KARIN MAIA MONTEIRO

AVALIAÇÃO TOXICOLÓGICA E FARMACOLÓGICA DO COMPLEMENTO NUTRICIONAL "TK3"

CAMPINAS

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ORIENTADOR: *PROF. DR. JOÃO ERNESTO DE CARVALHO*

CPQBA/UNICAMP

CO-ORIENTADORA: *DRA. ANA LÚCIA TASCA GÓIS RUIZ*

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Membros:

1. Prof(a). Dr(a) Carina Denny
2. Prof(a). Dr(a). Valdomiro Carlos Sgarbieri
3. Prof(a). Dr(a). João Ernesto de Carvalho

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a beleza da vida.

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"Não nos recordamos dos dias, recordamo-nos dos momentos."

Cesare Pavese

"Trago dentro do meu coração,
Como num cofre que não se pode
Fechar
De cheio,
Todos os lugares onde estive,
Todos os portos a que cheguei,
Todas as paisagens que vi através de
Janelas ou vigias,
Ou de tombadilhos, sonhando,
E tudo isso, que é tanto,
É pouco para o que eu quero."

Álvaro de Campos

"Muitas vezes as pessoas são egocêntricas, ilógicas e insensatas.

Perdoe-as assim mesmo.

Se você é gentil, as pessoas podem acusá-lo de egoísta, interesseiro.

Seja gentil assim mesmo.

Se você é um vencedor, terá alguns falsos amigos e alguns inimigos verdadeiros.

Vença assim mesmo.

Se você é honesto e franco, as pessoas podem enganá-lo.

Seja honesto assim mesmo.

O que você levou anos para construir, alguém pode destruir de uma hora para outra.

Construa assim mesmo.

Se você tem paz, é feliz, as pessoas podem sentir inveja.

Seja feliz assim mesmo.

Dê ao mundo o melhor de você, mas isso nunca pode ser o bastante.

Dê o melhor de você assim mesmo.

Veja você que, no final das contas, é entre você e Deus.

Nunca foi entre você e as outras pessoas."

Madre Tereza de Calcutá

"A caridade não brilha unicamente na dádiva.
Destaca-se nos mínimos gestos do cotidiano.
Está no sorriso de compreensão e tolerância;
na palavra que tranqüiliza; na gentileza
para com desconhecidos; no amparo a criança;
no socorro ao doente; na atenção para com quem fala;
no acatamento das confidências de um amigo;
no silêncio, ante os conceitos agressivos desse
ou daquele adversário; e no respeito perante os hábitos
e as cicatrizes do próximo."

Emmanuel

786-O	Linhagem tumoral renal
AMPc	Adenosina monofosfato cíclico
ALB	Albumina / *albumin*
ALP	Fosfatase alcalina / *alkaline phosphatase*
ALT	Alanina aminotransferase / *alanine aminotransferase*
ANOVA	Análise de variância entre grupos
AST	Aspartato aminotransferase / *aspartate aminotransferase*
ATP	Adenosina trifosfato
BCT	Tempo de coagulação / *blood clotting time/potential*
BUN	Uréia / *blood urea nitrogen*
C	Controle de célula
CGRP	*Calcitonin gene related peptide*
CHO	Coleterol total / *total cholesterol*
COBEA	Colégio Brasileiro de Experimentação Animal
COX	Cicloxigenase / *cyclooxygenase*
CRE	Creatinina / *creatinine*
DDR	Dose diária recomendada
DMSO	*Dimethyl sulfoxide*
DNA	Ácido desoxirribonucleico
EAC	Tumor ascítico de Ehrlich
EC	Células enterocromafins / *enterochromaffin cells*
EMS	*Eosinophilia-Myalgia-Syndrome*

FDA	*US-Food and Drug Administration*
GCP	Boas Práticas Clínicas / *Good Clinical Practice*
GLU	Glicose / *glucose*
GSH	Glutationa / *glutathione*
HBG	Concentração de hemoglobina / *haemoblogin concentration*
HCT	Hematócrito / *Haematocrit*
5-HT	5-hidroxitriptamina
HT-29	Linhagem tumoral de cólon
IC	Inibição de crescimento
INCA	Instituto Nacional do Câncer
i.p.	Intra-peritoneal
i.v. / e.v.	Endovenoso
L-NAME	Nω- L-arginina metil ester / *Nω- L-arginine methyl esther*
MCF-7	Linhagem tumoral de mama
MCH	Hemoglobina corpuscular média / *mean corpuscular haemoglobin*
MCHC	Concentração de hemoglobina corpuscular média / *mean corpuscular haemoglobin concentration*
MCV	Volume corpuscular médio / *mean corpuscular volume*
NaCl	Cloreto de sódio
NaOH	Hidróxido de sódio
NADPH	*Nicotinamide adenine dinucleotide phosphate*
NCI	*National Cancer Institute*
NCI-ADR/RES	Linhagem tumoral de mama com fenótipo de resistância à múltiplas drogas

NCI-H460	Linhagem tumoral de pulmão tipo não-pequenas células
NEM	N-etilmaleimida / *N-ethylmaleimide*
NO	Óxido nítrico / *nitric oxide*
NOAEL	*No-observed-adverse effects at the lowest dose level*
OECD	*Organisation for Economic Co-operation and Development*
OMS	Organização Mundial de Saúde
OVCAR-3	Linhagem tumoral de ovário
PC-3	Linhagem tumoral de próstata
PG	Prostaglandina / *prostaglandin*
pH	Potencial de hidrogênio iônico
PLC	Contagem de plaquetas / *platelet count*
p.o.	Oral / *per os*
POT	Potássio / *potassium*
RBC	Contagem de eritrócitos totais / *red blood cell count*
RNA	Ácido ribonucleico
ROS	*Reactive oxygen species*
s.c.	Sub-cutânea
SFB	Soro fetal bovino inativado
SH	Compostos sulfidrílicos / *sulfhydryl compounds*
SNC	Sistema Nervoso Central
SOD	Sódio / *sodium*
T	Média da absorbância da célula tratada
To	Controle das células no dia da adição das drogas

TCA	Ácido tricloroacético
TLD	*thymineless death*
TPR	Proteína total / total protein
TS	*Thymidylate synthase*
UACC-62	Linhagem celular de melanoma
UKCCCR	*United Kingdom Coordinating Committee on Cancer Research*
ULI	Índice de lesões ulcerativas / *ulcerative lesions index*
VIP	Peptídeo intestinal vasoativo
WBC	Contagem de leucócitos totais / *total leukocyte count*

PÁG.

RESUMO

O complemento nutricional "TK3" foi inicialmente estudado por um bioquímico alemão, Sr. Friedrich Lavitcshka, que desenvolveu uma formulação em 1969, inicialmente líquida, constituída basicamente pela associação de triptofano e timina. Seus estudos em modelos experimentais de tumores de pele induzidos por alcatrão em ratos não foram publicados. Entretanto, seus resultados, bem como relatos de casos provenientes do uso informal de tal suplementação, apontavam para efeitos benéficos superiores ao de um mero complemento nutricional ou "fortificante", como se costumava denominar tal categoria de substâncias.

A avaliação toxicológica deste complemento, como parte inicial deste estudo pré-clínico, revelou:

1. Estudos "in vitro": atividade não citotóxica de "TK3" em cultura de células humanas normais – fibroblastos – e tumorais;

2. Estudos agudos "in vivo": a administração aguda de "TK3" (5 g/Kg, p.o. e 2g/Kg, i.p.) em ratos *Wistar* não produziu quaisquer sinais clínicos de toxicidade. A análise macroscópica dos órgãos destes animais experimentais não revelou quaisquer alterações sugestivas de toxicidade;

3. Estudos sub-crônicos "in vivo": a administração doses-repetidas 90 dias de "TK3" (100 mg/Kg, 300 mg/Kg e 1000 mg/Kg, *p.o.*) em ratos *Wistar* de ambos os sexos igualmente não revelou quaisquer sinais de toxicidade, confirmados pela análise macroscópica dos órgãos. Finalmente, as análises hematológicas e bioquímicas não identificaram alterações indicativas de toxicidade.

A triagem farmacológica deste complemento revelou:

1. Em modelo de úlcera induzida por etanol em ratos , o tratamento com "TK3" (1000 mg/Kg, *p.o.*) reduziu o índice de lesão ulcerativa (86.2%). Esta atividade antiulcerogênica foi superior àquela apresentada pela administração dos componentes isoladamente (triptofano: 400mg/Kg [61.5%] e timina: 400 mg/Kg [+30.8%]);

2. Na avaliação da atividade antisecretora, em modelo de ligadura de piloro por 4 horas, a administração intraduodenal de "TK3" (1000 mg/Kg, *p.o.*) não reduziu o volume, nem a acidez total e nem aumentou o pH da secreção ácida gástrica basal;

3. Na triagem dos mecanismos de citoproteção gástrica, o pré-tratamento de ratos com indometacina (5 mg/Kg, *p.o.*) não inibiu o efeito protetor do "TK3" (1000 mg/Kg, *p.o.*) nas lesões induzidas por etanol;

4. O pré-tratamento de ratos com L-NAME (5 mg/Kg, *i.v.*) não inibiu o efeito protetor do "TK3" (1000 mg/Kg, *p.o.*) nas lesões induzidas por etanol;

5. Finalmente, o pré-tratamento de ratos com NEM (10 mg/Kg, *s.c.*) foi capaz de inibir em 50% o efeito protetor do "TK3" (1000 mg/Kg, *p.o.*) nas lesões induzidas por etanol, sugerindo a participação de compostos contendo grupos sulfidrila no mecanismo de ação do referido complemento.

ABSTRACT

The nutritional supplement "TK3" was originally studied by a German biochemist, Mr. Friedrich Lavitcshka, who first associated L-tryptophan and thymine, in a liquid formula, in 1969. His early studies using experimental tar-induced skin cancer models were not published even though they seemed to confirm the benefits reported by informal use of such association at that time. These reports suggested some pharmacological effects beyond the nutritional one.

The toxicological evaluation of this new association of substances, as part of its pre-clinical trial, revealed:

1. "In vitro" studies: no citotoxicity activity of "TK3" on normal (fibroblasts) and cancer human cells;

2. "In vivo" acute studies: acute administration of "TK3" (5 g/Kg, *p.o.* and 2g/Kg, *i.p.*) in Wistar rats revealed no signs of systemic toxicity or impending death;

3. "in vivo" sub-chronic studies: 90 day repeated-dose study of "TK3" (100 mg/Kg, 300 mg/Kg and 1000 mg/Kg, *p.o.*) in Wistar rats of both sexes revealed no clinical signs of toxicity, confirmed by both histopathological and biochemical analysis.

The pharmacological screening of this nutritional supplement revealed:

1. In the ethanol-induced gastric ulcer experimental model in rats, the treatment with "TK3" (1000mg/Kg, *p.o.*) reduced the ulcerative ulcer index (86.2%). This antiulcerogenic activity was superior to the ones presented by the isolated components administrated individually (L-tryptophan: 400mg/Kg [61.5%] and thymine: 400mg/Kg [+30.8%]);

2. In the evaluation of gastric secretion through pyloric ligation model in rats, the treatment with "TK3" (1000 mg/Kg, *i.d.*) had no significant effect over the gastric juice volume, nor the pH or hydrogenionic concentration of gastric content;

3. In the evaluation of possible gastric cytoprotective mechanisms of action, pre-treatment of experimental rats with indomethacin (5 mg/Kg, *p.o.*) did not inhibit the antiulcerogenic effect of "TK3" (1000 mg/Kg, *p.o.*) in the ethanol induced ulcer model;

4. Similarly, pre-treatment of experimental rats with L-NAME (5 mg/Kg, *i.v.*) did not inhibit the antiulcerogenic effect of "TK3" (1000 mg/Kg, *p.o.*) in the ethanol induced ulcer model;

5. Finally, pre-treatment of experimental rats with NEM (10 mg/Kg, *s.c.*) decreased 50% of the antiulcerogenic effect of "TK3" (1000 mg/Kg, *p.o.*) in the ethanol induced ulcer model, suggesting the participation of endogenous non-protein SH-containing compounds. However, as this reduction was not complete, there seems to be more aspects involved in this mechanism of action.

INTRODUÇÃO GERAL

Esta tese está de acordo com a informação CCPG 001/98, UNICAMP, que regulamenta o formato alternativo para dissertação e tese, permitindo a inserção de artigos científicos de autoria ou co-autoria do candidato. Assim sendo, esta tese é composta de dois estudos que se encontram em fase de submissão em revistas científicas.

O estudo toxicológico foi submetido à revista *Toxicology* e o estudo antiulcerogênico foi submetido à revista *Life Sciences*.

O objetivo geral deste trabalho foi realizar o estudo toxicológico do complemento nutricional "TK3" e avaliar as possíveis propriedades farmacológicas do referido complemento.

1.1- Complementos nutricionais

De acordo com a Agência Nacional de Vigilância Sanitária (ANVISA), complementos nutricionais são elaborados com a finalidade de complementar a dieta cotidiana de uma pessoa saudável, que deseja compensar um possível déficit de nutrientes, a fim de alcançar os valores da Dose Diária Recomendada (DDR). O Complemento Nutricional não substitui o alimento, não podendo ser utilizado como dieta exclusiva. A Portaria n°19, de 15 de março de 1995, elege o termo Complemento Nutricional em substituição aos termos complemento alimentar, suplemento alimentar e suplemento nutricional.

A ingestão de complementos nutricionais, de uma forma geral, deve levar em conta o nível de atividade física e a qualidade da alimentação do indivíduo a fim de se estabelecer as necessidades reais de uma possível complementação nutricional. Não podemos negligenciar, entretanto, a definitiva correlação entre dieta e saúde (Devereux, 2006; Miller Iii, 2006; Naghii & Fouladi, 2006; Wright et al., 2004; Ogilvie et al., 2000; Billman et al., 1999).

Pesquisas indicam que a incidência de câncer em todo o mundo poderia ser reduzida pela metade se fossem implantadas, em nível global, medidas que estimulem mudanças de comportamento, tais como redução do uso do tabaco, aumento da atividade física, controle da obesidade, consumo limitado de bebidas alcoólicas, uso de práticas

sexuais seguras, realização de exames de rotina, exposição solar controlada e, principalmente, consumo de alimentos saudáveis (Stein & Colditz, 2004).

O uso indiscriminado dos complementos nutricionais e vitamínicos, principalmente por parte de atletas e fisiculturistas em todo o mundo e daqueles que substituem um estilo de vida saudável pelo uso desses complementos, fez com que essa classe de compostos estivesse sujeita a aspectos legais. Apesar das inúmeras críticas sobre o uso de complementos nutricionais como adjuvantes em diversas patologias (Mudway et al., 2006; Huang et al., 2006; Chang et al., 2005), o crescente entendimento acerca dos mecanismos pelos quais fatores nutricionais podem afetar o sistema imunológico de um indivíduo, por exemplo, tem promovido um aumento do seu uso (Stephen & Avenell, 2006; Tam et al., 2003; Villamor et al., 2002; WHO, 2002).

A literatura contém inúmeros exemplos de estudos acerca dos possíveis efeitos benéficos da ingestão de aminoácidos livres como complementos nutricionais (Charlton, 2002). Infelizmente, a heterogeneidade das respostas terapêuticas em determinadas enfermidades parece gerar certa insegurança no que tange à prescrição destas substâncias ao público em geral (Choudry et al., 2006). Entretanto, sua ingestão tem apresentado resultados positivos como coadjuvante de diversas patologias, tais como infecções bacterianas (Taniguchi et al., 2006), doenças hepáticas (Mascarenhas & Mobarhan, 2004), entre outras. A Tabela 1 relaciona alguns exemplos do uso de aminoácidos como coadjuvantes no tratamento de patologias.

Tabela 1- Exemplos de aminoácidos utilizados como coadjuvantes no tratamento de patologias.

Aminoácido	Patologia	Referência
Glicina	Doença de Alzheimer	Fonteh et al., 2006
Alanina	*Glycogen Storage Disease type II (GSD II)*	Mundy et al., 2006
Glutamina	Câncer esofágico	Yoshida et al., 2001
Arginina	Doença Renal Crônica	Baylis, 2006
Selenocisteína	Doença Auto-imune da Tireóide	Duntas, 2006
Fenilalanina	Leucemia Mielóide Aguda	Furet et al., 2006
Prolina	Doença de Alzheimer	Bilikiewicz & Gaus, 2004.

A ingestão regular de fontes de bases purinas e pirimidínicas tem importantes efeitos no crescimento e desenvolvimento de células com rápida taxa de renovação, tais como as células do sistema imune (Jyonouchi et al., 2003), eritrócitos (Scopesi et al., 1999) e do trato gastrointestinal (Dancey et al., 2006). Como precursores de DNA e RNA, servem também a uma gama de funções no metabolismo celular (Szathmary, 2003).

1.1.1- Aminoácidos / Triptofano

Em química, um aminoácido é qualquer molécula que contém pelo menos um grupo funcional um carboxílico. Em bioquímica, este termo é usado para definir os alfa-aminoácidos, isto é, aqueles em que as funções amino e carboxilato estão ligadas ao mesmo carbono.

Aminoácidos essenciais são aqueles que não podem ser produzidos pelo corpo humano. Dessa forma, somente podem ser adquiridos pela ingestão de alimentos, vegetais ou animais. São eles Fenilalanina, Isoleucina, Leucina, Lisina, Metionina, Treonina, Triptofano e Valina (Bourre, 2006).

Os alfa aminoácidos apresentam como fórmula geral **R** - CH (NH2)-COOH onde **R** é um radical orgânico. No aminoácido glicina, o radical é o elemento **H**. Dependendo do radical **R**, os aminoácidos podem ser classificados em:

Aminoácidos ácidos: Apresentam radicais com grupo carboxílico. São hidrófilos. São eles: Ácido aspártico e ácido glutâmico.

Aminoácidos básicos: Apresentam radicais com o grupo amino. São hidrófilos. São eles: Arginina, Lisina e Histidina.

Aminoácidos polares neutros: Apresentam radicais que tendem a formar pontes de hidrogênio. São eles: Serina, Treonina, Cisteína, Tirosina, Asparagina e Glutamina.

Aminoácidos apolares: Apresentam radicais de hidrocarbonetos apolares ou hidrocarbonetos modificados, exceto a glicina. São radicais hidrófobos. A tabela 2 apresenta a relação de seus nomes e respectivas estruturas químicas.

Tabela 2- Relação de nomes e respectivas estruturas dos aminoácidos apolares.

Nome	**Estrutura**
Glicina	**H**- CH (NH_2) - COOH
Alanina	**CH_3**- CH (NH_2) - COOH
Leucina	**CH_3-CH_2-CH_2**-CH (NH_2)- COOH
Valina	**CH_3-CH(CH_3)**-CH (NH_2)- COOH
Isoleucina	**CH_3-CH_2-CH (CH_3)**-CH (NH_2)- COOH
Prolina	**-CH_2-CH_2-CH_2-** ligando o grupo amino ao carbono alfa
Fenilalanina	**C_6H_5-CH_2**-CH (NH_2)- COOH
Triptofano	**R aromático**- CH (NH_2)- COOH
Metionina	**CH_3-S-CH_2-CH_2**- CH (NH_2)- COOH

Na nomenclatura dos aminoácidos, a numeração dos carbonos da cadeia principal é iniciada a partir do carbono da carboxila. O triptofano ou ácido 2-amino-3-indolpropiônico pode ser representado pelas siglas Trp, Tri ou W (Figuras 1 e 2).

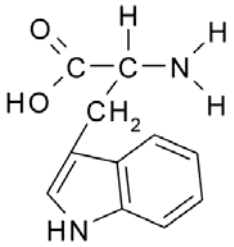

Fig. 1. Estrutura química do triptofano.

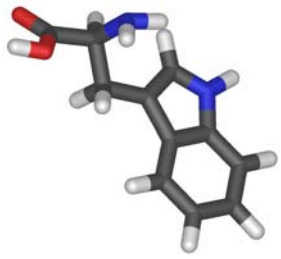

Fig. 2. Estrutura tridimensional do triptofano

O nitrogênio é uma importante fonte de elétrons para os seres vivos, através da amônia. Entretanto, só algumas bactérias conseguem converter nitrogênio em amônia. Na maior parte dos seres vivos, essa conversão é realizada por um sistema enzimático complexo, denominado nitrogenase, que utiliza NADPH como doador de elétrons e só é processado com um consumo muito grande de ATP (Syrtsova et al., 2000).

As proteínas são alfa-polímeros formados por alfa-aminoácidos. Alguns autores relatam que, para formar uma proteína, é necessária uma cadeia com mais de 70 aminoácidos (Dass & Choong, 2006). Os aminoácidos podem ser obtidos através da hidrólise de proteínas, quebrando-se as ligações peptídicas (ligação entre a carboxila de um aminoácido e o grupo amino de outro).

Os alfa-aminoácidos apresentam-se como sólidos com solubilidade variável em água. Possuem atividade óptica por apresentarem carbono assimétrico, em geral, na forma levógira. O grupo carboxílico (-COOH) na molécula confere ao aminoácido uma característica ácida enquanto o grupo amino (-NH2) uma característica básica. Por isso, os aminoácidos apresentam um caráter anfótero, ou seja, reagem tanto com ácidos como com bases formando sais orgânicos.

