UNIVERSIDADE ESTADUAL DE CAMPINAS

Ana Beatriz Albino de Almeida

ATIVIDADE ANTIULCEROGÊNICA E ANTIINFLAMATÓRIA INTESTINAL DA *Arctium lappa*

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Ana Beatriz Albino de Almeida e aprovada pela Comissão Julgadora.

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Funcional e Molecular, na área de Fisiologia

Orientadora: Profa. Dra Alba Regina Monteiro Souza Brito

2005

FICHA CATALOGRÁFICA ELABORADA PELA BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP

Almeida, Ana Beatriz Albino de

AL64a Atividade antiulcerogênica e antinflamatória intestinal da *Arctium lappa* /Ana Beatriz Albino de Almeida. -- Campinas, SP: [s.n.], 2004.

Orientadora: Alba Regina Monteiro Souza Brito
Tese (Doutorado) – Universidade Estadual de Campinas . Instituto de Biologia.

1. Úlcera gástrica. 2. Sesquiterpenos. 3. Bardana (Planta). I. Brito, Alba Regina Monteiro Souza. II. Universidade Estadual de Campinas. Instituto de Biologia. III.Título.

DATA DA DEFESA: 31/01/2005

BANCA EXAMINADORA

Profa. Dra. Alba Regina Monteiro Souza Brito (Orientadora) — Assinatura

Profa. Dra. Helena Coutinho Franco de Oliveira — Assinatura

Prof. Dr. Wagner Vilegas — Assinatura

Profa. Dra. Clélia Akiko Hiruma Lima — Assinatura

Prof. Dr. Juvenal Ricardo Navarro Góes — Assinatura

Prof. Dr. Luiz Claudio Di Stasi — Assinatura

Profa. Dra. Lourdes Campaner dos Santos — Assinatura

Resumo

Onodorpicrina, uma sesquiterpeno lactona, presente nas folhas de *Arctium Lapa* L. demonstrou através dos estudos de Barbosa et at (1993) erficaz atividade farmacológica. Com base nesses resultados e, devido ao fato, de nosso laboratório possuir estudos em relação à atividade antiulcerogênica de outras sesquiterpeno lactonas (desidrocrotonina e crotonina) obtidas das cascas de *Croton cajucara* Benth, decidimos estudar a atividade antiulcerogênica e antiinflamtória intestinal da fração semi-purificada (ONP), cujo composto majoritário é a onopordopicrina. Primeiramente estudamos o efeito da citotoxicidade da ONP avaliado através da viabilidade celular em fibroblastos (V79) de pulmão de Hamster chinês. A ONP apresentou IC_{50} aproximadamente de 15µM nos ensaios de MTT, vermelho neutro e conteúdo de ácido nucléico. Por ser uma substância com uma toxicidade relativa resolvemos estudar o efeito antitumoral dessa substância em linhagem celular de leucemia promielocítica (HL60) usada como modelo para estudos antitumorais. Da mesma forma que nos ensaios de citotoxidade, a ONP apresentou IC_{50} aproximadamente de 15 µM em ensaios de MTT e PTP. Para avaliar a atividade antiulcerogênica da ONP, foram utilizadas diferentes doses em modelos clássicos de indução de úlcera: etanol/HCl, estresse por imobilização e frio, indometacina e ligadura de piloro. Em todos os modelos a ONP apresentou atividade antiulcerogênica significativa. Nos procedimentos seguintes, avaliamos a atividade antisecretora da ONP através de ensaios de somatostatina e gastrina, nos quais a substância apresentou aumento da liberação e/ou produção de somatostatina e diminuição da produção e/ou liberação de gastrina. Em relação ao estudo da ONP em experimentos de muco e óxido nítrico, essa não apresentou diferença significativa. No entanto, promoveu diferença significativa no modelo de avaliação do envolvimento de radicais sulfidrila, indicando uma possível atividade antioxidante. Quando avaliada em modelo de colite ulcerativa induzida por TNBS, a ONP apresentou significativa proteção provavelmente pelo envolvimento da mieloperoxidase (MPO) e fator de necrose tumoral (TNF-alfa). Visto a atividade obtida com a ONP, resolvemos analisar a atividade do chá de Arctium lappa, o qual apresentou significativa envolvimento com óxido nítrico e grupamento sulfidrila para a obtenção da atividade antiulcerogência promovida pelo chá. Os resultados obtidos com a *Arctium lappa* são

promissores, por causa de sua significativa proteção contra úlceras induzidas por diferentes agentes, sugerindo um efeito antisecretor mediado por sua ação na secreção de Somatostatina e Gastrina e um efeito protetor proporcionado pela propriedade antioxidante presente na ONP. Os dados também revelaram que o pré-tratamento com ONP é capaz de reduzir a inflamação intestinal produzida através do modelo de indução de colite ulcerativa por TNBS em ratos. O efeito agudo antiinflamatório provavelmente está relacionado com a diminuição de neutrófilos e diminuição da produção de TNF-α na mucosa intestinal. Nossos resultados sugerem um significativo potencial terapêutico da ONP na area gastrointestinal.

Abstract

Onodorpicrin, a sesquiterpene lactone, present in the leaves of *Arctium lappa* L., showed through the studies of Barbosa et al (1993) effective pharmacological activity. Based on those results and due to the fact that our laboratory has studies in relation to the antiulcerogenic activity of other sesquiterpene lactone (dehydrocrotonin and crotonin) obtained by barks of *Croton cajucara* Benth, we decided to study the antiulcerogenic activity and bowel antiinflamatory of the semi-purified fraction (ONP), which majority compound is the onopordopicrin. Firstly we studied the effect of the citotoxicity of appraised ONP through the cellular viability in fibroblast (V79) of lung of Chinese Hamster. ONP presented IC_{50} approximately of 15μM in the MTT, red neutral and content of nucleic acid experiments. For being a substance with a relative toxicity we decided to study the effect antitumoral of that substance in cellular lineage of promyelocytic leukemia (HL60) used as model for antitumoral studies. In the same way that in the citotoxicity experiments, ONP presented IC50 approximately of 15 μM in experiments of MTT and PTP. To evaluate the antiulcerogenic activity of ONP, different doses were used in classic models of ulcer induction: ethanol/HCl, stress for immobilization and cold, indometacin and pylorus ligature. In all of the models ONP presented significant antiulcerogenic activity. In the following procedures, we evaluated the activity antisecretory of ONP through somatostatin and gastrin experiments, in which the substance presented increase of the liberation and/or somatostatin production and decrease of the liberation and/or gastrin production. In relation to the study of ONP in mucus experiments and nitric oxide, that one didn't present significant difference. However, it showed significant difference in the model of evaluation of the involvement of radicals sulphydryl, indicating a possible antioxidant activity. When evaluated in model of colitis ulcerative induced by TNBS, ONP presented significant protection probably for the involvement of the mieloperoxidase (MPO) and factor of necrosis tumoral (TNF-alpha). Checked the activity obtained with ONP, we decided to analyze the activity of the tea of *Arctium lappa*, which presented significant involvement with nitric oxide and sulphydryl group for the obtaining of the antiulcerogenic activity promoted by the tea. The results obtained with *Arctium lappa* are promising, because the significant protection against ulcers induced by different agents suggesting a

effect antisecretory mediated through somatostatin and gastrin secretion and protective effect by the antioxidant properties present in ONP. The data also reveals that pre-treatment with ONP is able to reduce intestinal inflammation in theTNBS model of colitis in rats.The acute antiinflamatory effects seem to be related to impairment of neutrophil function and absence of up-regulation of TNF-α production in intestinal mucosa. Our findings suggest that ONP shows an excellent potential for therapy in the gastrointestinal area

Ao meu pai (in memoriam), que no silêncio de sua presença amparou-me com seu doce e eterno amor.

À minha mãe pelo universo de sabedoria, fé, bondade, determinação, cuidado e amor que a sua presença representa em minha vida.

Ao André, irmão e amigo, fonte de alegria, parceiro de vida e de ideais.

À Profa Dra Alba pelos contínuos ensinamentos, amizade e profissionalismo. Minha gratidão por permitir, que esse importante momento faça parte da minha história de vida

Agradecimentos

Este trabalho não teria sido elaborado sem o auxílio de diversas pessoas e algumas entidades, às quais quero expressar meus sinceros agradecimentos:

Aos animais, às plantas e a população brasileira por exercerem papel fundamental para o desenvolvimento da ciência.

À Profa Dra Catalina Alarcon de La Lastra, Departamento de Farmacologia da Universidade de Sevilha-Espanha, pela competente orientação em meu trabalho realizado em seu laboratório e pela carinhosa recepção e amizade

Ao Prof Dr Antonio Zarzuelo, Departamento de Farmacologia de Granada-Espanha, pelo profissionalismo e valiosos ensinamentos científicos.

Ao Prof Dr José Roberto Trigo, Departamento de Zoologia da UNICAMP, pela importante orientação para obtenção do material fitoquímico e por disponibilizar seu laboratório para realização desses ensaios

À Profa Dra Marcela Haun, Dra Patrícia S Melo, Maristella Conte Anazetti, João B.F. Neto e Daniel, do Departamento de Bioquímica da UNICAMP, pela amizade e colaboração na realização dos ensaios in vitro em cultura de células.

Ao Prof Dr Wagner Villegas, Profa Dra Lourdes C. Santos, Marcio R. F. Tlene pela competente colaboração para obtenção do material fitoquímico.

À minha família (avós, tios e primos), que dia a dia acompanha-me acariciando-me com seus aconchegantes braços amorosos. Minha eterna gratidão e meu eterno amor a vocês que sempre caminharam ao meu lado dando-me continuamente a força, o estímulo e a coragem que sempre necessitei.

Aos meus amigos de laboratório, com os quais não dividi somente experimentos, mas tive o privilégio de poder dividir minha vida, minhas alegrias, angústias e ideais.

Aos meus amigos do laboratório da Espanha, os quais foram extensão dos meus queridos amigos de laboratório do Brasil, que, tais como os daqui, souberam com brilhantismo realizar os experimentos necessários para a conclusão da minha tese, bem como compartilhar minhas saudades, alegrias e conquistas.

Aos meus amigos não Unicampianos e nem Sevilhanos, que pertencem à minha vida há muito tempo e que doaram um pouquinho de si mesmos para eu poder compor o mosaico de minha alma.

Aos meus "novos" amigos, que nasceram em minha vida no período em que estava na UNICAMP e que, junto com os demais, teceram em meu ser as mais profundas alegrias, as trabalhosas conquistas e as queridas realizações.

Aos funcionários do Departamento de Fisiologia e Biofísica da UNICAMP, da Universidade de Sevilha e da Universidade de Granada, pelo contínuo carinho e auxílios prestados.

Aos docentes e colegas do Departamento de Fisiologia e Biofísica da Unicamp pela colaboração na minha formação profissional.

À CAPES pelo apoio financeiro para minha formação científica no Brasil e no Exterior.

Índice

I. Secreção Ácida Gástrica

Nos últimos 20 anos o estudo das doenças relacionadas à úlcera péptica gástrica e duodenal, tem aumentado significativamente devido à identificação de várias técnicas, as quais têm possibilitado o estudo mais detalhado da mucosa gástrica (Brzozowski, 2003).

Na média populacional o risco de complicação com úlcera aumenta quatro vezes, resultando em 1,25 hospitalizações adicionais para cada 100 pacientes por ano (Hawkey, 2000). A secreção ácida é o aspecto mais importante da função gástrica estudado na prática clínica. A influência de alterações nessa função tem sido considerada no desenvolvimento de drogas (Pohle et al., 2003).

As doenças ulcerativas do trato gastrointestinal dependem de duas condições: presença de ácido e predisposição das mucosas às lesões por fatores diversos. Não há maneiras estabelecidas de interferir farmacologicamente com as predisposições, genéticas ou não, da mucosa aos danos. Assim sendo, terapias preventivas continuam sendo o meio mais utilizado de controle da secreção ácida, pois reverter os danos causados à mucosa e controlar os eventos inflamatórios que se sucedem após a instalação das lesões são tarefas bem mais complexas (Brzozowski, 2003).

O processo de secreção consiste em três etapas, duas das quais são estimuladoras - fase cefálica e fase gástrica, e outra inibidora – fase intestinal. Estes estágios começam através de: fenômeno neurológico – pensamento, cheiro ou memória, alimentos, drogas e outras substâncias ingeridas (Chavez et al., 1996).

As alternativas terapêuticas disponíveis para regulação da secreção ácida buscam modificar a influência neural, por meio de cirurgias, ou alterar os mecanismos que envolvem os segundos mensageiros na célula parietal, através da utilização de antagonistas muscarínicos ou histamínicos; alterar o último evento da cascata de reações envolvidas com a secreção pode ser conseguido com o uso dos inibidores da bomba protônica (Hirschowitz et al., 1995). A produção do ácido depende, numa primeira etapa, da histamina, acetilcolina e gastrina, primeiros mensageiros do processo (Chavez et al., 1996).

Algumas vias de sinalização intracelular têm sido identificadas como importantes contribuintes na ativação da célula parietal, incluindo proteína quinase A (PKA), proteína quiinase C (PKC), calmodulina-Ca^{2+} (CaM), fosfatidil-inositol (IP3), e várias outras quinases (Yao, 2003).

A mucosa gástrica exposta a agentes ulcerogênicos necrotizantes como aspirina, indometacina, ácidos biliares, álcool e isquemia, desenvolve características morfológicas, alterações ultraestruturais e funcionais que refletem em injúrias. As lesões agudas da mucosa gástrica estão associadas com: 1)

ruptura da camada e superfície hidrofóbica da membrana, 2) injúria e esfoliação da superfície do epitélio com perda da barreira e função elétrica, 3) injúria profunda na camada da mucosa incluindo célula endotelial microvascular, zona de proliferação celular, e célula parietal e principal. Danos do endotélio microvascular levam a uma parada microvascular, cessando oxigênio e transporte de nutrientes. Todos esses eventos resultam na formação de erosão e ulceração na mucosa. A diferença entre erosão e úlcera é que a erosão se implanta na mucosa, enquanto a úlcera penetra na muscular da mucosa (Jones et al., 1999).

1. Bases Celulares da Secreção Ácida

Nos 25 primeiros anos do século vinte foram desvendados os principais eventos envolvidos com a estimulação da secreção ácida. A gastrina, um hormônio liberado a partir do antro (Edkins, 1906), e a acetilcolina liberada a partir do nervo vago (Loewi et al., 1921), foram respectivamente identificadas como agentes responsáveis pelas regulações periférica e central da secreção ácida; pouco antes desta última descoberta, o papel da histamina como potente secretagogo já havia sido descrito (Popielski, 1920). A secreção ácida no estômago é produzida pela célula oxíntica, uma dos diversos tipos de células presentes nas glândulas gástricas (Yao et al., 2003).

Os primeiros tratamentos das patologias existentes estiveram relacionados à ingestão de alimentos pelo homem. Os gregos tiveram um respeito especial à digestão do alimento. De acordo com suas teorias, o alimento no estômago era transformado em quimo através de quatro líquidos sistêmicos: sangue, muco, bile e bile preta (sangue esplênico) (Srodka, 2003). A terapêutica para regulação da secreção ácida, até 1973, dependia de intervenção cirúrgica ou da utilização de bloqueador vagal, o extrato de Atropa beladona. Após essa data surgiu a cimetidina, o primeiro antagonista de receptores H_2. Code et al (1955) estabeleceram que a histamina, melhor que a gastrina, era um quimio estimulador das células oxínticas dado que: 1) histamina age diretamente na célula oxíntica, 2) histamina foi detectada em grande quantidade na mucosa gástrica, localmente liberada por células ECL, possuindo a histamina descarboxilase para transformar histidina em histamina, 3) histaminase, que poderia destruir efetivamente histamina, não pode ser detectada na mucosa oxíntica, 4) histamina é liberada por estimulantes da secreção ácida como comida ou gastrina. Segundo esses autores, a ação da gastrina e da acetilcolina nos histaminócitos (agora conhecidos como células Enterocromafin – ECL) é responsável pela liberação de histamina, a qual é estimulada pelas glândulas oxínticas.

A introdução de antagonista histaminérgico modificou os tratamentos até então empregados na úlcera, assim como contribuiu no sentido de melhorar a compreensão da fisiologia regulatória das secreções gástricas. Esse antagonista de receptor H_2 não somente bloqueou a secreção ácida induzida

por histamina, como também aquela induzida por gastrina e, parcialmente, aquela induzida por mediação vagal. É importante ressaltar que as moléculas de histamina não se assemelham nem à gastrina e nem à acetilcolina; pode-se afirmar que a liberação de histamina é o maior evento regulatório na estimulação da secreção ácida (Sachs et al., 1994; Aihara et al., 2003)

Experimentos in vivo sugerem que a gastrina e a acetilcolina estimulam a célula enterocromafin "like" (ECL), a liberar histamina. ECL tem um "único maquinário" para controlar a síntese e a estocagem de histamina sob diferentes condições fisiológicas. A ativação de seus receptores causa influxo de cálcio seguido por liberação da histamina estocada (Prinz et al. 2003).

A importância das células ECL em condições fisiológicas tem sido determinada por inúmeros estudos, mas pouco se sabe à respeito da resposta das células ECL durante inflamação gástrica crônica, a qual geralmente é provocada por bactéria gram-negativa Helicobacter pylori (Wessler et al., 2002).

A célula parietal, com seus distintos receptores é bastante estudada; embora o potencial eletroquímico, que culmina com sua secreção seja conhecido, os mecanismos moleculares e suas origens ainda são pouco claros (Black, 1993). Exemplo disto são as interações dos vários agentes secretagogos com seus receptores específicos, envolvendo séries imensas de reações em cascata que conduzirão, finalmente, à secreção gástrica ou à sua inibição.

O desenvolvimento de potentes drogas anti-secretoras, como antagonistas do receptor de histamina e inibidores da bomba de proton, resultam na formação de bicarbonato de sódio, que é secretado na superfície das células epiteliais na cavidade gástrica (Tsukimi et al., 2001).

A liberação de gastrina, a partir da célula G antral, é o evento responsável pela estimulação de histamina a partir das células ECL in vivo, tornando-se fundamental para a estimulação da secreção ácida. A somatostatina, contida nas células D localizadas no fundo e antro do estômago, inibe a gastrina e, consequentemente, a estimulação da secreção ácida através de sua ligação às células ECL. A regulação da secreção ácida por células endócrinas gástricas envolve, portanto, interações positivas e negativas entre o trio vital de células G, ECL e D (Hakanson et al., 1986).

2. Regulação da Secreção Ácida

O Sistema Nervoso Central (SNC) regula a atividade parassimpática no plexo mioentérico da parede gástrica. A regulação é realizada por modulação da atividade dos núcleos do hipotálamo, através de peptídeos e transmissores químicos. A modificação da atividade vagal afeta a secreção ácida por meio de alterações nas complexas redes dos sistemas reflexos aferentes e eferentes (Tache et al., 1990).

O complexo dorsal vagal (CDV) tem um importante papel na regulação da integridade da mucosa gástrica, envolvendo proteção da mucosa e formação de úlcera. O neurônio aferente tem

função semelhante ao eferente no trato gastrointestinal; neuropeptídeos liberados a partir de terminações nervosas periféricas de neurônios aferentes primários podem induzir proteção da mucosa gástrica (Gyires, 2004)

A via neural estimula diretamente células parietais, células G, no antro, responsável pela liberação de gastrina, bem como célula ECL presente na glândula oxíntica, promovendo a liberação de histamina. A estimulação direta e indireta da célula parietal é mediada principalmente por três receptores: muscarínico colinérgico (M_3R), histaminérgico (H_2R) e receptor de gastrina/CCK-B (CCK_2R). Desses três receptores, entretanto, o H_2R parece ser o receptor chave na secreção ácida gástrica, pois antagonistas de H_2R não inibem somente a secreção ácida estimulada pela histamina, mas também a secreção ácida estimulada por gastrina e acetilcolina (Aihara et al.,2003).

A ativação de receptor de histamina resulta num aumento dos níveis intracelulares de AMP_c, o qual age como segundo mensageiro para a transferência do sinal na etapa final da secreção ácida. Por outro lado, a estimulação do receptor muscarínico M_3, pela acetilcolina, ou do receptor CCK_2R pela gastrina, resulta num sinal mediado pelo aumento intracelular de íons de cálcio livre (Aihara et al., 2003).

Embora a histamina tem sido reconhecida como um potente secretagogo na secreção ácida desde 1920, o seu papel fisiológico no estômago é novamente tema de debates, também a acetilcolina e gastrina são de grande importância na estimulação da produção ácida gástrica (Barocelli et al., 2003). Na última década, um número crescente de pesquisas tem enriquecido substancialmente o conhecimento sobre mecanismos que envolvem a histamina no controle da secreção ácida (Lewin et al., 1991).

A gastrina é liberada em resposta a produtos da digestão tais como aminoácidos; sua inibição é promovida pela somatostatina (produzida pelas células D) quando o pH cai abaixo de 3 no antro. O ácido aplicado diretamente à célula G inibe a secreção de gastrina; já na célula D, o mesmo procedimento estimula a secreção de somatostatina, o que consequentemente inibe a liberação de gastrina (Zavros et al., 2002).

A inibição da ativação do receptor de histamina H_2 é um meio efetivo e seguro de regular a secreção ácida gástrica. Ligantes colinérgicos não somente estimulam a liberação de histamina a partir das células ECL, como também estimulam diretamente a célula parietal por ativação do receptor M_3 na superfície celular. Por essa razão, um antagonista do receptor M_3 pode inibir completamente, tanto direta quanto indiretamente, a secreção ácida visto que as vias de estimulação da célula parietal não são distintas. (Wilkes et al., 1991).

3. Sistema de Receptores da Célula Parietal

A terapêutica da secreção ácida gástrica tem avançado nos últimos anos, devido ao aumento da especificidade de fármacos envolvidos na inibição da secreção ácida gástrica (Sachs, 2003). A úlcera péptica, o refluxo gastroesofágico e o sangramento na parte superior do estômago são condições que aumentam a demanda no cuidado à saúde. A supressão de ácido é uma terapia comum nessas situações. Estudos de custo comparando os diversos supressores de ácido incluem benefícios econômicos prevenindo complicações (Erstad, 2003).

Agentes farmacológicos, como antagonistas de receptor H_2 e inibidores da bomba, são freqüentemente utilizados no tratamento de doenças relacionadas à acidez tais como úlcera gastroduodenal e esofagite de refluxo. O aumento da compreensão do mecanismo preciso da secreção ácida gástrica no nível de receptor, enzimas e sistema de transdução de sinal citoplasmático, possibilita o desenvolvimento de uma efetiva farmacoterapia antisecretora (Aihara et al., 2003)

3.1. Receptor muscarínico e seu segundo mensageiro

Devido à eficácia de antagonistas do receptor H_2, o receptor muscarínico tem sido pouco estudado em relação à secreção ácida gástrica. Somente em 1971 foi aceito, na terapêutica, que esse receptor pudesse estar envolvido na regulação da secreção. O receptor muscarínico é conhecido por estar envolvido na regulação da contração do músculo liso em vários órgãos incluindo o trato gasrtointestinal (Eglen et al., 2001).

Cinco subtipos de receptor muscarínico (M_1-M_5) foram clonados. Receptores muscarínicos e seus subtipos têm sido encontrados na mucosa gástrica de muitas espécies, especificamente nas células parietais, principais e endócrinas. Contudo, muitas vezes, é difícil identificar o subtipo de receptor específico que media a ação muscarínica da acetilcolina no músculo liso, devido à falta de ligantes específicos a esses subtipos (Insuk et al., 2003). Mecanismos ligados aos receptores M_1, M_3 e M_5 envolvem ativação da fosfolipase (PL) C por liberação intracelular de cálcio, ativação da PLA_2 com elevação do ácido aracdônico, e liberação do cálcio intracelular dos reservatórios. Os receptores M_2 e M_4 inibem a adenilato ciclase e a ativação dos canais de potássio (Peralta et al., 1988). Esses receptores estão acoplados à proteína G, com sete alças transmembrânicas, estando o segmento N-terminal localizado na parte extracitoplasmática.

Esses receptores estão presentes tanto em células secretoras, quanto no músculo liso. Os receptores M_3 mediam neutransmissores parassimpáticos para as glândulas salivares e lacrimais, vasos sangüíneos, bexiga urinária e TGI (Cevill, et al, 2003). A liberação de histamina, a partir das células

ECL, parece ser sensível aos antagonistas M_1 e, por essa razão, explica-se a eficiência da pirenzepina na inibição da secreção ácida (Hirchowitz et al., 1995).

Receptores muscarínicos do tecido gástrico estão acoplados ao sistema de segundo mensageiro trifosfato de inositol (IP_3) estimulatório e aumento da secreção de íons de hidrogênio (H^+), pepsinogênio e muco (Seidler et al., 1991); alterações na membrana dos receptores e em seus acoplamentos com os agonistas tem sido intensivamente investigadas. A princípio, modificações sutis no sistema de segundo mensageiro podem resultar em alterações consideráveis na resposta fisiológica; exemplo disto é a interação sinérgica entre AMP_C e Ca^{2+}, como sistema de segundo mensageiro na célula parietal, podendo gerar redução da resposta celular (Pfeiffer et al., 1995).

A ação agonista sobre o receptor M_3 na célula parietal não depende somente da liberação temporária de Ca^{2+}, que é determinada pela ativação da PLC e formação de IP_3, mas depende ainda da entrada de cálcio modulada por outros compostos (Wilkes et al., 1991). O cálcio responde a um agonista, como por exemplo um hormônio, que usualmente consiste em duas fases: pico inicial causado por liberação de cálcio das vesículas sarcoplasmáticas e uma fase de "plateau" causada por influxo de cálcio (Zanner et al., 2002). Com a utilização de um antagonista colinérgico, o 4-DAMP, foi possível demonstrar a dissociação entre a liberação de cálcio, a partir dos estoques intracelulares, e a entrada de cálcio através da membrana da célula parietal. Por essa razão, acredita-se que hajam dois subtipos diferentes de receptor M_3 ou dois diferentes tipos de acoplamentos do mesmo receptor às proteínas, resultando em afinidades ligantes distintas (Kajimura et al., 1992).

Ainda que antagonistas muscarínicos tenham sido pouco utilizados na terapêutica de doenças relacionadas à acidez gástrica, tem sido demonstrado em cães que altas doses de antagonistas não seletivos são capazes de abolir a secreção ácida tão eficazmente quanto o antagonista H_2 (Hirchowitz et al., 1969). Os anticolinérgicos são drogas usualmente utilizadas na prática clínica; seus efeitos principais sobre a motilidade gastrointestinal são inibitórios devido a sua influência relaxante direta ou indireta no músculo liso. Essa ação, denominada resposta primária, é forte e duradoura (Romanski, 2003; Rang, et al, 1999; Adams, 2001). A eficácia do antagonismo seletivo M_1, obtido por exemplo, com pirenzepina, provavelmente se deve à inibição da secreção, por interferência com a liberação de histamina a partir das células ECL, ou por inibição pré-ganglionar através da ação direta na célula parietal, a qual possui receptor M_3 relativamente insensível a pirenzepina. A ação da estimulação vagal na célula parietal e na ECL pode ser demonstrada por meio da supressão efetiva promovida por atropina; outros antagonistas clinicamente efetivos como a pirenzepina e a telenzepina, tem maior seletividade sobre os receptores M_1, mas são menos eficazes que a atropina em suprimir os efeitos da estimulação vagal.

A estimulação de receptores muscarínicos M_3 promove uma elevação do cálcio; entretanto, essa elevação leva somente a um pequeno estímulo da secreção ácida (Negulescu et al., 1989). Por outro lado, estímulos ao receptor M_3 usualmente provocam uma resposta bifásica simples de $[Ca]_i$, isto é, um aumento inicial transitório devido à liberação a partir dos depósitos intracelulares, seguido de um "plateau" que é sustentado graças a um posterior influxo de cálcio. Este evento já havia sido demonstrado anteriormente em outro tipo de músculo liso, o ducto deferente de rato (Souza Brito et al., 1986). O efeito do antagonista muscarínico na regulação da secreção ácida coloca em discussão a importância do papel do cálcio na sua estimulação.

3.2. Receptor de Histamina e seu segundo mensageiro

A histamina é o principal estimulante parácrino da secreção ácida gástrica; ao ser liberada a partir da mucosa fúndica, presumivelmente das células ECL, estimula a secreção ácida por interação com receptor H_2 localizado na membrana da célula parietal ativando a bomba H, K-ATPase (Vuyuru et al., 1997). Receptores de histamina têm sido subdivididos em três subclasses: H_1, H_2 e H_3. O principal estoque da histamina se encontra nos macrófagos, basófilos e ECL. É considerada um composto biológico muito ativo, o qual participa da sinalização intracelular, podendo ser caracterizado como um neurotransmissor (Zimatkin et al., 1999).

Há consenso de que a histamina, a principal via estimulatória da cascata secretora, estimula receptores H_2 para ativar proteínas G_S, acopladas a adenilato ciclase, levando à produção de proteína quinase A (PKA) dependente de AMPc, embora alguns outros receptores demonstrem estar acoplados também à proteína G_S (Yokotani et al., 1994).

Após ativação da célula parietal por histamina, ocorre a secreção de ácido e o pH luminal da glândula diminui para aproximadamente 1, levando ao efluxo de prótons a partir da glândula do lúmem, resultando em subsequente diminuição intragástrica do pH. Entretanto, durante o fluxo massivo de prótons, o pH intracelular da célula parietal permanece estável em aproximadamente 7 (Sachs, 1997).

A descoberta de antagonistas histaminérgicos tem contribuído para esclarecer algumas das vias fisiológicas envolvidas na estimulação ácida. Por exemplo, na década de 60 considerava-se a gastrina como o maior mediador da secreção ácida; entretanto, esta idéia foi descartada pela demonstração do potencial de inibição da secreção gástrica por antagonistas dos receptores H_2 da histamina (Black et al., 1972). A utilização de antagonistas seletivos e competitivos para receptores H_2, como a cimetidina, ranitidina e lafutidina, novo antagonista de longa duração, resultou na supressão efetiva da secreção ácida em algumas doenças relacionadas à acidez. Antagonistas de receptores H_2 exercem efeitos

considerados na secreção ácida gástrica mais rapidamente que inibidores da bomba de próton (Fukushima et al, 2003). Antagonistas H_2 são também capazes de bloquear, ainda que parcialmente, a estimulação colinérgica, sugerindo que a ativação da secreção por gastrina e aquela produzida por estimulação do nervo vago, via acetilcolina, seja mediada inteiramente ou em parte pela liberação de histamina liberada a partir das células ECL (Hirshowitz et al., 1995), assim como exercem efeitos mais rapidamente sobre a secreção ácida que inibidores da bomba H, K-ATPase (Fukushima et al, 2003).

É aceito na clínica médica que cerca de 10% dos pacientes portadores de úlcera duodenal tem cicatrização mais lenta que o padrão de oito semanas, quando o tratamento é feito com antagonistas de receptores histaminérgicos. A base para esse fenômeno não é conhecida. Como são duas vias paralelas envolvidas na estimulação da célula, uma mediada vagalmente e a outra mediada por liberação de histamina a partir da célula parácrina, provavelmente a alteração na predominância da secreção por uma ou outra via seja devido à resistência ao antagonismo.O receptor H_2 de histamina na célula parietal é acoplado a ambas vias de sinalização Gs e Gq (Athmann et al, 2000).

Acredita-se que, no caso da histamina, o receptor H_2 esteja acoplado à proteína G_s, a qual ativa adenilato ciclase (ACase) produzindo AMPc, com subsequente ativa a proteína quinase A (PKA) dependente de AMPc. Quando a acetilcolina interage com o receptor M_3 a via de sinalização também envolve proteína G, já que este receptor está acoplado ao sistema G_s/G_i, provavelmente G_q, para ativar a PLC produzindo 1,4,5-trifosfato inositol (IP_3) e diacilglicerol (DAG). IP_3 leva à liberação de $[Ca^{2+}]i$ do retículo endoplasmático e o DAG juntamente com o Ca^{2+} ativam a PKC ou proteína quinase C promovendo a fosforilação da proteína (Urushidani et al., 1997). Essa elevação do cálcio associada freqüentemente a elevação do AMPc, possui um papel fisiológico para o acoplamento do receptor H_2 a proteína G_q (Athmann et al, 2000).

Algumas especulações foram feitas sobre outro subtipo de receptor histaminérgico, o receptor H_3. A R-α-metil histamina, um agonista seletivo do receptor H_3, foi capaz de inibir a secreção estimulada por gastrina; esta inibição foi revertida por um antagonista H_3, a tioperamida (Bado et al., 1991).

Vários estudos fornecem evidências funcionais para uma distribuição heterogênea de receptores histamínicos H_3 distribuídos na parede gástrica, os quais estão envolvidos na liberação de mediadores controladores da resposta secretora ácida (Boraccelli et al., 2003).

Alguns estudos explicam esses dados: a hipótese é que a histamina seria capaz de regular sua própria síntese e liberação, via autoreceptor H_3 (Hollande et al., 1993). Por outro lado, estudos prévios realizados em segmentos de mucosa do fundo de estômago de rato, indicam que a histamina, ou outro

agonista seletivo de receptor H_3, é capaz de inibir a secreção de somatostatina e, então, estimular a secreção de histamina (Schubert et al., 1993).

Recentemente, um novo subtipo foi adicionado à família dos receptores de histamina, denominado receptor H_4, já clonado, com informações armazenadas nos bancos de dados do genoma humano (Oda et al., 2000; Zhu et al., 2001). Devido à falta de agonista e antagonista seletivo o papel funcional dos receptores H_4 ainda se encontra obscuro (Haough, 2001).

3.3. Receptor de Gastrina e seu segundo mensageiro

Gastrina é um hormônio descoberto por J.S. Edkins em 1905 como um secretagogo ácido (Modlin et al., 1997). Os efeitos biológicos da gastrina são encontrados nas células do estômago, pâncreas, vesícula biliar e sistema nervoso central, através de sua ação em dois subtipos de receptores, colecistoquinina A (CCKA) e colecistoquinina B (CCKB) (Mantyh et al., 1994). Parte estrutural da gastrina tem homologia com a colecistoquinina (CCK) no aminoácido 5-terminal formando o local carboxiterminal; este domínio é necessário para a ligação com o receptor e, portanto, responsável por sua atividade biológica. Partes dos receptores CCKA e CCKB têm 48% de homologia e são conservados entre as espécies; no entanto as afinidades pela gastrina e CCK a esses receptores são diferentes. Receptor CCKA tem mais afinidade por CCK8 sulfatado que por gastrina. Já o receptor CCKB/gastrina, no qual se ligam gastrina 17 e CCK8 sulfatada e não sulfatada, tem igual afinidade (Wank et al., 1992). A gastrina é, portanto, capaz de estimular células parietais através de receptor de gastrina (CCK) ou indiretamente pela ativação de células ECL para liberação de histamina (Karen et al., 2003).

A crucial importância do hormônio local da gastrina foi confirmada por estudos descritos em camundongos deficientes de gastrina. O papel significante da gastrina para a manutenção do funcionamento normal da secreção de ácido foi corroborado ao ocorrer, através da reposição de gastrina, a restauração parcial da secreção ácida gástrica (Friis-Hansen et al., 1998).

O aumento de concentração de gastrina pode ser encontrado em pacientes com acloridria independente da causa, como aqueles que recebem tratamento com drogas que inibem a secreção ácida, como os inibidores da bomba protônica ou antagonista de receptor H_2 (Pohle et al., 2003).

A ativação do receptor da gastrina ativa a PLC, a qual induz à quebra do fosfatidilinositol 4, 5-bifosfato (PIP2) levando à formação de dois segundos mensageiros, IP3 e DAG. IP3 induz a liberação de cálcio pelo retículo sarcoplasmático ou por canais de íons da membrana, enquanto DAG ativa a PKC. O cálcio e a PKC desencadeiam outras ações intracelulares, propagando o sinal inicial em diferentes células alvos (Yassin et al., 1999).

A ação de agonistas nos receptores de gastrina tem mostrado que seu papel está relacionado à mudança da concentração intracelular de Ca^{2+} ($[Ca^{2+}]_i$). Outras alterações, verificadas a partir da interação da gastrina ao seu receptor, ocorrem através do estímulo produzido por este secretagogo sobre a célula parietal; exemplo clássico deste fato é a elevação do AMP_C. Alterações similares são observadas por estimulação muscarínica, mas estas não requerem necessariamente AMP_C. É possível que outras vias, em adição àquelas indutoras de modificações na $[Ca^{2+}]_i$, sejam ativadas por vias muscarínicas (Wilkes et al., 1991; Urushidani et al., 1997).

3.4. Receptor de Prostaglandina

A demonstração de que baixas concentrações de PG, do subtipo E_2, inibem a secreção ácida criou grande expectativa de que esses compostos possuiriam atividade antiúlcera.

O efeito biológico dos produtos da COX, PGs, é mediado por receptores de membranas específicos, denominados, receptores EP (EP_1, EP_2, EP_3 e EP_4), que são acoplados a proteínas-G de membrana, ligadas a diferentes vias de transdução de sinal intracelular (Sugimoto et al, 2000). Ligantes de PGE_2 a receptores EP_1, resultam na liberação intracelular de IP_3 e DAG, ligantes de EP_2 e EP_4, ativam o sistema adenil-ciclase –AMPc e ligantes EP_3 inibem o esse sistema (Pawlik et al, 2002).

Receptores de prostaglandina EP_2 e EP_4 são relacionados ao aumento da produção intracelular de AMPc através de protéinas G_S. Receptores EP_3 inibem a produção de AMPc pela via da proteína G inibitória (G_i), e receptores EP_1 estimulam o "turn over" de fosfatidil inositol (Ymamamoto et al., 1999).

