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CORRELAÇÃO ENTRE A DESNUTRIÇÃO PROTEICA E O PROCESSO DE FORMAÇÃO E REPARAÇÃO DE ÚLCERAS GÁSTRICAS: EFEITO PROTETOR/CURATIVO DO ÓLEO ESSENCIAL DE *Croton cajucara* BENTH (Euphorbiaceae)

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Ana Claudia Bensuaski de Paula e aprovada pela Comissão Julgadora.

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Orientador: Profa. Dra. Alba Regina Monteiro Souza Brito

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" O que quer que possa fazer ou sonhe em fazer, comece- o. Existe algo de genealidade, de poder e de magia na coragem"

(Goethe)

Aos meus pais

Sidney de Paula Filho
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ÍNDICE

ABSTRACT

It is known that the protein malnutrition can affect metabolism rates in humans, rodents and other animals. It is generally accepted that peptic ulcers are caused by a failure in the balance between gastric aggressive factors and mucosal defensive factors. Thus, the primary aim of this work was to verify the correlation between protein malnutrition and the gastric ulcer formation in rats fed diets with low (6%) or regular (17%) content of proteins. *Croton cajucara* Benth (Euphorbiaceae) is a specie widely used in Amazonian folk medicine for the treatment of a wide range of gastrointestinal symptoms. Thus, we also investigated the anti-ulcerogenic action of *C. cajucara* in the treatment of gastric ulcers in control and protein undernourished rats. It was observed that undernourished animals presented lower number of gastric injuries when compared with control animals in ethanol- and indomethacin-induced acute gastric ulcer. This may have occurred because undernourished animals produced greater amount of mucus and prostaglandin E_2 (PGE_2) when compared with the values obtained in control animals, even in the absence of the anti-ulcerogenic drugs. Besides, in placebo treated (12% Tween 80) undernourished rats submitted to pylorus ligature, a significant increase in gastric juice volume was observed when compared with control animals. After administration of essential oil from *C. cajucara* by intra-duodenal route, there was a significant reduction in the total acid content of both animal groups. The gastric juice volume was increased in the control group; however in undernourished rats, the gastric juice volume was higher before and remained high after treatment. In chronic experiments, where the animals were treated for 14 days with vehicle, standard drug (cimetidine) and with *Croton cajucara* essential oil, undernourished animals showed serious injuries in the gastric mucosa when compared with the gastric lesions of control animals. In acute and chronic ulceration, the essential oil was efficient in both animal groups to prevent and to heal these kinds of injuries. It was observed that *C. cajucara* essential oil caused citoprotection of the gastric mucosa of control and undernourished rats due to the increase of PGE_2 release and gastric mucus content.

It was also detected an increase of epidermal growth factor more pronounced in the gastric mucosa of control rats than malnourished rats that probably helped the healing of the gastric lesions. An increase in somatostatin and corticosterone levels and the decrease in gastrin levels were observed in both groups, control and undernourished animals.

However, the latter showed high levels of somatostatin and corticosterone hormones and low gastrin levels when compared to control rats. All these factors appear to contribute with the anti-ulcerogenic effect of the essential oil preventing and healing the gastric mucosal damage of both control and undernourished animals.

The results of this work confirmed healing effects previously obtained with *C. cajucara* essential oil in control rats. Additionally, it was possible to demonstrate that undernourished animals showed a resistance of the gastric mucosa against to ulcerogenic agents due to physiological changes found in their organisms.

RESUMO

É conhecido que a desnutrição protéica pode afetar o metabolismo de humanos, roedores e outros animais. É aceito que úlceras pépticas são causadas por um desequilíbrio entre fatores agressivos e fatores protetores da mucosa gástrica. Então, o objetivo primário deste trabalho foi verificar a correlação entre a desnutrição protéica e a formação de úlceras gástricas em ratos alimentados com baixo (6%) e regular (17%).

Croton cajucara Benth (Euphorbiaceae) é uma planta usada na medicina tradicional da Amazônia no tratamento das doenças do trato gastrointestinal. Assim, nós também investigamos a ação anti-ulcerogênica de *C. cajucara* no tratamento de úlceras gástricas em ratos controles e desnutridos. É observado que animais desnutridos apresentam um menor número de lesões gástricas quando comparado aos animais normais submetidos ao processo de úlcera gástrica induzida por etanol e indometacina. Isto se deve ao fato dos animais desnutridos produzirem uma maior quantidade de muco aderido a parede gástrica e de prostaglandina E_2 (PGE_2), quando comparado aos animais normais mesmo na ausência de droga anti-ulcerogênica. Na indução de úlcera pôr ligadura de piloro, os animais desnutridos tratados com o controle negativo 12% tween 80, mostraram um aumento significativo no volume do suco gástrico quando comparado aos animais normais. Após a administração (via intra-duodenal) do óleo essencial de sacaca, houve uma redução significativa na concentração de íons H^+ do conteúdo gástrico de ambos os grupos animais. O volume do suco gástrico esteve aumentado no grupo de animais normais, entretanto em ratos desnutridos o volume do suco gástrico manteve-se constante após o tratamento com sacaca, porém permanecendo com seus valores altos. Em experimentos crônicos, onde os animais foram tratados por 14 dias com o veículo, com a droga padrão (cimetidina) e com o óleo essencial de *Croton cajucara*, verificou-se que os animais desnutridos apresentaram sérias lesões na mucosa gástrica quando comparado às lesões gástricas dos animais normais. Em ambos os casos (ulceração aguda e crônica), o óleo essencial de sacaca revelou-se eficiente em impedir e em cicatrizar estas lesões gástricas em ambos os grupos animais.

Observou-se desta forma, que o efeito anti-ulcergênico do óleo essencial de *C. cajucara* se deve ao aumento de PGE_2 e muco aderido à parede gástrica conferindo a citoproteção do estômago. Aliado a isto, houve um aumento do fator de crescimento epidermal da mucosa gástrica, dos níveis de somatostatina e de corticosterona; e baixos níveis de gastrina, que favoreceram a cicatrização da mucosa gástrica de ambos os grupos animais. Todos estes fatores parecem agir conjuntamente no sentido de produzir um efeito anti-ulcerogênico do óleo essencial cicatrizando a mucosa gástrica de animais normais e desnutridos. Os resultados deste trabalho confirmaram o efeito cicatrizante do óleo essencial de *C. cajucara* obtido previamente com ratos normais. Adicionalmente, foi possível concluir que os animais com desnutrição protéica mostraram uma melhor prevenção da mucosa gástrica frente aos agentes ulcerogênicos, devido às alterações fisiológicas encontradas em seus organismos.

I-INTRODUÇÃO

1.1. O estômago e a secreção gástrica

As lesões ulcerosas agudas ou crônicas da mucosa gastroduodenal refletem a perda de equilíbrio dinâmico entre os elementos de defesa (secreção e ação de muco, prostaglandinas=PG´s e bicarbonato) e de agressão (secreção e ação do ácido e da pepsina) da mucosa, as quais atuam de modo antagônico (Chinzon & Zaterka, 1996).

A ulceração gástrica tem sido atribuída a causas diversas como estresse, hormônios, drogas, álcool, fumo e ingestão de determinados alimentos (Mc Grigan, 1991).

Desde que a secreção ácida foi descrita, tornou-se evidente que, de alguma forma, a presença de ácido no estômago é nociva, além de ser a principal responsável pela maioria das desordens que afetam este órgão (Sachs *et al.*, 1994).

Atualmente, entretanto já se sabe que a secreção ácida e sua regulação não podem ser considerados como os únicos fatores responsáveis pela úlcera péptica. O processo ulcerativo é complexo e exibe facetas múltiplas, as quais devem ser analisadas, como exemplo:

-o refluxo do conteúdo duodenal, que pode levar às ulceras gástricas. Apesar de resistir a um pH próximo de dois, as células epiteliais da mucosa gástrica não são resistentes às bases que rapidamente rompem a barreira gástrica mucoprotetora (Wallace & Granger, 1996; Kutchai, 1996);

-o esvaziamento gástrico acelerado pode levar às úlceras duodenais. Apesar de bastante resistente aos sais biliares, a mucosa duodenal não apresenta resistência ao ácido gástrico, sem a adequada neutralização (Kutchai, 1996);

-a supressão da secreção de muco ou bicarbonato compromete a barreira citoprotetora da mucosa gástrica; os efeitos do ácido clorídrico e da pepsina sobre a superfície do estômago podem produzir úlceras (Wallace & Granger, 1996);

-agonistas α-adrenérgicos, como a noradrenalina, além de produzirem vasoconstrição (impedindo a retirada do ácido coletado) e contração dos esfíncteres (que retém ácido no estômago), produzem ainda diminuição na secreção de bicarbonato (Kutchai, 1996), podendo induzir úlceras.

Desta forma, vários mecanismos estão implicados na patogênese das lesões gástricas agindo sinergicamente para produzir lesões crônica e aguda. Aumento na secreção do ácido gástrico e de pepsina, diminuição no fluxo sangüíneo gástrico, supressão da geração endógena

de prostaglandina, inibição do crescimento e proliferação celular da mucosa e alteração da motilidade gástrica, são alguns dos mecanismos envolvidos na ulcerogênese (Konturek *et al.*, 1992; Soll, 1993; Brzozowski *et al.*, 1993).

Além desses fatores relatados acima, há ainda agentes infecciosos como *Helicobacter pyllori,* implicados na etiologia das úlceras gastroduodenais. Porém, é relatado que esta bactéria está presente tanto na mucosa de indivíduos portadores de lesões gástricas, como também na mucosa de indivíduos sadios, os quais podem ou não desenvolver esta patologia. Assim, a presença da *H. pyllori*, as alterações na secreção ácida e a diminuição da citoproteção da mucosa gástrica, são considerados os principais responsáveis pela formação e desenvolvimento das úlceras pépticas. Segundo Brunton (1996); e estes mesmos fatores, por sua vez, ainda dificultam a cicatrização da lesão já instalada.

A cicatrização é o processo pelo qual um tecido lesado é substituído por um tecido conjuntivo vascularizado. O primeiro passo ao processo é a instalação de uma reação inflamatória, cujo exsudato de células fagocitárias reabsorve o sangue extravasado e os produtos que levam à destruição tecidual. No entanto, há também a proliferação fibroblástica e endotelial as quais originam o tecido conjuntivo cicatricial. O processo cicatricial pode ser influenciado por fatores locais e sistêmicos reduzindo, retardando ou impedindo, parcial ou totalmente, algumas fases ou todo o processo, pois mantém a reação inflamatória em andamento (Brasileiro Filho *et al.*, 1994).

A cicatrização da mucosa gástrica, após exposição à úlcera gástrica e duodenal, é um processo que envolve diferentes mecanismos, dos quais o mais importante parece ser a expressão de fatores de crescimento, especialmente do fator de crescimento epidermal, EGF (Konturek *et al.*, 1990; Konturek *et al.*, 1992, 1996; Podolsky, 1994). EGF é um peptídeo com 53 aminoácidos que não é expresso na mucosa intacta de roedores e humanos (Polk *et al.*, 1992; Calabro *et al.*, 1995). A expressão aumentada de EGF e de receptores de EGF foi relatada ainda em áreas de ulceração crônica (Wright *et al.,* 1990) e durante o reparo da mucosa gástrica após exposição ao ácido acético (Konturek *et al.*, 1996).

É citado que fatores de crescimento estimulam elementos celulares importantes da cicatrização da mucosa gástrica como angiogênese, formação de tecido de granulação e re-epitelização (Szabo & Vincze, 2000); esses mesmos autores provaram ainda a hipótese de que

a estimulação da proliferação celular conduz à angiogênese, resultando em rápida cicatrização da mucosa gástrica ulcerada.

Em adição, uma grande variedade de fatores endógenos está relacionada à patofisiologia da gastroproteção incluindo PG´s, gastrina, somatostatina, óxido nítrico e compostos sulfidrílicos, além dos fatores de crescimento anteriormente mencionados.

Assim, muitas investigações mostram a ocorrência de PG´s no estômago e sua importância na regulação da fisiologia do estômago (Peskar & Maricic, 1998). Análogos estáveis de prostaglandina (PGE_1), como o misoprostol, foram utilizados no tratamento da úlcera péptica, devido à sua atividade anti-secretória e citoprotetora (Konturek *et al.*, 1997); entretanto, essas drogas não são muito úteis devido aos seus efeitos adversos.

Novos compostos cicatrizantes de úlcera como a rebamipida mostraram ser efetivos na cicatrização da úlcera gástrica experimental por estimular a síntese de PG´s, especialmente PGE_2 a qual torna o trato gastrointestinal mais resistente à injúria aguda por aspirina, ácidos, sais biliares e estresse (Tarnawski *et al.*, 1998). A PGE_2, além de potente vasodilatadora e hiperalgésica, na mucosa gástrica é capaz de inibir parcialmente a secreção de ácido clorídrico e garantir, em grande parte, a manutenção do fluxo sangüíneo para esta mucosa e a vasodilatação garante também, em grande parte, a produção e secreção de muco e bicarbonato para a mucosa gástrica (Wallace *et al.*, 2000).

As PG´s são geradas na mucosa gástrica via atividade de uma enzima, a ciclooxigenase (Eberhart & Dubois, 1995) a qual existe como duas isoformas geneticamente diferentes: ciclooxigenase 1 (constitutiva), que exibe efeito citoprotetor da mucosa gástrica e ciclooxigenase 2 (induzida), que está relacionada com reações inflamatórias e tecidos danificados envolvendo várias citocinas, endotoxinas e fatores de crescimento (Kujubu *et al.*, 1991; O'Banion *et al.*, 1991; Feng *et al.*, 1995 and Brzozowski *et al.*, 1993); é sabido que essas duas isoenzimas estão envolvidas na recuperação da mucosa gástrica após indução de úlceras.

Sabe-se, desta forma, que a maior limitação do uso dos anti-inflamatórios não esteroidais (AINE) como a indometacina e aspirina, são os efeitos colaterais no trato gastrointestinal (TGI), incluindo-se a formação de lesões gástricas, aumento no número de úlceras existentes e interferência na cicatrização dessas úlceras.

Um número enorme de estratégias têm sido usada recentemente no desenvolvimento de novos AINE que não agridam o TGI. Exemplo disto é a pesquisa de drogas que inibem a isoforma da enzima ciclooxigenase 2 (COX-2) exercendo ação anti-inflamatória sem, no entanto, interferir com a síntese de PG´s no TGI ou inibir a COX-1. Assim, no método de indução de úlcera por indometacina, a mucosa gástrica apresenta-se com lesões hemorrágicas decorrentes da inibição da síntese de PG´s, com consequente redução dos mecanismos de citoproteção da mucosa gástrica mediados por estas substâncias (Ukawa *et al.*, 1998). Além deste fator, podem também ser agregados o aumento da motilidade gástrica e a ativação de neutrófilos que atuam conjuntamente na ulcerogênese (Evans, 1996).

No método de indução de úlceras por etanol, o contato direto deste agente com a mucosa gástrica causa a solubilização do muco protetor deixando a mucosa gástrica indefesa às ações hidrolítica e proteolítica do ácido clorídrico e da pepsina, respectivamente. Esta substância produz, ainda, um aumento na secreção do ácido gástrico e, por contato direto, altera a rede de vascularização local, rompendo vasos sanguíneos que irrigam a mucosa gástrica desencadeando processos de necrose no local (Mizui & Douteuchi, 1983; Lewis & Hanson, 1991; Tabata *et al.*,1996; Evans, 1996). Deste modo, de acordo com os mesmos autores, a hipersecreção gástrica, juntamente com a fragilidade da rede vascular e a dissolução da camada muco-protetora da mucosa gástrica, constituem os principais fatores envolvidos na instalação de uma úlcera induzida por etanol.

O modelo de ligadura de piloro, quando realizado em ratos, induz lesões gástricas principalmente por estímulo mecânico do conteúdo gástrico sobre a mucosa gástrica (Shay *et al.*, 1945; Lewis & Hanson, 1991). O acúmulo de ácido e do conteúdo gástrico em geral, promovido pela obstrução do piloro, gera por exemplo distensão exagerada da mucosa e hipersecreção ácida promovida pela liberação de acetilcolina proveniente de ramificações do nervo vago na mucosa gástrica. A acetilcolina induziria a secreção do ácido clorídrico atuando em receptores presentes nas células parietais. Ainda, de forma indireta, a acetilcolina promoveria a liberação de histamina e gastrina pelas células "enterocromafin-like" e células G produtoras de gastrina, respectivamente; histamina e gastrina iriam estimular também a secreção ácida, por sua ação em receptores H_2 e CCK-β, respectivamente das células parietais da mucosa gástrica (Shay *et al.*, 1945; Lewis & Hanson, 1991; Raffatullah *et al.*, 1994; Hirschowitz *et al.*, 1995; Pandolfino *et al.*, 2000). Neste método, parâmetros de secreção

gástrica tais como pH, volume e concentração de íons H^+ do conteúdo gástrico podem ser devidamente avaliados, quando se utiliza animais de experimentação.

Já a indução de úlcera crônica por ácido acético proporciona uma investigação da cicatrização do processo ulcerogênico. O ácido acético é conhecido por causar injúria ou evocar um processo inflamatório na parede do estômago de ratos. Esta úlcera aparenta ser uma úlcera intratável, comparada a aquelas que são vistas clinicamente, e de difícil produção em laboratórios (Monfort *et al.*, 1973). Esta classificação, como úlcera intratável, foi baseada na observação de sua persistência por mais de 150 dias sem intervenção adicional, e no agravamento da ulceração com o aparecimento de hemorragias e aumento das margens da lesão durante a cicatrização (Ito *et al.*, 1994).

1.2. Desnutrição e suas implicações

As causas da desnutrição estão ligadas a múltiplos fatores que, frequentemente, associam-se e incluem problemas relacionados ao meio ambiente, estrutura social, renda familiar e outros. Nos países subdesenvolvidos, um grande número de homens, mulheres e crianças é acometido por desnutrição leve, moderada ou grave

Um dos problemas sociais mais importantes encontrados no Brasil ainda hoje é a desnutrição, a qual é uma das causadoras de várias doenças em nosso país. Governantes tem como meta diminuir/acabar com a falta de alimento no país, visando resolver ou pelo menos amenizar o problema da desnutrição, podendo desta forma favorecer uma melhor condição de vida para milhares de crianças e adultos. Vários autores tem demonstrado alterações irreversíveis nos sistemas nervoso, endócrino, digestório, reprodutor e outros; estas alterações são encontradas tanto em crianças, como também em animais desnutridos experimentalmente (Cury *et al.*, 1984).

De acordo com Latorraca *et al.*, (1998), ratos alimentados cronicamente com dieta hipoprotéica (contendo 6% de proteínas), apresentam sinais comumente presentes na desnutrição protéica em humanos e em animais de laboratórios (Figura 1). Estes sinais são: redução de ganho de peso, hipoproteinemia, aumento de ácidos graxos livres e de glicogênio hepático. O teor plasmático de ácidos graxos livres está elevado na desnutrição protéica em animais e em humanos, baixando com a recuperação nutricional (Latorraca *et al.*, 1999).

A dieta nutricional é um componente de controle dos problemas gastrointestinais dos pacientes com úlcera péptica. Exemplos de diagnose de doenças no TGI, com notável implicação nutricional, incluem doenças inflamatórias intestinais, pancreatite, insuficiência

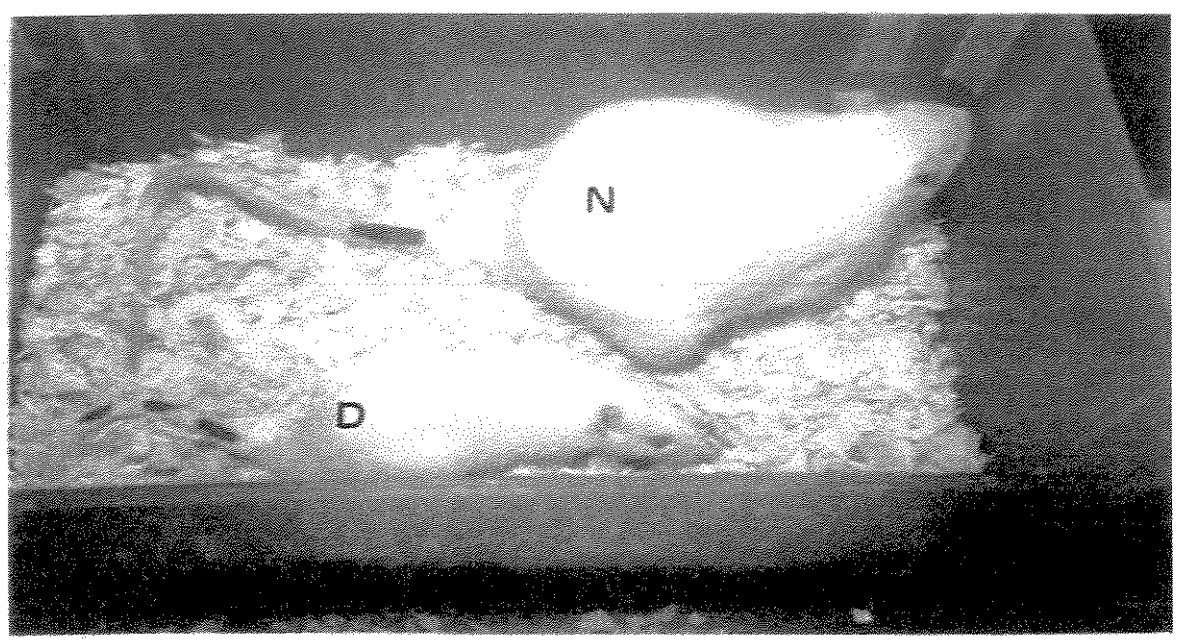

Figura 1: Modelo de Desnutrição intra-uterina. Animais normoprotéicos (N) alimentados com dieta contendo 17% de proteínas e hipoprotéicos (D) alimentados com dieta 6% de proteínas.

A desnutrição em humanos e outros mamíferos está associada a prejuízo da secreção de insulina e alterações no metabolismo de carboidratos (Carneiro *et al.*, 1995). Em ratos adultos submetidos à desnutrição protéica, aguda ou crônica, durante o período de crescimento após o desmame, observou-se redução na secreção de insulina em resposta à glicose e outras secreções (Carneiro *et al.*, 1995; Reis *et al.*, 1997).

O estado de deficiência nutricional induz uma variedade de distúrbios no metabolismo de mamíferos, um dos quais parece ser mediado através de disfunções das glândulas endócrinas. Desta forma, mudanças hormonais tem função de adaptação ao estresse agudo ou crônico causado pela alimentação deficiente em proteína visando preservar a homeostase do meio interno (Das *et al.*, 1998). Além disso, a restrição protéica durante a gravidez e lactação pode, adversamente, afetar a prole produzindo diminuição do peso corporal e do crescimento de órgãos e tecidos, dentre eles órgãos do TGI, com consequente redução da atividade das enzimas da mucosa do intestino delgado (Hales & Barker, 1992). A deficiência nutricional afeta rapidamente a divisão celular de órgãos que estão sofrendo diferenciação podendo, irreversivelmente, limitar seu crescimento e sua função (Lucas, 1990). Entretanto, como não existem estudos relacionados ao fato desta dieta deficiente em proteínas estar interferindo na formação de úlceras gastrointestinais, também na estrutura e função do sistema digestório, buscou-se estabelecer experimentalmente uma correlação entre a desnutrição protéica e o processo de formação de úlceras gástricas em animais.

1.3. Plantas Medicinais como Alternativa Terapêutica

A abordagem na investigação envolvendo plantas medicinais, objetiva a obtenção de novos medicamentos, incluindo-se técnicas de monitoramento químico das substâncias farmacologicamente ativas, presentes em extratos brutos ou em frações enriquecidas das espécies de plantas investigadas (Mcchesney, 1996).

É sabido que na pesquisa com plantas medicinais, para que uma espécie seja selecionada para estudo, não basta apenas uma indicação popular de uso medicinal. Fatores etnofarmacológicos, quando levados em consideração, podem reduzir o erro de se iniciar o estudo de espécies ou substâncias farmacologicamente inativas para o tratamento de certa doença. Assim, para um estudo bem elaborado de uma nova espécie de planta medicinal, faz-

se necessário, a conjunção de vários profissionais em equipes multidisciplinares, compostas por etnobotânicos, etnofarmacólogos, fitoquímicos, químicos de síntese, bioquímicos, agrotecnólogos, farmacólogos e toxicologistas, além de alunos de pós-graduação em programas de mestrado e doutorado, envolvidos em um trabalho multidisciplinar e interativo, na busca de substâncias ativas com potencial terapêutico (Souza Brito, 1996; Souza Brito & Nunes, 1997). É neste contexto que a realização deste trabalho buscou estabelecer não só uma relação do uso de dietas no estudo de úlceras gástricas, como também a atividade farmacológica do óleo essencial de *Croton cajucara* Benth na prevenção e no tratamento de lesões gástricas em animais tratados com dieta contendo 17% de proteínas e 6% de proteínas.

De acordo com Lewis & Hanson (1991), as principais classes químicas de compostos com atividade antiulcerogênica são terpenos, triterpenos, flavonóides, compostos fenólicos, taninos, alcalóides e glicosídeos, saponinas e polissacarídeos. Hiruma-Lima *et al.* (2002), descreveu que os constituintes majoritários do óleo essencial de *C. cajucara* são sesquiterpenos, no caso copaeno e cipereno.

Em geral, compostos obtidos de plantas com atividade antiulcerogênica exercem seus efeitos estimulando os fatores de proteção da mucosa gástrica, aumentando a produção de prostaglandinas e/ou estimulando a secreção de muco e bicarbonato. Outros mecanismos como inibição da secreção ácida gástrica (através da interação com diferentes receptores farmacológicos) ou ainda das enzimas e hormônios envolvidos com o processo secretor, são também encontrados (Lewis & Hanson, 1991; Borrelli & Izzo, 2000).

