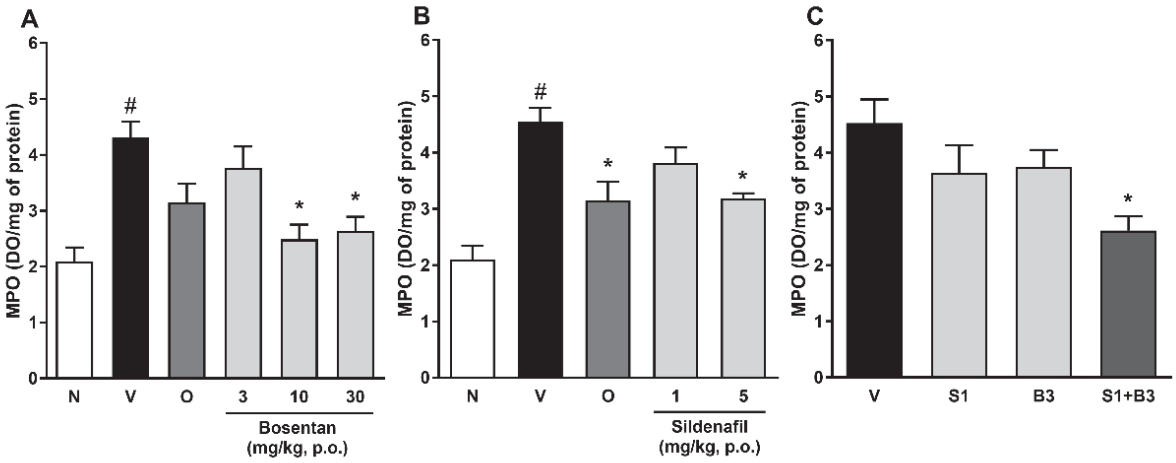


Fig. **4**. Effect of bosentan, sildenafil or low-dose combination on neutrophil infiltration on ischemic-reperfusion acute gastric ulcer in rats. **A-C**: Myeloperoxidase activity (MPO DO/mg of protein). Animals were orally pretreated with vehicle (V), omeprazole (O: 40 mg/kg) bosentan (3 (B3), 10 or 30 mg/kg), sildenafil (1 (S1) or 5 mg/kg) or low-doses combination bosentan (B3: 3 mg/kg) and sildenafil (S1: 1 mg/kg) 1h prior to ischemia induction. The results are expressed as mean ± S.E.M. (n=5). One-way ANOVA followed by Bonferroni's test.

*P<0.05 when compared to the vehicle group (V), #P<0.05 when compared to the naïve group (N).

3.3. Bosentan and sildenafil alone and in combination prevent endogenous antioxidant system depletion and reduce lipid peroxidation

Pretreatment with bosentan at doses of 10 mg/kg and 30 mg/kg was able to significantly prevented the gastric GSH depletion in 56.3% and 50.5% respectively when compared with the vehicle group (144.1 ± 6.3 GSH/g tissue) (Table 1). Sildenafil at a dose of 5 mg/kg was able to significantly prevented the gastric GSH depletion in 52.3% when compared to the vehicle group (142.8 ± 29.9 μg GSH/g tissue) (Table 2). The low-dose combination of bosentan 3 mg/kg and sildenafil 1 mg/kg significantly prevented the gastric GSH depletion in 42.2%, when compared with the vehicle group (144.8 ± 8.0 μg GSH/g tissue) (Table 3).

The GST activity was significantly preserved in 58% and 54.3% by pretreatment with bosentan at doses of 10 mg/kg and 30 mg/kg respectively, when compared with the vehicle group (133.1 ± 13.5 nmol/mg of protein/min-1) (Table 1). Sildenafil pretreatment at dose 5 mg/kg prevented the decrease of GST activity by 37.6%, when compared with the vehicle group (116.2 ± 10.1 nmol/mg of protein/min-1) (Table 2). The low-dose combination significantly prevented the decreased of GST activity by 30.2%, when compared with the vehicle group (120.5 ± 6.2 nmol/mg of protein/min-1) (Table 3).

The bosentan at 10 mg/kg and 30 mg/kg, sildenafil at dose 5 mg/kg and low dose combination pretreatments significantly prevented the increase of SOD activity, when compared to the respectively vehicle group (46.2 ± 4.0 U/mg of tissue) (Table 1), (50.4 ± 2.7 U/mg of tissue) (Table 2), and (40.6 ± 1.7 U/mg of tissue) (Table 3). The increase of CAT activity was prevented by pretreatment of bosentan at 10 mg/kg and 30 mg/kg, sildenafil at dose 5 mg/kg and low dose combination significantly, when compared to the respectively vehicle

group (3.8 ± 0.3 mmol/mg of protein/min^{-1}) (Table 1), (13.4 ± 0.2 mmol/mg of protein/min^{-1}), (Table 2), (15.4 ± 1.0 mmol/mg of protein/min^{-1}) (Table 3).

The LPO content was reduced by bosentan at 10 mg/kg and 30 mg/kg in 21.7% and 26.9% respectively, when compared with the vehicle group (4.3 ± 0.2 pmol/mg of protein). At dose 5mg/kg, the sildenafil pretreatment significantly reduced the LPO content in 36.2%, when compared to the vehicle group (4.1 ± 0.2 pmol/mg of protein) (Table 2). The low-dose combination of bosentan 3 mg/kg and sildenafil 1 mg/kg significantly reduced the LPO content in 31.9%, when compared to the vehicle group, (4.2 ± 0.4 pmol/mg of protein) (Table 3).

Table 1 – Effect of bosentan on GSH level, GST, SOD and CAT activity and LPO level on ischemic-reperfusion acute gastric ulcer in rats.

	Naïve	**Vehicle**	**Omeprazole**	**Bosentan (mg/kg)**		
				3	**10**	**30**
GSH (µg/g tissue)	234.0±11.9	144.1±6.3#	226.1±14.4*	117.4±24.9	225.3±9.0*	216.9±22.9*
GST (nmol/mg of protein/min^{-1})	242.1±19.9	133.1±13.5#	224.1±23.2*	156.2±1.4	210.3±18.5*	205.4±13.1*
SOD (U/mg of tissue)	29.2±1.6	46.2±4.0#	30.0±1.4*	41.0±1.2	34.4±3.9*	28.7±1.5*
CAT (mmol/mg of protein/min^{-1})	2.0±0.1	3.8±0.3#	2.8±0.1*	3.3±0.3	3.0±0.2*	2.8±0.1*
LPO (pmol/mg of protein)	2.1±0.25	4.3±0.28#	3.2±0.37*	3.1±0.3	2.5±0.27*	2.6±0.26*

The results are expressed as mean ± S.E.M. (n=6-8). One-way ANOVA followed by Bonferroni's test. *P<0.05 when compared to the vehicle group (V), #P<0.05 when compared to the naïve group (N).

Table 2 – Effect of sildenafil on GSH level, GST, SOD and CAT activity and LPO on ischemic-reperfusion acute gastric ulcer in rats.

	Naïve	Vehicle	Omeprazole	Sildenafil (mg/kg)	
				1	5
GSH (µg/g tissue)	284.6±15.6	142.8±29.9#	236.4±9.0*	184.9±13.9	217.5±7.9*
GST (nmol/mg of protein/min^{-1})	166.9±5.7	116.2±10.0#	177.0±15.4*	121.7±7.5	159.9±7.4*
SOD (U/mg of tissue)	35.6±1.0	50.4±2.7#	39.3±2.3*	47.4±3.9	39.6±1.48*
CAT (mmol/mg of protein/min^{-1})	10.1±0.7	13.4±0.2#	10.5±0.8*	12.3±0.8	10.4±0.9*
LPO (pmol/mg of protein)	2.3±0.2	4.0±0.2#	2.4±0.2*	4.3±0.4	2.6±0.4*

The results are expressed as mean ± S.E.M. (n=6-8). One-way ANOVA followed by Bonferroni's test. *P<0.05 when compared to the vehicle group (V), #P<0.05 when compared to the naïve group (N).

Table 3 – Effect of sildenafil, bosentan and combination on GSH level, GST, SOD and CAT activity on ischemic-reperfusion acute gastric ulcer in rats.

	Vehicle	Sildenafil (mg/kg)	Bosentan (mg/kg)	Sildenafil + Bosentan (mg/kg)
		1	3	1 + 3
GSH (µg/g tissue)	144.8±8.1	170.0±16.7	168.5±16.6	209.9±6.9*
GST (nmol/mg of protein/min^{-1})	120.5±6.2	121.7±8.9	139.7±6.4	156.9±4.2*
SOD (U/mg of tissue)	40.6±1.7	35.7±2.0	36.8±2.9	31.29±1.4*
CAT (mmol/mg of protein/min^{-1})	15.4±1.0	13.8±0.5	14.5±0.8	10.7±1.1*
LPO (pmol/mg of protein)	4.2±0.4	3.9±0.3	3.9±0.2	2.9±0.1*

The results are expressed as mean ± S.E.M. (n=6-8). One-way ANOVA followed by Bonferroni's test. *P<0.05 when compared to the vehicle group (V), #P<0.05 when compared to the naïve group (N).

4. DISCUSSION

The maintenance of blood flow in gastric ulcers is crucial for toxins removal and buffering the acid penetrates in lamina propria through the epithelium. The reactive hyperemia increases the protection of aggressive factors and allows the reconstitution of gastric epithelium [30]. Furthermore, vasoactive drugs such as bosentan and sildenafil, have vasodilation effects and increases stomach blood flow. These other effects include gastroprotection against the aggressor agents by maintaining protective factors such as mucus, endogenous antioxidant defenses and preventing inflammatory cell infiltration in the affected organ.

The analysis of the lesioned area showed that bosentan 10 or 30 mg/kg, sildenafil 5 mg/kg or combination of low doses protected the mucosa against IR injury, that can be observed by in the small lesioned area (Figure 1). The protection of bosentan and sildenafil in damaged tissue by IR is not limited to the stomach, such as in this study, as well seen in studies such protection occurs other organs, such as in the kidney [31], liver [32], skeletal muscle [33], testicles [34] e myocardium [35].

The gastric IR injury causes depletion of the stomach's defense mechanisms, such as the mucus barrier, which is the first defense against aggressive factors such as pepsin and HCl [2,36]. The pretreatment with bosentan, sildenafil or low-dose combination preserves the gastric mucus barrier during IR (Figure 3). These effects could associate with the vasodilator effect of bosentan and sildenafil that increase gastric blood flow, which maintains the mucus production, as well as sildenafil increases the intracellular cGMP concentration in gastric epithelial cells [2,37–39].

During IR lesion, endothelial cells produce and release ET-1, which stimulates VCAM-1 expression and neutrophil-like cells migration from the bloodstream to the tissues, performing the respiratory burst with a massive generation of ROS [40,41]. NO is involved, via activation of guanylyl cyclase and cGMP production, is responsible to inhibit expression of P-selectin on the vascular endothelium and the β-2 adhesion molecules on neutrophils [42]. Decreased neutrophil infiltration during IR injury is closely related to a minor gastric injury on both acute and chronic ulcer models [43–45]. Therefore, treatment with bosentan, sildenafil or low-dose combination decreased neutrophil infiltration into the stomach (Figure 4A, B and C), which may have contributed to lower oxidative stress and gastric damage [46,47].

Gastric IR injury causes an extensive depletion of non-enzymatic and enzymatic antioxidant defenses in the stomach [48]. During oxidative stress in the tissues, intracellular GSH is consumed to neutralize ROS and free radicals, preventing them from causing cell damage and death, such as membrane lipid peroxidation [48]. Lipid peroxidation in IR occurs due to the oxidation of membrane phospholipid double bonds by the superoxide anion generating lipoperoxide byproduct [49]. Therefore, increased lipoperoxide levels indicate major cellular damage by oxidative stress. Thus, both bosentan, sildenafil and the low-dose combination showed protection against oxidative stress in IR ulcers. Our results bosentan (Table 1), sildenafil (Table 2) or low-dose combination treatments (Table 3) prevents the GSH depletion and lipid peroxidation (LPO). Equally important, the GST enzyme is responsible for restoring the GSH depleted by gastric IR [50]. However, IR damage, depending on the intensity, may deplete GSH levels to the point that GST is unable to restore its amount due to cellular oxidative damage sustained [51,52]. In the stomach, the SOD enzyme performs the superoxide anion (O^{-}_{2}) dismutation into H_2O_2, converted next to H_2O and O_2 by the CAT enzyme [53]. Erythrocytes contain large amounts of SOD and CAT enzymes and IR injury causes vascular damage with blood leakage from vessels to tissue, increased activity of these enzymes along

with oxidative damage in injured tissue [54]. Thus, the lower activity of such enzymes in gastric tissue is justified by the smaller area IR injury, as seen in models of tissue damage by IR and NSAID [55,56]. Therefore, the minor SOD and CAT enzymes activity in the stomach by bosentan and sildenafil pre-treatments indicates a lower degree IR injury (Table 1-3).

5. CONCLUSION

Based on the results presented in this paper, we can conclude that vasoactive drugs such as bosentan and sildenafil or low-dose combination exhibit prominent gastroprotective effect against ischemia-reperfusion gastric injury. This protective effect occurs due to the maintenance of mucosal defenses, such as gastric mucus, endogenous antioxidant defenses and the reduction of factors that contribute to injury, such as neutrophil infiltration and cellular damage caused by oxidative stress.

Acknowledgments

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) - Research Grant: #3038751/2017-8, and was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

Author Contributions

D.M.F., B.B.L., P.G.C, and M.F.P.W contributed to the concept and design of the study.

Declarations of interest: The authors declare that they have no conflict of interest.

6. REFERENCES

1 Lanas A, Chan FKL, Kirsch JM, Hirsch-Reilly C. Peptic ulcer disease. Lancet. 2017 Aug;390(10094):613–24.

2 Yandrapu H, Sarosiek J. Protective Factors of the Gastric and Duodenal Mucosa: An Overview. Curr Gastroenterol Rep. 2015 Jun;17(6):24.

3 Casey KM, Quigley TM, Kozarek RA, Raker EJ. Lethal nature of ischemic gastropathy. Am J Surg. 1993 May;165(5):646–9.

4 Tang S, Daram SR, Wu R, Bhaijee F. Pathogenesis, Diagnosis, and Management of Gastric Ischemia. Clin Gastroenterol Hepatol. 2014 Feb;12(2):246-252.e1.

5 Shafii AE, Agle SC, Zervos EE. Perforated gastric corpus in a strangulated paraesophageal hernia: a case report. J Med Case Rep. 2009 Dec;3(1):6507.

6 Sahoo MR, Kumar AT, Jaiswal S, Bhujabal SN. Acute Dilatation, Ischemia, and Necrosis of Stomach without Perforation. Case Rep Surg. 2013;2013:1–4.

7 Sutcliffe AM, Clarke DL, Bradbury DA, Corbett LM, Patel JA, Knox AJ. Transcriptional regulation of monocyte chemotactic protein-1 release by endothelin-1 in human airway smooth muscle cells involves NF-κB and AP-1. Br J Pharmacol. 2009 Apr;157(3):436–50.

8 Schein M, Saadia R. Postoperative gastric ischaemia. Br J Surg. 1989 Aug;76(8):844–8.

9 Sharma A, Mukewar S, Chari ST, Wong Kee Song LM. Clinical Features and Outcomes of Gastric Ischemia. Dig Dis Sci. 2017 Dec;62(12):3550–6.

10 Chamoun F, Burne M, O'Donnell M, Rabb H. Pathophysiologic role of selectins and their ligands in ischemia reperfusion injury. Front Biosci. 2000 Nov;5:E103-9.

11 Abdallah DM, El-Abhar HS, Abdel-Aziz DH. TEMPOL, a membrane-permeable radical scavenger, attenuates gastric mucosal damage induced by ischemia/reperfusion: A key

role for superoxide anion. Eur J Pharmacol. 2009 Jan;603(1–3):93–7.

12 El-Abhar HS, Abdallah DM, Saleh S. Gastroprotective activity of Nigella sativa oil and its constituent, thymoquinone, against gastric mucosal injury induced by ischaemia/reperfusion in rats. J Ethnopharmacol. 2003 Feb;84(2–3):251–8.

13 S. Tarnawski A, Ahluwalia A, K. Jones M. The Mechanisms of Gastric Mucosal Injury: Focus on Microvascular Endothelium as a Key Target. Curr Med Chem. 2012;19(1):4–15.

14 Nehra AK, Alexander JA, Loftus CG, Nehra V. Proton Pump Inhibitors: Review of Emerging Concerns. Mayo Clin Proc. 2018;93(2):240–6.

15 Xie Y, Bowe B, Li T, Xian H, Balasubramanian S, Al-Aly Z. Proton Pump Inhibitors and Risk of Incident CKD and Progression to ESRD. J Am Soc Nephrol. 2016 Oct;27(10):3153–63.