Dentre os aminoácidos essenciais, o L-triptofano é bastante peculiar, pois, além de só ser obtido por fontes exógenas, é o menos abundante na dieta. Essas características são extremamente relevantes quando se considera o importante papel que seus produtos finais, desempenham na fisiologia (Figura 3). Um deles é o neurotransmissor serotonina (5-hidroxitriptamina, 5-HT) (van der Stelt et al., 2004) que, em conjunto com a dopamina, norepinefrina, e epinefrina, modula diversos processos fisiológicos e psicológicos (Ozer et al., 2006; Widner et al., 2002).

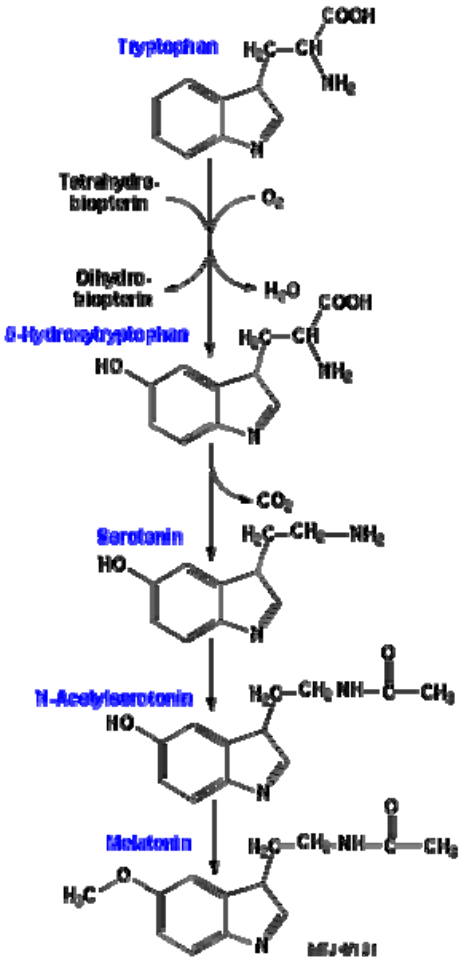

Fig. 3. Metabolismo parcial do triptofano.

A biossíntese de serotonina no corpo humano representa somente uma pequena parte da via metabólica do triptofano. Sob condições normais, não mais do que 2% do triptofano ingerido são utilizados nesta via, o que resulta numa produção diária de aproximadamente 10 mg de serotonina. A maior parte do triptofano é utilizada na síntese protéica, ao passo que sua maior via catabólica se dá através da *kinunerina* (Nemeth et al., 2006; Stone et al., 2003) e do ácido 3-hidroxiantranílico (Kema et al., 2000).

Uma vez absorvido, o triptofano pode seguir dois caminhos, permanecer na periferia ou ser transportado ao sistema nervoso central (SNC) onde será convertido em 5-HT. Romper a barreira hematoencefálica, entretanto, será o primeiro obstáculo por suas características pouco lipossolúveis, necessitando então o auxílio de proteínas transportadoras. Estas apresentam baixa seletividade pelos aminoácidos a serem transportados através da barreira, fazendo com que sejam selecionados, preferencialmente, aqueles aminoácidos cujas concentrações séricas estiverem mais elevadas. Isso explica o efeito paradoxal da dieta nos níveis de triptofano do líquor; na ocasião de uma dieta hiperproteica, o transportador será saturado pelos demais aminoácidos que terão acesso imediato ao SNC, enquanto que o triptofano, que é um dos menos abundantes na dieta, será aproveitado no metabolismo periférico (Verrey, 2003; Smith, 2000; Oldendorf & Szabo, 1976).

Quando é impossibilitado de alcançar o SNC, o triptofano será metabolizado de duas formas – uma reversível e outra não. A via reversível envolve a sua incorporação na síntese protéica no fígado e em outros tecidos (Munro, 1970). Esta via é dita reversível porque esses depósitos de triptofano podem ser mobilizados e posteriormente utilizados pelo SNC (Bloxam et al., 1974). A via irreversível envolve a enzima hepática L-triptofano-2,3-dioxigenase, também conhecida como triptofano pirrolase. A secreção dessa enzima é estimulada pelos níveis séricos de triptofano e de corticóides, ou seja, quanto mais elevados estes níveis, maior será a secreção da pirrolase (Young & Oravec, 1979). Um aumento na concentração do triptofano sérico aumentará a produção hepática desta enzima, diminuindo a meia-vida do aminoácido, ou seja, sua meia-vida é inversamente proporcional ao tamanho da dose administrada (Green et al., 1980).

1.1.1.1- Aplicação clínica do triptofano

O triptofano tem sido testado como antidepressivo em decorrência das hipóteses que associam a redução dos níveis de serotonina à etiologia da depressão (Bailara et al., 2006; Capuron & Dantzer, 2003; Bonkale et al., 2006). Entretanto, estudos

clínicos realizados por Shansis e colaboradores (2000) levantam a hipótese de que o triptofano seja mais eficaz em pacientes com depressões leves a moderadas.

A biodisponibilidade do triptofano é uma etapa limitante na síntese de serotonina e pode provocar alterações no humor em animais e seres humanos (Moore et al., 2000). Este fato tem sido explorado nas pesquisas onde a suplementação com este aminoácido (ou uma dieta com quantidades reduzidas de aminoácidos "competidores") é utilizada para alterar o *status* social e mental de primatas não-humanos (Moskowitz et al., 2001). As primeiras hipóteses de que baixos níveis de serotonina cerebral podem gerar comportamentos agressivos foram propostas por Kantak e colaboradores, em 1980, em experimentos com camundongos. Em estudos com pacientes esquizofrênicos, o triptofano administrado a pacientes agressivos reduziu o número de episódios de agressão (Monrad et al., 1983). Em indivíduos propensos ao comportamento agressivo, a restrição aguda do triptofano na dieta provocou uma tendência de aumento da agressividade (Cleare & Bond, 1995).

Estudos demonstrando o efeito do triptofano no sono revelam que este é mais efetivo em insônias leves e moderadas, e menos efetivo em insônias severas ou crônicas (Riemann et al., 2002; Hartmann & Greenwald, 1984). Seu pico de efeito, em humanos, é de aproximadamente 45 minutos após a sua administração (Poloni et al., 1974).

A ação antiulcerogênica do triptofano tem sido largamente estudada isoladamente e em comparação com os efeitos farmacológicos da melatonina (Brzozowska et al., 2002; Konturek et al., 1997).

1.1.1.2- Efeitos adversos do triptofano

Na década de 80, o triptofano já havia surgido no mercado de alimentos naturais, inclusive como adjuvante no tratamento de distúrbios do sono. No início dos anos 90, o FDA (*US-Food and Drug Administration*) restringiu de forma rigorosa a comercialização deste aminoácido como complemento nutricional, pois seu consumo foi relacionado a uma patologia muscular debilitante e incurável, a *Eosinophilia-Myalgia*

Syndrome (EMS). Entretanto, a relação desta patologia e a ingestão de L-triptofano não foram conclusivas (Daniels et al., 1995), sendo identificados diversos contaminantes responsáveis pelos efeitos tóxicos observados (Suzuki et al., 1996; Buss et al., 1996). Tal fato teve origem no emprego de metodologia alternativa para a purificação do referido aminoácido, por uma empresa petroquímica japonesa, a Showa Denko KK, que utilizou microorganismos (bactérias) geneticamente modificados, a fim de reduzir custos. Como resultado, mais de 1.500 pessoas ficaram permanentemente debilitadas e 37 foram a óbito pelo consumo de tais lotes de triptofano contaminados. Pesquisas posteriores identificaram tais impurezas (Simat et al., 1996).

1.1.2- Timina

Timina é um derivado pirimidínico, portanto, um constituinte do ácido nucléico. Entretanto, a grande maioria dos estudos realizados com esta substância concentra-se na área da Microbiologia, citando esta base como elemento fundamental para o desenvolvimento celular (Courcelle, 2005).

A timina, também conhecida como 5-metiluracila, é uma base nitrogenada que compõe o nucleotídeo, a principal estrutura que forma o ácido desoxirubonucléico, mais conhecido como DNA. Como o nome sugere, a timina pode ser formada a partir da metilação da uracila no 5° carbono. A estrutura da timina é formada por átomos que formam uma molécula num único anel. Este tipo de estrutura é chamada pirimidina.

A timina é a única base nitrogenada que existe apenas no DNA. As outras moléculas (guanina, citosina e adenina) também fazem parte do ácido ribonucléico (RNA). No RNA, a timina é substituída pela uracila. A Figura 4 apresenta a disposição das bases nitrogenadas tanto na molécula de RNA como na de DNA.

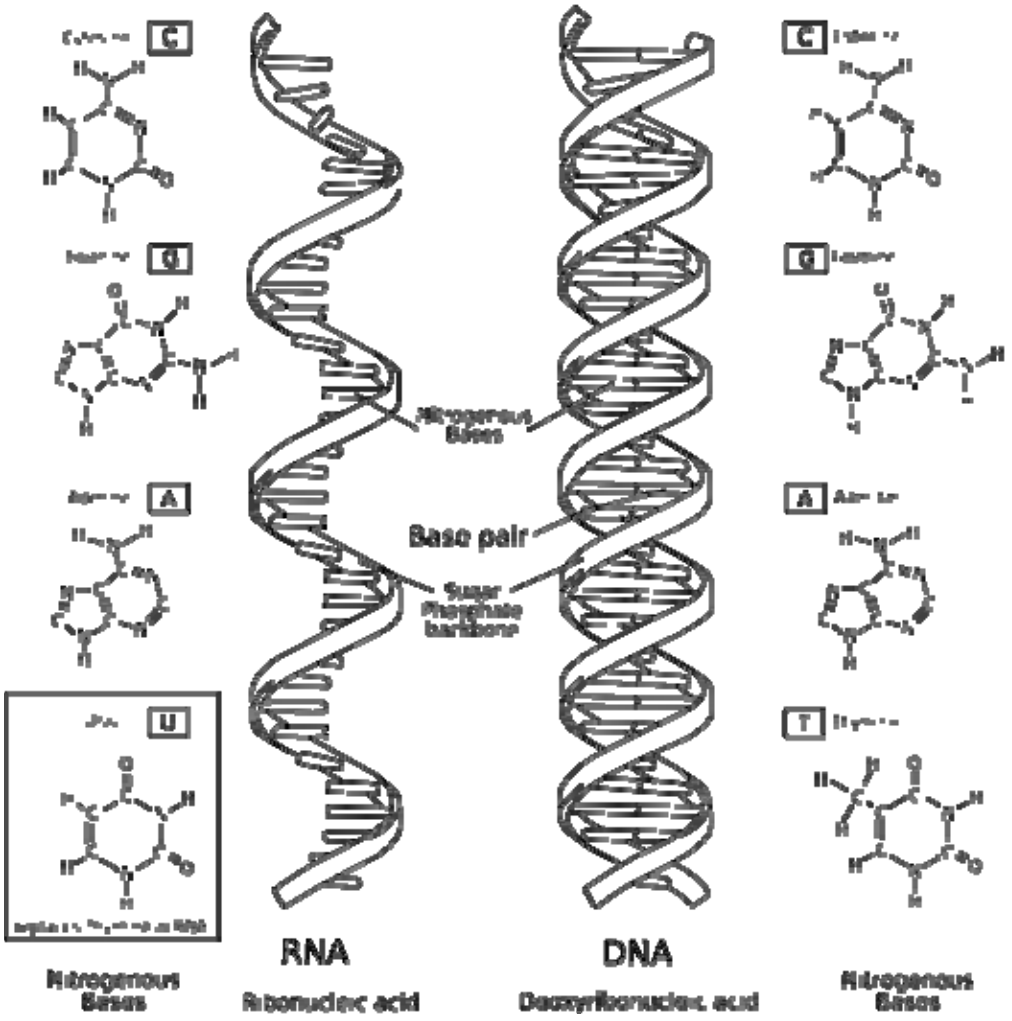

Fig. 4. Representação das moléculas de RNA e DNA.

1.1.3- Complemento Nutricional “TK3”

O complemento nutricional “TK3” foi inicialmente estudado por um bioquímico alemão, Sr. Friedrich Lavitcshka, que desenvolveu uma formulação em 1969, inicialmente líquida, constituída basicamente pela associação de triptofano e timina. Seus estudos em modelos experimentais de tumores de pele induzidos por alcatrão em ratos não foram publicados. Entretanto, seus resultados, bem como relatos de casos provenientes do uso informal de tal suplementação, apontaram para efeitos benéficos superiores ao de um

mero complemento nutricional ou "fortificante", como se costumava denominar tal categoria de substâncias.

O professor Lavitcshka passou a formulação do produto ao seu amigo Sr. Paul Huber, que utilizou seus ensinamentos e deu continuidade ao trabalho. Em 2003, estudos toxicológicos foram iniciados com o complemento nutricional "TK3" a fim de determinar a segurança deste produto como primeira etapa da fase pré-clínica.

1.2- Toxicologia Pré-Clínica: Introdução de novas substâncias no mercado

Em 1947, quando as atrocidades cometidas pelos nazistas durante a Segunda Guerrra Mundial tornaram-se públicas, a humanidade percebeu a urgência de se estabelecer Diretrizes Éticas Para Investigação Biomédica Envolvendo Seres Humanos (Taylor, 1949). Somente duas décadas depois, em 1964, a Declaração de Helsinki elaborada pela Organização Mundial de Saúde (OMS) estabeleceu os preceitos éticos para a pesquisa biomédica em seres humanos com o objetivo de estabelecer normas internacionais, além de facilitar a movimentação global de produtos farmacêuticos (*World Medical Organization*, 1996). Foram criadas, posteriormente, Diretrizes para as Boas Práticas Clínicas (GCP) em ensaios de produtos farmacêuticos (*World Health Organization*, 2002).

A Toxicologia Pré-Clínica representa etapa fundamental na introdução de novas substâncias no mercado farmacêutico. Essa etapa do processo de desenvolvimento de novos fármacos tem por objetivos principais rejeitar compostos com toxicidade inaceitável, identificar órgãos e tecidos-alvo, determinar a reversibilidade ou irreversibilidade dos efeitos adversos e identificar o(s) mecanismo(s) de ação tóxica (WHO, 1983).

Os efeitos adversos que podem advir da administração de um xenobiótico podem ser classificados em:

1. Ação farmacodinâmica principal excessiva ("overdose" ou sensibilidade aumentada);

2. Efeitos farmacológicos não relacionados com a atividade terapêutica;

3. Efeitos tóxicos da droga ou metabólitos não previsíveis pela ação farmacológica;

4. Reações mediadas pelo sistema imunológico e outras reações de hipersensibilidade.

A ação farmacodinâmica principal excessiva, bem como os efeitos farmacológicos não relacionados com a atividade terapêutica podem ser detectados através de ensaios direcionados especificamente para determinados sistemas, tais como, avaliação de atividade sobre o sistema nervoso central, pressão arterial, trato respiratório, motilidade digestiva, diurese, contração uterina, entre outros. Essa fase inclui a caracterização da atividade farmacológica do xenobiótico em diversos modelos animais, ensaios bioquímicos e em órgãos isolados. Após essa caracterização são iniciados os testes toxicológicos propriamente ditos, obedecendo-se, de certa forma, uma hierarquia na escala evolutiva dos chamados sistemas-teste, a saber, inicialmente, recomenda-se uma extensa compilação de dados da literatura, bem como de resultados de testes anteriores. Não havendo informações acerca da toxicidade neste ponto, deve-se partir para a realização de testes *in vitro* disponíveis, incluído determinação de pH eliminando-se, assim, quaisquer produtos que apresentem toxicidade exacerbada (Carvalho, 2004).

1.2.1- Toxicidade Aguda

Passa-se, então, para os testes *in vivo*, inicialmente realizados em roedores, tendo como objetivo determinar uma relação quantitativa entre as doses administradas e sinais de toxicidade, avaliando clinicamente os parâmetros de alterações de peso e consumo de alimentos, alterações na aparência e comportamento, índice de letalidade e forma de indução de óbito. Os parâmetros laboratoriais analisados incluem análise bioquímica, hematológica e histopatológica (OECD, 2001).

Com base nas informações obtidas neste primeiro teste, são determinadas as doses para o teste de toxicidade doses repetidas, 28 ou 90 dias, ainda em roedores.

1.2.2- Toxicidade Doses-Repetidas 28 / 90 dias

O Teste de Toxicidade Doses Repetidas, como já mencionado, pode ser realizado em uma espécie roedora, respeitando-se um "n" experimental de 10 animais por sexo/grupo e uma não roedora, que pode ser cães da raça Beagle ou *minipigs* (n=3). Todos devem possuir linhagens e raças definidas e a via de administração também deve ser a mesma preconizada para uso humano (OECD, 1998).

A duração deste estudo dependerá do período de uso pelo ser humano; estudo de quatro semanas para até 30 dias de uso por ano e estudo de 12 semanas quando se trata de tratamentos superiores a 30 dias de uso por ano. Devem ser utilizados, no mínimo, três níveis de dose da substância-teste e um grupo controle, tratado com o veículo.

Após a distribuição aleatória em grupos, os animais são tratados diariamente, aceitando-se o esquema de 5/7 dias/semana. Diariamente, todos os animais devem ser clinicamente analisados, levando-se em conta os parâmetros de aparência geral, sinais clínicos de toxicidade, alterações de comportamento, consumo de ração e ganho de peso, alterações do sistema nervoso central, autônomo e locomotor. Ao término do período experimental é realizada a coleta de sangue periférico para análises hematológicas e bioquímicas. Em seguida, é realizada a análise anatomopatológica e pesagem dos órgãos e tecidos e, aqueles que apresentarem alterações macroscópicas, deverão ser fixados e enviados para análise histopatológica.

Os seguintes parâmetros laboratoriais são analisados: eritrócitos totais, hemoglobina, hematócrito, volume corpuscular médio, hemoglobina corpuscular média e concentração de hemoblobina corpuscular média; leucócitos totais, neutrófilos, linfócitos, monócitos, eosinófilos, plaquetas e tempo de coagulação; aspartato transaminase; alanina transaminase; fosfatase alcalina; albumina, colesterol total e triglicérides; creatina fosfoquinase, lactato desidrogenase, amilase, glicemia, proteínas totais, lipoproteínas de alta densidade, ácido úrico e cálcio; fósforo, uréia, creatinina, sódio, potássio e magnésio.

1.3- Úlcera Gástrica

Entre as diversas regiões do organismo, o estômago é a que possui o ambiente mais peculiar principalmente pela quantidade elevada de ácido clorídrico, que mantém o pH entre 0,9 e 2,0. Esse ambiente ácido, além de participar da digestão, desempenha um papel de extrema importância impedindo a entrada de muitos microorganismos e, portanto, protegendo o organismo de agentes infecciosos. No entanto, isso faz com que as mucosas estomacal e duodenal fiquem expostas à ação do ácido e da pepsina, responsáveis pelo início do processo de digestão (Bighetti et al., 2002).

Por definição, entendem-se como úlceras pépticas as lesões do epitélio gastrointestinal que atingem a *muscularis mucosae* (Figura 5). Segundo sua localização, as úlceras pépticas podem ser classificadas em gástricas ou duodenais, sendo ambas histologicamente semelhantes.

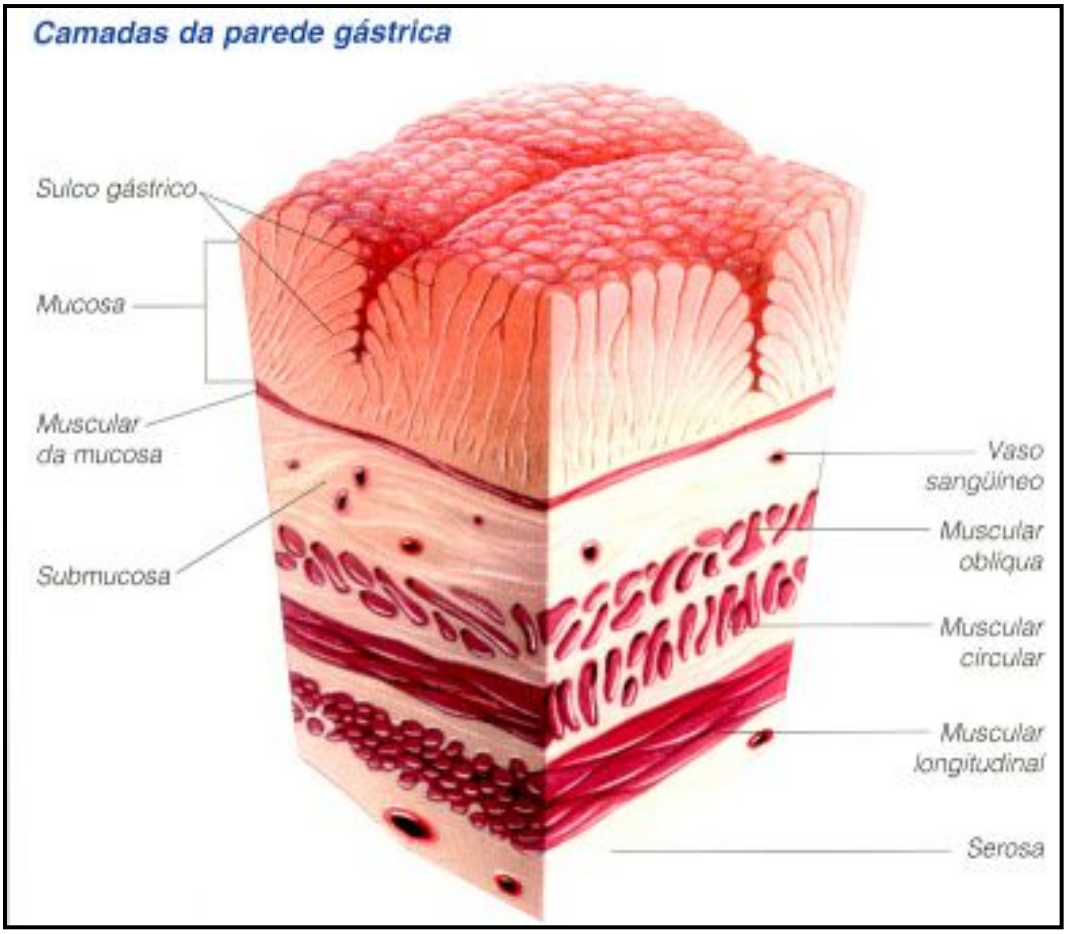

Fig. 5. Representação de corte da parede gástrica (http://www.gastronet.com.br)

Em condições normais, essa mucosa possui mecanismos de defesa contra a ação do ácido e da pepsina, além de protegê-la contra agentes agressivos exógenos como etanol (Rajendram & Preedy, 2005), fumo (Afridi, 2003; Ma et al., 1999), uso freqüente de drogas antiinflamatórias não esteroidais (Motilva et al., 2005), presença de *Helicobacter pylori* (Pajares & Gisbert, 2006; Konturek, 2003) e estresse (Bhatia & Tandon, 2005).