1.4. Espécie Utilizada

A espécie *Croton cajucara* Benth. pertencente à família Euphorbiaceae (Figuras 2 e 3) é comercializada no "Ver o Peso", de Belém do Pará sob a denominação popular de sacaca, sendo conhecida e usada pelos indígenas da região amazônica. O nome significa "feitiço" e seus usos na medicina tradicional indígena estão relacionados ao tratamento da malária (Simões *et al.*, 1979).

Figura 2 - *Croton cajucara* em seu habitat.

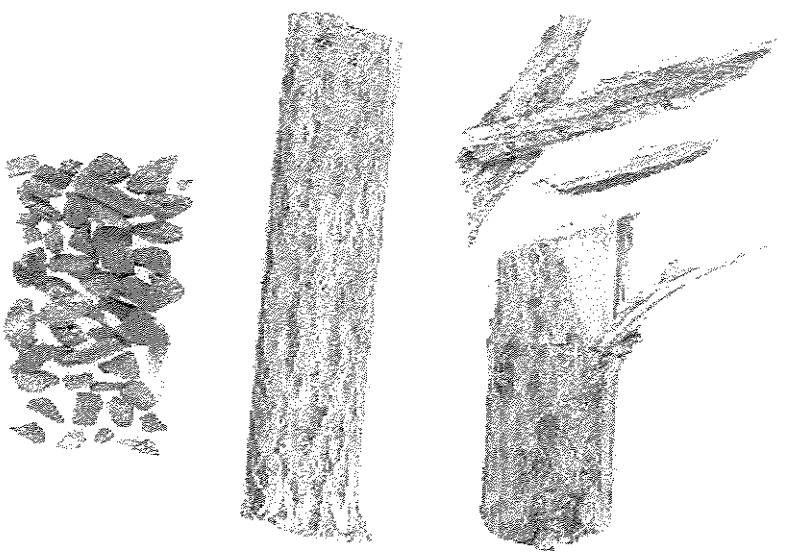

Figura 3- As cascas do caule de *Croton cajucara*.

A sacaca, um arbusto com 4 e 6 metros de altura, possui flores reunidas em inflorecências dispostas em racemos terminais de 6 a 9 cm de comprimento e os frutos são tri-loculares (Pio Correa, 1984). Esta planta é endêmica da região amazônica com centro de dispersão no Estado do Pará (Simões *et al.*, 1979). Em Belém, sua utilização está relacionada a problemas hepáticos tais como icterícia, hepatite, malária e diabetes. Além disso, existem também várias indicações na região amazônica do uso do chá das cascas e das folhas para dores estomacais, gastrites e úlceras gástricas (Simões *et al.*, 1979; Di Stasi *et al.*, 1989; Van den Berg, 1992) além de ser considerada como espécie útil no tratamento da diarréia, inflamação do fígado e por controlar os altos níveis de colesterol (Hiruma-Lima *et al.*, 2002). O chá das cascas desta planta é usado no tratamento de gastrites e úlcera péptica (Souza Brito & Nunes, 1997) e contém ao redor de 1% de uma rica mistura de sesquiterpenos (Nunes *et al.*, 1993).

Hiruma-Lima *et al.*, (1999) demonstraram o efeito anti-ulcerogênico do óleo essencial de *C. cajucara* em modelos de indução de lesões gástricas pôr estresse, indometacina, etanol/HCl e ligadura do piloro em camundongos. Os mesmos autores relatam que foi observado que o óleo essencial da sacaca apresentou baixa toxicidade, sugerindo que testes farmacológicos deveriam ser continuados.

Como não há estudo dos efeitos a longo prazo da desnutrição intra-uterina e a formação de úlceras pépticas, a proposta deste trabalho foi verificar se a desnutrição protéica influencia a formação de úlceras gástrica, bem como o metabolismo e crescimentos dos órgãos de animais alimentados com dietas contendo 6% comparando com um estudo de animais alimentados com outra dieta contendo 17% de proteínas. Paralelamente, um estudo farmacológico do efeito do óleo essencial de *C. cajucara* na prevenção e cicatrização de úlceras gástricas em animais normoproteicos e hipoproteicos foi realizado.

II- OBJETIVOS

2. OBJETIVOS

Considerando as indicações e a utilização popular de *Croton cajucara* Benth. no tratamento da gastrite e da úlcera gástrica e ainda os dados experimentais obtidos anteriormente em nosso laboratório, foram objetivos deste trabalho:

-Estudar a correlação existente entre a desnutrição protéica e o processo de indução, formação e cicatrização de úlceras gástricas;

-Determinar os níveis de corticosterona plasmática, no processo de úlcera gástrica aguda e crônica, no sentido de analisar se os mesmos estariam sendo afetados pela desnutrição protéica;

-Verificar o mecanismo preventivo e cicatrizante envolvido no processo de reparação das úlceras crônicas e agudas;

- Analisar se a resposta celular ao óleo essencial de *C. cajucara* no processo de reparação sofre, ou não, alteração na desnutrição protéica;

- Investigar a participação de hormônios do trato gastrointestinal como gastrina e somatostatina nestes modelos de lesão ulcerosa.

III- METODOLOGIA E RESULTADOS

Is gastric ulceration different in normal and malnourished rats?

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Abstract

Protein malnutrition can adversely affect all tissues. The aim of this study was to test the hypothesis that protein-deprivation influences gastric ulcer formation as well as metabolism and organ growth in rats. In the present study, there was a significative reduction in body and organ weight in rats fed a low protein diet ($p<0.001$). Malnourished rats were less susceptible to ulceration of the gastric mucosa in ethanol and indomethacin models of acute gastric ulcers when compared with in rats fed a normoproteic diet (17% of protein). Mucus production and PGE_2 formation were increased in malnourished rats, explaining the lower number of acute ulcers these animals. Ligation of the pylorus altered gastric juice composition (increased pH, gastric volume and decreased the total acid concentration) in low protein animal group compared to the group fed 17% of protein ($p<0.05$). The gastric mucosa of malnourished rats was more damaged than that of normal rats evaluated for 14 days after acetic acid injection ($p<0.001$). The resistance of malnourished rats to acute gastric lesions was due to an increase in PGE_2 release and mucus secretion, which protected the gastric mucosa. This phenomenon was not seen in sub chronic gastric ulceration.

Key words: anti-ulcer activity, malnourishment, citoprotection, gastric ulcers.

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Introduction

Malnutrition induces a variety of metabolic disturbances in man and other mammals, some of which may be mediated through dysfunction of the endocrine glands. Hormonal changes may play a major role adaptation to the acute or chronic stress of proteins deficiency (Das *et al.*, 1998). Malnutrition is associated with impared insulin secretion in response to glucose and other secretagogues, as well as alterations in carbohydrate metabolism (Okitolonda *et al.*, 1987; Carneiro *et al.*, 1995). During early life, malnutrition may have immediate and long-term consequences. Studies in animals have shown that maternal protein restriction during pregnancy and lactation can adversely affect offspring (Hales & Barker, 1992). Such malnutrition may affect the growth of different organ systems during intrauterine life, and may produce small but normal offspring, although during development, these may be selective effects on organ growth (Lucas, 1990). Generally, the earlier in life that malnutrition occurs, the more likely it is to exert, permanent effects on body weight and organ growth. Restriction of the nutrient supply affects rapidly dividing cells and can irreversibly limit organ growth and function. The sensitivity of organ and tissues to the effects of malnutrition at different stages of growth and differentiation has led to the concept of programming, in which a stimulus or insult during a critical period may have lasting or lifelong effects.

Maternal dietary restriction during pregnancy and lactation reduces the growth of gastrointestinal tract and lowers the activities of small intestinal mucosal enzymes (Young, 1987). However, there have been no studies of the long-term effects of perinatal malnutrition on the structure and function of the digestive system in adulthood. The purpose of this study was to test the hypothesis that protein-deprivation influences gastric ulcer formation as well as metabolism and organ growth in rats.

Material and Methods

Drugs

Ethanol, acetic acid and indomethacin were used in this study. Indomethacin was prepared in sodium bicarbonate (5%). Appropriate dilutions of these substances were prepared immediately before use.

Animals

Female Wistar rats (90 days old) were obtained from the animal facilities of the State University of Campinas. After the meet, pregnant females were randomly assigned to one of two groups. One group has maintained with diet containing 60 g of protein/Kg (lower protein/ LP diet) and the other group was fed with 170 g of protein/Kg (normal protein/ NP diet) from the first day of pregnancy until the end of lactation (Reeves et al., 1993 – Table I).

The LP and NP diets (6% and 17% of protein respectively) were based on the AIN-93 report (American Institute of Nutrition) which establishes dietary standards for nutritional studies with laboratory rodents.

During the experiment, the mothers were fed their respective diets *ad libitum* and had free access to water. The rats were housed on a 12h light-dark cycle at 24° C and the food intake was monitored daily. At 25 days of age, the pups were weaned and maintained on their mothers' diet. After 90 days, the normal and malnourished rats were used in experiments of anti-ulcerogenic activity and mechanism. All rats were weighed weekly for one month to obtain a growth curve. The experimental protocols were approved by the Institutional Animal Care and Use Committee and were done according to the recommendations of the Canadian Council on Animal Care (Olferd *et al.*, 1993).

Ethanol-induced ulcers. The ethanol-induced ulcer assay was done according to the method of Morimoto *et al.* (1991). Normal and malnourished male rats (weighing 150-200g and 90-120g, respectively) were fasted for 24h, before the experiment but had free access to water. One milliliter of 99,5% ethanol was given orally to each rat. 1 hour later after ethanol administration the rats were killed, the stomachs were removed and the ulcerative index was determined.

Acetic acid-induced gastric ulcers. The experiments were done as described by Takagi *et al.* (1969). Normal and malnourished rats were deprived of food for 24h. A laparotomy was done under anesthesia through a midline epigastric incision. After exposing the stomach, 0.05 ml of 30% v/v acetic acid, was injected into subserosal layer in the glandular part of anterior wall, with care taken not to disturb the blood vessels. Then the abdomen was closed and the rats then fed their diet for 14 days. The rats were subsequently sacrificed to assess ulcer healing. The stomach was removed and the gastric lesions were evaluated by examining the inner gastric surface with a

dissecting binocular microscope. The ulcer area (mm^2) and healing ratio (%) were determined in relation to the negative (100%) and positive (0%) control according toTakagi *et al.* (1969).

Indomethacin induced ulcers. Normal and malnourished rats were fasted for 24h, before the experiment. Indomethacin (30 mg kg^{-1}, dissolved in 5% bicarbonate) was administered subcutaneously to unanesthetized rats in each group, according to the method of Hayden *et al.* (1978). The rats were killed 4h later, the stomachs removed and the gastric lesions were determined as described above.

Shay ulcer. Normal and malnourished rats were fasted for 24 h, after which the pylorus was ligated as described by Shay *et al.* (1945). The rats were killed 4 h later and the stomachs were removed and inspected internally. The stomach contents were transferred to a graduated centrifuge tube and centrifuged at 2000 rpm for 10 min. The supernatant volume and pH were recorded with a digital pH meter (MARCONI- Model MA-RS232). The total acid content of the gastric secretion was also determined by titration to pH 7.0 with 0.05 N NaOH using a digital burette (MARCONI-HIRSCHMANN LABORGERATE- EM BURETTE DIGITAL-50 ml).

Determination of gastric wall mucus. Gastric wall mucus was determined according to Rafatullah *et al.* (1994). Normal and malnourished rats were fasted for 48h with free access to water, after which the pylorus was ligated as described above. The rats were subsequently sacrificed; the stomach was removed and opened along the larger curvature. Glandular segments from the stomach were removed and weighed. Each segment was immersed for 2 h in 10 ml of 0.1% (w/v) Alcian blue dissolved in 0.16 M sucrose solution, buffered with 0.05M sodium acetate, pH 5.8. Excess of dye was removed by washing the segments twice with 0.25 M sucrose solution during a period of 15 min and 45 min, respectively. Mucus-dye complex was extracted by placing the gastric walls in 10 ml of 0.5 M magnesium chloride with intermittent shaking (1 min) at 30 min intervals for 2 h. Four milliliters of blue extract were mixed with an equal volume of diethyl ether and shaken vigorously for 2 min. The emulsion obtained was centrifuged for 10 min at 3600 rpm and the absorbance of the aqueous layer was recorded at 580 nm. The Alcian blue extracted per gram of wet glandular tissue was calculated.

Assessment of prostaglandin synthesis. The rats were deprived of food for 24h prior to the experiment, which were done between 9:00 and 11:00 a.m. Groups of at least seven rats received indomethacin (20 mg kg^{-1}, s.c.) dissolved in 5% sodium bicarbonate, pH=8.3. Thirty minutes later, the animal were killed and the abdomen was opened. The stomach tissue was finely minced with scissors and then incubated at 37°C for 20 min. The prostaglandin (PGE_2) concentration in the buffer was measured by the enzymatic-immuno assay (RPN222 – Armersham) as previously described by Curtis *et al.* (1995).

Blood collection

Heparinized blood samples were obtained from the retroorbital plexus of normal and malnourished rats (Krous, 1980) and stored in a test tube at 0° C for 4h. The tubes were subsequently centrifuged (3000 rpm, 6° C) by 10 min. and the serum obtained was stored at -20° C until used.

Determination of corticosterone and somatostatin

Corticosterone (Mattingly, 1962) and somatostatin (Yago *et al.*, 1998) were determined by radioimmunoassay in serum from normal and malnourished rats.

Autopsy and organ weighing

After sacrifice, the organs of normal and malnourished rats were weighed to asses differences in their growth.

Reverse-transcriptase polymerase chain reaction (RT-PCR) for the detection of EGF mRNA

Acetic acid - induced gastric ulcers (Takagi *et al.*, 1969) were produced in normal and malnourished rats and the total RNA of mucosal specimens was extracted according Konturek *et al.* (1998) with Trizol reagent and stored at -80°C. The RNA was resuspended in RNAase-free tri-ethanol amine (TE) buffer and its concentration was estimated by the absorbance at 260 nm. The quality of each RNA preparation was determined by agarose-formaldehyde gel electrophoresis and ethidium bromide staining. The primers were synthesized by Biometra®

(Göttingen, Germany) and were based on the published cDNA sequences for rat EGF (epidermal growth factor) (Fan *et al.*, 1995; Saggi *et al.*, 1992): sense primer- 5'-GACAACTCCCCTAAGGCTTA-3' (nucleotides 2804-2823); and antisense primer-5'-CATGCACAGGCCACCATTGAGGCAGTACCCATCGTACGA-3' (nucleotides 3332-3370). To maximize the specificity of amplification, a hot start polymerase chain reaction was done in a Perkin Elmer Cetus DNA thermal cycler for 33 cycles. Aliquots (8 μl) of amplified polymerase chain reaction products were electrophoresed in 1.5% agarose gels stained with ethidium bromide and the bands the visualized and photographed under UV light. The primer sequences were specific, as ascertained by a computer-assisted search of version of GeneBank The specificity of the primer pair was also assessed by sequencing of the polymerase chain reaction products.

Photography

Samples of the stomachs were maintained in NaCl 0.9% (w/v) and the photos were taken under illumination.

Histology

Stomach samples from rats with indomethacin and ethanol-induced ulcers were fixed in Bouin for 24 h, dehydrated in an increasing ethanol series, cleared with xylene, embedded in Histosec (Merck-11609), and prepared for microtomy. The sections were then deparaffinized and rehydrated in a decreasing ethanol series prior to staining with hematoxylin-eosin (Yoshitake *et al.*, 1991; Junqueira & Junqueira, 1996) Photomicrographs were obtained with an Axiophot photomicroscope (Carl Zeiss, D-7082).

Statistical analysis

The results were expressed as mean ± SEM. Statistical significance was determined by Student t-test with the level of significance set at $p<0.05$. All statistical analyses were done using the Statistica software, version 5.1 (Statsoft, Inc.).

Results

Body weight

After weaning, normal and malnourished rats were weighed weekly for one month. The growth curve obtained, was indicative of a significant increase in body weight in normal rats compared to malnourished rats, $p<0,001$ (Figure 1A e 1B).

Organ weights in normal and malnourished rats

There was a significative decrease ($p<0,001$) in the organ weights of malnourished rats, compared to normal animals (Table II).

Dosage of corticosterone and somatostatin concentrations

The corticosterone levels before the ulcer induction were significantly higher in malnourished rats (44.0 ± 1.22 μg/100 ml of plasma) than in normal rats (11.0 ± 1.9 μg/100 ml of plasma; $p<0.001$). After gastric ulcer induced, the corticosterone levels were unchanged in both groups (42. 3 ± 1.4 μg and 10.3 ± 1.83 μg/100 ml of plasma ($p<0,001$) in malnourished and normal rats, respectively. The levels of somatostatin before and after ethanol induced damage of the gastric mucosa were the same in both groups of rats (10 ± 1.89 pmol/l and 10.2 ± 2.0 pmol/l, respectively).

Ethanol-induced ulcer

The oral administration of ethanol to normal rats produced the expected characteristic zonal necrotizing mucosal lesions. The lesion index in normal rats was 7.0 ± 0.53 mm^2 (n=17) while in malnourished rats the index was 3.0 ± 0.70 mm^2 (n=15) (p<0,001) (Figure 3).

Acetic acid-induced gastric ulcers

Table III shows that the gastric lesions caused by acetic acid were greater in malnourished animals than in normal rats, but these was no difference in the pH of the tissues. Lesion area was 26,3 ± 1,58 mm^2 (normal animals) and 51,9 ± 1,20 mm^2 (malnourished animals) (p<0.001).

Indomethacin- induced ulcers

Malnourished rats had a smaller number of indomethacin-induced ulcers than normal rats (7 ± 1.29 and 18 ± 1,32 mm^2, respectively, n=7 each; p<0.05). These results suggest a greater involvement of prostaglandin production in malnourished rats (Figure 4).

Prostaglandin synthesis and gastric wall mucus

Figure 2 shows that PGE_2 production was greater in malnourished rats compared to normal rats, and that indomethacin inhibited PGE_2 formation in both groups. Mucus production by the gastric mucosa of malnourished rats was two-fold greater than in normal rats (1.8 ± 1.2 g and 0.8 ± 0.34 g, respectively, n=8; p<0.001).

Shay ulcer

The administration of Tween 80 12% in pylorus ligated animals, induced a significantly altered the gastric volume, pH, and gastric acid content in malnourished animals compared to normal animals, p<0,05 respectively (Table IV).

RT-PCR for EGF mRNA

No mRNA EGF was detected in the gastric mucosa of normal and malnourished rats with acetic acid induced-ulcers.

Macroscopic gastric lesions after exposure to ethanol and indomethacin

The administration of absolute ethanol produced lesions with thick necrotic debris and severe hemorrhage in the glandular part of the stomach of normal whereas no such lesions were seen in malnourished rats (Figure 3). In normal rats, indomethacin-induced gastric ulcers showed punctuated necrotic lesions in the glandular portion of the stomach. Again, no such lesions were seen in malnourished rats (Figure 4).

Histological analysis of gastric lesions induced by ethanol and indomethacin

Histological examination of the oxyntic mucosa after the acute administration of absolute ethanol and indomethacin revealed damage in the gastric mucosa of normal and malnourished rats (Figures 5 and 6). In normal group surface epithelium, mucosal layer (with gastric fossets and glands) were harmed being these lesions less severe in malnourished rats. The muscle mucosal layer was not damaged in any group. Typically, erosion of the oxyntic mucosa was seen after 1 h of exposure to ethanol (Figure 5) and after 4 h of exposure to indomethacin (Figure 6).

Macro and microscopical gastric lesions after exposure to 30% acetic acid

The administration of 30% acetic acid produces large, deep ulcers in the glandular portion of the stomach in normal and malnourished rats, although the damage was greater in the latter group (Figure 7). Histological examination of the gastric ulcer produced by acetic acid showed that the damage was most severe in malnourished rats.

On the 15th day after acetic acid administration, the rats showed loss ulceration which had a punched-out appearance with a step wall and loss of the floor. These lesions were more severe in malnourished rats. The external surface of the ulcerated region was strongly adherent to the

liver, which formed part of the base of the ulcer. The margin of the ulcer was poorly defined and slightly elevated as a result of a submucosal edema. The surface epithelium, mucosal layer and muscle mucosa layer were damaged in normal and malnourished rats (Figure 8).

Discussion

Malnourishment initially affects tissues with a high cell turnover such as the intestinal mucosal; whereas the nervous system, stomach and pancreas are often the last to be affected (Sant'ana *et al.*, 1997). The administration of a low protein diet to the mother up to the end of the suckling period results in pups with a reduced body weight at birth and at weaning (Latorraca *et al.*, 1999). Malnutrition can also delay gastric emptying (Stenson 1999). Delayed gastric emptying also occurs in other illnesses and may be improved by supplements to induce weight gain. In this study, we examined the effects of perinatal protein restriction on the gastrointestinal tract. The body-weight gain of normal rats after weaning, was significantly higher than malnourished rats, indicating that maternal protein restriction during pregnancy and lactation can adversely affect offspring (Figure 1).

Protein restriction during critical periods of development can lead to generalized growth retardation and permanent reduction in the size of organs and tissues, including the pancreas and stomach (Desai *et al.*, 1995). The digestive system is affected in early life; and can have long-term effects on body weight. As shown here, most of the organs of malnourished rats weighed less than in normal rats (Table 2).

The malnourished rats had elevated plasma corticosterone levels compared to normal rats, probably as a result of the stress during pregnancy and lactation. Spadari and De Moraes (1988) reported that rats stressed by swimming sessions had increased plasma corticosterone levels. Stone *et al.* (1986) observed that stress reduced the sensitivity of the rat cerebral cortex to noradrenaline, suggesting that an increase in the plasma levels of adrenal cortical hormones could mediate the effects of stress. Corticosterone, the major adrenal steroid in the rat is released via the action of catecholamine during the repeated stress associated with malnutrition.

The gastrointestinal hormone, somatostatin, has a protective effect in various experimental models of gastric mucosal injury, i.e, ethanol and NSAIDs (Karmeli *et al.*, 1994). This protective effect is accompanied by a decreased generation of mucosal leukotrienes, substance P and vasoative peptide intestinal (VIP). Wallace *et al.* (1988) showed that

leukotrienes are potent mediators of microvascular damage. The leukotrienes alone do not cause extensive gastric mucosal damage and are therefore probably not the only mediators of gastric mucosal damage. As shown here the oral administration of ethanol, produced the characteristic zonal necrotizing mucosal lesions, probably via acid secretion and histamine release. Histological examination of the oxyntic mucosa also revealed damage to the gastric mucosal, in both groups of rats. The levels of somatostatin were low in normal and malnourished rats and did not protect the gastric tissue of these animals from damage.

In healthy human stomach and duodenum, a balance exists between the potential for gastric acid and pepsin to damage gastric mucosal cells and the ability of these cells to protect themselves from injury (Bagchi *et al.*, 1999). Disruption of this balance results from a breakdown of the normal mucosal defense mechanisms (Forsell, 1988). Several mechanisms are believed to be important in protecting gastric mucosa from damage, including mucus formation, mucosal blood flow, cell renewal and bicarbonate production. These factors help maintain the mucosal integrity. Oxygen free radicals are involved in the pathogenesis of gastric ulcer and can enhance lipidic peroxidation (D'Souza & Dhume, 1991).

The extent of ethanol-induced gastric mucosal damage in rats correlates with the content of degranulating mast cells (Diel *et al.*, 1986). Degranulating mast cells may be a source of several neuropeptides and inflammatory mediators, including histamine and leukotrienes. Ethanol-induced ulcers are not prevented by antisecretory agents such as cimetidine, but are inhibited by agents, such as PGE_2, that enhance mucosal defensive factors (Robert *et al.*, 1979). Malnourished rats generally had a lower number of ulcers than normal rats (Figure 2), perhaps because of this greater production of protective factors such as mucus and prostaglandins E_2 (PGE_2). Among the many factors that may contribute to the protective actions of prostaglandins in the stomach are the stimulation of phospholipid, mucus and bicarbonate secretion, the maintenance of gastric blood flow during exposure to an irritant factor, inhibition of inflammatory mediator release from mast cells and the inhibition of free radical production (Sun *et al.*, 1991).

The adaptation of malnourished rats to the stress of intra-uterine malnutrition may explain the lower number of ulcers. Thus, increased mucus and PGE_2 formation and higher plasma corticosterone levels are survival mechanisms against adverse factors. Several disease states increase the metabolic rate, including infection, in which the onset of fever is a hallmark of

increased metabolic rate (Weaver *et al.*, 1991). A hypermetabolic state may produce a significant and dangerous loss of body weight of malnourished rats with stress hormones contributing to the hypermetabolic state.

The gastrointestinal tract has a considerable ability to undergo adaptation (Klein & Mc Kenzie, 1993); and may grow in length, diameter and mucosal volume after resection (Dowling, 1988). Physiological stimuli leading to changes in gastrointestinal structure and function are well known.

Several studies have shown a severe decrease in gastric mucosal blood flow after treatment with indomethacin, a typical NSAID that inhibits cycloxygenase. This leads to a decrease in mucosal prostaglandin levels and a decrease in mucosal blood circulation (Trevethick *et al.*, 1995).

The use of indomethacin allows one to assess the capacity of the tissue to generate PGE_2 from endogenous arachidonic acid. The results generally correlate well with direct measurements of prostaglandin synthesis in gastrointestinal tissues (Curtis *et al.*, 1995).