16 Schoenfeld AJ, Grady D. Adverse Effects Associated With Proton Pump Inhibitors. JAMA Intern Med. 2016 Feb;176(2):172.

17 Gray SL. Proton Pump Inhibitor Use, Hip Fracture, and Change in Bone Mineral Density in Postmenopausal Women. Arch Intern Med. 2010;170(9):765.

18 Lo W, Chan WW. Proton Pump Inhibitor Use and the Risk of Small Intestinal Bacterial Overgrowth: A Meta-analysis. Clin Gastroenterol Hepatol. 2013 May;11(5):483–90.

19 Kwok CS, Arthur AK, Anibueze CI, Singh S, Cavallazzi R, Loke YK. Risk of Clostridium difficile Infection With Acid Suppressing Drugs and Antibiotics: Meta-Analysis. Am J Gastroenterol. 2012 Jul;107(7):1011–9.

20 Xie Y, Bowe B, Li T, Xian H, Yan Y, Al-Aly Z. Risk of death among users of Proton Pump Inhibitors: a longitudinal observational cohort study of United States veterans. BMJ Open. 2017 Jun;7(6):e015735.

21 Padol I, Huang JQ, Hunt RH. Anti-ulcerogenic properties of endothelin receptor

antagonists in the rat. Aliment Pharmacol Ther. 1999;13(4):537–44.

22 Said SA, Elmowafy AM. Role of endogenous endothelin-1 in stress-induced gastric mucosal damage and acid secretion in rats. Regul Pept. 1998;73(1):43–50.

23 Duggan A, Stack W, Hull M, Filipowicz B, Knifton A, Crome R, et al. Protection against aspirin-induced human gastric mucosal injury by bosentan, a new endothelin-1 receptor antagonist. Aliment Pharmacol Ther. 1999 May;13(5):631–5.

24 Krejci V, Hiltebrand LB, Erni D, Sigurdsson GH. Endothelin receptor antagonist bosentan improves microcirculatory blood flow in splanchnic organs in septic shock. Crit Care Med. 2003 Jan;31(1):203–10.

25 Medeiros JVR, Gadelha GG, Lima SJ, Garcia JA, Soares PMG, Santos AA, et al. Role of the NO/cGMP/K ATP pathway in the protective effects of sildenafil against ethanol-induced gastric damage in rats. Br J Pharmacol. 2008 Feb;153(4):721–7.

26 Moustafa YM, Khoder DM, El-Awady EE, Zaitone SA. Sildenafil citrate protects against gastric mucosal damage induced by indomethacin in rats. Eur Rev Med Pharmacol Sci. 2013 Jan;17(2):179–88.

27 Sawatzky DA, Megson IL, Rossi AG. Sildenafil offers protection against NSAID-induced gastric injury. Br J Pharmacol. 2005 Oct;146(4):477–8.

28 De Young LM, Kheifets JB, Ballaron SJ, Young JM. Edema and cell infiltration in the phorbol ester-treated mouse ear are temporally separate and can be differentially modulated by pharmacologic agents. Agents Actions. 1989 Mar;26(3–4):335–41.

29 Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of Cutaneous Inflammation: Estimation of Neutrophil Content with an Enzyme Marker. J Invest Dermatol. 1982 Mar;78(3):206–9.

30 Holzer P, Pabst MA, Lippe IT, Peskar BM, Peskar BA, Livingston EH, et al. Afferent nerve-mediated protection against deep mucosal damage in the rat stomach.

Gastroenterology. 1990 Apr;98(4):838–48.

31 Afyouni N, Halili H, Moslemi F, Nematbakhsh M, Talebi A, Shirdavani S, et al. Preventive role of endothelin antagonist on kidney ischemia: Reperfusion injury in male and female rats. Int J Prev Med. 2015;6(1):128.

32 Uhlmann D, Uhlmann S, Spiegel HU. Important role for endothelins in acute hepatic ischemia/reperfusion injury. J Invest Surg. 14(1):31–45.

33 Herbert KJ, Hickey MJ, Lepore DA, Knight KR, Morrison WA, Stewart AG. Effects of the endothelin receptor antagonist Bosentan on ischaemia/reperfusion injury in rat skeletal muscle. Eur J Pharmacol. 2001 Jul;424(1):59–67.

34 Turkili B, Kurcer Z, Dengiz GO, Kandemir NO, Mungan G, Ozacmak VH, et al. Role of angiotensin and endothelin in testicular ischemia reperfusion injury. Int J Urol. 2012 Mar;19(3):257–63.

35 Gupta SK, Saxena A, Singh U, Arya DS. Bosentan, the mixed ETA-ETB endothelin receptor antagonist, attenuated oxidative stress after experimental myocardial ischemia and reperfusion. Mol Cell Biochem. 2005 Jul;275(1–2):67–74.

36 Ichikawa T, Ishihar K. Protective Effects of Gastric Mucus. Gastritis and Gastric Cancer - New Insights in Gastroprotection, Diagnosis and Treatments. InTech; 2011. DOI: 10.5772/23951

37 Matsumaru K. Gastroenterology Bosentan , a novel synthetic mixed-type endothelin receptor antagonist , attenuates acute gastric mucosai lesions induced by indomethacin and HCI in the rat : Role of endogenous endothelin-1. 1997;164–70.

38 Duffin R, Shaw CA, Rossi AG. Sildenafil reduces alcohol-induced gastric damage: just say ‘NO.’ Br J Pharmacol. 2008 Feb;153(4):623–4.

39 Brown JF, Keates AC, Hanson PJ, Whittle BJ. Nitric oxide generators and cGMP stimulate mucus secretion by rat gastric mucosal cells. Am J Physiol Liver Physiol. 1993

Sep;265(3):G418–22.

40 El-Benna J, Hurtado-Nedelec M, Marzaioli V, Marie J-C, Gougerot-Pocidalo M-A, Dang PM-C. Priming of the neutrophil respiratory burst: role in host defense and inflammation. Immunol Rev. 2016 Sep;273(1):180–93.

41 Hassan M, Kashimura H, Matsumaru K. Gastric Mucosal Injury Induced by Local Ischemia ± Reperfusion in Rats Role of Endogenous Endothelin-1 and Free Radical. 1997;42(7):1375–80.

42 Davenpeck KL, Gauthier TW, Lefer AM. Inhibition of endothelial-derived nitric oxide promotes P-selectin expression and actions in the rat microcirculation. Gastroenterology. 1994 Oct;107(4):1050–8.

43 Kobayashi T, Ohta Y, Yoshino J, Nakazawa S. Teprenone promotes the healing of acetic acid-induced chronic gastric ulcers in rats by inhibiting neutrophil infiltration and lipid peroxidation in ulcerated gastric tissues. Pharmacol Res. 2001 Jan;43(1):23–30.

44 da Silva LM, Pezzini BC, Somensi LB, Bolda Mariano LN, Mariott M, Boeing T, et al. Hesperidin, a citrus flavanone glycoside, accelerates the gastric healing process of acetic acid-induced ulcer in rats. Chem Biol Interact. 2019 Aug;308:45–50.

45 Maria-Ferreira D, Da Silva LM, Mendes DAGB, De Almeida Cabrini D, Nascimento AM, Iacomini M, et al. Rhamnogalacturonan from Acmella oleracea (L.) R.K. Jansen: Gastroprotective and ulcer healing properties in rats. PLoS One. 2014;9(1). DOI: 10.1371/journal.pone.0084762

46 Gonon AT, Gourine A V, Middelveld RJ, Alving K, Pernow J. Limitation of infarct size and attenuation of myeloperoxidase activity by an endothelin A receptor antagonist following ischaemia and reperfusion. Basic Res Cardiol. 2001 Sep;96(5):454–62.

47 Zarpelon AC, Pinto LG, Cunha TM, Vieira SM, Carregaro V, Souza GR, et al. Endothelin-1 induces neutrophil recruitment in adaptive inflammation via TNFα and

CXCL1/CXCR2 in mice. Can J Physiol Pharmacol. 2012;90(2):187–99.

48 De-Faria FM, Almeida ACA, Luiz-Ferreira A, Takayama C, Dunder RJ, Da Silva MA, et al. Antioxidant action of mangrove polyphenols against gastric damage induced by absolute ethanol and ischemia-reperfusion in the rat. Sci World J. 2012;2012:1–9.

49 Sakamoto A, Tsuyoshi Ohnishi S, Ohnishi T, Ogawa R. Relationship between free radical production and lipid peroxidation during ischemia-reperfusion injury in the rat brain. Brain Res. 1991 Jul;554(1–2):186–92.

50 Hirokawa K, Kawasaki H. Changes in glutathione in gastric mucosa of gastric ulcer patients. Res Commun Mol Pathol Pharmacol. 1995 May;88(2):163–76.

51 Hoppenkamps R, Thies E, Younes M, Siegers C-P. Glutathione and GSH-dependent enzymes in the human gastric mucosa. Klin Wochenschr. 1984 Feb;62(4):183–6.

52 Dengiz GO, Odabasoglu F, Halici Z, Cadirci E, Suleyman H. Gastroprotective and Antioxidant Effects of Montelukast on Indomethacin-Induced Gastric Ulcer in Rats. J Pharmacol Sci. 2007;105(1):94–102.

53 Tandon R, Khanna HD, Dorababu M, Goel RK. Oxidative stress and antioxidants status in peptic ulcer and gastric carcinoma. Indian J Physiol Pharmacol. 2004 Jan;48(1):115–8.

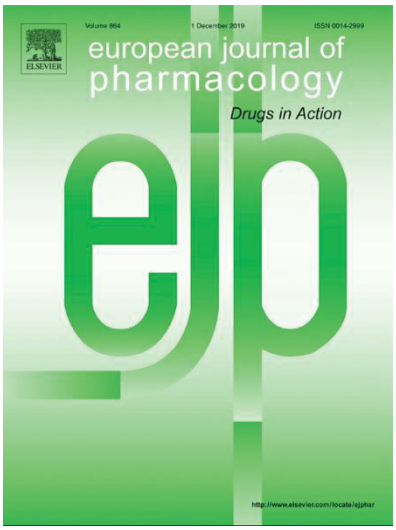
54 Scott MD, Eaton JW, Kuypers FA, Chiu DT, Lubin BH. Enhancement of erythrocyte superoxide dismutase activity: effects on cellular oxidant defense. Blood. 1989 Nov;74(7):2542–9.

55 Tas U, Ayan M, Sogut E, Kuloglu T, Uysal M, Tanriverdi H, et al. Protective effects of thymoquinone and melatonin on intestinal ischemia–reperfusion injury. Saudi J Gastroenterol. 2015;21(5):284.

56 ZHOU Y-L, WANG R, FENG X, ZHAO X. Preventive effect of insect tea against reserpine-induced gastric ulcers in mice. Exp Ther Med. 2014 Oct;8(4):1318–24.

3 MANUSCRITO 2

Author's Submitted Manuscript



Journal: European Journal of Pharmacology

Title: BOSENTAN, A NON-SELECTIVE ET_A/ET_B RECEPTOR ANTAGONIST EMERGE AS A VALUABLE THERAPY FOR GASTRIC WOUND HEALING

Corresponding Author: Maria Fernanda Werner

Co-Authors: Fernando T Dal Lin, master; Daniele Maria-Ferreira, PhD; Bruna B da Luz, master; Lia S Nakao, PhD;

BOSENTAN, A NON-SELECTIVE ET_A/ET_B RECEPTOR ANTAGONIST EMERGE AS A VALUABLE THERAPY FOR GASTRIC WOUND HEALING

Fernando Tonholi Dal Lin[a], Daniele Maria Ferreira[a], Bruna Barbosa da Luz[a], Lia Sumie Nakao[b], Maria Fernanda de Paula Werner[a*]

[a] *Department of Pharmacology, Federal University of Parana, Curitiba, PR, Brazil.*

[b] *Department of Basic Pathology, Federal University of Parana, Curitiba, PR, Brazil.*

Corresponding author at: Federal University of Parana, (UFPR), Biological Science Sector, Department of Pharmacology, PO Box 19031, Curitiba, PR 81531-980, Brazil. Tel.: +55 41 3361-1721, e-mail address: mfernanda.werner@ufpr.br (M.F. P. Werner).

ABSTRACT

The incidence of gastric ulcers and its complications remains a worldwide health problem. The long last inhibition of gastric acid secretion by conventional ulcer treatment with PPIs or H_2 antagonists, leads to diverse adverse effects and is associated with the development numerous pathologies. Beyond the gastric acid, the endothelins are one of the many signaling systems that can contribute significantly to the development and persistence of chronic gastric ulcer. The present study was conducted to investigate the potential new use of bosentan, a non-selective ET_A/ET_B, for the healing chronic gastric ulcers. Additionally, we showed the impact of bosentan on the acid gastric secretion. Chronic ulcers were induced in male Wistar rats by serosal application of 80% acetic acid, and treatments starting 2 days after ulcer induction. Rats were orally treated with vehicle, omeprazole or bosentan (3, 10 or 30 mg/kg), twice daily for 5 days. The gastric secretion was evaluated by pylorus ligation method. After the end of

treatment, the gastric tissue and blood samples were collected for analysis. The bosentan treatment accelerates the gastric healing, increased the cell proliferation and increased the gastric mucus layer on the 7th day following ulcer induction. In agreement with the ulcer healing results, bosentan treatments reduced both neutrophils infiltration, increased prostaglandin E2, and inflammatory cytokines levels (TNF-α and IL-1β) in ulcerated tissue. Immunoblotting analysis revealed that the relative protein expression of ET_A and ET_B receptors were increased in ulcer tissue in the vehicle-treated group. Nevertheless, in contrast to omeprazole, the volume, pH and total acidity of acid gastric secretion were unchanged by bosentan. Plasma samples revealed no alterations on biochemical markers of renal and hepatic function. The results indicate that the bosentan treatment accelerates the gastric mucosa healing without antisecretory mechanisms. Further, these findings suggest a potential off-label use of bosentan for healing gastric ulcers, avoiding the adverse effects of inhibition of gastric acid secretion.

Keywords: gastric ulcer; bosentan; endothelin; ET_A receptor; ET_B receptor; inflammation; cytokines.

1. INTRODUCTION

Chronic gastric ulcer is a necrotic lesion in gastric mucosa, with a delimited border that penetrates through the entire thickness of the epithelium and through the muscular layer [1]. Gastric ulcers emerge from the imbalance between aggressive factors (gastric acid hypersecretion e.g.) and protective factors (gastric mucus bicarbonate production, e.g.), and the impaired mucosal microcirculation of the mucosal layer plays a key role in the gastric ulcer development [2,3]. The main exogenous aggressive factors that develops gastric ulcer are the use of non-steroidal anti-inflammatory (NSAIDs) and *Helicobacter pylori* infection, and as risk factors are psychological stress and ethanol consumption [2–4].

The reduction of acid gastric secretion, by proton pump inhibitors (PPIs) or H_2 receptor antagonists, is the major purpose of the conventional pharmacological treatment, but the long-term inhibition of acid secretion leads to several health issues, such as vitamin B12 deficiency, anemia, osteoporosis-related fractures and gastrointestinal infections [5–7]. In addition to these adverse effects, studies showed that's gastric acid secretion is an increased risk for the development of numerous diseases, such as chronic kidney disease, dementia and spontaneous bacterial peritonitis [8–10]. Despite the advances in gastric ulcer treatment until the 90's, this disease remains a worldwide public health problem, which incidence and its complications varies across the world [11–13]. Therefore, there is a need for a new anti-ulcer drug, which presents a higher efficacy, low or no toxicity and no adverse effects associated with inhibition of gastric acid secretion. Thus, it is natural to think about new molecular targets, such as the endothelinergic system, which studies indicate its participation in ulcerogenesis [14–16].

The endothelin-1 (ET-1), produced by endothelial cells, was identified as the most potent vasoconstrictor peptide [17]. ET-1 effects are mediated through activation of the endothelin receptor A (ET_A) and endothelin receptor B (ET_B) is widely distributed in the tissues, including the gastric tissue [18–20]. Physiologically, the activation of these receptors by endothelins maintain the vascular tonus of the vessels and regulates the microcirculation in tissues, including the gastric tissue [21]. Nevertheless, the function of endothelins goes beyond a vasoconstrictor peptide, it performs as a cytokine-like molecule with multiple cellular functions [22].

Emerging evidences show that endothelins may play a critical role in pathogenesis and inflammation on numerous diseases, such as systemic sclerosis, asthma and chronic kidney disease [23–25]. Endothelins exacerbate the gastric lesions and contribute to the gastric mucosal inflammation and microcirculatory disturbances in animals [26,27]. Similarly in humans was reported an significant elevation of ET-1 in plasma and gastric tissue, by acute

ethanol consumption [28]. Consequently, pharmacological antagonism of ET_A or ET_B or both receptors was used in research in the role of endothelins in numerous pathological processes, reviewed by Barton [29]. Bosentan, a non-selective antagonist of ETA and ETB receptors, was initially approved by FDA for pulmonary arterial hypertension, and subsequently for off-label use for healing and prevent digital ulcers, a complication in patients with systemic sclerosis provoked by hemodynamic disturbances [30,31]. The off-label drug uses provide the initially evidences to drug repositioning researches programs, such as the Discovering New Therapeutic Uses for Existing Molecules Programme, by the National Centre for Advancing Translational Sciences in USA [32]. Compare to the new drug discovery the drug repositioning is remarkably superior in many aspects, such as a lower cost, time and a higher safety in the preclinical and clinical trials [33].