A úlcera gástrica é uma patologia complexa e multifatorial, cuja etiologia está relacionada com o desequilíbrio entre os fatores agressores e os defensores da mucosa gástrica. Durante o processo digestivo, essa mucosa está continuamente exposta ao ácido clorídrico e pepsina secretados. A produção de muco citoprotetor e bicarbonato criam uma barreira que neutraliza a ação do ácido sobre as células (Brzozowska et al., 2004). Tal

peculiaridade faz com que o controle da secreção, da digestão e da produção de fatores citoprotetores seja de extrema complexidade, com a participação do sistema nervoso central, plexo mioentérico, sistema nervoso autônomo, hormônios, neurotransmissores e autacóides.

1.3.1- Secreção Gástrica

A secreção ácida gástrica é regulada por mecanismos centrais e periféricos. A regulação central envolve a estimulação vagal pelo sistema nervoso central (mediada por receptores muscarínicos M_1) e conseqüente liberação de neurotransmissores das fibras pós-ganglionares no epitélio gástrico. A regulação periférica, por sua vez, compreende os eventos de exocitose em células endócrinas gástricas e intestinais, estimuladas principalmente pela histamina, acetilcolina e pela gastrina (Schubert, 2000).

Os neurônios entéricos secretam pelo menos uma dúzia de neurotransmissores cujas funções, da maioria deles, não são ainda totalmente conhecidas (Holzer, 2006). Acetilcolina e a noradrenalina são os mais estudados e provavelmente os mais influentes sobre esse sistema, onde o primeiro estimula suas funções enquanto o outro diminui. A adrenalina liberada pela glândula adrenal também exerce efeitos inibitórios sobre o trato gastrointestinal. Trifosfato de adenosina, serotonina, dopamina, colecistocinina, substância P, peptídeo intestinal vasoativo, somatostatina, leu-encefalina, met-encefalina também são neutransmissores liberados pelo plexo mioentérico onde alguns têm ações estimulantes e outros inibitórias (Guyton & Hall, 1996).

1.3.2- Mecanismos de Defesa Gástrica

Os mecanismos de defesa da mucosa gástrica podem ser didaticamente divididos em proteção pré-epitelial, epitelial e subepitelial (Guha & Kaunitz, 2002).

A proteção pré-epitelial é constituída pela barreira muco-bicarbonato, que auxilia na prevenção dos efeitos lesivos do conteúdo gástrico secretado. O muco, constituído principalmente por mucinas glicoproteicas, é secretado no lúmen

gastroduodenal pelas células epiteliais superficiais, pelas células do colo glandular e pelas glândulas submucosas de Brunner. É armazenado em grânulos no citoplasma das células mucosas do colo e nas células epiteliais superficiais, antes de ser liberado por exocitose (Allen & Flemstrom, 2005). Diversos secretagogos estimulam a secreção de mucinas no estômago. Dentre eles estão os agonistas muscarínicos, os agonistas β-adrenérgicos, a substância P, o peptídeo intestinal vasoativo (VIP), as prostaglandinas (E_1, E_2, $F_{2\beta}$, I_2) e a secretina. O Ca^{2+}, a proteína quinase C e o AMP_C, são os principais mediadores intracelulares envolvidos nos processos de secreção de mucinas (Forstner & Forstner, 1994). O bicarbonato é produzido e secretado pelas células epiteliais superficiais da mucosa gástrica e pelas células de Brunner duodenais, tornando a secreção mucosa alcalina (Takeuchi et al., 2005). A secreção gástrica do bicarbonato é estimulada pelo odor, paladar, visão e pensamento nos alimentos (fase cefálica), pelo ácido no lúmen gástrico (fase gástrica) e pelo ácido gástrico no lúmen duodenal (fase duodenal). O peptídeo intestinal vaso ativo, as prostaglandinas e os agonistas muscarínicos também estimulam esta secreção. A ativação do sistema nervoso simpático (em situações de estresse) é capaz de inibir a secreção gástrica de bicarbonato.

A proteção epitelial é garantida pelas propriedades intrínsecas das células epiteliais presentes na mucosa gástrica, graças à sua morfologia e características bioquímicas. Essas propriedades incluem a) o caráter hidrofóbico da mucosa que impede a difusão de íons hidrogênio, b) a presença de compostos sulfidrílicos não-proteicos, capazes de seqüestrar ânions superóxido e proteger diretamente a camada de muco dos radicais livres, e c) a capacidade de renovação tecidual através de migração e proliferação celular, apresentando um dos menores tempos de renovação dos tecidos corporais (Qiao et al., 2006).

A proteção subepitelial envolve fatores que permitem a manutenção dos processos de proteção pré-epiteliais. São eles o fluxo sangüíneo e a motilidade gastrointestinal. O fluxo sangüíneo garante o aporte adequado de oxigênio, nutrientes e hormônios, removendo os íons hidrogênio que penetram a barreira muco-bicarbonato e, nas células epiteliais, permitindo a rápida restituição de uma lesão epitelial (Clarke & Thompson, 2002; Sorbye & Svanes, 1994). Alterações na motilidade gastrointestinal

participam nos processos de defesa gástrica (Ephgrave et al., 1998). A redução do esvaziamento gástrico e a atividade motora do antro mediada por óxido nítrico endógeno refletem uma ação protetora deste mediador liberado por neurônios motores inibitórios. De forma similar, as prostaglandinas possuem atividade relaxante da musculatura gástrica (Holzer, 2000).

1.3.3- Mediadores da Defesa Gástrica

Dentre os mediadores endógenos relacionados à defesa gástrica destacam-se as prostaglandinas (principalmente PGE_2 e PGI_2), o óxido nítrico, os neuropeptídeos (CGRP) e as taquicininas (substância P, neurocinina A-NKA).

As prostaglandinas são eicosanóides sintetizados a partir do ácido araquidônico pela ação das cicloxigenases (Kaunitz & Akiba, 2004). São encontradas em altas concentrações no trato gastrointestinal (Wallace & Tigley, 1995) e são capazes de aumentar o fluxo sanguíneo, estimular a síntese de muco e bicarbonato, promover a manutenção dos grupos sulfidrílicos, bem como reduzir a secreção ácida gástrica e a motilidade gástrica (Martin & Wallace, 2006).

O óxido nítrico (NO) é sintetizado a partir dos átomos de nitrogênio no terminal guanidina da L-arginina. Existem duas classes de enzimas que estão envolvidas em sua síntese, a saber, a NO sintase constitutiva (com atividade cálcio-dependente) e a NO sintase indutível (com atividade independente de cálcio). A primeira está presente em altas concentrações na mucosa gástrica, em diversos tipos celulares, tais como células produtoras de muco, músculo liso, corpos celulares de neurônios do plexo mioentérico e células endoteliais. Essa ampla distribuição reflete a ampla participação do NO nos processos fisiológicos desse órgão, tais como a secreção de muco e bicarbonato, regulação do fluxo sanguíneo, modulação do tônus muscular e regulação da secreção ácida gástrica (Wallace, 2006). Entretanto, altas concentrações de NO produzidas pela ação da NO sintase indutível são lesivas à mucosa gástrica (Nishida et al., 1998).

Os mediadores que participam do chamado "sistema neural de emergência" são o peptídeo CGRP e as taquicinas (substância P e NKA), cujas ações são mediadas pelo NO. Esse sistema envolve a estimulação de fibras nervosas aferentes sensoriais presentes no trato gastrointestinal, plexo mioentérico, camada muscular circular e mucosa gástrica, facilitando os processos de defesa pré-epitelial (Holzer, 2000).

A maioria dos hormônios e neurotransmissores que atuam no sistema digestivo também possuem outras funções no organismo e, portanto, drogas que interagem com alguma dessas substâncias podem também exercer outras funções no organismo. Dessa forma, modelos experimentais utilizando o sistema digestório, ou partes dele, podem ser de grande utilidade para a descoberta de novas drogas. A Divisão de Farmacologia e Toxicologia (CPQBA/Unicamp) utiliza modelos experimentais de úlcera gástrica e de secreção estomacal, pois são modelos de custos relativamente baixo que necessitam basicamente de animais de laboratório e alguns reagentes.

1.4- Câncer

Segundo o *National Cancer Institute* (NCI), o termo câncer abrange todas as doenças nas quais células atípicas dividem-se descontroladamente. Células neoplásicas (ou cancerosas) são capazes de invadir tecidos adjacentes e ainda atingir a circulação sanguínea e sistema linfático, difundindo-se para outras partes do organismo. Existem diversos tipos e denominações gerais de câncer. Denomina-se carcinoma toda neoplasia maligna de origem epitelial (pele ou tecidos que revestem órgãos internos). Sarcoma, por sua vez, é a denominação geral para neoplasias malignas que têm origem nos tecidos ósseo, cartilaginoso, muscular, adiposo, vascular ou outro tecido conectivo ou de suporte. Leucemias são aquelas que têm origem em tecidos hematopoiéticos como a medula óssea e, como consequência, geram um aumento de células sanguíneas anormais circulantes. Finalmente, os linfomas e mielomas múltiplos são neoplasias malignas que têm origem nas células do sistema imune (*National Cancer Institute*, 1998).

Devido às novas técnicas de detecção, terapias de apoio e tratamento efetivo, o número de pessoas que sobrevivem ao diagnóstico de câncer nos Estados Unidos triplicou desde 1971 e têm crescido em média 2% a cada ano. Em 2001, essas pessoas totalizaram aproximadamente 10 milhões, o que representava, na época, 3,5% da população daquele país (Travis, 2006). Entretanto, o constante dilema dos oncologistas de todo o mundo parece ser a relação risco-benefício entre os efeitos citotóxicos da quimioterapia, que pode ser bem tolerada em pacientes mais jovens *versus* a inabilidade de cura em inúmeros casos, principalmente de tumores sólidos dada a não rara ineficácia dos fármacos existentes (Balis, 1998).

1.4.1- Epidemiologia

No Brasil, as estimativas para o ano de 2006 apontam que ocorrerão cerca de 472 mil casos novos de câncer. Os tipos mais incidentes, à exceção de pele não melanoma, serão os de próstata e pulmão no sexo masculino e mama e colo do útero no sexo feminino, acompanhando o mesmo perfil da magnitude observada no mundo. Em 2006 são esperados 234.570 casos novos para o sexo masculino e 237.480 para o sexo feminino. Estima-se que o câncer de pele não melanoma (116 mil casos novos) será o mais incidente na população brasileira, seguido pelos tumores de mama feminina (49 mil), próstata (47 mil), pulmão (27 mil), cólon e reto (25 mil), estômago (23 mil) e colo do útero (19 mil) (Instituto Nacional de Câncer-INCA, 2005). A Tabela 3 apresenta as estimativas do INCA para o ano de 2006 de novos casos de câncer, em homens e mulheres, segundo localização primária.

Tabela 3- Estimativas para o ano 2006 de número de casos novos por câncer, em homens e mulheres, segundo localização primária (INCA, 2005).

Localização primária	**Estimativa dos novos casos**		
Neoplasia maligna	**Masculino**	**Feminino**	**Total**
Mama Feminina	-	48.930	48.930
Traquéia, Brônquio e Pulmão	17.850	9.320	27.170
Estômago	14.970	8.230	23.200
Colo do útero	-	19.260	19.260
Próstata	47.280	-	47.280
Cólon e Reto	11.390	13.970	25.360
Esôfago	7.970	2.610	10.580
Leucemias	5.330	4.220	9.550
Cavidade Oral	10.060	3.410	13.470
Pele Melanoma	2.710	3.050	5.760
Outras Localizações	61.530	63.320	124.850
Subtotal	**179.090**	**176.320**	**355.410**
Pele não Melanoma	55.480	61.160	116.640
Todas as Neoplasias	**234.570**	**237.480**	**472.050**

Os tumores mais incidentes para o sexo masculino serão devidos ao câncer de pele não melanoma (55 mil casos novos), próstata (47 mil), pulmão (18 mil), estômago (15 mil) e cólon e reto (11 mil). Para o sexo feminino, destacam-se os tumores de pele não melanoma (61 mil casos novos), mama (49 mil), colo do útero (19 mil), cólon e reto (14 mil) e pulmão (9 mil) (INCA, 2005).

Pode-se facilmente concluir que, apesar do enorme progresso mundial na terapêutica das neoplasias malignas nos últimos 50 anos, a incidência desta patologia ainda demanda medidas efetivas e urgentes, tanto no que tange à prevenção como na descoberta de novos fármacos e métodos de diagnóstico (Ngoma, 2006).

1.4.2- Metodologias de triagem

A busca de novos quimioterápicos antineoplásicos tem utilizado, graças ao aprimoramento da metodologia de cultura de células, diversas linhagens celulares oriundas de tumores humanos, que possibilitaram o desenvolvimento da metodologia para triagem *in vitro*. Com esse objetivo, o Instituto Nacional do Câncer dos Estados Unidos desenvolveu um painel de células cancerígenas que, atualmente, conta com 60 linhagens oriundas de oito tipos de tumores sólidos (pulmão, melanoma, mama, rim, cólon, próstata, ovário, cérebro) e do sistema hematopoiético. Dessa forma, é possível avaliar diversos tipos de células neoplásicas, possibilitando a descoberta de drogas com maior especificidade. Outras vantagens dessa triagem são a rapidez e a eficiência do método, que em apenas uma semana permite avaliar um número elevado de substâncias (Rubistein et al., 1990; Skehan et al., 1990; Monks et al., 1991).

Um dos métodos desenvolvidos para avaliação *in vivo* no CPQBA foi o do tumor ascítico de Ehrlich (Sacoman et al., 2006). É um modelo prático e transponível para a análise de efeitos antineoplásicos de diversas moléculas e compostos. Após a inoculação intraperitoneal das células tumorais, o volume da ascite e o número de células aumentam progressivamente (Vincent & Nicholls, 1967). A ascite se forma provavelmente pela inflamação induzida pelo tumor, aumentando a permeabilidade vascular na reigião peritoneal (Fastaia & Dumont, 1976). Os animais vão a óbito após um curto período de tempo, em conseqüência da pressão mecânica exercida pelo fluido ascítico, da hemorragia intraperitoneal e da endotoxemia (Mayer, 1966). Além disso, a progressão do tumor de Ehrlich é caracterizada por profundas alterações na resposta imune e consumo elevado de glutamina, que está associado ao quadro de declínio da imunocompetência do organismo do animal (Melo et al., 2004). Esse modelo se mostra útil para demonstrar o efeito *in vivo* de um novo composto que, quando produz um ligeiro aumento no tempo de sobrevida dos animais, pode ser selecionado para as próximas etapas do estudo, em modelos que permitam uma melhor transposição de dados para a espécie humana.

CAPÍTULOS

CAPÍTULO 1 - "TK3": ASSOCIATION BETWEEN L-TRYPTOPHAN AND THYMINE – TOXICOLOGICAL STUDIES

Karin M. Monteiro[1,2], Paul Huber[5], Maria Christina M. Matias[1], Marcia A. Antonio[1,3], Alik T. F. Santos[1,2], Ana Possenti[1], Sirlene V. Tinti[1], Luiz A. Santos[1], Carina Denny[1], Luciana K. Kohn[1], Maria Carolina Bayeux[1,4], Cristiana Madjarof[1,4], Juliana Lessa[1,4], Ana Lúcia T. G. Ruiz[1], and João E. Carvalho[1,2]

[1]*Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas, Universidade Estadual de Campinas, P.O. Box 6171, ZIP Code 13083 970, Campinas, São Paulo, Brazil*

[2]*Departamento de Clínica Médica da Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Cidade Universitária Zeferino Vaz, ZIP Code 13081 970, Campinas, São Paulo, Brazil*

[3]*Universidade São Francisco, Curso de Farmácia, Campinas Campus, Rua Waldemar César da Silveira, 105 - Vila Cura D'Ars, ZIP Code 13045-510, Campinas, São Paulo, Brazil*

[4]*Departamento de Biologia Celular e Estrutural, Instituto de Biologia, Universidade Estadual de Campinas, Cidade Universitária Zeferino Vaz, ZIP Code 13083 863, Campinas, São Paulo, Brazil*

[5]*Lavilabor Produtos Naturais Ltda., Rod. Raposo Tavares, km 256, ZIP Code 18725000 Hollambra II, Paranapanema, SP, Brazil.*

*Corresponding author: Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas, Universidade Estadual de Campinas, P.O. Box 6171, ZIP Code 13083 970, Campinas, São Paulo, Brazil

Tel: +55-19-3884-7500 ext. 249, Fax: +55-19-3884-7811

E-mail address: farmatox@cpqba.unicamp.br

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Abstract

The main components of "TK3", a new nutritional supplement, are L-tryptophan and thymine. Pre-clinical acute and sub-chronic toxicity studies were investigated in rats to evaluate the safety of this association. Sighting *in vitro* studies of "TK3" were performed on normal (fibroblasts) and cancer human cells in order to anticipate possible hazards. *In vivo* studies consisted of a single intraperitoneal dose of "TK3" at 2000 mg/kg b.w. for acute systemic toxicity and a single oral dose of "TK3" at 5000 mg/kg b.w. studied over 14 days for acute toxicity. The sub-chronic (90-day) study involved daily oral doses of 100, 300 and 1000 mg/kg/day b.w. (5 days/week). Criteria to assess toxicity included daily clinical observations, body weight and food consumption, haematology, clinical biochemistry, gross examination at necropsy, organ weights and histopathology of vital organs at the termination of the experimental period. All animals survived the duration of the study and no significant toxicological effects were observed in any of the *in vivo* studies. Male rats in the high-dose group exhibited slightly higher WBC count compared to those of the controls. In conclusion, the results indicate no adverse effects of this nutritional supplementation in *Wistar* rats.

1- Introduction

L-tryptophan has been widely studied in the field of psychopharmacology as an important precursor of both serotonin and melatonin. Its use became highly controversial in 1989 when the US Food and Drug Administration (FDA) limited its availability as a food supplement for humans. This happened after a US epidemic of Eosinophilia-Myalgia-Syndrome (EMS), involving more than 1500 reported cases and at least 38 deaths (for review see Hertzman et al., 1991) associated with the use of L-tryptophan supplements made by a Japanese Company. It has been quite debated whether the epidemic was caused by L-tryptophan itself, or by a contaminant of the commercial product and the doubt raised led the FDA to restrict its use on humans.

The vital role of nucleotides in the cell has been described (Cooke & Robson, 2006; Plosky et al., 2002). In Pediatrics, for example, the benefits of dietary nucleotides include the synthesis of proteins and other macromolecules such as phospholipids, facilitating lipoprotein synthesis, during the early neonatal period, especially in preterm infants (Carver & Stromquist, 2006; Sanchez-Pozo et al., 1994). Thymine (5-methyluracil) is a pyrimidine derivative, a constituent of nucleic acids (Bentin et. al, 2006). Innumerous studies investigate the crosslinks between thymine and adenine or guanine basis and how both endogenous and exogenous agents, such as reactive oxygen species (ROS) may affect the integrity of these links (Xerri et al., 2006; Hong et al., 2006). However, little is known about its toxicity when administrated as a nutritional supplement.

Both tryptophan and thymine have already been studied as far as their so many biological properties. Based on a popular use of a nutritional supplement in which both elements seem to work together revealing some pharmacological effects, the present study was conducted. The purpose of this evaluation is to provide information relating to toxic effects and potential health hazards likely to arise from repeated exposures over a limited time period.