Subchronic gastric ulcers were more pronounced in malnourished rats because the low protein concentration either complicated the healing of these ulcers or favored gastric ulcer formation. Szabo and Vincze (2000) reported that growth factors stimulate important cellular elements involved in ulcer healing, such as angiogenesis, granular tissue formation and re-epithelization. Basic fibroblast growth factor accelerates the healing of experimental gastric ulcers, as well as recovery from chronic erosive gastritis (Motilva *et al.*, 1996). RT-PCR of the gastric mucosa of normal and malnourished rats with acetic acid induced gastric ulcers, failed to detect EGF mRNA. This result may reflect the absence of healing in the stomach of these rats. Konturek *et al.* (1998) found no increase the EGF mRNA content of the gastric mucosa in their experiments with acetic acid induced gastric ulcer.

The response of the gastrointestinal tract of protein-restricted rats is adaptive and involves little programming. Thus, the gastrointestinal tract responds to pathological and physiological challenges via its mucosa, which is constantly being renewed (Klein & Mckenzie, 1993). The extent of the tract mean that even with loss of a considerable portion, it is still able to support the nutritional needs of the animal (Weaver *et al.*, 1991).

Ligation of the pylorus caused a significantly increase in the volume and pH of the gastric juice in malnourished rats, and reduced the gastric acidity in these animals (Table 4). Prostanoids

are probably involved in the accumulation of fluid in the gastric lumen and mucus and PGE_2 can significantly increase the volume flow in the stomach of malnourished rats, as discussed above.

We conclude that the gastric mucosa of malnourished rats is less damaged by acute gastric ulcer than that in normal rats because the former have a greater production of PGE_2 and mucus in the gastric lumen, thereby increasing the volume of gastric juice. This situation is related to adaptations that occur during intra-uterine malnutrition. In subchronic experiments, malnutrition complicates the prevention of gastric ulcer formation and delays recovery. In malnourished rats, there is a serious ulcer formation because the stress and low protein content of the diet make the mucosa more suceptible to damage.

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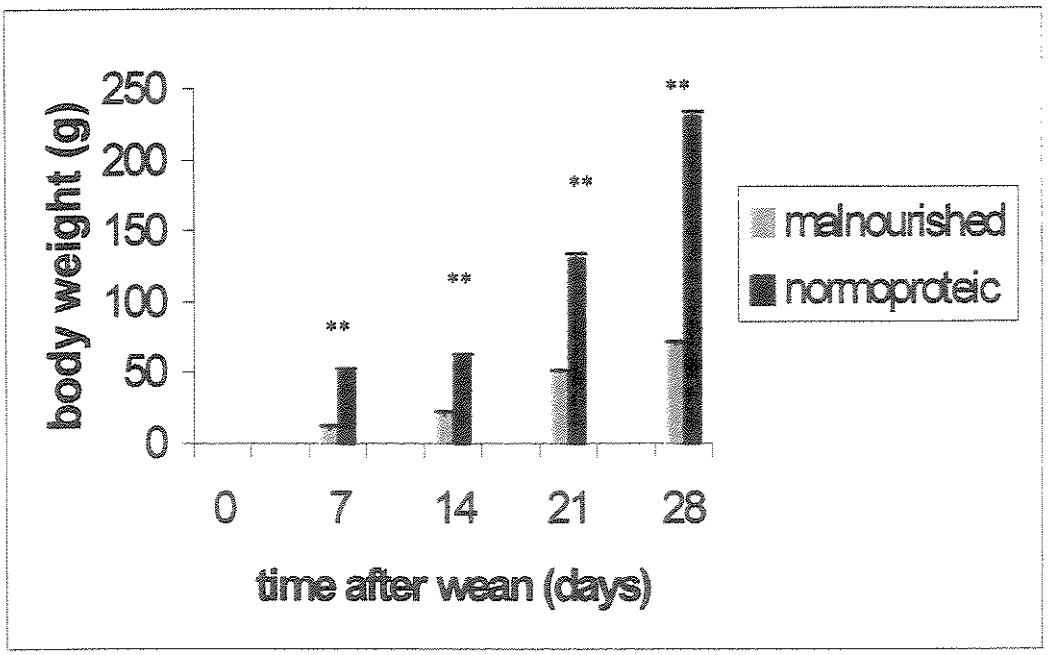


Figure 1: Determination of body weight (g) of normoproteic and malnourished animals since the wean (day 0). The date are the mean ± SEM of 16 animals in both animal groups. ** $p < 0,001$ to Student t- test.

Table I: The hipoproteic diet (6% of protein) was compared with the formulation offered by the American Institute of Nutrition to rodents in growthing, gestation and lactation conditions (AIN-93).

Ingredient	Normal protein (170g protein/kg)	Low protein (60g protein/kg)
Casein (840g protein/kg)	202.0	71.5
Maize Starch	397.0	480.0
Dextrinized maize starch	130.5	159.0
Sucrose	100.0	121.0
Soybean oil	70.0	70.0
Fiber	50.0	50.0
Mineral mix (AIN-93)*	35.0	35.0
Vitamin mix (AIN-93)*	10.0	10.0
L-Cystine	3.0	1.0
Choline Chlorhydrate	2.5	2.5

* For a detailed composition to see Reeves, 1993.

Table II: Organs weights of normal and malnourished animals, as described in the text.

Organs	*Organ weight (g/100 body weight)* *Normoproteic rats (n=15)*	*Organ weight (g/100 body weight)* *Malnourished rats (n=14)*
Stomach	1.10 ± 0.08	0.6 ± 0.06 (**)
Liver	3.30 ± 0.98	1.87 ± 0.09 (**)
Heart	0.58 ± 0.03	0.36 ± 0.01 (**)
Pancreas	0.45 ± 0.08	0.18 ± 0.01 (**)
Mesentery	0.85 ± 0.05	0.45 ± 0.02 (*)
Kidney	0.87 ± 0.02	0.32 ± 0.03 (**)

The values are means ± SEM. **p<0,001 and * p<0,05 Student's t-test

Table III: Gastric ulcer formation by the injection of 30 % acetic acid solution (0.05 ml) in normal and malnourished rat stomach walls treated 14 days later with 12% Tween 80.

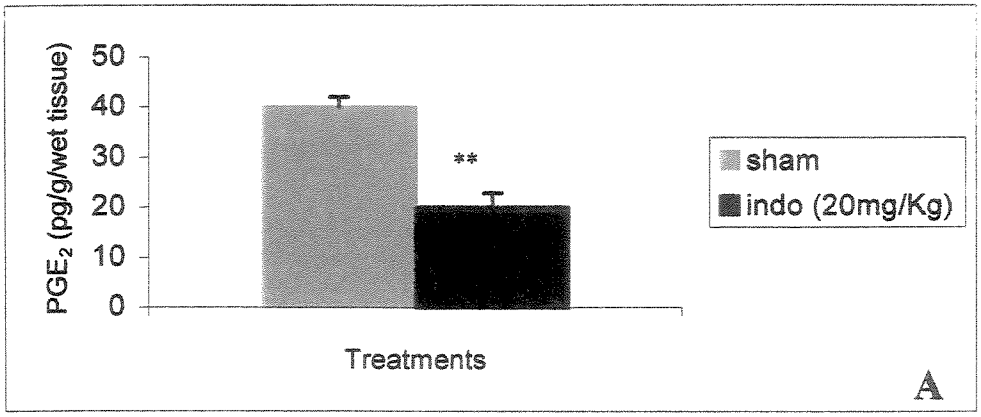
Parameter	*Normal Rats (n=11)*	*Malnourished Rats (n=13)*
Lesion area (mm^2)	26,3 ± 1,58	51,9 ± 1,20**
pH	3,0 ± 0,25	2,0 ± 0,80

The values are the mean ± SEM ,**p<0,001 (Student's test)

Table IV: Biochemical parameters: gastric juice volume, total gastric acid and pH in malnourished and normal rats following ligation of the pylorus.

Rats (n=7)	*pH (units)*	*Gastric Juice (ml/tempo)*	*Total Gastric Acid ($mEq\ ml^{-1}/4h$)*
Normal	3.17 ± 0.20	1.18 ± 0.27	7.57 ± 0.27
Malnourished	5.86 ± 0.17*	4.05 ± 0.31*	4.00 ± 0.31*

The values are the mean ± SEM, *p<0,05 (Student t- test)



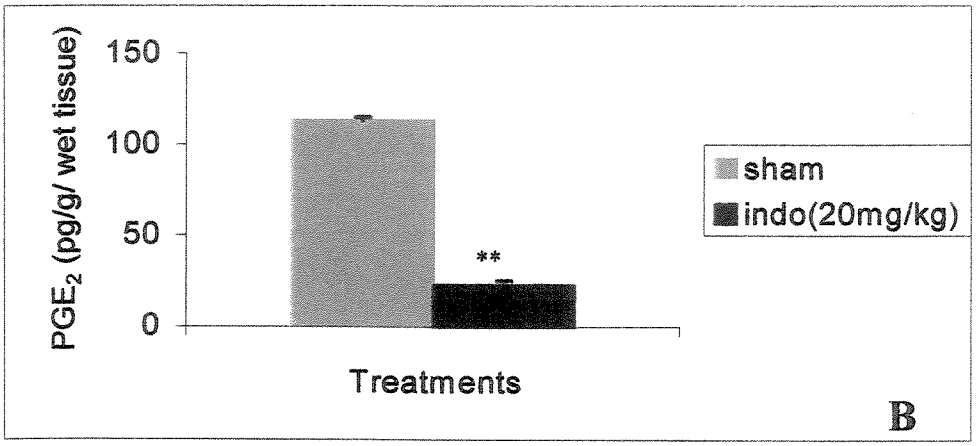


Figure 2: Gastric PGE_2 production in normal (A) and malnourished (B) rats in the absence and presence of indomethacin. Each column represents the mean ± SEM of 7 rats. **$p<0.001$ (Student t-test).

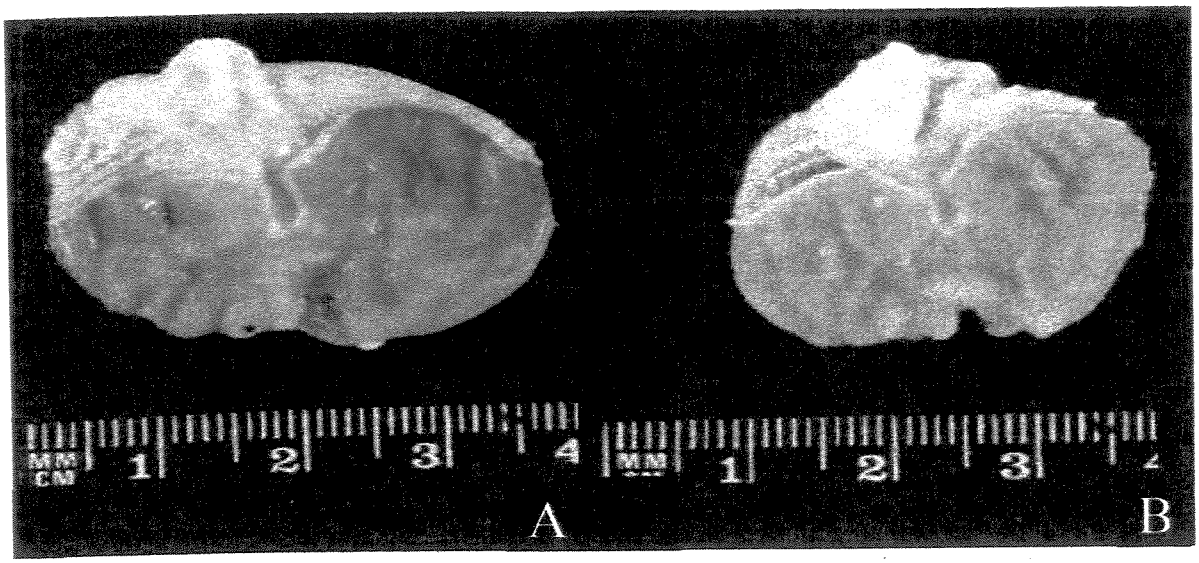


Figure 3: Gastric ulcers produced by absolute ethanol in normal (A) and malnourished rats (B) rats. Ulceration was observed 1h after the ingestion of ethanol. Severe hemorrhage can be seen in the glandular portion of the stomach in (A) but not in (B).

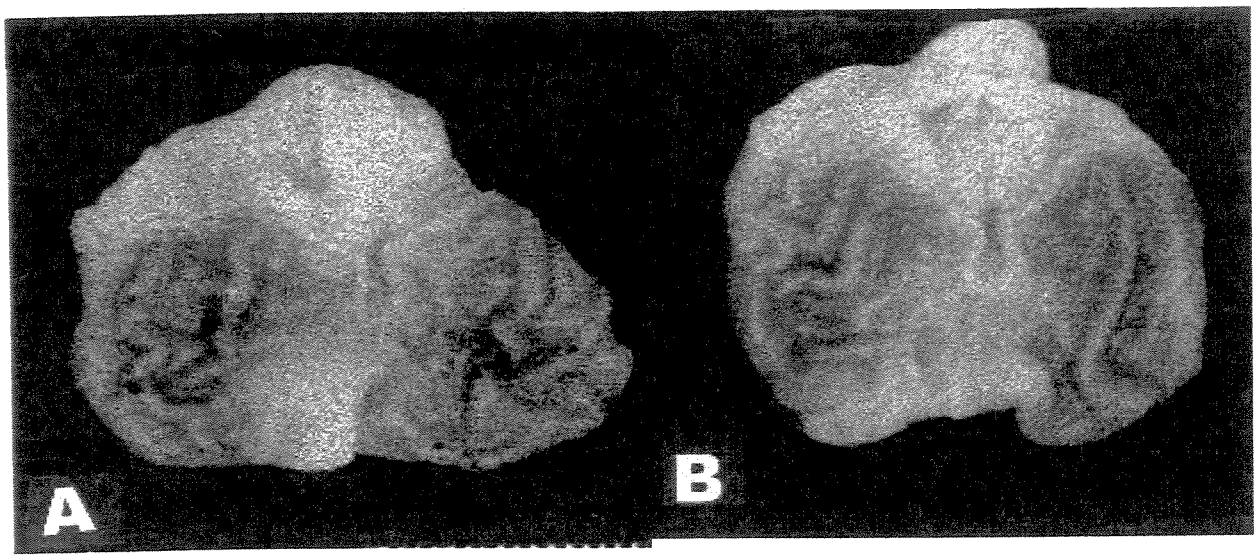


Figura 4: Gastric ulcers produced by indomethacin in normal (A) and malnourished (B) rats. Indomethacin-induced gastric ulcers showed punctuated necrotic lesions in the glandular portion of the stomach in (A) but not in (B).

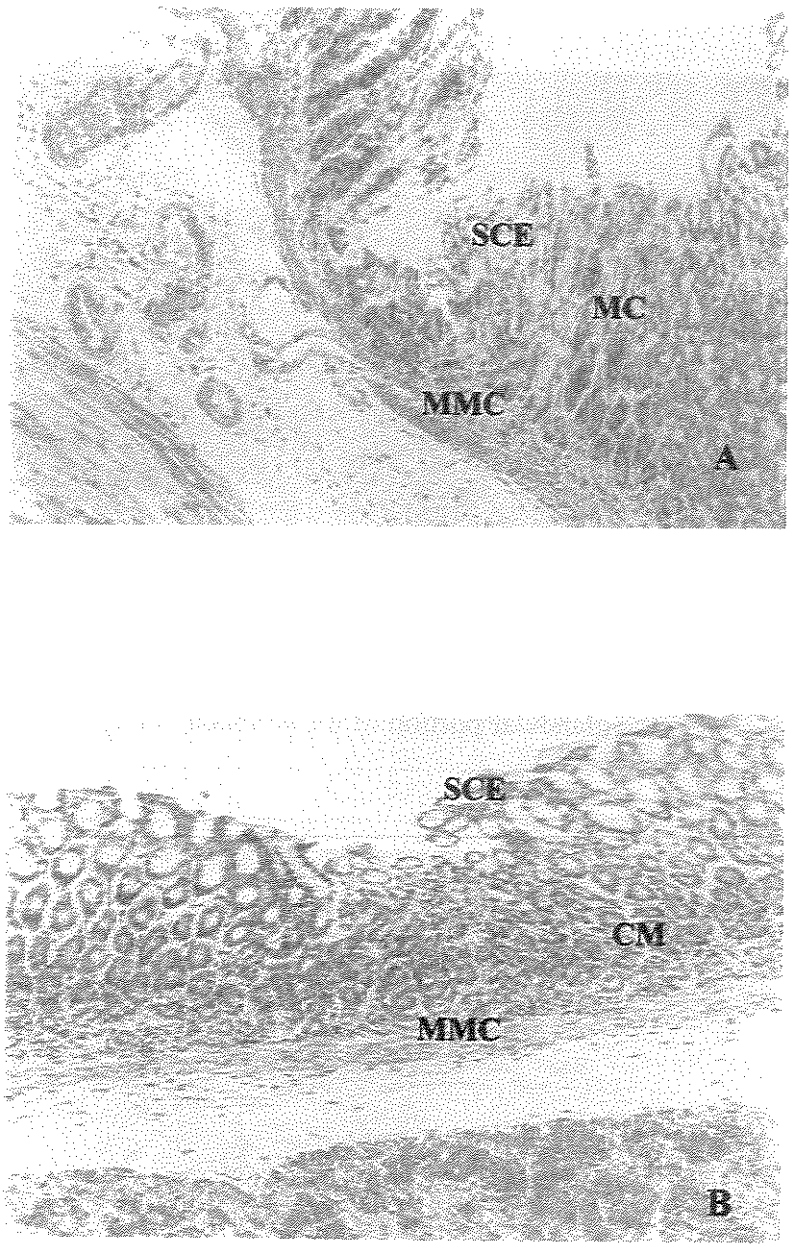


Figure 5: Histological appearance of a typical erosion in the oxyntic mucosa after 1 h of exposure to absolute ethanol in normal (A) rats compared to malnourished rats (B). In both groups, surface epithelium, mucosal layer were affected although this was less marked in malnourished rats. The muscle mucosal layer was not damaged in either group. Hematoxylin and eosin stain. X400.

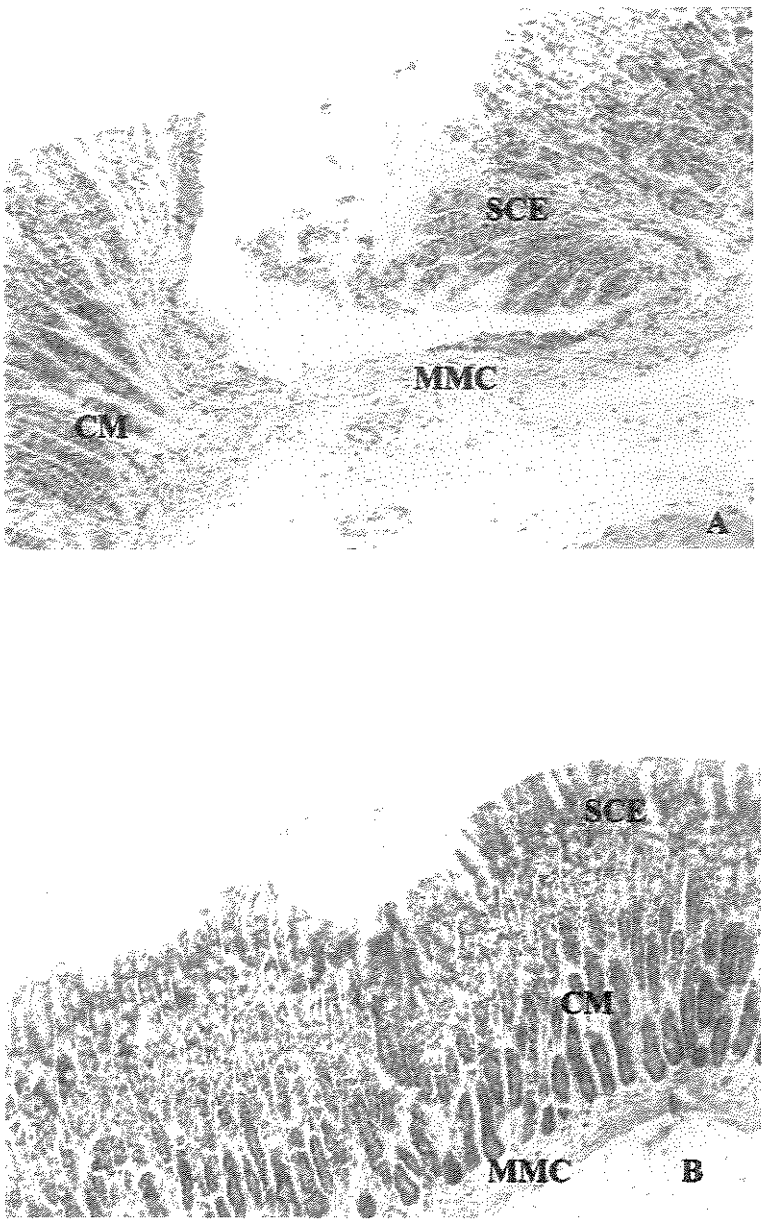


Figure 6: Histological appearance of a typical erosion in the oxyntic mucosa after 4 h of exposure to indomethacin in normal rats (A) compared to malnourished rats (B). In both groups, surface epithelium, mucosal layer were affected although this was less marked in malnourished rats. The muscle mucosal layer was not damaged in either group. Hematoxylin and eosin stain. X400

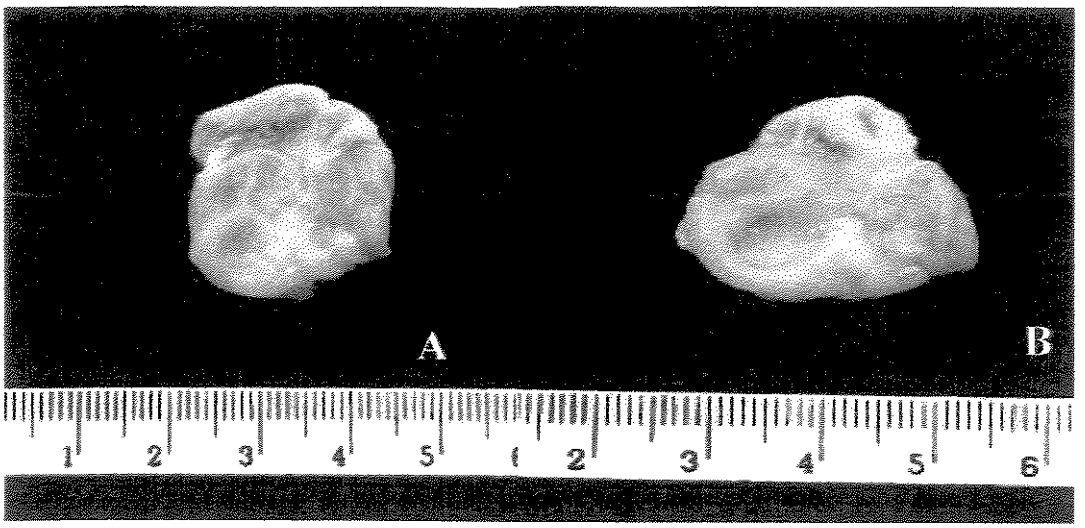


Figure 7: Gastric ulcers produced by the injection of 30% acetic acid solution (0.05 ml) into the stomach wall of rats. Panels A and B show the ulceration observed on the 14th day after treatment in normal and malnourished rats respectively. Note the large deep and ulcer in the glandular portion of the stomach and the thick necrotic debris at the bottom of the ulcer.

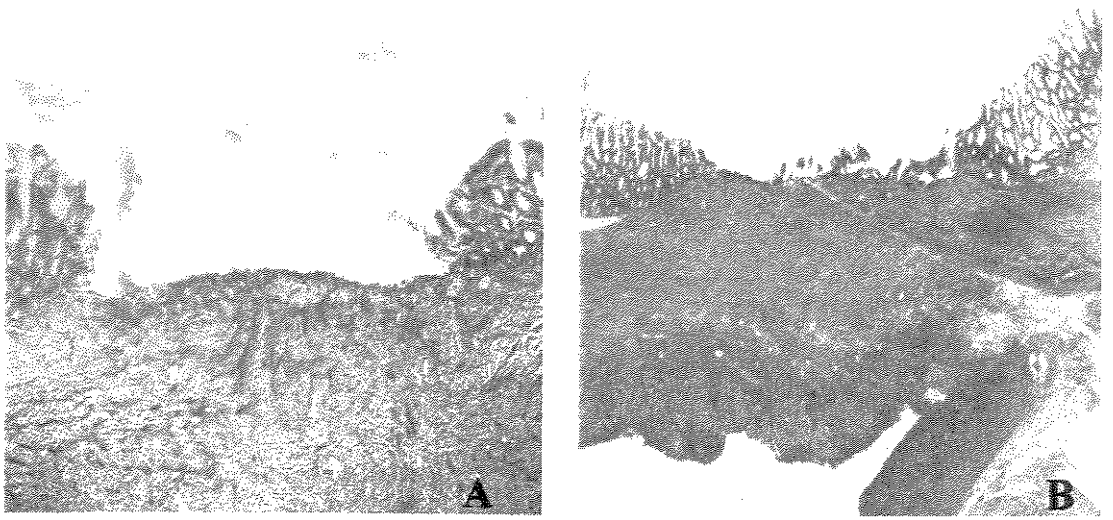


Figure 8: Histological appearance of gastric ulcers on the 14th day after the injection of 30% acetic acid solution (0.05 ml) into the stomach walls of normal (A) and malnourished (B) rats. Note the penetration of the fibrous tissue associated with healing in the wall gastric ulcer. Hematoxylin and eosin (magnification X400).