In acute gastric ulcers bosentan showed a notable gastroprotective effect, preventing the mucosal lesions and the reduction in gastric blood flow [28,34,35]. However, there is a lack of studies on the effect of bosentan in chronic gastric ulcers. Thus, it was hypothesized that bosentan, in addition to the gastroprotective effect in acute models, could have a healing effect on chronic gastric ulcers, which may contribute to a future drug repositioning.

2. MATERIAL AND METHODS

2.1. Animals

Male Wistar rats (180-200 g) supplied by BSU of Federal University of Paraná, Curitiba, Paraná, were used in all the *in vivo* experiments. The rats were housed in cages at 22 ± 2 °C 12h/12h cycles and free access to water and food (Nuvital®, Curitiba-PR, Brazil). All *in vivo* protocols were approved by Animal Ethics Committee of Biological Science Sector of Federal University of Paraná (CEUA/BIO-UFPR) on approval number 1054.

2.2. Induction of chronic gastric ulcer by acetic acid

The used model of chronic ulcers by acetic acid that proposed by Okabe, Roth & Pfeiffer [36], with some modifications. The animals were anesthetized with ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.) and the stomachs were exposed by an incision in the abdominal wall. A plastic cylinder of 6 mm of diameter was topically applied to the serosa stomach and 80% solution of acetic acid (*v/v,* 0.5 mL) was instilled into the cylinder. The acid was aspirated after 1 min and the stomach was washed with sterile saline. Afterward, the stomach was reinserted into the abdominal cavity and the incision was sutured. After anesthesia recovery, the animals were remained fasten, with water *ad libitum* until the next day. During the diet period, the food was offered twice daily for 1h for seven days. Forty-eight hours later the ulcer induction, the rats were orally treated with vehicle (water, 1 mL/kg), omeprazole (40 mg/kg) or bosentan (3, 10 or 30 mg/kg - Actelion Pharmaceuticals Ltd. Allschwil, Switzerland - PubChem CID: 104865), twice daily for 5 days. On the eighth day after ulcer induction, the animals were euthanized by a lethal dose of sodium thiopental and the stomachs were removed for the *ex vivo* experiments. The ulcer diameter was measured (mm^2).

2.3. Determination of EA_A/ET_B receptors expression by immunoblotting

The hole stomachs were homogenized in cold RIPA buffer (1% Triton X-100/1% sodium deoxycholate/0.1% $NaDodSO_4$/150 mM NaCl/50 mM Tris HCl, pH 7.4) with the addition of a proteinase inhibitor cocktail and phosphate inhibitor cocktail (Sigma, MO, USA) and centrifuged at 12.000 g for 10 min at 4ºC. The supernatants were collected, and protein concentration was estimated by Bradford protein assay kit (Biorad Laboratories, Hercules, CA, USA). An equal amount of protein (50 μg) was loaded on to 12% SDS-PAGE gel electrophoresis and wet-blotted onto a nitrocellulose membrane (Bio-Rad Laboratories, United States). The blocking of non-specific signals was minimized with 5% non-fat milk in TBST.

Then, the primary antibodies were incubated overnight at 4ºC, 1:200 rabbit anti-ET_A receptor (sc-33535 Santa Cruz Biotechnology, USA) 1:200 goat anti-ET_B receptor 1:200 (sc-21196, Santa Cruz Biotechnology), 1:4000 mouse anti-GAPDH (sc-32233, Santa Cruz Biotechnology, USA). After, the membranes were washed four times with TBST and the secondary antibodies were incubated for 1h at room temperature following 1:4000 mouse anti-rabbit IgG-HRP (sc-2357, Santa Cruz Biotechnology, USA), 1:7500 rabbit anti-goat IgG-HRP (sc-2768, Santa Cruz Biotechnology, USA), 1:8000 goat anti-mouse IgG-HRP (A4416, Sigma, USA). Then, the membranes were washed four times and SuperSignal West Pico chemiluminescent substrate was added (Thermo Fisher Scientific, IL, USA). The immunoreactive bands were detected by Amersham Imager 600 (GE life sciences, UK). To quantify the immunoreactive bands was used Image J® software.

2.4. Assessment of gastric ulcers and mucin-like glycoproteins content by histological analysis

The chronic gastric ulcer tissue was fixed in 10% formalin, 85% ethanol, 5% acetic acid for 24 h. After fixation, the tissues were dehydrated in ascending grades of ethanol, cleared in xylene and embedded in liquid paraffin wax. Sections of 5 μm of thickness were cut from blocks and mounted on homemade gelatin coated microscopic slides. The sections were dewaxed in xylene, rehydrated through graded series from ethanol to water and stained with hematoxylin and eosin (H&E) for pathological analysis, or periodic acid Schiff (PAS) for quantification of mucus content.

2.5. Immunohistochemistry for gastric epithelial cell proliferation

Paraffin-embedded sections were dewaxed in xylene, rehydrated through graded series from ethanol to water followed by antigen retrieval with citrate buffer (10 mM, pH 6.0).

To block were unreacted aldehydes the slides were incubated in 0.2 M glycine solution for 3 minutes. The endogenous peroxidase activity was blocked in 3% H_2O_2 in methanol for 10 minutes. The slides were incubated with the primary antibody 1:200 goat anti-PCNA (sc-9857, Santa Cruz Biotechnology, USA) overnight at 4 ºC in a humidified chamber. The HRP-conjugated secondary antibody 1:200 rabbit anti-goat HRP (sc-2768, Santa Cruz Biotechnology, USA) was incubated on the slides for 1 h in a humidified chamber and staining with chromogen diaminobenzidine solution (DAB substrate BD pharmigen™). The sections were counterstained with hematoxylin and mounted with Entellan® (Merk Millipore, USA).

2.6. Assessment of gastric inflammatory parameters

2.6.1. Determination of neutrophils infiltration by myeloperoxidase actividy

The actividy of neutrophil-specific myeloperoxidase was measured by the method of Bradley [37] and modified by De Young [38]. The ulcers samples were homogenized in 200 mM phosphate potassium buffer pH 6.5 and centrifuged at 9000 g for 20 min at 4 ºC. The pellets were resuspended with 1 mL of phosphate potassium buffer in the presence of 0.5% hexadecyltrimethylammonium (HTAB). The samples were sonicated for 1 min and centrifuged at 11000 g for 20 min at 4 °C. In 96 well plates, 30 µL of the supernatant was added in 220 µL of a solution of 100 µL of 80 mM potassium phosphate buffer, 85 µL of 22 mM sodium phosphate buffer and 15 µL 0.017% of H_2O_2. The reaction was started with the addition of 20 µL of tetramethylbenzidine (TMB) and incubated for 3 min at 37 °C. The reaction was interrupted by 30 µL of 1.46 M sodium acetate (pH = 3.0) and the enzyme levels were determined in a spectrophotometer at 620 nm. The results were expressed as a unit of optical density (O.D.)/mg protein.

2.6.2. Gastric cytokines and plasma PGE-2 determination by ELISA

The stomach homogenate samples were prepared as described above (*Section 2.3. Determination of ETA/ETB receptors expression levels by immunoblotting*). The levels of interleukin 1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) were measured by rat IL-1β Standard ABTS ELISA development and, murine TNF-α standard TMB ELISA development kit, respectively, strictly according to the manufacturer's protocol (Peprotech®, Rocky Hill, NJ, USA). The absorbances were measured in a Synergy HT microplate reader at 405 nm with wavelength correction at 605 nm for IL-1β and at 450 nm with wavelength correction at 620 nm for TNF-α (Bio-Tek, Winooski, VT, USA). The cytokines measured in the samples were extrapolated from a standard curve and results were expressed as pg/mL.

2.7. Acid gastric secretion determination by pylorus ligature

A pylorus ligature was carefully done in rats under general anesthesia [39]. Briefly, the pylorus was located and ligated with suture to maintain the gastric content into the stomach. The animals were orally treated with vehicle (water, 1 ml/kg), omeprazole (40 mg/kg, p.o.) or bosentan (3, 10 or 30 mg/kg, p.o.) 1 h before pylorus ligature. Four hours after pylorus ligature, the animals were euthanized, the stomachs were opened and the gastric acid secretion collected. Measurements of volume, pH and total acidity were evaluated.

2.8. Effect of bosentan on renal and hepatic functions.

After the oral treatments period in the chronic gastric ulcer model, blood samples were collected and centrifuged at 1500 g, the plasma samples were analyzed using a commercial kit (Bioclin/Quibasa, Belo Horizonte, MG, Brazil), for the creatinine, urea, aspartate aminotransferase (AST) alanine aminotransferase (ALT).

2.9. Statistical analysis

Results are expressed as mean ± standard error of the means (S.E.M). Therefore, statistical comparisons were performed by one-way analysis of variance (ANOVA), followed by Bonferroni's multi-comparison *pos-hoc* test for comparison of more than two groups. Unpaired *t*-test was used for comparison between two groups. The analyses were performed using GraphPad Prism® version 6.0 (GraphPad Software, San Diego, USA). Differences were considered as statistically significant when $*P \leq 0.05$.

3. RESULTS

3.1. ET_A and ET_B receptors are overexpressed in chronic gastric ulcer

The immunoblotting of ET_A and ET_B corresponds to a molecular weight of approximately 69 kDa and 50 kDa, respectively (Fig. 1A and 1B). The immunoblotting band densitometry showed a relatively increased expression on both ET_A and ET_B receptors by 5.9 and 6.9 folds, respectively, when compared acetic acid-induced gastric ulcer to *naïve* gastric tissue (Fig. 1C and D).

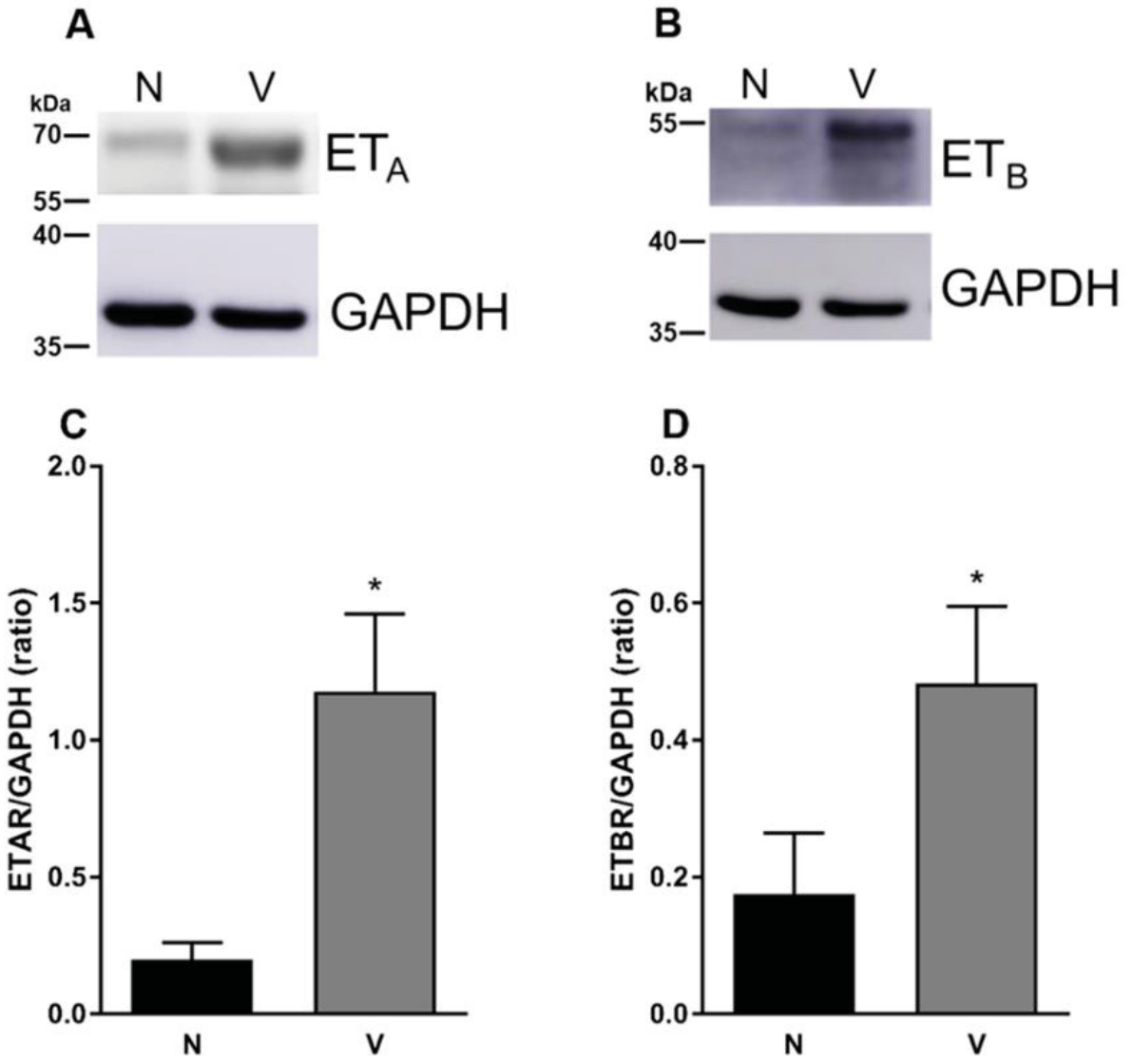
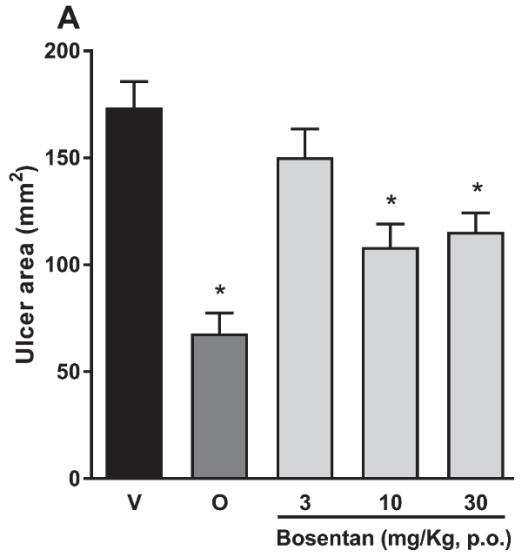


Fig. 1. Expression of endothelin ET_A and ET_B receptor protein by immunoblotting. (A) ET_A receptor bands. (B) ET_B receptor bands. (C) ET_A receptor expression levels. (D) ET_B receptor expression levels. Receptor expression levels of naïve group (N) and ulcerated vehicle treated group (V). Protein levels were expressed relative to GAPDH to account for protein loading differences per lane. The results are expressed as mean ± S.E.M. (n=3). Unpaired t-test. *P<0.05 when compared to naïve group (N).

3.2. Bosentan treatment increases gastric ulcer healing

The oral treatment of the animals with bosentan on doses 10 and 30 mg/kg significantly reduced ulcer area in 37.6% and 33.5% respectively, when compared to the vehicle group (173.6 mm^2) (Fig. 2A). On the other hand, the bosentan dose of 3 mg/kg did not reduce the ulcer area significantly. Oral treatment with omeprazole (40 mg/kg), a reference antiulcer drug, also resulted in a reduction of ulcer area by 60.9% compared to the vehicle group (Fig. 2A).

The representative macroscopic and microscopic photographs of gastric tissues reveal an extensive lesion induced by acetic acid in the vehicle group (Fig. 3B. Panel V). The omeprazole (Fig. 3B. Panel O), bosentan 10 mg/kg (Fig. 3B. Panel 10) and bosentan 30 mg/kg (Fig. 3B. Panel 30) photographs showed a gastric healing process by a minor central lesion area.


A
200
150
100
50
0
Ulcer area (mm2)
*
*
*
V
O
3
10
30
Bosentan (mg/Kg, p.o.)