As a general rule, in order to assess possible hazards in the consumption of any new substance or mixture of substances, it is necessary to perform a series of both *in vitro* and *in vivo* assays. Acute and sub-chronic oral toxicity data are used to satisfy hazard

classification and labeling requirements, for risk assessment for human health and environment, and when estimating the toxicity of mixtures. In our study, two *in vitro* tests as well as three *in vivo* ones were performed using "TK3".

In addition to that, all available information on the test substance was considered prior to conducting the toxicity tests. Such information included, for example, the identity and chemical structure of the substance; its physicochemical properties; the result of any other *in vivo* or *in vitro* toxicity tests on the substance; toxicological data on structurally related substances; the anticipated use(s) of the substance; and the likely regulatory data requirements.

2- Materials and methods

2.1- *Chemicals*

The substance tested in this investigation was a nutritional supplementation (TK3), which consists of L-tryptophan and thymine (5-methyluracil) on a fine powder. It was kindly donated by *LAVILABOR Produtos Naturais Ltda* laboratory. In order to perform the oral treatment, this powder was diluted in NaCl 0.9% saline solution (10 mL/kg) prior to each administration.

All other chemicals used were purchased from standard commercial suppliers and were of analytical grade quality (p.a.).

2.2- *Cells*

Human tumor cell lines UACC-62 (melanoma), MCF-7 (breast), NCI-H460 (lung, non-small cells), OVCAR-3 (ovarian), PC-3 (prostate), HT-29 (colon), 786-O (renal) and NCI-ADR/RES (breast expressing multiple drug resistance phenotype) were kindly provided by National Cancer Institute (NCI). Stock cultures were grown in a medium containing 5 mL of RPMI 1640 and supplemented with 5% of fetal bovine serum

(Gibco BRL Technologies). Human fibroblasts (FH) were obtained from biopsy material. Stock cultures were grown in a medium containing 10 mL of RPMI 1640 and supplemented with 10% of fetal bovine serum (Gibco BRL Technologies). Gentamicine (50 μg/ mL) was added to the experimental cultures.

2.3- *Animals*

Healthy adult (200-250g) male and female (nulliparous, non-pregnant) Wistar rats (*Rattus norvegicus*) acquired from the experimental animal center (CEMIB) of the University of Campinas were used. During maintenance period (at least seven days prior to the commencement of the study) all animals were group housed in polycarbonate cages, under a climate-controlled environment (22ºC ± 3ºC and relative humidity 30-70%) and a 12-hour light/dark cycle. The animals submitted to unlimited supply of conventional standard pelletized laboratory diet (Nuvilab®) and water.

Animal's welfare guidelines were adopted (Guide for the Care and Use of Laboratory Animals 1996, International Guiding Principles for Biomedical Research Involving Animals, 1985). The protocols employed are in agreement with Ethical Principles in Animal Research adopted by Brazilian College for Animal Experimentation (COBEA). Also, criteria for taking the decision of submitting the moribund animals (or those obviously in pain or showing signs of severe and enduring distress) to euthanasia was according to OECD Guidance Document on the Recognition, Assessment, and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation. Those animals were considered in the interpretation of the test results in the same as animals that died on test.

2.4-*"In vitro" exposure to TK3*

Cells in 96-well plates (100 μL cells/well) were exposed to various concentrations of samples in DMSO (Sigma Chemical Co.) (0.25, 2.5, 25, and 250 μg/mL, respectively) at 37ºC, 5% of CO_2 in air for 48 h. (The final concentration of DMSO did not

affect cell viability.) Then, a 50% solution of trichloroacetic acid (Sigma Chemical Co.) was added. After incubation for 30 min at 4ºC, washing and drying of the plates, cell proliferation was determined by spectrophotometric quantification (540 nm) of the cellular protein content using sulforhodamine B (Sigma Chemical Co.) assay described by Skehan et al. (1990). Cell proliferation estimative/evaluation formula is presented in Table 1.

2.5- *"In vivo" exposure to TK3*

Acute exposure 1 (AE1): animals were exposed to a single intraperitoneal (i.p.) injection of "TK3" – 2000 mg/kg.

Acute exposure 2 (AE2): animals were exposed to a single oral administration of "TK3" – 5000 mg/kg.

Sub-chronic exposure (SCE): animals were exposed to daily oral administrations of "TK3" – 100, 300 and 1000 mg/kg (dose per group), five times/week, for 13 weeks.

The doses of "TK3" for the sub-chronic studies were selected on the basis of the acute ones. Two control groups received only vehicle (0,9% NaCl saline solution) and no treatment at all (satellite group), respectively. The descending sequence of dose levels was selected in the attempt to demonstrate a dosage related response as well as no-observed-adverse effects at the lowest dose level (NOAEL).

Sixteen hours before the oral administration of substances, all animals were deprived from commercial diet, having free access to tapped water. Both single and repeated oral administrations were performed with the help of suitable intubation cannulas (gavage needles).

2.5.1- *Clinical observations and body weight*

In the AE2, after the administration of substances, all experimental animals were closely observed for the first 30 minutes and again 4 hours after the beginning of the study. After that, they were submitted to detailed clinical evaluation, once a day, for a period of 14 days. An observer unaware of the treatments recorded all data related to the mentioned observations.

In the SCE, the animals were orally treated daily in the morning period. Similarly, daily clinical evaluations were performed (by unaware observer) and recorded with special attention to the following parameters: changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity, changes in gait, posture and response to handling as well as presence of any bizarre behavior. At the end of the experimental period, sensory reactivity to stimuli of different types (e.g. auditory and proprioceptive stimuli), assessment of grip strength and motor activity assessment were performed. In addition, animals were weighed once a week.

2.5.2- *Haematology*

In the SCE, under intraperitoneal anesthesia (sodium phenobarbital, 100mg/kg) blood samples were obtained from retro orbital plexus and the following parameters were analyzed: haematocrit (HCT), haemoblogin concentration (HBG), red blood cell count (RBC), total (WBC) and differential leukocyte count, platelet count (PLC) and a measure of blood clotting time/potential. The above analyses were performed with the help of Beckmann Coulter® A^c.T diff^{TM} Analyser.

2.5.3- *Clinical biochemistry*

Clinical biochemistry determinations were performed to investigate major toxic effects in tissues and, specifically, effects on kidney and liver. Investigations of plasma or serum included: sodium (SOD), potassium (POT), glucose (GLU), total cholesterol (CHO),

blood urea nitrogen (BUN), creatinine (CRE), total protein (TPR) and albumin (ALB), and at least two enzymes indicative of hepatocellular effects (such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma glutamyl transpeptidase, and sorbitol dehydrogenase).

2.5.4- *Gross necropsy*

In all three studies (AE1, AE2 and SCE), all animals were subjected to a detailed gross necropsy, including careful examination of orifices as well as the cranial, thoracic and abdominal cavities and their contents. The liver, kidneys, adrenals, stomach, thymus, heart, spleen, pancreas, gonads and accessory sex organs and urinary bladder were trimmed of adherent tissues and weighed before being fixed in 10% neutral buffered formalin.

2.5.5- *Histopathology*

Tissues were trimmed into cassettes, processed and stained with hematoxylin and eosin (H and E) for light microscopic evaluation. Full histopathology was carried out on the preserved organs and tissues of all animals in the control and treated groups.

2.6- *Statistical analysis*

Data are presented as mean ± standard deviation of six animals per group. The means were compared by the analysis of variance (ANOVA). Differences were considered significant if the p value was less than 0.05.

3- Results

3.1- *"In vitro" exposure to TK3*

Since it is known that different cell lines display different sensitivities towards a cytotoxic compound, the use of more than one cell line is, therefore, considered necessary in the detection of cytotoxic compounds. Bearing this in mind, cell lines of different histological origin were used in the present study.

Figure 1a presents the effect of doxorrubicin (positive control) exposure on human cancer cell lines and 1b presents the effect of "TK3" exposure on human cancer cell lines and human fibroblasts. Both plots correlate the concentration-dependent effect over cell proliferation. Values between 100 and 0% indicate inhibition of growth whereas those values below zero line, which represents the protein content value at the beginning of test substance exposure, indicate cell death. Our results reveal no citotoxicity after the exposure of "TK3" in human cancer cell lines as well as human fibroblast cells.

3.2- *"In vivo" exposure to TK3*

3.2.1- *Effect of acute TK3 exposure on behaviour, body weight and food consumption*

In the AE1 (acute exposure 1), approximately 15 minutes after the intraperitoneal administration of 2000mg/kg of TK3, the experimental animals presented severe lethargy, which lasted for about 1-2 hours. After that, they gradually returned to the normal state of alertness and no further abnormal sign was recorded during the 14 days of observation. In the AE2 (acute exposure 2), no change was observed on the animals' behavior during the complete experimental period.

In both acute studies (AE1 and AE2), all animals gained weight during the study period (Figure 2a,b). For both sexes, no significant difference was observed on food consumption during the complete experimental period.

3.2.2- *Effect of sub-chronic TK3 exposure on behaviour*

At the end of the SCE (sub-chronic exposure), there was no difference between treated and control groups during the evaluation of sensory reactivity to stimuli of different types, assessment of both grip strength and motor activity.

No unscheduled deaths occurred in any of the studies (AE1, AE2 and SCE).

3.2.3- *Effect of sub-chronic TK3 exposure on body weight and food consumption*

In the SCE, no significant differences on body weight gain were observed in females; the same was not observed in males on the fifth week of treatment: the apparent decrease in body weight gain was also observed in the placebo-treated group, which eliminates the effect of oral treatment.

For both sexes, no significant difference was observed on food consumption during the complete experimental period.

3.2.4- *Effect of sub-chronic TK3 exposure on haematological parameters*

The hematological analyses are presented in both Tables 2 and 3. Few significant differences in hematological values were observed between treated and control groups. Compared to control groups, slight, statistically significant increase in white blood count in the highest dose group (1000 mg/kg) was observed in male rats as presented in Table 3.

3.2.5- *Effect of sub-chronic TK3 exposure on biochemical parameters*

The biochemical analyses, clinical chemistry values, are presented in both Tables 4 and 5. Increased serum levels of AST, ALT e ALP were observed in all experimental groups. This fact has probably no relation to the test substance since it was observed in all animals. As a whole, none of them presented significant alterations and were considered in the normal range of reference values.

3.2.6- *Effect of sub-chronic TK3 exposure on histopathology*

No relevant histopathological findings were observed. All organs revealed normal histological appearance, with no malformation or hyperplasia.

4- Discussion

The growing use of vitamin, mineral, herbal and nutritional supplements in the general population has become an important issue in the last decades. While deficiencies in such nutrients can be harmful to health, conflicting claims have been made about the health benefits of such supplementation (Kurpad, 2006; Thompson, 2005).

In this study we evaluated both "in vitro" and "in vivo" effects of a nutritional supplement ("TK3") consisting of L-tryptophan and thymine through a non-clonogenic assay and also by administrating different doses of the test substance to both male and female adult Wistar rats.

It has been known that thymine auxotrophic microorganisms undergo cell death in response to thymine starvation [thymineless death (TLD)] and there are similarities between TLD of bacteria and death of eukaryotic cells (Ahmad et al., 1988). This fact has been a target of some cancer cell studies, using genetic manipulation to induce thymidylate synthase (TS) deficiency, opening up a new pathway to turn the neoplastic cell a more fragile one (Houghton et al., 1994). Based on these facts, we investigated whether the nucleotide and even the essential amino acid supplementation would stimulate "in vitro" cancer cell growth. Our results reveal no cytostatic / cytotoxic response to the supplement exposure. On the other hand, the presence of L-tryptophan and thymine led to no stimulation of growth of the utilized cancer cell lines. A similar response was observed with the normal human fibroblasts, revealing no cytotoxicicty in the test substance concentration used.

The acute administration of "TK3" to adult Wistar rats was largely well tolerated except for the severe lethargy, which lasted for about 1-2 hours after the intraperitoneal administration. After that, all animals gradually returned to the normal state

of alertness and no further abnormal sign was recorded during the 14 days of observation. As a precursor of melatonin (Jaworek et al., 2003), the systemic acute effect of a high dose of L-tryptophan might have produced such a clinical sign with no additional detectable long-term side effect as the post-mortem analyses revealed no signs of toxicity.

No treatment-related changes in the appearance or behaviour of the animals were observed in the 90-day repeated-dose study. All groups showed normal growth and food consumption with minor differences between the groups.

Hematological measurements showed normal levels of all clinically relevant parameters of red cell function including red blood cells, hemoglobin, MCV, MCH and MCHC. All the hematological experimental values, except the white blood cells and blood clotting time remained in the normal range of adult Wistar rats (Loeb, 1999). The increase in white blood count, observed in male rats treated with the highest dose of "TK3" may be in accordance with an immunomodulatory property of both L-tryptophan and melatonin (Jaworek et al., 2003). Such a pharmacological response demands further investigation, probably with a more sensitive species.

In our study, liver function tests, done to assess for hepatotoxicity, were essentially normal. Serum aminotransferases, aspartate aminotransferases (AST) and alanine aminotransferases (ALT) remained with no significant alterations.

The blood urea nitrogen (BUN), renal electrolytes such as sodium, potassium, magnesium were all in the normal range for adult Wistar rats of both sexes and indicate there was no renal damage caused by "TK3" administration. All other parameters were within the normal range and changes were consistent with that observed for a heterogenic strain.

No treatment-related histological effects were observed in all vital organs analyzed. Moreover, no malformation, hyperplasia or degenerative lesions were detected. These results led us to conclude that the 90-day administration of up to 1000 mg/kd/day of "TK3" presented no toxic effects in Wistar rats.

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References

1. Ahmad, S. I., Kirk, S. H., Eisenstark, A. (1998) Thymine metabolism and thymineless death in prokaryotes and eukaryotes. Annual Review of Microbiology 52:591-625.

2. Bentin, T., Hansen, G. I., Nielsen, P. E. (2006) Structural diversity of target-specific hoompyrimidine peptide nucleic acids-DNA complexes. Nucleic Acids Research 00:1-10.

3. Carver, J. D., Stromquist, C. I. (2006) Dietary nucleotides and preterm infant nutrition. Journal of Perinatology 26:443-444.

4. Cooke, M.S., Robson, A. (2006) Immunochemical detection of UV-induced DNA damage and repair. Methods in Molecular Biology 314:215-228.

5. Hertzman, P.A., Falk, H., Kilbourne, E.M., Page S., Shulman, L.E. (1991) The eosinophilia-myalgia-syndrome – The Los-Alamos Conference. Journal of Rheumatology 18:867-873.

6. Hong, H., Cao, H., Wang, Y., Wang, Y. (2006) Identification and quantification of a guanine-thymine intrastrand cross-link lesion induced by Cu(II)/H_2O_2/ascorbate. Chemical Research in Toxicology 19(5):614-621.

7. Houghton, J. A., Harwood, F. G., Houghton, P. J. (1994) Cell cycle control processes determine cytostatic or cytotoxicity in thymineless death of colon cancer cells. Cancer Research 54(18):4967-4973.

8. Jaworek, J., Leja-Szpak, A., Bonior, J., Nawrot, K., Tomaszewska, R., Stachura, J., Sendur, R., Pawlik, W., Brzozowski, T., Konturek, S. J. (2003) Protective effect of melatonin and its precursor L-tryptophan on acute pancreatitis induced by caerulein overstimulation or ischemia/reperfusion. Journal of Pineal Research 34(1):40-52.

9. Kurpad, A. V. (2006) The requirements of protein & amino acid during acute & chronic infections. Indian Journal of Medical Research 124(2):129-148.

10. Loeb, W. F. (1999) The rat. In: The Clinical Chemistry of Laboratory Animals. Ed. W. F. Loeb & F. W. Quimby, E.D. Taylor & Francis, second edition, Philadelphia, P.A., p.33-48; 643-726.

11. Plosky, B., Samson, L., Engelward, B. P., Gold, B., Schlaen, B., Millas, T., Magnotti, M., Schor, J., Scicchitano, D. A. (2002) Base excision repair and nucleotide excision repair contribute to the removal of N-methylpurines from active genes. DNA Repair 1(8):683-969.

12. Sanchez-Pozo, A., Morillas, J., Molto, L., Robles, R., Gil, A. (1994) Dietary nucleotides influence lipoprotein metabolism in newborn infants. Pediatric Research 35(1):1107-1118.

13. Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J.T., Bokesch, H., Kenney, S., Boyd, M.R. (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. J National Cancer Institute 82:1107-1118.

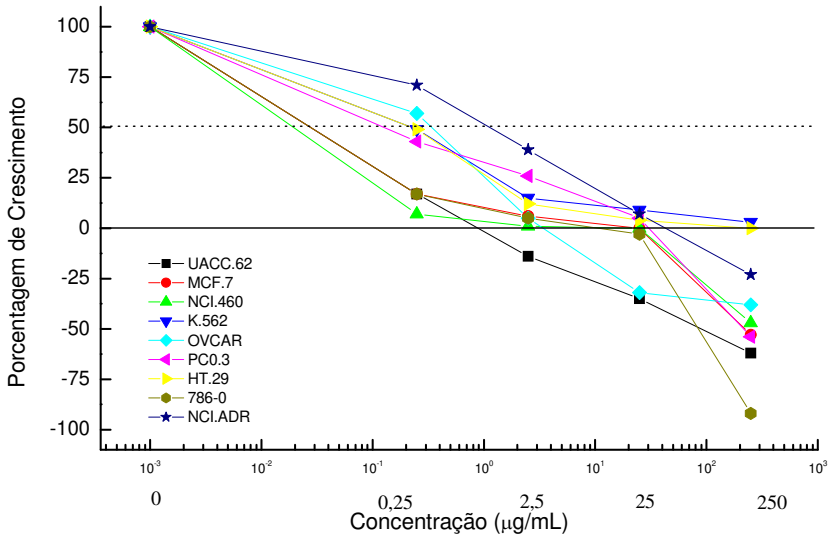
14. Thompson, J. (2005) Vitamins, minerals and supplements: part two. The Journal of Community Practice 78(10):366-368.

15. Xerri, B., Morell, C., Grand, A., Cadet, J., Cimino, P., Barone, V. (2006) Radiation-induced formation of DNA intrastrand crosslinks between thymine and adenine basis; a theoretical approach. Organic and Biomolecular Chemistry 4(21):3986-3992.

Figure Legends

Fig. 1. a) Effect of doxorrubicin (positive control) exposure on human cancer cell lines (Skehan, et al., 1990); b) Effect of "TK3" exposure on human cancer cell lines and human fibroblasts.

Fig. 2. Effect of exposure of "TK3" on body weight in Wistar rats. Acute intraperitoneal (a), acute oral (b) and sub-chronic (c;d) exposures.


Porcentagem de Crescimento
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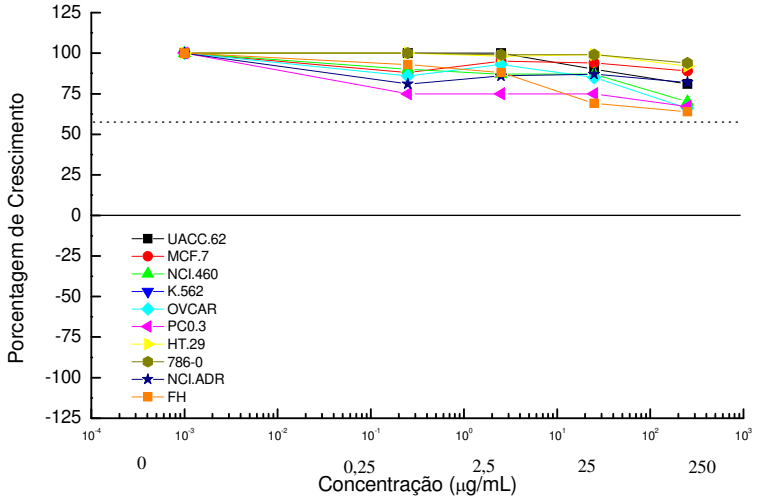
75
50
25
0
-25
-50
-75
-100
UACC.62
MCF.7
NCI.460
K.562
OVCAR
PC0.3
HT.29
786-0
NCI.ADR
0
0,25
2,5
25
250
Concentração (μg/mL)

Fig 1a.


Porcentagem de Crescimento
125
100
75
50
25
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UACC.62
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NCI.460
K.562
OVCAR
PC0.3
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0
0,25
2,5
25
250
Concentração (μg/mL)

Fig 1b.