Essential oil of *Croton cajucara* Benth protects against acute gastric ulcer in normal and malnourished rats

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Abstract

Croton cajucara Benth (Euphorbiaceae) is a well-known medicinal plant used in the Amazonian folk medicine for the treatment of a wide range of gastrointestinal symptoms. The essential oil from the bark of *C. cajucara* was investigated for its ability to prevent the formation of ulceration of the gastric mucosa in a model of experimentally induced gastric ulcer in rats that received a diet with 17% protein (control) and rats that received a diet with 6% protein (malnourished). When previously administered (p.o.) at the dose of 100 $mgkg^{-1}$, the essential oil significantly reduced ($p<0.01$) the gastric injury induced by indomethacin (70% after 4 h and 25% after 2 h); in the pylorus ligature model the essential oil induced alterations in gastric juice after 4 h (increased pH and gastric volume and decreased total acid concentration) in both groups, compared to the group treated with Tween (control group). We also observed that this drug induced a significant increase in PGE_2 production by glandular cells (50% compared to the control) in both groups, with the amount of gastric mucus increasing to levels two times higher than that of the control group ($p<0.001$ and $p<0.05$, respectively).Thus, the protective effect of essential oil from *C. cajucara* bark against induced gastric lesions was more effective in malnourished rats due to an increase in PGE_2 production and mucus secretion, corresponding to an increase in factors protecting the gastric mucosa.

Key words: *Croton cajucara*, antiulcerogenic activity, malnourished animals, medicinal plants, cytoprotection, essential oil.

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INTRODUCTION

Undernutrition in man and other mammals is associated with metabolic alterations. Maternal protein restriction during pregnancy and lactation can adversely affect the offspring (1). Protein restriction during critical periods of development can lead to generalized growth retardation and permanent reduction in the size and physiology of organs and tissues, including the pancreas and the stomach (2). There are no data describing malnutrition and the "repair" process of gastric ulcer.

Croton cajucara Benth is a well-known medicinal plant used in the Amazonian folk medicine to treat several illnesses such as diabetes and liver inflammation (5) and to control high cholesterol (3; 4). Other medicinal uses include a weak tea (prepared with only 5 g stem bark in 100 ml water) to be ingested in cases of heartburn, gastritis and peptic ulcer (6). The essential oil (up to 1.5 % dry bark) contains primarily sesquiterpenes (7). Oral administration of the essential oil displayed also a significant anti-ulcerogenic activity with no toxicological effects (8).

Since no studies of the anti-ulcer activity of *Croton cajucara* are available, the possible effects of this plant are currently being investigated using various agents which operate by distinct mechanisms of ulcerogenesis and that are used to induce gastric ulcers in animals. Thus, we analyzed the antiulcerogenic activity of the essential oil from *Croton cajucara* Benth in a model of acute gastric ulcer and its antiulcerogenic mechanism. The characteristics of the lesions were analyzed by morphology analysis.

MATERIAL AND METHODS

Animals

Female Wistar rats (90 days old) were obtained from the animal facilities of the State University of Campinas. After the mating pregnant females were separated at random and maintained on an isoenergetic diet containing 60 g protein/kg (lower protein/ LP diet) or 170 g protein/kg (normal protein/ NP diet) from the first day of pregnancy until the end of the lactation period (25).

The low protein and normal protein diets (6% and 17% protein, respectively) were based on the AIN-93 (report of the American Institute of Nutrition, establishing dietary standards for nutritional studies with laboratory rodents). The experimental protocols were approved by the

Animal Use and Care Committee of UNICAMP. All of the experiments were conducted in accordance with the recommendation of the Canadian Council on Animal Care (9).

During the experimental period, the mothers were fed their respective diets *ad libitum* and had free access to water.

The animals were kept under standard lighting conditions (12 h ligth-dark cycle) at a temperature of 24° C and food intake was monitored daily.

At 25 days of age, the pups were weaned and maintained on their mothers' diet. After 90 days the normal and malnourished animals were used in experiments for the determination of the anti-ulcerogenic activity of the *C. cajucara* essential oil.

Extraction, preparation and analysis of the essential oil

The stem bark of *Croton cajucara* Benth, was collected from an experimental plantation at Benfica, near Belém (Pará, Brazil). A voucher specimen (number 247) has been identified by Nelson A. Rosa and deposited in the IAN Herbarium, in Belém. The bark (20 kg) was subjected to steam distillation for 6 h, and the first fraction of 163 ml (F1) was collected after 3 h and a second fraction of 42 ml (F2) at the end of the process. Preliminary GC-FID and GC-MS analysis performed with a Hewlett Packard system using an HP-5 capillary column showed very similar patterns for the F1 and F2 fractions, which were composed mainly of $C_{15}H_{24}$ sesquiterpenes. α-Copaene (20.9%) and cyperene (29.0%) were the main components of F1, as confirmed by ^{13}C NMR spectra measured with a Varian spectrometer operating at 75.4 MHz and using benzene as solvent (8). Complete analysis of the samples are in progress. The F1 fraction was used for the pharmacological tests. F1 was emulsified with 12% Tween 80 before administration to the animals.

Drugs

The following drugs cimetidine (Tagamet® Smithkline), Tween 80® indomethacin and carbenoxolone (Sigma Chemical Co, U.S.A.) were used in this study. Indomethacin was prepared in sodium bicarbonate (5%), and essential oil was dissolved in 12% Tween 80®. All reagents were of a high purity grade. The substances and reagents were prepared immediately before use.

Anti-ulcerogenic activity

Shay ulcer. A total of 42 animals (normal and malnourished) were randomly divided into three groups and fasted for 24 hours, with free access to water. Thirty minutes after intraduodenal administration of essential oil (100 mg kg^{-1}body wt), cimetidine (100 mg kg^{-1}body wt) as positive control or a 12% solution of Tween 80 (10ml kg^{-1}body wt) as vehicle, was realized a pylorus ligature (10). The animals were killed four hours later, the abdomen was opened and the stomach was removed and inspected internally, and its content drained into a graduated centrifuge tube and centrifuged at 2000 rpm for 10 min. The supernatant volume and pH were recorded with a digital pHmeter (PA 200, Marconi S.A., Brazil). The total acid content of gastric secretion was also determined by titration to pH 7.0 with 0.05 M NaOH using a digital burette (E.M., Hirschmann Technicolor, Germany). Gastric lesions were evaluated by examining the inner gastric surface with a dissecting binocular microscope and the mucosal lesions were counted and scored.

Indomethacin ulcer. A total of 42 normal and malnourished rats were randomly divided into 3 groups each, and fasted for 24 h, with free access to water before the experiment.

One, two and four hours after oral administration of essential oil (100 mg kg^{-1}body wt), cimetidine (100 mg kg^{-1}body wt) or 12% Tween 80 (10 ml kg^{-1}body wt), 30 mg kg^{-1} body wt of indomethacin was subcutaneously administered to anaesthetized rats from each group (11). Indomethacin was dissolved in a 5% bicarbonate solution. The animals were killed 4 h later, the stomachs removed and opened and the gastric lesions were determined as described above.

Prostaglandin synthesis determination The normal and undernourished rats were deprived of food for 24 h prior to the experiment and all experiments were performed between 09:00 and 11:00 a.m. Groups consisting of at least 7 rats received one of the following solutions: 12% Tween 80 (vehicle, p.o.), indomethacin (20 mg kg^{-1}body wt, s.c.) used as a positive control, and essential oil (100 mg kg^{-1}body wt). The combination of essential oil and indomethacin was assayed and essential oil administration was followed 30 min after, by indomethacin treatment. Indomethacin was dissolved in 5% sodium bicarbonate solution, pH 8.3. Thirty min after this treatment, the animals were killed and their abdomen was opened. A sample of the corpus (full thickness) was excised, weighed and then suspended in 1 ml of 10 mM sodium phosphate buffer,

pH 7.4. The tissue was finely minced with a scissor and then incubated at 37° C, for 20 min. PGE_2 in the buffer was measured by enzyme immunoassay (EIA RPN222 – Armersham). The absorbance was read at 450 nm (12).

Determination of gastric wall mucus. Gastric wall mucus was determined by the method of Rafatullah et al (13). A total of 48 rats (normal and malnourished) were randomly divided into 3 groups each and fasted for 48h, with free access to water. Each group was orally treated with essential oil (100 mg kg^{-1}body wt), carbenoxolone (200 mg kg^{-1}body wt), and 12% Tween 80 (10 ml kg^{-1}body wt), respectively. After 1.5 h, animals were submitted to pylorus ligature as described previously. The animals were then sacrificed and their stomach were removed and opened along the larger curvature. Glandular segments from the stomach were removed and weigthed. Each segment was immersed for 2 h in 10 ml of 0.1% w/v Alcian blue, dissolved in 0.16 M sucrose solution buffered with 0.05 M sodium acetate, pH 5.8. Excess dye was removed by washing the segments twice with a 0.25 M sucrose solution for 15 and 45 min, respectively. The mucus-dye complex was extracted by placing the gastric walls in 10 ml of 0.5 M magnesium chloride and shaking intermittently for 1 at 30 min intervals for 2 h. Four milliliters of blue extract were mixed with an equal volume of diethyl ether and shaken vigorously for 2 min. The emulsion obtained was centrifuged for 10 min at 3600 rpm and the absorbance of the aqueous layer was recorded at 580 nm. Alcian blue extracted per gram of net glandular tissue was calculated.

Determination of gastric secretion A total of 36 normal and malnourished rats were randomly divided into three groups each and fasted for 24 hours with free access to water. The assay was performed by the method of Shay, with modifications (10). Immediately after pylorus ligature, essential oil (100 mg kg^{-1}), cimetidine (100 mg kg^{-1}) or a 12% Tween 80 solution (10 ml kg^{-1}) were administered intraduodenally. The animals were killed 3h later and the same procedures described for Shay ulcer were followed.

Morphological analysis

After sacrifice of the animals that were submitted to indomethacin induced ulcer (acute lesion), stomach samples were collected, fixed in Bouin for 24 h, dehydrated in an increasing

ethyl alcoholic series, cleared with xylene, embedded in Histosec (Merck –11609), and prepared for microtomy. The sections were then deparaffinized and rehydrated with a decreasing ethyl alcohol series. Next, the samples were stained with hematoxylin-eosin for morphologic analysis of the lesion (14). Hematoxylin was used for 15 min. The samples were washed in running water for 10 min, with sections changing in color from red to blue. The sections were stained with eosin for 10 min and for washed in water, dehydrated, placed in 95% alcohol, cleared and mounted in resin (15). Photomicrographs were obtained with an Axiophot Photomicroscope (Carl Zeiss, D-7082).

Statistical analysis

Results were expressed as mean ± S.E.M. Data were analyzed by one-way analysis of variance followed by Tukey and Scheffe's tests, with the level of significance set at $p<0.05$ and $p<0.001$. All statistical analysis were performed using the Systat software (version 5.0).

RESULTS

In the pylorus ligature tests, the administration of the essential oil (100 mg kg^{-1} body wt) by the intraduodenal route, produced a significant modification in gastric volume, pH and gastric acid content in normal and malnourished animals, $p<0.05$ and $p<0.001$, respectively (Table 2). Thus, the oil significantly reduced acidity and increased gastric juice volume and pH. Similarly, the positive control cimetidine administered by the same route (100 mg kg^{-1} body wt) also significantly reduced acidity gastric and increased gastric pH and juice volume.

Oral administration of essential oil for two and four hours at the dose of 100 mg/kg^{-1} body wt inhibited the appearance of gastric lesions induced by indomethacin in normal and undernutrition animals, suggesting the probable involvement of the oil in prostaglandin production. After one hour, administration of the essential oil at the same dose did not produce inhibition of gastric lesions in normal rats. However, malnourished animals had a lower number of ulcers than normal animals before and after this treatment (Figure 1).

The data obtained for gastric PGE_2 production using enzyme immunoassay (EIA) are shown in Figure 2. There was an increase in PGE_2 production by gastric tissue in response to the essential oil-treated normal (within 1 hour) and malnourished rats (within 4 hours), in oppose a significant decrease in gastric tissue from indomethacin treated normal and malnourished rats

(Figure 2). An important result was obtained when both groups of animals were treated with a combination of the two drugs, i.e., the increase in PGE_2 obtained by the treatment with essential oil was completely abolished by indomethacin.

We also studied the effect of *Croton cajucara* on free mucus production by gastric mucosa of normal and malnourished animals. Mucus production was doubled in both groups, but the malnourished animals showed a greater production of this defensive factor (Figure 3).

It was possible to verify that the positive control carbenoxolone (an antiulcerogenic drug obtained from *Glycyrrhiza glabra*) acts by increasing the production of mucus by the gastric mucosa and the essential oil of *Croton cajucara* Benth. also increased the production of gastric wall mucus.

Photos obtained with histologic studies of normal and hipoproteic stomach cells treated or not treated with essential oil; are shown in figures above. In the figure 4 is showed the erosion induced by acute treatment of indomethacin in the pyloric region of the stomach of normal (4A) and low protein (4B) rats pretreated with Tween 80. In (A) superficial coating epithelium and mucosal coat are destroyed, however in (B) only superficial coating epithelium is harmed. In figure 9, the pre-treatment of *Croton cajucara* Benth (100 $mgKg^{-1}$) protect stomach cells from erosion induced by indomethacin in normoproteic (A) and malnourished (B) rats. Finally, in figure 6 (sham group), the gastric wall upright of normoproteic (A) and malnourished (B) animals with superficial coating epithelium and mucosal coat preserved.

DISCUSSION

Protein undernutrition results when the body's needs for protein cannot be satisfied by the diet. Considering that all normal metabolic processes need proteins, it is believed that all tissues will be affected by a state of poor protein nutrition. The first tissues to suffer alterations with protein loss are those that exhibit a high rate of cellular turnover, like the intestinal mucosa and the last are those that show a low rate of cellular renewal, such as the nervous system, with the stomach and pancreas showing a loss of weight and size (16).

In a healthy human stomach and duodenum, an effective balance exists between the potential for gastric acid and pepsin to damage gastric mucosal cells and the ability of these cells to protect themselves from injury (17). Disruption of this balance results from a breakdown of the normal mucosal defense mechanisms (18). Several mechanisms are believed to be important

in protecting gastric mucosa from damage, such as mucus, mucosal blood flow, cell renewal and bicarbonate production acting together. These factors help to maintain the mucosal integrity. It has been suggested that oxygen free radicals are strongly involved in the pathogenesis of gastric ulcer that can result in enhanced lipid peroxidation (19). Among the many factors that may contribute to the protective actions of prostaglandins in the stomach are the stimulation of phospholipid, mucus and bicarbonate secretion, maintenance of gastric blood flow during the exposure to an irritant factor, inhibition of inflammatory mediator release from mast cells, and inhibition of free radical production (20).

Several studies have shown a severe decrease in gastric mucosal blood flow after treatment with indomethacin, a typical non steroidal anti-inflamatory (NSAID) that inhibits cycloxygenase, resulting in decreased mucosal prostaglandin levels and decrease mucosal blood circulation (21).

The essential oil of *Croton cajucara* Benth protected the gastric mucosa of normal and malnourished animals against the injury induced by indomethacin after 2 and 4 hours, showing that the anti-ulcerogenic effect of the essential oil of *C. cajucara* increased prostaglandin synthesis and/or release.

Our experiments of induction of acute ulcer by indomethacin demonstrated that malnutrition animals have a lower number of ulcers than normal animals (Figures 1 and 4). So, we can verify that the gastric mucosa of the malnutrition animals has a major protection against injure due to enhance mucosal defensive factors such as prostaglandin E_2 (PGE_2) and/or mucus synthesis.

Looking for the possible mechanisms that increase the mucosal protective factors, we investigated the effect of essential oil on PGE_2 production. Our data demonstrated that essential oil increases PGE_2 production, an effect which is completely abolished by pre-treatment with indomethacin. Therefore, essential oil has an anti-ulcerogenic effect by increasing gastric juice volume with a low acidity and enhancing PGE_2 synthesis in normal and malnourished animals. These two effects are observed when essential oil is administered before lesion induction, which means that the compound has a preventive anti-ulcerogenic effect (Figures 1 and 5).

The results of the present study showed that essential oil (100 mg kg^{-1} p.o.) from *C. cajucara* Benth. increased mucus synthesis and/or retention and increased the luminal PGE_2 concentration by 2-fold over basal levels in both animal groups (Figures 2 and 3).

Interesting results were obtained when these animals were treated with a combination of both drugs, essential oil and indomethacin. The increase in PGE_2 obtained in the treatment with essential oil was completely abolished by indomethacin, indicating that latter clearly inhibits the PGE_2 raise induced by essential oil (Figure 2).

This technique allows the determination of the capacity of the tissue to generate PGE_2 from endogenous arachidonic acid and has been shown to correlate well with measurements of prostaglandins synthesis by gastrointestinal tissues (12).

In the subsequent tests we showed the biochemical results obtained after submitting the animals to pyloric ligature. The intraduodenal administration of cimetidine and essential oil had similar effects on the parameters analyzed. So, like cimetidine, essential oil was also effective in reducing gastric acidity and in increasing the volume and pH of gastric juice in both groups of animals (Table 2).

There is evidence of an involvement of prostanoids in the accumulation of fluid in the gastric lumen, with PGE_2 causing a significant increase of volume flow in the stomach (22).

In the evolution of the experimental ulceration process a fully developed inflammatory response leading to cellular infiltration, extracellular matrix proliferation and establishment of a new microvascular supply may occur.

Necrotic tissue and debris must be removed for angiogenesis to occur. However, the inflammatory response leads to several alteration and changes associated with an inflammatory cell infiltrate (23). Motilva et al reported that neutrophils migrate to the interstitial space directed by chemotactic agents and, thereafter, release oxygen-derived free radicals and proteases, a process resulting in neutrophil-dependent inflammatory tissue injury. Although the inflammatory response is essential for maintaining normal health, excessive and/or inadvertent recruitment and metabolic activation of neutrophils results in neutrophil-dependent inflammatory tissue injury.

In a previous work from our laboratory (24) we suggested the hypothesis that essential oil from *C. cajucara* Benth (100mg/Kg $^{-1}$, p.o.) increased mucus synthesis and/or retention and increased the luminal PGE_2 concentration 2-fold over basal levels in normal animals. Here an interesting result was obtained when the malnourished animals were treated with this essential oil, i.e., the gastric ulcer healed more easily in malnourished than normal animals.

In conclusions, these results suggest that essential oil has good preventive effect on gastric ulcers, mainly by protecting the gastric mucosa by an increase in PGE_2 and mucus production, or that the efficacy of essential oil is based on its ability to strengthen defensive factors such as stimulants of mucus synthesis and to maintain the prostaglandin content of gastric mucosa at hight levels.

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Table 1: Composition of the normal protein and low protein diets

Ingredient	Normal protein (170g protein/kg)	Low protein (60g protein/kg)
Casein (840g protein/kg)	202.0	71.5
Maize Starch	397.0	480.0
Dextrinized maize starch	130.5	159.0
Sucrose	100.0	121.0
Soybean oil	70.0	70.0
Fiber	50.0	50.0
Mineral mix (AIN-93)*	35.0	35.0
Vitamin mix (AIN-93)*	10.0	10.0
L-Cystine	3.0	1.0
Choline Chlorhydrate	2.5	2.5

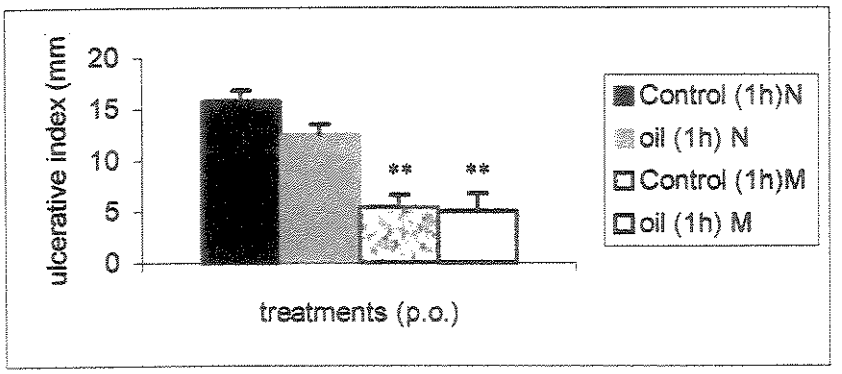
* For a detailed composition, see [25]

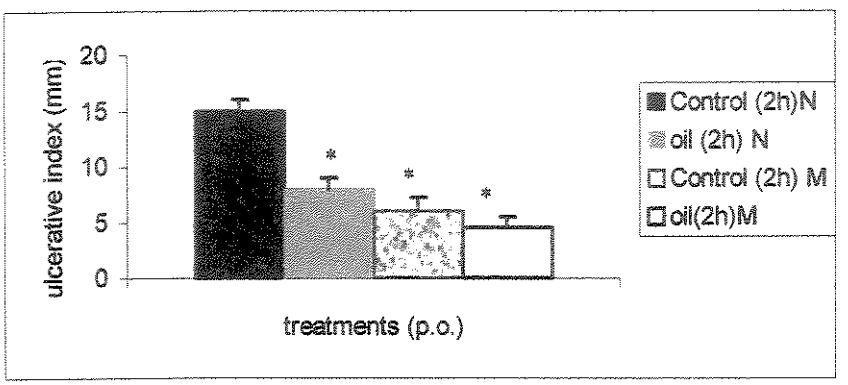
Table 2: Effects of essential oil (100 mg Kg^{-1}) given by the intraduodenal (i.d.) route on the biochemical parameters of gastric juice obtained from pylorus-ligated normal and malnourished rats.

Animals (n=7)	**Treatments**	pH (units/minutes)	Gastric Juice Volume (ml)	Total Gastric Acid (mEq ml^{-1}) 4 hours
Normal Rats	Control	3.17 ± 0.20	1.18 ± 0.27	7.57 ± 0.27
	Cimetidine	6.00 ± 0.17**	4.37 ± 0.24**	4.93 ± 0.24**
	Oil	5.50 ± 0.15*	3.83 ± 0.27*	5.96 ± 0.27**
Malnourished Rats	Control	2.86 ± 0.17	4.05 ± 0.31*	5.00 ± 0.31
	Cimetidine	4.17 ± 0.20**	5.21 ± 0.31**	4.40 ± 0.40*
	Oil	3.94 ± 0.17*	3.91 ± 0.40*	3.36 ± 0.30**

Data are expressed as mean ± SEM. ANOVA: $p<0.05$; pH $F_{(5,35)}= 6.905$; Gastric Juice Volume (ml) $F_{(5,40)} = 3.696$ and Total Gastric Acid (mEq ml^{-1}) $F_{(5,39)} = 3.812$.

Tukey' test *$p<0.05$ e **$p<0.001$.





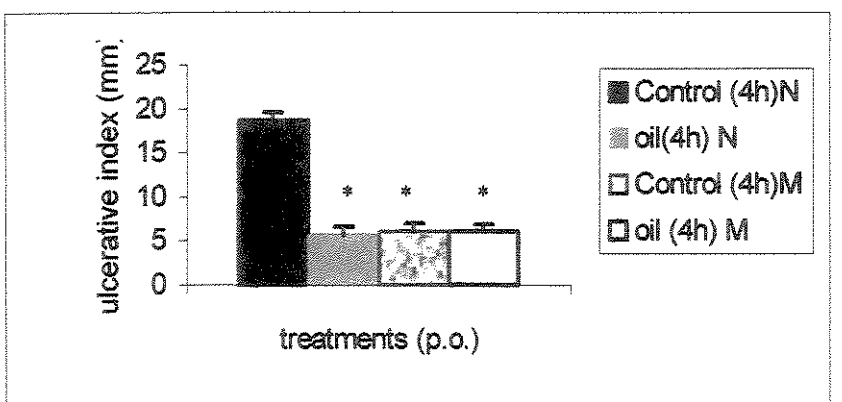
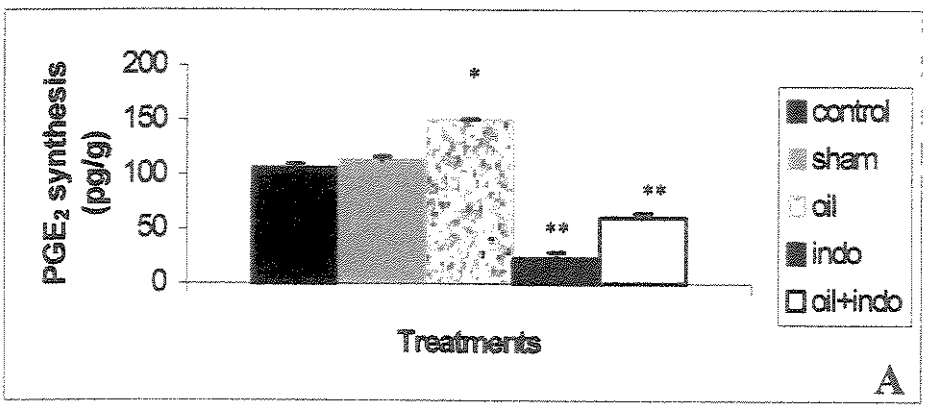


Figure 1: Effects of essential oil (100mg Kg^{-1} by oral route) for one, two and four hours of *Croton cajucara* Benth on the formation of gastric ulcer induced by indomethacin in normoproteic (N) and malnourished (M) rats. The results are expressed as mean ± SEM (n=7). ANOVA: $F_{(11,72)}$=2.680. Scheffe´s test: *p<0.05 and **p<0.001 in relation to control animals treated with 12% Tween 80.