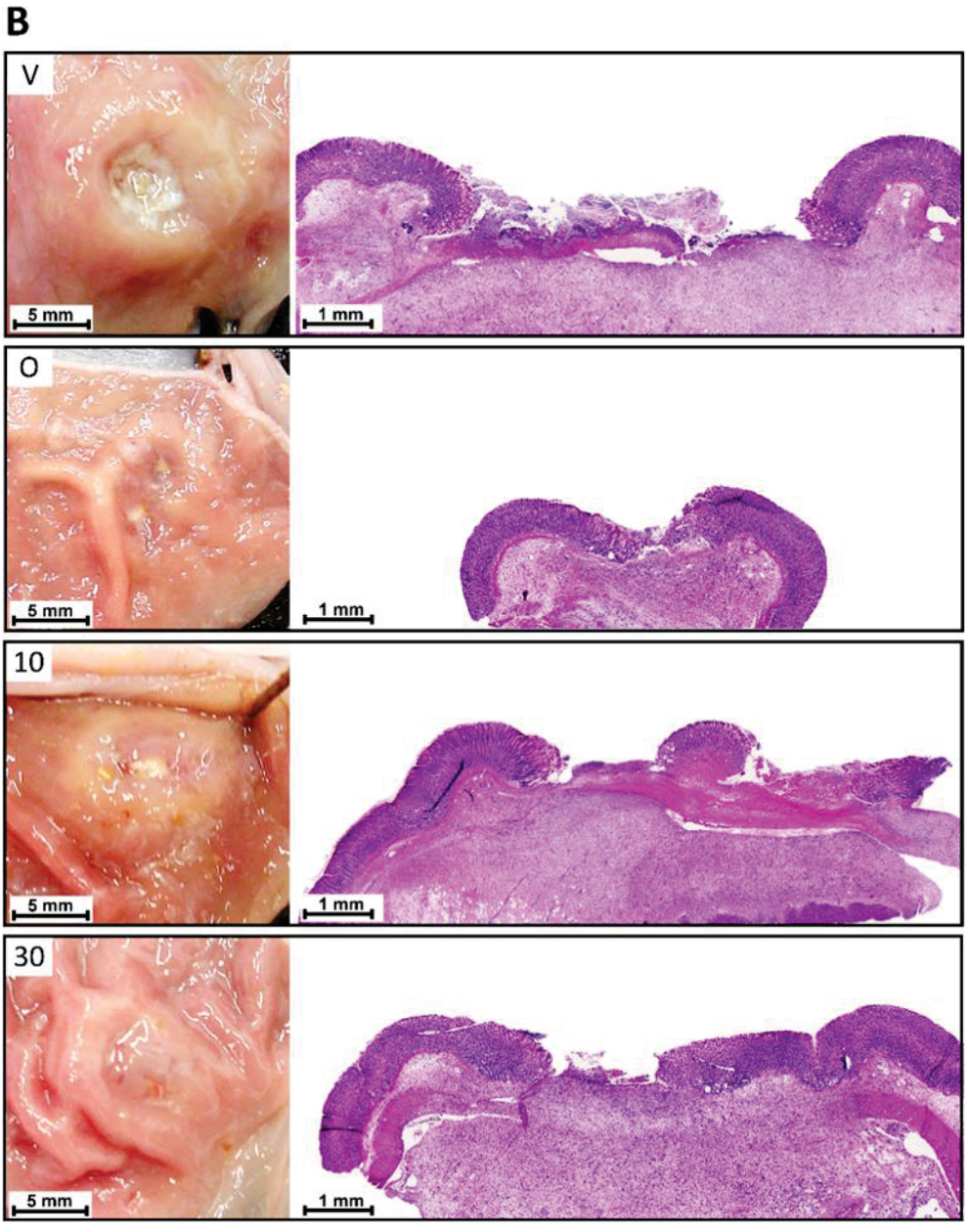
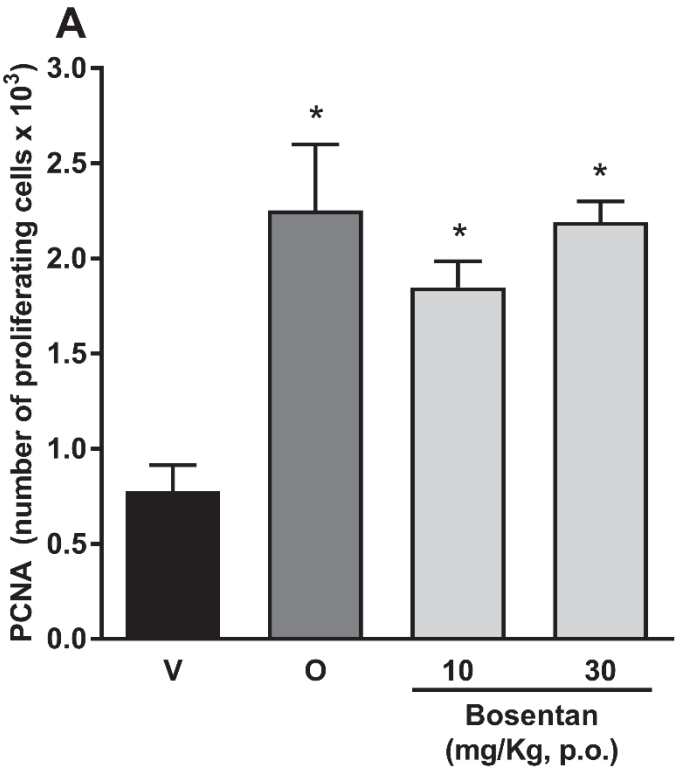

B
V
5 mm
1 mm
O
5 mm
1 mm
10
5 mm
1 mm
30
5 mm
1 mm

Fig. 2. Effect of bosentan on ulcer area on acetic acid-induced chronic gastric ulcer in rats. (A) Ulcer area measurement. (B) Representative macroscopic photograph and photomicrography of histological HE stained sections. Animals were orally treated with vehicle (V), omeprazole (O: 40 mg/kg) or bosentan (3, 10 or 30 mg/kg) twice daily for five days after the gastric ulcer induction. The results are expressed as mean ± S.E.M. (n=8). One-way ANOVA followed by Bonferroni's test. *P<0.05 when compared to the vehicle group.

3.3. Bosentan increases the epithelial cell proliferation

The PCNA immunohistochemical staining on chronic gastric lesions showed an immunoreactivity on the groups, characterized by brown nucleus color.

Oral treatment with omeprazole (40 mg/kg) or bosentan (10 or 30 mg/kg) showed a significant increase of positive PCNA immunoreactivity cells by 191%, 138%, and 183%, respectively, when compared to the vehicle group (0.78 x 10^3 number of proliferation cells) (Fig. 3A).


A
PCNA (number of proliferating cells x 10³)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
V
O
10
30
Bosentan
(mg/Kg, p.o.)

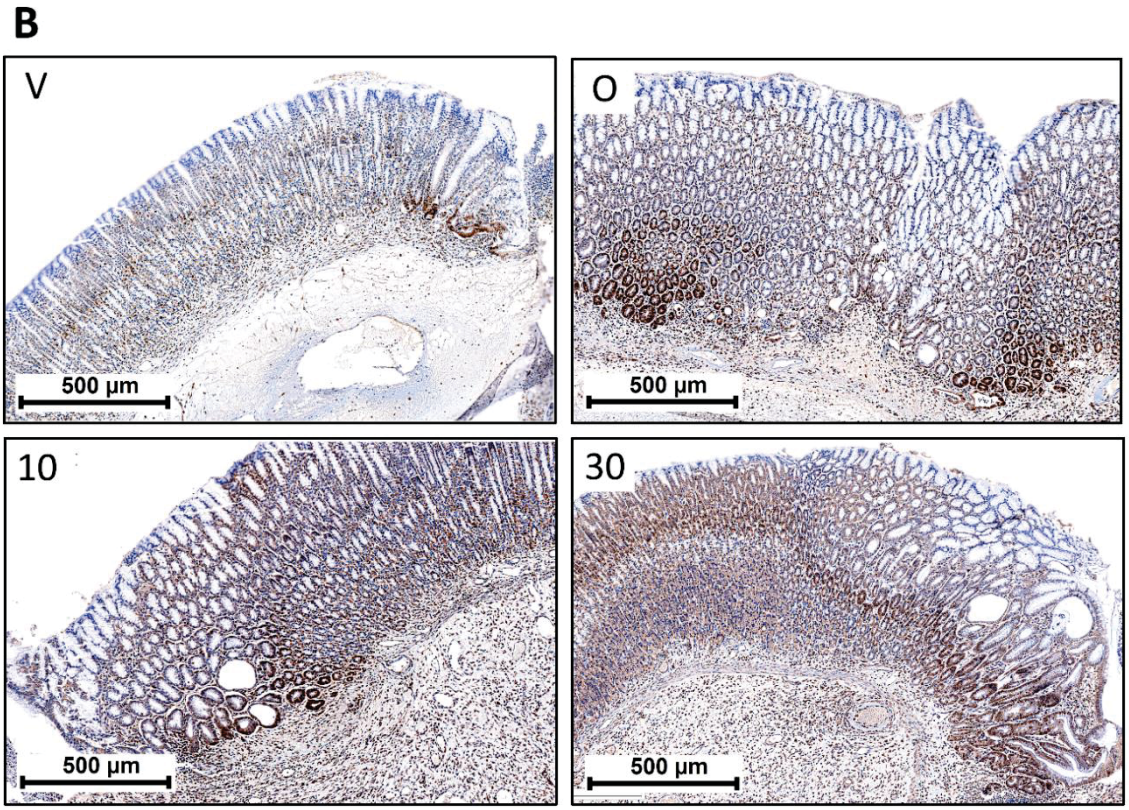

B
V
500 µm
O
500 µm
10
500 µm
30
500 µm

Fig. 3. Effect of bosentan on epithelial cell proliferation. (A) Quantification of gastric PCNA positive cells (B) Representative photomicrography of immunoreactivity of PCNA in gastric lesions induced by acetic acid. Animals were orally treated with vehicle (V), omeprazole (O: 40 mg/kg), bosentan (10: 10 mg/kg) or bosentan (30: 30 mg/kg) twice daily for five days after the gastric ulcer induction. The results are expressed as mean ± S.E.M. (n=3). One-way ANOVA followed by Bonferroni's test. *P<0.05 when compared to the vehicle group.

3.4. Bosentan increases the gastric mucin-like glycoproteins content

The representative microphotographs of periodic acid Schiff staining of groups showed the mucin-like glycoproteins staining (magenta color) on gastric mucosa (Fig. 4B). The mucin-like glycoproteins quantification showed a significant increase in omeprazole and bosentan groups (10 and 30 mg/kg) when compared to the vehicle group (25.9 ± 1.3 pixels x 10^4/field) (Fig. 4A).

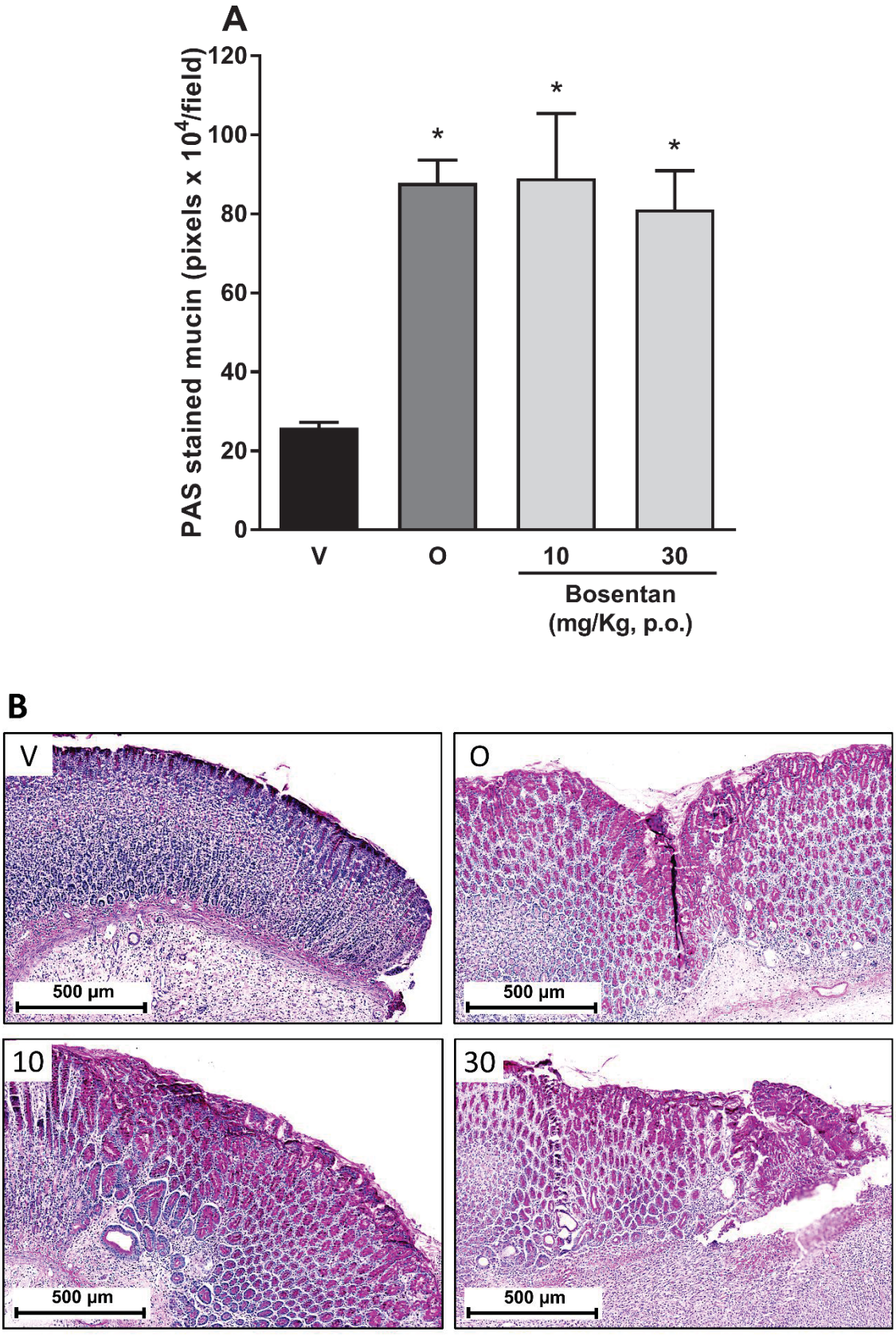


Fig. 4. Effect of bosentan treatment on gastric mucin-like glycoproteins layer on acetic acid-induced chronic gastric ulcer in rats. (A) Quantification of gastric mucin-like glycoproteins by

PAS. (B) Representative photomicrography of stomach lesions induced by acetic acid using the histochemical stained for mucin-like glycoproteins. Animals were orally treated with vehicle (Panel A), omeprazole (O: 40 mg/kg; Panel B), bosentan (10 mg/kg; Panel C) and bosentan (30 mg/kg; Panel D), twice daily for five days after the gastric ulcer induction.

3.5. Bosentan ameliorates the inflammatory parameters of the gastric ulcer

The myeloperoxidase actividy, an enzyme from inflammatory cell infiltration, from the gastric mucosa, was significantly reduced by 40.3% by omeprazole (40 mg/kg), 64.7% by bosentan (10 mg/kg) and by 59.7% by bosentan (30 mg/kg) when compared to the vehicle group (22.9 ± 3.3 O.D/mg of protein) (Fig. 5A). The plasma PGE2 quantifications reveled that the bosentan treatment (10 mg/kg) increased significantly the plasma PGE2, compared to the vehicle group (147.9 pg/mg of protein) (Fig. 5B).

The inflammatory cytokine TNF-α in ulcerated gastric tissue was decreased by oral treatment by omeprazole (40 mg/kg) and bosentan (10 mg/kg), in 39% and 67% respectively, when compared to the vehicle group (204.8 ± 13.3 pg/mg of protein) (Fig. 5C). Further, the IL-1β cytokine was significantly decreased by bosentan (10 mg/kg) in 70%, but not by omeprazole treatment (40 m/kg), when compared to the vehicle group (42.2 ± 7.9 pg/mg of protein) (Fig. 5D).