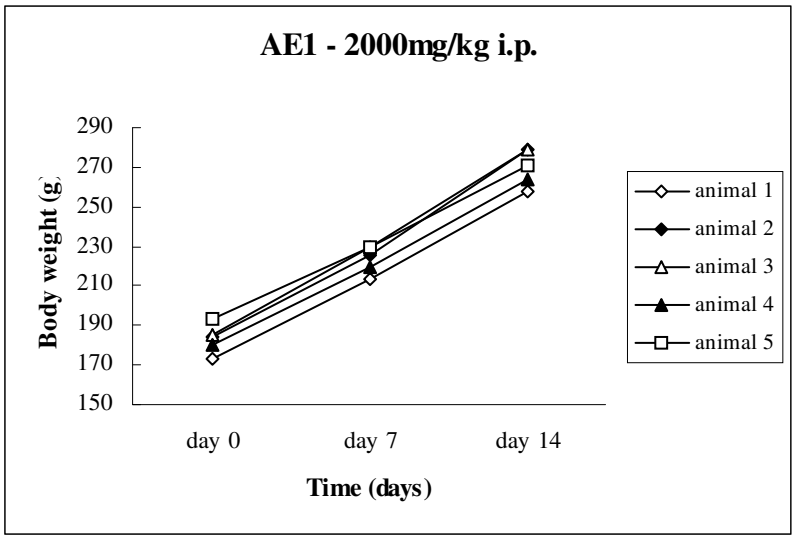
AE1 - 2000mg/kg i.p.
Body weight (g)
290
270
250
230
210
190
170
150
day 0
day 7
day 14
Time (days)
animal 1
animal 2
animal 3
animal 4
animal 5

Fig. 2a

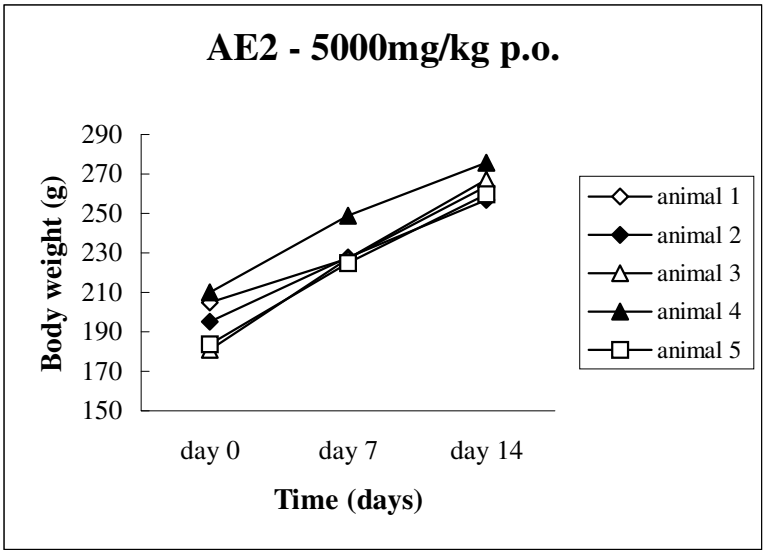
AE2 - 5000mg/kg p.o.
Body weight (g)
290
270
250
230
210
190
170
150
day 0
day 7
day 14
Time (days)
animal 1
animal 2
animal 3
animal 4
animal 5

Fig. 2b

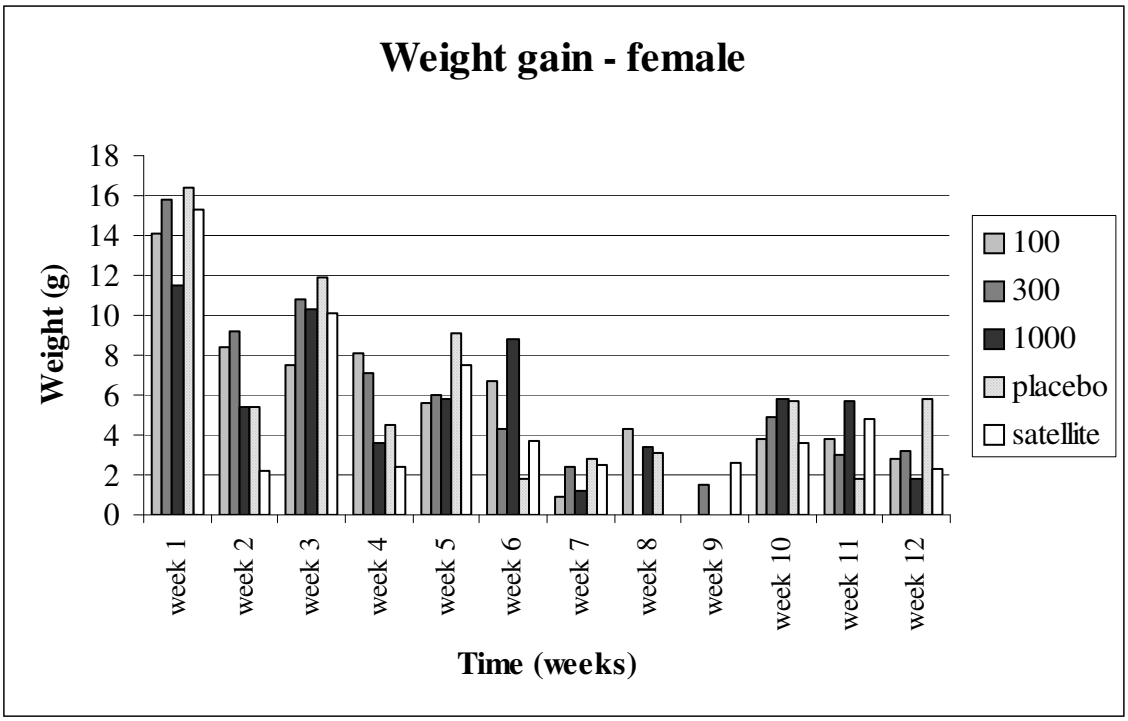
Weight gain - female
Weight (g)
18
16
14
12
10
8
6
4
2
0
week 1
week 2
week 3
week 4
week 5
week 6
week 7
week 8
week 9
week 10
week 11
week 12
Time (weeks)
100
300
1000
placebo
satellite

Fig. 2c

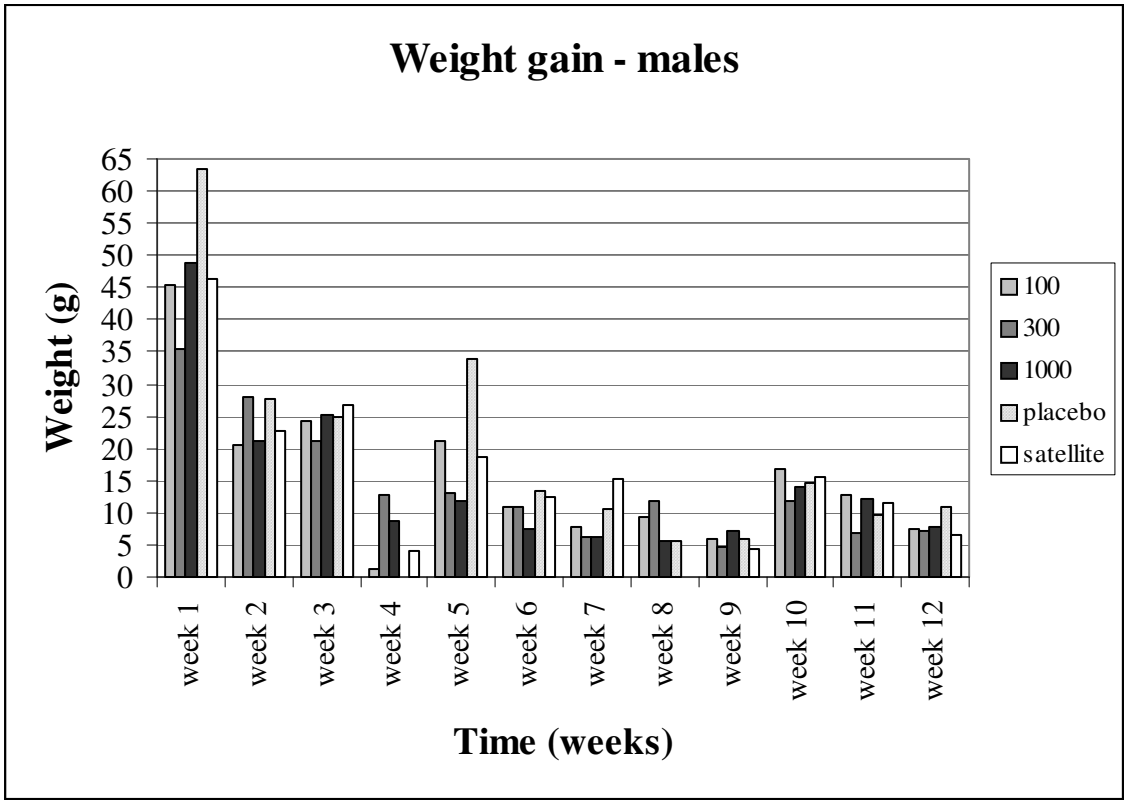
Weight gain - males
Weight (g)
65
60
55
50
45
40
35
30
25
20
15
10
5
0
week 1
week 2
week 3
week 4
week 5
week 6
week 7
week 8
week 9
week 10
week 11
week 12
Time (weeks)
100
300
1000
placebo
satellite

Fig. 2d

Table Legends

Table 1. Cell proliferation estimative/evaluation formula according to Skehan et al., 1990.

Table 2. Terminal hematological values of male and female Wistar rats orally treated with "TK3" and control for 90 days.

Table 3. Terminal hematological values of male and female Wistar rats orally treated with "TK3" and control for 90 days.

Table 4. Clinical chemistry values of male and female Wistar rats orally treated with "TK3" and control for 90 days.

Table 5. Clinical chemistry values of male and female Wistar rats orally treated with "TK3" and control for 90 days.

Hypothesis	Result	IC formula
If $T > C$	test substance stimulated cell growth	there is no IC
If $T >= T_0$ but $< C$	test substance is citostatic	formula is $100 X [(T- T_0)/(C- T_0)]$
If $T < T_0$	test substance is citocidal	formula is $100 X [(T- T_0)/T_0]$

Table 1

RBC Males-ANOVA: $F_{(4,45)}$=7.15 p<0.001. Duncan's Test *p<0.05 **p<0.001; HB Males-ANOVA: $F_{(4,45)}$=17.55 p<0.001. Duncan's Test *p<0.05 **p<0.001; HCT Males-ANOVA: $F_{(4,45)}$=6.67 p<0.001. Duncan's Test *p<0.05; HCM Males-ANOVA: $F_{(4,45)}$=12.28 p<0.001. Duncan's Test *p<0.05 **p<0.001; CHCM Males-ANOVA: $F_{(4,45)}$=26.91 p<0.001. Duncan's Test *p<0.001; RBC Females-ANOVA: $F_{(4,40)}$=8.42 p<0.001. Duncan's Test *p<0.05 **p<0.01 ***p<0.001; HB Females-ANOVA: $F_{(4,40)}$=3.10 p<0.05. Duncan's Test *p<0.05 HCT Females-ANOVA: $F_{(4,40)}$=9.88 p<0.001. Duncan's Test *p<0.01 **p<0.001; HCM Females-ANOVA: $F_{(4,40)}$=7.92 p<0.001. Duncan's Test *p<0.001; CHMC Females-ANOVA: $F_{(4,40)}$=9.89 p<0.001. Duncan's Test *p<0.001.

Group	**RBC** (**x 10^3 /µL**)	**HB** (**g/dL**)	**HCT** (**%**)	**MVC** (**fL**)	**HCM** (**pg**)	**CHMC** (**g/dL**)
Males						
100	9072 ± 483,00	15,23 ± 0,60	47,96 ± 1,88	52,91 ± 1,19	16,82 ± 0,71	31,78 ± 0,76*
300	9517 ± 218,96*	15,89 ± 0,60*	50,66 ± 1,81*	53,23 ± 1,46	16,70 ± 0,55	31,47 ± 0,43
1000	9723 ± 317,18**	17,44 ± 0,71**	50,89 ± 1,33*	52,40 ± 0,93	18,02 ± 0,46**	34,42 ± 1,17*
Placebo	8953 ± 441,57	15,28 ± 1,02	47,41 ± 2,36	53,08 ± 0,73	17,12 ± 0,44*	32,24 ± 0,92*
Sham	9140 ± 295,84	15,02 ± 0,46	48,70 ± 1,61	53,28 ± 1,24	16,43 ± 0,39	30,83 ± 0,38
Females						
100	8483 ± 124,58	14,86 ± 0,41*	47,07 ± 0,87**	55,49 ± 0,79	17,55 ± 0,37*	31,60 ± 0,66*
300	8274 ± 211,81	14,33 ± 0,45	45,49 ± 1,37**	54,99 ± 0,88	17,33 ± 0,38*	31,50 ± 0,40*
1000	7853 ± 329,70*	13,88 ± 0,53	43,48 ± 1,73*	55,38 ± 0,93	17,69 ± 0,57*	31,94 ± 0,95*
Placebo	8028 ± 404,62**	14,37 ± 0,62	44,90 ± 2,03**	55,94 ± 1,09	17,94 ± 0,52*	32,07 ± 1,10*
Sham	7448 ± 641,85	14,10 ± 1,11	40,84 ± 3,36	54,82 ± 0,29	18,96 ± 0,85	34,62 ± 1,51

RBC = red blood cell count HB = haemoglobin concentration HCT = hematocrit MCV = mean corpuscular volume

MCH = mean corpuscular haemoglobin MCHC = mean corpuscular haemoglobin concentration

Table 2

WBC Males-ANOVA: $F_{(4.45)}$=5,79 p<0.001. Duncan's Test *p<0.01; Lympho Males-ANOVA: $F_{(4.45)}$=3,88 p<0.01. Duncan's Test *p<0.05; Eosino Males-ANOVA $F_{(4.45)}$=46,47 p<0.001. Duncan's Test *p<0.01 ** p<0.001; BCT Males-ANOVA: $F_{(4,45)}$=6,25 p<0.001. Duncan's Test *p<0.001; Eosino Females-ANOVA: $F_{(4.40)}$=43,89 p<0.001. Duncan's Test *p<0.05 ** p<0.001;

Group	**WBC (n^o /µL)**	**Neutro (%)**	**Lympho (%)**	**Mono (%)**	**Eosino (%)**	**PC (x 10^3 /µL)**	**BCT (seconds)**
Males							
100	8510 ± 1670,6	15,7 ± 6,43	77,3 ± 6,62	3,9 ± 1,64	3,1 ± 1,37*	699,7 ± 132,08	35,3 ± 8,50
300	11200 ± 1400,7*	16,0 ± 3,92	78,5 ± 3,98	4,1 ± 2,02	1,4 ± 0,67**	756,8 ± 102,87	37,2 ± 9,22
1000	11820 ± 3518,2*	20,5 ± 5,20	71,4 ± 3,77*	3,1 ± 2,38	4,9 ± 1,22**	725,1 ± 88,93	59,6 ± 20,58*
Placebo	9220 ± 1004,8	17,4 ± 2,24	77,5 ± 2,46	5,0 ± 1,61	0,1 ± 0,30	758,7 ± 89,83	40,5 ± 10,31
Sham	8580 ± 567,1	20,1 ± 2,98	76,3 ± 3,26	3,2 ± 1,17	0,2 ± 0,40	762,6 ± 86,21	39,0 ± 3,52
Females							
100	9120 ± 1045,7	17,1 ± 5,10	76,6 ± 5,81	3,9 ± 1,81	1,6 ± 0,66*	768,7 ± 114,60	33,2 ± 5,53
300	9310 ± 1758,1	22,4 ± 5,37	70,7 ± 4,47	3,4 ± 1,50	3,5 ± 1,28**	774,9 ± 102,10	34,4 ± 3,77
1000	8260 ± 910,2	17,7 ± 4,22	73,7 ± 5,23	3,8 ± 1,40	4,8 ± 0,75**	779,4 ± 182,90	40,3 ± 8,04
Placebo	9150 ± 1250,0	18,0 ± 4,84	69,0 ± 6,13	5,4 ± 1,85	0,2 ± 0,40	798,9 ± 76,23	40,5 ± 10,31
Sham	8840 ± 2594,3	18,0 ± 6,06	77,0 ± 7,40	5,0 ± 1,41	0,6 ± 0,48	773 ± 48,38	37,4 ± 2,15

WBC = white blood cell count Neutro = neutrophil Lympho = lymphocyte Mono = monocyte Eosino = eosinophil

PC = platelet count BCT = blood clotting time/potential

Table 3

AST Males-ANOVA: $F_{(4,45)}$=7.80 p<0.001. Duncan's Test *p<0.001; Albumin Males-ANOVA: $F_{(4,45)}$=70.63 p<0.001. Duncan's Test *p<0.001; Cholesterol Males-ANOVA: $F_{(4,45)}$=13.09 p<0.001. Duncan's Test *p<0.001; ALP Females-ANOVA: $F_{(4,45)}$=3.87 p<0.01. Duncan's Test *p<0.01; Albumin Females-ANOVA: $F_{(4,45)}$=24.39 p<0.001. Duncan's Test *p<0.001; Cholesterol Females-ANOVA: $F_{(4,45)}$=7.45 p<0.001. Duncan's Test *p<0.05 **p<0.001; Triglycerides Females-ANOVA: $F_{(4,41)}$=3.41 p<0.05. Duncan's Test *p<0.01.

Group	**AST (U/L)**	**ALT (U/L)**	**ALP (U/L)**	**Albumin (g/dL)**	**Cholesterol (mg/dL)**	**Triglycerides (mg/dL)**
Males						
100	135,1 ± 32,75*	42,40 ± 14,68	242,0 ± 97,61	3,36 ± 0,20*	80,8 ± 14,72*	63,8 ± 20,38
300	82,9 ± 21,46	31,15 ± 9,17	208,7 ± 84,89	4,42 ± 0,31*	72,4 ± 8,24*	71,0 ± 13,59
1000	102,1 ± 15,01	37,80 ± 12,50	189,0 ± 92,57	3,35 ± 0,19*	54,7 ± 10,38	55,8 ± 20,23
Placebo	103,6 ± 11,39	36,90 ± 4,88	244,0 ± 56,21	4,7 ± 0,23*	58,6 ± 13,11	93,0 ± 30,83
Sham	97,8 ± 14,43	37,3 ± 6,75	174,9 ± 38,59	3,85 ± 0,11	48,2 ± 6,91	72,2 ± 24,04
Females						
100	81,7 ± 20,53	37,30 ± 16,04	94,9 ± 54,80	3,43 ± 0,24*	65,1 ± 13,23*	70,5 ± 27,67
300	96,7 ± 54,35	45,80 ± 14,52	72,8 ± 10,72	4,16 ± 0,33	72,8 ± 8,43	73,8 ± 11,82
1000	81,7 ± 14,93	40,40 ± 6,58	96,7 ± 29,35	3,49 ± 0,32*	55,7 ± 10,17**	73,8 ± 19,95
Placebo	91,4 ± 22,61	40,00 ± 9,32	140,2 ± 54,15*	4,68 ± 0,40*	76,1 ± 15,04**	92,4 ± 10,50*
Sham	88,8 ± 19,65	36,60 ± 6,39	85,0 ± 25,08	4,06 ± 0,25	50,1 ± 12,65	60,2 ± 19,28

Table 4

Phosphorus Males-ANOVA: $F_{(4,45)}$=8.39 p<0.001. Duncan's Test *p<0.05; Urea Males-ANOVA: $F_{(4,45)}$=7.01 p<0.001. Duncan's Test *p<0.01; Creatinine Males-ANOVA: $F_{(4,45)}$=18.56 p<0.01. Duncan's Test *p<0.001;

Sodium Males-ANOVA: $F_{(4,45)}$=6.04 p<0.001. Duncan's Test *p<0.01 **p<0.001; Potassium Males-ANOVA: $F_{(4,45)}$=11.20 p<0.001. Duncan's Test *p<0.05 **p<0.01 ***p<0.001; Magnesium Males-ANOVA: $F_{(4,45)}$=8.13 p<0.001. Duncan's Test *p<0.01; Phosphorus Females-ANOVA: $F_{(4,45)}$=14.04 p<0.001. Duncan's Test *p<0.001; Urea Females-ANOVA: $F_{(4,45)}$=10.38 p<0.001. Duncan's Test *p<0.01 **p<0.001; Creatinine Females-ANOVA: $F_{(4,45)}$=2.31 p<0.05. Duncan's Test *p<0.01; Sodium Females-ANOVA: $F_{(4,45)}$=3.71 p<0.05. Duncan's Test *p<0.05 **p<0.001; Magnesium Females-ANOVA: $F_{(4,45)}$=4.7 p<0.05. Duncan's Test *p<0.001.