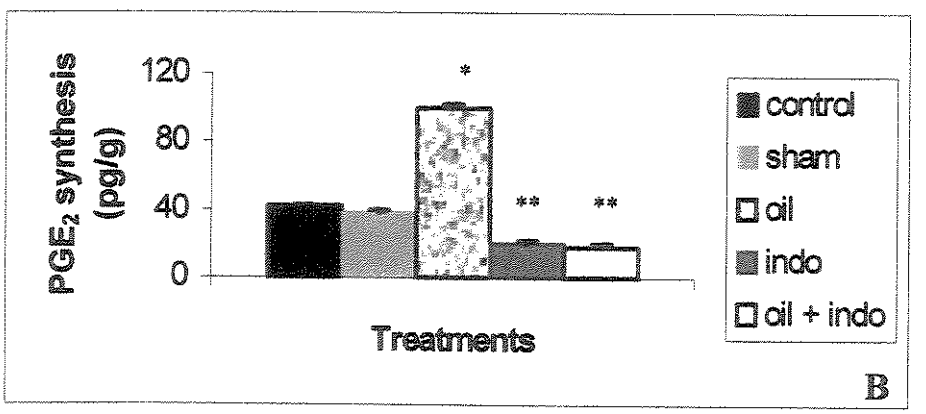
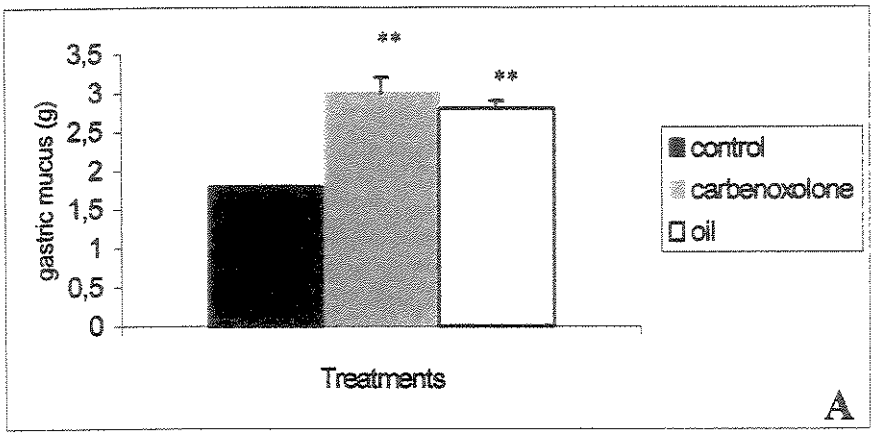


Figure 2: Effects of oral administration of essential oil and indomethacin for one hour on gastric PGE_2 production in malnourished (A) and normoproteic (B) rats. Each group is the mean ± SEM for 7 animals. ANOVA: $F_{(4,30)}$= 27.4 ($p<0.05$) followed by Tukey's test, * $p<0.05$ and ** $p<0.001$.



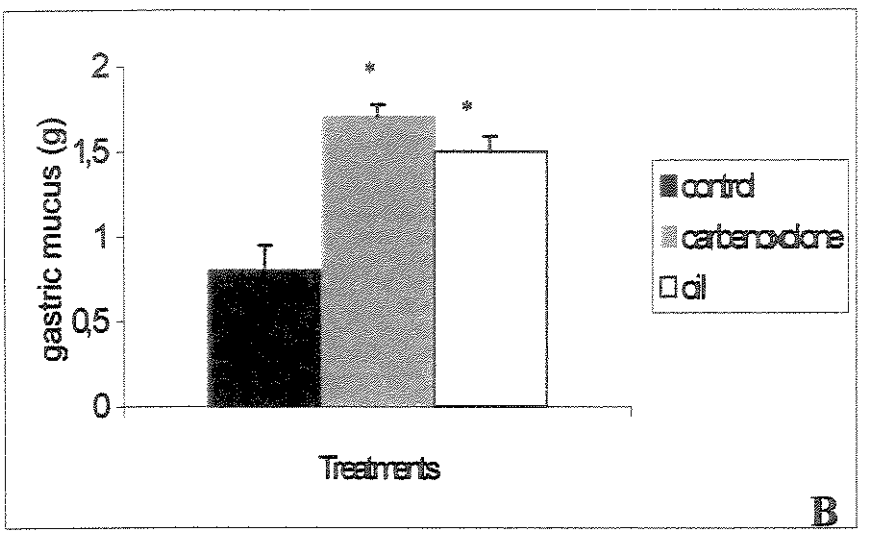


Figure 3: Effects of essential oil of *Croton cajucara* Benth. and positive control (carbenoxolone) on the production of gastric wall mucus in malnourished (ANOVA: $F(7,16)= 6.58$, $p<0.05$) (A) and normal (ANOVA: $F_{(7,16)}=6,15$, $p<0.05$) (B) animals. Results are expressed as means ± SEM for 8 animals, followed by Tukey'test where *$p<0.05$ and **$p<0.001$.

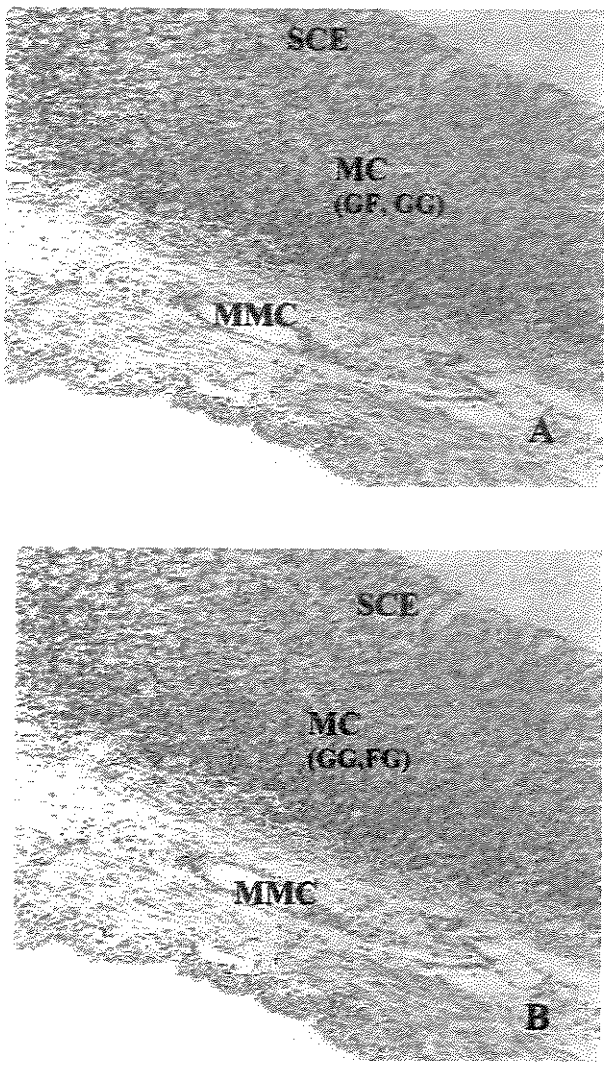


Figure 4: Photograph showing sham animals. In (A) representing the normal rats and in (B) low protein rats. In both groups the superficial coating epithelium (SCE), mucosal coat (MC) with gastric fossets (GF) and gastric glands (GG) and muscularis mucosal coat (MMC) are upright. HE 400X.

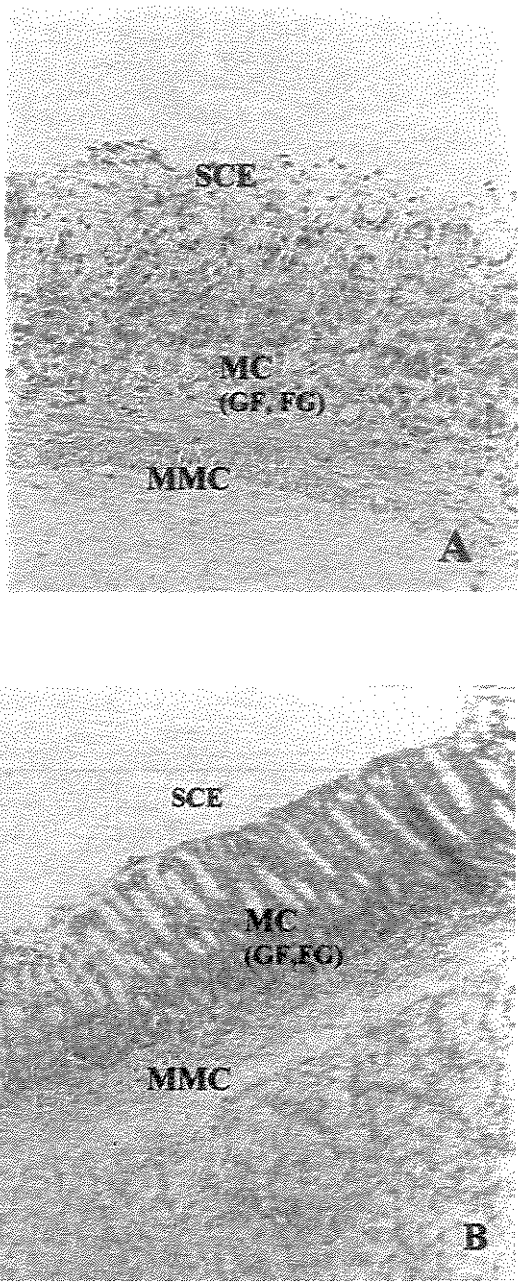


Figure 5: Photograph showing that pre-treatment with the essential oil of *Croton cajucara* Benth ($100mgkg^{-1}$ by oral route) protects stomach cells from the erosion induced by indomethacin in normal (A) and low protein (B) rats (pyloric region). It can be observed that the superficial coating epithelium (SCE), mucosal coat (MC with GF gastric fossets and GG gastric gland) and MMC muscularis mucosal coat are upright. HE 400X.

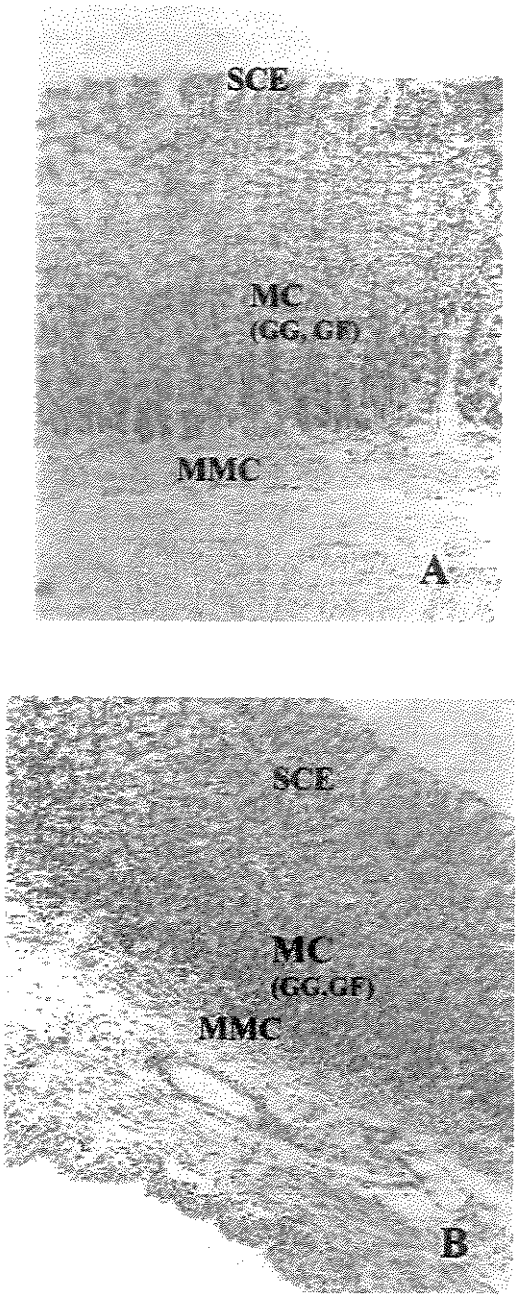


Figure 6: Photograph on the gastric wall upright (pyloric region) from normal (A) and low protein (B) animals belonging to the sham group. SCE, superficial coating epithelium; MC, mucosa coat (with gastric fossets and gastric gland) and MMC, muscularis mucosa coat. HE 400X.

Gastric antiulcer mechanism of *Croton cajucara* detected by RT-PCR, somatostatin and gastrin dosage in normoproteic and malnourished rats

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Abstract: Performance of the anti-ulcerogenic activity of *Croton cajucara* essential oil was analyzed in normoproteic and malnourished rats. In acetic acid and ethanol induced gastric ulcers were determined the ulcerative lesion index, the morphological examination and the "repair" process involved with these ulcers. Serum somatostatin (SMT) and gastrin were also examined. The data shown that a single oral administration of essential oil by day during 14 days (100 mg kg^{-1}) accelerated the healing of chronic gastric ulcer (49,37% and 69,82%) in normoproteic and malnourished rats respectively, when compared to negative control ($P<0.001$). This new drug also prevents the ethanol induced gastric ulcers in both animal groups ($P<0.001$); this action is similar to positive control, lanzoprazole ($P<0.05$). In normoproteic rats exposed to acetic acid and treated with essential oil, growth factors such as epidermal growth factor (EGF) was increased several-fold in the gastric lumen compared with the value measured in intact gastric mucosa in these animals. The morphological examination comproved these results showing gastric erosions with regenerated gastric surface epithelium and glandular areas. The serum somatostatin (SMT) values were increased in normoproteic and malnourished animals ($P<0.001$); however, serum gastrin levels were reduced in both experimental animal groups ($P<0.05$) with the essential oil treatment. The protective and curative effects of *C. cajucara* essential oil on induced gastric lesions were effective in normoproteic and malnourished rats. This manner, our studies indicate the essential oil as a potential new anti-ulcerogenic drug with a potent citoprotector activity.

Key words: malnourished, essential oil, *Croton cajucara*, medicinal plants, proteic nutrition.

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Introduction

Undernurition during early life may have both immediate and long-term consequences. In early intrauterine life undernutrition tends to produce selective restriction of organ growth and function (Widdowson 1970). Restriction of nutrient supply affects rapidly dividing cells and can lead to irreversible limitation of organ growth and function (Lucas, 1990).

Young *et al.*, 1987 had demonstrate that the maternal dietary restriction during pregnancy and lactation reduces the activities of small intestinal mucosal enzymes. However, there have been no studies of the effects of undernutrition on the structure and function of the digestive system in adulthood.

Proteic malnutrition is also associated with impaired insulin secretion and alterations in carbohydrate metabolism (Carneiro *et al.*, 1995). This manner, an elevated free fatty acid and corticosterone level, decrease serum insulin level and a poor insulin response to glucose were observed in this experimental model (Latorraca *et al.*, 1999).

Croton cajucara Benth, a herb which grows exclusively in the Amazon forest, is used in folk medicine as an agent for the treatment of diabetes, diarrhoea, gastrointestinal disorders and liver diseases (Hiruma-Lima *et al.*, 1999); the bark and leaves of the plant are commonly used in the form of tea or as powdered and dried pills for gastrointestinal disease (Souza Brito & Nunes, 1997). Cajucarindide and isocajucarinalide, two new clerodane diterpenes, have also been isolated from the bark of *C. cajucara*. It was recently demonstrated that the essential oil from *C. cajucara* has important antiulcerogenic properties without significant acute toxicological effects (Hiruma-Lima *et al.*, 2002).

The aim of this work was to perform of the anti-ulcerogenic activity of the *Croton cajuacara* essential oil in acetic acid and ethanol induced gastric ulcer in normoproteic and malnourished rats, and the "repair" process involved with these ulcers. Serum somatostatin (SMT) and gastrin were examined too.

Material and methods

Animals

Female Wistar rats (90days old) were obtained from the State University Campinas animal facilities. After several meet of 1 male/ 4 female (by one week), the pregnant females were separated at random and maintained on an isoenergetic diet containing 60 g protein/Kg (lower protein/ LP diet) or 170 g protein/Kg (normal protein/ NP diet) form the first day of pregnancy until the end of the lactation period (Reeves *et al.*, 1993-Table1).

During the experimental period, the mothers were fed on their respective diets that were put *ad libitum* and had free access to water. The animals were kept under standard lighting conditions (12h ligth-dark cycle) at a temperature of 24°C and the food intake was monitored daily.

At 25 days of age, the pups were weaned and maintained on their mothers' diet. After ninety days the normal and malnourished animals were used in experiments of anti-ulcerogenic activity and action mechanisms.

Drugs

The following drugs were used: cimetidine (Tagamet® Smithkline), lanzoprazole (Losec ®, Merrel Lepetit), Tween 80®, acetic acid (Sigma Chemical Co, U.S.A.) and ethanol (Sigma Chemical Co, U.S.A.). All reagents were of a high grade of purity. The substances and reagents were prepared immediately before use.

Essential oil of *Croton cajucara* Benth preparation

The stem bark of *Croton cajucara* Benth was collected from an experimental plantation in Benfica, near Belém, Pará, Brazil. A voucher specimen (number 247) has been identified by Nelson A. Rosa and deposited in the IAN Herbarium in Belém Brazil. The air-dried and milled bark (20 Kg) was subjected to steam distillation for 6h. Preliminary GC-FID and GC-MS analyses performed with an Hewlet Packard system using a HP-5 capillary columm showed very similar patterns for both F1 and F2 fractions, which were composed mainly of C_{15} H_{24} sesquiterpenes. α-copaene (20,9%) and cyperene (29,0%) were the main components of F1, as confirmed by ^{13}C NMR spectra measured in a varian spectrometer operating at 75,4 MHz and

using benzene as solvent (Hiruma-Lima *et al.*, 1999). Complete analyses of the samples are in progress. The F1 fraction was used for the pharmacological tests. F1 was emulsified in 12% Tween 80 before administration to the animals.

Etanol-induced ulcer. The ethanol-induced ulcer assay was carried out in rats according to the method of Morimoto *et al.*, (1991). A total of 32 animals male normoproteic and malnourished (weighing 150-200g and 90-120g, respectively) were randomly divided into 3 groups each and fasted for 24h, with free access to water before the experiment. One millilitre of 99,5% ethanol was orally administered to the animals which 1h previously had been treated with essential oil (100 $mgkg^{-1}$), lanzoprazole (20mg/kg^{-1}) or 12% Tween 80 (10ml/kg^{-1}). 1/2 hour after ethanol administration the animals were killed by cervical deslocation, the stomachs were removed and opened and the ulcerative index was determined.

Acetic acid-induced gastric ulcers. The experiment were performed according to the method of Takagi *et al.*, (1969). Male normoproteic and malnourished rats weighing 150-200 and 90-120 respectively at the time of operation were used in this experiment. Under anaesthesia laparotomy was performed through a midline epigastric incision. After exposing the stomach, 30% acetic acid, 0,05ml (v/v) per animal were injected into subserosal layer in the glandular part of anterior wall, with care taken not to disturb the blood vessels. Then the abdomen was closed and the animal was fed normally. Essential oil (100mg/kg^{-1}), cimetidine (100mg/kg^{-1}) and vehicle (10 ml/kg^{-1}) was administered orally for 14 consecutive days, once a day, beginning 2 days after the surgery. On day following the last drug administration, the animals were sacrificed by cervical dislocation at proper intervals to assess the healing process of the ulcer. The stomach was removed and the gastric lesions were evaluated by examining the inner gastric surface with a dissecting binocular microscope. Subsequently the ulcer area (mm^2) and curative ratio (%) were measured.

Blood collection

The heparinized blood samples of normoproteic and malnourished rats were obtained from the retroorbital plexus (Krous, 1980). Immediately after the blood collection, this was

submitted to centrifugation (3000 rpm, at 6^0C) by 10 minutes. After the centrifugation, the serum obtained was reserved in - 20°C until the experiments.

Somatostatin dosage

After induction of gastric ulcer by ethanol, the somatostatin hormone was measured in serum of normoproteic and malnourished rats (Krous, 1980) through of radioimmunoassay kits to somatostatin (RB-306, EURO –DIAGNÓSTICA) according Yago *et al.*, 1998.

Gastrin dosage

After induction of gastric ulcer by ethanol, the gastrin hormone was measured in serum of normoproteic and malnourished rats (Krous, 1980) through of radioimmunoassay kits to gastrin (CIS bio international- GASK-PR) according Mayer et al., 1974.

Reverse-transcriptase polymerase chain reaction for detection of mRNA for EGF (RT-PCR technique to epidermal growth factors)

The acetic acid induced gastric ulcers (Takagi *et al.*, 1969) in normoproteic and malnourished animals previously to molecular biology experiments. So, this method was performed according Konturek *et al.*, 1998, where total RNA was isolated from the mucosal specimens and stored at -80°C. A rapid isothiocyanate/phenol chloroform single step extraction kit from Stratagene® was used. Following precipitation, the RNA was resuspended in Rnase-free buffer (TE) and the concentration was estimated by absorbance at 260 nm wave length. The quality of each RNA preparation was determined by agarose-formaldehyde gel electrophoresis and ethidium bromide staining. Primers were synthesized by Biometra® (Göttingen, Germany) and the nucleotide sequences of the rat EGF primers were based on the published cDNA sequences encoding rat EGF (Fan et al., 1995; Saggi et al., 1992) that was 5'-GACAACTCCCCTAAGGCTTA-3' (nucleotides 2804-2823); the EGF antisense primer was 5'-CATGCACAGGCCACCATTGAGGCAGTACCCATCGTACGA-3' (nucleotides 3332-3370). To maximize amplification specificity, a hot start polymerase chain reaction was performed in a Perkin Elmer Cetus DNA thermal cycler for 33 cycles (94°C for 1 min, 60°C for 45 s, 72°C for 2

min) using AmpliWax® PCR Gen 50 beads. Ths manner, 8 µl aliquots of amplified polymerase chain reaction product were electrophoresed on a 1.5% agarose gel stained with ethidium bromide and visualized under UV light. The gel was then photographed under UV transillumination. Oligonucleotides primer sequences are specific, as ascertained by computer-assisted search of the update version of GeneBank. In addition to size analysis by agarose gel electrophoresis, specificity of the primer pair for EGF was assessed by sequencing of polymerase chain reactions products.

Morphological analyses

After sacrifice of the animals that were submitted to ethanol/acetic acid induced ulcer, the stomach samples were collected, fixed in Bouin for 24 h, dehydrated in an increasing ethyl alcoholic series, cleared with xylene, embedded in Histosec (Merck –11609), and prepared for microtomy. The sections were then deparaffinized and rehydrated with a decreasing ethyl alcohol series. Next, the samples were stained with hematoxylin-eosin for morphologic analysis of the lesion (Yoshitake *et al.*, 1991). Hematoxylin was used for 15 min. The samples were washed in running water for 10 min, with sections changing in color from red to blue. The sections were stained with eosin for 10 min and washed in water, dehydrated, placed in 95% alcohol, cleared and mounted in resin (Junqueira & Junqueira, 1996). Photomicrographs were obtained with an Axiophot in Photomicroscope (Carl Zeiss, D-7082). The lesions were counted by histomorfometric analyses.

Statistical analysis

Results were expressed as mean ± S.E.M. One-way analysis of variance were followed by Scheffe's test or Tukey's test with the level of significance set at $P<0.05$ and $P<0.001$. All statistical analyses were performed using the Statistica 5.1 software (StatSoft, Inc).

Results

In ethanol induced acute gastric ulcer, the essential oil of *Croton cajucara* prevented the appearance of gastric ulcer (Figure 1) when compared with the negative control 12% Tween 80 ($p<0.05$). In this figure, the new anti-ulcerogenic drug was as efficient as the positive control (lanzoprazole) at the same dose and route. The malnourished animals, had a lower number of

ulcers than normoproteic animals with lanzoprazole and essential oil treatment, but also without anti-ulcerogenic drugs.

After the essential oil treatment, it was possible to note that the serum gastrin levels were diminished in both animal groups ($P<0.05$) when compared with the negative control. The serum gastrin levels found in normoproteic and malnourished animals treated with essential oil, were similar to treatment with the positive control lanzoprazole (Figure 2).

The levels of somatostatin hormone were increased in normoproteic and malnourished serum rats after the treatment with the essential oil when compared to negative control ($P<0.001$); these results were similar to lanzoprazole (positive control) in the same experiment (Figure 3). So, was shown that the essential oil has anti-ulcerogenic effect, however its action was increased by the inhibitory action of somatostatin, that decreased the acid secretion.

In figure 4 the morphological examination of the oxyntic mucosa of normoproteic and malnourished animals submitted to ethanol induced gastric ulcer, showed dangerous erosion in normoproteic group, where the surface epithelium and gastric mucosa were seriously destroyed. In malnourished animal groups, only surface epithelium was destroyed, showing a lower lesion in this group when compared to normoproteic rats. In this acute experiment, the lesion area of normoproteic animals was 6.43 ± 0.40 while malnourished had the lesion area of 3.65 ± 0.98 μm^2 ($P<0.001$), when these animals were both treated with 12%Tween 80. After the essential oil treatment, the lesions were reduced in both groups to 3.52 ± 0.80 μm^2 and 2.80 ± 0.98 μm^2 respectively ($P<0.05$) (Figure 5).

In chronic treatment where the ulcerogenic agent was the acetic acid, the essential oil, showed healing action in the gastric ulcer formation in normoproteic and malnourished rats ($P<0.05$) at the same dose and route (Figure 6). In this experiment the essential oil was as efficient as the positive control cimetidine ($P<0.05$). However in this test, the malnourished rats treated with 12% Tween 80 had an increase in lesion area ($P<0.001$) differently of the ethanol-induced gastric ulcer described above.