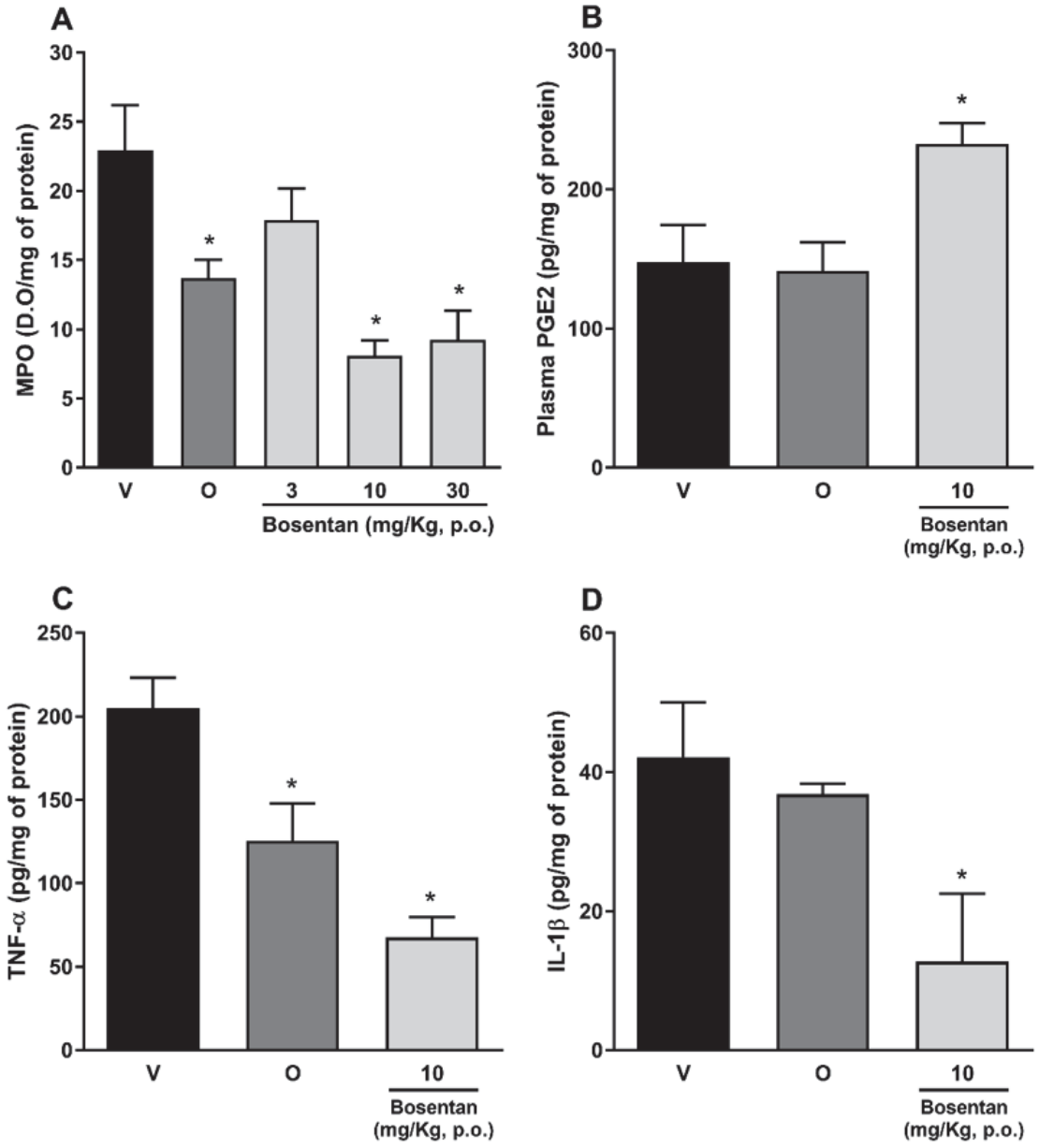


Fig. 5. Effect of bosentan on inflammatory parameters. (A) Neutrophil infiltration measurement by myeloperoxidase activity. (B) Plasma PGE2. (C) TNF-α. (D) IL-1β. Animals were orally treated with vehicle (V), omeprazole (O: 40 mg/kg) or bosentan (3, 10 or 30 mg/kg) twice daily for five days after the gastric ulcer induction. The results are expressed as mean ± S.E.M. (n=8). One-way ANOVA followed by Bonferroni's test. *P<0.05 when compared to the vehicle group.

3.6. Evaluation of acid gastric secretion

Oral treatment with bosentan (10 or 30 mg/kg) did not alter the acid gastric secretion volume (Fig. 6A), pH (Fig. 6B) or total acidity (Fig. 6C), when compared to the vehicle group. On the other hand, omeprazole caused a significant inhibition on all parameters of acid gastric secretion compared to the respective vehicle group.

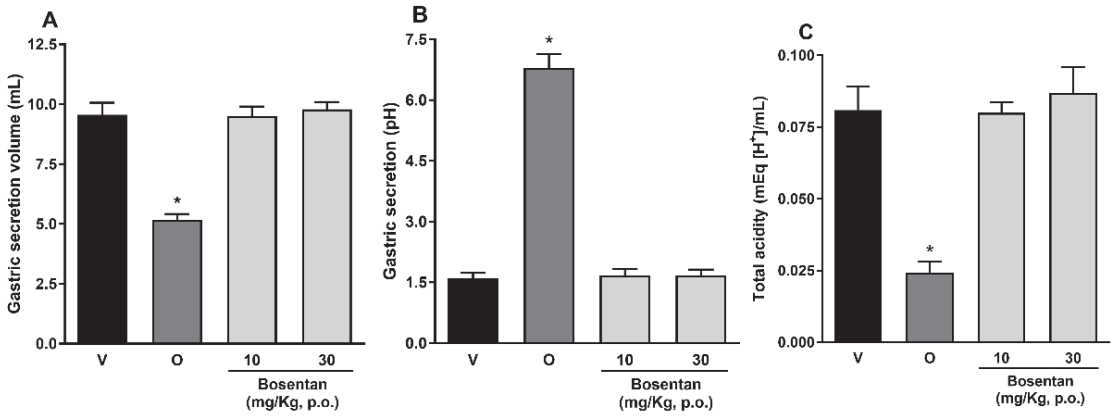


Fig. 6. Effect of bosentan on hypersecretion induced by acute pylorus ligation for 4 h in rats. (A) Gastric secretion volume. (B) Gastric secretion pH. (C) Total acidity. Animals were orally treated with vehicle (V), omeprazole (O: 40 mg/kg) or bosentan (10 or 30 mg/kg). The results are expressed as mean ± S.E.M. (n=7). One-way ANOVA followed by Bonferroni's test. **P*<0.05 when compared to the vehicle group.

3.7. Analysis of renal e hepatic functions

Biochemical analyses reveal that no oral treatments with omeprazole or bosentan caused alterations on biomarkers of renal and hepatic functions (Table 1).

Table 1. Effect of bosentan on biochemical analysis of markers of renal and hepatic function in plasma on acetic acid-induced chronic gastric ulcer in rats.

Group	Creatinine (mg/dL)	Urea (mg/dL)	AST (U/L)	ALT (U/L)
Vehicle (1 mL/kg p.o.)	0.71 ± 0.08	32.33 ± 1.58	125.8 ± 9.12	57.61 ± 2.15
Omeprazole (40 mg/kg p.o.)	0.69 ± 0.04	30.13 ± 0.92	129.9 ± 13.80	51.85 ± 1.77
Bosentan (3 mg/kg p.o.)	0.74 ± 0.07	29.26 ± 2.90	105.5 ± 9.22	55.14 ± 1.52
Bosentan (10 mg/kg p.o.)	0.76 ± 0.05	30.91 ± 1.01	96.68 ± 6.68	51.30 ± 1.65
Bosentan (30 mg/kg p.o.)	0.90 ± 0.09	34.04 ± 3.49	94.85 ± 5.16	51.39 ± 0.81

The results were expressed as mean ± S.E.M. (n=8). One-way ANOVA followed by Bonferroni's test.

4. Discussion

Gastric ulcers have a global incidence and their complications can lead to hospitalization and increased risk of death. Beyond the numerous adverse effects of reducing gastric acidity, by the use of PPIs such as omeprazole, there is an increased risk for the development of various diseases [8–10]. Due to these facts, is notorious the need for pharmacological treatments for chronic gastric ulcers, that avoid the adverse effects and associated risks. Considering the participation of endothelins in ulcerogenesis, the number of studies of endothelin receptors antagonists in chronic gastric ulcer is still scarce.

In other diseases, such as scleroderma which patients present high levels of ET-1 and digital ulcers, clinical trials showed a healing effect in digital ulcers by chronic oral treatment with bosentan [31]. According to with these healing effects of bosentan, our group showed that oral treatment with bosentan accelerates the gastric lesion healing, by minor ulcer area and a higher PCNA-positive cell on the gastric epithelial layer, a tissue marker of cell proliferation [40]. The gastric mucus production contributes crucially for epithelial cell proliferation,

protecting the epithelial layer against the chloridric acid injuries and allowing the gastric healing [41]. In this study, an increased in mucin-like glycoproteins on ulcer borders in bosentan group treatment indicated a gastroprotective effect, similar effect to that observed with treatment with the omeprazole.

During the ulcer formation occurs recruitment and activation of neutrophils and macrophages, leading to ROS generation and pro-inflammatory cytokines production, such as TNF-α and IL-1β [40,42,43]. Neutrophils synthesize ET-1 on physiological and inflammatory processes, which has both autocrine and paracrine actions on activation of ET_B receptors present on these cells, which induce the expression of the integrin CD11b, dependent of TNF-α, contributing to the neutrophils infiltration on inflamed tissues [44]. A reduction of neutrophils infiltration in gastric tissue is related to the reduction of ROS generation and ulcer healing [45]. In this study, the marker of neutrophils infiltration (MPO) was decreased by bosentan treatment on doses 10 and 30 mg/kg, which indicates a minor inflammatory cells infiltration on ulcer area and corroborates with the minor ulcer area. Increased plasma PGE2 from bosentan treatment may indicate increased angiogenesis due to up-regulation of VEGF by prostaglandins [46,47]. Treatment with a COX-2 selective inhibitor decreased PGE2 level and decreased the VEGF expression [48].

Furthermore, in gastric ulcer the macrophage migration with inflammatory cytokines IL-1β and TNF-α production, play an important role in gastric ulcerogenesis [49,50]. Drugs and natural compounds that decrease these cytokines production, attenuates gastric inflammation and accelerates the healing of gastric ulcer lesions [51,52]. In other animal models, such as inflammatory pain model, the endothelin antagonists treatment also showed a decrease of IL-1ß e TNF-α production and a minor disease index, denoting an involvement of endothelin on inflammation persistence [53].

Our results showed primarily the increase expression of ET_A and ET_B receptors in chronic gastric ulcer. In other *in vitro* and *in vivo* studies inflammatory process, the ET_A and ET_B receptors also showed increased expression, such as in severe pulmonary hypertension patients and, in smokers and chronic obstructive patients [54,55]. In this study by Milara [55] bosentan treatment in human small intrapulmonary arteries, previously exposed to cigarette's smoke or ET-1, restored the normal the upregulation of ET_A and ET_B expressions and attenuates the ROS generation. Wang [56] showed an upregulation of c-Jun N-terminal kinase and DNA binding of c-Jun and C/EBPβ by ET-1 treatment, which induces overexpression of ET_A and ET_B receptors, following apoptosis activation. TNF-α signaling also activates and induces the overexpression of the c-Jun N-terminal kinase, which causes a significant increase in ET-1 production [57–60].

These studies and our results indicating a positive regulation of ET_A and ET_B receptors in pathologies, it may be possible to establish a link between the elevation of ET-1 in gastric tissue, reported in humans by Iaquinto [28] and in rats by Michida [15], with increased expression of ET_A and ET_B receptors reported in chronic ulcer of this study.

The ET-1 contributes to the development of inflammatory response involving the expression of proinflammatory cytokines including IL-1, IL-6 and TNF-α, but little is known about ET-1 synthesis in this context [61]. Some studies indicated that immune cells like macrophages and lymphocytes, due to inflammatory stimulation, synthesize ET-1[62–65]. Bosentan exhibited a notable anti-inflammatory, anti-nociceptive and joint protection effects, in acute and chronic arthritis mice models denoting the involving of ETs in these processes [66].

The long last inhibition of gastric acid secretion, with PPIs or H_2 antagonists, lead to adverse effects and increased risk for the development of numerous diseases, such as chronic kidney disease and dementia [9,50]. Interestingly, we have demonstrated that bosentan

successfully accelerated the gastric mucosa healing without antisecretory mechanisms, maintaining the physiological acid gastric secretion, avoiding the long last inhibition of gastric acid secretion adverse effects. Based on these results, bosentan may be a new alternative and off-label use for the treatment of gastric ulcers.

5. CONCLUSION

Our results show, in the chronic gastric model, that bosentan accelerated the ulcer healing, was also able to decrease inflammatory parameters without inhibiting the acid gastric secretion. The overexpression on both ET_A/ET_B receptors on the chronic gastric ulcer denotes the involvement of endothelinergic system and ulcerogenesis.

Acknowledgments

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) - Research Grant: #3038751/2017-8, and was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

Author Contributions

D.M.F., B.B.L., N.S.L, and M.F.P.W contributed to the concept and design of the study.

Declarations of interest: The authors declare that they have no conflict of interest.

6. REFERENCES

1 Tarnawski AS. Cellular and molecular mechanisms of gastrointestinal ulcer healing. Dig Dis Sci. 2005;50(SUPPL. 1):24–33.

2 S. Tarnawski a., Ahluwalia A. Molecular mechanisms of epithelial regeneration and neovascularization during healing of gastric and esophageal ulcers. Curr Med Chem. 2012;19(1):16–27.

3 Magierowski M, Magierowska K, Surmiak M, Hubalewska-Mazgaj M, Kwiecien S, Wallace JL, et al. The effect of hydrogen sulfide-releasing naproxen (ATB-346) versus naproxen on formation of stress-induced gastric lesions, the regulation of systemic inflammation, hypoxia and alterations in gastric microcirculation. J Physiol Pharmacol. 2017;68(5):749–56.

4 Lin TF, Hsu PI. Second-line rescue treatment of Helicobacter pylori infection: Where are we now? World J Gastroenterol. 2018;24(40):4548–53.

5 Nehra AK, Alexander JA, Loftus CG, Nehra V. Proton Pump Inhibitors: Review of Emerging Concerns. Mayo Clin Proc. 2018;93(2):240–6.

6 Vaezi MF, Yang YX, Howden CW. Complications of Proton Pump Inhibitor Therapy. Gastroenterology. 2017;153(1):35–48.

7 Dial MS. Proton pump inhibitor use and enteric infections. Am J Gastroenterol. 2009;104 Suppl(March):S10–6.

8 Lazarus B, Chen Y, Wilson FP, Sang Y, Chang AR, Coresh J, et al. Proton pump inhibitor use and the risk of chronic kidney disease. JAMA Intern Med. 2016;176(2):238–46.

9 Haenisch B, von Holt K, Wiese B, Prokein J, Lange C, Ernst A, et al. Risk of dementia in elderly patients with the use of proton pump inhibitors. Eur Arch Psychiatry Clin Neurosci. 2015 Aug;265(5):419–28.

10 Xu HB, Wang HD, Li CH, Ye S, Dong MS, Xia QJ, et al. Proton pump inhibitor use and risk of spontaneous bacterial peritonitis in cirrhotic patients: A systematic review and meta-analysis. Genet Mol Res. 2015;14(3):7490–501.

11 Tonolini M, Ierardi AM, Bracchi E, Magistrelli P, Vella A, Carrafiello G. Non-perforated peptic ulcer disease: multidetector CT findings, complications, and differential diagnosis. Insights Imaging. 2017 Oct;8(5):455–69.

12 Fashner J, Gitu AC. Diagnosis and Treatment of Peptic Ulcer Disease and H. pylori Infection. Am Fam Physician. 2015 Feb;91(4):236–42.

13 Uyanikoğlu A, Danalioğlu A, Akyuz F, Ermiş F, Güllüoğlu M, Kapran Y, et al. Etiological factors of duodenal and gastric ulcers. Turkish J Gastroenterol. 2012;23(2):99–103.

14 Wallace JL, Cirino G, De Nucci G, McKnight W, MacNaughton WK. Endothelin has potent ulcerogenic and vasoconstrictor actions in the stomach. Am J Physiol. 1989;256(4 Pt 1):G661-6.

15 Michida T, Kawano S, Masuda E, Kobayashi I, Nishimura Y, Tsujii M, et al. Role of endothelin 1 in hemorrhagic shock-induced gastric mucosal injury in rats. Gastroenterology. 1994;106(4):988–93.

16 Kitajima T, Tani K, Yamaguchi T, Kubota Y, Okuhira M, Mizuno T, et al. Role of Endogenous Endothelin in Gastric Mucosal Injury Induced by Hemorrhagic Shock in

Rats. Digestion. 1995;56(2):111–6.

17 Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature. 1988;332(6163):411–5.

18 Huang S. Endothelin Receptors in Gastrointestinal Smooth Muscle Unclear action. 2005;547–57.

19 Watanabe H, Miyazaki H, Kondoh M, Masuda Y, Kimura S, Yanagisawa M, et al. Two distinct types of endothelin receptors are present on chick cardiac membranes. Biochem Biophys Res Commun. 1989 Jun;161(3):1252–9.

20 Emori T, Hirata Y, Marumo F. Specific receptors for endothelin-3 in cultured bovine endothelial cells and its cellular mechanism of action. FEBS Lett. 1990 Apr;263(2):261–4.

21 Wallace JL, Keenan CM, MacNaughton WK, Webb McKnight G. Comparison of the effects of endothelin-1 and endothelin-3 on the rat stomach. Eur J Pharmacol. 1989;167(1):41–7.

22 Krämer BK, Nishida M, Kelly RA, Smith TW. Endothelins. Myocardial actions of a new class of cytokines. Circulation. 1992 Jan;85(1):350–6.

23 Elisa T, Antonio P, Giuseppe P, Alessandro B, Giuseppe A, Federico C, et al. Endothelin Receptors Expressed by Immune Cells Are Involved in Modulation of Inflammation and in Fibrosis: Relevance to the Pathogenesis of Systemic Sclerosis. J Immunol Res. 2015;2015:1–11.