Group	**Phosphorus (mg/dL)**	**Urea (mg/dL)**	**Creatinine (mg/dL)**	**Sodium (mmol/L)**	**Potassium (mmol/L)**	**Magnesium (mg/dL)**
Males						
100	6,27 ± 0,60*	47,3 ± 4,78	0,64 ± 0,08*	140,4± 1,8**	4,78 ± 0,25***	2,08 ± 0,32
300	5,27 ± 0,45	50,7 ± 3,85	0,61 ± 0,07*	141,4± 1,6*	4,37 ± 0,33**	3,18 ± 0,97*
1000	6,30 ± 0,23*	44,3 ± 6,20	0,69 ± 0,05*	141,9± 1,8*	4,28 ± 0,36*	1,76 ± 0,23
Placebo	6,18 ± 0,46*	56,2 ± 8,99**	0,61 ± 0,03*	141,8± 1,2*	4,47 ± 0,15***	2,63 ± 0,67
Sham	5,70 ± 0,49	48,9 ± 6,74	0,44 ± 0,08	144,8± 3,2	3,97 ± 0,16	2,29 ± 0,03
Females						
100	6,38 ± 0,47*	42,6 ± 6,04	0,60 ± 0,04	139,8± 1,5*	4,75 ± 0,12	2,16 ± 0,37
300	5,19 ± 0,48	49,4 ± 4,18*	0,62 ± 0,04	141,3± 1,1*	4,39 ± 0,31	2,30 ± 0,05
1000	6,48 ± 0,29*	41,7 ± 5,81	0,66 ± 0,07*	141,4± 1,7	4,60 ± 0,31	1,79 ± 0,20*
Placebo	5,63 ± 0,55	55,3 ± 7,01**	0,61 ± 0,03	141,5± 1,4	4,45 ± 0,12	2,13 ± 0,20
Sham	5,38 ± 0,51	42,0 ± 4,36	0,56 ± 0,13	143,3± 3,3	4,59 ± 0,69	2,35 ± 0,39

Table 5

CAPÍTULO 2- Association between L-Tryptophan and Thymine: Efficacy on the Inhibition of Stomach Ulcerative Lesions Caused by Ethanol Ingestion

K. M. Monteiro*[1,2], P. Huber[4], M. C. M. Matias[1], M. A. Antonio[1,3], A. T. S. Fernandes[1,2], A. Possenti[1], S. V. Tinti[1], A. L. G. Ruiz[1], and J. E. Carvalho[1,2]

[1]*Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas, Universidade Estadual de Campinas, P.O. Box 6171, ZIP Code 13083 970, Campinas, São Paulo, Brazil*

[2]*Departamento de Clínica Médica da Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Cidade Universitária Zeferino Vaz, ZIP Code 13081 970, Campinas, São Paulo, Brazil*

[3]*Universidade São Francisco, Curso de Farmácia, Campinas Campus, Rua Waldemar César da Silveira, 105 - Vila Cura D'Ars, ZIP Code 13045-510, Campinas, São Paulo, Brazil*

[4]*Lavilabor Produtos Naturais Ltda., Rod. Raposo Tavares, km 256, ZIP Code 18725000 Hollambra II, Paranapanema, SP, Brazil.*

*Corresponding author: Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas, Universidade Estadual de Campinas, P.O. Box 6171, ZIP Code 13083 970, Campinas, São Paulo, Brazil

Tel: +55-19-3884-7500 ext. 249, Fax: +55-19-3884-7811

E-mail address: farmatox@cpqba.unicamp.br

Running Title: L-Tryptophan and Thymine as antiulcerogenic agents

Abstract: L-Tryptophan has been widely studied in the field of psychopharmacology as an important precursor of both serotonin and melatonin. Its protective effect over the gastric mucosa has equally been described. However, the association between L-tryptophan and a nucleotide base – thymine – has presented some pharmacological effects that seem to go beyond the nutritional ones. The aim of this study was to evaluate the possible beneficial effects of this new association of substances. The experimental model utilized was the acute ulceration induced in rats, considered to be a reliable tool to study the pathogenesis of acute gastric mucosal ulceration. For all experiments using this model, repetitive-dose oral administration of the test substances were performed. The nutritional supplement was orally administered in a dose of 1000 mg/Kg body weight revealing a gastroprotective activity compared to the drug used as positive control – carbenoxolone (200 mg/Kg). This effect was better than the one observed after the oral administration of two components separately. The research of probable mechanisms of action suggests the participation of sulfhydryl substances since there was a reduction in the ulcer healing capacity of the supplement after the subcutaneous administration of N-ethylmaleimide (10 mg/Kg), although this may not be the sole responsible for the antiulcerogenic activity observed. Our findings seem to be in accordance with the antioxidizing activity and scavenging properties of tryptophan even though further studies remain to be established in order to elucidate the beneficial participation of the nucleotide base in the gastroprotective activity of this association.

Keywords: L-tryptophan, thymine, gastroprotective activity, ethanol-induced ulcer.

INTRODUCTION

Humans are inevitably exposed to compounds that are not essential for life or "natural" from the standpoint of evolution. Some of these compounds may be acutely toxic, potentially toxic following activation, or may exhibit long-term effects such as cancer promotion. The complete elimination of these compounds is likely unachievable for a number of reasons, including the natural occurrence of some toxic agents within the so many substances we are exposed to nowadays. Over the last decades, nutrition researchers have developed diets in an attempt to promote optimal immunity in health individuals and maintain normal immune defenses in compromised patients.

Our growing understanding about the mechanisms through which nutritional factors may affect the immune system, for example, has promoted an increase in their use as adjuvant in the treatment of several diseases (WHO/FAO, 2002; Rique et al., 2002) despite the huge criticism against its use (Chang et al., 2005).

There are somewhat heterogeneous therapeutic responses of free amino acids as dietary supplements within a particular disorder, which rise some concern regarding the safety of amino acid supplements for the general public. However, examples of amino acids used as therapeutic agents are widely available (May, 2002).

Among these, tryptophan (L-tryptophan) is a peculiar compound. To begin with, as an essential amino acid, it is required exclusively from exogenous sources, not being synthesized in vivo. Second, because it is the least abundant in a regular diet. The major part of tryptophan is utilized for protein synthesis (Kema et al., 2000). Its characteristics are extremely relevant when we take into consideration the importance of its final products, serotonin and melatonin, over the physiologic equilibrium. Biosynthesis of serotonin in the human body represents only a minor metabolic route for tryptophan. Under normal conditions, it accounts for not more than 2% of ingested tryptophan. Together with dopamine, norepinephrine, and epinephrine, 5-hydroxytryptamine (serotonin) modulates several physiological and psychological processes including an individual's mental state or mood (Widner et al., 2002; Sandyk, 1992).

Melatonin, a pineal hormone, is known to scavenge oxygen free radicals. More recently, studies have revealed that since endogenous melatonin levels fall markedly in advanced age, the loss of this antioxidant may contribute to the incidence or severity of some age associated neurodegenerative diseases (Reiter, 1998). Its immunoneuroendocrine function has also been described and studied with positive results (Maestroni, 1993; Caroleo, 1992).

Several specific nutritional substrates have been shown to augment and/or modulate host immune function. Some enteral formulas enriched with specific immune-modulating nutrients are presently available for clinical use in Japan. Such nutrients include n-3 fatty acids, arginine, glutamine and nucleotides as a whole (Saito, 2004). A dietary requirement for a source of purine and/or pyrimidine bases has been identified as necessary for normal development of cellular immune responses (Holen, 2006). Nucleotides are precursors of DNA and RNA and also serve as a multitude of other important functions in cellular metabolism (Carver, 1994). Dietary nucleotides may also be effective in macrophage activation of the T helper/inducer populations (Rudolph et al., 1990). The importance of dietary nucleotides in maintaining optimal T-helper cell functions for humoral immune responses in response to T-dependent antigens have also been described (Jyonouchi, 1994; Van Buren et al., 1994). Thymine (5-methyluracil) is a pyrimidine derivative, a constituent of nucleic acids. An interesting fact is that most of the researches involving this isolated nucleotide are related to microbiological studies (Courcelle, 2005).

Both tryptophan and thymine have already been studied as far as their so many biological properties. However, little is known about this association. Based on a popular use of a nutritional supplement in which both elements seeming to reveal possible pharmacological effects, the present study was conducted. Taken these together, the purpose of this study is to evaluate the mechanisms through which this new association protects against gastric ulcer, using absolute ethanol as the ulcerogenic agent.

MATERIALS & METHODS

Animals

Healthy adult (200-250g) male Wistar rats (*Rattus norvegicus*) acquired from the experimental animal center (CEMIB) of the University of Campinas were used. During maintenance period (at least seven days prior to the commencement of the study) all animals were group housed in polycarbonate cages, under a climate-controlled environment (22ºC ± 3ºC and relative humidity 30-70%) and a 12-hour light/dark cycle. The animals submitted to unlimited supply of conventional standard pelletized laboratory diet (Nuvilab®) and water.

Animal's welfare guidelines were adopted (Guide for the Care and Use of Laboratory Animals 1996, International Guiding Principles for Biomedical Research Involving Animals, 1985). The protocols employed are in agreement with Ethical Principles in Animal Research adopted by Brazilian College for Animal Experimentation (COBEA).

Test substance and chemicals

The substance tested in this investigation was a nutritional supplement (TK3), which consists of L-tryptophan and thymine (5-methyluracil) on a fine powder. All test substances were kindly donated by *LAVILABOR Produtos Naturais Ltda* laboratory. In order to perform the oral treatment, this powder was diluted in 0.9% saline solution (10 mL/Kg) prior to each administration.

Ethanol (Merck), carbenoxolone (SIGMA Chem. Co. - USA), N-ethylmaleimide (NEM – SIGMA), Indomethacin (IND - SIGMA), Nω- L-arginine methyl esther (L-NAME - SIGMA) were all reagent grade (p.a.).

Administration of substances - Oral Treatment

The oral treatments for the antiulcerogenic assays were carried out according the following pattern:

Repetitive-dose administration (double treatment): The animals were fasted for 16 hours before receiving the first treatment. In order to avoid jeopardizing especially the absorption of the L-tryptophan by the amino acids present in the animals' regular diet, there

was a 60-minute delay in the offering of the commercial diet. After an 8-hour feeding period, the animals were, again, submitted to a 16-hour fasting period, after which the second treatment was given (Figure 1). In a sequence, one hour after, all rats received orally 1 mL of absolute ethanol (ulcerogenic agent).

Ulcer induction

For induction of gastric mucosa ulcerative lesions, 5-6 rats were randomly assigned to each treatment. In accordance with Robert (1979), the studied groups consisted of saline solution 0,9% as negative control, carbenoxolone as positive control, and the different dose levels of the test substance. Absolute ethanol was the ulcerogenic agent. Details of the experiments are given in Figure 1.

One hour after the last oral treatment, all rats received orally 1 mL of absolute ethanol. Finally, after an elapsed time of one hour, the animals were euthanized by cervical dislocation and their stomachs were opened along the line of the greater curvature. After washing the stomachs with saline solution, the ulcerative lesions were assessed. Ulcerations were the linear necrohemorrhagic lesions present in the glandular part of the stomach. They were scored by examining the stomach using a simple magnifier.

The degree of the antiulcerogenic activity at the experimental groups was assessed according to Gamberini et al. (1991). The presence of hemorrhage, folding loss, edema and discoloration as well as the number of lesions, considering their size, were counted and scored as presented in Table 1. The ulcerative lesions index (ULI) was calculated by the sum of all the pathological parameters observed.

The percentage of inhibition of ulcerative lesions was calculated by comparing the lesions in the experimental treatment group of animals with those in the negative control (NaCl 0,9% saline solution) treatment group, using the following formula:

$$\text{ULI\%} = \frac{\text{average control} - \text{average test sample}}{\text{average control}} \times 100$$

Estimation of the effective dose

In order to estimate the effective dose of the supplement, the ethanol-induced ulcer assay was performed using three different doses: 100, 300 e 1000 mg/Kg body weight.

Indomethacin Treatment (IND)

In order to evaluate the participation of endogenous prostaglandin in the protection by our supplement, the method described by Martin et al. (1994) was used. Thirty minutes prior to the last administration of the samples, 10 mg/Kg b.w. of indomethacin was given intraperitoneally (volume 10 ml/Kg b.w.). In a sequence, all animal groups received orally their respective treatment (last administration of the samples). The rats were then treated in the ethanol model (1 mL/animal) as previously explained. The ulcerative lesions index (ULI) was calculated according to Gamberini et al. (1991).

Nω- L-arginine methyl esther treatment (L-NAME)

The role of the nitric oxide in this cytoprotection was studied by administering intravenously (volume 1 mL/Kg b.w.) L-NAME (Nω- L-arginine methyl ester) at a dose of 5 mg/Kg b.w. After thirty minutes, all animal groups received orally their respective treatment. One hour later, the rats were submitted to the ethanol-induced model. The ulcerative lesions index (ULI) was calculated according to Gamberini et al. (1991).

Treatment with N-ethylmaleimide (NEM)

In order to evaluate the participation of sulfhydryl (SH) compounds on the protection of stomach ulcerative lesions by ethanol, a previous administration of a subcutaneous injection (volume 2,5 mL/Kg b.w.) of N-ethylmaleimide (NEM) (10 mg/Kg b.w.) was performed. After thirty minutes, all animals received their respective oral treatment. One hour after treatments all rats were equally submitted to the ethanol-induced ulcer model. The ulcerative lesions index (ULI) was calculated according to Gamberini et al. (1991).

Determination of gastric secretion

The assay was performed by the method of Shay et al. (1945) with a few modifications. All groups of animals (n=7) fasted for 24 h, had with free access to water. Immediately after pylorous ligature, "TK3" (1000 mg/Kg), cimetidine (100 mg/Kg) as positive control, or NaCl 0,9% saline solution (10 mL/Kg) as negative control were administered intraduodenally. The animals were submitted to euthanasia 4 hours later by cervical dislocation. Their abdomen and the oesophagus were ligated in order to avoid leaking of their content. The stomachs were carefully removed, washed, dried and the gastric content collected to determine the total amount of gastric-juice acid (mL) and pH values. Distilled water (2 mL) was added and the resultant solution centrifuged at 2000 rpm for 10 min. Total acid in the gastric secretion was determined in the supernatant volume by titration to 7,0 with 0,1M NaOH.

Statistical analysis

Data are presented as mean ± standard deviation of six animals per group. The means were compared by the analysis of variance (ANOVA). Differences were considered significant if the *p* value was less than 0.05.

RESULTS

Estimation of the effective dose – Antiulcerogenic activity

The effective dose for the antiulcerogenic activity was determined through a single protocol using increasing doses of 100, 300 and 1000 mg/Kg body weight of the nutritional supplement on a repetitive-dose basis. The dose level groups were compared with carbenoxolone (200 mg/Kg – positive control) and 0,9% saline solution (10 mL/Kg – negative control). The data are presented in Table 2.

In this model, the protective effect of a repetitive administration of "TK3" against the ulcerogenic agent (ethanol) was best with the dose of 1000mg/Kg. From that point on, all other tests were performed using this dose.

Single x Double dose

As a nutritional supplement with antiulcerogenic properties, there was no significant difference whether "TK3" was administered only once to the experimental animals before the administration of ethanol or on a repetitive-dose basis (data not shown).

L-Tryptophan x Thymine x "TK3"

In this acute experimental model of ulcer, "TK3" presented growing gastric ulcer inhibition indexes as long as higher doses were orally administered to the experimental animals. In order to verify the possible synergism between these two substances (L-tryptophan and thymine), the same experimental model was utilized. The oral treatment doses were: "TK3" 1000 mg/Kg, L-tryptophan 400 mg/Kg b.w. and thymine 400 mg/Kg b.w. on a repetitive basis. The choice of the last doses was based on the proportion of the two elements in the original formula. As it can be observed in Figure 2, the antiulcerogenic activity was significantly higher than that of both L-tryptophan and thymine administered separately.

Evaluation of the cytoprotection mechanisms

Indomethacin treatment

In the ethanol induced ulcer model, when a previous administration of low dosages of indomethacin (cyclooxygenase inhibitor) is performed, there is a significant reduction in the amount of prostaglandins in the gastric mucosa. Therefore, as the test substance maintained its antiulcerogenic effect (Figure 3), its mechanism of action does not seem to be related to this group of substances.

L-NAME treatment (double treatment)

By blocking the metabolic route of the nitric oxide via L-NAME (an inhibitor of NO synthase), we evaluated the antiulcerogenic activity of the nutritional supplement "TK3". As presented in Figure 4, pretreatment with L-NAME showed no attenuation of the gastroprotection, indicating that this pathway probably does not involve the nitric oxide.

NEM treatment

As important protective agents of the gastric mucosa we find the non-proteic SHcontaining compounds, mainly represented by glutathione (GSH) (Repetto and Llesuy, 2002). Pretreatment of the experimental animals with N-ethylmaleimide (NEM), a blocker of SHs, significantly reduced the anticulcerogenic activity of the studied nutritional supplement ("TK3") suggesting a strong participation of endogenous SHs in the gastroprotective effects of this new association (Figure 5).

Determination of gastric secretion

Ligation of the pylorous produced accumulation of gastric juice that was used to analyze the gastric biochemical parameters after intraduodenal administration of "TK3". As presented in Table 3, volume, pH and hydrogenionic concentration of gastric acid content were not altered by i.d. treatment.

DISCUSSION

Under normal conditions, the integrity of the gastric environment is the result of equilibrium between the aggressive agents, constantly present in the mucosa, and the physiological mechanisms of protection (Bighetti et al., 2002). Mucus secretion, gastroduodenal bicarbonate production (Hogan et al., 1994), prostaglandin synthesis (Peskar and Maricic, 1998), cholecystokinin and somatostatin (Brzozowski et al., 1998), cellular regeneration, and normal tissue microcirculation are involved in the protection against ulcer formation. Ethanol-induced damages are produced by disturbance of mucosal microcirculation, ischaemia, free radicals, endotelin release, degranulation of mast cells, inhibition of prostaglandins and decrease of mucus protection (Samonina et al., 2004).

Based on this knowledge, we looked forward to finding the mechanisms involved in the antiulcerogenic activity presented by the nutritional supplement "TK3" using the absolute ethanol-induced model.

In our study, "TK3" was able to reduce the ulcerative lesion index in all experimental models tested. The results in the pyloric ligation model lead us to believe that this antiulcerogenic activity is not related to the mechanisms that control the gastric acid

secretion. Indeed, in this model, "TK3" modified neither the gastric juice volume, nor the pH or hydrogenionic concentration of gastric content. This result suggests that the antiulcerogenic properties of "TK3" are due to cytoprotective mechanisms.

Among these, prostaglandins, especially those of series E, F and I, protect the gastric mucosa through various pathways, such as the reduction of acid secretion, stimulation of the production of bicarbonate and mucus, and increasing the peripheral blood flow (Atay et al., 2000).

Another important element in the gastric cytoprotection is the nitric oxide. It is an endogenous substance that increases the blood flow of the mucosa and inhibits the release of oxygenated metabolites and proteases from leucocytes (Coruzzi et al., 2000). Nitric oxide also modulates the secretion of some cellular factors (Elliot et al., 1995).

Finally, there are the endogenous sulfhydryl compounds (SH) responsible for binding to free radicals produced as a result of tissue injury. These compounds may also protect mucus, since mucus subunits are united by disulfide bridges that, if reduced, render mucus water-soluble chacteristics (Avila et al., 1996).

In the attempt to understand the mechanism of action, these three important cytoprotective pathways were blocked. Preteatment with NEM (SH blocker) was the only one that presented a significant reduction (around 50%) in the antiulcerogenic activity of the studied supplement. On the other hand, pretreating the experimental animals with either indomethacin (cyclooxygenase inhibitor) or L-NAME (NO blocker) had no effect over the pharmacological activity of the nutritional supplement "TK3".

The gastroprotective activity presented by the nutritional supplement "TK3" in the ethanol-induced ulcer model strongly suggests the participation of endogenous non-protein SH-containing compounds. However, there seems to be more aspects to be investigated in this mechanism of action since the reduction in the antiulcerogenic activity was not complete.

Melatonin is probably synthesized in the enterochromaffin cells (EC) of the GI mucosa, after oral or parenteral administration of its substrate, L-tryptophan (Jaworek et al., 2003). The connection between melatonin and L-tryptophan in the mechanism of gastric mucosal integrity and in gastroprotection against several irritants has

already been described since pretreatment with this indole or its precursor applied exogenously, prevented the formation of acute gastric lesions induced by ethanol, stress, aspirin and ischemia-reperfusion (Brzozowski et al., 1997; Cho et al., 1989; De-La-Lastra et al., 1997; Kato et al., 1998; Konturek et al., 1997; Konturek et al., 1997).

Some reports suggest that the gastroprotective effect of melatonin against injury is due to the activation of the cyclooxygenase (COX)-prostaglandin (PG) system (Brzozowska et al., 2002; Kato et al., 2002). It is, therefore, suggested that PG and NO play important roles in the ulcer healing by melatonin (Brzozowska et al., 2002). Other studies, however, suggest this indole inhibits indomethacin-induced gastroduodenal ulcerations via a mechanism probably unrelated to endogenous PG (Melchiorri et al., 1997; Bandyopadhyay et al., 2000). It seems that the ultimate hypothesis consider that endogenous PG and NO are definitely involved in the healing properties of melatonin, although this may not be the sole mechanism for the ulcer healing activity of this hormone and its presursor, L-tryptophan (Brzozowska et al., 2002).

Our results are in agreement with the literature data regarding the benefits of the ingestion of tryptophan in gastric disorders. Nevertheless, the tryptophan-treated group presented a lower level of gastro protection when compared to the "TK3" (L-tryptophan+thymine) treated one. In addition to that, the probable participation of endogenous SH-compounds in the mechanism of action of this nutritional supplement does not seem to be the sole responsible for that activity.

By precipitating proteins, ethanol blocks the gastric cytoprotection, releasing free radicals and decreasing concentration of sulfhydryl compounds in the mucosa cells (Repetto and Llesuy, 2002). The ulcerogenic effects of this agent play a crucial role in altering gastric mucosal defense mechanisms, destroying the mucus and bicarbonate protective layer and releasing free radicals after precipitating proteins (Mozsik and Javor, 1988). On the other hand, the cytoprotective phenomenon is a multifactorial, dynamic process (MacMath, 1990). The complex interplay of mucosal defense factors, endogenous and exogenous stimuli, induction of humoral responses, and ultimately the success or failure of cellular repair, is a rich tool not only in the search of new gastro protective drugs

but also in the understanding of the mechanisms of action of correlate substances, such as anti-inflammatory ones, pain killers, and even antineoplastic ones.