In the experiments of RT-PCR where we analyzed the intact mucosa of normoproteic and malnourished rats, the mRNA was not identified (data not shown). Increased expression of epidermal growth factor (EGF) was however identified in areas where the gastric ulcer was treated with the essential oil treatment by 14 days (Figure 7). In this chronic experiment, the normoproteic rats had a lesion area of 6.23 ± 1.22 μm^2 and malnourished rats had a lesion area of

11.12 ± 1.98 μm^2 (P<0.001) after the 12% Tween 80 treatment (Figure 8). With the essential oil treatment, these lesions were healing until 4.89 ± 0.98 μm^2 and 3.25 ± 1.90 μm^2 to normoproteic and malnourished rats, respectively (P<0.001) (Figure 9), where is possible to note that the surface ephitelium had a initial regeneration (9A); there is also an inflammatory infiltrate formation and angiogenesis is in progress showing the initial healing and mucosa recuperation (9B).

Discussion

Ethanol induce oxidative stress in rats gastrointestinal mucosa, increasing the lipid peroxidation and DNA fragmentation, leading to gastric lesions (Baghi *et al.*, 1999). Ethanol also depress tissue levels of DNA, RNA and proteins, leading to alterations in blood flow, contributing to necrotic and hemorrhage aspect of the gastric tissues (Szabo, 1987). Moreover, it is well known that ethanol-induced ulcers are not inhibited by anti-secretor agents as cimetidine, but are inhibited by agents who enhance mucosal defensive factors such as prostaglandin E_2 (Robert *et al.*, 1979). Our data suggested that, essential oil significantly reduce the lesions index in normoproteic and malnourished rats being as effective as lanzoprazole, used as positive control in these experiments; however the malnourished rats had a lower number of ulcer than normoproteic animals without the use of anti-ulcerogenic drugs (Figure 1). Omeprazole, a proton pump inhibitor (H^+, K^+- ATPase), was expected; accelerate to dose dependently the healing of ethanol-induced gastric ulcers in rats (Ito *et al.*, 1994).

Proton pump inhibitors have been introduced in therapy for the management of peptic ulcer disease and other acid-related disorders of the upper gastrointestinal tract. We have also found that serum gastrin levels in normoproteic and malnourished rats treated with *Croton cajucara*, were similar to serum gastrin levels of animals treated with lanzoprazole in our experiments. Both drugs (essential oil and lanzoprazole) inhibited the gastric secretion induced by gastrin in normoproteic and malnourished rats and, also, serum gastrin levels in both animal groups were lower than negative control treated with 12% Tween 80 (Figure 2). Gastrin has been indicated to possess throphic action such as the proliferation of gastric mucosal cells (Willems *et al.*, 1972; Johnson and Guthrie, 1974; Johnson *et al.*, 1975; Hansen *et al.*, 1976) in addition to the stimulation of gastric acid secretion. So, the anti-ulcer effect obtained by omeprazole may be due to the inhibition of gastric acid secretion. However, it is now widely accepted that the

success of pharmacological treatments aiming to prevent or heal peptic ulcer disease does not reside only on the inhibition of acid secretion, but depends also on the enhancement of mucosal protective factors.

In this regard, omeprazole protected the gastric mucosa against necrotizing agents and hemorrhagic shock, these effects being unrelated to the inhibition of acid secretion (Konturek *et al*; 1983) but associated with a significant strengthening of gastric mucus barrier (Blandizzi *et al*., 1994). In addition, a large variety of endogenous factors, including prostaglandins, growth factors, gastrin, somatostatin, sensory peptides, nitric oxide (NO), and sulfhydryl compounds, have been implicated in the pathophysiology of gastroprotection.

In our experiments of radioimunoassay, a cyclic tetradecapeptide, somatostatin, was shown to be increased in normoproteic and malnourished rats treated with essential oil when compared with the animals treated with 12%tween 80 ($P<0.001$). The serum somatostatin values were more pronounced in malnourished animals ($P<0.001$), explaining the low number of ulcers in these animals because this hormone has inhibitory action in the gastric secretion (Figure 3). Karmeli *et al*., 1994 show that in the stomach somatostatin serves as a paracrine regulator of both acid and gastrin release, exerting some of its actions via interference in cAMP pathways. The same authors had demonstrated other effects of somatostatin in the stomach including inhibition of pepsinogen secretion, gastric emptying, and stimulation of gastric mucus output. It was clear that the protective effect of somatostatin was accompanied by decrease generation of mucosal leukotrienes, and decrease mucosal levels of substance P, VIP and gastrin. Degranulating mast cells may be a source of several neuropeptides and inflammatory mediators, among them, which are histamine and leukotrienes. The extent of ethanol-induced gastric mucosal damage in rats correlates with increased amounts of degranulation mast cells (Diel *et al*., 1986). Pretreatment of rats with somatostatin prior to ethanol administration significantly decreased mucosal damage, reducing leukotriene generation as well as the gastric acid secretion (Diel & Szabo, 1986). This effect may contribute to the gastroprotective effects of somatostatin against acute necrotizing agents or stress conditions.

Among the mechanisms suggested to explain the antiulcerogenic effects of somatostatin in prevention or decrease of ethanol-induced mucosal damage are inhibition of gastric acid secretion and of gastrin liberation, reduction of blood flow, generation of endogenous prostaglandins, stimulation of gastric mucus secretion, and possible blocking of histamine

release from mast cells (Lucey & Yamada, 1989). In this way, the preventive effect caused by essential oil in ethanol-induced gastric ulcer in normoproteic and malnourished animals, explain the increase of somatostatin level blocking the gastrin liberation or reducing the gastrin levels and consequently the gastric lesions. Moreover, prostanoids as PGE_2 production and mucus secretion in the accumulation of fluid in the gastric lumen, contributes to increase the mucosal defensive mechanisms.

Morphological examination of the oxyntic mucosa after ethanol induced gastric ulcer, revealed damage only to the surface epithelium in malnourished animals, and in the normoproteic groups, this erosion arrived until the gastric mucosa (gastric glands and fossets are destroyed) (Figure 4). After the essential oil treatment, these lesions are absent, showing the anti-ulcerogenic effect of the essential oil obtained from *C. cajucara* (Figure 5). The oral administration of cimetidine (100 mg Kg^{-1}) and essential oil in the same dose and route for 14 consecutive days, accelerated the healing of gastric mucosa in normoproteic and malnourished rats producing a remission of 49% and 70% respectively of chronic gastric lesions (Figure 6). In the same figure, it is possible to verify that the lesion of malnourished rats is more dangerous (52 mm^2) than the lesion of the normoproteic animals (26 mm^2). Nevertheless, this bigger healing in malnourished rats can be due to a major liberation of gastroprotective factors (PGE_2 and mucus) and an increase in somatostatin levels which inhibits gastrin secretion (which caused dangerous gastric ulcers) as described above. The morphological examination of normoproteic rats showed serious lesions in the gastric mucosa, however the subchronic treatment with 100 mg kg^{-1} by oral route by 14 days, recovered the gastric mucosa, reducing the gastric lesions. In malnourished rats, the chronic lesions are more pronounced than in normoproteic rats. The gastric mucosa of these animals showed perfured lesions, that were recovered with a diary oral treatment of *C. cajucara* oil by 14 consecutive days (Figure 6).

When we studied the healing of the gastric mucosa after exposure to acetic acid induced gastric ulcer, it was possible to note that a complex study involving different mechanisms in this process are in progress; the most important appear to be the growth factors, especially epidermal growth factor (EGF) and transforming alpha growth factor (TGF α) (Konturek, 1990; Konturek et al., 1992; Podolsky, 1994). EGF is a 53-amino acid peptide that originates mainly from the salivary glands and no EGF mRNA has been detected in the intact gastric mucosa of rodents and humans (Polk *et al.*, 1992; Calabro *et al.*, 1995). In experiments of RT-PCR where we analyzed

the intact mucosa of normoproteic and malnourished rats, the mRNA was not identified according described by Konturek et al., 1998 (data not shown). Increased expression of EGF was, however, reported in the gastric mucosa surrounding areas of chronic ulcerations induced by acetic acid during repair of this gastric mucosa that were exposed to this dangerous agent and treated with 100 mg Kg^{-1} (oral route) by 14 days. In this way, increased EGF mRNA has been found in the damaged mucosa of normoproteic rats (when compared with malnourished rats) that have been treated with the new anti-ulcerogenic drug obtained of *Croton cajucara* Benth (Figure 7). This manner, the mechanism of action of *C. cajucara* in the chronic gastric ulcer occurred through enhancement the growth factor expression (EGF), that can explain the pharmacological results obtained with the data or gastric lesions index (ILU) (Figure 6). In this case the healing exhibited by essential oil of *C. cajucara* detected by RT-PCR was more efficient in the gastric mucosa of normoproteic rats after the subchronic treatment with this new drug, probably due to the protein deficiency that induces low expression of this epidermal growth factor (EGF) in malnourished rats.

It is of interest that the growth factors, especially EGF, whose mRNA was not expressed in the intact mucosa, was expressed in the mucosa of normoproteic rats submitted to ulcerogenic process and treated with the essential oil.

As both EGF and TGF α are effective to protect gastric mucosa from gastric lesions caused by acetic acid, it is reasonable to assume that these factors limit the extent of mucosal damage caused by ulcerogenic agent and probably contribute to the early recovery of the mucosa (Konturek, 1990). It has been related that the unregulated expression of gastric mucous from acetic acid damage, both EGF and TGFα shown to enhance mucosal cell migration, cell proliferation and DNA synthesis as well as mucus secretion and gastric blood flow. This does not exclude the growth factors from the mediation of the mucosa recovery, stimulating prostaglandin-cyclo-oxygenase activity and/or the mucosal generation of prostaglandins according the results obtained with the gastroprotectives factors (Hiruma-Lima *et al.*, 2002) how prostaglandin E_2 and mucus; these factors have synergic actions with EGF in the gastric mucosa recuperation (Konturek *et al.*, 1998).

We can conclude that the *C. cajucara* essential oil prevents and heal gastric ulcers. The mechanisms of this medicinal plant are related with PGE_2 expression, inhibition of gastric mucus secretion; increase somatostatin levels and decrease gastrin levels in the stomach. These

mechanisms and EGF expression maintain the gastric mucosa intact and prevent the gastric ulcer formation.

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Table 1: The hipoproteic diet (6% of protein) was compared with the formulation offer by American Institute of Nutrition to rodents in growthing, gestation and lactation conditions (AIN-93).

Components(g/Kg)	*AIN- 93 G*	*Hipoproteic*
Casein *	**202.0**	**71.5**
Maize Starch	**397.0**	**480.0**
Dextrinized maize starch	**130.5**	**159.0**
Sucrose	**100.0**	**121.0**
L-cystine	**3.0**	**1.0**
Soybean oil	**70.0**	**70.0**
Mineral mix (AIN-93GMX)[2]	**35.0**	**35.0**
Vitamin mix (AIN-93GVX)[2]	**10.0**	**10.0**
Fiber	**50.0**	**50.0**
Choline Chlorhydrate	**2.5**	**2.5**

***For a detailed composition, see Reeves *et al.*, 1993.**

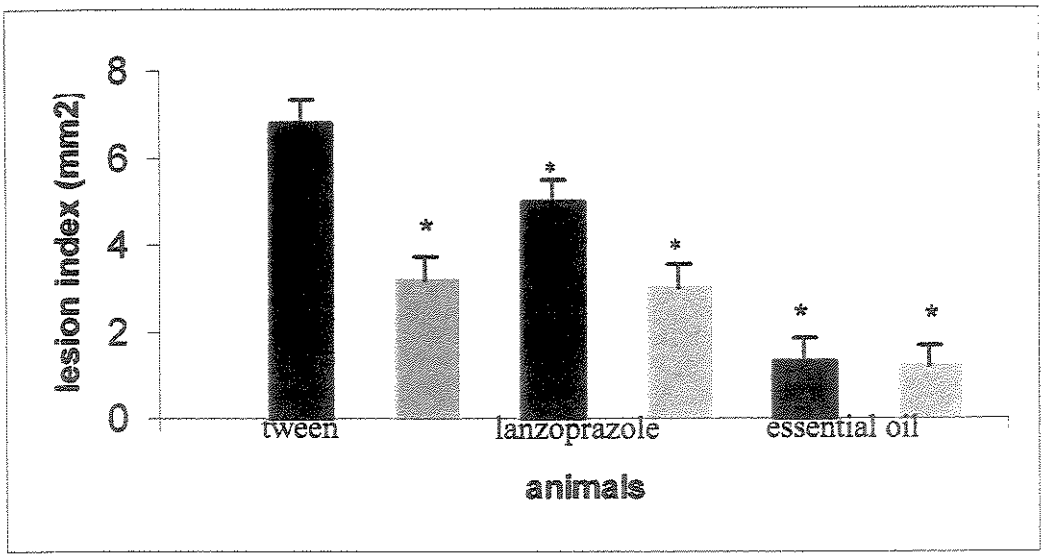

Figure 1: Effects of lanzoprazole and essential oil of *Croton cajucara* Benth. in gastric ulcers induced by ethanol in normoproteic rats (black column) and malnourished rats (gray column). The results are expressed as the mean ± SEM. (N=17 and N=15, respectively).

ANOVA: $F_{(5,26)}$= 5.483, following of Scheffe´s test:*p<0,05

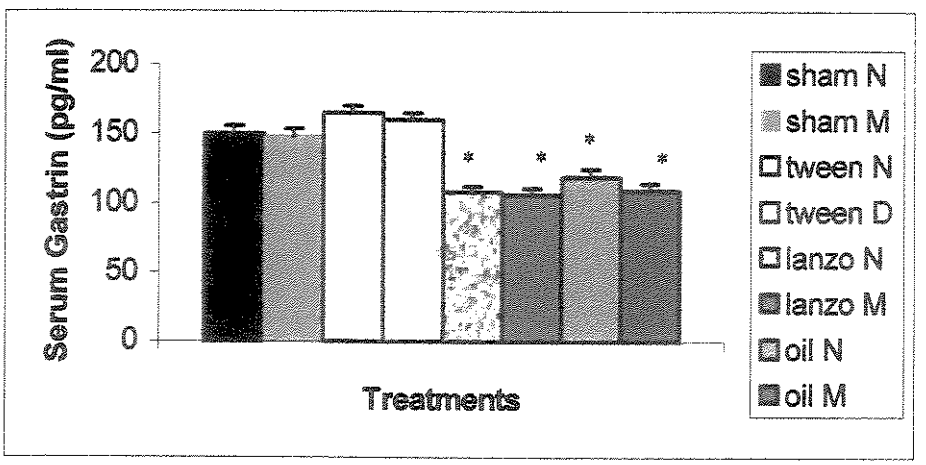

Figure 2: Serum gastrin levels (pg/ml) in normoproteic (N) and malnourished (M) rats before (sham group) and after ethanol induced gastric ulcer, pre treated with negative control (tween), positive control (lanzoprazole) and 100mg kg^{-1} (oral route) of essential oil of *Croton cajucara* Benth. Mean ± SEM of 5 animals. ANOVA: $F_{(7,35)}$= 41,29 following Tukey's test *p<0.05.

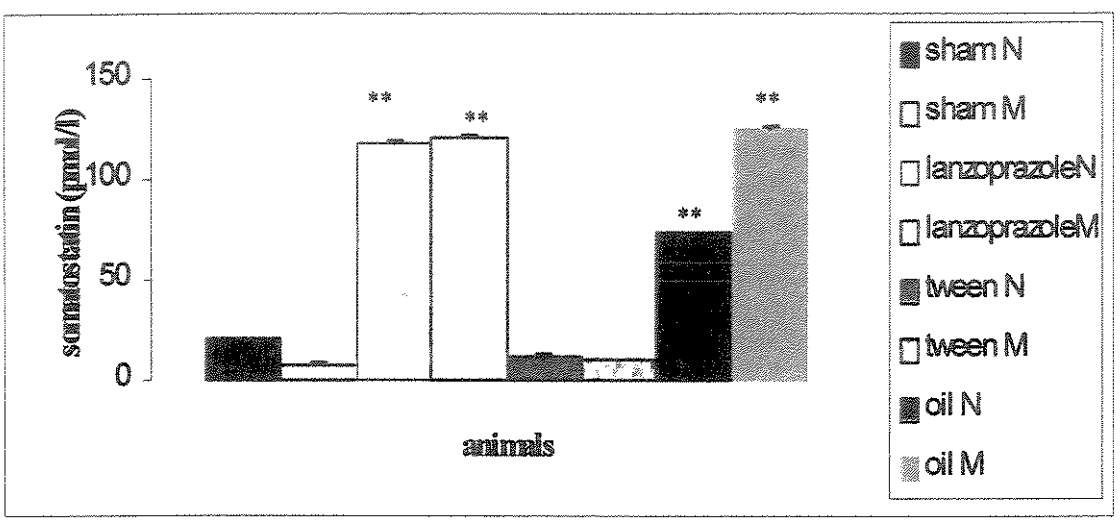

Figure 3: Serum somatostatin dosage (pmol/l) of normal (N) and malnourished (M) animals before (sham group) and after the ulcer induction by ethanol pre treated with negative control (tween), positive control (lanzoprazole) and 100 mg/kg of essential oil of *Croton cajucara* Benth.

The date are expressed as mean ± EPM of 5 animals ANOVA: $F_{(7,35)} = 32,4$ following Tukey'test **P<0.001.

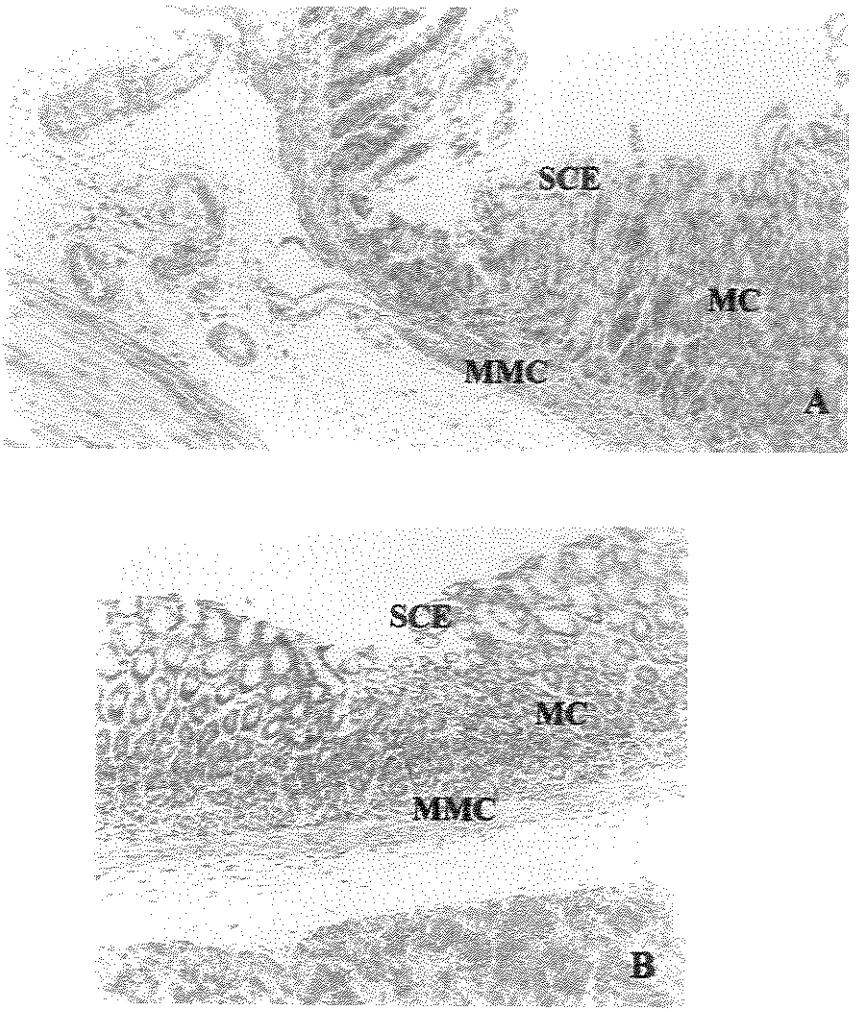

Figure 4: Morphologic appearance of the erosion in the oxyntic mucosa of normoproteic (A) and malnourished (A) rats after exposure to pre-treatment with 12% Tween 80 by 1 hour and posteriori treatment with ethanol (1/2h). In (B) surface epithelium (SCE) and mucosa layer (MC) are destroyed and in (B) the erosion is little, only surface epithelium is damaged. Hematoxilin and eosin stain, 400X.

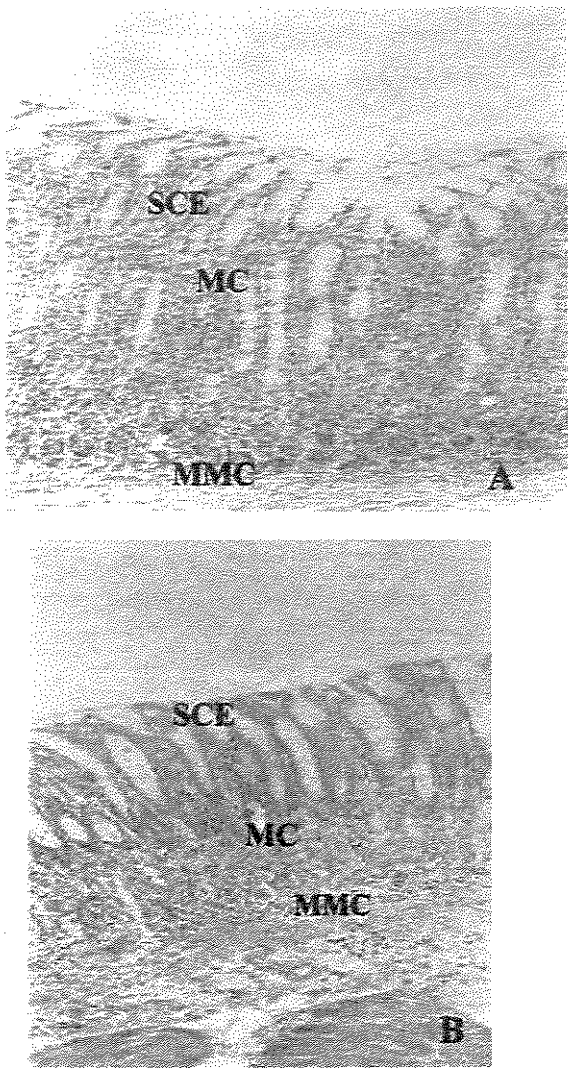

Figure 5: Essential oil of *Croton cajucara* (100 $mgkg^{-1}$) protected the acute erosion induced by ethanol in normoproteic (A) and malnourished (B) rats. It can be seen that surface epithelium (SCE), mucosa layer (MC) and muscle mucosa layer (MMC) are intact. Hematoxilin and eosin stain, 400X.

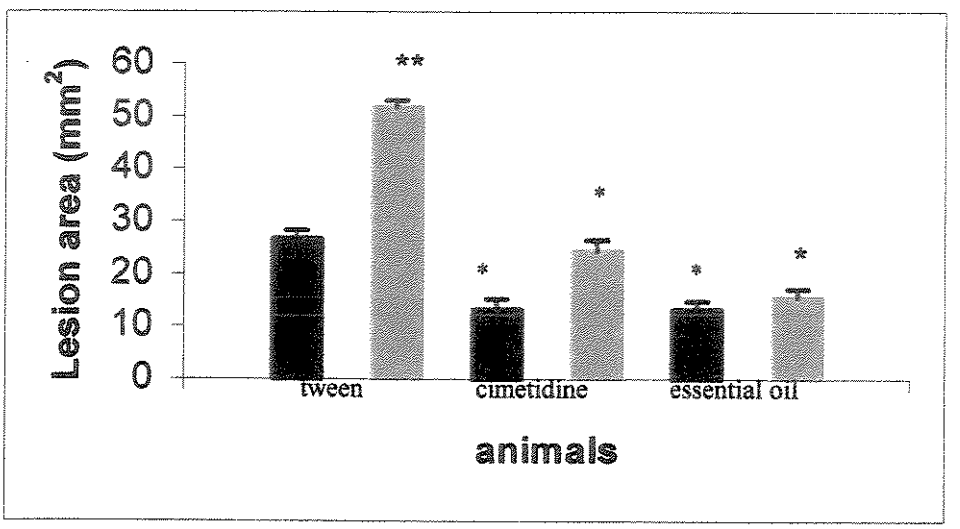

Figure 6: Effects of vehicle, cimetidine and essential oil of *Croton cajucara* Benth. in the healing of chronic gastric ulcer induced by acetic acid in normal (black column) and malnourished (gray column) rats. Expressed as mean ± SEM, Scheffe's test *P<0.05 and **p<0.001 (d≠a, b,c,e,f,) (N= 8).