24 Coyle TB, Metersky ML. The Effect of the Endothelin-1 Receptor Antagonist, Bosentan,

on Patients with Poorly Controlled Asthma: A 17-week, Double-Blind, Placebo-Controlled Crossover Pilot Study. J Asthma. 2013 May;50(4):433–7.

25 Meyers KEC, Sethna C. Endothelin antagonists in hypertension and kidney disease. Pediatr Nephrol. 2013 May;28(5):711–20.

26 Morales RE, Johnson BR, Szabo S. Endothelin induces vascular and mucosal lesions, enhances the injury by HCl/ethanol, and the antibody exerts gastroprotection. FASEB J. 1992 Mar;6(6):2354–60.

27 Hassan M, Kashimura H, Matsumaru K. Gastric Mucosal Injury Induced by Local Ischemia ± Reperfusion in Rats Role of Endogenous Endothelin-1 and Free Radical. 1997;42(7):1375–80.

28 Iaquinto G, Giardullo N, Taccone W, Leandro G, Pasquale L, De Luca L, et al. Role of endogenous endothelin-1 in ethanol-induced gastric mucosal damage in humans. Dig Dis Sci. 2003;48(4):663–9.

29 Barton M. Aging and endothelin: Determinants of disease. Life Sci. 2014 Nov;118(2):97–109.

30 Rubin LJMD, David B. Badesch, M.D., Robyn J. Barst, M.D., Nazzareno Galiè MD, Carol M. Black MD, Anne Keogh MD, Tomas Pulido MD, Adaani Frost MD, et al. Bosentan Therapy for Pulmonary Arterial Hypertension. N Engl J Med. 2002 Mar;346(12):896–903.

31 Matucci-Cerinic M, Denton CP, Furst DE, Mayes MD, Hsu VM, Carpentier P, et al. Bosentan treatment of digital ulcers related to systemic sclerosis: results from the RAPIDS-2 randomised, double-blind, placebo-controlled trial. Ann Rheum Dis.

2011;70(1):32–8.

32 Langedijk J, Mantel-Teeuwisse AK, Slijkerman DS, Schutjens M-HDB. Drug repositioning and repurposing: terminology and definitions in literature. Drug Discov Today. 2015 Aug;20(8):1027–34.

33 Sun W, Sanderson PE, Zheng W. Drug combination therapy increases successful drug repositioning. Drug Discov Today. 2016 Jul;21(7):1189–95.

34 Duggan A, Stack W, Hull M, Filipowicz B, Knifton A, Crome R, et al. Protection against aspirin-induced human gastric mucosal injury by bosentan, a new endothelin-1 receptor antagonist. Aliment Pharmacol Ther. 1999 May;13(5):631–5.

35 Matsumaru K. Gastroenterology Bosentan , a novel synthetic mixed-type endothelin receptor antagonist , attenuates acute gastric mucosai lesions induced by indomethacin and HCI in the rat : Role of endogenous endothelin-1. 1997;164–70.

36 Okabe S, Roth JLA a., Pfeiffer CJ. A method for experimental, penetrating gastric and duodenal ulcers in rats - Observations on normal healing. Am J Dig Dis. 1971;16(3):277–84.

37 Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of Cutaneous Inflammation: Estimation of Neutrophil Content with an Enzyme Marker. J Invest Dermatol. 1982 Mar;78(3):206–9.

38 De Young LM, Kheifets JB, Ballaron SJ, Young JM. Edema and cell infiltration in the phorbol ester-treated mouse ear are temporally separate and can be differentially modulated by pharmacologic agents. Agents Actions. 1989 Mar;26(3–4):335–41.

39 Shay H, Komarov S, Fels S, Meranze D, Gruenstein M. A simple method for the uniform

production of gastric ulceration in the rat. Gastroenterology. 1945;5:43–61.

40 Maria-Ferreira D, Da Silva LM, Mendes DAGB, De Almeida Cabrini D, Nascimento AM, Iacomini M, et al. Rhamnogalacturonan from Acmella oleracea (L.) R.K. Jansen: Gastroprotective and ulcer healing properties in rats. PLoS One. 2014;9(1). DOI: 10.1371/journal.pone.0084762

41 Kobayashi T, Ohta Y, Yoshino J, Nakazawa S. Teprenone promotes the healing of acetic acid-induced chronic gastric ulcers in rats by inhibiting neutrophil infiltration and lipid peroxidation in ulcerated gastric tissues. Pharmacol Res. 2001 Jan;43(1):23–30.

42 Kwiecień S, Brzozowski T, Konturek SJ. Effects of reactive oxygen species action on gastric mucosa in various models of mucosal injury. J Physiol Pharmacol. 2002 Mar;53(1):39–50.

43 Wallace JL, Arfors KE, McKnight GW. A monoclonal antibody against the CD18 leukocyte adhesion molecule prevents indomethacin-induced gastric damage in the rabbit. Gastroenterology. 1991 Apr;100(4):878–83.

44 Koehl B, Nivoit P, El Nemer W, Lenoir O, Hermand P, Pereira C, et al. The endothelin B receptor plays a crucial role in the adhesion of neutrophils to the endothelium in sickle cell disease. Haematologica. 2017 Jul;102(7):1161–72.

45 Ferrat M, Moulahoum H, Boumaza BMA, Mouzaoui S, Périanin A, Djerdjouri B. Gadolinium chloride attenuates acetic acid-evoked colitis in mice by reducing neutrophil infiltration and pro-oxidative enzyme activity. Naunyn Schmiedebergs Arch Pharmacol. 2019 Mar;392(3):299–311.

46 Majima M, Hayashi I, Muramatsu M, Katada J, Yamashina S, Katori M. Cyclo-

oxygenase-2 enhances basic fibroblast growth factor-induced angiogenesis through induction of vascular endothelial growth factor in rat sponge implants. Br J Pharmacol. 2000 Jun;130(3):641–9.

47 Yoshida S, Amano H, Hayashi I, Kitasato H, Kamata M, Inukai M, et al. COX-2/VEGF-Dependent Facilitation of Tumor-Associated Angiogenesis and Tumor Growth in vivo. Lab Investig. 2003 Oct;83(10):1385–94.

48 WU G, LUO J, RANA J, LAHAM R, SELLKE F, LI J. Involvement of COX-2 in VEGF-induced angiogenesis via P38 and JNK pathways in vascular endothelial cells. Cardiovasc Res. 2006 Feb;69(2):512–9.

49 Stephen AO, James O, Ikoojo ER, Sunday AO. Effects of Selenium Treatment on Healing of Acetic Acid Induced Gastric Ulcer in Albino Wistar Rats. 2016;4(1):18–22.

50 Larivière R, Gauthier-Bastien A, Ung R-V, St-Hilaire J, Mac-Way F, Richard DE, et al. Endothelin type A receptor blockade reduces vascular calcification and inflammation in rats with chronic kidney disease. J Hypertens. 2017 Feb;35(2):376–84.

51 Almasaudi SB, Abbas AT, Al-Hindi RR, El-Shitany NA, Abdel-dayem UA, Ali SS, et al. Manuka Honey Exerts Antioxidant and Anti-Inflammatory Activities That Promote Healing of Acetic Acid-Induced Gastric Ulcer in Rats. Evidence-Based Complement Altern Med. 2017;2017:1–12.

52 Rozza AL, Meira de Faria F, Souza Brito AR, Pellizzon CH. The Gastroprotective Effect of Menthol: Involvement of Anti-Apoptotic, Antioxidant and Anti-Inflammatory Activities. PLoS One. 2014 Jan;9(1):e86686.

53 Serafim KGG, Navarro SA, Zarpelon AC, Pinho-Ribeiro FA, Fattori V, Cunha TM, et

al. Bosentan, a mixed endothelin receptor antagonist, inhibits superoxide anion-induced pain and inflammation in mice. Naunyn Schmiedebergs Arch Pharmacol. 2015;388(11):1211–21.

54 Bauer M, Wilkens H, Langer F, Schneider SO, Lausberg H, Schäfers H-J. Selective upregulation of endothelin B receptor gene expression in severe pulmonary hypertension. Circulation. 2002 Mar;105(9):1034–6.

55 Milara J, Gabarda E, Juan G, Ortiz JL, Guijarro R, Martorell M, et al. Bosentan inhibits cigarette smoke-induced endothelin receptor expression in pulmonary arteries. Eur Respir J. 2012 Apr;39(4):927–38.

56 Wang J, Yorio T, Ma H-Y, Krishnamoorthy RR, He S. A feed-forward regulation of endothelin receptors by c-Jun in human non-pigmented ciliary epithelial cells and retinal ganglion cells. PLoS One. 2017;12(9):e0185390.

57 Reinhard C. Tumor necrosis factor alpha -induced activation of c-jun N-terminal kinase is mediated by TRAF2. EMBO J. 1997 Mar;16(5):1080–92.

58 Narayan S, Prasanna G, Krishnamoorthy R, Zhang X, Yorio T. Endothelin-1 Synthesis and Secretion in Human Retinal Pigment Epithelial Cells (ARPE-19): Differential Regulation by Cholinergics and TNF-α. Invest Ophthalmol Vis Sci. 2003;44(11):4885–94.

59 Marsden PA, Brenner BM. Transcriptional regulation of the endothelin-1 gene by TNF-alpha. Am J Physiol. 1992 Apr;262(4 Pt 1):C854-61.

60 Virdis A, Schiffrin EL. Vascular inflammation: a role in vascular disease in hypertension? Curr Opin Nephrol Hypertens. 2003 Mar;12(2):181–7.

61 Yeager ME, Belchenko DD, Nguyen CM, Colvin KL, Ivy DD, Stenmark KR. Endothelin-1, the Unfolded Protein Response, and Persistent Inflammation. Am J Respir Cell Mol Biol. 2012 Jan;46(1):14–22.

62 Shinagawa S, Okazaki T, Ikeda M, Yudoh K, Kisanuki YY, Yanagisawa M, et al. T cells upon activation promote endothelin 1 production in monocytes via IFN-γ and TNF-α. Sci Rep. 2017 Dec;7(1):14500.

63 Ehrenreich H, Anderson RW, Fox CH, Rieckmann P, Hoffman GS, Travis WD, et al. Endothelins, peptides with potent vasoactive properties, are produced by human macrophages. J Exp Med. 1990 Dec;172(6):1741–8.

64 Wahl JR, Goetsch NJ, Young HJ, Van Maanen RJ, Johnson JD, Pea AS, et al. Murine macrophages produce endothelin-1 after microbial stimulation. Exp Biol Med (Maywood). 2005 Oct;230(9):652–8.

65 McKenna S, Gossling M, Bugarini A, Hill E, Anderson AL, Rancourt RC, et al. Endotoxemia Induces IκBβ/NF-κB–Dependent Endothelin-1 Expression in Hepatic Macrophages. J Immunol. 2015 Oct;195(8):3866–79.

66 Imhof A-K, Glück L, Gajda M, Bräuer R, Schaible H-G, Schulz S. Potent anti-inflammatory and antinociceptive activity of the endothelin receptor antagonist bosentan in monoarthritic mice. Arthritis Res Ther. 2011;13(3):R97.

4 CONCLUSÃO E CONSIDERAÇÕES FINAIS

Dentre as conclusões desse trabalho, considera-se que não se pode ignorar os constantes estudos que denotam os riscos associados a inibição da secreção ácida gástrica, que configura o tratamento farmacológico convencional para úlceras gástricas. Visto a complexidade fisiopatológica da ulcerogênese, outros sistemas além da secreção ácida gástrica possuem um potencial a ser explorado, como o sistema endotelinérgico, em que estudos já demonstraram seu envolvimento em diversas doenças crônicas.

A partir dos achados nesse trabalho, conclui-se que tanto o sistema endotelinérgico quanto a via do óxido nítrico são potenciais vias a serem consideradas para tratamento de úlceras gástricas agudas. A base para tal constatação é de que fármacos vasodilatadores como a bosentana (antagonista duplo de receptores de ET_A/ET_B) e a sildenafila (inibidor da fosfodiesterase 5) exibiram relevantes efeitos gastroprotetores no modelo de úlcera por isquemia-reperfusão. Dentre eles, obteve-se: a manutenção dos fatores protetores da mucosa gástrica, como o muco gástrico e as defesas antioxidantes, menor grau de dano celular por estresse oxidativo e menor grau inflamatório no tecido gástrico. De modo interessante, esses efeitos também foram observados com pré-tratamento em associação da menor dose não eficaz da bosentana e sildenafila, as quais isoladamente não possuem efeito significativo. Assim, essa combinação demonstra um efeito gastroprotetor sinérgico de ambas as drogas vasodilatadoras.

Este trabalho demonstrou de modo inédito que na úlcera crônica gástrica há uma expressão aumentada dos receptores endotelinérgicos ET_A/ET_B, o que demonstra a participação de tal sistema na ulcerogênese. Tal dado é corroborado com os resultados desse trabalho, de que o tratamento com a bosentana acelerou a cicatrização de úlceras crônicas em ratos, assim como diminuiu os parâmetros inflamatórios no tecido gástrico. Esses efeitos foram obtidos com a manutenção da secreção ácida gástrica, o que contorna os efeitos adversos do uso dos IBPs. Portanto, o uso *off-label* de fármacos vasoativos para úlceras gástricas pode ser uma alternativa promissora, devido a sua segurança comprovada pelo uso clínico em doenças crônicas, evitando-se os efeitos adversos da terapia com IBPs.

5 REFERÊNCIAS

AMBACH, A. et all. Low-dose combination therapy of severe digital ulcers in diffuse progressive systemic sclerosis with the endothelin-1 receptor antagonist bosentan and the phosphodiesterase V inhibitor sildetnafil. **Journal der Deutschen Dermatologischen Gesellschaft = Journal of the German Society of Dermatology : JDDG**, v. 7, n. 10, p. 888–891, 2009.

ANGGRAHINI, D. W. et al. Vascular endothelial cell-derived endothelin-1 mediates vascular inflammation and neointima formation following blood flow cessation. **Cardiovascular Research**, v. 82, n. 1, p. 143–151, 1 abr. 2009.

ARO, P. et al. Peptic ulcer disease in a general adult population: The kalixanda study: A random population-based study. **American Journal of Epidemiology**, v. 163, n. 11, p. 1025–1034, 2006.

AYDINLI, B. et al. The role of sildenafil citrate in the protection of gastric mucosa from nonsteroidal anti-inflammatory drug-induced damage. **Ulusal Travma ve Acil Cerrahi Dergisi**, v. 13, n. 4, p. 268–273, 2007.

BADIOLA, N. et al. The Proton-Pump Inhibitor Lansoprazole Enhances Amyloid Beta Production. **PLoS ONE**, v. 8, n. 3, p. e58837, 8 mar. 2013.

BARTON, M.; TRAUPE, T.; HAUDENSCHILD, C. C. Endothelin, hypercholesterolemia and atherosclerosis. **Coronary artery disease**, v. 14, n. 7, p. 477–90, nov. 2003.

BELLISAI, F. et al. Evaluation of the Effect of Bosentan Treatment on Proinflammatory Cytokine Serum Levels in Patients Affected by Systemic Sclerosis. **International Journal of Immunopathology and Pharmacology**, v. 24, n. 1, p. 261–264, jan. 2011.

BISHOP et al. Protection against aspirin-induced human gastric mucosal injury by bosentan, a new endothelin-1 receptor antagonist. **Alimentary Pharmacology and Therapeutics**, v. 13, n. 5, p. 631–635, 1999.

BOBIK, A. et al. Growth factor activity of endothelin on vascular smooth muscle. **American Journal of Physiology-Cell Physiology**, v. 258, n. 3, p. C408–C415, mar. 1990.

BREMNES, T. et al. Regulation and Intracellular Trafficking Pathways of the Endothelin Receptors. **Journal of Biological Chemistry**, v. 275, n. 23, p. 17596–17604, 9 jun. 2000.

BRIYAL, S.; PHILIP, T.; GULATI, A. Endothelin-A Receptor Antagonists Prevent Amyloid-β-Induced Increase in ETA Receptor Expression, Oxidative Stress, and Cognitive Impairment. **Journal of Alzheimer's Disease**, v. 23, n. 3, p. 491–503, 14 fev. 2011.

BROCK, G. B. et al. Efficacy and safety of tadalafil for the treatment of erectile dysfunction: results of integrated analyses. **The Journal of urology**, v. 168, n. 4 Pt 1,

p. 1332–6, out. 2002.

BRUECKNER, C. S. et al. Effect of sildenafil on digital ulcers in systemic sclerosis: analysis from a single centre pilot study. **Annals of the rheumatic diseases**, v. 69, n. 8, p. 1475–8, 2010.

BURNIER, M. et al. Endothelin Receptors Antagonists as Renal Protective Agents. **Clinical Medicine Reviews in Vascular Health**, v. 2, p. 41–49, 18 fev. 2010.

CAMPBELL, H. E. Clinical Monograph for Drug Formulary Review: Erectile Dysfunction Agents. **Journal of Managed Care Pharmacy**, v. 11, n. 2, p. 151–171, mar. 2005.