A literature survey revealed that the study of thymine is mainly related to microbiology. Little is known, however, about its pharmacological properties. Our results suggest a synergism between this nucleotide and L-tryptophan since their association produced a better antiulcerogenic effect than the one observed with this amino acid administered alone.

Due to the relevance of the oxidative stress being associated with cell aging (Sohal et al., 2002) and chronic diseases such as Alzheimer (Halliwell, 2001), cancer (Hagen et al., 1994), atherosclerosis (Halliwell, 2000a) and diabetes (Chowienczyk et al., 2000), extensive research is needed in order to find the successful antioxidant treatments to delay or prevent the onset of those diseases (Galli et al., 2002; Halliwell, 2000b). Therefore, the evidence that suggests the participation of the antioxidant SH-compounds in the gastroprotective activity of the nutritional supplement "TK3" lead us to continue conduct future studies involving other potential pharmacological properties of such an association.

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REFERENCES

Atay, S., Tarnawski, A.S., Dubois, A., 2000. Eicosanoids and the stomach. Prostaglandins and other Lipids Mediators 61:105-124.

Avila, J.R., de la Lastra, C.A., Martin, M.J., Motilva, V., Luque, I., Delgado, D., Esteban J., Herrerias, J., 1996. Role of endogenous sulfhydryls and neutrophil infiltration in the pathogenesis of gastric mucosal injury induced by piroxicam in rats. Inflammation Research 45:83-88.

Bandyopadhyay, D., Biswas, K., Bandyopadhyay, U., Reiter, R.J., Banerjee, R.K., 2000. Melatonin protects against stress-induced gastric lesions by scavenging the hydroxyl radical. Journal of Pineal Research 29:143-151.

Bighetti, A.E., Antonio, M.A., Carvalho, J.E., 2002. Regulation and modulation of gastric secretion. Revista de Ciências Médicas 11(1):55-60.

Brzozowska, I., Konturek, P.C., Brzozowski, T., Konturek, S.J., Kwiecien, S., Pajdo, R., Drozdowicz, D., Pawlik, M., Ptak, A., Hahn, E.G., 2002. Role of prostaglandins, nitric oxide, sensory nerves and gastrin in acceleration of ulcer healing by melatonin and its precursor, L-tryptophan. Journal of Pineal Research Apr 32(3):149-162.

Brzozowski, T., Konturek, P.C.H., Konturek, S.J., Kwiecien, S., Pajdo, R., Brzozowska, I., Hahn, E.G., 1998. Involvement of endogenous cholecystokinin and somatostatin in gastroprotection induced by intraduodenal fat. Journal of Clinical Gastroenterology 27(Suppl 1):S125-137.

Brzozowski, T., Konturek, P.C., Konturek, S.J., Pajdo, R., Bielanski, W., Brzozowska, I, Stachura J, Hahn EG., 1997. The role of melatonin and L-tryptophan in prevention of acute gastric lesions induced by stress, ethanol, ischemia, and aspirin. Journal of Pineal Research Sep;23(2):79-89.

Caroleo, M., Frasca, D., Nistico, G., Doria, G., 1992. Melatonin as immunomodulator inimmunodeficient mice. Immunopharmacology 23:81-89.

Carver, J.D., 1994. Dietary nucleotide: cellular immune, intestinal and hepatic system effects. Journal of Nutrition 124:144S-8S.

Chang, S.S., Ivey, B., Smith Jr., J.A., Roth, B.J., Cookson, M.S., 2005. Performance enhancing supplement use in patients with testicular cancer. Urology 66:242-245.

Cho, C.H.H., Pang, S.F., Chen, B.W., Pfeifer, C.J., 1989. Modulating action of melatonin on serotonin-induced aggravation of ethanol ulceration and changes of mucosal blood flow in rat stomachs. Journal of Pineal Research 6:89-97.

Chowienczyk, P.J., Brett, S.E., Gopaul, N.K., Meeking, D., Marchetti, M., Russel-Jones, D.L., Anggard, E.E., Ritter, J.M., 2000. Oral treatment with an antioxidant (raxofelast) reduces oxidative stress and improves endothelial function in men with type II diabetes. Diabetologia 43:974-977.

Coruzzi, G., Adami, M., Morini, G., Pozzoli, C., Cena, C., Bertinaria, M., Gasco, A., 2000. Antisecretory and gastroprotective activities of compounds endowed with H2 antagonists and nitric oxide (NO) donor properties. Journal of Physiology-Paris 94(1):5-10.

Courcelle, J., 2005. Recs preventing wrecks. Mutation Research 577:217-227.

De-La-Lastra, C.A., Cabeza, J., Motilva V., Martín, M.J., 1997. Melatonin protects against gastric ischemia-reperfusion injury in rats. Journal of Pineal Research 23:47-52.

Elliot, S.N., McKnight, W., Cirino, G., Wallace, J.L., 1995. A nitric oxide-releasing nonsteroidal anti-inflammatory drug accelerates gastric ulcer healing in rats. Gastroenterology Aug;109(2):524-30.

Galli, R.L., Shukitt-Hale, B., Youdim, K.A., Joseph, J.A., 2002. Fruit polyphenolics and brain aging: nutritional interventions targeting age-related neuronal and behavioral deficits**.** Annals of the New York Academy of Sciences 959:128-132.

Gamberini, M.T., Skorupa, L.A., Souccar, C., Lapa, A.J., 1991. Inhibition of gastric secretion by a water extract from *Baccharis triptera*, Mart. Memórias do Instituto Oswaldo Cruz 86:Suppl.II, 137-139.

Hagen, T.M., Huang, S., Curnnutte, J., Fowler, P., Martinez, V., Wehr, C.M., Ames, B.N., Chisari, F.V., 1994. Extensive oxidative DNA damage in hepatocytes of transgenic mice with chronic active hepatitis destined to develop hepatocellular carcinoma. Proceedings of the National Academy of Sciences, USA 91:12808-12812.

Halliwell, B., 2000a. Lipid peroxidation, antioxidants and cardiovascular disease: how should we move forward? Cardiovascular Research 47:410-418.

Halliwell, B., 2000b. The antioxidant paradox. Lancet 355:1179-1180.

Halliwell, B., 2001. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. Drugs Aging 18:685-716.

Hogan, D.L., Ainsworth, N.A., Isenberg, J., 1994. Review article: Gastroduodenal bicarbonate secretion. Alimentary Pharmacology & Therapeutics 8:475-478.

Holen, E., Bjørge, O.A., Jonsson, R., 2006. Dietary nucleotides and human immune cells. II. Modulation of PBMC growth and cytokine secretion. Nutrition 22:90-96.

Jaworek, J., Leja-Szpak, A., Bonior, J., Nawrot, K., Tomaszewska, R., Stachura, J., Sendur R., Pawlik, W., Brzozowski, T., Konturek, S.J., 2003. Protective effect of melatonin and its precursor L-tryptophan on acute pancreatitis induced caerulein overestimulation or ischemia/reperfusion. Journal of Pineal Research 34:40-52.

Jyonouchi, H., 1994. Nucleotide actions on humoral immune responses. Journal of Nutrition 124:138S-143.

Kato, K., Murai, I., Asai, S., Matsuno, Y., Komuro, S., Kaneda, N., Iwasaki, A., Ishikawa, K., Nakagawa, S., Arakawa, Y., Kuwayama, H., 1998. Protective role of melatonin and the pineal gland in modulating water immersion restraint stress ulcer in rats. Journal of Clinical Gastroenterology 27:110-115.

Kato, K., Murai, I., Asai, S., Takahashi, Y., Nagata, T., Komuro, S., Mizuno, S., Iwasaki, A., Ishikawa, K., Arakawa Y., 2002. Circadian rhythm of melatonin and prostaglandin in modulation of stress-induced gastric mucosal lesions in rats. Alimentary Pharmacology & Therapeutics 16:29-34.

Kema, I.P., de Vries, E.G.E., Muskiet, F.A.J., 2000. Clinical chemistry of serotonin and metabolites. Journal of Chromatography B 747:33-48.

Konturek, P.C., Konturek, S.J., Brzozowski, T., Dembinski, A., Zembala, M., Mytar, B., Hahn, E.G., 1997. Gastroprotective activity of melatonin and its precursor, Ltryptophan, against stress-induced and ischaemia-induced lesions is mediated by scavenge of oxygen radicals. Scandinavian Journal of Gastroenterology. May;32(5):433-438.

Konturek, P.C., Konturek, S.J., Majka, J., Zembala, M., Hahn, E.G., 1997. Melatonin affords protection against gastric lesions induced by ischemia-reperfusion possibly due to its antioxidant and mucosal microcirculatory effects. European Journal of Pharmachology 322:73-77.

MacMath, T.L., 1990. Alcohol and gastrointestinal bleeding. Emergency Medicine Clinics of North America Nov 8(4):859-872.

Maestroni, G.J., 1993. The immunoneuroendocrine role of melatonine. Journal of Pineal Research 14:1-10.

May, P.E., Barber, A., D'Olimpio, J.T., Houribane, A., Abumrad, N.N., 2002. Reversal of cancer-related wasting using oral supplementation with a combination of beta-hydroxybeta-methylbutyrate, arginine, and glutamine. American Journal of Surgery 183(4):471-47.

Melchiorri, D., Sewerynek, E., Reiter, R.J., Ortiz, G.G., Poeggeler, B., Nisticò G., 1997. Suppressive effect of melatonin administration on ethanol-induced gastroduodenal injury in rats in vivo. British Journal of Pharmacology 121:264−270.

Mozsik, G., Javor, T., 1988. A biochemical and pharmacological approach to the genesis of ulcer disease. I. A model study of ethanol-induced injury to gastric mucosa in rats. Digestive Diseases Sciences 33:92-105.

Peskar, B.M., Maricic, N., 1998. Role of prostaglandins in gastro protection. Digestive Diseases Sciences 43(Suppl 9):23S-29.

Reiter, R.J., 1998. Oxidative damage in the central nervous system: protection by melatonin. Progress in Neurobiology 56(3):359-384.

Repetto, M.G., Llesuy, S.F., 2002. Antioxidant properties of natural compounds used in popular medicine for gastric ulcers. Brazilian Journal of Medicinal and Biological Research 35:523-534.

Rique, A.B.R., Soares, E.A., Meirelles, C.M., 2002. Nutrição e exercício na prevenção e controle das doenças cardiovasculares. Revista Brasileira Medicina Esporte 8(6):244-254.

Robert, A., 1979. Cytoprotection by prostaglandins. Gastroenterology 77:761-767.

Rudolph, F.B., Kulkarni, A.D., Fanslow, W.C., Pizzini, R.P., Kumar, S., Van Buren, C.T., 1990. Role of RNA as a dietary source of pyrimidines and purines in immune function. Nutrition 6:45-52.

Saito, H., 2004. [Immunonutrition] Nippon Geka Gakkai Zasshi. Feb;105(2):213-217.

Samonina, G.E., Kopylova, G.N., Lukjanzeva, G.V., Zhuykova, S.E., Smirnova, E.A., German, S.V., Guseva, A.A., 2004. Antiulcer effects of amylin: a review. Pathophysiology 11:1-6.

Sandyk, R., 1992. L-tryptophan in neuropsychiatric disorders: a review. International Journal of Neuroscience 67:127-144.

Shay, H., Komarov, S.A., Fels, S.S., Meranze, D., Gruestein, M., Siplet, H., 1945. A simple method for the uniform production of gastric ulceration in the rat. Gastroenterology 5, 43-61.

Sohal, R.S., Mockett, R.J., Orr, W.C., 2002. Mechanisms of aging: an appraisal of the oxidative stress hypothesis. Free Radical Biology and Medicine 33:575-586.

Van Buren, C.T., Kulkarni, A.D., Rudolph, F.B., 1994. The role of nucleotides in adult nutrition. Journal of Nuttition 124:160S-164.

Widner, B., Laich, A., Sperner-Unterweger, B., Ledochowski, M., Fuchs, D., 2002. Neopterin production, tryptophan degradation, and mental depression - what is the link? Brain, Behavior, and Immunity Oct;16(5):590-595.

World Health Organization, 2002. The scientific basis for diet, nutrition and prevention of type 2 diabetes. Geneva: WHO/FAO;1-53.

Figure Legends

Fig. 1. Experimental design for the induction of stomach ulcer, in rats, by oral administration of absolute ethanol (Gamberini et al., 1991).

Fig. 2. Effect of oral administration of carbenoxolone, L-tryptophan (Try), thymine (Thy) and "TK3" in the ethanol-induced experimental model in rats (ANOVA: $F(4.23)=43.76$ $p<0.001$. Duncan's Test* $p<0.001$).

Fig. 3. Effect of oral administration of both carbenoxolone and the nutritional "TK3" in the ethanol-induced experimental model in rats previously treated with indomethacin (5 mg/Kg, i.p.) (ANOVA: $F(4.24)=16.04$ $p<0.001$. Duncan's Test* $p<0.001$).

Fig. 4. Effect of oral administration of the nutritional "TK3" in the ethanol-induced experimental model in rats previously treated with L-NAME (5mg/Kg, e.v.) (ANOVA: $F3.17)=67.74$ $p<0.001$. Duncan's Test * $p<0.001$).

Fig. 5. Effect of oral administration of the nutritional "TK3" in the ethanol-induced experimental model in rats previously treated with N-ethylmaleimide (10 Mg/Kg, s.c.) (ANOVA: $F(3.19)=21.77$ $p<0.001$. Duncan's Test* $p<0.001$).

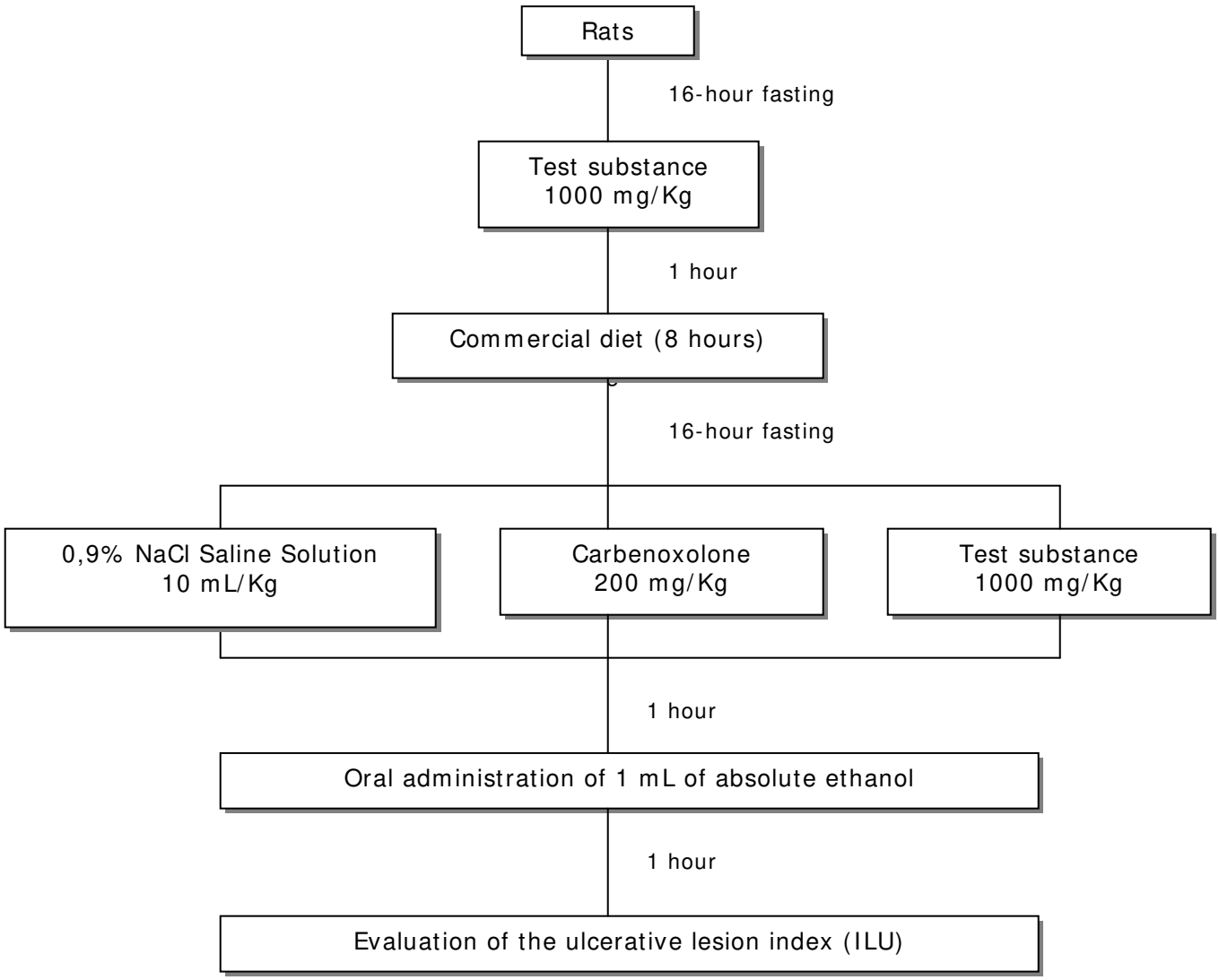
Rats
16-hour fasting
Test substance
1000 mg/Kg
1 hour
Commercial diet (8 hours)
16-hour fasting
0,9% NaCl Saline Solution
10 mL/Kg
Carbenoxolone
200 mg/Kg
Test substance
1000 mg/Kg
1 hour
Oral administration of 1 mL of absolute ethanol
1 hour
Evaluation of the ulcerative lesion index (ILU)

Fig. 1

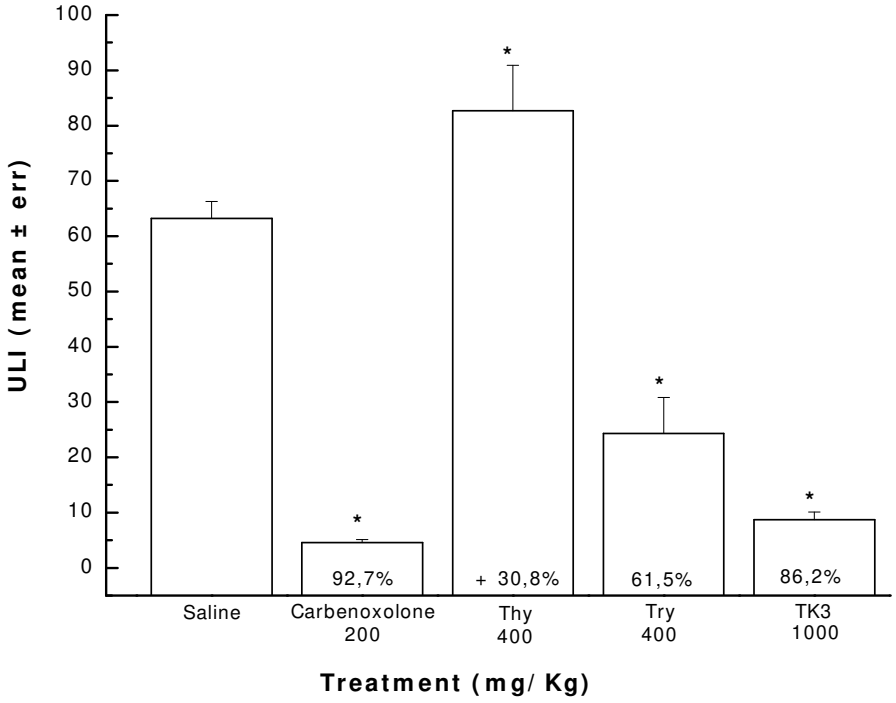
100
90
80
70
60
50
40
30
20
10
0
ULI (mean ± err)
*
*
*
*
92,7%
+ 30,8%
61,5%
86,2%
Saline
Carbenoxolone 200
Thy 400
Try 400
TK3 1000
Treatment (mg/ Kg)

Fig. 2

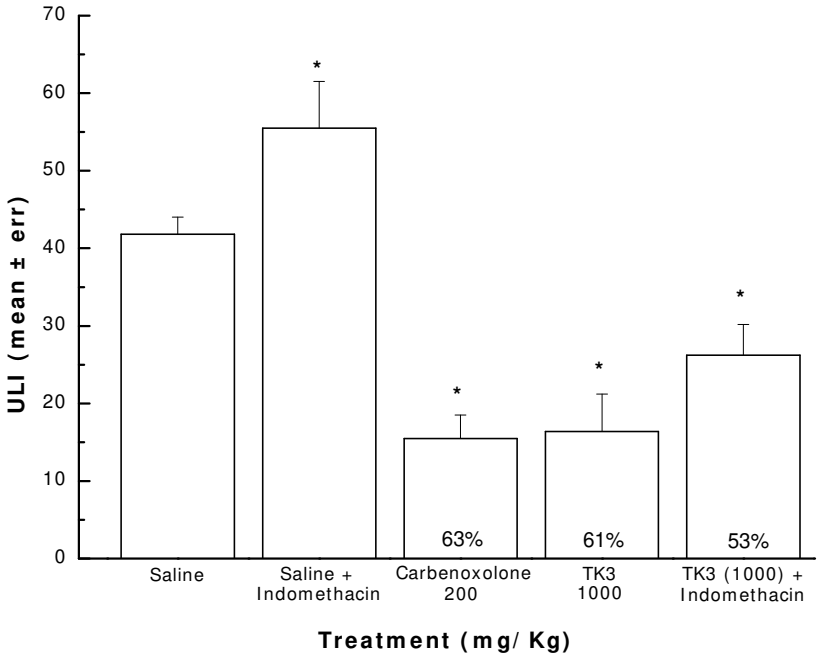
70
60
50
40
30
20
10
0
ULI (mean ± err)
*
*
*
*
63%
61%
53%
Saline
Saline + Indomethacin
Carbenoxolone 200
TK3 1000
TK3 (1000) + Indomethacin
Treatment (mg/ Kg)