Quando eventos inflamatórios ocorrem na mucosa gástrica, fatores teciduais do tipo IL-1β e TNF-α em concentrações altas, aumentam a produção de PGE_2 local, os quais têm levado a prejudicar a secreção de histamina pela ECL através de receptores EP_3 (Lindstrom et al., 1998)

3.5. Receptor de Fator de Crescimento Epidermal (EGF)

Esse membro da família de receptores da tirosina quinase está presente na célula parietal de mamíferos. Através da interação com receptores celulares da superfície, EGF estimula proliferação e migração celular, além de manter a integridade da mucosa gastrointestinal (Chun et al., 2001). EGF e seu homólogo, o fator de crescimento, TGFα, parecem modular as funções da célula parietal de maneira autócrina (Beauchamp et al., 1989).

O tratamento crônico com EGF aumenta a produção ácida da cultura de célula parietal, enquanto que a estimulação aguda de receptor EGF inibe a secreção ácida estimulada por histamina, através do acoplamento a proteína G_i (Chew et al., 1994). EGF sozinho causou estimulação transitória da secreção ácida idêntica à produzida pelo carbacol (Lewis et al., 1990), sem afetar a $[Ca^{2+}]_i$ da célula parietal ou àquelas estimuladas pela via do carbacol (Wang et al., 1996).

O fator de crescimento epidermal é um dos maiores mecanismos de defesa da mucosa gástrica, de renovação e de proliferação celular, responsável por manter a integridade da mucosa, reparar e cicatrizar a úlcera. EGF e TGF-alfa interagem com um receptor em comum, chamado receptor de fator de crescimento epidermal (EGFR). EGF é um potente peptídeo mitogênico, o qual tem um papel crucial em promover migração epitelial celular gástrica e proliferação. O aumento da produção local de EGF pode levar a uma super expressão do EGFR (Fugiwara et al., 1997).

O balanço entre a perda celular e a renovação do fluxo sanguíneo na mucosa mantém a estrutura e a função do estômago, além de estar associado às doenças gástricas e cicatrizações de lesões gastrointestinais (Fugiwara et al., 2000). Esse balanço necessita ser regulado, pois a perda excessiva de célula pode resultar em atrofia ou ulceração, enquanto que a proliferação celular ou prolongamento da vida celular leva à hiperplasia (Jones et al., 1999).

Geralmente, a função do peptídeo do fator de crescimento como mediador da proliferação e/ou diferenciação celular através de alguns fatores de crescimento (ex. TGF-beta) tem mostrado inibir a proliferação em certos tipos de célula. A ligação desses peptídeos a receptores específicos transmembrânicos na superfície de células alvo inicia a cascata de transdução de sinal, a qual culmina com a ativação de genes específicos levando à divisão celular ou diferenciação (Jones et al., 1999).

O receptor majoritário do peptídeo de fator de crescimento encontrado no trato gastrointestinal possui atividade intrínseca tirosina quinase no domínio intracelular. Tirosina quinase, asssociada com vários fatores de crescimento, pode ter um importante papel na regulação da proliferação celular da mucosa gástrica após injúria. EGF-R e sua subsequente fosforilação para EGF-R tirosina quinase ativa tem sido demonstrado no início da fase de reparação da injúria na mucosa gástrica (Relan et al., 1996).

3.6. Receptor de Somatostatina

A somatostatina está distribuída no hipotálamo e outros lugares do cérebro, nervo perférico e trato gastrointestinal. O principal efeito fisiológico da somatostatina no sistema digestivo é a inibição da secreção ácida gástrica. Esse hormônio pode inibir o peristaltismo do estômago, intestino, vesícula biliar e proliferação de células da mucosa, reduzirá ainda fluxo sanguíneo no trato gastrointestinal, absorção de águas, eletrólitos, glicose, aminoácido e triglicerídeo no intestino delgado, além de deprimir secreção de ácido gástrico, pepsina, bili e secreção de gastrina e outros hormônios gastrointestinais (Sun et al., 2003).

A regulação periférica da secreção ácida depende principalmente da integração dos hormônios e da liberação de transmissores a partir dos 3 tipos de célula: ECL, G e D do fundo e do antro (Calam et al., 2001).

O efeito inibitório da somatostatina é preferencialmente devido ao bloqueio da liberação de histamina que a inibição direta da célula parietal (Komasaka et al, 2002). Athmann et al (2000), reportaram que a somatostatina não inibe o aumento dos níveis de cálcio na célula parietal estimulado pela histamina, gastrina ou carbacol, mas abole a resposta do cálcio celular da célula ECL produzida pela gastrina.

A somatostatina é um peptídeo regulatório, que age via receptores específicos para inibir funções de células alvo por mecanismos endócrinos, parácrinos e neurócrinos (Ancha et al., 2003). Cinco distintos subtipos de receptores de somatostatina têm sido clonados incluindo SST_1, SST_2, SST_3, SST_4 e SST_5 (Yamada et al, 1993). Esses receptores pertencem a superfamílias da proteína G, a qual está acoplada à superfície celular com sete domínios trans-membrânicos. Os cinco subtipos têm em comum a via de sinalização intracelular, que tem a habilidade de inibir a adenilatociclase e ativar a fosfatase fosfotirosina (Patel, 1999).

A ação da somatostatina em receptores de somatostatina (SST_2), os quais estão predominantes em células ECL, levam à inibição da liberação de histamina induzida por gastrina, devido a redução do aumento de cálcio intracelular. Esse mecanismo é provavelmente responsável pelo efeito inibitório da somatostatina na secreção ácida (Prinz et al 1994; Komasaka et al, 2002).

O receptor SST_2 é acoplado à proteína G_i, que inibe a via acoplada ao G_s, e em algumas células, tais como as ECL, podem também inibir vias acopladas ao G_q. A inibição da via de sinalização na célula ECL, que depende da G_q, explica o efeito do receptor SST_2 no bloqueio da estimulação da secreção ácida pela gastrina (Athmann et al 2000).

II. Mecanismos de Defesa do Estômago

O mecanismo de defesa da mucosa gastrointestinal contra fatores agressores, como ácido clorídrico, ácido biliar e drogas antiinflamatórias não esteroidais, consiste principalmente de fatores funcionais humorais e neurais. A secreção de muco alcalino, microcirculação da mucosa e motilidade agem como fatores funcionais, enquanto prostaglandina e óxido nítrico agem como fatores humorais; neurônios sensoriais sensitivos, como a capsaisina, agem como fatores neurais (Tsukimi et al. 2001; Repetto et al., 2002).

Uma das vias pelas quais mediadores inflamatórios podem alterar a integridade da mucosa é por influência de vários componentes de defesa da mucosa, que são a combinação de fatores que permitem a mucosa resistir à exposição de substâncias com grande limite de pH, temperatura e osmolaridade, às

solucões com propriedades detergentes (bile), e aos produtos que contém bactéria capazes de provocar a reação inflamatória local e sistêmica (Wallace et al., 1996)

Proteção à curto prazo, conhecida como citoproteção adaptativa, foi originalmente introduzida por Robert e colaboradores para descrever a ação protetora a certos agentes irritantes (etanol 20%, NaCl 5%...); esta ação protetora é atribuída à ação de prostaglandinas endógenas e óxido nítrico.

A defesa da mucosa tem sido bem caracterizada no estômago por resistir aos danos promovidos pelo efeito do ácido e da pepsina.

Os vários componentes de defesa da mucosa podem ser visualizados e organizados hierarquicamente correspondendo ao arranjo anatômico da mucosa, como se segue:

- o primeiro nível consiste de fatores secretados dentro do lúmen incluindo ácido, bicarbonato, muco e fosfolipídeos ativos superficiais. A principal função do ácido gástrico é reduzir o número de bactérias ingeridas no intestino delgado. (Wallace et al., 2001);
- no segundo nível encontra-se o epitélio, o qual é resistente às injúrias induzidas por ácido, formando uma barreira impermeável à difusão passiva; sendo capaz de promover reparos se sua continuidade for rompida;
- a microcirculação da mucosa e sua associação aos nervos aferentes sensoriais dentro da mucosa e submucosa encontram-se no terceiro nível; o refluxo de ácido ou toxinas para dentro da mucosa resulta em elevação, neuronalmente mediada, do fluxo de sangue da mucosa para limitar os danos e facilitar o reparo;
- o sistema imune consiste de várias “células alarme”, tais como mastócitos e macrófagos, que ao detectarem a entrada de um material estranho dentro da mucosa, organizam uma resposta inflamatória apropriada; este seria o quarto nível de arranjo anatômico da mucosa.
- no quinto e último nível de organização estariam fatores de reparo/crescimento: quando os danos da mucosa se estendem profundamente, estes fatores são capazes de proporcionar: a) crescimento e regeneração das glândulas gástricas; b) regeneração da inervação do sistema nervoso extrínseco e intrínseco; e c) restabelecimento da microcirculação.

1. Ácido

A função primária do ácido gástrico é bactericida em relação às bactérias ingeridas. Com exceção de Helicobacter pilory, o ácido gástrico é bastante efetivo na minimização da colonização bacteriana do estômago. Hipocloridria e acloridria predispõem à exacerbação da severidade de infeções bacterianas e de certas infeções parasitárias. Evidências indiretas da importância da ação bacteriana do ácido gástrico podem ser obtidas a partir dos estudos com a cimetidina, nos quais demonstrou-se

presença de diarréia em pacientes, que tomaram drogas antisecretoras por um período de tempo prolongado (Wallace et al., 1996).

A H. pylori não só possui um mecanismo de sobrevivência em meio ácido, como também é capaz de alterar a secreção ácida. O mecanismo responsável por elevar a secreção ácida gástrica em pacientes infectados com H. pylori não está completamente elucidado, mas há evidências de alteração na liberação de gastrina e de somatostatina, quando essas bactérias colonizam o antro gástrico. A elevação da secreção ácida, como resultado da infeção da H. pilory, pode contribuir na patogênese da úlcera duodenal (Geibel et al., 2001).

O HCl é secretado pelas células parietais presentes na mucosa gástrica. A pepsina é derivada do pepsinogênio que, quando em contato com o HCl, é liberado pelas células principais da mucosa gástrica. A regulação dessa secreção é através de uma interação de mecanismos neuronal (acetilcolina), hormonal (gastrina) e parácrino (histamina, somatostina). Receptores de cada um desses agentes acoplados aos seus respectivos receptores têm sido encontrados na célula parietal. O efeito estimulatório da acetilcolina e gastrina é mediado pelo aumento do cálcio citosólico, enquanto a histamina é mediada pela ativação da adenilato ciclase e GMPc (Shamburek et al., 1992).

Após a ativação da célula parietal pela histamina, ocorre estimulação da secreção ácida e diminuição do pH luminal para aproximadamente 1, levando ao efluxo de prótons a partir da glândula do lumen resultando na diminuição do pH intragástrico (Geibel et al., 2001)

A célula parietal é altamente especializada possuindo várias características morfológicas distintas que justificam suas atividades funcionais. A membrana plasmática apical é formada por uma série de pequenos canais (canalículos) que se formam a partir da superfície e se projetam no interior da célula com freqüente interconexação. No espaço citoplasmático há uma abundância de estruturas membranosas, ricas em H,K-ATPase, que possuem forma morfológica vesicular e tubular, denominadas por isso como estruturas tubulovesículares (Yao et al, 2003).

2. Muco e Bicarbonato

A função da chamada “barreira muco-bicarbonato” na proteção da mucosa a partir das injúrias induzidas por ácido e pepsina, é um dos aspectos mais controversos da defesa da mucosa. Há algumas dúvidas em relação à função do muco como um agente de restrição à movimentação de bactérias para a superfície epitelial: as bactérias se prenderiam ao muco e, eventualmente, seriam excretadas nas fezes (Wallace et al., 2000).

O mecanismo pelo qual bicarbonato é secretado a partir de células epiteliais também é controverso. Através da difusão de CO_2 nas células ocorre a conversão e formação de bicarbonato. Uma

das hipóteses é que o bicarbonato é secretado pela membrana apical através da troca de ânion, outra hipótese relevante, é que o bicarbonato é transportado através do sangue pela membrana basolateral, através de um transportador sódio-bicarbonato em resposta à diminuição do pH intracelular resultando numa exposição luminal ácida (Kaunitz et al., 2001).

Outro fator significativamente importante para a mucosa gástrica é o muco, o qual se apresenta de forma viscosa, elástica, aderente, como um gel transparente, que contém 95% de água e 5% de glicoproteína, recobrindo toda a superfície da mucosa gastrointestinal. O muco é capaz de agir como antioxidante e reduzir danos da mucosa promovidos por radicais livres (Repetto et al., 2002).

Foi proposto que o muco seria o agente responsável por uma contínua "cobertura" na superfície da mucosa, a qual secreta bicarbonato pelo epitélio; por essa razão, o muco agiria como camada por onde ocorre difusão do ácido luminal e sua conseqüente neutralização. Essa hipótese é sustentada pela demonstração do gradiente de pH que atinge níveis abaixo de 2, enquanto que a superfície do epitélio permanece com pH próximo à neutralidade (Garner et al., 1984).

Entretanto, essa teoria vem sendo questionada por diversas razões:

- é questionável a formação de uma camada contínua de muco na superfície do estômago; (Morris et al., 1984);
- um gradiente de pH não pode ser detectado na superfície da mucosa se o pH luminal for reduzido, como é freqüente em situações fisiológicas (Bahari et al., 1982);
- existem circunstâncias experimentais nas quais o gradiente de pH não pode ser detectado, pois o epitélio resiste às injúrias induzidas por uma alta concentração de ácido, isto é, a barreira de muco-bicarbonato é funcionalmente redundante (Wallace, 1989);
- discute-se que altas concentrações de ácido são encontradas nas glândulas gástricas na região da célula parietal e que essa é também uma região de alta concentração de pepsina; nesse local, no entanto, não há células secretoras de muco e, portanto, as células expostas a uma alta concentração de ácido e pepsina devem possuir mecanismos adicionais de resistência às lesões (Wallace et al., 1996);
- se o muco forma uma camada contínua sobre a superfície da mucosa, como a condução do ácido poderia ocorrer, a partir das glândulas gástricas, em direção ao lúmen? Discute-se, assim, que o movimento do ácido em direção oposta esteja retardado em função da presença do muco (Wallace et al., 1996).

3. Epitélio

O estômago possui várias formas de se proteger quando exposto continuamente a altas concentrações de ácido; uma das mais importantes é o epitélio gástrico. Ele é freqüentemente renovado, sendo as células "velhas" deslocadas em direção ao lúmen. O epitélio gástrico humano renova-se completamente a cada 2-4 dias. A habilidade em permitir que as células velhas sejam repostas por células mais jovens, sem quebra significante da barreira, é atribuída a um processo de extensão celular, ou seja, as células vizinhas gradualmente "apertam" as células envelhecidas na base (Wallace et al., 1996).

Nos organismos multicelulares as células são organizados em comunidades, rodeadas por um fluido intersticial de volume extremamente limitado. Comunicação local entre as células adjacentes ocorre freqüentemente através de junções gap em células que são fisicamente conectadas ou através da liberação de moléculas de sinalização parácrina (ex. ATP, glutamato, óxido nítrico), que se difundem para seus receptores alvos através de microambientes extracelulares (Hofer et al., 2004).

O aumento da permeabilidade vascular causa danos à mucosa. A permeabilidade vascular aumentada pode representar um estado transitório hemodinâmico entre vasos normais e danificados, que podem ser potencialmente reversíveis. Contudo, edemas severos podem ser observados em locais de inflamação, onde substâncias inflamatórias, como histamina e prostaglandina, são recrutadas. Sob tais condições é possível que o dano vascular cause reação inflamatória no local extravascular e progressivamente leve a danos teciduais (Hase et al, 2003).

4. Fluxo Sangüíneo da Mucosa

O fluxo sangüíneo da mucosa gástrica é regulado e modificado por sistemas e fatores locais metabólicos como prostaglandina, leucotrienos e outros mediadores químicos endógenos na mucosa (Kawano et al., 2000)

Um dos papéis do fluxo sanguíneo na mucosa é suprir de oxigênio, nutrientes e hormônios a mucosa gástrica, além de participar da regulação da saída do ácido, da produção de muco, da secreção de bicarbonato, da remoção dos produtos e da retrodifusão de íons hidrogênio; esses eventos substancialmente contribuem para a manutenção fisiológica da integridade da mucosa. Redução do fluxo sanguíneo está envolvido com as lesões da mucosa gástrica causadas por stress, etanol e DAINES (Kawano et al., 2000). De fato, o aumento do fluxo sanguíneo na mucosa gástrica diminui sensivelmente o dano causado por diversos agentes nocivos (Brzozowski et al., 1997).

A microcirculação é um importante nível da defesa da mucosa e é modulada significativamente pelo sistema nervoso e por mediadores inflamatórios. Difusão de ácido ou toxina na mucosa resulta em elevação mediada por neurônio sensorial aferente do fluxo sanguíneo que é crítico, limitando danos e

facilitando a reparação. O sangue dilui e/ou neutraliza o ácido/toxina e previne o acúmulo de concentrações citotóxicas na mucosa (Wallce et al., 2001).

Essa resposta hiperêmica promovida por irritante luminal é mediada via liberação do peptídeo liberador do gene calcitonina (CGRP) dos neurônios sensoriais aferentes, o qual causa liberação de NO endotélio vascular, causando dilatação das arteríolas da submucosa. O resultados é o aumento do fluxo sanguíneo, que auxilia a diluir, neutralizar e remover toxinas e ácidos (Wallace et al., 2001).

Nervos aferentes sensoriais, os quais possuem suas terminações abaixo do epitélio, podem detectar a entrada de ácido e, possivelmente, de outras toxinas, para dentro da mucosa; isso resulta em ativação desses nervos. Ao retornar para o SNC, os impulsos nervosos gerados afetam diretamente o tônus das arteríolas da submucosa; o tônus desses vasos é que irão regular o fluxo sangüíneo (Holzer, 1991).

O fluxo sanguíneo na mucosa mantém a estrutura e a função do estômago e está associado a doenças gástricas e cicatrização de lesões gastrointestinais. Ele é regulado e modificado por sistemas e fatores locais metabólicos como prostaglandina, leucotrienos e outros mediadors químicos endógenos na mucosa (Kawano et al., 2000)

5. Reconstituição

O termo "reconstituição" refere-se ao processo de reparo epitelial da mucosa, que envolve migração rápida de células cicatrizantes aos locais lesionados na base da membrana desprotegida. As células gástricas estão ligadas à membrana basal da célula epitelial e este local é bastante sensível aos danos induzidos por ácido (Paimela et al., 1995).

Tendo ocorrido dano na mucosa, forma-se uma "capa mucóide" sobre o local lesionado, que consiste de fragmentos celulares, muco e plasma (incluindo proteínas tais como fibrina e albumina). A capa mucóide provém de um microambiente que conduz à reconstituição epitelial; o pH da capa mucóide é mantido em torno de 5 (Wallace et al., 1991). A manutenção desse pH relativamente alto, nesse microambiente, é dependente de um suprimento contínuo de sangue na região. É comum que o plasma proveniente dos vasos sangüíneos seja responsável por tamponar algum ácido que se difunda de dentro da capa mucóide; mantendo assim o pH nesse microambiente. A capa mucóide serve para "capturar" o plasma e para interromper, ainda que brevemente, o fluxo sangüíneo; sem a capa ocorre uma queda do pH. A progressão dos danos do epitélio superficial acabará por abranger também a mucosa, causando uma lesão hemorrágica grave (wallace et al., 1996).

A cicatrização na mucosa requer reconstituição da estrutura glandular epitelial (re-epitelização), restauração da lâmina própria incluindo uma rede microvascular na mucosa, nervos e células de tecidos conectivos (Jones et al., 1999).

A cicatrização de úlcera é acompanhada de um aumento do fluxo sanguíneo gástrico na área da úlcera e por um significativo aumento de gastrina plasmática e citocinas pró-inflamatórias como TNF-α e IL-1β. Com o progresso da cicatrização da úlcera ocorre declínio gradual do fluxo sanguíneo, da gastrina plasmática e citocinas pró-inflamatórias. Foi concluído que a hipergastrinemia durante o período anterior à cicatrização da úlcera pode ser atribuído à supressão da acidez gástrica e expressão de fatores de crescimento como: EGF, TGF- α e HGF, os quais controlam a proliferação celular (Brzozowski et al., 2001).

Esses fatores de crescimento promovem a proliferação e migração de células epiteliais para a cratera da úlcera, levando a reepitelização dessa cratera e maturação das glândulas. Na base da úlcera ocorre granulação de tecido sofrendo contínua remodelação. A reparação do dano causado a mucosa gástrica requer angiogênese – formação de novos microvasos. Isso facilita a entrada de nutrientes e oxigênio na área, permitindo a proliferação celular e migração. Angiogênese é importanate para o reparo tanto de dano agudo da mucosa ou crônico durante a cicatrização de úlcera gastroduodenal. Fator de crescimento vascular endotelial (VEGF) e fator de crescimento do fibroblasto (bFGF) são fortes fatores angiogênicos em células endoteliais vascular (Jones et al., 1998). A angiogênese na granulação de tecido facilita a remodelação por liberar oxigênio e nutrientes. Células inflamatórias são substituídas por fibroblastos e microvasos na fase final da cicatrização (Chan et al, 2001).

Evidências têm sugerido que certos fatores de crescimento podem contribuir para esse processo; por exemplo: o fator de crescimento fibroblástico (FGF) tem sido demonstrado comportar-se como uma influência positiva na reconstituição. Paimela (1993) demonstrou que a aplicação luminal de protamina ou suramina, as quais interferem com a ação de bFGF endógeno, inibiu significantemente a recuperação funcional da célula epitelial após danos da mucosa induzidos por salina hipertônica. Por outro lado, sucralfato aplicado luminalmente, pode se ligar ao bFGF e, desse modo, prevenir essa degradação pelo ácido, permitindo a reconstituição epitelial que pode ocorrer no pH luminal de 3.

6. Prostaglandinas

Prostaglandina é uma das substâncias endógenas, que mais contribui para o reparo da mucosa. É produzida em grande quantidade pela COX-2 (Konturek et al, 2001), resultando numa expressão local de outras substâncias protetoras (EGF, TGF-alfa, bFGF, fator de crescimento endotelial vascular e NO), que podem contribuir para a cicatrização da úlcera. A ciclooxigenase é uma enzima conversora de

ácido araquidônico, presente na membrana fosfolipídica, a PGE_2, PGD_2, PGI_2, PGJ_2 e TX_2 (Peleg et al, 2002).

Prostanóides, tais como PGI_2, produzido em grande parte pelo endotélio, agem via receptor fosfatidil-inositol (IP), levando à prevenção da agregação plaquetária causando vasodilatação, natriurese e inibição da secreção ácida gástrica. A PGE_2 induz contração uterina, vasoconstrição e redução da secreção ácida gástrica. PGD_2 aumenta fluxo sanguíneo gástrico e renal, inibe secreção gástrica de H^+, e agregação plaquetária. PGD_2 pode ser transformada em PGJ_2 que é reconhecida como estímulo de reparação e cicatrização de tecido e indutora de NO-sintase. TXA_2 aumenta o nível plasmático de cálcio, resultando na agregação plaquetária e potente vasoconstrição (Pawlik et al, 2002).

Ainda que alguns mediadores químicos participem na produção coordenada e efetiva da resposta da mucosa à injúria, há uma influência dominante da prostaglandina e do óxido nítrico nesse processo; tanto PG´s quanto NO, são moduladores essenciais na defesa da mucosa. Essas substâncias influenciam cada um dos componentes de defesa da mucosa inibindo a secreção ácida, estimulando a secreção do muco e de bicarbonato, e elevando o fluxo sangüíneo da mucosa, o qual promoverá tamponamento da acidez e remoção de algumas toxinas que atravessam a mucosa, proporcionando a aceleração da cicatrização da úlcera (Wallace et al., 2000). Outra importante ação relatada é o efeito inibitório na atuação de mastócitos e na aderência de leucócitos ao endotélio vascular. É provável que atuam como imunomoduladores. A supressão da síntese e/ou liberação de prostaglandina ou de NO rende à mucosa maior susceptibilidade à injúria (Whittle et al., 1990).

A prostagandina tem ação citoprotetora por estimular muco e secreção de bicarbonato, mantendo fluxo sanguíneo e aumentando a resistência de células epiteliais contra injúrias causadas por citotoxinas (Hawkey et al. 1985). Um dos mecanismos através do qual a prostaglandina diminui a resposta inflamatória e reduz a severidade do dano na mucosa é através da modulação da atividade de imunócitos na mucosa. Por exemplo, prostaglandina E_2 tem sido um potente supressor de TNF-α o qual é liberado por macrófagos, reduzindo a expressão do gene TNF-α nestas células (Kunkel et al., 1988). DAINES, por outro lado, aumentam a liberação de TNF-α por macrófagos, justamente por impedir a formação de PG (Santucci et al., 1994). Prostaglandina também regula a liberação de outras citocinas como: IL-1 originado de macrófagos, e reduz a produção de potentes quimitotáxicos, como leucotrieno B_4 originados dos neutrófilos (Werthein et al., 1993)

Observação recente realizada por Gretzer et al (1998), reportou que a expressão da COX-2 tem um importante papel na cicatrização de úlcera gástrica, e que derivados da PG a partir de COX-2, podem estar envolvido na citoproteção adaptativa induzida por agentes irritantes moderados, quando uma grande área da mucosa é lesada. Drogas antiinflamatórias não-esteroidais (DAINES), inibem a

produção de prostaglandina responsável pela proteção da mucosa gástrica e possuem um efeito tópico direto irritante (Ballinger et al, 2001). O ácido gástrico provavelmente exacerba os danos na mucosa induzidos por DAINES devido a vários fatores: a) o ácido converte lesões superficiais em necroses profundas na mucosa por interferir com processo de reconstituição; b) a presença do ácido prejudica a presença de hemostasia; c) o ácido interfere com processo de cicatrização da úlcera (Chan et al., 2001). O efeito deletério de DAINES clássicos na cicatrização da úlcera pode ser reproduzido por inibidores seletivos de COX-1 e COX-2, sugerindo que ambas isoformas de COX são importantes fontes de PG durante a cicatrização de úlcera (Brzozowski et al, 2000).

A diminuição do fluxo sanguíneo na área ulcerada, a expressão excessiva de citocinas e a falha da atividade de enzimas antioxidantes na mucosa induzidas por DAINES, podem contribuir para retardar a cicatrização da úlcera gástrica e agravamento de danos gástricos induzidos por estresse.

7. Óxido Nítrico

Nos últimos anos o NO assumiu um importante papel como modulador endógeno de inúmeras funções fisiológicas. Essa molécula está envolvida na regulação do fluxo sangüíneo, manutenção do tônus vascular, integridade da mucosa, controle de agregação plaquetária e modulação da atividade de mastócitos (Motilva, et al, 2001). As três vias enzimáticas produtoras de NO são: NO-sintase neuronal, (NOSn), endotelial (NOSe), constitutiva (NOSc) e induzida (NOSi), que tem sido caracterizada no TGI. A NOSi, a qual produz grande quantidade de NO, causa injúria e, portanto, inibição específica dessas enzimas causa benefícios. A NOSc mantém a barreira da mucosa intacta (Kubes et al, 2000).

A inibição de NO aumenta o estresse oxidativo, o qual ativa mastócito. Essas células são encontradas em grandes quantidades no TGI, as quais liberam mediadores como histamina e fator ativador de plaquetas, causando aumento da permeabilidade epitelial; esse evento é rapidamente revertido pela liberação exógena de NO (Kanwar et al,1994).

É bem conhecido que o óxido nítrico (NO) media uma variedade de efeitos inibitórios no TGI: relaxamento do fundo do estômago, relaxamento não-adrenérgico e não-colinérgico (NANC) relaxamento do esfíncter gastrointestinal, fase descendente de reflexo peristáltico, inibição tônica da motilidade intestinal (Kortezova et al, 2004). Wiklumb et al (1993) demonstraram que a liberação de NO/NO_2 é inibida não somente pelo bloqueio de NO-sintase, remoção de cálcio, mas também após o bloqueio de receptor muscarínico pela atropina. Ward et al (1996) também sugerem que receptor muscarínico participaria na regulação da produção de NO. Buntzen et al (1996) encontraram que a administração exógena de acetilcolina causa relaxamento dependente de NO.

A óxido nítrico sintase (NOS) também existe nas formas induzida (iNOS) e constitutiva (Miller et al., 1995). No estômago, a forma constitutiva é encontrada no endotélio (eNOS) e neurônio entérico (nNOS). Estímulos apropriados, tais como na exposição à endotoxina, fazem com que a iNOS possa ser detectada (Brown et al., 1994). Isso sugere que o NO, derivado da iNOS, poderia exercer efeito citoprotetor no estômago; por outro lado, sugere-se que essa enzima produz níveis citotóxicos de NO (Tepperman et al., 1994), o que permanece pouco claro.

A respeito da importância do NO e da PG para a defesa da mucosa, tem sido demonstrado que estes mediadores podem regular a síntese um do outro: o NO pode estimular a atividade da COX, aumentando consequentemente a síntese de PG (Salvemini et al, 1993). Isso levanta a possibilidade de que drogas possam ser desenvolvidas através da estimulação de produção de PG's e/ou NO, as quais aumentariam a habilidade da mucosa gástrica para resistir às lesões induzidas por irritantes presentes no lúmen.

NOSi pode ser um mecanismo endógeno de aumento de níveis de NO local, minimizando disfunções intestinais, e possivelmente contribuindo para o reparo da mucosa pelo aumento do fluxo sanguíneo, pela redução de infiltrado inflamatório e pelo aumento da capacidade antioxidante (Kubes et al., 2000). Por outro lado, a NOSi, a qual produz quantidades relativamente grandes de NO, em condições patológicas, contribui para o dano e disfunção da mucosa (Wallace et al., 2000).

Uma das importantes fontes oxidantes é a infiltração de neutrófilo, e o NO é um reconhecido anti-aderente molecular. Outro mecanismo pelo qual o NO reduz o estresse oxidativo é por inibir o recrutamento de neutrófilo no local da inflamação. NO pode reduzir a fonte primária oxidante por inibir a infiltração de neutrófilo, inibir a maquinária responsável pela produção de neutrófilos na oxidação. Embora NO per se não seja deletério, ele pode reagir com o superóxido produzindo peroxinitrito, o qual causa injúria celular. NOSi produz peroxinítrito (Kubes et al., 2000).

No trato gastrointestinal, o NO participa de mecanismos de defesa, mas também contribui para danos da mucosa gástrica. O resultado desse duplo papel no desenvolvimento de drogas que modulam a síntese de NO tem sido amplamente estudada (Muscará et al., 1999).

Os dois maiores tipos de doenças gastrointestinais são caracterizados pelo envolvimento de NO: desordens na motilidade e doenças inflamatórias ulcerativas. NO é um mediador chave para relaxamento do músculo liso por nervos não adrenérgicos e não-colinérgicos; isso é extremamente importante para regular o trânsito do quimo e o tônus do esfincter (Muscará et al., 1999). NO produzido em grande quantidade [via isoforma NOSi] tem sido sugerido ser importante na mediação da injúria do tecido associado à ulceração gastroduodenal e doenças inflamatórias intestinas, bem como injúrias no tecido gastrointestinal (Miller et al., 1994).

III. Helicobacter pylori

H. pylori é um bacilo gram negativo, que infecta a camada da mucosa do estômago em humanos. Desde o seu descobrimento em 1984, a bactéria foi associada à gastrite e úlcera péptica. Cerca de 15-20% da população infectada por essa bactéria, pode desenvolver úlcera péptica ou câncer gástrico (Goddard et al., 2003).

A H. pylori é uma das principais causadoras de úlcera péptica, e um importante fator de risco no desenvolvimento do câncer gástrico. A terapia antimicrobiana ideal tem sido caracterizada pela erradicação de cerca de 90% da bactéria e mínima taxa de efeito colateral. O aumento da eficácia da terapia contra H. pylori vem sendo recentemente testado num centro multidisciplinar com mais de 1000 pacientes; é uma nova terapia com mais de 10 dias de tratamento. Essa alternativa levou a erradicação de 97% da bactéria em paciente com úlcera gástrica em comparação com a taxa obtida com 7 dias de terapia tripla (Hassan et al., 2003).

O dito "no acid no ulcer" tinha no passado resumido o conceito da patogênese da úlcera péptica. Entretanto, foi reconhecida que a infecção por H. pylori é uma das maiores causas de ulceração duodenal e gástrica (Peura et al., 1996).

O mecanismo pelo qual a H. Pylori infecta a mucosa gástrica ainda não está bem esclarecido. A úlcera duodenal pode ser efetivamente tratada pela forte supressão do ácido sugerindo que ela seja é uma doença relacionada à hipersecreção ácida. (El Omar et al., 1995). Essas anormalidades incluem aumento basal e estímulo de secreção de ácido, redução do efeito inibitório da somatostatina na liberação da gastrina, e inibição da secreção gástrica na resposta da distensão antral. Hassan et al. (2003) têm proposto que a alta concentração de amônia produzida pela H. pylori impede a secreção de somatostatina pela célula D antral, prejudicando o controle inibitório da liberação de gastrina. Por outro lado, H. pylori associada à gastrite antral pode afetar célula D e célula G, por estimular produção de citocinas. Resultados in vitro mostram que algumas citocinas pró-inflamatórias como a interleucina 8 e TNF-α, afeta a liberação de somatostatina e gastrina (Chan et al., 2002). TNF- α parece ser um dos principais fatores para muitas formas de danos na mucosa gástrica, incluindo a associação com a infecção por H. pylori e o uso de DAINES (Wallace et al., 2001).

Fatores ambientais também afetam a célula parietal e sua capacidade de secretar ácido, através da infecção e má nutrição, cujos fatores predispõem o organismo à úlcera gástrica em resposta a infecção por H. pylori. (Chan et al., 2002).

No tratamento da infecção de H. pylori tem sido usado à tripla terapia convencional, a qual consiste em bismuto, metronidazol e tetraciclina. Entretanto o uso de inibidores da bomba protônica

(IBP), ou ranitidina citrato bismuto (RCB) e dois antibióticos (amoxilina e claritromicina, metronidazol e claritromicina ou amoxilina e metronidazol) é uma das terapias mais utilizadas. A rápida resistência antimicrobiana tem substancialmente reduzido a eficácia da terapia tripla (IBP ou RCB). No presente mais de 12% de H. pylori isoladas nos USA são resistentes à claritromicina e tendendo a se estender por demais países desenvolvidos. A resistência à claritromicina é devido à mutação do RNA da H. pylori. Por outro lado, a resistência ao metronidazol é prevalente em países desenvolvidos (Chan et al., 2002).

É conhecido a resistência da H pylori aos dois grupos de drogas: nitromidazóis (tinidazol e metronidazol) e macrolídeos (claritromicina e azitromicina). Claritromicina parece ser um importante componente da erradicação da terapia, devido ao bom resultado obtido quando usado sozinho ou com associação. No Brasil, a resistência primária da claritromicina é baixa (menos que 5%), enquanto que em outros países é maior que 10% (Bellelis et al,.2004).

IV. Doenças inflamatórias intestinais

Entre as doenças gastrointestinais, as mais obscuras são as doenças inflamatórias intestinal (DII), as quais atualmente se referem a duas diferentes doenças conhecidas como: doença de Crohn e colite ulcerativa (Hanauer, 1993). A doença de Crohn e a colite ulcerativa são patologias caracterizadas principalmente por um processo inflamatório crônico no intestino de humanos e infiltração por leucócitos inflamatórios tais como, neutrófilo, macrófago e linfócitos no trato gastrointestinal. As doenças inflamatórias intestinais (DII) têm sido objetivo de extensas pesquisas nos últimos anos (Egan e Sandborn, 1998).

Embora estas pesquisas ainda não tenham apresentado dados definitivos sobre a etiologia desta doença ou de um tratamento totalmente eficaz, é possível observar que provavelmente existe relação entre esta patologia e o processo inflamatório crônico do intestino (Gálvez et al., 2000). Uma das grandes limitações na pesquisa de DII tem sido a necessidade de bons modelos dessas doenças (Kim et al, 1992). Dos inúmeros modelos animais alguns se tornaram valiosas ferramentas para o estudo da patogênese e para a descoberta de novas terapêuticas. O modelo de ácido sulfônico 2,4,6-trinitrobenzeno (TNBS) tem sido extensivamente utilizado nessa consideração.

A administração intracolônica de TNBS dissolvido em etanol 50 %, promove uma resposta inflamatória local caracterizada como colite aguda em animais, a qual é semelhante àquela desenvolvida em humanos; inclusive, está demonstrado, que o referido modelo desencadeia sintomas clássicos como inibição do apetite, perda de peso do animal, diarréia, hiperemia intensa na mucosa intestinal, ulceração na região do cólon e, em muitos casos, rompimento do segmento colônico (Morris

et al., 1989). Isso sugere que esse modelo de colite seja adequado para o estudo de eventos que ocorram no tempo da inflamação e do reparo (Yamada, Y. et al., 1992)

O aparecimento das DII pode estar relacionada diretamente à uma resposta anormal e desordenada do sistema imunológico mediante um estimulo inócuo, envolvendo diversos mediadores da inflamação (Nassif et al., 1996). Um intenso desequilíbrio bioquímico relacionado à tentativa de regulação do sistema imunológico também contribuiria para a instalação da DII (Gálvez, 2000). Por outro lado, radicais livres derivados do oxigênio e também do nitrogênio tem sido propostos como agentes envolvidos no aparecimento e desenvolvimento da DII, os quais seriam indicativos de estresse oxidativo no tecido intestinal (Harris et al., 1992).