CERRATO, R. et al. Endothelin-1 increases superoxide production in human coronary artery bypass grafts. **Life Sciences**, v. 91, n. 13–14, p. 723–728, out. 2012.

CHEN, Y. et al. Endothelin-1 receptor antagonists prevent the development of pulmonary emphysema in rats. **European Respiratory Journal**, v. 35, n. 4, p. 904–912, 1 abr. 2010.

CHUNG, L. Therapeutic Options for Digital Ulcers in Patients with Systemic Sclerosis. **JDDG**, v. 5, n. 6, p. 460–465, jun. 2007.

CHUNG, L.; FIORENTINO, D. Digital ulcers in patients with systemic sclerosis. **Autoimmunity Reviews**, v. 5, n. 2, p. 125–128, fev. 2006.

DAI, X. et al. Increased O 2 ·− Production and Upregulation of ET B Receptors by Sympathetic Neurons in DOCA-Salt Hypertensive Rats. **Hypertension**, v. 43, n. 5, p. 1048–1054, maio 2004.

DASHWOOD, M. R.; TSUI, J. C. S. Further evidence for a role of endothelin-1 (ET-1) in critical limb ischaemia. **Journal of Cell Communication and Signaling**, v. 5, n. 1, p. 45–49, 27 mar. 2011.

DAVENPORT, A. P. International Union of Pharmacology. XXIX. Update on endothelin receptor nomenclature. **Pharmacological reviews**, v. 54, n. 2, p. 219–26, jun. 2002.

DAVENPORT, A. P. et al. Endothelin. **Pharmacological reviews**, v. 68, n. 2, p. 357–418, 2016.

DE LEEST, H. et al. Costs of treating bleeding and perforated peptic ulcers in The Netherlands. **The Journal of rheumatology**, v. 31, n. 4, p. 788–91, abr. 2004.

DEL VALLE, J. Peptic Ulcer Disease and Related Disorders. In: KASPER, D. et al. (Eds.). . **Harrison's Principles of Internal Medicine**. 19. ed. New York, NY: McGraw Hill Education, 2015. p. 1911–1932.

DONATE, P. B. et al. Bosentan, an endothelin receptor antagonist, ameliorates collagen-induced arthritis: The role of TNF-α in the induction of endothelin system genes. **Inflammation Research**, v. 61, n. 4, p. 337–348, 2012.

DONG, F. et al. Endothelin-1 enhances oxidative stress, cell proliferation and reduces

apoptosis in human umbilical vein endothelial cells: role of ET B receptor, NADPH oxidase and caveolin-1. **British Journal of Pharmacology**, v. 145, n. 3, p. 323–333, jun. 2005.

DUERRSCHMIDT, N. et al. Endothelin-1 Induces NAD(P)H Oxidase in Human Endothelial Cells. **Biochemical and Biophysical Research Communications**, v. 269, n. 3, p. 713–717, mar. 2000.

DUFFIN, R.; SHAW, C. A; ROSSI, A G. Sildenafil reduces alcohol-induced gastric damage: just say “NO”. **British journal of pharmacology**, v. 153, n. 4, p. 623–4, 2008.

DUPUIS, J.; HOEPER, M. M. Endothelin receptor antagonists in pulmonary arterial hypertension. **European Respiratory Journal**, v. 31, n. 2, p. 407–415, 1 fev. 2008.

ELMARAKBY, A. A. et al. NADPH Oxidase Inhibition Attenuates Oxidative Stress but Not Hypertension Produced by Chronic ET-1. **Hypertension**, v. 45, n. 2, p. 283–287, fev. 2005.

ERIKSSON, S. et al. Omeprazole and H2-receptor antagonists in the acute treatment of duodenal ulcer, gastric ulcer and reflux oesophagitis: a meta-analysis. **European journal of gastroenterology & hepatology**, v. 7, n. 5, p. 467–75, maio 1995.

FASHNER, J.; GITU, A. C. Diagnosis and Treatment of Peptic Ulcer Disease and H. pylori Infection. **American family physician**, v. 91, n. 4, p. 236–42, 15 fev. 2015.

FORD, R. L. et al. Endothelin-A Receptor Inhibition After Cardiopulmonary Bypass: Cytokines and Receptor Activation. **The Annals of Thoracic Surgery**, v. 86, n. 5, p. 1576–1583, nov. 2008.

FRANCIS, S. H.; BUSCH, J. L.; CORBIN, J. D. cGMP-Dependent Protein Kinases and cGMP Phosphodiesterases in Nitric Oxide and cGMP Action. **Pharmacological Reviews**, v. 62, n. 3, p. 525–563, 1 set. 2010.

FRANCIS, S. H.; CORBIN, J. D. Sildenafil: efficacy, safety, tolerability and mechanism of action in treating erectile dysfunction. **Expert Opinion on Drug Metabolism & Toxicology**, v. 1, n. 2, p. 283–293, 16 ago. 2005.

FREEDMAN, R. R.; GIRGIS, R.; MAYES, M. D. Acute effect of nitric oxide on Raynaud’s phenomenon in scleroderma. **The Lancet**, v. 354, n. 9180, p. 739, ago. 1999.

FRIES, J. W. U. MicroRNAs as markers to monitor endothelin-1 signalling and potential treatment in renal disease: Carcinoma − proteinuric damage − toxicity. **Biology of the Cell**, p. boc.201800059, 8 abr. 2019.

FUJITANI, Y. et al. Suppression of endothelin-1-induced mitogenic responses of human aortic smooth muscle cells by interleukin-1 beta. **Journal of Clinical Investigation**, v. 95, n. 6, p. 2474–2482, 1 jun. 1995.

GAGNEMO-PERSSON, R. et al. Growth of the Parathyroid Glands in Omeprazole-Treated Chickens. **Scandinavian Journal of Gastroenterology**, v. 29, n. 6, p. 493–497, 8 jan. 1994.

GAGNEMO-PERSSON, R. et al. Chicken Parathyroid Hormone Gene Expression in Response to Gastrin, Omeprazole, Ergocalciferol, and Restricted Food Intake. **Calcified Tissue International**, v. 61, n. 3, p. 210–215, 1 set. 1997.

GARCÍA-GONZÁLEZ, M. A. et al. Association of PSCA rs2294008 gene variants with poor prognosis and increased susceptibility to gastric cancer and decreased risk of duodenal ulcer disease. **International Journal of Cancer**, v. 137, n. 6, p. 1362–1373, set. 2015.

GIULIANO, F. et al. Safety of sildenafil citrate: review of 67 double-blind placebo-controlled trials and the postmarketing safety database. **International Journal of Clinical Practice**, v. 64, n. 2, p. 240–255, 9 nov. 2009.

GONON, A. T. et al. Limitation of infarct size and attenuation of myeloperoxidase activity by an endothelin A receptor antagonist following ischaemia and reperfusion. **Basic research in cardiology**, v. 96, n. 5, p. 454–62, set. 2001.

GRATTON, J.-P. et al. ET B Receptor and Nitric Oxide Synthase Blockade Induce BQ-123–Sensitive Pressor Effects in the Rabbit. **Hypertension**, v. 30, n. 5, p. 1204–1209, nov. 1997.

GRESSER, U.; GLEITER, C. H. Erectile dysfunction: comparison of efficacy and side effects of the PDE-5 inhibitors sildenafil, vardenafil and tadalafil--review of the literature. **European journal of medical research**, v. 7, n. 10, p. 435–46, 29 out. 2002.

GRIMELIUS, L. et al. The Parathyroid Glands in Experimentally Induced Hypergastrinemia in the Rat. **Scandinavian Journal of Gastroenterology**, v. 12, n. 6, p. 739–744, 23 set. 1977.

GUERRA-MORA, J. R. et al. Effects of Sildenafil and Tadalafil on Edema and Reactive Oxygen Species Production in an Experimental Model of Lung Ischemia-Reperfusion Injury. **Transplantation Proceedings**, v. 49, n. 6, p. 1461–1466, jul. 2017.

HACHULLA, E. et al. Natural history of ischemic digital ulcers in systemic sclerosis: single-center retrospective longitudinal study. **The Journal of rheumatology**, v. 34, n. 12, p. 2423–30, dez. 2007.

HACHULLA, E. et al. Efficacy of sildenafil on ischaemic digital ulcer healing in systemic sclerosis: the placebo-controlled SEDUCE study. **Annals of the Rheumatic Diseases**, v. 75, n. 6, p. 1009–1015, jun. 2016.

HAENISCH, B. et al. Risk of dementia in elderly patients with the use of proton pump inhibitors. **European Archives of Psychiatry and Clinical Neuroscience**, v. 265, n. 5, p. 419–428, 24 ago. 2015.

HANSEN, P. R. Role of Neutrophils in Myocardial Ischemia and Reperfusion. **Circulation**, v. 91, n. 6, p. 1872–1885, 15 mar. 1995.

HASSAN, M.; KASHIMURA, H.; MATSUMARU, K. Gastric Mucosal Injury Induced by Local Ischemia ± Reperfusion in Rats Role of Endogenous Endothelin-1 and Free Radical. v. 42, n. 7, p. 1375–1380, 1997.

HAUG, C. et al. Increased endothelin release by cultured human smooth muscle cells from atherosclerotic coronary arteries. **Cardiovascular Research**, v. 31, n. 5, p. 807–813, maio 1996.

HELLSTROM, W. J. G. et al. Vardenafil for treatment of men with erectile dysfunction: efficacy and safety in a randomized, double-blind, placebo-controlled trial. **Journal of andrology**, v. 23, n. 6, p. 763–71, 2002.

HELLSTROM, W. J. G. et al. Sustained efficacy and tolerability of vardenafil, a highly potent selective phosphodiesterase type 5 inhibitor, in men with erectile dysfunction: results of a randomized, double-blind, 26-week placebo-controlled pivotal trial. **Urology**, v. 61, n. 4 Suppl 1, p. 8–14, abr. 2003.

HILTON, D. et al. Absence of abdominal pain in older persons with endoscopic ulcers: a prospective study. **The American Journal of Gastroenterology**, v. 96, n. 2, p. 380–384, fev. 2001.

HO, P. M. Risk of Adverse Outcomes Associated With Concomitant Use of Clopidogrel and Proton Pump Inhibitors Following Acute Coronary Syndrome. **JAMA**, v. 301, n. 9, p. 937, 4 mar. 2009.

HOUDE, M.; DESBIENS, L.; D'ORLÉANS-JUSTE, P. Endothelin-1. In: [s.l: s.n.]. p. 143–175.

HUMBERT, M. Successful treatment of systemic sclerosis digital ulcers and pulmonary arterial hypertension with endothelin receptor antagonist bosentan. **Rheumatology**, v. 42, n. 1, p. 191–193, 1 jan. 2003.

HUNT, R. H. et al. Helicobacter pylori in developing countries. World Gastroenterology Organisation Global Guideline. **Journal of gastrointestinal and liver diseases : JGLD**, v. 20, n. 3, p. 299–304, set. 2011.

IAQUINTO, G. et al. Role of endogenous endothelin-1 in ethanol-induced gastric mucosal damage in humans. **Digestive Diseases and Sciences**, v. 48, n. 4, p. 663–669, 2003.

INAN, M. et al. Protective effect of sildenafil on liver injury induced by intestinal ischemia/reperfusion. **Journal of Pediatric Surgery**, v. 48, n. 8, p. 1707–1715, ago. 2013.

INCEBIYIK, A. et al. Does sildenafil have protective effects against ovarian ischemia-reperfusion injury in rats? **Archives of Gynecology and Obstetrics**, v. 291, n. 6, p. 1283–1288, 22 jun. 2015.

INOUE, A. et al. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. **Proceedings of the National Academy of Sciences**, v. 86, n. 8, p. 2863–2867, 1 abr. 1989.

ISKIT, A. B. et al. Endothelin receptor antagonist bosentan improves survival in a murine caecal ligation and puncture model of septic shock. **European Journal of Pharmacology**, v. 506, n. 1, p. 83–88, dez. 2004.

IVEY, M. E.; OSMAN, N.; LITTLE, P. J. Endothelin-1 signalling in vascular smooth muscle: Pathways controlling cellular functions associated with atherosclerosis. **Atherosclerosis**, v. 199, n. 2, p. 237–247, ago. 2008.

JUMP, R. L. P.; PULTZ, M. J.; DONSKEY, C. J. Vegetative Clostridium difficile Survives in Room Air on Moist Surfaces and in Gastric Contents with Reduced Acidity: a Potential Mechanism To Explain the Association between Proton Pump Inhibitors and C. difficile-Associated Diarrhea? **Antimicrobial Agents and Chemotherapy**, v. 51, n. 8, p. 2883–2887, 1 ago. 2007.

KANTOR, E. D. et al. Trends in Prescription Drug Use Among Adults in the United States From 1999-2012. **JAMA**, v. 314, n. 17, p. 1818, 3 nov. 2015.

KELLER, S. et al. INHIBITION OF ENDOTHELIN-1-MEDIATED UP-REGULATION OF INOS BY BOSENTAN AMELIORATES ENDOTOXIN-INDUCED LIVER INJURY IN CIRRHOSIS. **Shock**, v. 25, n. 3, p. 306–313, mar. 2006.

KEMPENICH, J. W.; SIRINEK, K. R. Acid Peptic Disease. **Surgical Clinics of North America**, v. 98, n. 5, p. 933–944, out. 2018.

KITADA, K. et al. Inhibition of Endothelin ETB Receptor System Aggravates Neointimal Hyperplasia after Balloon Injury of Rat Carotid Artery. **Journal of Pharmacology and Experimental Therapeutics**, v. 331, n. 3, p. 998–1004, 1 dez. 2009.

KITADA, K.; OHKITA, M.; MATSUMURA, Y. Pathological Importance of the Endothelin-1/ET B Receptor System on Vascular Diseases. **Cardiology Research and Practice**, v. 2012, p. 1–7, 2012.

KLENIEWSKA, P. et al. Influence of Block of NF-Kappa B Signaling Pathway on Oxidative Stress in the Liver Homogenates. **Oxidative Medicine and Cellular Longevity**, v. 2013, p. 1–8, 2013.

KNOLL, M. R. et al. Action of Pure Ethanol and Some Alcoholic Beverages on the Gastric Mucosa in Healthy Humans: A Descriptive Endoscopic Study. **Endoscopy**, v. 30, n. 03, p. 293–301, 17 mar. 1998.

KO, J. K.; CHO, C. H. Alcohol drinking and cigarette smoking: a “partner” for gastric ulceration. **Zhonghua yi xue za zhi = Chinese medical journal; Free China ed**, v. 63, n. 12, p. 845–54, dez. 2000.

KORN, J. H. et al. Digital ulcers in systemic sclerosis: Prevention by treatment with bosentan, an oral endothelin receptor antagonist. **Arthritis and Rheumatism**, v. 50, n. 12, p. 3985–3993, 2004.

KOWAL-BIELECKA, O. et al. Update of EULAR recommendations for the treatment of systemic sclerosis. **Annals of the Rheumatic Diseases**, v. 76, n. 8, p. 1327–1339, ago. 2017.

KREJCI, V. et al. Endothelin receptor antagonist bosentan improves microcirculatory blood flow in splanchnic organs in septic shock. **Critical care medicine**, v. 31, n. 1, p. 203–10, jan. 2003.

KROBOT, K. et al. Effect of inappropriate initial empiric antibiotic therapy on outcome of patients with community-acquired intra-abdominal infections requiring surgery. **European Journal of Clinical Microbiology & Infectious Diseases**, v. 23, n. 9, 21 set. 2004.

KURATA, J. H. et al. A prospective study of risk for peptic ulcer disease in Seventh-Day Adventists. **Gastroenterology**, v. 102, n. 3, p. 902–9, mar. 1992.

LAM, J. R. et al. Proton Pump Inhibitor and Histamine 2 Receptor Antagonist Use and Vitamin B 12 Deficiency. **JAMA**, v. 310, n. 22, p. 2435, 11 dez. 2013.

LANAS, A. et al. The changing face of hospitalisation due to gastrointestinal bleeding and perforation. **Alimentary Pharmacology & Therapeutics**, v. 33, n. 5, p. 585–591, mar. 2011.

LANAS, A. We Are Using Too Many PPIs, and We Need to Stop: A European Perspective. **American Journal of Gastroenterology**, v. 111, n. 8, p. 1085–1086, ago. 2016.

LANAS, A. et al. Peptic ulcer disease. **The Lancet**, v. 390, n. 10094, p. 613–624, ago. 2017.

LATIES, A.; SHARLIP, I. Ocular Safety in Patients Using Sildenafil Citrate Therapy for Erectile Dysfunction. **The Journal of Sexual Medicine**, v. 3, n. 1, p. 12–27, jan. 2006.

LAU, J. Y. et al. Systematic Review of the Epidemiology of Complicated Peptic Ulcer Disease: Incidence, Recurrence, Risk Factors and Mortality. **Digestion**, v. 84, n. 2, p. 102–113, 2011.