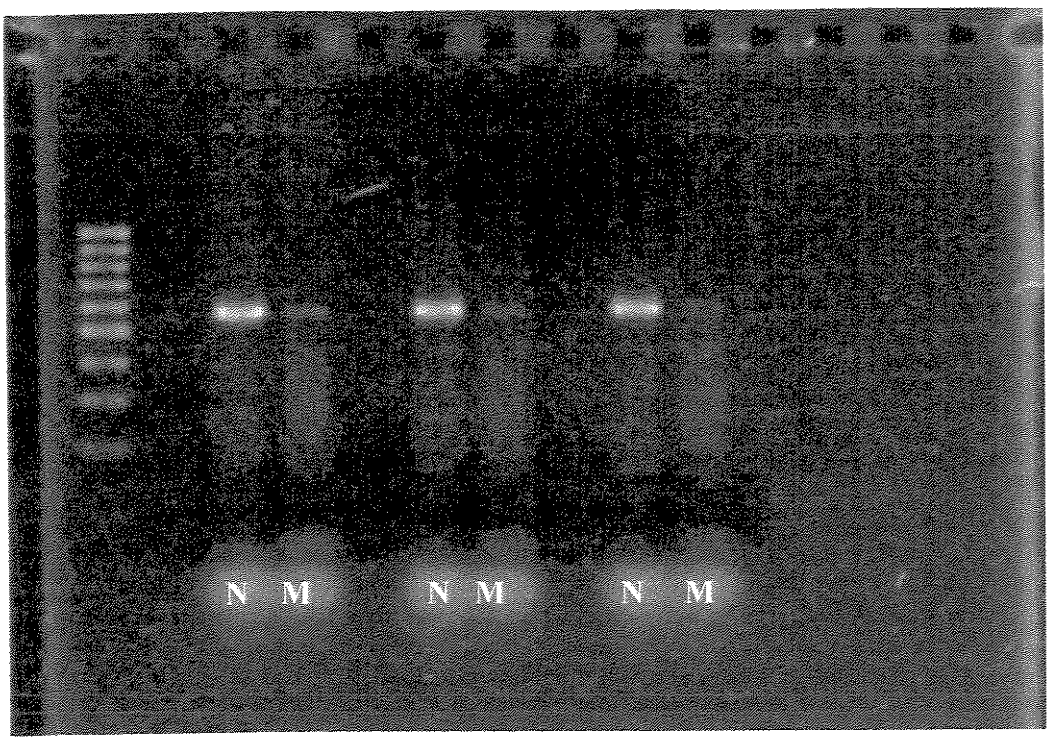

Figure 7: Messenger RNA expression to epidermal growth factor in gastric mucosal of normoproteic (N) and malnourished (M) rats after the treatment with the essential oil of *Croton cajucara* Benth. The treatment resulted in expression of mRNA to epidermal growth factors in both groups being more pronounced in the group treated with AIN-93 diet (17% of protein),

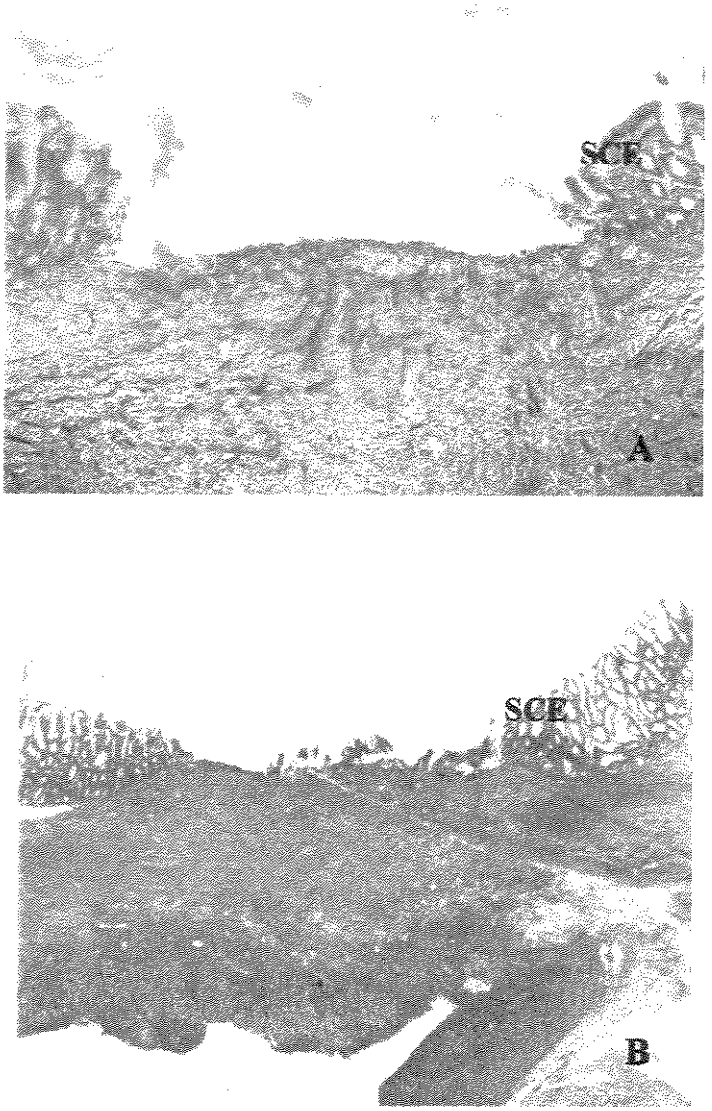

Figure 8: Morphologic appearance of gastric ulcer observed on the 14th day after the injection of 30% acetic acid solution, 0.05 ml into the stomach walls of normoproteic (A) and malnourished (B) animals. In (A) surface epithelium, mucosa layer and mucularis mucosa layer are destroyed and in (B) a typical penetration into the fibrous tissue wall of the healed ulcer can be seen in deficient protein animals. Hematoxylin and eosin stain, 400X.

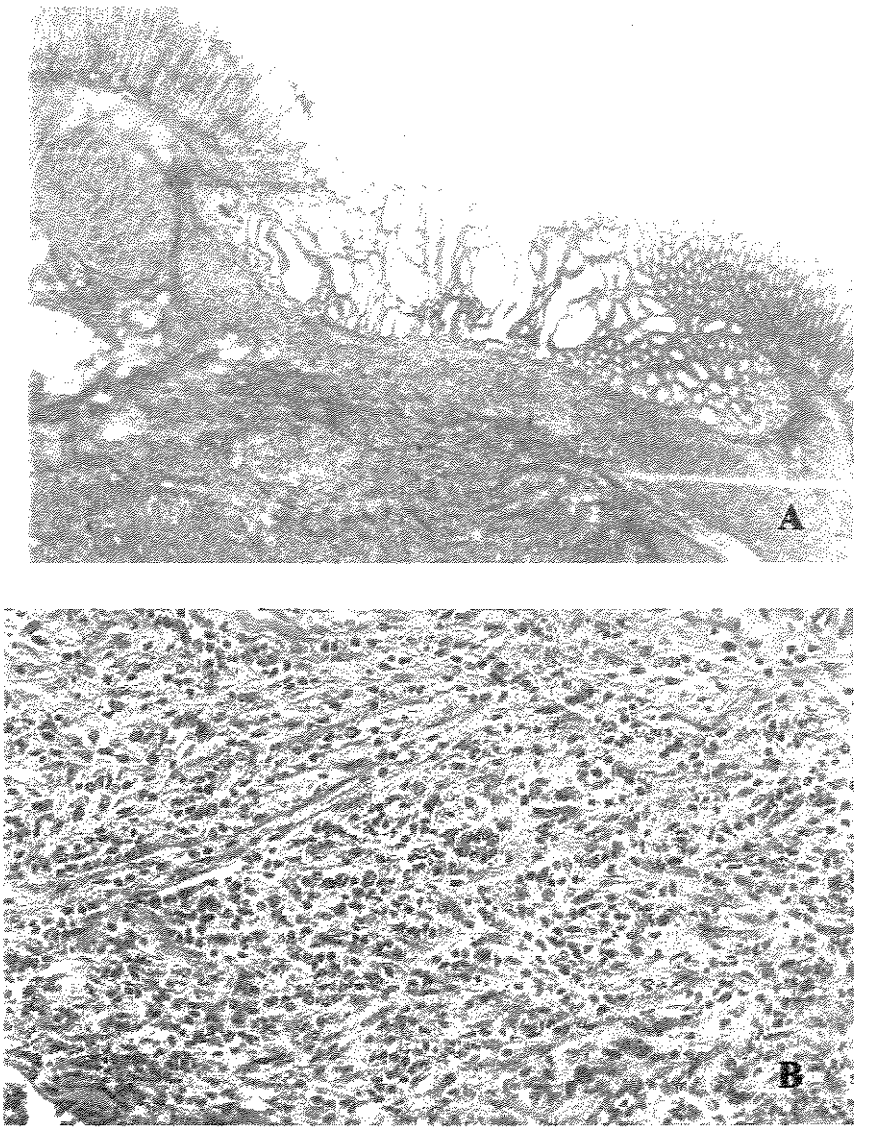

Figure 9: Healed acetic acid induced gastric ulcer. Depressed gastric mucosa is covered with initial regenerated gastric surface epithelium (A) with essential enhanced proliferation in the regeneration zone of the glands, angiogenesis and inflammatory infiltrate is shown this regeneration (B) with normal rats.

IV- DISCUSSÃO GERAL

1. Desnutrição Protéica

São várias as causas do retardo no crescimento intra-uterino; entre elas estão mal-formações congênitas, erros inatos do metabolismo (considerados fatores intrínsecos), insuficiência vascular uterino-placentária (causando déficit assimétrico no crescimento fetal) e desnutrição materna (levando ao prejuízo no desenvolvimento de todos os órgãos) (Nóbrega, 1996). Esse mesmo autor relata que, apesar das diversas maneiras de reproduzir experimentalmente o quadro de desnutrição durante a fase gestacional, na maioria delas verificam-se sérios comprometimentos no peso corporal e em diversos órgãos dos filhotes ao nascer; isto ocorre porque há um retardo na divisão celular evidenciado pelo menor conteúdo de DNA, RNA e proteínas desses órgãos. Além disso, observa-se que existe um menor número de filhotes por ninhada em ratas com desnutrição protéica, considerando que este fato se deve ao aborto e/ou reabsorção fetal ou ainda a uma diminuição dos hormônios materno-hipofisários necessários à manutenção da gravidez.

Hales & Barker (1992) têm sugerido que a desnutrição materna pode levar ao retardo generalizado no crescimento e redução permanente no tamanho dos órgãos e tecidos, incluindo o pâncreas e o estômago. Esse crescimento diferenciado se dá por interferência do teor protéico da dieta no metabolismo geral do animal (Masanés *et al.*, 1999).

Em nosso estudo observou-se que os animais alimentados com dieta hipoprotéica apresentaram um importante retardo no crescimento quando comparado aos seus respectivos controles. Essas características foram mantidas quando analizou-se também o peso dos órgãos desses animais. A associação entre retardo no crescimento total do animal e de seus órgãos (Latorraca *et al.*, 1998) está de acordo com os estudos relatados anteriormente, confirmando assim sinais importantes da desnutrição, presentes nesse modelo animal. Um fator que pode ser complicante na geração da desnutrição é o período no qual a restrição protéica é desenvolvida; este fato se confirmou como uma dificuldade, pois muitos filhotes não conseguem sobreviver. Em nosso caso, optou-se pela obtenção da desnutrição intra-uterina, pois nela são observadas as características causadas pela privação protéica com maior intensidade, além de permitir um índice significativo de animais nascidos.

De acordo com Weaver *et al.* (1998) a restrição de proteína na dieta materna durante os períodos de prenhez e lactação diminui o crescimento dos órgãos em geral e do trato gastrointestinal do rato, reduzindo a atividade de enzimas do intestino delgado. Entretanto, não há estudos relacionando dados de ratos desnutridos recém–nascidos e adultos com mudança na estrutura e função do sistema digestório. O trato gastrointestinal desses animais responde a mudanças patológicas e fisiológicas através de constante renovação da mucosa gástrica. Segundo os mesmos autores, a resposta do trato gastrointestinal de ratos que conviveram com uma restrição protéica, torna-se um comportamento adaptativo, que permite a preservação de órgãos vitais para o desenvolvimento animal.

Milner (1971) e Carneiro *et al.* (1995) observaram que a restrição protéica leva ainda a alterações nos níveis plasmáticos de hormônios e de outros parâmetros bioquímicos tais como glicose, ácidos graxos livres=AGL, proteínas séricas totais, etc. Mais recentemente, Latorraca *et al.* (1998) confirmaram esses dados.

As características citadas, em associação com alterações hormonais tais como aumento de corticosterona, diminuição de "Insulin-like growth factor" ou IGF-I, aumento de epinefrina e alterações nos níveis de hormônio de crescimento (Shils *et al.*, 1999), permitiram selecionar o modelo animal de desnutrição intra-uterina para nosso estudo, pois o mesmo reproduz o papel da restrição protéica no processo de formação e desenvolvimento de úlceras gástricas.

Após a indução de úlceras os parâmetros bioquímicos (glicose, ácidos graxos livres e proteínas séricas totais) sofreram uma redução significativa em seus valores com relação à observação feita nos mesmos parâmetros antes da indução de úlceras nos dois grupos animais, sem que houvesse alteração no número e no tamanho das úlceras. Apesar de ter havido redução nos níveis de ácidos graxos livres de animais normo e hipoprotéicos, os animais desnutridos continuaram apresentando níveis de ácidos graxos livres significativamente mais elevados quando comparado aos seus respectivos controles.

No organismo íntegro, os corticosteróides apresentam efeitos sobre o metabolismo de carboidratos e proteínas, que podem ser resumidos como diminuição da utilização de glicose, aumento da clivagem protéica e ativação da lipólise nos adipócitos aumentando a liberação de ácidos graxos livres, gerando aminoácidos e glicerol para a gliconeogênese Esses efeitos estão associados a inúmeras ações catabólicas, inclusive atrofia do tecido linfóide e massa muscular

diminuída. Assim, além das alterações hormonais e enzimáticas, a desnutrição dificulta a oferta de aminoácidos e, consequentemente, o processo de síntese protéica (Shils *et al.*, 1999).

Após o processo de restrição protéica os animais desnutridos apresentaram aumento nos níveis de corticosterona. Já após o processo de indução de úlcera, este parâmetro não sofreu alteração quando comparado ao respectivos controles, que por sua vez mantiveram os níveis de corticosterona plasmática baixos. A princípio seria possível sugerir que os animais desnutridos seriam mais susceptíveis ao aparecimento de úlceras; mas conforme verificado em nossos estudos observou-se o contrário, ou seja, os animais normoprotéicos apresentaram mucosa gástrica com maior número de lesões.

De acordo com Pelletier (1995) o estado de desnutrição é caracterizado por infecções frequentes e deficiência nutricional específica as quais, separadas ou juntas, causam perda de apetite e de peso exercendo influência em todos os tecidos. O trato gastro intestinal é danificado havendo, também, comprometimento das funções renal e hepática. A desnutrição afeta ainda a morfologia da mucosa intestinal, causando alterações bioquímicas e de permeabilidade da membrana da célula entérica (Nóbrega, 1996). Em nosso modelo de restrição protéica pode-se observar, no entanto uma menor incidência de úlcera; a despeito de todas essas características classicamente demonstradas.

Petzke *et al.* (1999), discutiram que ainda não está claro porque a dieta com restrição protéica (abaixo dos níveis requeridos, de 5-10%) aumentaria a capacidade de quebrar radicais livres de oxigênio. Neste contexto, é interessante notar que a característica clínica clássica da doença de Kwashiorkor é o resultado das baixas concentrações de glutationa hepática (GSH), diminuindo os danos peroxidativos e garantindo a integridade da membrana. Talvez, em nosso modelo, os níveis alterados de hormônios que servem para a adaptação do processo da desnutrição estejam influenciando na concentração de glutationa e isto poderia, pelo menos em parte, explicar a menor incidência de úlceras em animais desnutridos.

De acordo com Hum *et al.* (1992) a glutationa hepática tem a função de destruir intermediários reativos do metabolismo oxidativo, incluindo aqueles gerados de moléculas endógenas de drogas ou do processo de carcinogênese. Vale salientar ainda que a nutrição exerce forte influência sob o conteúdo da GSH hepática e de cisteína (precursor direto de GSH), as quais estão diminuídas após alimentar ratos com dieta hipoprotéica.

2. Plantas Medicinais na Terapêutica da Úlcera Gástrica

O uso de produtos naturais com propriedades terapêuticas é tão antigo quanto a civilização humana e, por muito tempo, produtos naturais foram as únicas fontes de drogas (De Pasquale, 1984). Nos últimos anos, tem aumentado o interesse no uso terapêutico de produtos naturais, especialmente daqueles derivados de plantas (Rates, 2001).

Na medicina tradicional, várias espécies vegetais têm sido usadas para o tratamento de desordens gastrointestinais, incluindo-se úlceras gástricas (Satyavati *et al.*, 1987). A primeira droga de origem vegetal, efetiva contra a úlcera gástrica, foi a carbenoxolona, descoberta em resultados de uma pesquisa com a planta comumente usada pelos indígenas, a *Gliycyrrhiza glabra* pertencente à família das LEGUMINOSAE (Brown *et al.*, 1959). Dezenas de outras espécies já foram descritas com propriedades semelhantes (Lewis & Hanson 1991) muitas das quais estudadas em nosso laboratório (Cota *et al.*, 1999; Gracioso *et al.*, 2000).

A espécie *Croton cajucara* Benth. (Euphorbiaceae), uma planta medicinal da Amazônia (comumente chamada de Sacaca), tem dentre seus usos, indicação de planta útil no tratamento de problemas gastrointestinais (Di Stasi *et al.*, 1989).

De acordo com Hiruma-Lima *et al.* (1999) o óleo essencial de *C. cajucara* Benth. aumenta a síntese e/ou retenção de muco, aumentando a concentração de PGE_2 na mucosa gástrica em duas vezes quando comparada com os níveis basais; isto levaria a um aumento dos fatores de proteção da mucosa e diminuição dos fatores agressivos, restaurando assim o equilíbrio entre eles.

A Prostaglandina E_2 (PGE_2) é responsável pela estimulação da síntese de muco nas células epiteliais gástricas formando uma camada viscoelástica. Esta estimulação se dá através de uma interação da PGE_2 com um subtipo de receptor para prostaglandinas, o EP_4, lembrando que a referida interação favorece a ativação da via cAMP/PKA (proteína quinase A), aumentando a produção de cAMP em resposta à PGE_2 (Takahashi *et al.*, 1999). No modelo de indução de úlcera pôr etanol, o uso do óleo essencial de *Croton cajucara* Benth (na dose de 100 mg/Kg, 60 minutos antes da administração do agente lesivo), preveniu de maneira significativa ($p<0,05$) a formação de úlceras em animais alimentados com dieta normo e hipoprotéica na ordem de 81% e 82,4% respectivamente.

Nossos dados mostraram que o óleo essencial inibe significativamente as lesões gástricas induzidas pelo etanol absoluto em animais normo e hipoprotéicos. Por outro lado, desde que fora estabelecido o fenômeno de citoproteção por Robert *et al.*(1979) algumas observações foram feitas com base na técnica microscópica do processo de ulcerogênese em estômago de ratos. Esta é uma avaliação histológica bastante cuidadosa, já que a avaliação macroscópica sozinha é algumas vezes enganosa, pois a dimensão dos danos de células epiteliais é quase sempre subestimada. A avaliação macroscópica da mucosa gástrica de ratos normais submetidos à ulceração por agentes ulcerogênicos, apresenta visível injúria na região glandular do estômago. Assim um estudo histológico é requerido para avaliação acurada da extensão dos danos.

Resultados histológicos recentes mostraram que a mucosa gástrica dos animais tratados com etanol apresenta danos em algumas partes da camada mucosa sem, no entanto, destruir a camada muscular da mucosa. Desta forma, os animais normoprotéicos apresentaram lesões (cuja área foi de 6.43 ± 0.40 μm^2) que atingiram as fossetas e as glândulas gástricas na região mucosa do estômago; já no grupo de animais desnutridos essas lesões (com valores de 3.65 ± 0.98 μm^2 de área) se restringiram à região das fossetas não chegando a destruir as glândulas gástricas. No estudo histológico foi analisada ainda a ação do óleo essencial de *C. cajucara* em previnir a formação de danos na mucosa. Foi possível observar que o óleo preveniu a formação de lesões por etanol na mucosa de animais normo (2.80 ± 0.98 μm^2) e hipoprotéicos (1.52 ± 0.80 μm^2) restabelecendo todo o epitélio de revestimento superficial, conservando mucosa e submucosa; glândulas gástricas recuperaram ainda a distribuição normal, embora o epitélio de revestimento não esteja completamente restaurado, este fato evitou a formação de uma lesão necrótica grave. A avaliação macroscópica da mucosa gástrica dos animais desnutridos não acusava nenhum tipo de lesão; no entanto, um estudo mais minucioso desta região mostrou que o etanol promove destruição tecidual superficial da mucosa no estômago desses animais.

O controle positivo usado nesse ensaio foi o omeprazol, um inibidor da bomba de prótons; desta forma, o uso do óleo de *C. cajucara* Benth. como agente anti-ulcerogênico em nossos experimentos, mostrou um efeito citoprotetor bastante significativo quando comparado com o controle, sendo 3 vezes mais potente que o omeprazol, uma droga utilizada há tempo na terapia de úlceras gástricas.

Segundo Bagchi *et al.* (1999) a administração de etanol 80% induz estresse oxidativo nas mucosas gástrica e intestinal de animais, aumentando a peroxidação lipídica e a fragmentação do

DNA, levando ao aparecimento de lesões. Sabe-se que o etanol é oxidado para acetaldeído que, por sua vez, é oxidado para acetato (Wang, 1999).

Ainda nesse modelo de indução de úlcera aguda, observou-se que animais desnutridos tiveram um índice de lesão ulcerativa (ILU) menor do que os animais alimentados com dieta normoprotéica. É possível sugerir aqui que o baixo conteúdo protéico da dieta desses animais pode ter potencializado um possível efeito antioxidante na mucosa gástrica protegendo-a e impedindo a formação de um maior número de lesões, independentemente do uso de drogas anti-ulcerogênicas, ou ainda que as alterações nos níveis de hormônios provocadas pela desnutrição nos animais modulem a resposta da mucosa gástrica ao etanol.

O etanol diminui os níveis teciduais de DNA, RNA e proteínas, levando a uma estase do fluxo sanguíneo na área injuriada; que contribui para o desenvolvimento do aspecto necrótico e hemorrágico da injúria tecidual (Szabo *et al.*, 1987). Além disso, é conhecido que as úlceras induzidas por etanol não são inibidas por agentes antisecretores tais como cimetidina, mas sim por agentes que aumentam os fatores de defesa da mucosa como prostaglandina E_2 , PGE_2 (Robert *et al*, 1979).

Vários estudos mostraram uma diminuição severa no fluxo sanguíneo da mucosa após tratamento com indometacina, um típico AINE que inibe a ciclooxigenase, resultando na diminuição dos níveis de prostaglandina. Estes agentes também destroem a integridade da barreira mucosa, provocando um aumento da permeabilidade da mucosa gástrica aos íons H^+ e Na^+ e uma queda na diferença do potencial transmucoso, resultando na indução de erosões e úlceras (Trevethick *et al.*, 1995). Uma possibilidade, então, é que mucosa gástrica dos animais desnutridos teria níveis de PGE_2 ainda mais elevados, conferindo assim maior proteção à mesma. Neste sentido, analisou-se o provável efeito citoprotetor do óleo essencial de *C. cajucara* em relação à produção de PGE_2 pela mucosa gástrica.

Em nossos experimentos de úlcera aguda, utilizando como agente injuriante a indometacina, verificou-se que o óleo essencial, na dose de 100mg/Kg por uma hora em contato com a mucosa gástrica de ambos os grupos animais, não foi capaz de produzir uma redução significativa dos danos da mucosa gástrica de animais normo e hipoproteicos causados pelo anti-inflamatório; no entanto, sempre ocorreu formação de um menor número de lesões na mucosa dos animais desnutridos, $p<0,05$ que independe do uso de drogas, mostrando que deve haver

algum outro fator preventivo predominante impedindo/amenizando a formação dessas lesões dos animais em estudo.

O óleo essencial de *C. cajucara,* durante duas e quatro horas em contato com a mucosa gástrica de animais normo e hipoproteicos, foi significativamente efetivo ($p<0,05$) na prevenção da formação de úlceras gástricas induzidas por AINE´s. Assim, um maior tempo de contato da droga teste com a mucosa gástrica garante citoproteção, indicando a possibilidade de um aumento local de prostaglandinas (PGE_2) que confirma os dados obtidos nos experimentos realizados com etanol.

O estudo histológico dos experimentos de indução de úlcera com indometacina revelaram que as úlceras de animais desnutridos (área de lesão 2.24 ± 0.10 μm^2) mostraram epitélio de revestimento superficial, em sua maior parte, íntegro apenas com alguma erosão. Já com relação aos dados obtidos com animais normais (área de lesão 4.84 ± 0.19 μm^2) ocorreram lesão no epitélio de revestimento superficial e camada mucosa. Após o tratamento dos animais desnutridos (área de lesão 1.17 ± 0.09 μm^2) com o óleo essencial de sacaca foi possível preservar a integridade do epitélio superficial de revestimento gástrico. No entanto, com relação aos animais normais tratados com o óleo essencial de *C. cajucara* (área de lesão 2.19 ± 0.16 μm^2), o epitélio foi restabelecido e observou-se presença de muco na camada muscular da mucosa. Neste caso, apareceu também tecido conjuntivo que muitas vezes é o responsável pelo reparo de lesões gástricas.

Como neste caso foi observado, macro e microscopicamente, uma espessa camada de muco na parede do estômago dos animais de nossos experimentos, é provável que a ação do óleo essencial de *C. cajucara* Benth tenha ocorrido através do aumento dos elementos protetores da mucosa gástrica, tais como a PGE_2 confirmando os dados obtidos por Hiruma-Lima *et al.*, (1999). Citoproteção (gástrica e intestinal) é definida como a propriedade que muitas PGE_2 apresentam em inibir, nas mucosas do estômago e intestino, os processos inflamatórios e necrotizantes, quando estas mucosas são expostas a agentes nocivos.

O óleo essencial de *C. cajucara* aumentou significativamente a produção de PGE_2 nos animais alimentados com dieta hipoprotéica quando comparados aos animais tratados com tween e aqueles do grupo sham ($P<0.05$); esse efeito, entretanto, foi drasticamente reduzido quando os animais foram tratados com indometacina ($P<0.001$). O efeito do AINE não foi revertido quando associado ao óleo essencial da sacaca. Vale a pena salientar aqui que os animais hipoprotéicos

pertencentes aos grupos sham e Tween possuem uma produção de PGE_2 alta mesmo sem o tratamento com drogas anti-úlcera, onde o aumento da produção de muco e PGE_2 previniram a formação de úlcera aguda.