A terapia atual das DII consiste da utilização de drogas com atividade antiinflamatória e/ou antioxidante como superóxido desmutase, sulfasalazina e o ácido 5-aminosalicilico; porém, até o momento, nenhuma droga foi totalmente eficaz em promover a cura destas lesões ou então capaz de impedir seu aparecimento (Egan e Sandborn, 1998). Drogas sintéticas ou de origem natural, que apresentam atividade antiinflamatória e antioxidante, tem sido extensamente testadas experimentalmente para esta patologia (Gálvez et al, 2000).

V. Espécie Estudada

A Arctium lappa L., pertencente à família Asteraceae, é popularmente conhecida como "GOBÔ" ou "BARDANA". A espécie é originária do Japão, de algum valor como forragem devido às suas grandes folhas, sendo às vezes cultivada para tal fim; geralmente, a cultura tem por objetivo obter as raízes napiformes, as quais são servidas nas refeições como legumes após dupla decocção. Mesmo assim, as raízes de bardana só são comestíveis enquanto novas, pois logo se ramificam e tornam-se lenhosas. No Japão elas constituem, entretanto, um dos legumes mais comumente usados (Pio Correa, 1984)

A Arctium lappa L. (figure 1) alcança de 50 cm a 2 m de altura; é planta erecta, ramosa, pubescente-cotonosa, folhas alternas, pecioladas, as inferiores cordiformes e as superiores ovadas, flores purpúreas reunidas em capítulos grandes dispostos em corymbos na extremidade do caule e dos ramos; fruto achenio oblongo-subtrigono com papilho de pêlos muito caducos (Pio Correa, 1984).

Figure 1

Estudos farmacológicos e toxicológicos vêm sendo realizados com a espécie Arctium lappa L, os quais revelaram uma diversidade de ações terapêuticas, como: atividade antioxidante (Liu & Ng, 2000; Pin Der, 1998), inibição da interação entre HIV-1 gp 120 e receptor CD4 imobilizado (Collins et al., 1997) e inibição do fator de necrose tumoral (Smith et al., 1999). Barbosa et al. (1993) encontrou, durante o processo de purificação das substâncias presentes nessa espécie, uma lactona sesquiterpênica, identificada como onopordopicrina, a qual se encontrava de forma majoritária em uma das frações do extrato clorofórmico.

Durante esses últimos anos mais de 500 lactonas sesquiterpênicas foram isoladas e identificadas a partir de espécies variadas. Embora inúmeras revisões tenham descrito a biogênese, distribuição química e quimiotaxonomia das sesquiterpeno lactonas, sua atividade biológica nos inúmeros sistemas tem sido pouco relatada.

De acordo com Szabo (1984) a citoproteção gástrica pode ser mediada por no mínimo dois diferentes mecanismos: um deles envolve prostaglandinas e o outro, envolvendo compostos com radicais sulfidrila presentes na mucosa. O estudo realizado por Giordano et al (1992) sobre a relação estrutura-atividade de uma sesquiterpeno lactona evidencia que o mecanismo de citoproteção gástrica deve ser mediado pela produção de prostaglandina, bem como pela reação de Michael entre compostos contendo radicais sulfidrila na mucosa com aceptores de Michael presentes na molécula em estudo.

Esses resultados podem ser utilizados para compreender o papel estrutural da sesquiterpeno lactona na atividade citoprotetora e proporcionar um guia para o design de compostos com essa atividade farmacológica. Portanto, por se tratar de uma sesquiterpeno lactona, que apresenta atividade antioxidante, uma possível eficácia em relação à atividade antiulcerogênica foi aventada. Para isso, utilizamos o mesmo método de obtenção do material fitoquímico utilizado por Barbosa et al. (1993).

V.1. Preparação do Material

Folhas de Arctium lappa foram coletadas em Mogi Mirim, SP, Brasil e depositadas no Herbário da UNICAMP (voucher número 131.966). As folhas frescas foram trituradas em 70% EtOH, filtradas e o solvente foi evaporada à vácuo. O resíduo resultante foi diluído em EtOH:H_2O (2:1, v/v), e extraído três vezes com eter. O extrato etéreo foi tratado com Na_2SO_4 anidro, evaporado, diluído em EtOH:H_2O (2:1, v/v) e extraído três vezes com hexano. O extrato hexânico foi removido e a camada polar foi extraída três vezes com $CHCl_3$. O extrato clorofórmico foi tratado Na_2SO_4 anidro, evaporado à vácuo. A fração semi purificada (ONP), a qual contém onopordopicirna como composto majoritário, foi obtida a partir do extrato clorofórmico (100 mg) por adsorção cromatográfica usando uma coluna de Silica Gel 60 (0,0063-0,200 mm). A coluna foi eluída com clorofórmio e o gradiente de polaridade obtido por adição de 1-4% MeOH. A fração foi analisada por GC-MS em sistema Hewlett Packard-6890 GC com uma coluna capilar (30 m x 0.25 mm x 0.25µm, HP-5MS, Crossbond 5% fenyl 95%-dimetilpolisiloxano, HP) acoplado diretamente a Hewlett Packard 5973 com um detector de massa seletiva. A temperatura da injeção foi de 250°C; programa de temperature de 40-300°C at 4°C/min, operado em splitless mode por 1.5 min. O gás utilizado foi o He em fluxo constante de 1ml/min e um volume de amostra foi de 1µl. Onopordopicrina ($C_{19}H_{24}O_6$ - Figure 2) foi eluída a 57.686 min. A fragmentação de massa foi de acordo com a citada por Barbosa Filho et al. (1993): m/z (abundância relativa) M^+ 348(<1), 281(8), 207(29), 147(43), 119(88), 91(52) e 85(100).

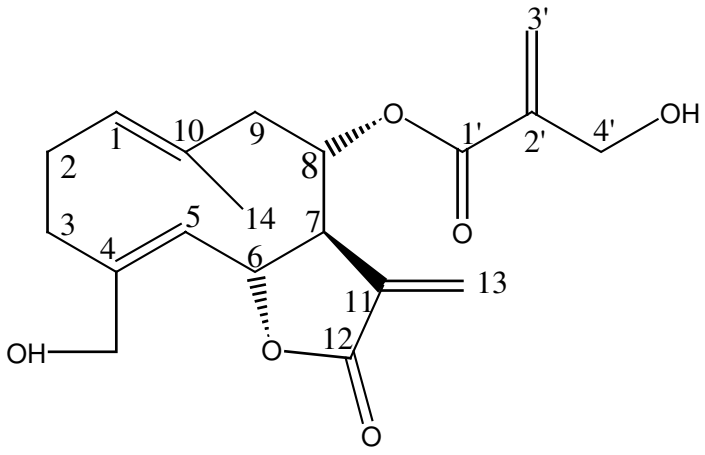


Figure 2

Dentro de um estudo farmacológico envolvendo lactonas, que vem sendo realizado no Laboratório de Produtos Naturais do Departamento de Fisiologia e Biofísica do IB-UNICAMP, o o objetivo geral é o de encontrar substâncias, que possam semelhante potência em relação à atividade farmacológica às substâncias já estudadas, sem apresentar os efeitos tóxicos que acompanham as mesmas, os quais foram observados em estudos de citotoxicidade de curto e médio prazo.

O objetivo específico deste trabalho foi o de estudar a atividade faramacológica da fração que contém ONP de forma majoritária, obtidda a partir das folhas de Arctium lappa, investigando:

- sua atividade antiulcerogênica em modelos experimentais de úlcera induzida por diferentes agentes em camundongos e ratos;
- seus mecanismos de ação antiulcerogênica, em modelos experimentais diversos;
- sua atividade antiinflamatória intestinal em modelo de colite ulcerativa aguda induzida por ácido sulfônico trinitrobenzeno (TNBS)

Screening of Biological Activities of the *Arctium lappa*

[1]Ana Beatriz Albino de Almeida*, [1]Leonia Maria Batista, [1]Luciana Di Pietro Magri, [2]Maristella Conte Anazetti, [2]Patricia Silva Melo, [3]Joseilson A. Paiva, [4]José Roberto Trigo, [2]Marcela Haun, [1]Alba R. M. Souza Brito. [1]Departamento de Fisiologia e Biofísica, IB/Universidade Estadual de Campinas; [2]Departamento de Bioquímica, IB/Universidade Estadual de Campinas; [3]Departamento de Química, IQ/Universidade Estadual de Campinas; [4]Departamento de Zoologia, IB/Universidade Estadual de Campinas, Campinas-SP-Brazil.

*Ana Beatriz Albino de Almeida
Departamento de Fisiologia e Biofísica , Instituto de Biologia,
Universidade Estadual de Campinas (UNICAMP)
CP 6109,
CEP 13083-970
Campinas, SP, Brazil
Fax: +55(19)3788-6185
E-mail:anabia5@yahoo.com.br

Abstract

1. Onopordopicrin, a sesquiterpene lactone isolated from the leaves of *Arctium lappa*, has good pharmacological activity (Barbosa et al., 1993). Based on these results, and because we have studied the antiulcerogenic activity of other lactones, we investigated the biological activity of a semi-purified fraction (ONP) of this plant, which contains onopordopicrin as majority compound.
2. The cytotoxicity of ONP, was assessed in Chinese hamster lung V79 fibroblasts , a cell line commonly used in cytotoxicity studies. Cell viability was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), total nucleic acid content and neutral red uptake assays. ONP had IC_{50} values of 17.5 µM, 12.5 µM and 15 µMin neutral red uptake, nucleic acid content and MTT reduction assays, respectively.
3. The antitumoral effects of ONP were evaluated by different endpoints of cytoxicity on promyelocytic leukemia cell line (HL60). Cell viability was determined by MTT reduction and phosphatase activity (PTP) assays. The IC_{50} for ONP was 16.3 µM in both of these assays. This value was similar to that for V79 cells.
4. The antiulcerogenic effects of the ONP, at different doses, were assessed classic models of gastric ulcer induced by ethanol/HCl (12.5, 25, 50, 100, 200 or 400 mg/kg), indomethacin/bethanechol (50, 100, 200 or 400 mg/kg), hypothermic restraint stress (50 mg/kg) and pylorus ligature (50 mg/kg) in male Swiss mice. ONP was antiulcerogenic in all of the models, suggesting a possible antisecretory action combined with a cytoprotective effect. Based on these findings, the effect of

ONP on the gastric wall mucus, obtained from pylorus-ligated mice was also evaluated. ONP didn't present significant differences in the mucus production.

5. The results obtained with ONP are promising, because the significant protection against ulcers induced by different agents suggested an antisecretory and a protective effect thet was probably mediated through somatostatin and gastrin secretion, and/or by the antioxidant properties present in ONP substance.

Keywords*Arctium lappa*, gastric ulcer, : onopordopicrin, sesquiterpene lactone

Introduction

Lewis & Hanson (1991) and Borrelli & Izzo (2000) discussed the wide variety of chemical compounds with antiulcer activity isolated from medicinal plants. we have also described antiulcer activities for several sesquiterpene lactones, such as trans-dehydrocrotonin (Souza Brito et al., 1998; Hiruma-Lima et al., 1999), cordatin (Hiruma-Lima et al., 2000), aparisthman (Hiruma-Lima et al., 2001), and semi-synthetic crotonin (Albino de Almeida et al., 2003).

Arctium lappa L., which belongs to the family Asteraceae and is popularly known as "Gobô" or "Bardana", is native to Japan. This plant is generally cultivated for theist edible roots to alimentation (Pio Correa, 1984). Pharmacological and toxicological studies have shown that *Arctium lappa* L. (Asteraceae), shows a diversity of therapeutics actions as antioxidant activity, inhibition of interaction between HIV-1 gp 120 and receptor CD4, tumour necrosis factor inhibition, antiinflamatory effect (Lin et al., 1996). Barbosa et al. (1993) identified in this plant asesquiterpene lactone (onopordopicrin)), which presented significative analgesic effect. Therefore, for the fact to treat of a semi purified fraction (ONP) obtained from leaves of *Arctium lappa*, which contains onopordopicrin as majority compound, we decided to investigate its biological activity.

Screening tests for toxicity are routinely used in drug development programs to determine a given concentration is also toxic. Cell viability assays are commonly done with V79

fibroblasts, a cell line that is well characterized and widely used in mutagenicity and toxicity studies (Cingi et al., 1991).

Lactone sesquiterpene has a common characteristic: pharmacological activity associated with high toxicity. Thus, we decided to investigate the effect of ONP on cancer cells. The inability of most cancer cells to mature into non-replicating adult cells means that they remain in a highly proliferative state and outgrow their normal cellular counterparts. Defects in the intrinsic ability of haematopoietic progenitor cells to undergo cell death may allow the cell to acquire further mutations and eventually become malignant. Such defects could account for the resistance to cell death, seen in leukemic cells (Brady, 2003). One approach to control this proliferation is to use antineoplastic agents that can kill cells by inducing cell death or differentiation (Tsftsoglou et al 2003). The human leukemia cell line HL60 can be induced to undergo cell death by several chemical and biological agents, including sesquiterpene lactones. The finding that neoplastic cells can be induced to undergo cell death or differentiation indicates that the malignant state is not irreversible and suggests that certain cancers could be treated with agents that have this activity (Anazetti et al., 2003).

In this work, we will investigate the cytotoxicity, antiulcerogenic and antitumoral effect of a semi-purified fraction of this plant.

Methods

Animals

Male Swiss mice (30-35 g), all obtained from the Central Animal House of the State University of Campinas (CEMIB/UNICAMP), were used in these experiments. The animals were fed normal rodent chow (NUVILAB CR-a®), with free access to tap water, and were always fasted before the experiments because the drugs or test substances were administered orally.

Preparation of semi-purified fraction (ONP)

Leaves of *A. lappa* were collected in Mogi Mirim, SP, Brazil and a voucher herbarium specimen was deposited in the herbarium of the State University of Campinas (voucher no. 131.966). Fresh leaves were triturated in aqueous 70% ethanol, filtered, and the solvent then evaporated under vacuum. The resulting residue was diluted in EtOH:H_2O (2:1, v/v), and extracted three times with ether. The ethereal layer was treated with anhydrous Na_2SO_4, evaporated, diluted in EtOH:H_2O (2:1, v/v) and extracted three times with hexane. The hexane layer was set removed, and the polar layer was extracted three times with $CHCl_3$. The chloroform layer was then treated with anhydrous Na_2SO_4, evaporated under vacuum. Semi-purified fraction (ONP), which it contais onopordopicrin as majoritary compound, was obtained from chloroform extract (100 mg) by adsorption cromathography using a column packed with Silica Gel 60 (0,0063-0,200 mm) as the stationary phase. The column was eluted with chloroform and a polarity gradient obtained by adding 1-4% MeOH. The fraction was analyzed by GC-MS in a Hewlett Packard-6890 GC system with a

fused capillary column (30 m x 0.25 mm x 0.25μm, HP-5MS, Crossbond 5% phenyl 95%-dimethylpolysiloxane, HP) coupled directly to Hewlett Packard 5973 with a selective mass detector. The injection temperature was 250°C; temperature program of 40-300°C at 4°C/min, operated in the splitless mode for 1.5 min. The carrier gas was He at a constant flow of 1ml/min and the sample volume was 1μl. Onopordopicrin ($C_{19}H_{24}O_6$ - Figure 1) eluted at 57.686 min. The mass fragmentation agreed with that reported by Barbosa Filho et al. (1993): m/z (relative abundance) M^+ 348(<1), 281(8), 207(29), 147(43), 119(88), 91(52) and 85(100).

V79 fibroblast cultures

The cytotoxic effect of ONP, expressed as cell viability, was assessed in Chinese hamster V79lung fibroblasts, a cell line that has been used in othercytotoxicity studies of plant products (Souza-Brito et al., 1998; Rodriguez et al., 1999). The fibroblasts were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum, 100 IU ofpenicillin/ml and 100 μg of streptomycin/ml in a humidified incubator with a 5% CO_2 atmosphere at 37°C. The cells were plated onto 96-well plates at a density of 3 x 10^4 /ml. The medium was removed 48 h after cell seeding and replaced with one containing ONP (5-50 μM), initially dissolved in methanol and then diluted in DMEM. The final concentration of methanol in the test and control media was 1%. The cells were exposed for 24 h to the test medium with or without ONP (control). Each drug concentration was tested in eight replicates and in each of three experiments. At the end of the incubation, three independent endpoints for cytotoxicity, MTT reduction, total nucleic acid content and neutral red uptake, were evaluated and and the toxicity IC_{50}.

Nucleic acid content

The number of cells in control and treated wells was estimated from their total nucleic acid content according to Cingi et al. (1991). The cells were washed twice with cold phosphates; buffered saline (PBS) and a soluble nucleotide pool was extracted with cold ethanol. The cell monolayers were then lysed by incubation in 0.5 M NaOH for 1 h at 37°C. The absorbance of the NaOH fraction at 260 nm was used as an index of cell number (Bianchi et al., 1990) .

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide reduction (MTT reduction)

The tetrazolium reduction assay was performed using the method of Denizot and Lang (1986). Briefly, fibroblasts were washed once with phosphate-buffered saline (PBS) before addition of 0.1 ml of serum-free medium containing MTT (1 mg/ml) to each well. Following incubation for 4 h, the supernatant was removed and the blue formazan product obtained was dissolved in 1 ml of ethanol with stirring for 15 min in a microtitre plate shaker. The resultingabsorbance was read at 570 nm.

Neutral red uptake

The neutral red uptake assay was performed as described by Borenfreund and Puerner (1984). After 4 h of incubation with serum-free medium containing 50 μg of neutral red/ml, the cells were quickly washed with PBS and then 0.1 ml of an aqueous solution of 1% (v/v) acetic acid: 50% (v/v) ethanol was added to each well to extract the dye. After rapid shaking on a microtitre plate shaker, the absorbance was read at 540 nm.

HL60 leukemia cell culture

Human leukemia cells (HL60) were kindly provided by Dr. Rui Curi (Laboratory of Cellular Metabolism and Regulation, Department of Physiology and Biophysics, Institute of Biomedical Sciences (ICB), University of São Paulo). The cells were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU of penicillin/mL and 100 µg of streptomycin/mL in a humidified atmosphere of 5% CO_2 in air at 37ºC. The cells were passaged twice a week and were used in the exponential growth phase at passages 12-26. To assess viability, the cells were seeded (3 x 10^5 cells/ml) in 96 wellplates and incubated with different concentrations of ONP for 72 h. Cell viability was determined by MTT reduction as described above and by their phosphatase activity (Anazetti et al., 2003).

Phosphatase activity

After incubation with ONP for 72 h, the phosphatase activity of HL60 cells was assayed as described by Anazetti et al. (2003). The culture medium was carefully removed from the wells and p-nitrophenyl phosphate dissolved in 1 M acetate buffer, pH 5,5 at a concentration of 75 mM was added. After incubation for 30 min on a microplate shaker at room temperature, the reaction was stopped by adding NaOH. The resulting absorbance was read at 405 nm.

Acute gastric lesions

The antiulcerogenic activity of different doses of ONP (12.5, 25, 50, 100, 200 or 400 mg/kg) was assessed in experimental models of acute gastric ulcers. In all experiments, the doses of ONP were diluted in 12% Tween 80 and a positive control group was used. After

animal sacrifice, the stomachs were removed and opened along the greater curvature to determine the ulcerative index (UI) as described by Szelenyi and Thiemer (1978).

Ethanol/HCl-induced ulcers

This assay was done in groups of 7 mice each using the method of Mizui et al. (1983). Mice were randomly allocated into eight groups and fasted for 24 h before the experiment. Ethanol 60%/HCl 0.1 N was administered orally to mice treated with ONP (12.5, 25, 50, 100, 200 or 400 mg/kg) or vehicle (12% Tween 80, 10 ml/kg) 50 min previously. One hour after the administration of ethanol/HCl, the mice were killed and the stomachs were removed, opened and the ulcerative index was determined.

Hypothermal-restraint stressinduced ulcers

The antiulcerogenic activity of ONP was assessed in hypothermal restraint stress-induced gastric ulcers in mice according to the method of Levine (1971), with some modifications. Mice were fasted for 36 h and then received an oral dose of ONP (50 mg/kg), cimetidine (100 mg/kg) or vehicle (12% Tween 80, 10 ml/kg). Thirty minutes after the treatments, gastric ulceration was induced by immobilizing the mice in a closed cylindrical cage at 4°C. After 3 h, the mice were killed by cervical dislocation and the stomachs removed and examined for ulcer.

NSAID-induced gastric ulcers in cholinomimetic-treated mice

This assay was done in groups of 6 mice fasted for 24 h with free access to water. Thirty minutes after the oral administration of ONP (50 - 400 mg/kg), cimetidine (100 mg/kg) or vehicle (12% Tween 80, 10 ml/kg), a solution of indomethacin (30 mg/kg, dissolved in 5%

sodium bicarbonate) was administered subcutaneously to each group as described by Rainsford et al. (1978). Acetyl-β-methylcholine chloride (bethanechol, 7.5 mg/kg) was administered intraperitoneally 15 min before indomethacin. The mice were killed 4 h later and the stomachs removed and opened, to assess the gastric lesions.

Gastric wall mucus determination

A slightly modifies procedure of Corne et al (1974) was followed. Male Swiss mice were fasted for 24 h. The pylorus was ligated and the mice were treated intraduodenally with carbenoxolone (200 mg/kg), ONP (50 mg/kg) or vehicle (12% Tween 80, 10 ml/kg). The glandular segments of stomachs opened along their greater curvatures were removed, weighed,and immediately transferred to 10 ml of 0.1% (w/v) Alcian blue dye solution. The amount of Alcian blue extracted per g (wet weight) of glandular tissue was then calculated from a standard curve After reding the absorbance at 596 nm in a spectrophotometer (LKB).

Statistical Analysis

The results are expressed as the mean ± SEM. Statistical comparisons was determined by one-way analysis of variance followed by the Dunnett test, with the level of significance set at $p<0.05$. All statistical analyses were done using Statistic 5.1 software (StatSoft, Inc.).

Results and Discussion

Cell viability assays measure the “killing capacity” or the “metabolic capacity” of a given chemical, are often used in toxicity studies with an unknown compound (Cingi et al., 1991).

The cytotoxic effects of ONP were measured by the MTT reduction, nucleic acid content and neutral red uptake assays in V79 fibroblasts. As shown in Fig. 1, the toxicity of ONP was similar in the nucleic acid content (IC_{50} = 12.5 µM), neutral red uptake (IC_{50} = 17.5 µM) and MTT reduction (IC_{50} =15 µM) assays. These results indicated a loss of viability in V79 fibroblasts after a 24 h exposure to ONP (Fig. 1). This loss of viability was concentration-dependent and involved cell detachment and death.

The balance between the therapeutic *versus* the toxicological effects of a compound is an important parameter in assessing its applicability as an antiulcer or any other pharmacological agent such as antitumor agent. The toxicological assays described here were done in cultured cells, which can be used to evaluate cytotoxicity (Ekwall et al., 1988) and target organ toxicity. In some cases, cultured cells may also provide information about the lethal dose *in vivo* (Shrivastava et al., 1991).

Many cytotoxic drugs kill malignant cells by inducing cell death. Leukemic cells (HL60) provide a model for studying the mechanisms and relationships involved in the induction of cell death by antitumor agents (Anazetti et al., 2003). Cultured mammalian cells provide an inportant tool for evaluating the cytotoxicity of compounds with of potential therapeutic activity (Pailard et al., 1999). The effect of ONP on the growth and viability of HL60 cells was assessed by the MTT reduction and phosphatase activity assays. As shown in Fig 3, ONP decreased the cell viability in both assays in a concentration-dependent manner. The IC_{50} value for the two assays was 16,3 µM. Thus, ONP inhibited HL60 cell growth *in vitro* after a 72 h exposure to the compound .

Sesquiterpene lactones have functional groups that are responsible for their biological effects, including antitumor activity. Other diterpenes isolated from *C. incanus* and *Laetia corymbulosa* are also cytotoxic in several tumor cell lines (Beutler et al., 2000). The IC_{50} values for ONP in V79 and HL60 cells were similar. These results provide a basis for studies aimed at of chemically modifying the structure of ONP to decrease the cytotoxicity in normal cells (V79) while maintaining or increasing the antitumor activity.

To establish a general profile of the antiulcerogenic activity of ONP, the compound was administered orally at different doses in several gastric ulcer models in mice and rats. The gross appearance of gastric injury after administering ethanol provides a valuable basis for screening of effects of different compounds and also for establishing the mode of their action (Szelenyl et al., 1988). When the gastric mucosal defense is compromised, exogenous noxious agents (e.g., ethanol, aspirin), together with HCl and pepsin, penetrate into the mucosa and damage the mucosal microvessels (Atay et al., 2000). For this reason, we examined the activity of ONP (12.5, 25, 50, 100, 200 or 400 mg/kg) on ethanol/HCl-induced ulcers. Pretreatment with ONP (25 - 400 mg/kg) was effective in to inhibiting ulcer formation and indicated gastroprotective effect, except at dose of 12.5 mg/kg (Table 1). Szabo et al (1985) and Murakami et al (1985) proposed that prostaglandins may prevent the increase in vascular permeability in response to ethanol, thereby maintaining the vascular integrity and adequate mucosal perfusion. The ability of prostaglandins to increase mucus and bicarbonate secretion may also be important. The antiulcerogenic effect of ONP may involve prostaglandins or other protective agents in the gastric mucosa. In addition, the reduction in oxygen and nutrient delivery caused by ethanol releases pro-inflammatory and vasoactive mediators (serotonin, leukotriene C_4, platelet activating factor) that in turn

potentiate the ischemic necrosis (Tarnawski et al., 1991, 1992). Accordingly, these results indicate that oxygen-derived free radicals may be involved in the pathogenesis of ethanol-induced gastric mucosal damage and, consequently, that ONP may have antioxidant activity. Pretreatment with ONP (50 mg/kg) was more effective in inhibiting ulcer formation than semi-synthetic crotonin, a sesquiterpene lactone from *Croton cajucara* with significant antiulcerogenic activity in the ethanol-induced ulcer model (Albino de Almeida et al., 2003). Based on these results, we hypothesized that ONP acted on the gastric mucosa as an antisecretory agent. To test this hypothesis, we examined the antiulcerogenic action of ONP in indomethacin and stress–induced ulcer models, as well as the action of ONP on some parameters of gastric secretion and mucus production.

To investigate the possible role of endogenous prostaglandins in the protection induced by ONP, a subcutaneous dose of indomethacin (30 mg/kg) and an intraperitoneal dose of bethanecol (7.5 mg/kg) were administered 30 min after pretreatment with ONP (50, 100, 200 or 400 mg/kg). The protective effect of orally administered ONP was significantly greater than the control group at doses of 50 - 200 mg/kg (Table 1). However, the protection decreased at the highest dose of ONP. A similar effect has been observed with semi-synthetic crotonin (Albino de Almeida et al., 2003). Prostaglandins regulate the secretion of mucin and of surface active phospholipids by mucous cells, when cyclooxygenase is inhibited by indomethacin, these are quantitative and qualitative decreases in mucosal barrier function (Sarosiek et al., 1986; Atay et al., 2000). The suppression of prostaglandin synthesis by NSAIDs increases the susceptibility of the mucosa to injury, inhibits epithelial cell proliferation, interferes with cell migration (essential for re-epithelialization), and inhibits angiogenesis (Tarnawski, 1993), especially

in the presence of decreased nitric oxide synthesis (Wallace et al., 1996). The gastric injury induced by the suppression of prostaglandin synthesis can be prevented by the administration of nitric oxide (NO) (Wallace et al., 1996; Maricic et al., 1998). The effect of ONP on indomethacin-induced ulcers suggest an action on mucus production, a reduction in gastric acid secretion, and the involvement of NO. For this reason, we examined the effect of ONP on mucus production and gastric acid secretion.

The antisecretory and cytoprotective actions of ONP were assessed hypothermic-restraint stress-induced ulcers in mice. Pretreatment with ONP (50 mg/kg, p.o.) significantly inhibited the formation of gastric lesions by hypothermic restraint-stress by 65% (Table 1). Cimetidine (100 mg/kg), a reference antiulcerogenic agent, also significantly inhibited (75%) the ulceration induced in this model. Disturbances of the gastric mucosal microcirculation and alterations in gastric secretion and abnormal motility have been considered responsible for stress-induced mucosal lesions and gastric mucus depletion (Goa et al., 1987). According with these author, the most important factor in the genesis of stress treated ulcer is an increase in gastric acid secretion, often refered to as the “aggressive factor”.

Stress causes sympathetic stimulation of the stomach, which induces direct arteriolar vasconstriction and gradually reduces the blood flow to the stomach, leading to local hypoxia and ischemia (Takeuchi et al., 1990). Ischemia increases the leakage of O_2 from the mitochondrial electron transport chain (Blake et al., 1987) and facilitates the availability of “redox-active” copper and iron. This leakage of O_2 can cause lipidi peroxidation and lead to a loss of membrane fluidity, impaired ion transport and membrane integrity, and a

loss of cellular functions (Halliwell et al., 1990). Together these factors play a key role in stress-induced gastric ulceration.

The involvement of histamine in hypothermal-restraint stress-induced ulceration is indicated by the observation that cimetidine, an H_2 receptor antagonist, completely inhibited the ulceration caused by hypothermic-restraint stress. The cytoprotective action of ONP could therefore involve antagonism of histaminergic and/or cholinergic receptors. Semi-synthetic crotonin showed similar inhibition at a dose of 100 mg/kg (Albino de Almeida et al., 2003). These results confirm the antiulcerogenic effect of ONP and indicate the need to evaluate the biochemical aspects of gastric acid secretion and mucus production by the stomach mucosa.

Mucus production was evaluated after ligature of the pylorus. Gastric mucus exists in two forms: those adherent to the mucosa, referred to as barrier mucus, and those not adherent in the gastric juice, refered to as soluble mucus or more accurately as free mucus, as not all the mucus in the gastric contents is in solution. Free mucus probably represents shed barrier mucus and has no physiological role (Bolton et al., 1978). We measured only adherent mucus. Treatment with ONP did not significant changes the mucus production(Fig 4).

The results of this study show that ONP has as important protective action against gastric ulcers induced by various agents. This protection may involve antisecretory and antioxidant activities of ONP. Overall, ONP had better cyprotective activity than semi-synthetic crotonin, and the antiulcerogenic action of these two compounds apparently involves

different mechanisms. Further studies are necessary to determine the full range of mediators involved in the antiulcerogenic activity of ONP.

Acknowledgments

Financial Support: CAPES and FAPESP.

References

Albino de Almeida AB, Melo PS, Hiruma-Lima CA, Gracioso JS, Carli L, Nunes DS, Haun M, Souza Brito AR. (2003) Antiulcerogenic effect and cytotoxic activity of semi-synthetic crotonin obtained from Croton cajucara Benth.Eur J Pharmacol. Jul 11;472(3):205-12

Anazetti, M.C.; Melo, P.S.; Durán, N.; Haun, M. (2003) Comparative cytotoxicity of dimethylamide-crotonin in the promyelocytic leukemia cell line (HL60) and human peripheral blood mononuclear cells. Toxicology. 188: 261-274.

Atay, S.; Tarnawski, A.S.; Dubois, A. (2000) Eicosanoids and the stomach. Prostaglandin & other Lipid Mediators 61, 105-124.

Barbosa Filho, JM, Costa M, Gomes C, Trolin G (1993). Isolation of onopordopicrin, the toxic constituent of *Arctium lappa* L. *J Braz. Chem. Soc.*, 4**,** 186-187.

Beutler, J.A.; McCall, K.L.; Herbert, K.; Johnson, T.; Shoemaker, R.H.; Bouyd, M.R. (2000) Cytotoxic clerodane diterpene ester from Laetia corymbulosa. Phytochemistry. 55: 233-236.

Bianchi V, Fortunat E (1990) Cellular effects of an anionic surfactant detected in (V79) fibroblasts by different cytotoxicity tests. *Toxicol. In Vitro* 4, 9–16.

Blake, D.R.; Allen, R.E.; Lunee, J. (1987) Free Radicals in biological systems – A review oriented to inflammatory process. Br. Med. Bull. 43: 371-385.

Bolton, J.P.; Palmer, D.; Cohen, M.M. (1978) Stimulation of mucus and nonparietal cell secretion by the E_2 Prostaglandins. Digestive Disease. 23(4): 359-364.

Borefreund, E Puerner, JA (1984) A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR90). *J. Tissue Cult. Methods* 9, 7–9.

Borrelli F, Izzo AA (2000) The plant kingdom as a source of anti-ulcer remedies. Phytother Res 14: 581-591

Brady, H.J.M. (2003) Apoptosis and leukemia. British J. Haematol. 123: 577-585.

Cingi MR, De Angelis I, Fortunati E, Reggiani D, Bianchi V, Tiozzo R, Zucco F (1991), Choice and standardization of test protocols in cytotoxicology—a multicenter approach. *Toxicol. In Vitro* 5, 119–125

Corne, S.J., Morrisey, S.; Woods, R.J. (1974) A method for the quantitative estimation of gastric barrier mucus. J. Physiol. 242, 116P-117P.

Costa et al (1993) ou Barbosa

Denizot F, Lang, R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* 89, 271–277

Ekwall, B. Ekwall, K (1988) Comments on the use of diverse cell systems in toxicity testing. *ATLA* 15, 193–200.

Goa, K.L.; Monk, J.P. (1987) Emprostil: A preliminary review of its pharmacodynamic and pharmacokinetic properties and therapeutic efficacy in the treatment of peptic ulcer disease. Drugs 3: 539-559.

Halliwell, B., Gutteridge, J.M.C. (1990) Role of free radicals and catalytic metal ions in human disease: Na overview. Methods Enzymol. 186: 1-85.

Hiruma-Lima CA, Spadari-Bratfisch RC, Grassi-Kassisse DM, Brito AR. (1999) Antiulcerogenic mechanisms of dehydrocrotonin, a diterpene lactone obtained from Croton cajucara .Planta Med. 65(4):325-30.

Hiruma-Lima, C.A., Gracioso, J.S., Toma, W., De Paula, A.C.B., Almeida, A.B., Brasil, D.S.B., Muller, A.H. & Souza Brito, A.R.M. (2000). Evaluation of the gastroprotective activity of cordatin, a diterpene isolated from *Aparisthmium cordatum* (Euphorbiaceae). Biol.Pharm. Bull., 23(12): 1465-1469.

Hiruma-Lima, C.A.; Gracioso, J.S.; Paula, A.C.B.; Almeida, A.B.; Toma, W.; Brasil, D.S.B.; Muller, A.H. and Souza Brito, A.R.M. (2001). Gastroprotective effect of aparisthman, a diterpene isolated from Aparisthmium cordatum, on experimental gastric ulcer models in rats and mice.Phytomedicine 8(2):94-100.

Levine RJ (1971) A method for rapid production of stress ulcer in rats. In: C.J. Pfeiffer, Editor, *Peptic Ulcer*, Munksgaard, Copenhagen 92–97.

Lewis DA, Hanson PJ (1991) Anti-Ulcer Drugs of Plant Origin. In: Ellis GP, West GB Prog Med Chemistry 28: 201-231

Lin CC, Lu JM, Yang JJ, Chuang SC, Ujie T (1996) Anti-inflammatory and radical scavenging effects of *Arctium lappa*. *Am. J. Chin. Med.* 24**,** 127-137.

Maricic, N.; Ehrlich, K.; Respondek, M.; Peskar, B.M. (1998) Rat gastric ischemia/ reperfusion injury: role of nitric oxide and relation to COX-1 and COX-2. Gastoenterology 114, A216

Morimoto, Y, Shimohara, K, Oshima S, Sukamoto, T (1991) Effects of the new anti-ulcer agent KB-5492 on experimental gastric mucosal lesions and gastric mucosal defensive factors, as compared to those of tepreone and cimetidine. *Jpn. J. Pharmacol.* 57**,** 495–505

Murakami, M.; Mizuno, N.; Saita, H.; Ashida, Y.; Inada, M.; Miyake, T. (1985) Effect of mild irritants on the gastric mucosal blood flow and potential difference in the rat. Gastroenterology 88, 1512.

Pailard, F.; Finot, F.; Mouche, I. Prenez, A.; Vericat, J.A. (1999) Use of primary cultures of rat hepatocytes to predict toxicity in the early development of new chemical entities. Toxicol. In Vitro 13: 693-700.

Pio Correa, M. (1984). In: *Dicionário de Plantas Úteis do Brasil*. pp. 269. Rio de Janeiro: Imprensa Nacional.

Rainsford, KD (1978) Inhibition by leukotriene inhibitors, and calcium and platelet-activating factor antagonists, of acute gastric and intestinal damage in arthritic rats and in cholinomimetic-treated mice. *J. Pharm. Pharmacol.* 51, 331–339.

Rodriguez, JA, Haun, M (1999) Cytotoxicity of *trans*-dehydrocrotonin from *Croton cajucara* on V79 cells and rat hepatocytes. *Planta Med.* 65, 1–5.

Sarosiek, J.; Mizuta, K.; Slomiany, A. (1986) Effects of acetylsalicylic acid on gastric mucin viscosity permeability to hydrogen íon, and susceptibility to pepsin. Biochem Pharmacol 35, 4291-5

Shrivastava R, John GW, Rispat G, Chevalier A, Massingham R (1991) Can the in vivo maximum tolerated dose be predicted using in vitro techniques—a working hypothesis. *ATLA* 19, 393–402

Souza Brito AR, Rodriguez JA, Hiruma-Lima CA, Haun M, Nunes DS. (1998) Antiulcerogenic activity of trans-dehydrocrotonin from Croton cajucara. Planta Med. 64(2):126-9.