LAXMI, V. et al. Inhibitory effects of sildenafil and tadalafil on inflammation, oxidative stress and nitrosative stress in animal model of bronchial asthma. **Pharmacological Reports**, v. 71, n. 3, p. 517–521, jun. 2019.

LAZARATOS, S. et al. Bosentan antagonizes the effects of endothelin-1 on rat gastric blood flow and mucosal integrity. **Life Sciences**, v. 56, n. 9, p. PL195–PL200, jan. 1995.

LI, L. et al. Endothelin-1 Stimulates Arterial VCAM-1 Expression Via NADPH Oxidase-Derived Superoxide in Mineralocorticoid Hypertension. **Hypertension**, v. 42, n. 5, p. 997–1003, nov. 2003.

LIN, C.-C. et al. Up-regulation of COX-2/PGE2 by endothelin-1 via MAPK-dependent NF-κB pathway in mouse brain microvascular endothelial cells. **Cell Communication and Signaling**, v. 11, n. 1, p. 8, 2013.

LOOMIS, E. D. Endothelin Mediates Superoxide Production and Vasoconstriction through Activation of NADPH Oxidase and Uncoupled Nitric-Oxide Synthase in the Rat Aorta. **Journal of Pharmacology and Experimental Therapeutics**, v. 315, n. 3, p. 1058–1064, 24 ago. 2005.

LOPES, P. J. et al. Correlation Between Serum Magnesium Levels and Hepatic Encephalopathy in Immediate Post Liver Transplantation Period. **Transplantation**

Proceedings, v. 45, n. 3, p. 1122–1125, abr. 2013.

LÓPEZ-SEPÚLVEDA, R. et al. Red wine polyphenols prevent endothelial dysfunction induced by endothelin-1 in rat aorta: role of NADPH oxidase. **Clinical Science**, v. 120, n. 8, p. 321–333, abr. 2011.

LU, C. L. et al. Silent peptic ulcer disease: Frequency, factors leading to “silence,” and implications regarding the pathogenesis of visceral symptoms. **Gastrointestinal Endoscopy**, v. 60, n. 1, p. 34–38, 2004.

MALATY, H. M.; GRAHAM, D. Y. Importance of childhood socioeconomic status on the current prevalence of Helicobacter pylori infection. **Gut**, v. 35, n. 6, p. 742–745, 1 jun. 1994.

MALMI, H. et al. Incidence and complications of peptic ulcer disease requiring hospitalisation have markedly decreased in Finland. **Alimentary Pharmacology & Therapeutics**, v. 39, n. 5, p. 496–506, mar. 2014.

MASCLEE, G. M. C. et al. Risk of Upper Gastrointestinal Bleeding From Different Drug Combinations. **Gastroenterology**, v. 147, n. 4, p. 784- 792.e9, out. 2014.

MASCLEE, G. M. C. et al. Increased Risk of Microscopic Colitis With Use of Proton Pump Inhibitors and Non-Steroidal Anti-Inflammatory Drugs. **American Journal of Gastroenterology**, v. 110, n. 5, p. 749–759, maio 2015.

MATSUMARU, K. Gastroenterology Bosentan , a novel synthetic mixed-type endothelin receptor antagonist , attenuates acute gastric mucosai lesions induced by indomethacin and HCI in the rat : Role of endogenous endothelin-1. p. 164–170, 1997.

MATUCCI-CERINIC, M. et al. Bosentan treatment of digital ulcers related to systemic sclerosis: results from the RAPIDS-2 randomised, double-blind, placebo-controlled trial. **Annals of the rheumatic diseases**, v. 70, n. 1, p. 32–8, 2011.

MEDEIROS, J. V. R. et al. Role of the NO/cGMP/K(ATP) pathway in the protective effects of sildenafil against ethanol-induced gastric damage in rats. **British journal of pharmacology**, v. 153, n. 4, p. 721–7, 2008.

MEYER, M. R.; BARTON, M.; PROSSNITZ, E. R. Functional heterogeneity of NADPH oxidase-mediated contractions to endothelin with vascular aging. **Life Sciences**, v. 118, n. 2, p. 226–231, nov. 2014.

MIAN, M. O. R. et al. Preservation of Endothelium-Dependent Relaxation in Atherosclerotic Mice with Endothelium-Restricted Endothelin-1 Overexpression. **Journal of Pharmacology and Experimental Therapeutics**, v. 347, n. 1, p. 30–37, 1 out. 2013.

MOHEY, V. et al. Sildenafil obviates ischemia-reperfusion injury–induced acute kidney injury through peroxisome proliferator–activated receptor γ agonism in rats. **Journal of Surgical Research**, v. 201, n. 1, p. 69–75, mar. 2016.

MONTANI, D. et al. Pulmonary veno-occlusive disease. **European Respiratory Journal**, v. 33, n. 1, p. 189–200, 1 jan. 2009.

MONTANI, D. et al. Pulmonary arterial hypertension. **Orphanet Journal of Rare Diseases**, v. 8, n. 1, p. 97, 2013.

MONTEZANO, A. C. et al. Nicotinamide Adenine Dinucleotide Phosphate Reduced Oxidase 5 (Nox5) Regulation by Angiotensin II and Endothelin-1 Is Mediated via Calcium/Calmodulin-Dependent, Rac-1-Independent Pathways in Human Endothelial Cells. **Circulation Research**, v. 106, n. 8, p. 1363–1373, 30 abr. 2010.

MOTTE, S.; MCENTEE, K.; NAEIJE, R. Endothelin receptor antagonists. **Pharmacology & Therapeutics**, v. 110, n. 3, p. 386–414, jun. 2006.

MOUSTAFA, Y. M. et al. Sildenafil citrate protects against gastric mucosal damage induced by indomethacin in rats. **European Review for Medical and Pharmacological Sciences**, v. 17, n. 2, p. 179–188, 2013.

NAJM, W. I. Peptic Ulcer Disease. **Primary Care: Clinics in Office Practice**, v. 38, n. 3, p. 383–394, set. 2011.

NOSHAD, H. et al. Arterial atherosclerosis in patients with chronic kidney disease and its relationship with serum and tissue endothelin-1. [corrected]. **Iranian journal of kidney diseases**, v. 3, n. 4, p. 203–9, out. 2009.

O'DONOGHUE, M. L. et al. Pharmacodynamic effect and clinical efficacy of clopidogrel and prasugrel with or without a proton-pump inhibitor: an analysis of two randomised trials. **The Lancet**, v. 374, n. 9694, p. 989–997, set. 2009.

OKTAR, B. K. et al. Endothelin-1-induced PMN infiltration and mucosal dysfunction in the rat small intestine. **American Journal of Physiology-Gastrointestinal and Liver Physiology**, v. 279, n. 3, p. G483–G491, set. 2000.

PADOL, I.; HUANG, J. Q.; HUNT, R. H. Anti-ulcerogenic properties of endothelin receptor antagonists in the rat. **Alimentary Pharmacology and Therapeutics**, v. 13, n. 4, p. 537–544, 1999.

PIECHOTA-POLAŃCZYK, A.; GORĄCA, A. Influence of specific endothelin-1 receptor blockers on hemodynamic parameters and antioxidant status of plasma in LPS-induced endotoxemia. **Pharmacological reports : PR**, v. 64, n. 6, p. 1434–41, 2012.

PIECHOTA, A.; GORACA, A. Influence of nuclear factor-κB inhibition on endothelin-1 induced lung edema and oxidative stress in rats. **Journal of physiology and pharmacology : an official journal of the Polish Physiological Society**, v. 62, n. 2, p. 183–8, abr. 2011.

RAPOPORT, R. M. Acute nitric oxide synthase inhibition and endothelin-1-dependent arterial pressure elevation. **Frontiers in Pharmacology**, v. 5, 1 abr. 2014.

RICHTER, J. E. A critical review of current medical therapy for gastroesophageal reflux disease. **Journal of clinical gastroenterology**, v. 8 Suppl 1, p. 72–80, 1986.

ROSENSTOCK, S. J.; JØRGENSEN, T. Prevalence and incidence of peptic ulcer disease in a Danish County--a prospective cohort study. **Gut**, v. 36, n. 6, p. 819–24, jun. 1995.

ROSS, L.; NIKPOUR, M. Gaining the Upper Hand on Systemic Sclerosis Digital Ulcers. **The Journal of Rheumatology**, v. 46, n. 6, p. 548–549, 1 jun. 2019.

RUBANYI, G. M.; POLOKOFF, M. A. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. **Pharmacological reviews**, v. 46, n. 3, p. 325–415, set. 1994.

SAIDA, K.; MITSUI, Y.; ISHIDA, N. A novel peptide, vasoactive intestinal contractor, of a new (endothelin) peptide family. Molecular cloning, expression, and biological activity. **The Journal of biological chemistry**, v. 264, n. 25, p. 14613–6, 5 set. 1989.

SALONIA, A.; RIGATTI, P.; MONTORSI, F. Sildenafil in erectile dysfunction: a critical review. **Current Medical Research and Opinion**, v. 19, n. 4, p. 241–262, 22 jan. 2003.

SARAC, M. et al. Comparison of the therapeutic effects of sildenafil citrate, heparin and neuropeptides in a rat model of acetic acid-induced gastric ulcer. **Life Sciences**, v. 186, n. July, p. 102–110, 2017.

SCALERA, F. et al. Effect of endothelin-1 on intracellular glutathione and lipid peroxide availability and on the secretion of vasoactive substances by human umbilical vein endothelial cells. **European journal of clinical investigation**, v. 32, n. 8, p. 556–62, ago. 2002.

SCHNEIDER, L. A. et al. Molecular mechanism of mast cell–mediated innate defense against endothelin and snake venom sarafotoxin. **The Journal of Experimental Medicine**, v. 204, n. 11, p. 2629–2639, 29 out. 2007.

SCHUMOCK, G. T. et al. National trends in prescription drug expenditures and projections for 2016. **American Journal of Health-System Pharmacy**, v. 73, n. 14, p. 1058–1075, 15 jul. 2016.

SCHWESINGER, W. Operations for peptic ulcer disease: paradigm lost. **Journal of Gastrointestinal Surgery**, v. 5, n. 4, p. 438–443, ago. 2001.

SHIOTANI, A. et al. Single nucleotide polymorphism markers for low-dose aspirin-associated peptic ulcer and ulcer bleeding. **Journal of Gastroenterology and Hepatology**, v. 29, p. 47–52, dez. 2014.

SHIOTANI, A.; FUJITA, Y.; NISHIO, K. Low-Dose Aspirin-Associated Upper and Mid Gastrointestinal Tract Damage and Gene Polymorphism. **Current Pharmaceutical Design**, v. 21, n. 35, p. 5066–5072, 30 out. 2015.

SIDHARTA, P. N.; VAN GIERSBERGEN, P. L. M.; DINGEMANSE, J. Safety, tolerability, pharmacokinetics, and pharmacodynamics of macitentan, an endothelin receptor antagonist, in an ascending multiple-dose study in healthy subjects. **The Journal of Clinical Pharmacology**, p. n/a-n/a, ago. 2013.

SONNENBERG, A. Time Trends of Ulcer Mortality in Non-European Countries. **The American Journal of Gastroenterology**, v. 102, n. 5, p. 1101–1107, maio 2007a.

SONNENBERG, A. Time Trends of Ulcer Mortality in Europe. **Gastroenterology**, v. 132, n. 7, p. 2320–2327, jun. 2007b.

SONNENBERG, A. Review article: historic changes of Helicobacter pylori -associated diseases. **Alimentary Pharmacology & Therapeutics**, v. 38, n. 4, p. 329–342, ago. 2013.

SONNENBERG, A.; EVERHART, J. E. Health impact of peptic ulcer in the United States. **The American journal of gastroenterology**, v. 92, n. 4, p. 614–20, abr. 1997.

SØREIDE, K. et al. Perforated peptic ulcer. **The Lancet**, v. 386, n. 10000, p. 1288–1298, set. 2015.

SPENCE, R. W.; CELESTIN, L. R. Gynaecomastia associated with cimetidine. **Gut**, v. 20, n. 2, p. 154–7, fev. 1979.

SUERBAUM, S.; MICHETTI, P. Helicobacter pylori Infection. **New England Journal of Medicine**, v. 347, n. 15, p. 1175–1186, 10 out. 2002.

TAIBI, G. et al. Sildenafil protects epithelial cell through the inhibition of xanthine oxidase and the impairment of ROS production. **Free Radical Research**, v. 44, n. 2, p. 232–239, 8 jan. 2010.

TANIKAWA, C. et al. A genome-wide association study identifies two susceptibility loci for duodenal ulcer in the Japanese population. **Nature Genetics**, v. 44, n. 4, p. 430–434, 4 abr. 2012.

TARGOWNIK, L. E. et al. Use of proton pump inhibitors and risk of osteoporosis-related fractures. **CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne**, v. 179, n. 4, p. 319–26, 2008.

THAKALI, K. et al. Endothelin-1-induced contraction in veins is independent of hydrogen peroxide. **American Journal of Physiology-Heart and Circulatory Physiology**, v. 289, n. 3, p. H1115–H1122, set. 2005.

VAEZI, M. F.; YANG, Y. X.; HOWDEN, C. W. Complications of Proton Pump Inhibitor Therapy. **Gastroenterology**, v. 153, n. 1, p. 35–48, 2017.

VAN VLIET, E. P. M. et al. Inappropriate prescription of proton pump inhibitors on two pulmonary medicine wards. **European Journal of Gastroenterology & Hepatology**, v. 20, n. 7, p. 608–612, jul. 2008.

VIRDIS, A.; SCHIFFRIN, E. L. Vascular inflammation: a role in vascular disease in hypertension? **Current opinion in nephrology and hypertension**, v. 12, n. 2, p. 181–7, mar. 2003.

VISWANATHA SWAMY, A. H. . et al. Cardioprotective effect of ascorbic acid on doxorubicin-induced myocardial toxicity in rats. **Indian Journal of Pharmacology**, v. 43, n. 5, p. 507, 2011.

VLACHOPOULOS, C. et al. Acute effect of sildenafil on inflammatory markers/mediators in patients with vasculogenic erectile dysfunction. **International Journal of Cardiology**, v. 182, p. 98–101, mar. 2015.

VON BRANDENSTEIN, M.; RICHTER, C.; FRIES, J. W. U. MicroRNAs: Small but

amazing, and their association with endothelin. **Life Sciences**, v. 91, n. 13–14, p. 475–489, out. 2012.

WANDALL, J. H. Effects of omeprazole on neutrophil chemotaxis, super oxide production, degranulation, and translocation of cytochrome b-245. **Gut**, v. 33, n. 5, p. 617–621, 1 maio 1992.

WANG, G. et al. Sildenafil Protects against Myocardial Ischemia-Reperfusion Injury Following Cardiac Arrest in a Porcine Model: Possible Role of the Renin-Angiotensin System. **International Journal of Molecular Sciences**, v. 16, n. 11, p. 27015–27031, 12 nov. 2015.

WANG, P.-Y. et al. Impact of Blood Type, Functional Polymorphism (T-1676C) of the COX-1 Gene Promoter and Clinical Factors on the Development of Peptic Ulcer during Cardiovascular Prophylaxis with Low-Dose Aspirin. **BioMed Research International**, v. 2014, p. 1–6, 2014.

WEDGWOOD, S. et al. Role for Endothelin-1–Induced Superoxide and Peroxynitrite Production in Rebound Pulmonary Hypertension Associated With Inhaled Nitric Oxide Therapy. **Circulation Research**, v. 89, n. 4, p. 357–364, 17 ago. 2001.

XIE, K. et al. Hydrogen Gas Presents a Promising Therapeutic Strategy for Sepsis. **BioMed Research International**, v. 2014, p. 1–9, 2014.

XU, H.; LIN, L.; YUAN, W.-J. Antiarrhythmic effect of endothelin-A receptor antagonist on acute ischemic arrhythmia in isolated rat heart. **Acta pharmacologica Sinica**, v. 24, n. 1, p. 37–44, jan. 2003.

YAMANE, K. et al. Significance of plasma endothelin-1 levels in patients with systemic sclerosis. **The Journal of rheumatology**, v. 19, n. 10, p. 1566–71, out. 1992.

YANAGISAWA, M. et al. **A novel potent vasoconstrictor peptide produced by vascular endothelial cellsNature**, 1988. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/2451132>

YEAGER, M. E. et al. Endothelin-1, the Unfolded Protein Response, and Persistent Inflammation. **American Journal of Respiratory Cell and Molecular Biology**, v. 46, n. 1, p. 14–22, jan. 2012.

ZAHRAN, M. H. et al. Sildenafil activates antioxidant and antiapoptotic genes and inhibits proinflammatory cytokine genes in a rat model of renal ischemia/reperfusion injury. **International Urology and Nephrology**, v. 47, n. 11, p. 1907–1915, 16 nov. 2015.

ZHANG, M. Seroepidemiology of Helicobacter pylori infection in elderly people in the Beijing region, China. **World Journal of Gastroenterology**, v. 20, n. 13, p. 3635, 2014.