É sugerido que a citoproteção conferida pelo aumento na síntese de PG's endógenas seja um fator importante na manutenção da integridade da mucosa gástrica, devido à manutenção do fluxo sanguíneo da parede gástrica e também à atividade antisecretora. Além disso, é relatado que o aumento de diferentes formas de PG's, principalmente a PGE_2, leva ao aumento da produção de muco e bicarbonato pela mucosa gástrica formando a barreira citoprotetora (Robert *et al.*, 1979).

Desta forma, nossos resultados mostraram que o óleo essencial de *C. cajucara* (utilizado em experimentos com animais normo e hipoprotéicos), promoveu um aumento duas vezes maior da produção de PGE_2 em animais desnutridos quando comparado aos níveis de PGE_2 dos animais normais que receberam apenas o veículo, $p<0,001$. Este aumento foi completamente abolido pela administração prévia de indometacina aos animais, demonstrando que a elevação da PGE_2 gástrica foi promovida pela administração oral do óleo essencial da espécie em estudo. Este tipo de técnica permitiu determinar a capacidade de amostras de tecido em gerar PGE_2 à partir de ácido araquidônico e tem mostrado uma boa correlação com as medidas de síntese de PGE_2 pelo tecido mucoso (Curtis *et al.*, 1995).

Sabe-se que agentes injuriantes como etanol depletam o muco gástrico, possivelmente por mobilizar mucopolissacarídeos da mucosa dentro do lúmen (Koo *et al.*, 1996). De fato a exposição contínua da mucosa gástrica íntegra a agentes que a danificam, pode causar inibição da biossíntese de muco, levando à redução da produção deste fator defensivo e degradação do epitélio gástrico, resultando na depleção do estoque intramucoso e eventualmente em severos danos no tecido. Os mesmos autores discutem, entretanto, que ainda não está claro se agentes anti-ulcerogênicos abasteceriam a ação citoprotetora através da preservação do muco intramucoso.

Em nossos experimentos dos mecanismos envolvidos com a ação anti-ulcerogênica do óleo essencial de *C. cajucara* prevenindo e/ou cicatrizando a formação de lesões gástricas, verificou-se que tanto os animais normo como os hipoprotéicos tiveram um aumento significativo ($p<0,001$ e $p<0,05$, respectivamente) na produção de muco aderido à parede gástrica. O óleo essencial da sacaca causou um aumento na produção de muco bastante próximo

ao muco produzido nos animais do grupo controle positivo, isto é, tratados com carbenoxolona. Ressalta-se ainda que a quantidade de muco produzida por grama de tecido glandular pelos animais desnutridos foi maior do que a quantidade de muco/g de tecido glandular produzida pelos animais normais.

O muco é sintetizado e liberado de células epiteliais gástricas, adere à superfície da mucosa como uma fina (<80 μm) mais contínua camada de gel (Garner *et al.*, 1994). Estes autores descrevem que a superfície da camada de muco forma uma fronteira dinâmica entre conteúdo gástrico e epitélio de revestimento; é esta camada de muco que torna a mucosa resistente à ulcerogênese. Portanto, o tratamento e/ou a prevenção da formação de úlcera gástrica, deveu-se à ação do óleo essencial de *C. cajucara* através da síntese e liberação de muco, sendo este dado confirmado pela presença de muco e de células mucosas próxima ao epitélio de revestimento superficial da mucosa gástrica (dados não mostrados). Pode-se sugerir, ainda que o óleo essencial assim como o omeprazol (controle positivo), possa estar ativando a produção de genes para o EGF e, conseqüentemente, restaurando o epitélio gástrico. Como foi relatado em nossos resultados, é nítido que a biossíntese de PGE_2 produzida pela mucosa de animais desnutridos é maior do que aquela produzida pela mucosa de animais normais. Essa maior quantidade de produção de PGE_2 e muco garantem a integridade da parede gástrica desses animais, de uma maneira bastante eficiente quando comparada à mucosa dos animais normais.

Nóbrega (1996) descreve que a concentração de ácido clorídrico (HCl) gástrico em animais desnutridos é baixa, o que pode favorecer uma maior proteção da mucosa gástrica do estômago desses animais. Assim, pode-se sugerir que um outro provável fator defensivo da mucosa gástrica de animais desnutridos seja devido a menor liberação de secreção ácida gástrica, a qual garante a integridade dessa mucosa. Isto pode ser importante no sentido de auxiliar a desvendar o menor número de lesões que apareceram em nossos resultados, quando foi analisado o estômago de animais alimentados com dieta hipoprotéica.

As úlceras crônicas, produzidas por injeção de uma solução de ácido acético na parede do estômago, foram caracterizadas como úlceras intratáveis basicamente por duas razões. A primeira delas é o fato das mesmas persistirem por mais de 150 dias sem qualquer procedimento adicional; segundo, à extensão das ulcerações e as hemorragias aparentes nas bordas das lesões são confirmadas, mesmo após a ocorrência do processo cicatrizante (Monfort *et al.*, 1973; Takagi *et al.*, 1969). O tratamento de úlceras crônicas da mucosa estomacal dos ratos normais e

desnutridos com o óleo essencial de *C. cajucara* na dose de 100 mg/Kg/dia, durante 14 dias, foi tão ou mais efetivo do que cimetidina inibindo as lesões causadas pelo ácido acético.

Nos nossos experimentos crônicos, tendo como agente lesivo o ácido acético, utilizamos como controle positivo a cimetidina, um antagonista de receptor de histamina H_2 (Ito *et al.* 1994). De acordo com os resultados obtidos observou-se que a área de lesão gástrica em animais normais e desnutridos, tratados com Tween após a injeção de ácido acético, foi da ordem de 26 e 52 mm^2, respectivamente. Após o tratamento da úlcera com o óleo de *C. cajucara*, as lesões apresentaram-se bastante reduzidas chegando a 14 e 15 mm^2, respectivamente.

Assim o óleo essencial de *C. cajucara* Benth. cicatrizou a úlcera induzida pelo ácido acético em ambos os grupos de experimentação, ou seja, em animais normo e hipoprotéicos. Sendo assim, o efeito cicatrizante máximo foi obtido em animais desnutridos, onde o óleo produziu uma taxa de cura da ordem de 70%, enquanto em animais normais este mesmo tratamento levou à uma cicatrização que não atingiu 50%. Esses dados mostram que o tratamento com o óleo, em animais normais, tem um efeito da mesma magnitude que aquele da cimetidina; entretanto, quando esse efeito foi analisado em relação aos animais desnutridos, verificou-se um efeito ainda mais significativo devido, provavelmente, à potencialização do efeito antioxidante na mucosa gástrica dos animais desnutridos, como discutido anteriormente, ou ainda aos altos níveis de corticosterona (alterando o metabolismo de carbohidratos, proteínas e outros hormônios), somados a uma maior produção de muco e PGE_2 pôr parte da mucosa gástrica destes animais.

O estudo histológico do processo de úlcera crônica revelou que os animais desnutridos tratados com Tween (área de lesão 11.12 ± 1.98 μm^2) tiveram epitélio de revestimento superficial, camada mucosa e camada muscular da mucosa completamente destruídos. Esta úlcera é considerada perfurada pois tecidos do fígado e intestino, além do tecido gástrico, foram atingidos. Com o tratamento dos animais com o óleo parte deste tecido está sendo regenerado, pois já aparece o infiltrado inflamatório com posterior início de tecido de granulação (regenerativo). No caso dos animais normais (área de lesão 6.23 ± 1.22 μm^2, $p<0.001$) o mesmo acontece, ou seja a lesão gástrica atingiu camadas mais profundas destruindo por completo a muscular da mucosa.

A transição da fase de cicatrização inicial para a final é caracterizada pela migração de células epiteliais regeneradas que são responsáveis pela re-epitalização da úlcera e pela intensa proliferação de células epiteliais na margem da úlcera (Halter *et al.*, 1995).

Brasileiro Filho *et al.* (1994) descreveram que o prejuízo na cicatrização de lesões na mucosa de animais desnutridos deve-se, em parte a uma diminuição da resistência a agentes patogênicos causadores de infecções, devido, principalmente, ao prejuízo na formação e multiplicação celular de leucócitos, anticorpos e opsoninas. Leucopenia, neutropenia, defeitos intrínsecos dos leucócitos e neutrófilos, além de deficiências na síntese de moléculas de adesão no endotélio ou nos fagócitos, acompanham retardo na cicatrização também por facilitar as infecções.

A inibição da acidez gástrica ou pepsina não acelera a cicatrização das úlceras, ao passo que agentes que estimulam a reepitelização ou granulação do tecido do estômago exercem uma significativa aceleração na cicatrização, principalmente na fase inicial (Halter *et al.*, 1995).

O óleo essencial exerce ação antiulcerogênica, já que ambos os grupos de animais usados nesses experimentos de ulcerogênese crônica, mostraram início de cicatrização através da presença de infiltrado inflamatório e indício de tecido de granulação. Esse tecido é considerado de reparo, mas há ainda presença dos capilares neoformados que já são canalizados.

A formação de novos vasos (angiogênese) no tecido de granulação é crucial para o processo de cicatrização porque eles facilitam a distribuição de nutrientes e a oxigenação para a base da úlcera. Conseqüentemente, a fibrinogênese é mais pronunciada e persiste a infiltração leucocitária. Brasileiro Filho *et al.* (1994) descrevem que a desnutrição, especialmente a deficiência em proteínas, retarda a cicatrização por interferir diretamente nos processos de síntese do colágeno como ocorre em úlceras crônicas.

Assim, nossos resultados histológicos mostraram que tanto animais normo como hipoprotéicos apresentaram a formação de erosão na parede gástrica em modelos de úlcera aguda. No entanto, a indução de úlceras pelo ácido acético produziu lesões bastante extensas em ambos os grupos animais, atingindo não só tecido gastrointestinal mas também hepático; ainda assim, o óleo essencial mostrou ter efeito curativo conforme descrito acima. Desta forma, confirmou-se a atividade anti-ulcerogênica do composto em estudo, indicando ainda que um dos mecanismos de ação do óleo essencial desta espécie de planta está baseado na síntese e liberação de muco e prostaglandinas.

A fase inicial da úlcera gástrica mostra necrose tecidual com atração de macrófagos e leucócitos polimorfonucleares. Esta fase termina quando o tecido de granulação se forma acima da depressão ulcerosa (Halter *et al.*, 1995). Durante a fase de cicatrização rápida o tecido de granulação resiste à remodelação contínua e mudanças na composição celular. Inicialmente células inflamatórias e macrófagos são abundantes, enquanto que nos estágios finais predominam fibroblastos.

Skarstein (1996) reportou que há mudanças no fluxo sanguíneo da mucosa ao redor da úlcera em seus animais de experimentação, sugerindo que a maioria das PG's, as quais causam vasodilatação, foram sintetizadas em maior quantidade ao redor da úlcera do que em outras partes da mucosa gástrica. O grande suprimento sanguíneo local vem refletir na reepitelização, a qual requer um abundante fornecimento de glicose e oxigênio (Sato *et al.*, 1995).

De acordo com Brzozowski *et al.* (1998), a cicatrização dos danos da mucosa gástrica após exposição ao ácido acético é um processo complexo que envolve diferentes mecanismos, onde um dos mais importantes parecem ser os fatores de crescimento, especialmente o EGF.

Em nossos resultados foi possível observar que houve a expressão de EGF no grupo de animais normais tratados por 14 dias com o óleo essencial de *Croton cajucara* Benth. Verificou-se ainda que o EGF esteve aumentado muitas vezes nos animais alimentados com a dieta contendo 17% de proteínas, quando comparado ao EGF expresso na mucosa gástrica de animais que se alimentaram da dieta que contém apenas 6% de protéina.

Tarnawski *et al.* (2000) relataram que o omeprazol (utilizado como controle positivo), restaurou completamente a arquitetura da mucosa através da ativação de genes para o EGF e seus receptores (EFG-R), o qual é responsável por promover crescimento, migração e proliferação de células epidermais, proporcionando mecanismos de cicatrização da úlcera com conseqüente restauração da mucosa. Este é um dos mecanismos de defesa da mucosa gástrica requerido para manter a integridade da mesma. Ainda, o tratamento com omeprazol acelerou a diminuição do tecido de granulação. Isto ocorreu devido à diminuição substancial de ácido e pepsina, os quais danificam a formação de tecido de granulação no leito da úlcera. Este fato foi implementado pela redução da quantidade de células inflamatórias e aceleração da maturação do tecido de granulação.

Desta forma o mecanismo de ação do óleo essencial de *C. cajucara* no processo de ulceração gástrica crônica deu-se também através da expressão de fatores de crescimento, no caso através do fator de crescimento epidermal, o qual não se expressa na mucosa intacta.

Estes dados obtidos com técnicas de biologia molecular comprovam os resultados farmacológicos que envolvem a quantificação do índice de lesão ulcerativa apresentados anteriormente, onde a cicatrização mais eficiente, por parte do óleo essencial da sacaca, deu-se na mucosa gástrica dos animais tidos como normoprotéicos após um tratamento crônico com o óleo essencial da sacaca.

É de grande interesse que os fatores de crescimento, especialmente o EGF, para o qual o mRNA não esteve expresso na mucosa gástrica intacta, seja expresso na mucosa de animais submetidos ao processo de ulcerogênese. O EGF (além do TGF α) é efetivo em proteger a mucosa gástrica da indução de lesões por ácido acético e isto é importante pois estes fatores limitam os danos extensivos da mucosa causados pelo agente ulcerogênico e contribuem para a recuperação desta mucosa através da cicatrização (Konturek *et al.*, 1990; Konturek *et al.*, 1998).

Também tem sido demonstrado que os fatores de crescimento medeiam a recuperação da mucosa gástrica por estimular a atividade da geração de prostaglandinas (Mori *et al.*, 1987). Fatores de crescimento estão, desta forma, continuamente envolvidos na reconstituição de estruturas epiteliais incluindo tecido conectivo/vasos e células musculares lisas, favorecendo o substrato da matrix extracelular na migração e diferenciação celular.

A administração oral do óleo essencial de sacaca, acompanhada de aumento da expressão do gene do EGF detectada por RT-PCR, sugerem uma função importante do EGF em mediar o reparo da mucosa e o processo de cicatrização por parte da droga teste aumentando a expressão deste peptídio de integridade da mucosa.

O mecanismo de ação gastroprotetor do óleo essencial de sacaca envolveu aumento da síntese de PGE_2 e muco, assim como o mecanismo cicatrizante envolve a expressão de EGF. Isto indica que PG's, muco e EGF local devem ter função sinérgica na mediação do processo de reparo da mucosa gástrica injuriada.

Nos experimentos de ligadura do piloro, foi avaliada a atividade do óleo essencial de *Croton cajucara* em alterar parâmetros bioquímicos do suco gástrico de ratos normo e hipoprotéicos.

O método de ligadura de piloro, com administração intraduodenal das amostras vegetais, foi estabelecido no intuito de verificar a atividade sistêmica das amostras. Esta forma de administração evita um contato direto das substâncias com a mucosa e com o suco gástrico como acontece quando as drogas são administradas por via oral. Assim, numa análise bioquímica do suco gástrico dos animais normais foi possível observar que a cimetidina (100 mg/Kg) causou alterações no pH, no volume e na concentração de íons hidrogênio do conteúdo gástrico, assim como o óleo essencial que, administrado pelas mesmas dose e via, provocou significativo aumento no volume gástrico, diminuição da concentração de íons hidrogênio e do pH. Em animais desnutridos o óleo essencial foi efetivo tanto na redução da acidez, quanto no aumento do volume gástrico, assim como a cimetidina. Ainda foi possível notar que os valores de volume gástrico obtidos para os animais desnutridos tratados somente com tween estiveram significativamente aumentados ($p<0,05$) com relação ao volume dos animais normais. Isto provavelmente se deveu ao aumento da produção de muco da mucosa gástrica desses animais, observado nos experimentos de muco aderido à parede gástrica anteriormente relatado. É possível inferir que as ações anti-secretoras do óleo podem ser devidas a um efeito sistêmico (anulando a hipótese deste efeito ser devido ao contato do óleo com a mucosa gástrica), confirmando a propriedade antiulcerogênica da planta.

3. Ação de hormônios do tratogastrointestinal

Além dos experimentos já relacionados, os níveis plasmáticos dos hormônios do trato-gastrointestinal de animais normo e hipoprotéicos foram avaliados para verificar a interferência destes hormônios nos mecanismos de gastroproteção e/ou no processo de formação de úlcera gástrica. Os hormônios avaliados foram somatostatina (SMT) e gastrina em ambos os grupos de animais, normo e hipoprotéicos, frente ao processo de ulcerogênese e em condições basais.

A somatostatina é um tetradecapeptídeo cíclico amplamente distribuído através do TGI. Está confinada principalmente às células D da mucosa do estômago e do pâncreas; no estômago, a somatostatina é considerada como um regulador da liberação de ácido e de gastrina, da secreção de pepsinogênio e do esvaziamento gástrico (Karmeli *et al.*, 1994).

A gastrina é produzida em células G, no estômago e duodeno, estimula receptores de Colecistocinina-β (CCK-β) da célula parietal, induzindo o aumento da secreção ácida através do

aumento dos níveis intracelulares de Ca^{++}, assim como a acetilcolina. Acredita-se que a gastrina seja secretada pela presença de proteínas nos alimentos, Ca^{2+}, Mg^{2+}, Al^{3+}, além da estimulação vagal e da alcalinização do antro (Dockroy *et al.*, 1995; Konturek *et al.*, 1996; Kutchai, 1996).

Ambos os grupos animais testados foram submetidos ao processo de ulceração aguda tendo como agente lesivo o etanol absoluto (Morimoto *et al.*, 1991). Esses animais foram pré-tratados com Tween, lanzoprazol e com o óleo essencial de sacaca, e o sangue desses animais foi submetido à dosagens de SMT e gastrina por radioimunoensaio (RIE). Foi possível verificar que os animais desnutridos, tratados com Tween, tiveram níveis de SMT plasmática semelhante aos níveis de SMT obtidos com animais normais, ou seja níveis baixos deste hormônio. Após o tratamento dos animais normo e hipoprotéicos com lanzoprazol os níveis de SMT estiveram bastante elevados mas não diferiram entre si. Com o pré-tratamento do óleo essencial de *C. cajucara* os níveis de SMT, em ambos os grupos, continuaram elevados. Um aumento significativo nas dosagens de SMT no grupo de animais desnutridos foi observada quando as mesmas foram comparadas às dosagens dos animais normais ($P<0.001$).

Karmeli *et al.* (1994) discutem que o etanol, quando administrado via oral, causa hiperemia local, edema, necrose, hemorragia submucosa, bem como distúrbios circulatórios. A extensão dos danos causados pelo etanol na mucosa gástrica de ratos está relacionada ao aumento da desgranulação de mastócitos, que deve ser a fonte de vários neuropeptídios e mediadores inflamatórios, entre os quais estão histamina e leucotrienos nos mastócitos; estariam ainda, substância P e peptídio intestinal vasoativo (VIP) que levam ao aumento dos danos induzidos pelo etanol na mucosa gástrica de roedores. Sabe-se que a SMT previne os danos da mucosa devido à diminuição da secreção ácida. Os mesmos autores enfatizaram que o efeito protetor da SMT, neste estudo, foi devido à diminuição da geração de leucotrienos na mucosa e diminuição dos níveis de substância P e VIP.

Ainda Lucey & Yamada (1989) e Diel & Szabo (1986) sugeriram que os mecanismos para explicar o efeito anti-ulcerogênico da SMT estão relacionados à inibição da secreção ácida gástrica, redução do fluxo sanguíneo esplâncnico, geração de prostaglandina endógena, estimulação da secreção de muco gástrico e possível bloqueio da liberação de histamina de mastócitos.

Nas análises das dosagens de gastrina por RIE em nossos experimentos pudemos observar que os valores deste hormônio no soro de animais normo e hipoprotéicos tratados com

o óleo essencial de *C. cajucara*, foram similares aos valores encontrados nos dois grupos animais (normo e hipoprotéicos) tratados com o controle positivo lanzoprazol ($p<0.001$). Esses valores hormonais foram menores do que os valores de gastrina encontrados nos animais tratados com o controle negativo de Tween 80 a 12% ($p<0.001$). A gastrina aparece em adição na estimulação da secreção ácida gástrica; entretanto não seria correto afirmar que a prevenção/tratamento de úlceras gástricas esteja somente relacionada à inibição da secreção ácida; é importante lembrar que estes efeitos envolvem também um aumento dos fatores protetores da mucosa gástrica (Konturek *et al.*, 1983, Blandizzi *et al.*, 1994).

Neste caminho, os efeitos preventivos causados pelo óleo essencial de *C. cajucara* na metodologia de indução de úlcera por etanol, explicam o aumento dos níveis de SMT bloqueando a liberação de gastrina ou reduzindo seus níveis e, consequentemente, as lesões gástricas. Entretanto, vários fatores endógenos, incluindo PG's, EGF, gastrina, somatostatina, peptídeos sensoriais, óxido nítrico e compostos sulfidrílicos, estão implicados na patofisiologia da gastroproteção. Assim, é possível relacionar nossos resultados com óleo essencial de *Croton cajucara* e alguns desses mecanismos, como o aumento da produção de muco, PG's, EGF e aumento de SMT e diminuição de gastrina; ou ainda sugerir outros conforme discutido acima. De qualquer forma, a SMT funcionou como um peptídio modulador da ulcerogênese, já que os animais desnutridos tiveram menor índice de lesão ulcerativa (conforme relatado anteriormente) apresentando valores significativamente altos de SMT plasmática.

A análise histológica da mucosa gástrica de animais normais e desnutridos pôde revelar a extensão dos danos causados por agentes indutores de úlcera aguda e úlcera crônica. Desta forma, além das figuras contendo cortes histológicos, foi possível quantificar a profundidade dessas lesões. Mais uma vez foi confirmada a hipótese de que, no processo de ulceração aguda, os estômagos dos animais que se alimentaram com a dieta contendo 6% de proteínas, apresentaram lesões menos graves do que os estômagos daqueles animais que se alimentaram com dieta contendo 17% de proteína, devido ao aumento de fatores citoprotetores da mucosa gástrica. No entanto, com relação à ulceração crônica, foi possível observar que os animais normoprotéicos tiveram uma melhor cicatrização da mucosa gástrica quando comparado aos dados obtidos com os animais desnutridos. Isto pode ser explicado pelo fato de que animais normais, em nossos experimentos de biologia molecular, expressam melhor o fator de crescimento epidermal garantindo a cicatrização da mucosa gástrica.

Desta forma, concluiu-se que o óleo essencial de *Croton cajucara* previniu e cicatrizou úlceras pépticas, sendo o efeito descrito obtido através de mecanismos de citoproteção com aumento da produção de PGE_2 e muco em ambos os grupos estudados, e por mecanismos que garantem a reepitelização da mucosa gástrica (estimulando a angiogênese) aumentando a expressão de EGF em animais tratados com dieta contendo 6% de proteínas e, principalmente, naqueles alimentados com dieta contendo 17% de proteínas.

Ainda, os altos níveis de lípides no plasma dos animais desnutridos, o baixo conteúdo de DNA/RNA, o aumento da produção de muco na mucosa gástrica dos animais desnutridos e a liberação acentuada de corticosterona circulante, agravada pelo fator estresse, seriam caminhos que poderiam conduzir a uma melhor compreensão do mecanismo que estaria protegendo a mucosa gástrica dos animais com restrição protéica. Este fato é importante pois os mesmos poderiam ter um papel preponderante na taxa de formação de úlceras gástricas. Também, a indicação popular das cascas de sacaca como agente antiulcerogênico possui fundamentos farmacológicos evidenciados pelo óleo essencial com atividade cicatrizante e citoprotetora na mucosa gástrica tanto em animais que receberam dieta 17% quanto aqueles que receberam dieta 6%, sugerindo que o teor protéico na dieta não é um fator importante para que o óleo exerça seu poder cicatrizante ou protetor.

V- CONCLUSÕES

Como última análise é possível concluir que os animais desnutridos apresentam um menor índice de lesão gástrica aguda quando comparado aos animais normoprotéicos. Isto se deu através de vários modelos de úlcera devido ao aumento de fatores citoprotetores da mucosa gástrica como muco e prostaglandina E_2 (PGE_2). Em contrapartida, as lesões crônicas formadas nos animais desnutridos foram mais graves do que no grupo de animal normoprotéico.

Com relação ao estudo do efeito anti-ulcerogênico do óleo essencial obtido das cascas de *Croton cajucara* Benth. em modelos de úlcera aguda, verificamos uma prevenção na formação de lesões gástricas em ambos os grupos animais. Com relação ao tratamento de úlcera crônica, o óleo essencial de sacaca mostrou seu efeito cicatrizante através do aumento de fator de crescimento epidermal (EGF), aliado ao aumento da produção de muco e PGE_2 e dos níveis séricos de somatostatina com consequente redução dos valores de gastrina.

Assim a indicação popular das cascas de sacaca como agente antiulcerogênico possui fundamentos farmacológicos evidenciados pelo óleo essencial com atividade cicatrizante e citoprotetora na mucosa gástrica, tanto em animais que receberam dieta 17% quanto aqueles que receberam dieta 6%, sugerindo que o teor protéico na dieta não é um fator importante para que o óleo exerça seu poder cicatrizante ou protetor.

VI- REFERÊNCIAS BIBLIOGRÁFICAS

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