Szabo, S.; Trier, J.S.; Brown, A.; Schnoor, J. (1985) Early vascular injury and increased vascular permeability in gastric mucosal injury caused by ethanol in the rat. Gastroenterology 88, 228-236.

Szelenyl, I.; Brune, K. (1988) Possible role of oxygen free radicals in ethanol-induced gastric mucosal damage in rats. Digestive Diseases and Sciences. 33 (7), 865-871.

Takeuchi, K.; Furukawa, O.; Okada, M.; Niida, H.; Okabe, S. (1990) Influence of stress on gastric alkaline secretion in rats. J. Pharmacol. Exp. Ther. 252: 1228-1233.

Tarnawaski, A.; Stachura, D.T.G. (1991) Indomethacin impairs quality of experimental gastric ulcer healing: a quality histologic and ultrastructural analysis. In: Garner A, O'Brian PEJ, editors. Mechanism of injury, protection and repair of the upper gastrointestinal tract. Chester: Wiley & Sons 521-31.

Tarnawski A. (1993) Cellullar mechanisms of gastric ulcer healing. In: Domscke W, Konturek SJ, editors. The stomach. Berlin: Spring 177-192

Tarnawski, A.; Arakawa, T. (1992) Preventing GI damage with cytoprotective drugs. Contemp. Int. Méd. 4, 95-109.

Tsiftsoglou, A.S.; Pappas, I.S.; Vizirianakis, I.S. (2003) Mechanisms involved in the induced differentiation of leukemia cells. Pharmacol. Therapeutics 100: 257-290.

Wallace, J.L; Granger, D.N. (1996) The cellular and molecular basis of gastric mucosal defense. FASEB J. 10, 823-57.

Figure Legend

Fig 1 Chemical Structure of onopordopicrin

Fig 2 Viability of V79 cells after treatment with ONP. The endpoints evaluated were neutral red uptake (NRU), MTT reduction (MTT) and nucleic acid content (NAC). The points are the mean±SD.

Fig 3 Cytotoxicity of ONP on HL60 cells. The endpoints evaluated were MTT reduction (MTT) and phosphatase activity (PTP). The points are the mean±SD.

Fig 4. Effects of ONP on mucus production obtained from pylorus-ligated mice

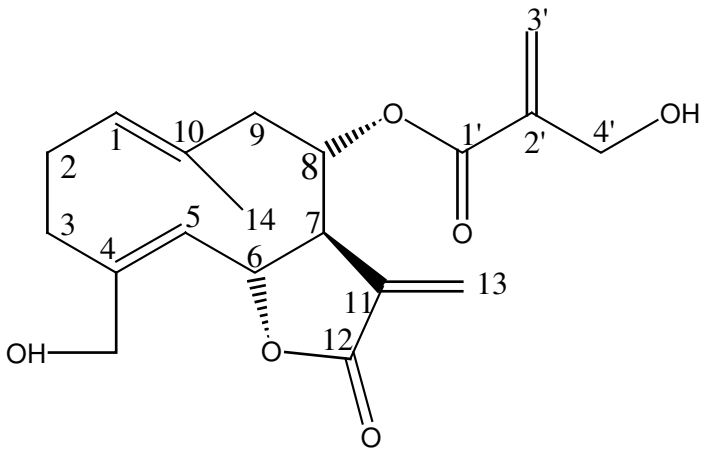


Fig 1

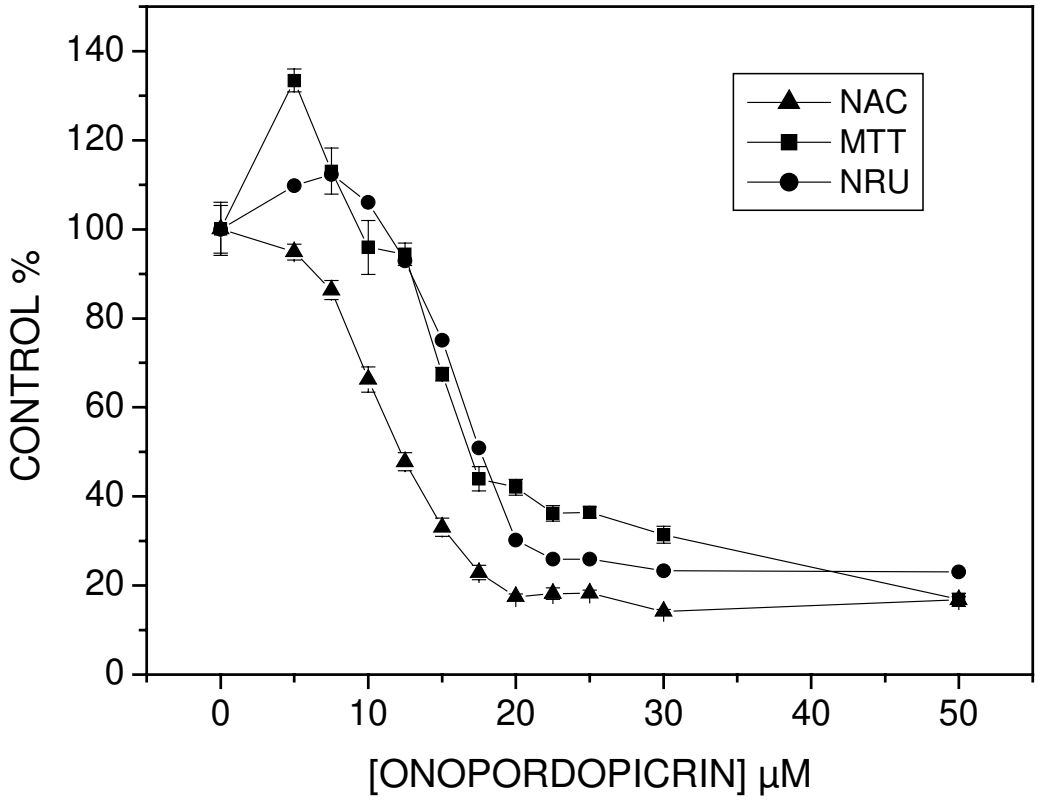

140
120
100
80
60
40
20
0
CONTROL %
0
10
20
30
40
50
[ONOPORDOPICRIN] µM
NAC
MTT
NRU

Fig 2

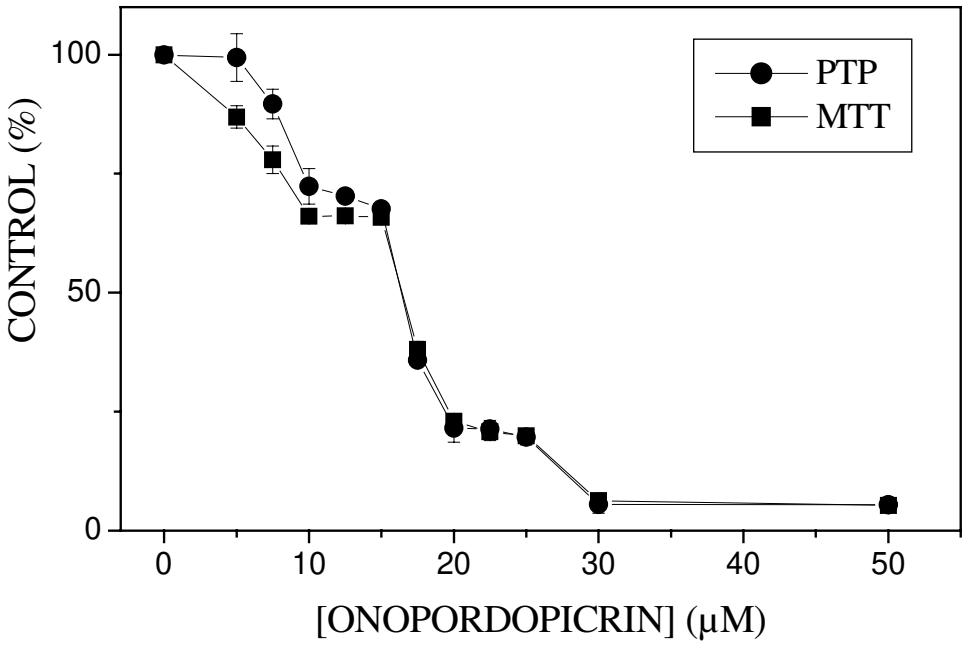


Fig 3

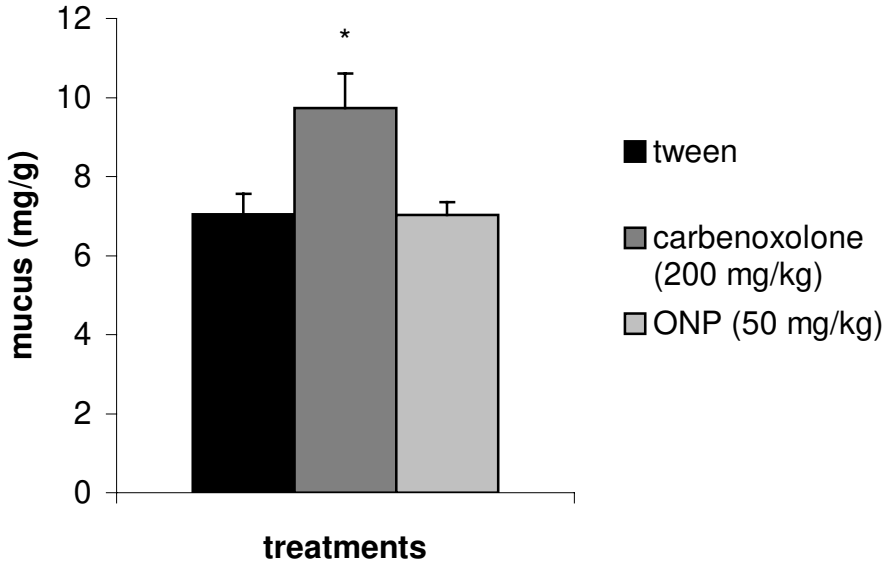

12
10
8
6
4
2
0
mucus (mg/g)
*
treatments
tween
carbenoxolone (200 mg/kg)
ONP (50 mg/kg)

Fig 4

Table 1. Effects of ONP on different gastric lesions in mice. The results are reported as the means ± S.D.

Method	Treatment	Dose (mg/kg)	ILU	Inhibition (%)
Ethanol/HCl	Control	-	22.84±14.29	-
	lanzoprazole	30	12.54±7.19*	45.09
	ONP	12.5	12.8±8.79	43.97
		25	5±3.74**	78.11
		50	5.25±3.45**	77.02
		100	3.87±2.66**	83.03
		200	4.12±1.36**	81.94
		400	4.5±1.32**	80.30
Indomethacin/ bethanechol	Control	-	10.57±4.69	-
	Cimetidine	100	4.05±2.41**	61.63
	ONP	50	3.42±2.44**	67.58
		100	4.22±2.39**	60.10
		200	6.71±2.54*	43.28
		400	15.12±3.05	-
Hypothermic restraint stress	Control	-	10.58±3.32	-
	Cimetidine	100	2.66±2.10**	75.34
	ONP	50	3.75±2.63**	65.33

*P<0.05 and **P≤0.001 compared to the corresponding control group (Dunnett's test)

Mechanisms involved in the antiulcerogenic activity of the *Arctium lappa* L. (Asteraceae)

[1*]Ana Beatriz Albino de Almeida, [1]Leonia Maria Batista, [1]Luciana Di Pietro Magri, [2]Joseilson Alves de Paiva, [2]José Roberto Trigo, [1]Alba R. M. Souza Brito. [1]Departamento de Fisiologia e Biofísica and [2]Departamento de Zoologia, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brazil.

Correspondence

*Ana Beatriz Albino de Almeida

Departamento de Fisiologia e Biofísica , Instituto de Biologia,

Universidade Estadual de Campinas (UNICAMP)

CP 6109

CEP 13083970

Campinas, SP, Brazil

E-mail:anabia5@yahoo.com.br

FAX: +55(19) 37886185

Summary

1. Extracts of the *Arctium lappa* L. (Asteraceae), have a variety of therapeutic actions, including antioxidant activity, inhibition of tumoral necrosis factor, antiinflammatory and analgesic effects. The sesquiterpene lactone, onopordopicrin, has been obtained from this species. Since sesquiterpene lactones have important antiulcerogenic activity, we examined the mechanisms of action of a semi-purified fraction from the leaves of *A. lappa* on ethanol-induced ulcer model in mice, which it contains onopordopicrin as majoritary compound. For this reason we denominated this fraction as ONP.
2. Mice were treated with 12% Tween 80 (negative control), a standard antiulcer drug (positive control) or ONP.
3. ONP reduced ethanol/HCl-induced gastric ulcers in mice by in comparison to the control. Since ONP (50 mg/kg) was equipotent to lanzoprazole (30 mg/kg), the protection seen with ONP treatment could be related to an antisecretory action of ONP.
4. For this reason, the effect of ONP (50 mg/kg) on the biochemical parameters of gastric juice obtained from pylorus-ligated mice was also evaluated. ONP (50 mg/kg) and cimetidine (100 mg/kg) showed significant decreases in total acid concentration, with a consequent in increase pH compared to the control group. The decrease in total acid concentration was not followed by changes in gastric volume for either ONP or cimetidine.
5. ONP (50 mg/kg) and lanzoprazole (30 mg/kg), significantly ($p<0.05$) increased the serum somatostatin levels (pmol/L) (82.1 ± 8.19, 87.7 ± 22.1 and 12.7 ± 8.02, mean±SD), but

decreased the serum gastrin levels (μU/ml) (62.6 ± 13.5, 46.6 ± 7.05 and 361.5 ± 14.3, mean±SD), respectively. These data confirmed that an antisecretory mechanism was involved with the antiulcerogenic effect of ONP.

6. The synthase protective effect of ONP was unaltered by the nitric oxide synthase inhibitor L-NAME, N^G-nitro-L-arginine methyl ester, (24.7 ± 17.1, 5 ± 23.5, 5.6 ± 3.7), but was attenuated by N-methylmaleimide, a sulphydril groups blocker (12.7 ± 4.4, 50.5 ± 23.9, 42.9 ± 18.1).
7. Thus, the protective action of ONP involves somatostatin secretion and inhibition of gastrin release, and an antioxidant effect.

Keywords:, *Arctium lappa*, gastric ulcer, lactone sesquiterpen, onopordopicrin,

Introduction

The mechanisms of defense and repair in the gastric mucosa involve a variety of mediators, including nitric oxide, sulfhydryl group, gastrin and somatostatin. Nitric Oxide (NO) can protect at subdilatory concentrations (Payne *et al*., 1993). The exacerbation of vascular injury following NO inhibition cannot be entirely accounted for by its vasoactive properties since a not all vasoconstrictors (unlike NO inhibitors) necessarily enhance mucosal damage in models of inflammation (Andrews *et al*., 1994). A more likely explanation for the protective effect of NO is that it works as an antioxidant during the inflammatory response. NO rapidly reacts with superoxide anion to inactivate the latter *in vitro* (Rubanyi *et al*., 1991).

The role of sulfhydryl groups in gastric mucosal protection is unclear; although gastric mucus glycoproteins may be involved (Robert *et al*., 1979). Tissue injury produced by noxious agents may result in the accumulation of toxic free radicals in mucosal cells. The gastric mucosa contains unusually high concentrations of reduced glutathione (Boyd *et al*., 1981), the major component of the endogenous, non-protein sulfhydryl pool. Ethanol lowers the concentration of non-protein sulfhydryls in gastric mucosa, and sulfhydryl blocking agents prevent the cytoprotective effect of prostaglandin $F_{2\beta}$ and decrease non-protein sulfhydryl levels (Szabo *et al*., 1981).

The gastric epithelium is folded into glands, which are the distinctive feature of the gastric mucosa. The corpus epithelium contains endocrine cells that regulate acid secretion through paracrine mechanisms, the most important being histamine-releasing enterochromaffin-like (ECL) cells and somatostatin-releasing D-cells. Parietal and ECL cells are absent in the glands of the pyloric antral region of the stomach, but there is an additional endocrine cell type, the gastrin

(G) cell (Walsh *et al*., 1994). Gastrin is the major secretory product of the antral endocrine cell system and is a major stimulant of acid secretion. The G- and D-cells of the pyloric antral mucosa act in concert as transepithelial transducers to monitor the luminal nutrients (protein and amino acids) and pH, respectively. Somatostatin is secreted from antral D-cells when the luminal pH falls below 3.5, and acts by a paracrine mechanism to suppress G-cell function, thereby completing a negative feedback loop controlling acid secretion (Wu *et al*., 1990; Dimaline *et al*., 1991).

Arctium lappa L., of the family Asteraceae, is popularly known as "GOBÔ" or "BARDANA"., and is original by from Japan. This plant includes is cultivated for its roots which are edible (Pio Correa, 1984). Pharmacological and toxicological studies have shown that *A. lappa* L. has a variety of therapeutic action, includings as antioxidant and antiinflammatory activities (Lin *et al*., 1996), platelet activating factor (PAF) antagonists (Iwakami *et al*., 1992), hepatoprotection (Lin *et al*., 2002) and analgesic activity (Barbosa *et al*., 1993). A sesquiterpene lactone known as onopordopicrin has been isolated from this species (Barbosa *et al*., 1993). Previous studies in our laboratory (Souza Brito *et al*., 1998; Hiruma-Lima *et al*., 2000; Hiruma-Lima *et al*., 2001; Almeida *et al*., 2003) have show that this sesquiterpene lactone has an important antiulcerogenic activity. In this study, we examinated the mechanisms of action a semi-purified fraction from the leaves of *A. lappa*, which it contains onopordopicrin as majority compound. For this reason we denominated its as ONP. This is the same fraction studied by Barbosa Filho *et al* (1993).

Methods

Animals

Male Wistar rats (150-250 g) and Male Swiss mice (30-35 g) obtained from the Central Animal House at UNICAMP were houses on a 12 h light/dark cycle at 22 ± 1 ºC and 55% humidity. The animals received a certified Nuvilab CR-a® (Nuvital) diet and water ad libitum but were fasted prior to all assays because standard drugs or ONP were always administered orally (by gavage – 10 ml/kg) using a 12% Tween 80 as the vehicle solution.. All of the experiments were done according to the recommendations of the Canadian Council on Animal Care (Olfert *et al*., 1993).

Preparation of semi-purified fraction (ONP)

Leaves of *A. lappa* were collected in Mogi Mirim, SP, Brazil and a voucher herbarium specimen was deposited in the Herbarium of the State University of Campinas (voucher number 131.966). Fresh leaves were triturated in aqueous 70% EtOH, filtered, and the solvent then evaporated under vacuum. The resulting residue was diluted in EtOH:H_2O (2:1, v/v), and extracted three times with ether. The ethereal layer was treated with anhydrous Na_2SO_4, evaporated, diluted in EtOH:H_2O (2:1, v/v) and extracted three times with hexane. The hexane layer was set removed, and the polar layer was extracted three times with $CHCl_3$. The chloroform layer was then treated with anhydrous Na_2SO_4, evaporated under vacuum. Semi-purified fraction (ONP), which it contais onopordopicrin as majoritary compound, was obtained from chloroform extract (100 mg) by adsorption cromathography using a column packed with Silica Gel 60 (0,0063-0,200 mm) as the stationary phase. The column was eluted with chloroform and a polarity gradient obtained by adding 1-4% MeOH. The fraction was analyzed by GC-MS in a Hewlett Packard-6890 GC

system with a fused capillary column (30 m x 0.25 mm x 0.25μm, HP-5MS, Crossbond 5% phenyl 95%-dimethylpolysiloxane, HP) coupled directly to Hewlett Packard 5973 with a selective mass detector. The injection temperature was 250°C; temperature program of 40-300°C at 4°C/min, operated in the splitless mode for 1.5 min. The carrier gas was He at a constant flow of 1ml/min and the sample volume was 1μl. Onopordopicrin ($C_{19}H_{24}O_6$ - Figure 1) eluted at 57.686 min. The mass fragmentation agreed with that reported by Barbosa Filho *et al*. (1993): m/z (relative abundance) M^+ 348(<1), 281(8), 207(29), 147(43), 119(88), 91(52) and 85(100).

Pylorus ligature

Male Swiss mice were fasted for 24 h. The pylorus was ligated by the method of Shay *et al*. (1945) and the mice received 12% Tween 80, cimetidine (100 mg/kg) or ONP (50 mg/kg) intraduodenally. Intraduodenal administration was used to ensure the systemic action of the substances. The mice were sacrificed 4 h later and the stomachs were removed and the contents drained into a graduated centrifuge tube via a small incision. The volume and pH of gastric secretion were determined and the total acid output was calculated by titrating the pH to 7.0 with 0.05 N NaOH.

Blood collect

Blood samples were collected by punction of the retroorbital plexus (Krous *et al*., 1980). Immediately after collect, the blood was centrifuged (3000 rpm, at 6^0C, 10 min) and the serum was stored in 20 ^{o}C until used.

Quantification of gastrin and somatostatin

The serum levels of somatostatin and gastrin in rats with ethanol-induced ulcers treated with 12% Tween 80 or ONP (50 mg/kg) were measured by radioimmunoassay using commercial kits (RB-306, EURO –DIAGNÓSTICA and CIS bio international- GASK-PR), as described by Arimura *et al*. (1978) and Slingerland *et al*. (1984), respectively.

Involvement of nitric oxide (NO) in the cytoprotection by onopordopicrin

These experiments were done as described by Sikiric *et al*. (1997). Male Swiss mice fasted for 24 h with free access to water were allocated to various groups. The control group received an intravenous injection of saline solution and the others, an injection of L-NAME (10 mg/kg), an NO synthase inhibitor, by the same route. After 30 min, both all groups received 12% Tween 80 or ONP (50 mg/kg) orally. After a further 50 min, the mice given ethanol/HCl orally and then sacrificed 1 h later. The stomachs were removed and opened along the greater curvature and the ulcerative index was determined.

Ethanol-induced gastric mucosal lesions in N-methylmaleimide-pretreated mice (Matsuda et al., 1999)

To investigate the involvement of endogenous sulfhydryls groups in the protection activity by ONP, *N*-methylmaleimide (10 mg/kg) was injected subcutaneously 30 min before the oral administration of ONP (50 mg/kg) on 12% Tween 80. After 50 min, the mice received ethanol/HCl orally. One hour later, the mice were sacrificed 1 h later and their stomachs were removed and opened along the greater curvature. The ulcerative index was calculated.

Statistical Analysis

The results are expressed as the mean ± SEM. Statistical comparisons was determined by one-way analysis of variance followed by the Tukey test, with the level of significance set at $p<0.05$. All statistical analyses were done using Statistic 5.1 software (StatSoft, Inc.).

Results and Discussion

Since there may be discrepancies between the results of macroscopic and microscopic histological evaluations of ulcers (Lacy *et al*., 1999), is this study, only macroscopic examination of the stomach was used to assess the extend of damage and the protective effect of ONP. Generally, only deep necrosis, and not the desquamation of surface cells, is prevented by agents that protect against ethanol-induced gastric injury in rats (Szabo *et al*., 1987). When the gastric mucosal defense is compromised, exogenous noxious agents (e.g. ethanol, aspirin), together with HCl and pepsin, penetrate the mucosa and damage the mucosal microvessels (Atay *et al*., 2000). For this reason, in another work we showed the protective effect of various doses of ONP (25, 50, 100, 200 and 400 mg/kg) in a model of ethanol/HCl-induced ulcers.

Szabo *et al*. (1985) and Trier *et al*. (1987) showed that the instillation of ethanol caused severe vascular injury (with increased vascular permeability) within 1-3 min, and that this damage consistently preceded the development of macroscopic hemorrhagic lesions in rat glandular mucosa. ONP may have exerced its protective action in this period. Szabo *et al*. (1985) proposed that prostaglandins might could the increase in vascular permeability in response to ethanol and could help to maintain the vascular integrity and adequate mucosal perfusion. The ability of

prostaglandin to increase mucus and bicarbonate secretion may also be important. According to consideration carry out on antiulcerogenic effect of ONP we can to verify its cytoprotective and antisecretory action through of possible involvement with prostaglandin or by mucus production.

Ethanol can stimulate the gastric and oral secretion of gastrin and release of histamine via a reflex both way involving sensory terminals present in the gastric and oral mucosa. Although gastric acid is not a primary cause of gastric mucosal damage plays a permissive role in the damage induced by ethanol (Tarbawski *et al*., 1983). Based on the involve mechanism of gastric acid in ethanol-induced injury, we investigated the influence of ONP on the biochemical parameters of gastric acid (pH, total gastric acid content, gastric juice volume) using the pylorus ligature method. The intraduodenal administration of ONP (50 mg/kg) or cimetidine (100 mg/kg) immediately after ligation of the pylorus, resulted in a decrease in the total acid output/4 h, with a consequent increase in pH, but no change in gastric juice volume (Table 1). The reduction in acid output suggested that the protective action of ONP may involve the inhibition of gastric secretion. Based on these findings, we investigated the ability of ONP to stimulate the secretion of somatostatin and gastrin, two important hormones involved in gastric acid secretion.

There are several mechanisms involved in the action of somatostatin in the stomach. The modulation of mast cell function by somatostatin has been implicated in the protection of the gastric mucosal against ethanol induced damage (Szabo *et al*., 1986). The vasoactive properties of somatostatin on gastrointestinal blood flow and other vascular systems have been studied. Somatostatin direct and indirect actions on blood vessels. In the latter case, somatostatin can inhibit the release of vasodilatory substances such as bradykinin, glucagon, acetylcholine, histamine and vasoactive intestinal polypeptide, and thus indirectly cause vasoconstriction and a decrease in mesenteric blood flow (Lucey *et al*., 1989). In rats pretreated with ONP (50 mg/kg),

these was an increase in serum somatostatin levels (Table 2). This finding confirmed that the protective mechanism of ONP involving the inhibition of gastric secretion was partly mediated via the secretion of somatostatin.

In the stomach, somatostatin serves as a paracrine regulator of acid and gastrin release via a mechanism that may involve in cAMP pathways (Makhlouf *et al*., 1990). In addition to stimulating acid secretion, gastric also stimulates the secretion of bicarbonate by the pancreas and the release of insulin and calcitonin. Indirectly, gastrin has trophic effect on the digestive mucosal and has a role in the motility of the digestive tract (Varro *et al*., 1990). As shown in table 2, there was a decrease in the serum gastrin levels of rats the treated with ONP (50 mg/kg). Thus the antisecretory activity of ONP also involves a decrease in gastrin release.

Szelenyi *et al*. (1988) demonstrated that and superoxide dismutase, a scavenger of superoxide radicals, sulfur-containing organic compounds, certain inorganic compounds can protect the gastric mucosa against ethanol-induced injury. In models of acute inflammatorion the inhibition of endogenous NO exacerbates injury (Masuda *et al*., 1995) whereas exogenous NO protects the gastrointestinal mucosa against noxious stimuli (Wallace *et al*., 1994). An improvement in blood flow associated with increased NO delivery could be important in countering the compromised blood flow resulting from by HCl (Kitagawa *et al*., 1990), ethanol (Masuda *et al*., 1995), and indomethacin (Wallace *et al*., 1994).

To investigate the possible role of NO and sulfhydryl groups in the protection effect by ONP, the NO synthase inhibitor L-NAME and the sulfhydryl blocker N-methylmaleimide were administered separately 30 min before ONP (50 mg/kg) orally. As shown in table 3, L-NAME did not significantly affect the protection by ONP, indicating that NO is not involved in this phenomenon. In contrast, pretreating the mice with *N*-methylmaleimide, a sulfhydryl-blocker,

reduced the protection afforded by ONP (Table 4). Thus, the protective action of ONP on ethanol/HCl-induced ulcers also involve gastric lipid peroxidation and an increase in the level of gastric nonprotein sulfhydryl (NP-SH) compounds. An increase in the gastric NP-SH content limits the production of oxygen-derived free radicals (Itoh, 1985).

Conclusion

In conclusion, the ability of ONP to protect against gastric lesions involves the stimulation of somatostatin secretion and inhibition of gastrin release, as well as an antioxidant effect.

References

ALMEIDA, A.B.A., MELO, P.S., HIRUMA-LIMA, C.A., GRACIOSO, J.S., CARLI, L., NUNES, D.S., HAUN, M., SOUZA BRITO, A.R.M. (2003) Antiulcerogenic effect and cytotoxic activity of semi-synthetic crotonin obtained from *Croton cajucara* Benth. *Eur. J. Pharmacol.* **472,** 205-212.

ANDREWS, F.J., MALCONTENI-WILSON, C., O'BRIEN, P.E. (1994). Protection against gastric ischemia-reperfusion injury by nitric oxide generators. *Dig. Dis. Sci.*, **39,** 366-373.

ARIMURA, A., LUNDQVIST, G. (1978). Radioimmunoassay of somatostatin. *Metabolism.* **27,** 1139-1144.

ATAY, S., TARNAWSKI, A.S., DUBOIS, A. (2000). Eicosanoids and the stomach. *Prostaglandin Other Lipid Mediat.* **61,** 105-124.

BARBOSA FILHO, J.M., COSTA, M., GOMES, C., TROLIN, G. (1993). Isolation of onopordopicrin, the toxic constituent of *Arctium lappa* L. *J Braz. Chem. Soc.*, **4,** 186-187.

BOYD, S.C., SASAME, H.A., BOYD, M.R. (1981). Gastric glutathione depletion and acute ulcerogenesis by diethylmaleate given subcutaneously to rats. *Life Sci*. **29,** 2987-2992.

DIEL, F., SZABO, S. (1986). Dose-dependent effects of linear and cyclic somatostatin in ethanol-induced gastric erosions: the role of mast cells and increased vascular permeability in the rat. *Regul. Pept.* **13,** 235–243.

DIMALINE, R., EVANS, D., VARRO, A., DOCKRAY, G.J. (1991). Reversal by omeprazole of the depression of gastrin cell function by fasting in the rat. *J. Physiol.* **433,** 483-493.

HIRUMA-LIMA, C.A., GRACIOSO, J.S., TOMA, W., PAULA, A.C., ALMEIDA, A.B.A., BRASIL, D.D., MULLER, A.H., SOUZA BRITO, A.R.M. (2000). Evaluation of the gastroprotective activity of cordatin, a diterpene isolated from *Aparisthmium cordatum* (Euphorbiaceae). *Biol. Pharm. Bull.* **23,** 1465-1469.

HIRUMA-LIMA, C.A., GRACIOSO, J.S., TOMA, W., PAULA, A.C., ALMEIDA, A.B.A., BRASIL, D.D., MULLER, A.H., SOUZA BRITO, A.R.M. (2001). Gastroprotective effect of aparisthman, a diterpene isolated from *Aparisthmium cordatum*, on experimental gastric ulcer models in rats and mice. *Phytomedicine* **8,** 94-100.

ITOH, M. (1985). Role of oxygen-derived free radicals in hemorrhagic shock-induced gastric lesions in the rat. *Gastroenterology* **88,** 1162-1167.

IWAKAMI, S., WU, J.B., EBIZUKA, Y., SANKAWA, U. (1992). Platelet activating factor 8 (PAF) antagonists contained in medicinal plants: lignans and sesquiterpenes. *Chem. Pharm. Bull.* **40,** 1196-1198.

KITAGAWA, H., TAKEDA, F., KOHEI, H. (1990). Effect of endothelium-derived relaxing factor on the gastric lesion induced by HCl in rats. *J. Pharm. Exp. Ther.* **253,** 1133-1137.

KROUS S. (1980). Research applications. In: *The Laboratory Rat*. Ed. Baker HJ, Hussel J & Weisbroth SH. pp. 2-28. New York: Academic Press.

LACY, E.R., ITO, S. Microscopic analysis of ethanol damage to rat gastric mucosa after treatment with a prostaglandin. *Gastroenterology* **83,** 619-625.

LIN, C.C., LU, J.M., YANG, J.J., CHUANG, S.C., UJIE, T. (1996). Anti-inflammatory and radical scavenging effects of *Arctium lappa*. *Am. J. Chin. Med.* **24,** 127-137.

LIN, S.C., LIN, C.H., LIN, Y.H., CHEN, C.F., CHEN, I.C., WANG, L.Y. (2002). Hepatoprotective effects of *Arctium lappa* Linneu on liver injury induced by chronic ethanol consumption and potentiated by carbon tetrachloride. *J. Biomed. Sci.* **9,** 401-409.

LUCEY, M.R., YAMADA, T. (1989). Biochemistry and physiology of gastrointestinal somatostatin. *Dig. Dis. Sci*. **34,** 5S–13S.

MAKHLOUF, G.M., SCHUBERT, M.L. (1990). Gastric somatostatin: a paracrine regulator of acid secretion. *Metabolism*. **39,** 138-142.

MASUDA, E., KAWANI, S., NAGANO, K. (1995). Endogenous nitric oxide modulates ethanol-induced gastric mucosal injury in rats. *Gastroenterology* **108,** 58-64.

MATSUDA, H., LI, Y., YOSHIKAWA, M. (1999). Gastroprotections of escins Ia, Ib, IIa, and IIb on ethanol-induced gastric mucosal lesions in rats. *Eur. J. Pharmacol.* **373,** 63-70.

MORIMOTO, Y., SHIMOHARA, K., OSHIMA, S., SUKAMOTO, T. (1991). Effects of the new anti-ulcer agent KB-5492 on experimental gastric mucosal lesions and gastric mucosal defensive factors, as compared to those of teprenone and cimetidine. *Jpn. J. Pharmacol.* **57,** 495-505.

OLFERT, E.D., CROSS, B.M., MCWILLIAM, A.A. (1993). Guide to the Care and use of Experimental Animals. pp. 1-213. Otawa: Canadian Council on Animal Care.

PAYNE, D., KUBES., P. (1993). Nitric oxide donors reduce the rise in reperfusion-induced intestinal mucosal permeability. *Am. J. Physiol.*, **265,** G189-195.

PIO CORREA, M. (1984). In: *Dicionário de Plantas Úteis do Brasil*. pp. 269. Rio de Janeiro: Imprensa Nacional.

ROBERT., A. (1979). Cytoprotection by prostaglandins. *Gastroenterology*. **77,** 761-767.

RUBANYI, G.M., HO, E.H., CANTOR E.H. (1991). Cytoprotective function of nitric oxide: inactivation of superoxide radicals produced by human leukocytes. *Biochem. Biophys. Res. Commun.* **181,** 1392-1397.

SHAY, H., KOMAROV, S.A., FELS, S.S., MERANZE, D., GRUENSTEIN, M., SIPLET, H. (1945). A simple method for the uniform production of gastric ulceration in the rat. *Gastroenterology* **5,** 43-61.

SIKIRIC, P., SEIWERTH, S., GRABAREVIC, Z., RUCMAN, R., PETEK, M., JAGIC, V., TURKOVIC, B., ROTKVIC, I., MISE, S., ZORICIC, I., KONJEVODA, P., PEROVIC, D., JURINA, L., SEPAROVIC, J., HANZEVACKI, M., ARTUKOVIC, B., BRATULIC, M., TISLJAR, M., GJURASIN, M., MIKLIC, P., STANCIC-ROKOTOV, D., SLOBODNJAK, Z., JELOVAC, N., MAROVIC, A. (1997). The influence of a novel pentadecapeptide, BPC 157, on N^G-nitro-L-arginine methylester and L-arginine effects on stomach mucosa integrity and blood pressure. *Eur. J. Pharmacol.* **332,** 23-33.

SLINGERLAND, D.W., CARDERALLI, J.A., BURROWS, B.A., MILLER, A. (1984). The utility of serum gastrin levels in assessing the significence of low serum B_{12} levels. *Arch. Intern. Med*. **144,** 1167-1168.

SOUZA BRITO, A.R.M., RODRIGUEZ, J.A., HIRUMA-LIMA, C.A., HAUN, M., NUNES, D.S. (1998). Antiulcerogenic activity of trans-dehydrocrotonin from *Croton cajucara*. *Planta Med.* **64,** 126-129.

SZABO, S., BROWN, A. (1987). Prevention of ethanol-induced vascular injury and gastric mucosal lesions by sucralfate and its components: possible role of endogenous sulfhydryls. *Proc. Soc. Exp. Biol. Med.* **185,** 493–497

SZABO, S., TRIER, J.S., BROWN, A., SCHNOOR, J. (1985). Early vascular injury and increased vascular permeability in gastric mucosal injury caused by ethanol in the rat. *Gastroenterology* **88,** 228–236.

SZABO, S., TRIER, J.S., FRANKEL, P.W. (1981). Sulfhydryl compounds may mediate gastric cytoprotection. *Science* **214,** 200-202.

SZELENYI, I., BRUNE, K. (1988). Possible role of oxygen free radicals in ethanol-induced gastric mucosal damage in rats. *Dig. Dis. Sci.* **33,** 865-871.

SZELENYI, I., THIEMER, K. (1978). Distention ulcer as a model for testing of drugs for ulcerogenic side effects. *Arch. Toxicol.* **41,** 99-105.

TARNAWSKI, A., HOLLANDER, D., STACHURA, J., KRAUSE, W.J. (1983). Arachidonic acid protection of gastric mucosa against alcohol injury: sequential analysis of morphologic and functional changes. *J. Lab. Clin. Med.* **102,** 340-351.

TRIER, J.S., SZABO, S., ALLAN, C.H. (1987). Ethanol-induced damage to mucosal capillaries of rat stomach. Ultrastructural features and effects of prostaglalndin $F_{2\beta}$ and cysteamine. *Gastroenterology* **92,** 13-22.

VARRO, A., NEMETH, J., BRIDSON, J., LONOVICS, J., DOCKRAY, G.J. (1990). Modulation of posttranslational processing of gastrin precursor in dogs. *Am. J. Physiol.* **258,** G904-G909.

WALLACE, J.L., REUTER, B.K., CIRINO, G. (1994). Nitric oxide-releasing non-steroidal anti-inflammatory drugs: a novel approach for reducing gastrointestinal toxicity. *J Gastroenterol Hepatol* **9,** S40-S44.

WALSH, J.H. (1994). Gastrin. In: *Gut Peptides*. Ed. Walsh, J.H. & Dockray, G.J. pp.75-121. New York: Raven Press.

WU, S.V., GIRAUD, A., MOGARD, M., SUNII, K., WALSH, J.H. (1990). Effects of inhibition of gastric secretion on antral gastrin and somatostatin gene expression in rats. *Am. J. Physiol*. **258,** G788-793.

Figure legend

Fig 1. Chemical structure of onopordopicrin

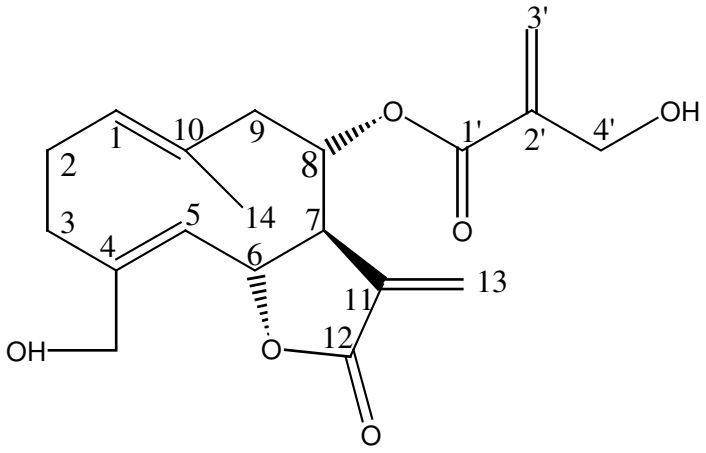


Fig 1

Table 1. Effects of onoporodopicrin on the biochemical parameters of gastric juice obtained from pylorus-ligated mice.

Treatment	**Dose (mg/kg, po)**	**pH**	**Total gastric acid (mEq/ml/ 4 h)**	**Gastric juice volume (ml)**
12% Tween 80	-	3.1±0.3	23.1±12.6	0.246±0.03
Cimetidine	100	4.9±0.9***	10.2±2.9**	0.171±0.02
Onoporodopicrin	50	4.2±1.1*	14±2.9*	0.243±0.03

The results are expressed as the mean±SEM ANOVA: $F_{pH\ (2,24)} = 11.838$, $F_{total\ gastric\ acid\ (2,24)} = 7.008$, $F_{gastric\ juice\ volume\ (2,24)}=2.555$ for $p<0.05$ followed by Tukey test. *$p<0.05$ **$p<0.01$ ***$p<0.001$ compared to the control (Tween 80) group.

Table 2. Effect of onpordopicrin on somatostatin and gastrin gastric secretion in mice.

Treatments	**Dose (mg/kg, po)**	**Somatostatin (pmol/L)**	**Gastrin (μU/ml)**
12% Tween 80	-	12.7 ± 4	361.5 ± 8.2
Sham	-	20.8 ± 0.8	336.6 ± 7.5
Lanzoprazole	30	87.7 ± 11*	46.6 ± 4.07*
Onopordopicrin	50	82.1 ± 4.1*	62.6 ± 6.04*

The results are expressed as mean ± SEM. ANOVA: $F_{somatostatin\ (3,11)} = 35.08$, $F_{gastrin\ (3,10)} = 620.7$ for $p<0.05$ followed by Tukey test. *$p<0.001$ compared to the (Tween 80) group.

Table 3. Involvement of nitric oxide (NO) in the cytoprotection afforded by onopordopicrin.

Treatments / Route	**n**	**UI**	**Inhibition (%)**
12%Tween 80 (po) + 0.9% saline (10 mg/kg, iv)	9	24.7 ± 5.7	-
12% Tween 80 (po) + L-NAME (10 mg/kg, iv)	11	55 ± 6.2**	0
ONP (50 mg/kg, po) + L-NAME (10 mg/kg, iv)	9	5.6 ± 1.2*	77.3

The results are expressed as the mean±SEM. ANOVA: $F_{(2,26)}$=23.804 for $p<0.05$, followed by Tukey test. *$p<0.05$ **$p<0.001$ compared to the control group.

Table 4. Effect of N-methylmaleimide (10 mg/kg, s.c.) and the antiulcerative action of onopordopicrin (50 mg/kg, p.o.) in mice.

Treatments / Route	**n**	**ILU**	**Inhibition (%)**
12% Tween 80 (po)+0.9% Saline (10 mg/kg, sc)	11	12.7 ± 1.3	-
12% Tween 80 (po) + N-methylmaleimide (sc)	11	50.5 ± 6.6*	0
Onopordopicrin (po) + N-methylmaleimide (sc)	10	42.9 ± 5.7*	0

The results are expressed as the mean ± SEM. ANOVA: $F_{(2,29)}$=15.898 for $p<0.05$, followed by Tukey test. *$p<0.001$ compared to the control group.

Arctium lappa attenuates mucosal damage due to colitis induced by trinitrobenzene sulphonic acid in rats.

[*1]Ana Beatriz Albino de Almeida, [2]Marina S.Hidalgo, [2]Antonio Ramón Martín, [3]Wagner Villegas, [3]Lourdes C Santos, [3]Marcio R F Tiene, [1]Alba R Monteiro Souza Brito, [2]Catalina Alarcon de la Lastra,. [1]Departament of Physiology-State University of Campinas (UNICAMP)-Campinas-SP/Brazil, [2]Departament of Pharmacology - Faculty of Pharmacy-University of Seville-Seville/Spain, [3]Departament of Chemistry – IQ/Paulista State University (UNESP)-Araraquara-SP/Brazil.

*Correspondence to:
Ana Beatriz Albino de Almeida
Department of Physiology-IB
State University of Campinas – UNICAMP
Cidade Universitária Zeferino Vaz, s/n
PO: 6109 – Campinas-SP-Brazil
Fone: (55)19-37886192
e-mail: anabia5@yahoo.com.br

Abstract

Arctium lappa (Asteraceae), commonly known as "bardana", has a diversity of therapeutics actions as antioxidant activity, inhibition of tumour necrosis factor (TNFα), antiinflamatory and analgesic effects (Lin et al., 1996). The pathological features of inflammatory bowel disease (IBD) such Crohn's disease and ulcerative colitis are marked by the presence of mucosal ulceration, and the infiltration of neutrophils and lymphocytes in mucous membranes. Oxygen free radicals, neutrophils and proinflammatory cytokines are clearly involved in the pathogenesis of IBD. Accordingly, in this study, we examined the effects of semi-purified fraction (ONP), which have as main constituent the sesquiterpene lactone onopordopicrin, on acute experimental trinitrobenzene sulphonic acid (TNBS)-induced colitis in rat. ONP (25 and 50 mg/Kg/day) was administered by oral gavage 48,24 and 1h prior to the induction of colitis and 24h later. The animals were sacrificed 48h after induction of colitis. Colons were removed and lesions were blindly score according to macroscopic and histological scales. Inflammation response was assessed by neutrophil infiltration, determined by histology and MPO activity. Indeed, we evaluated mucosal tumor necrosis factor TNF-α levels, cyclooxigenases (COX)-1 and –2 expression by imunohistochemistry and Western Blotting was also assessed. Inflammation following TNBS was characterized by increased colonic wall thickness, oedema, diffuse inflammatory cell infiltration in the mucosa and necrosis. At 2 days treatment with 25 or 50 mg/kg of ONP ameliorated colonic damage score. The levels of mucosal TNF-α were significantly elevated in the colon at 48h after TNBS instillation. In contrast, the levels of cytokines were significantly lower in rats treated with 25 and 50 mg/kg of ONP. Treatment of TNBS-treated rats with ONP significantly reduced the degree of polymorphonuclear neutrophil infiltration. However gluthation total remained unchanged. Compared with inflamed colon, unchanges in staining for COX-1 was observed in

colon of ONP-treated rats, in contrast COX-2 expression was increased. In conclusion, our results demonstrate that ONP is protective in acute experimental colitis. The acute inflammatory effect seems to be related to up-regulation of TNF-α production in intestinal mucosa. Our findings suggest that onopordopicrin shows an excellent potential for therapy in the gastrointestinal area.

Keywords: onopordopicrin, *Arctium lappa*, Colitis, TNBS (trinitrobenzene sulphonic acid).

1. Introduction

The pathological features of inflammatory bowel disease, such Crohn's disease and ulcerative colitis, are marked by the presence of mucosal ulceration, and the infiltration of neutrophils and lymphocytes in mucous membranes (Zhou et al., 1999). The development of these diseases may be a result of uncontrolled or inadequately down-regulated cellular immune responses in the intestinal mucosa to a hitherto unknown agent, probably a constituent of the luminal contents (Duchmann et al., 1996). This leads to mucosal injury, breakdown of the epithelial barrier function, and increased influx of luminal contents to the lamina propria, which might further exaggerate the uncontrolled immune response (Stallmach et al., 1999). Activated immune cells, primarily represented by neutrophils, macrophages and cytotoxic T cells play the role of aggressors that attack and destroy the intestinal barrier either directly through physical contact or directly through the release of reactive oxygen and nitrogen metabolites, cytotoxic proteins, lytic enzymes, or cytokines such as tumour necrosis factor (TNF-α) and interleukin (IL)-1β (Kruidenier et al., 2002; Nitta et al., 2002; Abreu, 2002).

Arctium lappa (Asteraceae), commonly known as "bardana", has a diversity of therapeutics actions as antioxidant activity, antiinflamatory and analgesic effects. However, there is no report related to its activity on experimental Ulcerative Colitis (UC), thus here we investigated the effects of ONP on the colon injury caused by intracolonic administration of 2,4,6- trinitrobenzenesulphonic acid (TNBS) in rats. The inflammation response was assessed on the basis of histology and myeloperoxidase (MPO) activity, as an index of quantitative inflammation and neutrophil

infiltration in the mucosa. Mucosal TNF-α production, immunohistochemical study and western blot analysis for COX-1 and –2.

2. Materials and methods

2.1. Experimental animals

Male Wistar rats supplied by Animal Services, Faculty of Medicine, University of Seville, Spain, and weighing 180–220 g, were placed singly in cages with wire-mesh floors in a room with temperature, 24–25 °C, humidity, 70–75%, and lighting, 12-h light/12-h dark, and were fed a normal laboratory diet (Panlab, Barcelona, Spain). The rats were deprived of food for 24 h prior to the induction of colitis, but were allowed free access to tap water throughout. They were randomly assigned to groups of 8–14 animals. The experiments followed a protocol approved by the local animal Ethics Committee and the local Government. All experiments were in accordance with the recommendations of the European Union regarding animal experimentation (Directive of the European Council 86/609/EC).

2.2. Preparation of semi-purified fraction (ONP)

Leaves of *A. lappa* were collected in Mogi Mirim, SP, Brazil and a voucher herbarium specimen was deposited in the herbarium of the State University of Campinas (voucher no. 131.966). Fresh leaves were triturated in aqueous 70% ethanol, filtered, and the solvent then evaporated under vacuum. The resulting residue was diluted in EtOH:H_2O (2:1, v/v), and extracted three times with ether. The ethereal layer was treated with anhydrous Na_2SO_4, evaporated, diluted in EtOH:H_2O (2:1, v/v) and extracted three times with hexane. The hexane layer was set removed, and the polar layer was extracted three times with $CHCl_3$. The chloroform layer was then treated with anhydrous Na_2SO_4, evaporated under vacuum. Semi-purified fraction (ONP), which it contais onopordopicrin

as majoritary compound, was obtained from chloroform extract (100 mg) by adsorption cromathography using a column packed with Silica Gel 60 (0,0063-0,200 mm) as the stationary phase. The column was eluted with chloroform and a polarity gradient obtained by adding 1-4% MeOH. The fraction was analyzed by GC-MS in a Hewlett Packard-6890 GC system with a fused capillary column (30 m x 0.25 mm x 0.25μm, HP-5MS, Crossbond 5% phenyl 95%-dimethylpolysiloxane, HP) coupled directly to Hewlett Packard 5973 with a selective mass detector. The injection temperature was 250°C; temperature program of 40-300°C at 4°C/min, operated in the splitless mode for 1.5 min. The carrier gas was He at a constant flow of 1ml/min and the sample volume was 1μl. Onopordopicrin ($C_{19}H_{24}O_6$ - Figure 1) eluted at 57.686 min. The mass fragmentation agreed with that reported by Barbosa Filho et al. (1993): m/z (relative abundance) M^+ 348(<1), 281(8), 207(29), 147(43), 119(88), 91(52) and 85(100).

2.3. Induction of colitis

Colitis was induced according to the procedure described by Morris et al. (1989). Briefly, rats were lightly anesthetized with ether following a 24-h fast, and then a medical-grade polyurethane cannula for enteral feeding (external diameter 2 mm) was inserted into the anus and the tip was advanced to 8 cm proximal to the anus verge. TNBS (Sigma, Spain) dissolved in 50% ethanol was instilled into the colon through the cannula (10 mg in a volume of 0.25 ml to induce acute). The animals were maintained in a head-down position for a few minutes to prevent leakage of the intracolonic instillate. Control groups were created for comparison with TNBS/ethanol instillation: rats in the normal group received 12% Tween 80 instead of the TNBS solution, and the ethanol control group received 0.25 ml 50% ethanol.

In the acute model of colitis, ONP was diluted in 12% Tween 80 and administered (p.o.) at doses 25 and 50 mg/kg, 48, 24 and 1 h prior to the induction of colitis and 24 h later. Control groups

received vehicle in a comparable volume (10 ml/kg body weight). The animals were killed with cervical deslocation after induction of colitis. The rats were checked daily for behaviour, body weight, and stool consistency.

2.4. Assessment of colitis

An independent observer who was blind to the treatment evaluated severity of the colitis. For each animal, the distal 10-cm portion of the colon was removed and cut longitudinally, slightly cleaned in physiological saline to remove fecal residues and weighed. Macroscopic inflammation scores were assigned, based on clinical features of the colon (score 0–10), the presence of adhesions (score 0–2), and/or stool consistency (score 0–1) according to the criteria of Bobin-Dubigeon et al. (2001). Pieces of inflamed colon were collected and frozen in liquid nitrogen to quantify myeloperoxidase activity.

2.5. Histological studies

For light microscopy we used tissue samples from the distal colon of each animal fixed in 4% buffered paraformaldehyde, dehydrated in increasing concentrations of ethanol, and embedded in paraffin. Thereafter, sections of tissue were cut at 5 μm on a rotary microtome (Leica Ultracut), mounted on clean glass slides and dried overnight at 37°C. The sections were cleared, hydrated, and stained with hematoxylin and eosin, Alcian blue or Giemsa for histological evaluation of colon damage, mucus content and neutrophil infiltration, respectively, according to standard protocols, and the slides were coded to prevent observer during evaluation. All tissue sections were examined with an Olympus BH-2 microscope for characterization of histopathological changes.

Photographs of colon samples were digitized using a Kodak D290 Zoom camera Eastman Kodak, USA and Motic®Images 2000 release 1.1 (MicroOptic Industrial Group; B1 Series System Microscopes). Analysis of the figures was carried out with the Adobe®Photoshop® Version 5.0 (Adobe Systems) image analysis program.

2.6. Immunohistochemical study

Colon tissues were fixed in 4% buffered paraformaldehyde, dehydrated with increasing concentrations of ethanol, embedded in paraffin, and sectioned. Sections (5 μm thick) were mounted on slides, cleared, and hydrated. All sections were treated with a buffered blocking solution (3% bovine serum albumin in phosphate-buffered saline, PBS) for 15 min. The sections were then co-incubated with primary anti-EGF receptor antibody (1:400 in PBS, v/v), at room temperature for 2 h, followed by washing with PBS and co-incubation with secondary antibody (anti-sheep IgG, peroxidasic conjugated, Sigma) (1:400 in PBS, v/v), at room temperature for 1 h. Thereafter, the sections were washed as before and with Tris–HCl 0.05 M, pH 7.66, then co-incubated with a 3,3'-diaminobenzidine solution in the dark, at room temperature for 30 min. The sections were washed with Tris–HCl, mounted with glycerine and observed with an Olympus BH-2 microscope.

2.7. Assessment of leukocyte involvement

Myeloperoxidase activity was assessed as a marker of neutrophil infiltration according to the methods of Grisham et al. (1990). One sample from the distal colon was taken from all animals. Samples were excised from each animal and rapidly rinsed with ice-cold saline, blotted dry, and frozen at -70 °C. The tissue was thawed, weighed and homogenized in 10 volumes 50 mM PBS, pH=7.4. The homogenate was centrifuged at 20,000×*g*, 20 min, 4 °C. The pellet was again homogenized in 10 volumes 50 mM PBS, pH=6.0, containing 0.5% hexadecyl-trimethylammonium bromide (HETAB) and 10 mM EDTA. This homogenate was subjected to one cycle of freezing/thawing and brief sonication. A sample of homogenate (0.5 μl) was added to a 0.5-ml reaction volume containing 80 mM PBS, pH 5.4, 0.5% HETAB and 1.6 mM 3,3',5,5'-tetramethylbenzidine (TMB). The mixture was incubated at 37 °C for 5 min and the reaction was started by the addition of 0.3 mM H_2O_2. Each tube containing the complete reaction mixture was

incubated for exactly 3 min at 37 °C. The reaction was terminated by the sequential addition of catalase (20 μg/ml) and 2 ml 0.2 M sodium acetate, pH=3.0. The changes in absorbance at 655 nm were measured with a spectrophotometer. One unit of myeloperoxidase activity was defined as the amount of enzyme present that produced a change in absorbance of 1.0 U/min at 37 °C in the final reaction volume containing the acetate. The results were quantified as U/mg protein.

2.8. TNF-α levels

Distal colon samples were weighed (100 mg) and homogenized, after thawing, in 0.3 ml phosphate buffer saline solution (PBS pH 7.2) at 4 °C. They were centrifuged at 12 000 rpm for 10 min. Mucosal TNF-α level was assayed with a quantitative TNF-α enzyme immunoassay (ELISA) kit (Quantikine®M, R&D Systems). The TNF-α values were expressed as pg/mg protein.

2.9. Western Blotting

Colonic mucosal samples (~100 mg) of all groups were homogenized in 5 volumes of boiling lysis buffer [1% SDS, 1.0 mM sodium vanadate, 10 mM tris (hydroxymethyl)aminomethane (Tris).HCl, pH 7.4] centrifuged at 12000 g, 15 min, 4ºC. Protein concentration of the homogenate was determined following Bradford´s colorimetric method. The tissue homogenates, 30 and 50 μg of protein per lane, were electrophoresed in 10% SDS-polyacrilamide gel, and transferred onto nitrocellulose membranes by electroblotting using a wet transfer cell. Equal loading was confirmed by Ponceau S staining. The blots were pretreated in Tris-buffered saline containing 5% nonfat dry milk, 0.5% octylphenylpolyethylenglycol (Nonidet®) and incubated with specific primary antibodies for COX-1 (M-20) at a dilution of 1:2000, COX-2 (M-19) at a dilution of 1:400 (Santa Cruz Biotechnology®). Filters were washed three times for 15 min each other and incubated with a horseradish peroxidase-conjugated

secondary antibody against goat IgG (Santa Cruz Biotechnology®) and anti-rabbit IgG (Sigma-Aldrich®). Immunocomplexes were detected by the SuperSignal West Pico chemiluminescent Kit (Pierce, USA). Thereafter, the developed membrane was exposed to an X-ray film (Kodak, Germany).

2.11. Statistical analysis

All values in the figures and text are expressed as arithmetic means±standard error of the mean (S.E.M.) or standard deviation (SD). The data were evaluated with Graph Pad Prism® Version 2.01 software. The statistical significance of differences for each parameter among the groups was evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's test. *P* values of <0.05 were considered statistically significant.

3. Results

Rats treated with TNBS and ethanol showed prostration, piloerection and hypomotility. Macroscopic inspectium of the cecum, colon and rectum showed evidence of severe colonic mucosal damage, with oedema, deep ulcerations and haemorrhage. The animals were severely anorexic, with a marked decrease in average food intake compared to that of the vehicle-treated group (Table 1). The cecum, colon and rectum showed evidence of damage with mucosal haemorrhage, oedema and deep ulceration. Lesion in the distal colon were quantified according to a macroscopic damage score (Fig 2). A significant increase en the weight/length of the rat colon, an indicator of inflammation, was also observed in TNBS- and ethanol-treated rats (280±90 mg/cm) in comparison with vehicle-treated rats (Table 1)

The histopatological features included necrosis, oedema and diffuse inflammatory cell infiltration in the mucosa. There was focal ulceration of the colonic mucosa extending through the muscularis mucosae, desquamated areas or loss of epithelium and mucin depletion. The architecture of the crypts was distorted and the lamina propria was thickened in peripheral areas of distorted crypts, specially in basal areas. An infiltrate consisting of polymorphonucler leukocytes, lymphocytes, and eosinophils was observed. There was also granulation tissue in the submucosa (Fig 3D, 4D and 5D). Some areas showed accumulation of mucus and cell remnants, however, Alcian blue positive-positive cells were less numerous. In addition, the mucin layer of the epithelium was missing (Fig 3D).

Treatment of rats with ONP (25 or 50 mg/kg) significantly attenuated the extend and severity of the colonic injury, reducing the macroscopic damage score ($p<0.05$), although this effect was not dose dependent (Fig. 2). Histologically, there was attenuation of the extend and severity of the histological signs of cell damage and we did not see inflammatory cells in the lamina propria (Fig 4E and F). In some areas, the epithelium remained intact and the mucin layer was clearly visible. Alcian blue-positive cells were less numerous, however, in ulcerative areas, exfoliation of epithelial cells, dilated crypts, inflammatory cells and congestion vascular were observed (Fig 3E and F).

MPO is an enzyme found in neutrophils and its activity in the colon is linearly related to neutrophil infiltration (Villegas et al., 2003). The colitis caused by TNBS and ethanol was characterized by an increase in MPO activity (1.74±0.03 U/mg tissue) (Table 2). This was consistent with the histological findings. Treatment of TNBS-treated rats with ONP (25 or 50 mg/kg) reduced significantly the degree of polymorphonuclear neutrophil infiltration. The levels of TNF-α were

significantly elevated in the colon at 48 h after TNBS instillation. In contrast, the levels of cytokines were significantly lower in rats treated with ONP (Table 3).

In normal colon, specific immunosignals for COX-1 were obtained in surface epithelium, and in the upper half of the crypts. Mononuclear cells of the lamina propria and the regional lymphatic nodules as well as cells of the muscularis mucosae showed COX-1 specific immunosignals (Fig 6B). COX-2 specific immunolabelling was occasionally observed in colonocytes of the normal surface epithelium of matched control colon as shown in Fig 7B.

Compared with normal colon, unchanged in the cellular distribution of COX-1, significant changes in the cellular distribution of COX-2 were observed in animals treated with TNBS in that colonocytes of the surface and the crypt epithelium were only weakly decorated by the COX-1 specific antiserum (Fig 6D), whereas prominent COX-2 expression was found in cells of surface epithelium and in cells of the inflammatory infiltrate (Fig 7D). Compared with inflamed colon, no significant changes in the cellular localization and the degree of positive staining for COX-1 were observed in the colon of ONP (25 or 50 mg/kg)-treated rats (fig 6E, F and Fig 8) whereas COX-2 expression was increased in apical epithelial cells of inflamed colon from ONP-treated rats (Fig7 E, F and Fig 9)

1. Discussion

Ulcerative colitis (UC) is an inflammatory bowel disease that approximately 60,000 patients in Japan and 500,000 in the US suffer from, though its pathogenesis is unknown. Disruption of immune systems in the intestinal tract is suggested to be involved in the etiology of UC, thus immune suppressive agents and antibiotics are currently used as tentative drugs. However, prolonged and chronic UC may progress to colorectal cancer (Murakami et al., 2003). The

pathological hallmark of inflammatory bowel disease (IBD) is marked by the presence of mucosal ulceration, infiltration of the mucosa with neutrophils and abdominal discomfort or pain with altered bowel habits (diarrhea, constipation or altercating episodes of diarrhea and constipation) (De Ponti et al., 2001). Reactive oxygen metabolites mediate cell injury. Oxygen derived free radicals are generated by several sources, including stimulated polymorphonuclear cells, eosinophils, xanthine oxidase, colonic bacteria, and epithelial lipooxygenase, all of which are present in the inflamed bowel of inflammatory bowel disease patients (Karmeli et al., 1995). Several strategies for drug intervention specifically directed at the attenuation of oxidative stress have been considered (Babbs, 1992). These include blocking of O_2^- formation by phagocytes scavenging of O_2^- before it can react with iron, binding iron so that it does not start the oxidation-reduction cycle, and scavenging OH or HOCl. The primary defense against oxidative insult to tissue includes superoxide dismutase, catalase, and gluthathione peroxidase (Babbs, 1992).

The present study confirmed that treatment with 25 or 50 mg/kg of ONP was able to reduce the severity and extent of the acute colonic damage induced by TNBS and ethanol (Fig 2,4). The decrease in the extent of colitis induced by this substance was accompanied by a lower incidence of diarrhea and of loss of weight of the animals and decrease in the incidence of adhesions. The presence of adhesions between the colon and adjacent organs, which results from transmural inflammation, is a common feature of TNBS colitis (Morris et al., 1989). The reduction in the incidence of adhesions suggests a beneficial effect of ONP on the extent of the inflammatory process in this experimental model (Table 1). Another sesquiterpene lactone, dehydroleucodine (DhL), of the guaianolide type isolated from *Artemisis dougalsiana* Besser shows a pharmacological cytoprotective effects and significantly prevents the formation of gastric and duodenal lesions induced by ethanol induced necrosis (Giordano et al., 1990). Intracolonic

administration of TNBS/ethanol resulted in extensive haemorrhagic and ulcerative damage to the proximal colon. Pretreatment for 1h with DhL (40 mg/kg) significantly reduced the extent of TNBS-induced colonic damage. ONP had similar result than DhL, but obtained significantly reduction of colonic damage with lower dose (25 mg/kg) than DhL.

The protective effect of mucus as an active barrier may be attributes large to viscous and gel-forming properties, which are derived from mucin glycoprotein constituents (Villegas et al., 2003). Our results reveal that ONP increased the amount of mucus stained Alcian blue (acid glucoproteins such as sialomucins) colon mucosa (Fig 3). Alcian blue-positive cells seem to be associated with regenerative processes of the mucosa, while reduction in the amount stained has been related to decreased resistance of mucosa and paralleled by alterations in the normal pathway of maturation of the mucin in globet cells (Alarcon de la Lastra et al., 1994). Wendel et al (1999) have demonstrated that DhL and other related sesquiterpene lactone, like ONP, significantly prevent the formation of gastric lesions induced by various necrotizing agents. Furthermore, he attributed this cytoprotective activity to the presence of a non-hindered Michael acceptor in the molecules assayed and suggests that mechanism of protection would be, at least in part, through an increase of prostaglandin synthesis and mucosal glycoprotein synthesis (Giordano et al., 1992).

The role of eicosanoids in the intestinal inflammatory process is not completely understood: several eicosanoids are increased in IBD and a positive correlation exists between tissue levels of these eicosanoids and disease activity. COX-1 expression is detected in both normal and inflamed gastrointestinal mucosa; in contrast, COX-2 is expressed in epithelial cells in the upper portions of the crypts and on the surface in Crohn`s colitis and ulcerative colitis, in villous epithelial cells in Crohn’s ileitis and is not detectable in the epithelium of the normal ileum or colon (Cipolla et al.,

2002). Abnormal metabolism of arachidonic acid is another vital factor in the IBD pathogenesis. As the crucial synthethase in the arachidonic acid metabolic pathway, COX-2 could be activated to produce excessive PGE_2 and TBX_2, two important inflammatory mediators, in the inflammatory bowel disease, which contribute to the bowel hyperemia, oedema and even dysfunction. Administration of either COX-2 or thromboxane synthase inhibitors has been shown to be useful for the treatment of IBD (Kankuri et al., 2001). Since, prostanoids play a pivotal role in IBD, attempts to ameliorate the severity of the colonic inflammatory process with non-selective COX inhibitors: non-steroidal anti-inflammatory drugs (NSAIDs) have been made (Murakami et al., 2003). However, the outcome of the trials reported aggravation, and flare of the colonic inflammatory condition in patients administered with NSAIDs; the probable reason documented was non-specific inhibition of both isoforms of COX enzymes (Karmeli et al., 2000; Murakami et al., 2003). COX-2 expression is one of the key steps in various inflammatory pathogeneses in the digestive tracts, such as colitis (Agoff et al., 2000; Singer et al., 1998), and leads to an accelerated synthesis of PGH_2, and then of PGE_2, $PGF_{2\alpha}$, and PGD_2 (Murakami et al., 2003). We determined the expression of COX-1 and –2 by immunohistochemistry. Our data showed that ONP did not interfered with COX-1 activity, however affect COX-2 activity when incubated with microsomes derived from recombinant human COX-2 (Fig 6,7,8 and 9).

The pathogenesis of colitis was also associated with TNF-α and interleukin-1β, since these cytokines are present in colon tissues and can be detected immunohistochemically in the inflamed tissues (Carty et al., 2000). Reactive oxygen and nitrogen species have been reported to be involved in the development of UC in tests that modified the environmental components, including proteins and DNA bases (D`Odorico et al., 2001). Inflammatory cytokines such as interferon-γ and TNF-α have been implicated in the expression of inducible nitric oxide synthase (iNOS) (Kankuri

et al., 1999), which generates excess amounts of nitric oxide (NO) from inflammatory lekocytes in colitis tissue (Obermeier et al., 1999). There is good evidence that TNF-α and IL-1 help to propagate the extension of a local or systemic inflammatory process. TNF- α (and IL-1) causes the activation and translocation of NF-κB into the nucleus (Wooley et al., 1993)**.** In our experiment the levels of TNF-α were significantly increased in the colon at 48 h after TNBS instillation. In contrast, the levels of cytokines were significantly lower in rats treated with 25 and 50mg/kg of ONP

Neutrophils have been considered to play a crucial role in the development and full manifestation of gastrointestinal inflammation, as they represent a major source of free radicals in the inflamed colonic mucosa (Grisham, 1994). Colon inflammation is usually characterized by extensive infiltration of colon tissue by polymorphonuclear leukocytes, which is more marked in bronchoalveolar lavage fluid during acute, infectins exacerbations (Cuzzocrea et al., 2001). Neutrophil activation represents an important source of reactive oxygen and nitrogen species play a key role in inflammatory bowel disease (Grisham, 1994). MPO derived from activated neutrophils transforms H_2O_2 into toxic tissue oxidant hypochlorous (HOCl), responsible inactivation of alpha-1-antitrypsin (AT) a glycoprotein, which in turn, results in the elastase (lysosymal enzyme)-mediated digestion of intracellular tissue matrix (Grisham, 1994). In our experiments, the level of myeloperoxidase activity was significantly increased in TNBS control animals killed 48 h after TNBS enema, indicating that neutrophil-derived free radicals were involved in the pathogenesis of TNBS-induced colon injury. However, treatment of TNBS-treated rats with ONP significantly reduced the degree of polymorphonuclear neutrophil infiltration.

In conclusion, the data from the present study reveal that pre-treatment with ONP is able to reduce intestinal inflammation in the TNBS model of colitis in rats. The acute antiinflamatory effects

seem to be related to impairment of neutrophil function and absence of up-regulation of TNF-α production in intestinal mucosa. Our findings suggest that ONP shows an excellent potential for therapy in the gastrointestinal area.

Acknowledgments

The author thank to Department of Pharmacology of the University of Seville-Spain, Department of Chemistry of the Paulista State University-Brazil and Department of Physiology of the State University of Campinas-Brazil. This study was supported by CAPES, special funds from the Ministry of Science and Technology (Brazil).

References

Abreu MT, Yang H (2003) Defining subtypes of Crohn's disease patients: the ground work for translational research in inflammatory bowel disease. J Clin Gastroenterol. 36 (1), 3-4.

Agoff SN, Brentnall TA, Crispin DA, Taylor SL, Raaka S, Haggitt RC, Reed MW, Afonina IA, Rabinovitch PS, Stevens AC, Feng Z, Bronner MP (2000) The role of cyclo-oxygenase 2 in ulcerative colitis-associated neoplasia. *Am. J. Pathol.* **157** (2000), pp. 737–745.

Alarcón de la Lastra C, Martín MJ, Motilva V (1994) Antiulcer and gastroprotective effect of quercetin: a gross and histologic study. *Pharmacology* 48**,** 56–62.

Babbs CF. (1992) Oxygen radicals in ulcerative colitis. Free Radic Biol Med. 13(2), 169-81. Review.

Bobin-Dubigeon C, Collin X, Grimaud N, Robert JM, Le Baut G, Petit JY (2001) Effects of tumor necrosis factor-α synthesis inhibitors on rat trinitrobenzene sulphonic acid-induced chronic colitis. *Eur. J. Pharmacol.* 431, 103–110

Carty E, De Brabander M, Feakins RM, Rampton DS (2000) Measurement of in vivo rectal mucosal cytokine and eicosanoid production in ulcerative colitis using filter paper. *Gut* 46, 487–492.

Cipolla G, Crema F, Sacco S, Moro E, de Ponti F, Frigo G. (2002) Nonsteroidal anti-inflammatory drugs and inflammatory bowel disease: current perspectives. Pharmacol Res. 2002 Jul;46(1):1-6. Review.

Cuzzocrea S, Mazzon E, Serraino I, Dugo L, Centorrino T, Ciccolo A, Sautebin L, Caputi AP (2001) Celecoxib, a selective cyclo-oxygenase-2 inhibitor reduces the severity of experimental colitis induced by dinitrobenzene sulphonic acid in rats. *Eur. J. Pharmacol.* 431, 91–102

De Ponti F, Tonini M.(2001) Irritable bowel syndrome: new agents targeting serotonin receptor subtypes. Drugs. 61(3), 317-32. Review.

D'Odorico A, Bortolan S, Cardin R, D'Inca' R, Martines D, Ferronato A, Sturniolo GC. (2001) Reduced plasma antioxidant concentrations and increased oxidative DNA damage in inflammatory bowel disease. Scand J Gastroenterol. 2001 36 (12),1289-94.

Giordano OS, Guerreiro E, Pestchanker MJ, Guzman J, Pastor D, Guardia T.(1990) The gastric cytoprotective effect of several sesquiterpene lactones.J Nat Prod. 53(4), 803-9.

Grisham MB, Beniot JN, Granger DN (1990) Assessment of leucocyte in involvement during ischemia and reperfusion on the intestine. In: L. Packer and A.E. Glazer, Editors, *Methods in Enzymology. Oxygen Radicals in Biological Systems*, Academic Press, San Diego, 729–741

Grisham MB, Specian RD, Zimmerman TE.(1994) Effects of nitric oxide synthase inhibition on the pathophysiology observed in a model of chronic granulomatous colitis.J Pharmacol Exp Ther. 271(2):1114-21.

Kankuri E, Asmawi MZ, Korpela R, Vapaatalo H, Moilanen E. (1999) Induction of iNOS in a rat model of acute colitis. Inflammation. 23(2), 141-52.

Kankuri E, Vaali K, Korpela R, Paakkari I, Vapaatalo H, Moilanen E (2001) Effects of a COX-2 preferential agent nimesulide on TNBS-induced acute inflammation in the gut. *Inflammation* 25**,** 301–309.

Karmeli F, Cohen P, Rachmilewitz D (2000) Cyclo-oxygenase-2 inhibitors ameliorate the severity of experimental colitis in rats. *Eur. J. Gastroenterol. Hepatol.* 12, 223–231

Karmeli F, Eliakim R, Okon E, Samuni A, Rachmilewitz D. (1995) A stable nitroxide radical effectively decreases mucosal damage in experimental colitis. Gut. 37(3), 386-93.

Kruidenier L, Verspaget HW (2002) Oxidative stress as a pathogenic factor in inflammatory bowel disease—radicals or ridiculous?. *Aliment. Pharmacol. Ther.* 16, 1997–2015.

Martín AR, Villegas I, La Casa C, Alarcón de la Lastra C (2003) The cyclo-oxygenase-2 inhibitor, rofecoxib, attenuates mucosal damage due to colitis induced by trinitrobenzene sulphonic acid in rats European Journal of Pharmacology 481(2-3), 281-291.

Morris GP, Beck PL, Herridge MS, Depew WT, Szewczuk MR, Wallace JL (1989) Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology* 96, 795–803

Nitta M, Hirata I, Toshina K, Murano M, Maemura K, Hamamoto N, Sasaki S, Yamauchi H, Katsu K (2002) Expression of the EP4 prostaglandin E2 receptor subtype with rat dextran sodium sulphate colitis: colitis suppression by a selective agonist, ONO-AE1-329. *Scand. J. Immunol.* 56, 66–75

Obermeier F, Kojouharoff G, Hans W, Scholmerich J, Gross V, Falk W. (1999) Interferon-gamma (IFN-gamma)- and tumour necrosis factor (TNF)-induced nitric oxide as toxic effector molecule in chronic dextran sulphate sodium (DSS)-induced colitis in mice. Clin Exp Immunol. 116(2), 238-45.

Stallmach A, Wittig B, Giese T, Pfister K, Hoffmann JC, Bulfone-Paus S, Kunzendorf U, Meuer SC, Zeitz M. (1999) Protection of trinitrobenzene sulfonic acid-induced colitis by an interleukin 2-IgG2b fusion protein in mice. *Gastroenterology*, 117(4), 866-76.

Villegas C. La Casa, Orjales A, Alarcón de la Lastra C. (2003) Effects of dosmalfate, a new cytoprotective agent, on acute and chronic trinitrobenzene sulphonic acid-induced colitis in rats. *Eur. J. Pharmacol.* 460, 209–218

Wendel GH, Maria AO, Mohamed F, Dominguez S, Scardapane L, Giordano OS, Guerreiro E, Guzman JA.(1999) Effect of dehydroleucodine in experimental colitis in rats and mice. Pharmacol Res. 40(4):339-44.

Wooley PH, Dutcher J, Widmer MB, Gillis S. (1993) Influence of a recombinant human soluble tumor necrosis factor receptor FC fusion protein on type II collagen-induced arthritis in mice. J Immunol. 151(11), 6602-7.

Fig 1. Chemical Structure onopordopicrin (ONP)

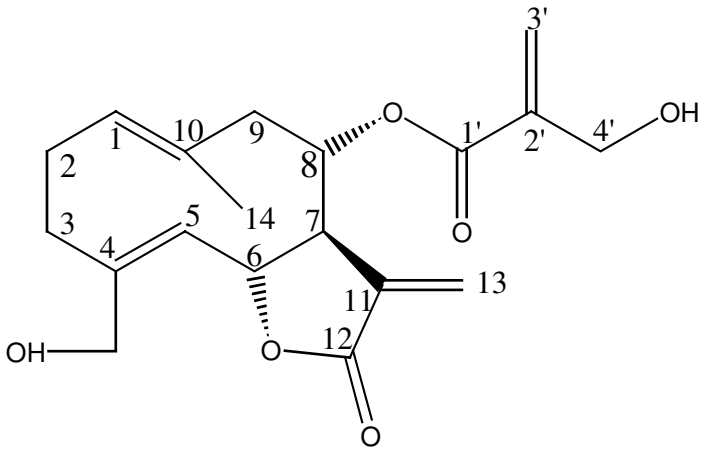
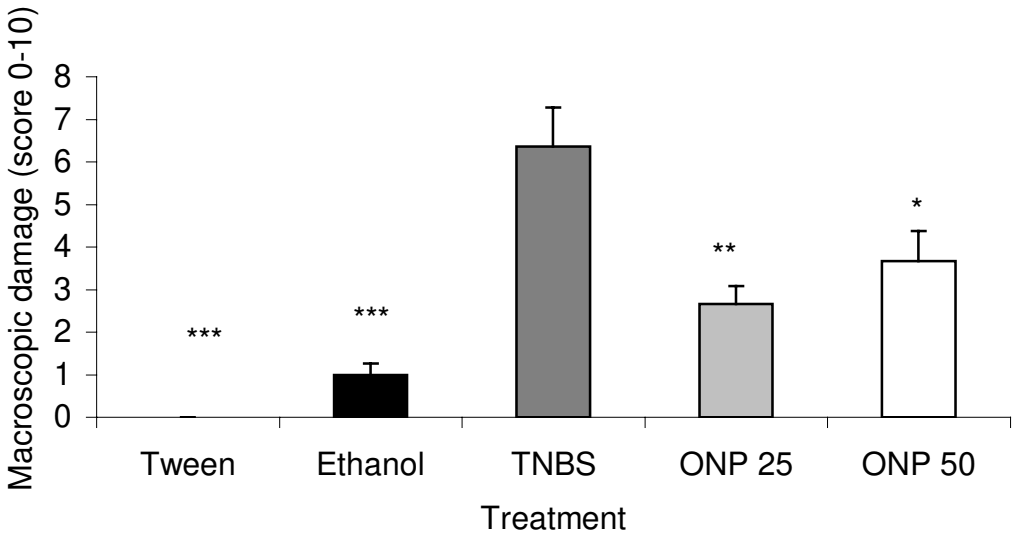


Fig 2. Effects of acute administration of onopordopicrin on the colon damage score. Colonic macroscopic damage resulting from TNBS (10 mg/animal) instilled into rat colon was scored in Section 2. The data are expressed as the means±SD. *p<0.05 **p<0.01 ***p<0.001vs TNBS group.



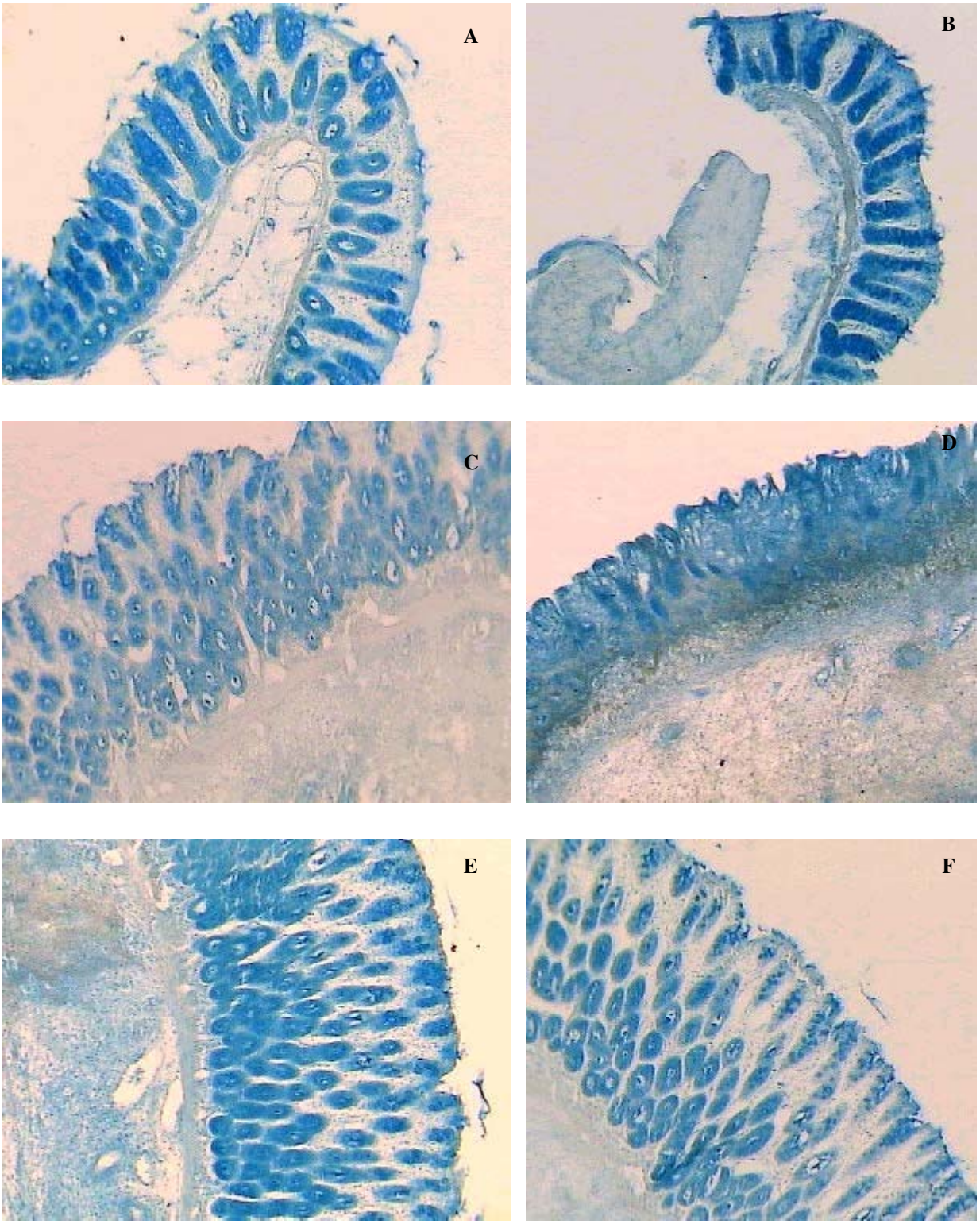


Fig 3. Acute colitis model induced by TNBS: effect of onopordopicrin on colon injury. Histological appearance of rat colonic mucosa after Alcian Blue stain: sham (A), treated with tween (B), ethanol (C), TNBS (D), ONP 25 mg/kg (E) and ONP 50 mg/kg (F). Histopathological features of the colon in association with colitis. (A and B) No histological modification was present in the sham and tween animals. (C) Mucosal injury was produced after ethanol

administration. (D) Mucosal injury was produced after TNBS administration, characterized by necrosis of epithelium, focal ulceration of the mucosa and diffuse infiltration of inflammatory cells in the mucosa and submucosa. (E and F) Treatment with onopordopicirin (25 and 50 mg/kg) reduced the morphological alterations associated with TNBS administration, protecting the mucosal architecture; some areas showed accumulation of mucus and cell remnants. Alcian blue positive cells. Original magnification 10x.

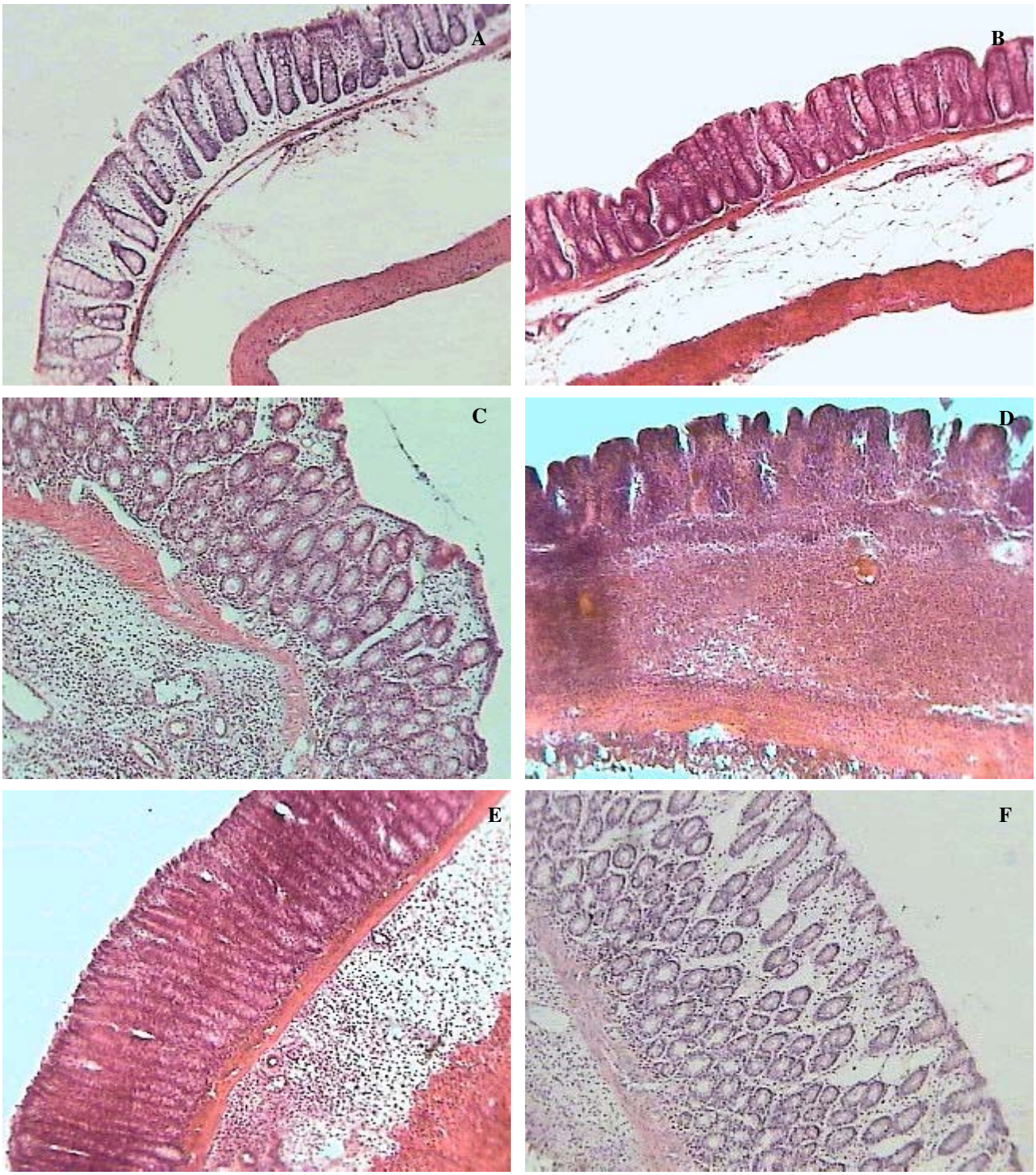


Fig 4. Acute colitis model induced by TNBS: effect of onopordopicrin on colon injury. Histological appearance of rat colonic mucosa: sham (A), treated with tween (B), ethanol (C), TNBS (D), ONP 25 mg/kg (E) and ONP 50 mg/kg (F). Histopathological features of the colon in association with colitis. (A and B) No histological modification was present in the sham and tween animals. (C) Mucosal injury was produced after ethanol administration. (D) Mucosal injury was produced after TNBS administration, characterized by necrosis of epithelium, focal ulceration of the mucosa and diffuse infiltration of inflammatory cells in the mucosa and submucosa. (E and F) Treatment with onopordopicirin (25

and 50 mg/kg) reduced the morphological alterations associated with TNBS administration, protecting the mucosal architecture.Hematoxylin and eosin stain. Original magnification 10x.

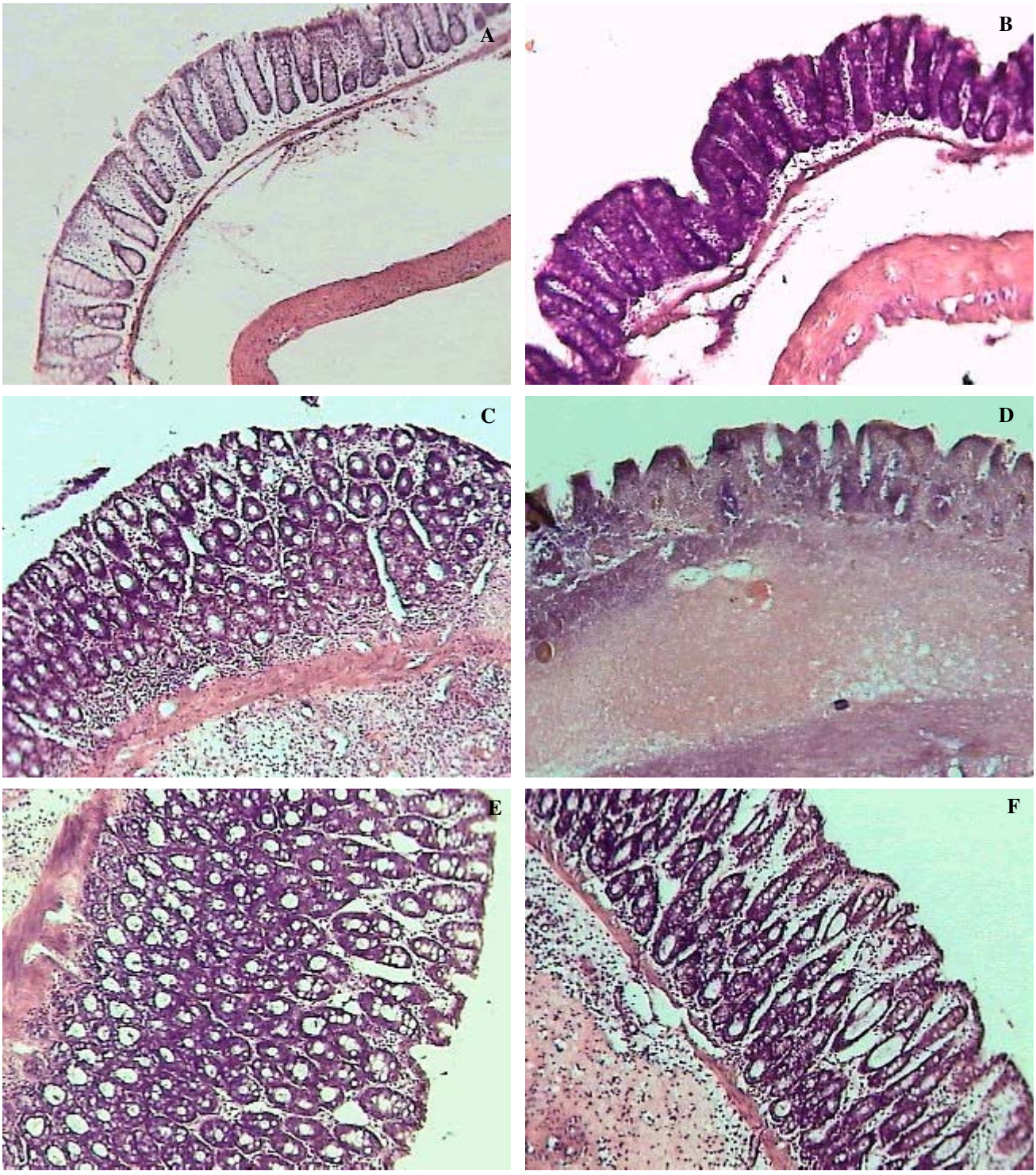


Fig 5. Rat colon segments stained with Giemsa: sham (A), treated with tween (B), ethanol (C), TNBS (D), ONP 25 mg/kg (E) and ONP 50 mg/kg (F). Infiltration of inflammatory cells was highly observes in the colonic mucosa of TNBS-treated animals. Original magnifications 10x.

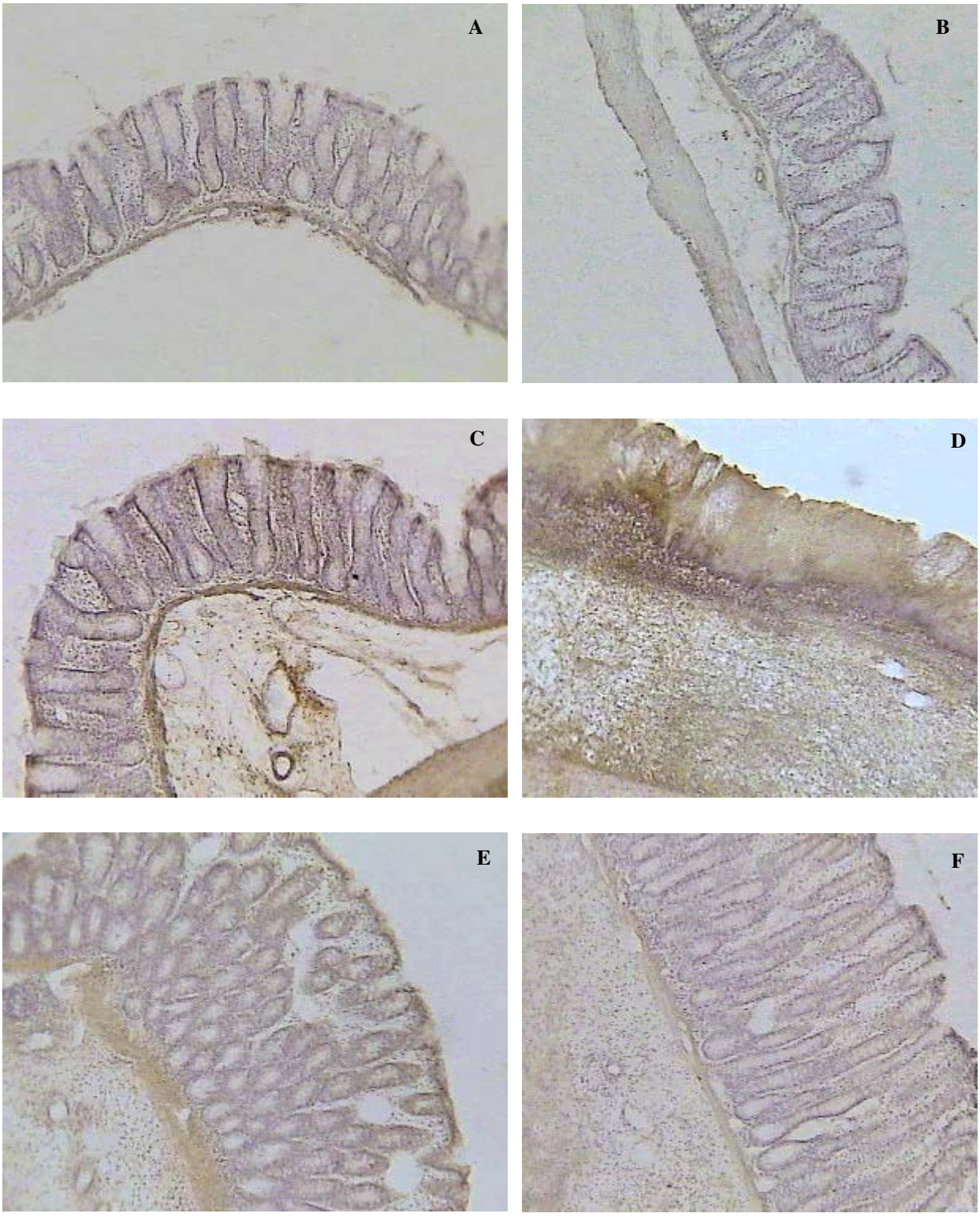


Fig 6. Immunohistochemical localization of COX-1 isoenzyme in sections of colon. Negative control (A). In normal colon, colonocytes of the upper half of the crypts were found to be COX-1 positive (B). COX-1 expression in endocrine cells is particularly evident in this image of normal colonic mucosa (B and C). COX-1 expression in the

colon of TNBS-control rats (D). COX-1 expression of inflamed colon treated with onopordopicrin 25 mg/kg (E) and 50 mg/kg (F). Original magnifications 10x.

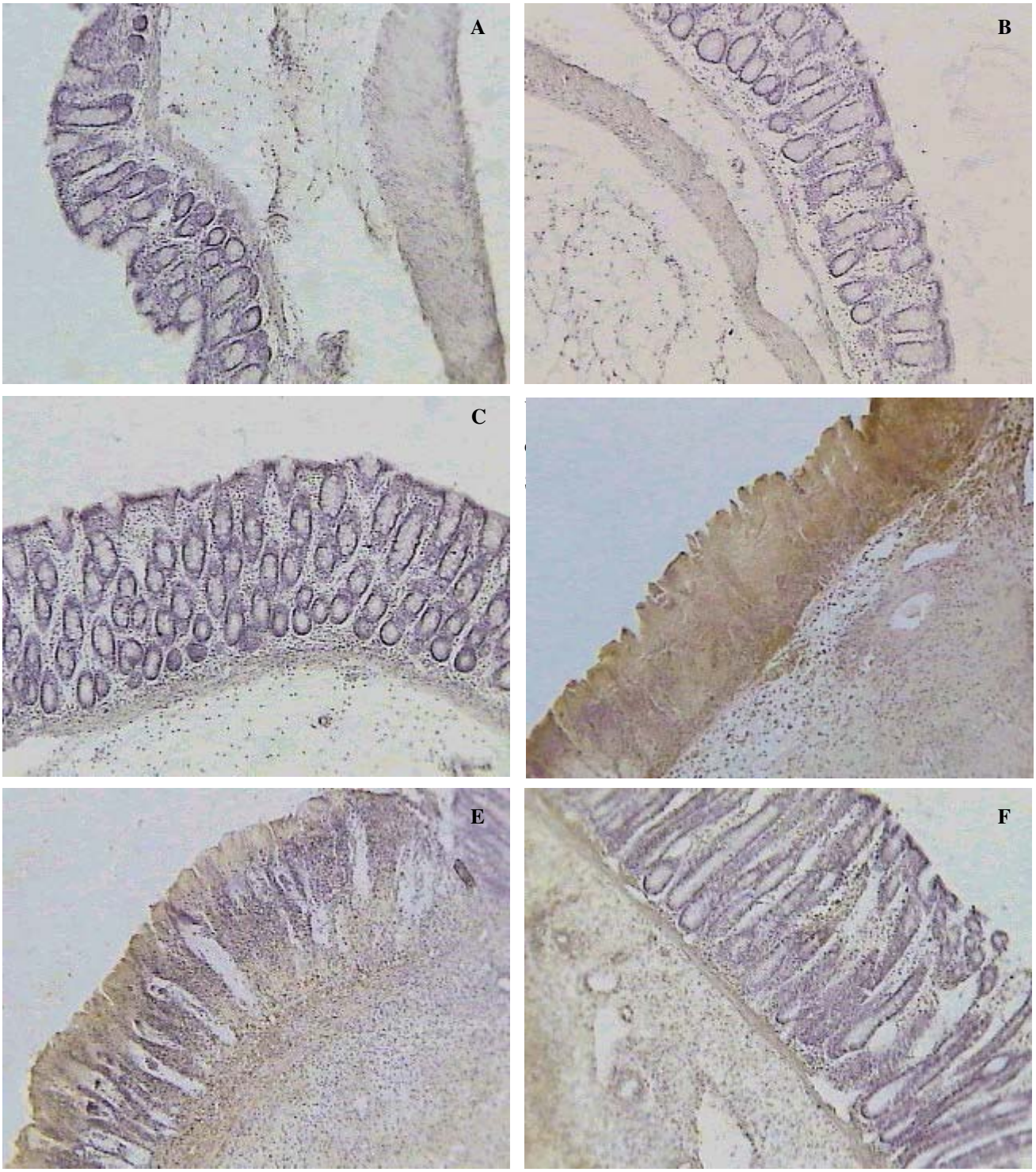
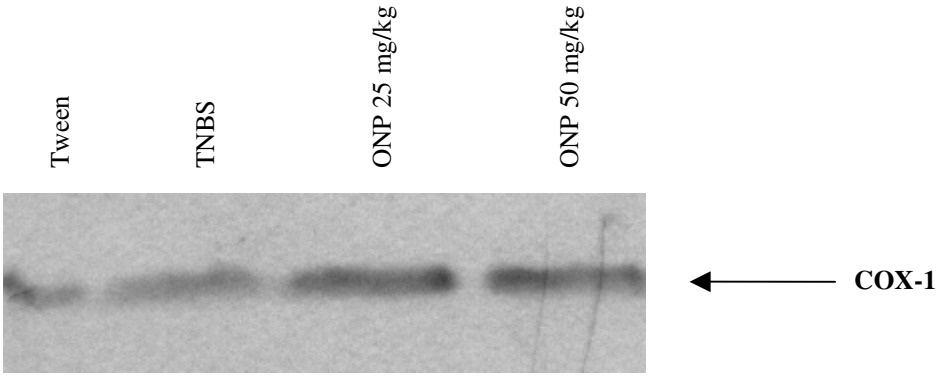
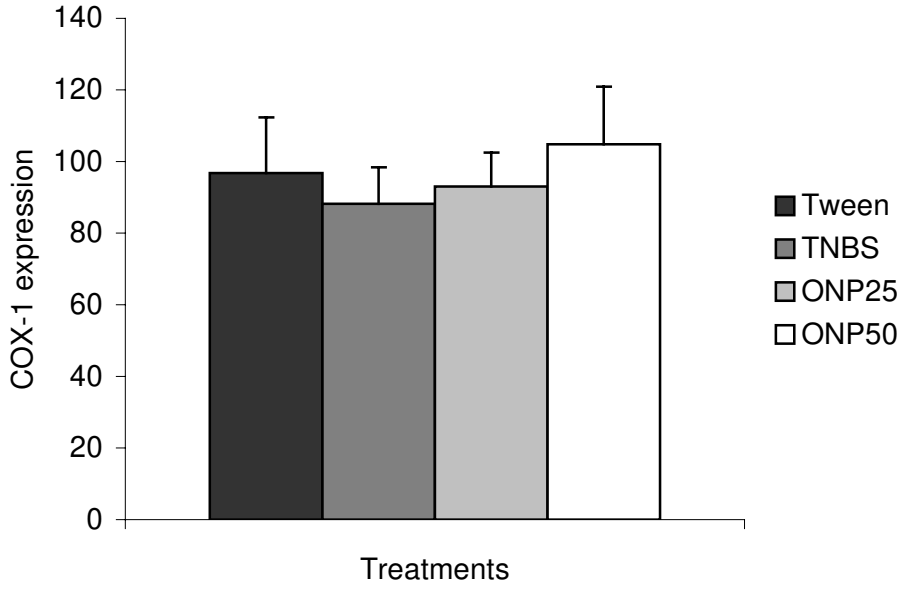


Fig 7. Immunohistochemical localization of COX-2 isoenzyme in sections of colon. Negative control (A). COX-2 expression in normal colonic mucosa (B and C). COX-2 is strongly expressed in the colon of TNBS-control rats (D). COX-2 expression did not decreased in apical epithelial cells of inflamed colon treates with onopordopicrin 25 mg/kg (E) and 50 mg/kg (F). Original magnifications 10 x.

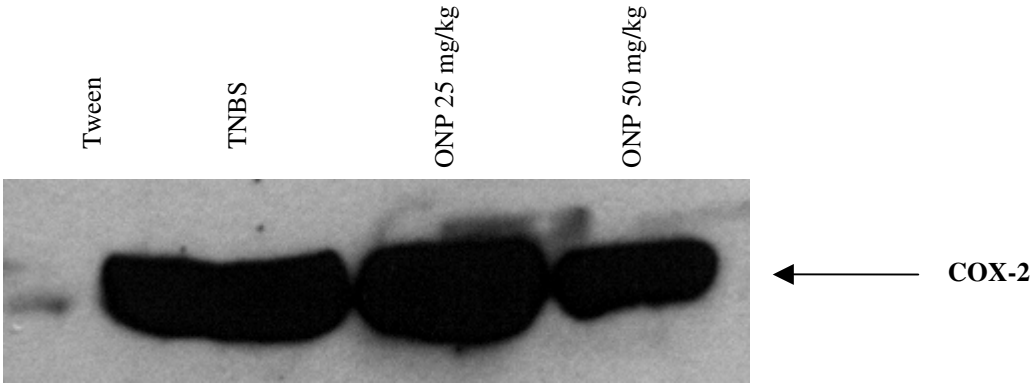


A

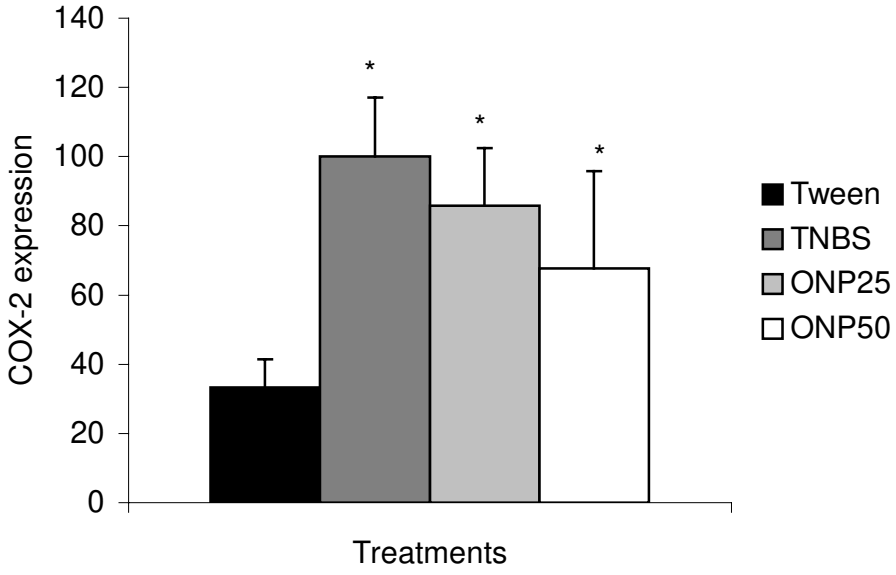


B

Fig 8. Effect of onopordopicrin on COX-1 expression in rat colon. Representative Western blotting of COX-1 (A) as well as the densitometric analysis (B) shows the effect of onpordocpicrin (25 or 50 mg/kg) on COX-1 expression evaluated in rat colon tissue after colitis induction. The results were expressed as means ± SD compared with control group, followed by Dunnett's test. *p<0.01. ANOVA: $F_{(3,428)} = 29.50$ p<0.05.



A



B

Fig 9. Effect of onopordopicrin on COX-2 expression in rat colon. Representative Western blotting of COX-2 (A) as well as the densitometric analysis (B) shows the effect of onpordocpicrin (25 or 50 mg/kg) on COX-2 expression evaluated in rat colon tissue after colitis induction. The results were expressed as means ± SD, compared with control group followed by Dunnett's test. *$p<0.01$. ANOVA: $F_{(3,389)} = 697.92$, $p<0.05$.

Table 1 Parameters quantified after administration of onopordopicrin (25 and 50 mg/kg) in rats with acute colitis induced by TNBS intracolonic instillation

Group	Body weight changes (g)	Food consumption (g/rat day)	Adhesions (score 0-2)	Diarrhoea (score 0-1)	Colon weight/colon length (mg/cm)
Tween	14±5.38	0.78±0.078	0±0	0±0	98±7
EtOH	$30\pm17.55^{+++}$	0.49±0.05	1±0.27	$0\pm0^{+}$	$130\pm10^{+}$
TNBS	$-21\pm8.19^{**}$	0.54±0.09	$6.36\pm0.93^{**}$	$1.42\pm0.78^{++}$	$280\pm90^{***}$
ONP 25	-13±4.78	$0.20\pm0.074^{***,+}$	$2.67\pm0.42^{++}$	0.83±0.75	$260\pm53^{**}$
ONP 50	$-17\pm2.64^{*}$	$0.08\pm0.02^{***,+++}$	$3.67\pm0.71^{+}$	0.85±0.69	$240\pm40^{**}$

Data are expressed as means±SEM $^{*}p<0.5$ $^{**}p<0.01$ $^{***}p<0.001$ significantly different from Tween; $^{+}p<0.05$ $^{++}p<0.01$ $^{+++}p<0.001$ significantly different from TNBS

Table 2. Mieloperoxidase activity (MPO, U/mg tissue) after onopordopicrin administration (25 and 50 mg/kg) in rats with acute colitis produced by TNBS intracolonic instillation (10 mg/kg)

Group	MPO (U/mg tissue)
Tween	0.82±0.18
Ethanol	1.63±0.53
TNBS	$1.74\pm0.03^{***}$
ONP 25 mg/kg	$0.97\pm0.09^{++}$
ONP 50 mg/kg	$1.18\pm0.08^{+}$

Data are expressed as the means±SD $^{***}p<0.001$ significantly different from Tween, $^{+}p<0.05$ $^{++}p<0.01$ significantly different from TNBS

Table 3. Tumor necrosis factor alpha levels (TNF-α, pg/mg protein) after onopordopicrin administration (25 or 50 mg/kg) in rats with acute colitis produced by TNBS intracolonic instillation (10 mg/kg)

Group	TNF-α (pg/mg protein)
Tween	0.07±0.02
TNBS	$0.69\pm0.14^{***}$
ONP 25 mg/kg	$0.12\pm0.02^{++}$
ONP 50 mg/kg	$0.26\pm0.04^{+}$

The results were expressed as means ± SD, compared with TNBS group followed by Dunnett's test. $^{***}p<0.001$ significantly different from Tween, $^{+}p<0.05$ $^{++}p<0.01$ significantly different from TNBS

Antiulcerogenic activity of tea from leaves of *Arctium lappa* L. (Asteraceae)

[*1]Ana Beatriz Albino de Almeida, [1]Leonia Maria Batista, [1]Luciana Di Pietro Magri, [2]Wagner Villegas, [2]Lourdes C. Santos, [2]Marcio R. F. Tiene, [1]Alba R. M. Souza Brito. [1]Departamento de Fisiologia e Biofísica, Instituto de Biologia-Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brazil. [2]Departamento de Química, Instituto de Química – Universidade Estadual Paulista (UNESP), Araraquara, SP, Brazil.

*Correspondence
Ana Beatriz Albino de Almeida
Departamento de Fisiologia e Biofísica – Instituto de Biologia
Universidade Estadual de Campinas – UNICAMP
CP6109
CEP 13083-970, Campinas, SP, Brasil
E-mail:anabia5@yahoo.com.br
Fone: +(55)19 37886192

Abstract

Arctium lappa (Asteraceae), commonly known as "bardana", has a diversity of therapeutics actions, includings antioxidant activity, inhibition of tumour necrosis factor and antiinflammatory and analgesic effects. Previous studies have shown that onopordopicrin (ONP), a sesquiterpene lactone isolated from the leaves of this species, has an antisecretory effect in the gastric mucosa that involve the stimulation of somatostatin release and the inhibition of gastrin secretion, as well as a, cytoprotective action that probably involves antioxidant activity. In this study, we examined the actions of other compounds presents in the tea made from the leaves of this species. The antiulcerogenic activity of tea was examined in ethanol/HCl-, piroxicam-, hypothermic restraint stress-, pylorus ligature-, N^G-nitro-L-arginine-methyl-ester and N-methylmaleimide-induced gastric ulcers in mice. At doses of 50, 100 and 200 mg/kg, the *Arctium lappa* tea reduced the damage in ethanol/HCl-induced gastric ulcers by 64, 46 and 69%, respectively, compared to the reduction of 75% seen with lanzoprazole (30 mg/kg, positive control). In hypothermic restraint stress-induced ulcers, the *Arctium lappa* tea (50 mg/kg) also significantly reduced the formation of gastric lesions (from 12.2±4.2 to 5.9±3.0; $p<0.05$). In pylorus-ligated mice, cimetidine (100 mg/kg, p.o., positive control) significantly decreased the total acid concentration, with a consequent pH increase, whereas the *A. lappa* tea had no effect on these parameters. The *Arctium lappa* tea (50 mg/kg) and cimetidine (100 mg/kg), significantly prevented the formation of gastric lesions in piroxicam-induced ulcers (4.85±3.33 and 5±3.24, respectively, compored to 11±0.81 in the control mice; $p<0.05$). The action of tea (50 mg/kg) had no effect on the enhanced gastric lesions caused by L-NAME (10 mg/kg), a nitric oxide synthase inhibitor, and *N*-methylmaleimide (10 mg/kg), a sulfhydryl group blocker, in ethanol/HCl-induced gastric lesions in mice. These results indicate that the cytoprotective action of *A. lappa* tea involves the stimulation of prostaglandin production and on antioxidant activity of components present in this tea.

Keywords: *Arctium lappa*, gastric ulcer, tea

Introduction

Arctium lappa (Asteraceae), commonly known as "bardana", has a diversity of therapeutics actions, includings antioxidant activity, inhibition of tumour necrosis factor and antiinflammatory and analgesic effects. Previous studies have shown that onopordopicrin (ONP), a sesquiterpene lactone isolated from the leaves of this species, has an antisecretory effect in the gastric mucosa thatinvolve the stimulation of somatostatin release and the inhibition of gastrin secretion, as well as a, cytoprotective action that probably involves antioxidant activity. In this study, we examined the actions of other compounds presents in the tea made from the leaves of this species. The antiulcerogenic activity of tea was examined in ethanol/HCl-, piroxicam-, hypothermic restraint stress-, pylorus ligature-, N^G-nitro-L-arginine-methyl-ester and N-methylmaleimide-induced gastric ulcers in mice.

Material and Methods

Animals

Male Swiss mice (30-35 g) obtained from the Centro Multidisciplinar e Integrado de Bioterismo (CEMIB-UNICAMP) were housed on a 12 h light/dark cycle at 22±1ºC and 55% humidity, with free access to water and a certified diet (CR-a®, Nuvital, Nuvilab). The mice were fasted for 24-36 h prior to all assays because various drugs and the *Arctium lappa* tea were administered orally (by gavage – 10 ml/kg) using a saline solution as vehicle. All of the experiments were done in accordance with the recommendations of the Canadian Council on Animal Care [19].

Preparation of tea from *Arctium lappa* leaves

One kilogram of *Arctium lappa* leaves was macerated for one week with 70% ethanol. Rotaevaporation yielded 30 g of hydroaclcoholic extract. Liquid-liquid partitioning in a chloroform:water mixture (1:1, v/v) yielded 17 g of chloroform extract and 13 g of aqueous extract. The chloroform extract was fractionated by column chromatography an silica gel and the column was eluted with a gradient of chloroform and methanol. Sixty-seven fractions (75 ml each) were collected and analysed by this layer chromatography on silica gel plates were developed with iodine vapor and anisaldehyde. Fraction three was analyzed by RMN ^{1}H and 13C and found to contain a mixture of triterpenes that were identified as ursolic acid and oleanolic acid.

Ethanol/HCl-induced ulcers

This assay done using groups of 7 mice each as described by Morimoto et al. (1991). The mice were randomly allocated into eight groups and fasted for 24 h before the experiment. Ethanol (60%)/HCl 0.1 N was orally administered to mice treated with *Arctium lappa* tea(50, 100 or 200 mg/kg) or saline 50 min previously. One hour after the administration of ethanol/HCl, the mice were killed and the stomachs were removed and opened along the greater curvature**.** The results were expressed as an ulcerative lesion index (ULI), as described by Szelenyi et al, (1978).

Pylorus ligature

Male Swiss mice were fasted for 24 h and the pylorus was ligated by the method of Shay et al (1945). The mice received saline, cimetidine (100 mg/kg) or *Arctium lappa* tea (50 mg/kg) intraduodenally. Intraduodenal treatment was used to ensure the systemic action of the test substances. Four hours later, the mice were killed, the stomachs were removed, and the contents were drained into a graduated centrifuge tube via a small incision. The volume of gastric secretion was recorded, the pH was determined and the total acid output was calculated by titrating the pH to 7.0 with 0.05 N NaOH.

Assessment of the role nitric oxide (NO) in the cytoprotection afforded by *Arctium lappa* tea

This experiment was done as described by Sikiric et al. (1997). Fasted male Swiss mice were allocated into groups. The control group received an intravenous injection of saline solution and the others, an injection of L-NAME (N^N-nitro –L- arginine methyl ester, 10 mg/kg) a nitric oxide synthase inhibitor, by the same route. After 30 min the mice received saline or *Arctium lappa* tea (50 mg/kg) orally. Fifty min later, the mice were given orally ethanol/HCl orally. The mice were killed after 1h and their stomachs were removed and opened along the greater curvature to determine the lesion index.

Ethanol-induced gastric mucosal lesions in N-methylmaleimide-pretreated mice (Matsuda et al., 1999)

The gastric mucosal lesions were induced, measured and scored as described before. To investigate the involvement of endogenous sulfhydryls in the protective effects of *Arctium lappa*, *N*-methylmaleimide (10 mg/kg) was subcutaneously injected 30 min

before the administration of tea of leaves of *Arctium lappa* (50 mg/kg). After 30 min, all animal groups received orally the respective treatment (saline or tea of leaves of *Arctium lappa*). After 50 min, the animals were orally administrated with ethanol/HCl. These animals were sacrificed 1 h later and their stomachs were removed and opened along the greater curvature. The lesion index was determined as previously indicated.

Hypothermal-restraint stress ulcers

The antiulcerogenic activity of *Arctium lappa* tea was assessed in hypothermic restraint stress-induced gastric ulcers as described by Levine (1971)**,** with some modifications. Mice were fasted for 36 h and then received an oral dose of *Arctium lappa* tea (50 mg/kg), cimetidine (100 mg/kg) or vehicle (saline, 10 ml/kg). Thirty minutes later, gastric ulceration was induced by immobilizing the mice in a closed cylindrical cage at 4°C. After 3 h, the mice were killed by cervical dislocation and the stomachs were removed and examined for ulcers.

Piroxicam-induced gastric ulcers in treated mice

Mice (7 groups, three groups) were fasted for 24 h with free access to water. Thirty minutes after the oral administration of *Arctium lappa* tea (50 mg/kg), cimetidine (100 mg/kg) or vehicle (saline, 10 ml/kg) on piroxicam (30 mg/kg) was administered subcutaneously to each group as described by Rainsford et al. (1978). The mice were killed 4 h later, and the stomachs were removed, opened, and the gastric lesions were determined.

Statistical analysis

The results were expressed as the mean ± SD. Statistical comparisions were done using one-way analysis of variance ANOVA followed by the Dunnett test, with the level of significance set at $p<0.05$. All statistical analyses were done using Statistic 5.1 software (StatSoft, Inc.).

Results and Discussion

The integrity of the gastric mucosa depends on a good equilibrium between aggressive and defensive mucosal factors (Mózsik et al., 2001). Aggressive agents may be endogenous (HCl, pepsin) or exogenous (chemicals, drugs, xenobiotics, physical stress, etc.), whereas defensive factors are predominantly endogenous and include mucus

secretion, prostaglandin generation, gastric mucosal blood flow, SH-groups, metabolic adaptation and mucosal biochemistry (Mózsik et al., 2001). As a result, gastric mucosal damage develops when the equilibrium between aggressive defensive factors (Mózsik et al., 1993).

Increased gastric acid production is not a primary cause but plays a permissive role in the gastric mucosal damage induced by ethanol (Tarnawski et al., 1983). Antiulcer agents, such as proton pump inhibitors (e.g. lanzoprazole) and histamine H_2 receptor antagonists (e.g. cimetidine), inhibit acid secretion (Kinoshita et al., 1997). When the gastric mucosal defense is compromised, exogenous noxious agents (e.g. ethanol, aspirin), together with HCl and pepsin, penetrate into the mucosa and damage the mucosal microvessels. The resulting reduction in oxygen and nutrient delivery causes the release of pro-inflammatory and vasoactive mediators (serotonin, endothelin, leukotriene, platelet activating factor) that in turn exacerbate the ischemic necrosis (Tarnawski et al., 1992).

The oral administration of ethanol/HCl to the control group produced the characteristic zonal necrotic mucosal lesions. *Arctium lappa* tea (50, 100 and 200 mg/kg) and lanzoprazole (30 mg/kgf, positive control) significantly inhibited ulcer formation in this model by 75, 64, 46 and 69% respectively (Figure 1A). The reduction seen with *Arctium lappa* tea suggested that part of the protective mechanism could involve the inhibition of gastric and secretion, such as occurs with lanzoprazole.

Stress-induced ulcers are probably caused by the release of histamine, by enhanced acid secretion and by a reduction in mucus production (Pal and Nagchaudhury, 1991). The involvement of histamine in hypothermal-restraint stress ulceration was supported by the observation that cimetidine, an H_2 receptor antagonist, completely inhibited the ulceration induced by hypothermic restraint stress (Figure 1B). Pre-treatment with *Arctium lappa* tea (50 mg/kg) significantly protected the gastric mucosa against ulcers in this model, as also observed for cimetidine when compared with the control. This finding suggested that *Arctium lappa* tea inhibited gastric mucus depletion and/or decrease acid secretion.

Based on this result, the effect of *Arctium lappa* tea (50 mg/kg) on the biochemical parameters of gastric juice obtained from pylorus-ligated mice was assessed. Cimetidine (100 mg/kg) strongly inhibited gastric acid secretion as shown by decrease in the total acid content of gastric juice. In contrast, *Arctium lappa* tea did not alter the biochemical parameters of the gastric juice (Table 1). This finding indicates that the protective action of *Arctium lappa* tea was independent of acid production.

Szabo et al. (1985) proposed that prostaglandins might prevent the increased vascular permeability in response to ethanol and maintain the vascular integrity and adequate mucosal perfusion. The ability of protaglandins to increase mucus and bicarbonate secretion may also be important (Nosál'ová et al., 1998). Non-steroidal anti-inflammatory drug (NSAID) interfere with gastric mucosal defense at pre-epithelial, epithelial, and post-epithelial levels (Scheiman, 1992). Prostaglandins regulate the secretion of mucin and surface active phospholipids by mucous cells (Atay et al., 2000), and aspirin and other NSAIDs significantly inhibit basal bicarbonate secretion from gastric and duodenal mucosa, an effect that is reversed by exogenous prostaglandin E_2 (Selling et al., 1987). *Arctium* lappa tea (50 mg/kg) significantly inhibited ulcer formation in the piroxicam-induced ulcer model (Figure 1C). This result confirmed our hypothesis that a cytoprotective mechanism was involved with the antiulcerogenic effect of *Arctium lappa* tea. Motilva et al. (1996) suggested that prostaglandins make the mucosa more resistant to damage by (a) stimulating bicarbonate and mucus production, (b) maintaining an adequate blood flow, (c) inhibiting the removal of mast cell-derived inflammatory mediators, and (d) decreasing the production of free radicals. The toxicity of NSAID is partly mediated by free radicals, decreased mitochondrial oxidation and reduced nitric oxide (NO) production. NO is important for maintaining the integrity of the epithelium became of its role in regulatory mucosal blood flow and the delivery of oxygen to epithelial cells (Atay et al., 2000). The inhibition of NO synthesis exacerbates NSAID injury, and NO donors reduce NSAID toxicity in animal models (Wallace et al., 1994).

Gastric cytoprotection involves several endogenous mediators, including prostaglandins and sulfhydryl substances in the mucosa (Szabo and Szelenyi, 1987). Pretreatment with prostaglandins at doses that do not inhibit acid secretion reduces damage to the glandular gastric mucosa of rats caused by various noxious agents, including

concentrated solutions of ethanol, acid and alkali. This phenomenon has been refered to as cytoprotection (Szabo et al., 1983). Pretreatment with *N*-methylmaleimide, a sulfhydryl-blocker, reduced the protection afforded by *Arctium lappa* tea (50 mg/kg) (Figure 2), which suggested that endogenous sulfhydryls may be involved in the protective mechanism of *Arctium lappa* tea. Szabo and Trier (1984) proposed that a major target of such protection may be the microvasculature, since cytoprotection is accompanied by the preservation of the intact blood vessels and blood flow in the gastric mucosa. Ethanol-induced gastric damage is associated with the depletion of endogenous sulfhydryls, and pretreatment with sulfhydryl-blockers prevented the gastroprotection of sulfhydryl-containing substances (Szabo and Brown, 1987). Loguercio et al. (1991) demonstrated that ethanol, at a concentration that causes gross damage to the gastric mucosa, significantly affected the tissue sulfhydryl levels in human stomach, with a decrease in the total SH, glutathione, and cysteine levels in the gastric body and antrum. Since ethanol-induced gastric mucosal damage is associated with the generation of toxic oxygen metabolites (Szelenyi et al., 1988), the decrease in nonprotein glutathione levels could reflect the oxidation of reduced glutatione (Loguercio et al., 1991).

NO is synthesized from L-arginine by three isoenzymes that are divided into two functional classes based on their sensitivity to calcium. The constitutive forms (cNOS) are expressed continuously under normal physiological conditions, and bind calmodulin in a reversible, calcium-dependent fashion (Morschl et al., 2001). The inhibition of cNOS leads to vasoconstriction, platelet aggregation, increased vascular permeability, and the adhesion of neutrophils to the vascular endothelium, all of which are processes involved in the development of the vascular endothelial dysfunction (Moncada et al., 1995; 1997). As shown here, the administration of L-NAME, a non-selective NO synthase inhibitor, prior to ethanol significantly enhanced the development of gastric mucosal lesions in normal mice. Pretreatment with L-NAME tended to aggravate ethanol-induced mucosal lesions. It had tendency to increase the length and the score, and reduced the protection afforded by *Arctium lappa* tea (50 mg/kg) (Figure 3). This finding indicates that NO may be not involved in the protective action of *Arctium lappa* tea.

MacNaughton et al (1989) reported that ethanol-induced gastric damage can be significantly reduced by NO, probably because NO has an important role in regulating

the gastric wall blood flow (Pique et al., 1989), and gastric acid and mucus secretion (Brown et al., 1993). Kubes et al (2000) suggested that the improved blood flow associated with increased NO delivery was an important factor in countering the damaging effects of HCl (Kitagawa et al., 1990), ethanol (Masuda et al., 1995) and indomethacin (Wallace et al., 1994) to the lumen. NO can also protect at subdilatory levels (Payne et al., 1993). In addition, the exacerbation of injury following NO inhibition cannot be accounted for entirely by its vasoactive properties since not all vasoconstrictors necessarily enhance mucosal damage in models of inflammation (Andrews et al., 1994).

In conclusion, the cytoprotective action of *arctium lappa* tea probably involves enhanced prostaglandin production and the presence of antioxidants in the tea.

References **(Só estão presentes algumas referências)**

Andrews FJ, Malcontenti-Wilson C, O'Brien PE (1994) Protection against gastric ischemia-reperfusion injury by nitric oxide generators. Dig Dis Sci 39: 366-373.

Atay S, Tarnawski AS, Dubois A (2000) Eicosanoids and the stomach. Prostaglandin and other lipid mediators. 61: 105-124.

Brown JF, Keates AC, Hanson PG, Whittle BJR (1993) Nitric oxide generators and cGMP stimulate mucus secretion by rat gastric mucosal cells. Am J Physiol 265: G418-G422

Kinoshita M, Saito N, Tamaki H (1997) Antisecretory and antiulcer effect of T-330, a novel reversible proton pump inhibitor, in rats. European Journal of Pharmacology 321, 325-332.

Kitagawa H, Takeda F, Kohei H (1990) Effect of endothelium-derived relaxing factor on the gastric lesion induced by HCl in rats. J Pharm Exp Ther 253: 1133-1137

Kubes P, McCafferty DM (2000) Nitric oxide and intestinal inflammation. Journal of Physiology 109: 150-158.

MacNaughton WK, Cirino G, Wallace JL (1989) Endothelium-derived relaxing factor (nitric oxide) has protective action in the stomach. Life Science 45, 1869-1876.

Masuda E, Kawani S, Nagano K (1995) Endogenous nitric oxide modulates ethanol-induced gastric mucosal injury in rats. Gastroenterology 108: 58-64.

Matsuda H, Li Y, Yoshikawa M. (1999) Gastroprotection of escins Ia, Ib, IIa, and IIb on ethanol-induced gastric mucosal lesions in rats. Eur J Pharmacol 373: 63-70.

Morimoto Y, Shimohara K, Oshima S, Sukamoto T. (1991) Effects of the new anti-ulcer agent KB-5492 on experimental gastric mucosal lesions and gastric mucosal defensive factors, as compared to those of teprenone and cimetidine. Jpn J Pharmacol 57: 495-505.

Morschl E, Pavo I, Varga G, Nemcsik J, Laszlo F, Whittle BJR (2001) Endogenous bacteria-triggered inducible nitric oxide synthase activation protects the ovariectomized rat stomach. Journal of Physiology-Paris 95: 137-140.

Mózsik G, Karádi O, Király A, Debreceni A, Figler M, Nagy L, Pár A, Pár G, Sutó G, Vincze A The key-role vagal nerve and adrenals in the cytoprotection and general gastric mucosal integrity. Journal of Physiology – Paris (2001) 95: 229-237.

Mózsik G, Káradi O, Király A, Matus Z, Sutó G, Tóth G, Vincze A. (1993) Vagal nerve and the gastric mucosal dence. Journal of Physiology (Paris) 87: 329-334.

Murakami M, Mizuno N, Saita H, Ashida Y, Inada M, Miyake T (1985) Effect of mild irritants on the gastric mucosal blood flow and potential difference in the rat. Gastroenterology 88, 1512.

Olfert ED, Cross BM, McWilliam AA. In: Guide to the Care and use of Experimental Animals. Canadian Council on Animal Care 1993. p. 1-213.

Payne D, Kubes P. (1993) Nitric oxide donors reduce the rise in reperfusion-induced intestinal mucosal permeability. Am J Physiol; 265: G189-G195.

Pique JM, Whittle, BJR, Esplugues, JV (1989) The vasodilator role of endogenous nitric oxide in the rat gastric microcirculation. Eur J Pharmacol 174: 293-296.

Scheiman JM (1992) Pathogenesis of gastroduodenal injury due to nonsteroidal anti-inflammatory drugs: implications for prevention and therapy. Semin Arthritis Rheum 21: 201-210.

Selling JA, Hogan DL, Aly A (1987) Indomethacin inhibits duodenal mucosal bicarbonate secretion and endogenous prostaglandin E_2 output in human subjects. Ann Intern Med 16:368-71.

Shay H, Komarov SA, Fels SS, Meranze D, Gruenstein M, Siplet H. (1945) A simple method for the uniform production of gastric ulceration in the rat. Gastroenterology; 5: 43-61.

Sikiric P, Seiwerth S, Grabarevic Z, Rucman R, Petek M, Jagic V, Turkovic B, Rotkvic I, Mise S, Zoricic I, Konjevoda P, Perovic D, Jurina L, Separovic J, Hanzevacki M, Artukovic B, Bratulic M, Tisljar M, Gjurasin M, Miklic P, Stancic-Rokotov D, Slobodnjak Z, Jelovac N, Marovic A (1997) The influence of a novel pentadecapeptide, BPC 157, on N^G-nitro-L-arginine methylester and L-arginine effects on stomach mucosa integrity and blood pressure. Eur J Pharmacol; 332: 23-33.

Szabo S, Brown A (1987) Prevention of ethanol-induced vascular injury and gastric mucosal lesions by sucralfate and its components: possible role of endogenous sulfhydryls. Proc Soc Exp Biol Med 185: 493-497.

Szabo S, Gallagher GT, Horner HC, Frankel PW, Underwood RH, Konturek SJ, Brzozowski T, Trier JS (1983) Role of the adrenal cortex in gastric mucosal protection by prostaglandins. Sulfhydryls, and cimetidine in the rat. Gastroenterology 85: 1384-90

Szabo S, Trier JS, Brown A, Schnoor J (1985) Early vascular injury and increased vascular permeability in gastric mucosal injury caused by ethanol in the rat. Gastroenterology 88: 228-236.

Szabo S, Szelenyi I (1987) Cytoprotection in gastrointestinal pharmacology. Trends Pharmacol.Sci. 8:149-154.

Szabo S, Trier JS (1984) Pathogenesis of acute gastric mucosal injury: sulfhydryls as a protector, adrenal cortex as a modulator, and vascular endothelium as a target. In: Mechanisms of mucosal protection in the upper gastrointestinal tract, ed. By A.Allen, G. Flemstrom, A.Garner, W. Silen and L.A.Turnberg, pp. 287-293, Raven Press, New York, 1984.

Szelenyi I, Brune K (1988) Possible role of oxygen free radicals in ethanol-induced gastric mucosal damage in rats. Dig Dis Sci 33: 865-871.

Szelenyi I, Thiemer K. (1978) Distention ulcer as a model for testing of drugs for ulcerogenic side effects. Arch Toxicol 41: 99-105.

Tarnawski A (1993) Cellular mechanisms of gastric ulcer healing. In: Domschke W, Konturek SJ, editors. The stomach. Berlin: Spring, pp. 177-92.

Tarnawski A, Arakawa T (1992) Preventing GI damage with cytoprotective drugs. Contemp Int Med 4: 95-109.

Tarnawski A, Hollander D, Gergely H, Stachura J (1983) Comparison of antacid, sucralfate, cimetidine and ranitidine in protection of gastric mucosa against ethanol injury. Gastroenterology 84: 1331.

Wallace JL, Reuter B, Cicala C (1994) Novel nonsteroidal anti-inflammatory drug derivates with markedly reduced ulcerogenic properties in the rat. Gastroenterology; 107: 173-179

Wallace JL, Reuter BK, Cirino G (1994) Nitric oxide-releasing non-steroidal anti-inflammatory drugs: a novel approach for reducing gastrointestinal toxicity. J. Gastroenterol Hepatol 9: S40-S44.

Wallace JL, Whittle, BJR (1985) Acceleration of recovery of gastric epithelial integrity by 16, 16-dimethyl prostaglandin E_2. British Journal of Pharmacology 86: 837-842.

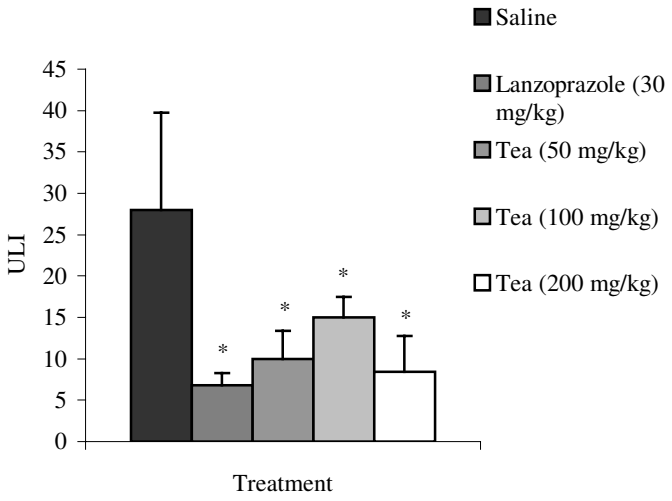
Figure legends

Figure 1. Inhibition of ethanol/HCl-induced ulcers (A), hypothermal-restraint stress-induced ulcers (B) and piroxicam-induced gastric ulcers (C) by tea from leaves of *Arctium lappa*.

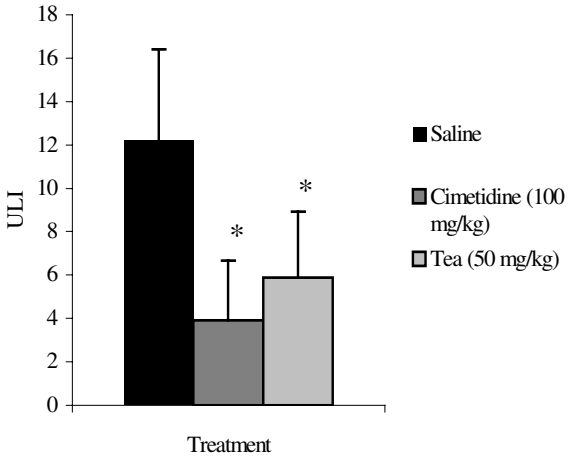
Figure 2. Role of sulfhydryl groups in the gastric protection provived by t
.ea from the leaves of *Arctium lappa*

Figure 3. Lack of involvement of NO in the gastric protection produced by tea from the leaves of *Arctium lappa*

A


Saline
Lanzoprazole (30 mg/kg)
Tea (50 mg/kg)
Tea (100 mg/kg)
Tea (200 mg/kg)
ULI
45
40
35
30
25
20
15
10
5
0
*
*
*
*
Treatment

B


Saline
Cimetidine (100 mg/kg)
Tea (50 mg/kg)
ULI
18
16
14
12
10
8
6
4
2
0
*
*
Treatment

C

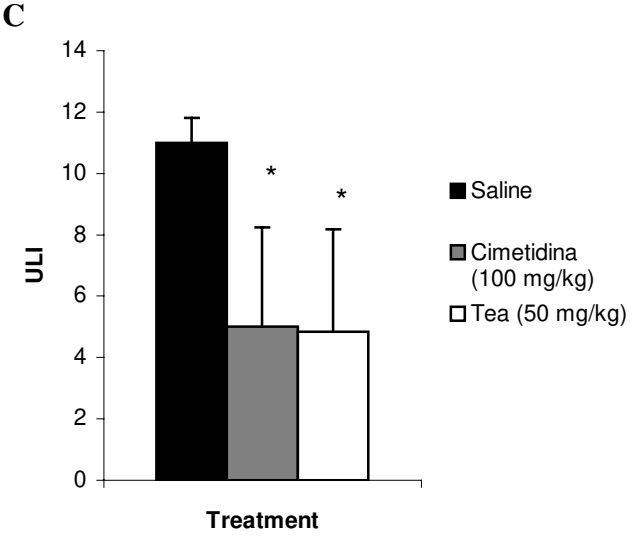

Saline
Cimetidina (100 mg/kg)
Tea (50 mg/kg)
ULI
14
12
10
8
6
4
2
0
*
*
Treatment

Fig 1

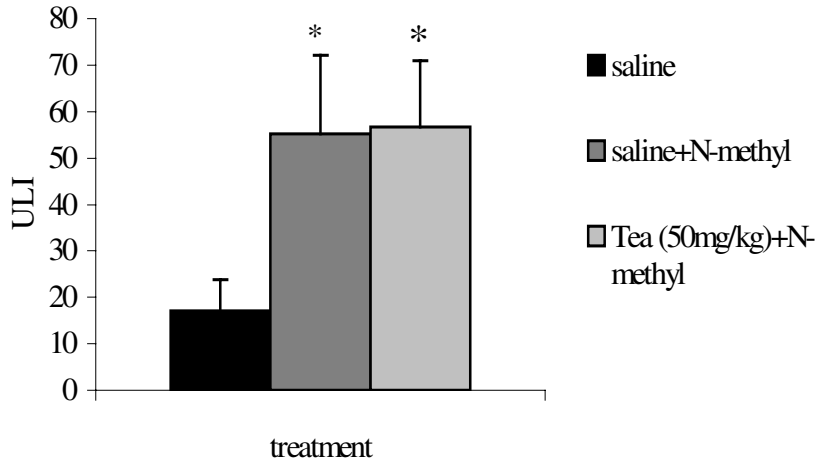

80
70
60
50
40
30
20
10
0
ULI
*
*
saline
saline+N-methyl
Tea (50mg/kg)+N-methyl
treatment

Fig 2

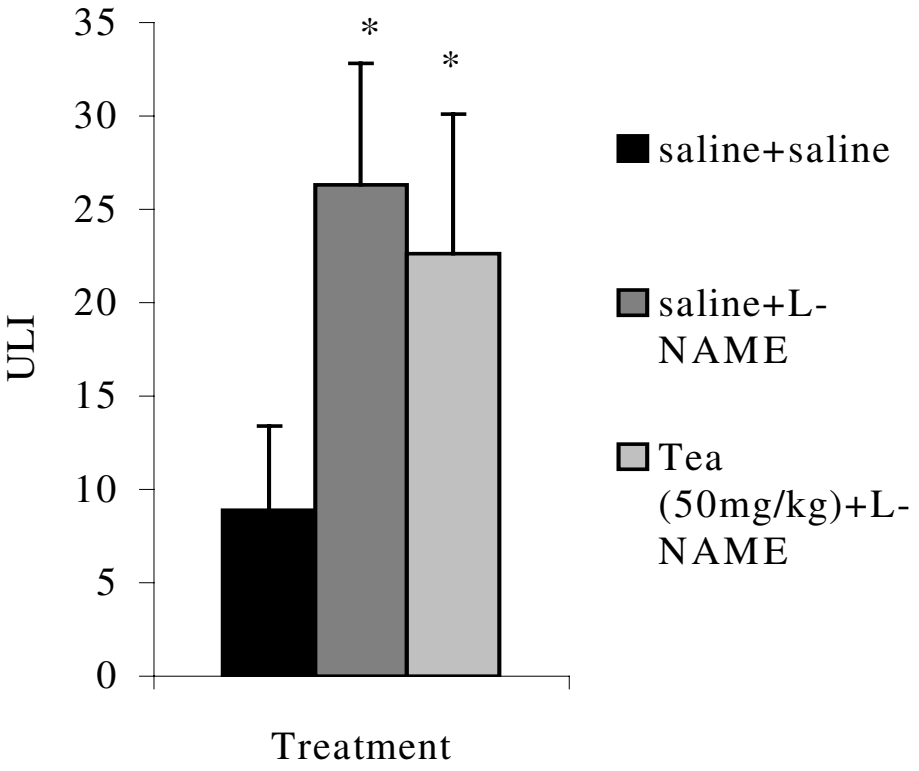

35
30
25
20
15
10
5
0
ULI
*
*
Treatment
saline+saline
saline+L-NAME
Tea (50mg/kg)+L-NAME

Fig 3

Table 1. Influence of tea from the Leaves of *Arctium lappa* on selected biochemical parameters of the gastric secretion in mice

Treatment	Dose (mg/kg)	pH	Volume (ml)	total acid production (mEq/4 h/ml)
Saline	-	2.9±0.9	0.36±0.37	5.58±2.49
Cimetidine	100	4.0±0.5*	0.15±0.07	11.7±7.76*
Tea	50	3.5±1.3	0.29±0.17	8.25±5.04

The results are expressed as the mean ±SD. ANOVA: $F_{pH\ (2,23)} = 2.49$; $F_{volume\ (2,23)} = 1.69$; $F_{[total\ acid]} = 2.63$ $p<0.05$ following Dunnett test*$p<0.05$ compared to the saline group

- Os resultados obtidos com a *Arctium lappa* são promissores, por causa de sua significativa proteção contra úlceras induzidas por diferentes agentes, sugerindo um efeito antisecretor mediado por sua ação na secreção de Somatostatina e Gastrina e um efeito protetor proporcionado pela propriedade antioxidante presente na ONP.
- Os dados também revelaram que o pré-tratamento com ONP é capaz de reduzir a inflamação intestinal produzida através do modelo de indução de colite ulcerativa por TNBS em ratos. Esse efeito agudo antiinflamatório provavelmente está relacionado com a diminuição de neutrófilos e diminuição da produção de TNF-α na mucosa intestinal.

Assim, nossos resultados sugerem um significativo potencial terapêutico da ONP na área gastrointestinal. Entretanto são necessários estudos adicionais para:

a) prosseguir na investigação de mecanismos de ação ainda não explorados
b) identificar as substâncias presentes na fração semi-purificada
c) isoladamente estudar as substâncias contidas nessa fração.

Referência Bibliográfica

Abreu MT, Yang H (2003) Defining subtypes of Crohn's disease patients: the ground work for translational research in inflammatory bowel disease. *J Clin Gastroenterol*. 36 (1), 3-4.

Adams HR (2001) Cholinergic Pharmacology: Autonomic Drugs In Veterinary Pharmacology and Therapetics. HR Adams (ed.) Iowa State University press, Ames pp. 117-136.

Agoff SN, Brentnall TA, Crispin DA, Taylor SL, Raaka S, Haggitt RC, Reed MW, Afonina IA, Rabinovitch PS, Stevens AC, Feng Z, Bronner MP (2000) The role of cyclo-oxygenase 2 in ulcerative colitis-associated neoplasia. *Am. J. Pathol.* 157 (2000), pp. 737–745.

Alarcón de la Lastra C, Martín MJ, Motilva V (1994) Antiulcer and gastroprotective effect of quercetin: a gross and histologic study. *Pharmacology* 48, 56–62.

Albino de Almeida AB, Melo PS, Hiruma-Lima CA, Gracioso JS, Carli L, Nunes DS, Haun M, Souza Brito AR. (2003) Antiulcerogenic effect and cytotoxic activity of semi-synthetic crotonin obtained from Croton cajucara Benth. *Eur J Pharmacol*. 11;472(3):205-12

Anazetti, M.C.; Melo, P.S.; Durán, N.; Haun, M. (2003) Comparative cytotoxicity of dimethylamide-crotonin in the promyelocytic leukemia cell line (HL60) and human peripheral blood mononuclear cells. *Toxicology*. 188: 261-274.

Andrews FJ, Malcontenti-Wilson C, O'Brien PE (1994) Protection against gastric ischemia-reperfusion injury by nitric oxide generators. Dig Dis Sci 39: 366-373.

Arimura A, Lundqvist G (1978). Radioimmunoassay of somatostatin. *Metabolism.* 27, 1139-1144.

Atay S, Tarnawski AS, Dubois A (2000) Eicosanoids and the stomach. Prostaglandin and other lipid mediators. 61: 105-124.

Athmann C, Zeng N, Scott DR, Sachs G (2000) Regulation of parietal cell calcium signaling in gastric glands. *Am. J. Physiol.* 279, G1048–G1058

Babbs CF. (1992) Oxygen radicals in ulcerative colitis. *Free Radic Biol Med.* 13(2), 169-81. Review.

Ballinger A, Smith G (2001) COX-2 inhibitors vs. NSAIDs in gastrointestinal damage and prevention *Expert Opin Pharmacother*. 2(1):31-40

Barbosa Filho JM, Costa M, Gomes C, Trolin G (1993) Isolation of Onopordopicrin, the toxic constituent of *Arctium lappa* L. *J Braz Chem Soc* 4, 186-7

Barocelli E, Ballabeni V (2003) Histamine in the control of gastric acid secretion: a topic review. *Pharmacol Res*. 47(4):299-304

Beutler, J.A.; McCall, K.L.; Herbert, K.; Johnson, T.; Shoemaker, R.H.; Bouyd, M.R. (2000) Cytotoxic clerodane diterpene ester from Laetia corymbulosa. Phytochemistry. 55: 233-236.

Bianchi V, Fortunat E (1990) Cellular effects of an anionic surfactant detected in (V79) fibroblasts by different cytotoxicity tests. *Toxicol. In Vitro* 4, 9–16.

Blake, D.R.; Allen, R.E.; Lunee, J. (1987) Free Radicals in biological systems – A review oriented to inflammatory process. *Br. Med. Bull*. 43: 371-385.

Bobin-Dubigeon C, Collin X, Grimaud N, Robert JM, Le Baut G, Petit JY (2001) Effects of tumor necrosis factor-α synthesis inhibitors on rat trinitrobenzene sulphonic acid-induced chronic colitis. *Eur. J. Pharmacol.* 431, 103–110

Bolton, J.P.; Palmer, D.; Cohen, M.M. (1978) Stimulation of mucus and nonparietal cell secretion by the E_2 Prostaglandins. Digestive Disease. 23(4): 359-364.

Borefreund, E Puerner, JA (1984) A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR90). *J. Tissue Cult. Methods* 9, 7–9.

Borrelli F, Izzo AA (2000) The plant kingdom as a source of anti-ulcer remedies. Phytother Res 14: 581-591

Boyd SC, Sasame HA, Boyd MR (1981) Gastric glutathione depletion and acute ulcerogenesis by diethylmaleate given subcutaneously to rats. *Life Sci*. 29, 2987-2992.

Brady, H.J.M. (2003) Apoptosis and leukemia. British J. Haematol. 123: 577-585.

Brown JF, Keates AC, Hanson PG, Whittle BJR (1993) Nitric oxide generators and cGMP stimulate mucus secretion by rat gastric mucosal cells. Am J Physiol 265: G418-G422

Brzozowski T (2003) Experimental production of peptic ulcer, gastric damage and cancer models and their use in pathophysiological studies and pharmacological treatment - Polish achievements. *J Physiol Pharmacol*. 3, 99-126

Brzozowski T, Konturek PC, Konturek SJ (2000) Gastroprotective and ulcer healing effects of nitric oxide-releasing non-steroidal anti-inflammatory drugs. *Dig Liver Dis* 32, 583-594.

Brzozowski T, Konturek PC, Konturek SJ (2001) Classic NSAID and selective cyclooxygenase (COX)-1 and COX-2 inhibitors in healing of chronic gastric ulcers. *Microsc Res Tech* 53:343-353.

Buntzen S, Nordgren S, Hulten L, Delbro D (1996) The role of nitric oxide in the acetylcholine-induced relaxation of the feline internal anal sphincter, in vitro. *Scand J Gastroenterol* 31, 1189–94.

Calam J, Baron JH (2001) ABC of the upper gastrointestinal tract Pathophysiology of duodenal and gastric ulcer and gastric cancer. *BMJ* 323, 980-982

Carty E, De Brabander M, Feakins RM, Rampton DS (2000) Measurement of in vivo rectal mucosal cytokine and eicosanoid production in ulcerative colitis using filter paper. *Gut* 46, 487–492.

Cavill D, Waterman SA, Gordon TP (xxxx) Antibodies Raised Against the Second Extracellular Loop of the Human Muscarinic M3 Receptor Mimic Functional Autoantibodies in Sjo¨gren's Syndrome *Scand J Immun* 59, 261–266.

Chan FK, Sung JJ (2001) Role of acid suppressants in prophylaxis of NSAID damage *Best Pract Res Clin Gastroenterol* 15(3): 433-45

characterization of novel type of histamine receptor preferentially expressed in leukocytes. *J Biol Chem* 275: 36781-36786.

Chavez, RR (1996) Gastric acid. *Rev. Gastroenterol. Peru*, 16(3): 249-253.

Christoph A, Ningxin Z, Scott DR, Sachs G (2000) Regulation of parietal cell calcium signaling in gastric glands *Am J Physiol Gastrointest Liver Physiol* 279, G1048-G1058.

Chun Yu Wong, B. , Ping Wang, W. , Hau Leung So, W. , Shin, V. Y. , Man Wong, W. , Man Yee Fung, F. , Shiu Lam Liu, E. , Mo Hiu, W. , Kum Lam, S. & Hin Cho, C. Epidermal growth factor and its receptor in chronic active gastritis and gastroduodenal

ulcer before and after *Helicobacter pylori* eradication . *Alimentary Pharmacology & Therapeutics* 15 (9), 1459-1465.

Cingi MR, De Angelis I, Fortunati E, Reggiani D, Bianchi V, Tiozzo R, Zucco F (1991), Choice and standardization of test protocols in cytotoxicology—a multicenter approach. *Toxicol. In Vitro* 5, 119–125

Cipolla G, Crema F, Sacco S, Moro E, de Ponti F, Frigo G. (2002) Nonsteroidal anti-inflammatory drugs and inflammatory bowel disease: current perspectives. *Pharmacol Res.* 46(1):1-6. Review.

Coleman RA, Smith WL, Narumiya S (1994) Classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacological Review* 46, 205–229.

Collins, RA; Ng, TB; Fong, WP; Wan, CC; Yeung, HW (1997) A comparison of human immunodeficiency virus type 1 inhibition by partially purified aqueous extracts of Chinese medicinal herbs. *Life Science* Including Pharmacology Letters, 60 (23): PL345-PL351.

Corne, S.J., Morrisey, S.; Woods, R.J. (1974) A method for the quantitative estimation of gastric barrier mucus. *J. Physiol.* 242, 116P-117P.

Cuzzocrea S, Mazzon E, Serraino I, Dugo L, Centorrino T, Ciccolo A, Sautebin L, Caputi AP (2001) Celecoxib, a selective cyclo-oxygenase-2 inhibitor reduces the severity of experimental colitis induced by dinitrobenzene sulphonic acid in rats. *Eur. J. Pharmacol.* 431, 91–102

Das D, Bandyopadhyay D, Bhattacharjee M, Banerjee RK (1997) Hydroxyl radical is the major causative factor in stress-induced gastric ulceration. 23 (1), 8-18.

De Ponti F, Tonini M.(2001) Irritable bowel syndrome: new agents targeting serotonin receptor subtypes. *Drugs*. 61(3), 317-32. Review.

Denizot F, Lang, R (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods* 89, 271–277

Diel F, Szabo S (1986). Dose-dependent effects of linear and cyclic somatostatin in ethanol-induced gastric erosions: the role of mast cells and increased vascular permeability in the rat. *Regul. Pept.* 13, 235–243.

Dimaline R, Evans D, Varro A, Dockray GJ (1991). Reversal by omeprazole of the depression of gastrin cell function by fasting in the rat. *J. Physiol.* 433, 483-493.

D'Odorico A, Bortolan S, Cardin R, D'Inca' R, Martines D, Ferronato A, Sturniolo GC. (2001) Reduced plasma antioxidant concentrations and increased oxidative DNA damage in inflammatory bowel disease. *Scand J Gastroenterol.* 2001 36 (12),1289-94.

Eglen RM, Choppin A, Watson N (2001) Therapeutic opportunities from muscarinic receptor research. *TINS* 22, 409-414.

Ekwall, B. Ekwall, K (1988) Comments on the use of diverse cell systems in toxicity testing. *ATLA* 15, 193–200.

Friis-Hansen L, Sundler YL, Gillespie PJ, Saunders TL, Greeson T (1998) Impaired gastric acid secretion in gastrin-deficient mice. *Am. J. Physiol*. 274, G561-G568.

Fukushima Y, Ishikawa T, Saitoh T, Tateishi K, Ogihara T, Fujishiro M, Shojima N, Honda M, Kushiyama A, Anai M, Sakoda H, Ono H, Onishi Y, Otsuka H, Katagiri H, Nagai R, Omata M, Asano T. (2003) Extremely early onset of ranitidine action on human histamine H2 receptors expressed in HEK293 cells. *Digestion.* 68(2-3),145-52

Fukushima Y, Otsuka H, Ishikawa M, Asano T, Anai M, Katsube T, Ogawa K, Kajiwara T, Ohkawa S, Ishikawa T, Omata M, Saitoh T. (2001) Potent and long-lasting action of lafutidine on the human histamine H(2) receptor.*Digestion.* 64(3), 155-60.

Giordano OS, Guerreiro E, Pestchanker MJ, Guzman J, Pastor D, Guardia T.(1990) The gastric cytoprotective effect of several sesquiterpene lactones.*J Nat Prod.* 53(4), 803-9.

Goa, K.L.; Monk, J.P. (1987) Emprostil: A preliminary review of its pharmacodynamic and pharmacokinetic properties and therapeutic efficacy in the treatment of peptic ulcer disease. *Drugs* 3, 539-559.

Gretzer B, Ehrlich K, Maricic N, Lambrecht N, Respondek M, Peskar BM (1998) Selective cyclooxygenase-2 inhibitors and their influence on the protective effect of a mild irritant in the rat stomach. *Br J Pharmacol* 123, 927-935.

Grisham MB, Beniot JN, Granger DN (1990) Assessment of leucocyte in involvement during ischemia and reperfusion on the intestine. In: L. Packer and A.E. Glazer,

Editors, *Methods in Enzymology. Oxygen Radicals in Biological Systems*, Academic Press, San Diego, 729–741

Grisham MB, Specian RD, Zimmerman TE.(1994) Effects of nitric oxide synthase inhibition on the pathophysiology observed in a model of chronic granulomatous colitis.*J Pharmacol Exp Ther*. 271(2), 1114-21.

Halliwell, B., Gutteridge, J.M.C. (1990) Role of free radicals and catalytic metal ions in human disease: Na overview. *Methods Enzymol*. 186, 1-85.

Harwkey CJ (2000) Nonsteroidal Anti-inflammatory Drug Gastropathy. *Gastroenterology* 119, 521-535.

Hase S, Yokota A, Nakagiri A, Takeuchi K (2003) Prostaglandin E2 aggravates gastric mucosal injury induced by histamine in rats through EP1 receptors *Life Sci.* 74(5), 629-41.

Hawkey CJ, Rampton DS. (1985) Prostaglandins and the gastrointestinal mucosa: are they important in its function, disease, or treatment? *Gastroenterology* 89, 1162–1188,

Hinkle KL, Bane GC, Jazayeri A, Samuelson LC (2003) Enhanced calcium signaling and acid secretion in parietal cells isolated from gastrin-deficient mice. *Am J Physiol Gastrointest Liver Physiol*. 284(1), G145-53.

Hirota, M.; Inoue, M.; Ando, Y.; Morivo, Y (1990) Inhibition of stress-induced gastric mucosal injury by a long acting superoxide dismutase that circulates bound to albumin. *Arch. Biochem. Biophys*. 280, 269-273.

Hiruma-Lima CA, Spadari-Bratfisch RC, Grassi-Kassisse DM, Brito AR. (1999) Antiulcerogenic mechanisms of dehydrocrotonin, a diterpene lactone obtained from Croton cajucara .*Planta Med*. 65(4), 325-30.

Hiruma-Lima CA, Toma W, Gracioso Jde S, de Almeida AB, Batista LM, Magri L, de Paula AC, Soares FR, Nunes DS, Souza Brito AR. (2002) Natural trans-crotonin: the antiulcerogenic effect of another diterpene isolated from the bark of Croton cajucara Benth. *Biol Pharm Bull.* 25(4), 452-6

Hiruma-Lima, C.A., Gracioso, J.S., Toma, W., De Paula, A.C.B., Almeida, A.B., Brasil, D.S.B., Muller, A.H. & Souza Brito, A.R.M. (2000). Evaluation of the gastroprotective

activity of cordatin, a diterpene isolated from *Aparisthmium cordatum* (Euphorbiaceae). *Biol.Pharm. Bull*., 23(12), 1465-1469.

Hiruma-Lima CA, Gracioso JS, Toma W, Paula AC, Almeida ABA, Brasil DD, Muller AH, Souza Brito ARM (2001). Gastroprotective effect of aparisthman, a diterpene isolated from *Aparisthmium cordatum*, on experimental gastric ulcer models in rats and mice. *Phytomedicine* 8, 94-100.

Hough LB (2001) Genomics meets histamine receptors: new subtypes, new receptors. *Mol Pharmacol* 59, 415-419.

Wallace JL, Ma L.(2001) Inflammatory mediators in gastrointestinal defense and injury *Exp Biol Med* (Maywood). 226(11), 1003-15. Review

Itoh M (1985). Role of oxygen-derived free radicals in hemorrhagic shock-induced gastric lesions in the rat. *Gastroenterology* 88, 1162-1167.

Iwakami S, Wu JB, Ebizuka Y, Sankawa U (1992) Platelet activating factor 8 (PAF) antagonists contained in medicinal plants: lignans and sesquiterpenes. *Chem. Pharm. Bull.* 40, 1196-1198.

Iwakami S, Wu JB, Ebizuka Y, Sankawa U (1992). Platelet activating factor 8 (PAF) antagonists contained in medicinal plants: lignans and sesquiterpenes. *Chem. Pharm. Bull.* 40, 1196-1198.

Kankuri E, Asmawi MZ, Korpela R, Vapaatalo H, Moilanen E. (1999) Induction of iNOS in a rat model of acute colitis. *Inflammation.* 23(2), 141-52.

Kankuri E, Vaali K, Korpela R, Paakkari I, Vapaatalo H, Moilanen E (2001) Effects of a COX-2 preferential agent nimesulide on TNBS-induced acute inflammation in the gut. *Inflammation* 25, 301–309.

Kanwar S, Wallace JL, Befus D, Kubes P (1994) Nitric oxide synthesis inhibition increases epithelial permeability via mast cells. *Am J Physiol* 266, G222–G229

Karmeli F, Cohen P, Rachmilewitz D (2000) Cyclo-oxygenase-2 inhibitors ameliorate the severity of experimental colitis in rats. *Eur. J. Gastroenterol. Hepatol.* 12, 223–231

Karmeli F, Eliakim R, Okon E, Samuni A, Rachmilewitz D. (1995) A stable nitroxide radical effectively decreases mucosal damage in experimental colitis. Gut. 37(3), 386-93.

Kinoshita M, Saito N, Tamaki H (1997) Antisecretory and antiulcer effect of T-330, a novel reversible proton pump inhibitor, in rats. *European Journal of Pharmacology* 321, 325-332.

Kitagawa H, Takeda F, Kohei H (1990) Effect of endothelium-derived relaxing factor on the gastric lesion induced by HCl in rats. *J Pharm Exp Ther* 253, 1133-1137

Komasaka M, Horie S, Watanabe K, Murayama T (2002) Antisecretory effect of somatostatin on gastric acid via inhibition of histamine release in isolated mouse stomach. *Eur J Pharmacol*. 452(2):235-43.

Konturek C, Bielanski W, Konturek SJ, Hahn EG (1999) *Helicobacter pylori* associated gastric pathology. *J. Physiol. Pharmacol.* 50, 695–710

Kortezova NI, Shikova LI, Milusheva EA, Itzev DE, Bagaev VA, Mizhorkova ZN (2004) Muscarinic modulation of nitrergic neurotransmission in guinea-pig gastric fundus *Neurogastroenterol Motil* 16, 155–165

Krous S (1980). Research applications. In: *The Laboratory Rat*. Ed. Baker HJ, Hussel J & Weisbroth SH. pp. 2-28. New York: Academic Press.

Kruidenier L, Verspaget HW (2002) Oxidative stress as a pathogenic factor in inflammatory bowel disease—radicals or ridiculous?. *Aliment. Pharmacol. Ther.* 16, 1997–2015.

Kubes P, McCafferty DM (2000) Nitric oxide and intestinal inflammation. *Am J Med*. 109(2): 150-8.

Kunkel SL, Spengler M, May MA, Spengler R, Larrick J, Remick D. Prostaglandin E_2 regulates macrophage-derived tumor necrosis factor gene expression. J Biol Chem 263:5380–5384, 1988

Lacy ER, Ito S (1982) Microscopic analysis of ethanol damge to rat gastric mucosa after treatment with a prostaglandin. Gastroenterology 83, 619-625.

Levi S., Goodlad RA, Lee CY (1990) Inhibitory effect of nonsteroidal anti-inflammatory drugs on mucosal cell proliferation associated with gastric ulcer healing *Lancet* 2, 840-3.

Levine RJ (1971) A method for rapid production of stress ulcer in rats. In: C.J. Pfeiffer, Editor, *Peptic Ulcer*, Munksgaard, Copenhagen 92–97.

Lewin MJ, Bado A (1991) Receptor regulating acid secretion. *Scand. J. Gastroenterol*., 180, 53-57.

Lewis DA, Hanson PJ (1991) Anti-Ulcer Drugs of Plant Origin. In: Ellis GP, West GB *Prog Med Chemistry* 28, 201-231

Lin CC, Lu JM, Yang JJ, Chuang SC, Ujie T (1996) Anti-inflammatory and radical scavenging effects of *Arctium lappa*. *Am. J. Chin. Med.* 24, 127-137.

Lin SC, Lin CH, Lin YH, Chen CF, Chen IC, Wang LY (2002). Hepatoprotective effects of *Arctium lappa* Linneu on liver injury induced by chronic ethanol consumption and potentiated by carbon tetrachloride. *J. Biomed. Sci.* 9, 401-409.

Lindstrom E, Hakanson R (1998) Prostaglandins inhibit secretion of histamine and pancreastatin from isolated rat stomach ECL cells. *Br. J. Pharmacol.* 124, 1307–1313.

Liu, F; Ng, TB (2000) Antioxidative and Free Radical Scavenging Activities of Selectes Medicinal Herbs. Life Sciences Including Pharmacology Letters. 66(8): 725-735.

Lucey MR, Yamada T (1989). Biochemistry and physiology of gastrointestinal somatostatin. *Dig. Dis. Sci*. 34, 5S–13S.

MacNaughton WK, Cirino G, Wallace JL (1989) Endothelium-derived relaxing factor (nitric oxide) has protective action in the stomach. *Life Science* 45, 1869-1876.

Maity, S.; Vedasiromoni, J.R.; Ganguly, D.K. (1995) Anti-ulcer effect of the hot water extract of black tea (*Camellia sinensis*). *Jounal Ethnopharmacology* 46, 167-174.

Makhlouf GM, Schubert ML (1990). Gastric somatostatin: a paracrine regulator of acid secretion. *Metabolism*. 39, 138-142.

Muscará MN, Wallace JL (1999) *Am J Physiol Gastrointest Liver Physiol* 276: G1313-G1316

Maricic, N.; Ehrlich, K.; Respondek, M.; Peskar, B.M. (1998) Rat gastric ischemia/ reperfusion injury: role of nitric oxide and relation to COX-1 and COX-2. *Gastroenterology* 114, A216

Martín AR, Villegas I, La Casa C, Alarcón de la Lastra C (2003) The cyclo-oxygenase-2 inhibitor, rofecoxib, attenuates mucosal damage due to colitis induced by trinitrobenzene sulphonic acid in rats *European Journal of Pharmacology* 481(2-3),

281-291.

Martin MJ, Jimenez MD, Motilva V.(2001) New issues about nitric oxide and its effects on the gastrointestinal tract. *Curr Pharm Des* 7(10), 881-908

Masuda E, Kawani S, Nagano K (1995) Endogenous nitric oxide modulates ethanol-induced gastric mucosal injury in rats. Gastroenterology 108: 58-64.

Matsuda H, Li Y, Yoshikawa M. (1999) Gastroprotection of escins Ia, Ib, IIa, and IIb on ethanol-induced gastric mucosal lesions in rats. Eur J Pharmacol 373: 63-70.

Miller M J S, Grisham MB (1995) Nitric oxide as a mediator of inflammation? You had better believe it! *Med. Inflamm.* 4: 387-396.

Minamo JF, Serrano JS, Pascual J, Sancibrian M (1987) Effects of GABA on gastric acid secretion and ulcer formation in rats.

Morimoto K, Sugimoto Y, Katsuyama M, Oida H, Tsuboi K, Kishi K, Kinoshita Y, Negishi M, Chiba T, Narumiya S, Ichikawa A (1997) Cellular localization of mRNAs for prostaglandin E receptor subtypes in mouse gastrointestinal tract. *American Journal of Physiology* 272, G681–G687

Morimoto Y, Shimohara K, Oshima S, Sukamoto T. (1991) Effects of the new anti-ulcer agent KB-5492 on experimental gastric mucosal lesions and gastric mucosal defensive factors, as compared to those of teprenone and cimetidine. Jpn J Pharmacol 57, 495-505.

Morris GP, Beck PL, Herridge MS, Depew WT, Szewczuk MR, Wallace JL (1989) Hapten-induced model of chronic inflammation and ulceration in the rat colon. *Gastroenterology* 96, 795–803

Morschl E, Pavo I, Varga G, Nemcsik J, Laszlo F, Whittle BJR (2001) Endogenous bacteria-triggered inducible nitric oxide synthase activation protects the ovariectomized rat stomach. Journal of Physiology-Paris 95: 137-140.

Motilva V, López A, Martín MJ, LaCasa C, Alacón de la Lastra C (1996) Cytoprotective activity of cisapride on experimental gastric mucosal lesions induced by ethanol. Role of endogenous prostaglandins. *Prostaglandins* 52, 63–74

Mózsik G, Karádi O, Király A, Debreceni A, Figler M, Nagy L, Pár A, Pár G, Sutó G, Vincze (2001) A The key-role vagal nerve and adrenals in the cytoprotection and general gastric mucosal integrity. *J Physiol – Paris* 95, 229-237.

Mózsik G, Káradi O, Király A, Matus Z, Sutó G, Tóth G, Vincze A. (1993) Vagal nerve and the gastric mucosal dence. Journal of Physiology (Paris) 87: 329-334.

Mózsik, G.; Király, Á; Suto, G.; Vincze, A. (1992) ATP breakdwn and resynthesis in the development of gastrointestinal mucosal damage and its prevention in animals and human. *Acta Physiol.* Hung. 80, 39-80.

Murakami, M.; Mizuno, N.; Saita, H.; Ashida, Y.; Inada, M.; Miyake, T. (1985) Effect of mild irritants on the gastric mucosal blood flow and potential difference in the rat. Gastroenterology 88, 1512.

Neuropeptides and gastric mucosal homeostasis.Gyires K. *Curr Top Med Chem.* 2004;4(1):63-73.

Nitta M, Hirata I, Toshina K, Murano M, Maemura K, Hamamoto N, Sasaki S, Yamauchi H, Katsu K (2002) Expression of the EP4 prostaglandin E2 receptor subtype with rat dextran sodium sulphate colitis: colitis suppression by a selective agonist, ONO-AE1-329. *Scand. J. Immunol.* 56, 66–75

Obermeier F, Kojouharoff G, Hans W, Scholmerich J, Gross V, Falk W. (1999) Interferon-gamma (IFN-gamma)- and tumour necrosis factor (TNF)-induced nitric oxide as toxic effector molecule in chronic dextran sulphate sodium (DSS)-induced colitis in mice. *Clin Exp Immunol.* 116(2), 238-45.

Ohno, T.; Ohtsuki, H.; Okabe, H. (1985) Effects of 16,16-dimethyl prostaglandin E_2 on ethanol-induced and aspirin-induced gastric damage in the rat. Scanning electron microscopic astudy. *Gastroenterology* 88, 353-361.

Olfert ED, Cross BM, McWilliam AA. In: Guide to the Care and use of Experimental Animals. Canadian Council on Animal Care 1993, 1-213.

Pailard, F.; Finot, F.; Mouche, I. Prenez, A.; Vericat, J.A. (1999) Use of primary cultures of rat hepatocytes to predict toxicity in the early development of new chemical entities. *Toxicol. In Vitro* 13: 693-700.

Paimela H, Goddard PJ, Silen W. Present views on restitution of gastrointestinal epithelium. Dig Dis Sci 40:2495–2496, 1995.[

Pal S, Nagchaudhury AK (1991) Studies on the anti-ulcer activity of a *Biyophyllum pinnatum* leaf extract in experimental animals. *J. Ethnopharmacol.* 33, 97–102

Payne D, Kubes P. (1993) Nitric oxide donors reduce the rise in reperfusion-induced intestinal mucosal permeability. Am J Physiol; 265: G189-G195.

Peleg II, Wilcox CM (2002) Role of eicosanoids, cyclooxygenases, and nonsteroidal antiinflammatory drugs in colorectal tumorigenesis and chemoprevention. *J. Clin. Gastroenterol.* 34, 117–125

Pio Correa, M. (1984). Dicionário da Plantas Úteis do Brasil e das Exóticas Cultivadas. Imprensa Nacional, Vol. IV, 581.

Pique JM, Whittle, BJR, Esplugues, JV (1989) The vasodilator role of endogenous nitric oxide in the rat gastric microcirculation. *Eur J Pharmacol* 174: 293-296.

Pohle T, Domschke W (2003) Gastric function measurements in drug development *Br J Clin Pharmacol*. 56(2):156-64.

Prinz C, Sachs G, Walsh JH, Coy DH, Wu SV (1994) The somatostatin receptor subtype on rat enterochromaffin-like cells. *Gastroenterology* 107, 1067–1074

Prinz C, Zanner R, Gratzl M (2003) Physiology of Gastric Enterochromaffin-Like Cells. *Annu. Ver. Physiol.* 65, 371-382.

Rainsford, KD (1978) Inhibition by leukotriene inhibitors, and calcium and platelet-activating factor antagonists, of acute gastric and intestinal damage in arthritic rats and in cholinomimetic-treated mice. *J. Pharm. Pharmacol.* 51, 331–339.

Rang HP, Dale MM, Ritter JM (1999) Pharmacology. Churchill Lingstone Edinburgh 1999

Robert, A.; Lancaster, C.; Davis, J.P. Field, S.O.; Wiekrema Sinha, A.J.; Thornurgh, B.A. (1985) Cytoprotection by prostaglandin occurs in spite of penetration of absolute ethanol into the gastric mucosa. Gastroenterology 88, 328-333.

Robert A (1979). Cytoprotection by prostaglandins. *Gastroenterology*. 77, 761-767.

Rodriguez, JA, Haun, M (1999) Cytotoxicity of *trans*-dehydrocrotonin from *Croton cajucara* on V79 cells and rat hepatocytes. *Planta Med.* 65, 1–5.

Romanski KW (2003) The rebound excitation triggered by anticholinergic drugs from ovine pyloric antrum, small bowel and gallbladder. *J Physiol Pharmacol*. 54(1):121-33.

Rubanyi GM, Ho EH, Cantor EH (1991). Cytoprotective function of nitric oxide: inactivation of superoxide radicals produced by human leukocytes. *Biochem. Biophys. Res. Commun.* 181, 1392-1397.

Sachs, J. (1997) *Pharmacotherapy* 17, 22-37

Santucci L, Fiorucci S, Giansanti M, Brunori PM, DiMatteo FN, Morelli A. Pentoxifylline prevents indomethacin-induced acute mucosal damage in rats: role of tumour necrosis factor-α. Gut 35:909–915, 1994

Sarosiek, J.; Mizuta, K.; Slomiany, A. (1986) Effects of acetylsalicylic acid on gastric mucin viscosity permeability to hydrogen íon, and susceptibility to pepsin. *Biochem Pharmacol* 35, 4291-5

Scheiman JM (1992) Pathogenesis of gastroduodenal injury due to nonsteroidal anti-inflammatory drugs: implications for prevention and therapy. *Semin Arthritis Rheum* 21, 201-210.

Scheiman, J.M. (1996) NSAIDs, gastrointestinal injury, and cytoprotection. Gastroenterology Clin. North Am. 25, 279-298.

Selling JA, Hogan DL, Aly A (1987) Indomethacin inhibits duodenal mucosal bicarbonate secretion and endogenous prostaglandin E_2 output in human subjects. *Ann Intern Med* 16, 368-71.

Shay H, Komarov SA, Fels SS, Meranze D, Gruenstein M, Siplet H. (1945) A simple method for the uniform production of gastric ulceration in the rat. Gastroenterology; 5: 43-61.

Shrivastava R, John GW, Rispat G, Chevalier A, Massingham R (1991) Can the in vivo maximum tolerated dose be predicted using in vitro techniques—a working hypothesis. *ATLA* 19, 393–402

Sikiric P, Seiwerth S, Grabarevic Z, Rucman R, Petek M, Jagic V, Turkovic B, Rotkvic I, Mise S, Zoricic I, Konjevoda P, Perovic D, Jurina L, Separovic J, Hanzevacki M, Artukovic B, Bratulic M, Tisljar M, Gjurasin M, Miklic P, Stancic-Rokotov D, Slobodnjak Z, Jelovac N, Marovic A (1997) The influence of a novel pentadecapeptide, BPC 157, on N^G-nitro-L-arginine methylester and L-arginine effects on stomach mucosa integrity and blood pressure. Eur J Pharmacol; 332: 23-33.

Slingerland DW, Carderalli JA, Burrows BA, Miller A (1984). The utility of serum gastrin levels in assessing the significence of low serum B_{12} levels. *Arch. Intern. Med.* 144, 1167-1168.

Smith, M; Boon, HS (1999) Counseling cancer patients about herbal medicine. Patient Education and Couseling, 38(2), 109-120.

So I, Yang DK, Kim HJ, Min KW, Kang TM, Kim SJ, Kim KW, Park KH, Jeon JH, Choi KH, Kim IG (2003) Five subtypes of muscarinic receptors are expressed in gastric smooth muscles of guinea pig. *Exp Mol Med.* 35(1),46-52

Souza Brito AR, Rodriguez JA, Hiruma-Lima CA, Haun M, Nunes DS. (1998) Antiulcerogenic activity of trans-dehydrocrotonin from Croton cajucara. Planta Med. 64(2),126-9.

Stallmach A, Wittig B, Giese T, Pfister K, Hoffmann JC, Bulfone-Paus S, Kunzendorf U, Meuer SC, Zeitz M. (1999) Protection of trinitrobenzene sulfonic acid-induced colitis by an interleukin 2-IgG2b fusion protein in mice. *Gastroenterology*, 117(4), 866-76.

Sugimoto Y, Narumiya S, Ichikawa A (2000) Distribution and function of prostanoid receptors: studies on knockout mice. *Prog. Lipid Res.* 39. 289-314.

Szabo S, Brown A (1987) Prevention of ethanol-induced vascular injury and gastric mucosal lesions by sucralfate and its components: possible role of endogenous sulfhydryls. Proc Soc Exp Biol Med 185: 493-497.

Szabo S, Gallagher GT, Horner HC, Frankel PW, Underwood RH, Konturek SJ, Brzozowski T, Trier JS (1983) Role of the adrenal cortex in gastric mucosal protection by prostaglandins. Sulfhydryls, and cimetidine in the rat. Gastroenterology 85: 1384-90

Szabo S, Szelenyi I (1987) Cytoprotection in gastrointestinal pharmacology. Trends Pharmacol.Sci. 8:149-154.

Szabo S, Trier JS (1984) Pathogenesis of acute gastric mucosal injury: Sulfhydryls as a protector, adrenal cortex as a modulator, and vascular endothelium as a target. In: Mechanisms of mucosal protection in the upper gastrointestinal tract, ed. By A.Allen, G. Flemstrom, A.Garner, W.Silen and LATurnberg, pp. 287-293, Raven Press, New York, 1984.

Szabo S, Trier JS, Brown A, Schnoor J (1985) Early vascular injury and increased vascular permeability in gastric mucosal injury caused by ethanol in the rat. Gastroenterology 88: 228-236.

Szabo S. (1989) Mechanisms of mucosal protection. In: Hollander D, Tarnawski A, editors. Gastric cytoprotection: a clinician's guide 49-60.

Szabo, S. (1984) Role of Sufhydryls and early vascular lesions in gastric mucosal injury. Recent advances in gastrointestinal cytoprtection. Akademiai Kiodo, Budapest, 17.

Szabo S, Brown A. (1987). Prevention of ethanol-induced vascular injury and gastric mucosal lesions by sucralfate and its components: possible role of endogenous sulfhydryls. *Proc. Soc. Exp. Biol. Med.* 185, 493–497

Szabo S, Trier JS, Brown A, Schnoor J. (1985). Early vascular injury and increased vascular permeability in gastric mucosal injury caused by ethanol in the rat. *Gastroenterology* 88, 228–236.

Szabo S, Trier JS, Frankel PW (1981). Sulfhydryl compounds may mediate gastric cytoprotection. *Science* 214, 200-202.

Szabo, S.; Szelenyi, I. (1987) "Cytoprotection" in gastrointestinal pharmacology, Trends Pharm. Sci. 8, 149-154.

Szelenyi I, Brune K (1988) Possible role of oxygen free radicals in ethanol-induced gastric mucosal damage in rats. *Dig Dis Sci* 33: 865-871.

Szelenyi I, Thiemer K. (1978) Distention ulcer as a model for testing of drugs for ulcerogenic side effects. Arch *Toxicol* 41: 99-105.

Tagore A, Gonsalkorale WM, Pravica V, Hajeer AH, McMahon R, Whorwell PJ, Sinnott PJ, Hutchinson IV. Interleukin-10 (IL-10) genotypes in inflammatory bowel disease. Tissue Antigens 54:386–390, 1999

Takeuchi, K.; Furukawa, O.; Okada, M.; Niida, H.; Okabe, S. (1990) Influence of stress on gastric alkaline secretion in rats. J. Pharmacol. Exp. Ther. 252, 1228-1233.

Tarnawaski, A.; Stachura, D.T.G. (1991) Indomethacin impairs quality of experimental gastric ulcer healing: a quality histologic and ultrastructural analysis. In: Garner A, O'Brian PEJ, editors. Mechanism of injury, protection and repair of the upper gastrointestinal tract. Chester: Wiley & Sons 521-31.

Tarnawski A (1993) Cellular mechanisms of gastric ulcer healing. In: Domschke W, Konturek SJ, editors. The stomach. Berlin: Spring, pp. 177-92.

Tarnawski A, Arakawa T (1992) Preventing GI damage with cytoprotective drugs. Contemp Int Med 4: 95-109.

Tarnawski A, Hollander D, Gergely H, Stachura J (1983) Comparison of antacid, sucralfate, cimetidine and ranitidine in protection of gastric mucosa against ethanol injury. *Gastroenterology* 84, 1331.

Tarnawski A. (1993) Cellullar mechanisms of gastric ulcer healing. In: Domscke W, Konturek SJ, editors. The stomach. Berlin: Spring 177-192

Tarnawski, A. Erickson, R.A. (1991) Sucralfate, 24 years later: current concepts of its protective and Therapeutic actions. *Europ. J. Gastroenterol Hepatol* 3, 795-810.

Tarnawski A, Hollander D, Stachura J, Krause WJ (1983). Arachidonic acid protection of gastric mucosa against alcohol injury: sequential analysis of morphologic and functional changes. *J. Lab. Clin. Med.* 102, 340-351.

Tarnawski, A.; Arakawa, T. (1992) Preventing GI damage with cytoprotective drugs. Contemp. Int. Méd. 4, 95-109.

Tarnawski, A.; Brzozowski, T.; Sarfeh, I.J. (1988) Prostaglandin protection of human isolated gastric glands against indomethacin and ethanol injury: evidence for direct cellular action of prostaglandin. J. Clin. Invest. 81, 1081

Tarnawski, A.; Hollander, D.; Stachura, J.; Krause W.J. Gergely, H. (1985) Prostaglandin protection of the gastic mucosa against alcohol injury – a dynamic time-related process. Role of the mucosal proliferative zone. Gastroenterology 88, 334-352.

Teresa P, Konturek PC, Konturek JW, Konturek SJ, Brzozowski T, Czenikiewicz M, Plonka M, Bielanski W, Areny H (2002) Impact of *Helicobacter pylori* and nonsteroidal anti-inflammatory drugs on gastric ulcerogenesis in experimental animals and in humans *Eur J Pharmacol.* 449(1-2):1-15

Trier JS, Szabo S, Allan CH (1987). Ethanol-induced damage to mucosal capillaries of rat stomach. Ultrastructural features and effects of prostaglalndin $F_{2\beta}$ and cysteamine. *Gastroenterology* 92, 13-22.

Tsiftsoglou, A.S.; Pappas, I.S.; Vizirianakis, I.S. (2003) Mechanisms involved in the induced differentiation of leukemia cells. *Pharmacol. Therapeutics* 100, 257-290.

Urushidani, T, Forte JG (1997) Signal transduction and activation of acid secretion in the parietal cell. *J Membr Biol* 159, 99-111

Varro A, Nemeth J, Bridson J, Lonovics J, Dockray GJ (1990). Modulation of posttranslational processing of gastrin precursor in dogs. *Am. J. Physiol.* 258, G904-G909.

Villegas C. La Casa, Orjales A, Alarcón de la Lastra C. (2003) Effects of dosmalfate, a new cytoprotective agent, on acute and chronic trinitrobenzene sulphonic acid-induced colitis in rats. *Eur. J. Pharmacol.* 460, 209–218

Wallace JL, Reuter B, Cicala C (1994) Novel nonsteroidal anti-inflammatory drug derivates with markedly reduced ulcerogenic properties in the rat. *Gastroenterology;* 107, 173-179

Wallace JL, Reuter BK, Cirino G (1994) Nitric oxide-releasing non-steroidal anti-inflammatory drugs: a novel approach for reducing gastrointestinal toxicity. J. Gastroenterol Hepatol 9: S40-S44.

Wallace JL, Whittle, BJR (1985) Acceleration of recovery of gastric epithelial integrity by 16, 16-dimethyl prostaglandin E_2. *British Journal of Pharmacology* 86, 837-842.

Wallace JL, Reuter BK, Cirino G (1994). Nitric oxide-releasing non-steroidal anti-inflammatory drugs: a novel approach for reducing gastrointestinal toxicity. *J Gastroenterol Hepatol* 9, S40-S44.

Wallace, J.L; Granger, D.N. (1996) The cellular and molecular basis of gastric mucosal defense. FASEB J. 10, 823-57.

Wallace, J.L; Whittle, B.J.R. (1985) Acceleration of recovery of gastric epithelial integrity by 16, 16-dimethylprostgalndin E_2. *Br. J. Pharmacol*. 86, 837-842.

WALSH, J.H. (1994). Gastrin. In: *Gut Peptides*. Ed. Walsh, J.H. & Dockray, G.J. pp.75-121. New York: Raven Press.

Ward SM, Dalziel HH, Khoyi MA, Westfall AS, Sanders KM, Westfall DP (1996) Hyperpolarization and inhibition of contraction mediated by nitric oxide released from enteric inhibitory neurones in guinea-pig taenia coli. *Br J Pharmacol* 118: 49–56.

Wendel GH, Maria AO, Mohamed F, Dominguez S, Scardapane L, Giordano OS, Guerreiro E, Guzman JA.(1999) Effect of dehydroleucodine in experimental colitis in rats and mice. *Pharmacol Res*. 40(4):339-44.

Wertheim WA, Kunkel SL, Standiford TJ, Burdick MD, Becker FS, Wilke CA, Gilbert AR, Strieter RM. Regulation of neutrophil-derived IL-8: the role of prostaglandin E_2, dexamethasone, and IL-4. *J Immunol* 151:2166–2175, 1993

Wessler S, Rapp UR, Wiedenmann B, Meyer TF, Schoneberg T (2002) B-Raf/Rap 1 signaling, but not c-Raf-1/Ras, induces the histidine decarboxylase promoter in Helicobacter pylori infection. *FASEB J.* 16, 417-419.

Wiklund CU, Wiklund NP, Gustafsson LE (1993) Modulation of neuroeffector transmission by endogenous nitric oxide – a role for acetylcholine receptor-activated nitric oxide formation, as indicated by measurements of nitric oxide/nitrite release. *Eur J Pharmacol* 240, 235–42.

Wooley PH, Dutcher J, Widmer MB, Gillis S. (1993) Influence of a recombinant human soluble tumor necrosis factor receptor FC fusion protein on type II collagen-induced arthritis in mice.
J Immunol. 151(11), 6602-7.

Wu SV, Giraud A, Mogard M, Sunii K, Walsh JH (1990). Effects of inhibition of gastric secretion on antral gastrin and somatostatin gene expression in rats. *Am. J. Physiol*. 258, G788-793.

Yao X, Forte JG (2003) Cell Biology of Acid Secretion by the Parietal Cell. *Annu. Ver. Physiol.* 65, 103-131.

Zavros Y, Rieder G, Ferguson A, Samuelson LC, Merchant JL (2002) Genetic or chemical hypochlorhydria is associated with inflammation that modulates parietal and G-cell populations in mice. *Gastroenterology* 122, 119–133.

Zhu Y, Michalovich D, Wu H (2001) Cloning, expression, and pharmacological characterization of a novel human histamine receptor. *Mol Pharmacol* 59, 434-441.

Zimatkin SM, Anichtchik OV (1999) Alcohol-Histamine Interactions. *Alcohol & Alcoholism.* 34(2), 141-147.