Fig. 3

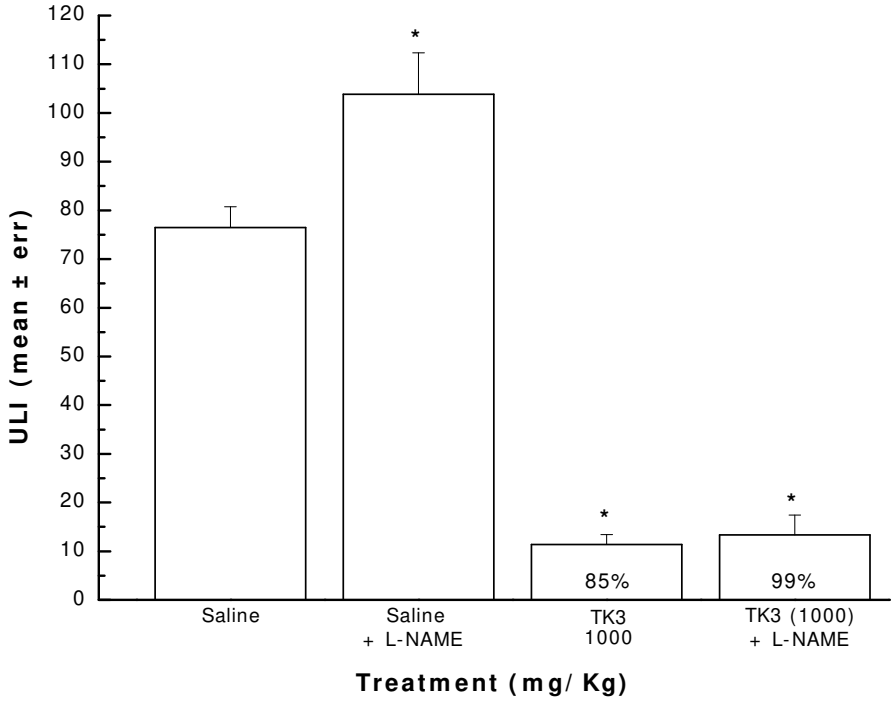
ULI (mean ± err)
120
110
100
90
80
70
60
50
40
30
20
10
0
*
*
*
85%
99%
Saline
Saline
+ L-NAME
TK3
1000
TK3 (1000)
+ L-NAME
Treatment (mg/ Kg)

Fig. 4

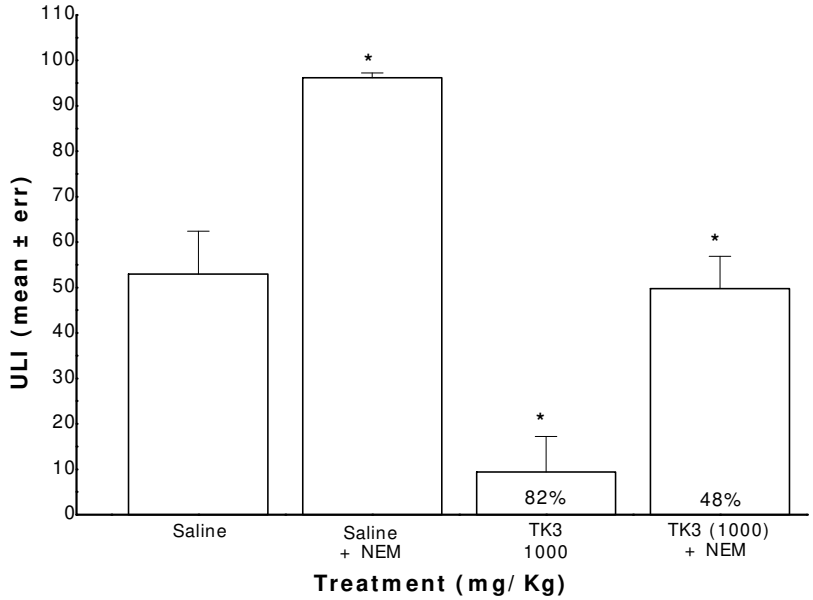
110
100
90
80
70
60
50
40
30
20
10
0
ULI (mean ± err)
*
*
*
82%
48%
Saline
Saline + NEM
TK3 1000
TK3 (1000) + NEM
Treatment (mg/ Kg)

Fig. 5

Table Legends

Table 1. Index of ulcerative lesions (ULI) according to Gamberini et al. (n* = number of ulcers found.)

Table 2. Protective effect of repetitive doses of "TK3" treatments on the ethanol inductive model of stomach ulcer in the rat. (ANOVA $F(4.23) = 41.06$ $p<0.001$. Duncan's Test *$p<0.001$, referred to saline).

Table 3. Effect of intraduodenal administration of "TK3" (1000 mg/Kg) on volume, pH and hydrogenionic concentration of gastric content in pyloric ligation model. a,b ANOVA $F(4,28)$: 12.24 $p<0.001$. Duncan's Test a $p<0.01$; b $p<0.001$. c ANOVA $F(4,28)$: 9.53 $p<0.001$. Duncan's Test $p<0.001$. d ANOVA $F(4,28)$: 6.84 $p<0.001$. Duncan's Test $p<0.05$.

Lesions	**Scoring**
Loss of normal morphology	One point
Mucosa discoloration	One point
Mucosa edema	One point
Hemorrhages	One point
Petechial point (until 9)	Two points
Petechial point (10 or more)	Three points
Ulcers (up to 1 mm)	n* x 2 points
Ulcers (1mm or more)	n* x 3 points
Perforated ulcers	n* x 4 points

Table 1

Treatment (mg/Kg)	**ULI**	**Inhibition of ULI (%)**
0,9 NaCl Saline Solution (10 mL/Kg)	88.0	-
Carbenoxolone (200)	5.7	93.5*
TK3 (100)	82.8	5.9
TK3 (300)	33.0	62.5*
TK3 (1000)	13.8	84.3*

Table 2

Treatments	**Dose (mg/Kg)**	**Volume (mL)**	**pH**	**$[H^+]$ {mEq/L/4h}**
TK3	1000	5.3 ± 0.7^a	2.7 ± 0.4	47.14 ± 8
Cimetidine	100	2.9 ± 0.13^b	4.7 ± 0.7^c	18.2 ± 2.7^d
0,9% Saline		7.9 ± 0.2	2.3 ± 0.2	32 ± 4

Table 3

CONCLUSÃO GERAL

A avaliação toxicológica do complemento nutricional denominado "TK3", como parte de seus ensaios pré-clínicos, não revelou citotoxicidade para nenhuma das linhagens avaliadas, até a concentração máxima utilizada de 250 µg/mL. Tais resultados apontam para a segurança de seu uso e refutam a argumentação de que tal complemento poderia servir de substrato para o desenvolvimento tumoral, uma vez que não houve estímulo para o crescimento das linhagens tumorais humanas.

A avaliação toxicológica em roedores, aguda e sub-crônica (90 dias), não demonstrou quaisquer efeitos tóxicos relevantes. O achado clínico digno de nota observado, após a administração intraperitoneal de 2000 mg/kg, foi uma severa letargia, reversível após uma hora desta administração, sem quaisquer outros efeitos clínicos ou laboratoriais adversos. Tal fato sugere a conversão do L-triptofano em melatonina. Após administrações repetidas por 90 dias, foi observado um aumento na contagem total de leucócitos nos machos que receberam 300 e 1000 mg/kg. A possível atividade imunomoduladora deste complemento está em conformidade com relatos das propriedades imunoestimulantes do L-triptofano e da melatonina.

Como ferramenta de estudo, foi explorada a avaliação antiulcerogênica do "TK3". Os resultados obtidos demostraram que, além da ação protetora gástrica já relatada para o L-triptofano, a associação com a timina potencializa tal efeito, chegando a índices semelhantes aos atingidos pela substância utilizada como controle positivo do experimento (Carbenoxolona, 200 mg/kg). No processo de triagem do provável mecanismo de ação citoprotetora, os resultados obtidos demonstraram a participação de compostos sulfidrílicos não-proteicos, uma vez que a administração prévia de NEM foi capaz de reduzir em 50% a atividade antiulcerogênica do referido complemento.

A atividade antitumoral em modelo ascítico de Ehrlich, em camundongos, demonstrou uma melhora na avaliação clínica dos animais. Apesar de não ter sido observada diferença significativa na sobrevida, tal resposta aponta para uma ação benéfica como coadjuvante. Um efeito similar já foi relatado em estudos clínicos com 22 pacientes terminais portadores de carcinomas metastáticos (Costello, 1975) que receberam diariamente 5-fluorotritptofano (200 mg/dia) e apresentaram, em sua maioria, importante melhora em sua qualidade de vida. De acordo com a literatura, a ação imunomoduladora do

L-triptofano tem sido observada em infecções, doenças auto-imunes e neoplasias malignas que envolvem ativação da resposta celular (tipo Th1) (Schrocksnadel et al., 2006), o que poderia ser um dos aspectos relacionados com a melhor qualidade de vida. Entretanto, nossos resultados revelam que a associação deste aminoácido com a timina apresenta um efeito superior àquele observado nos animais portadores de Tumor Ascítico de Ehrlich que receberam os componentes isoladamente (Apêndice).

Considerando, portanto, os objetivos iniciais deste trabalho, ou seja, a avaliação de segurança e triagem farmacológica do complemento nutricional "TK3" foi possível concluir que se trata de uma associação com potencial para estudos clínicos por não apresentar toxicidade evidente ou exacerbada. Concomitantemente, dada à relevância dos diversos tratamentos coadjuvantes de pacientes portadores de neoplasias malignas (Lev et al., 2006; Paull et al., 2006; Lipton, 2006; Yoshida et al., 2001) , os resultados obtidos representam estímulo para uma investigação mais detalhada acerca dos possíveis mecanismos de ação e confirmação das atividades antioxidante e imunomoduladora.

REFERÊNCIAS BIBLIOGRÁFICAS

Afridi, M. A., 2003. Tobacco use as contributory factor in peptic ulcer disease. Journal of College of Physicians and Surgeons Pakistan 13(7):385-387.

Allen, A. & Flemstrom, G., 2005. Gastroduodenal mucus bicarbonate barrier: protection against acid and pepsin. American Journal of Physiology – Cell Physiology 288(1):C1-19.

Bailara, K. M., Henry, C., Lestage, J., Launay, J. M., Parrot, F., Swendsen, J., Sutter, A. L., Roux, D., Dallay, D., Demotes-Mainard, J., 2006. Decreased brain tryptophan availability as a partial determination of post-partum blues. Psychoneuroendocrinology. 31(3):407-13. Epub 2005 Nov 21.

Balis, F. M., 1998. The Goal of Cancer Treatment. Oncologist 3(4):V.

Baylis, C., 2006. Arginine, arginine analogs and nitric oxide production in chronic kidney disease. Nature Clinical Practice Nephrology 2(4):209-220.

Bhatia, V. & Tandon, R. K., 2005. Stress and the gastrointestinal tract. Journal of Gastroenterology and Hepatology 20:332-339.

Bighetti, A. E., Antonio, M. A., Carvalho, J. E., 2002. Regulação e modulação da secreção gástrica. Revista de Ciências Médicas 11:55-60.

Bilikiewicz, A., Gaus, W., 2004. Colostrinin (a naturally occurring, proline-rich, polypeptide mixture) in the treatment of Alzheimer's disease. Alzheimer's Disease 6(1): 17-26.

Billman, G. E., Kang, J. X., Leaf, A., 1999. Prevention of sudden cardiac death by dietary pure omega-3 polyunsaturated fatty acids in dogs. Circulation 99(18):2452-2457.

Bloxam, D.L., Warren, W.H., White, P.J., 1974. Involvement of the liver in the regulation of tryptophan availability: possible role in the responses of liver and brain to starvation. Life Sciences 14:1321-1329.

Bonkale, W. L., Turecki, G., Austin, M. G., 2006. Increased tryptophan hydroxylase immunoreactivity in the dorsal raphe nucleus of alcohol-dependent, depressed suicide subjects is restricted to the dorsal subnucleus. Synapse 60(1):81-85.

Bourre, J. M., 2006. Effects of Nutrients (in Food) on the Structure and Function of the Nervous System: Update on Dietary Requirements for Brain. Part 1: Micronutrients. The journal of nutrition, health & aging 10(5):377-385.

Brzozowska, I., Konturek, P. C., Brzozowski, T., Konturek, S. J., Kwiecien, S., Pajdo, R., Drozdowicz, D., Pawlik, M., Ptak A., Hahn, E. G., 2002. Role of prostaglandins, nitric oxide, sensory nerves and gastrin in acceleration of ulcer healing by melatonin and its precursor, L-tryptophan. Journal of Pineal Research 32(3):149.

Brzozowska, I., Targosz, A., Sliwowski, Z., Kwiecien, S., Drozdowicz, D., Pajdo, R., Konturek, P. C., Brzozowski, T., Pawlik, M., Konturek, S. J., Pawlik, W. W., Hahn, E. G., 2004. Healing of chronic gastric ulcers in diabetic rats treated with native aspirin, nitric oxide (NO)-derivative of aspirin and cyclooxygenase (COX)-2 inhibitor. Journal of Physiology and Pharmacology 55(4):773-790.

Buss, W. C., Stepanek, J., Bankhurst, A. D., Mayeno, A. N., Pastuszyn, A., Peabody, D., 1996. EBT, a tryptophan contaminant associated with eosinophilia myalgia syndrome, is incorporated into proteins during translation as an amino acid analog. Autoimmunity 25(1):33-45.

Carvalho, J. E., 2004. Toxicidade pré-clínica: fitoterápicos e alimentos com propriedades funcionais ou de saúde. Conferência Sul-Americana de Medicina Veterinária - ABMA.

Capuron, L., Dantzer, R., 2003. Cytokines and depression: the need for a new paradigm. Brain, Behavior and Immunity Suppl 1:S119-24.

Charlton, K. E., 2002. Eating well: ageing gracefully! Asia Pacific Journal of Clinical Nutrition 11 Suppl 3:S607-S617.

Chang, S. S., Ivey, B., Smith Jr, J. A., Roth, B. J., Cookson, M. S., 2005. Performance-enhancing supplement use in patients with testicular cancer. Urology 66:242-245.

Choudry, H. A., Pan, M., Karinch, A. M., Souba, W. W., 2006. Branched-chain amino acid-enriched nutritional support in surgical and cancer patients. Journal of Nutrition 136(1 Suppl):314S-318S.

Clarke, D. L., Thomson, S. R., 2002. Attenuated gastric mucosal blood flow predicts non-healing of benign gastric ulcers. European Surgical Research 34(6):432-436.

Cleare, A.J. & Bond, A.J., 1995. The effect of tryptophan depletion and enhancement on subjective and behavioural aggression in normal male subjects. Psychopharmacology 118:72-81.

Courcelle, J., 2005. Recs preventing wrecks. Mutation Research, 577:217-227.

Costello, C. 1975. Carcinoid tumor metastases. Prospective study of twenty-two patients. American Journal of Surgery 130(6):756-759.

Dancey, C. P., Attree, E. A., Brown, K. F., 2006. Nucleotide supplementation: a randomised double-blind placebo controlled trial of IntestAidIB in people with Irritable Bowel Syndrome. Nutrition Journal 5:16.

Daniels, S. R., Hudson, J. I., Horwitz, R. I., 1995. Epidemiology of potential association between L-trytophan ingestion and eosinophilia-myalgia syndrome**.** Journal of Clinical Epidemiology 48(12):1413-1427; discussion 1429-1440.

Dass, C.R. & Choong, P. F., 2006. Biophysical delivery of peptides: Applicability for cancer therapy. Peptides Sep 21; [Epub ahead of print].

Devereux, G., 2006. The increase in the prevalence of asthma and allergy: food for thought. Nature Reviews Imunology 6(11):869-874.

Duntas, L. H., 2006. The role of selenium in thyroid autoimmunity and cancer. Thyroid 16(5):455-460.

Ephgrave, K. S., Brasel, K. J., Cullen, J. J., Broadhurst, K. A., 1998. Gastric mucosal protection from enteral nutrients: role of motility. Journal of the American College of Surgeons 186(4):434-440.

Fastaia, J. & Dumont, A., 1976. Pathogenesis of Ascites in Mice with Peritoneal Carcinomatosis. Journal of National Cancer Institute 56:547-549.

Fonteh, A. N., Harrington, R. J., Tsai, A., Liao, P., Harrington, M.G., 2006. Free amino acid and dipeptide changes in the body fluids from Alzheimer's disease subjects. Amino Acids Oct 10; [Epub ahead of print].

Forstner, J. F. & Forstner, G. G., 1994. Gastrointestinal mucus. In: Johnson, L.R., editor. Physiology of the gastrointestinal tract. 3rd ed. New York: Raven Press; 1255-1283.

Furet, P., Bold, G., Meyer, T., Roesel, J., Guagnano, V., 2006. Aromatic interactions with phenylalanine 691 and cysteine 828: a concept for FMS-like tyrosine kinase-3 inhibition. Application to the discovery of a new class of potential antileukemia agents. Journal of Medicinal Chemistry 49(15):4451-4454.

Green, A.R., Aronson, J.K., Curson, G., Woods, H.F., 1980. Metabolism of an oral tryptophan load. I. Effects of dose and pretreatment with tryptophan. British Journal of Clinical Pharmacology 10:603-610.

Guha, S. & Kaunitz, J. D., 2002. Gastroduodenal mucosal defense: an integrated protective response. Current Opinion in Gastroenterology 18(6):650-657.

Guyton, A. C., Hall, J. E., 1996. Textbook of Medical Physiology. Guyton and Hall ed. 9th ed. W.B. Saunders Company. Philadelphia.

Hartmann, E. & Greenwald, D., 1984. Tryptophan and human sleep: an analysis of 43 studies, In: Schlossberger, H.G.,; Kohen, W.; Linzen, B. & Steinhart, H. (eds.) – Progress in Tryptophan and Serotonin Research. Berlin, Walter and Gruyter, 1984, p. 297-304.

Holzer, P., 2006. Efferent-like roles of afferent neurons in the gut: Blood flow regulation and tissue protection. Autonomic Neuroscience: basic & clinical 125(1-2):70-75. Epub 2006 Mar 20.

Holzer, P., 2000. Gastroduodenal mucosal defense. Current Opinion in Gastroenterology 16(6):469-78.

Huang, H. Y., Caballero, B., Chang, S., Alberg, A. J., Semba, R. D., Schneyer, C. R., Wilson, R. F., Cheng, T. Y., Vassy, J., Prokopowicz, G., Barnes, G. J. 2nd, Bass, E. B., 2006. The efficacy and safety of multivitamin and mineral supplement use to prevent cancer and chronic disease in adults: a systematic review for a National Institutes of Health state-of-the-science conference. Annals of Internal Medicine 145(5):372-385. Epub 2006 Jul 31.

INCA, 2005. Brasil. Ministério da Saúde. Secretaria de Atenção à Saúde. Instituto Nacional de Câncer. Coordenação de Prevenção e Vigilância. Estimativa 2006: Incidência de Câncer no Brasil.

Jyonouchi, H., Sun, S., Winship, T., Kuchan, M. J., 2003. Dietary ribonucleotides increase antigen-specific type 1 T-helper cells in the regional draining lymph nodes in young BALB/cJ mice. Nutrition 19(1):68-69.

Kantak, K. M., Hegstrand, L. R., Eichelman, B., 1980. Dietary tryptophan modulation and aggressive behavior in mice. Pharmacology Biochemistry and Behavior 12(5):675-679.

Kaunitz, J. D. & Akiba, Y., 2004. Gastroduodenal mucosal defense: role of endogenous mediators. Current opinion in gastroenterology 20(6):526-532.

Kema, I.P., de Vries, E.G.E., Muskiet, F.A.J., 2000. Clinical chemistry of serotonin and metabolites. Journal of Chromatography B 747:33-48.

Konturek, J. W., 2003. Discovery by Jaworski of *Helicobacter Pylori* and its pathogenetic role in peptic ulcer, gastritis and gastric cancer. Journal of Physiology and Pharmacology 54(S3):23-41.

Konturek, P. C., Konturek, S. J., Brzozowski, T., Dembinski, A., Zembala, M., Mytar, B., Hahn, E. G., 1997. Gastroprotective activity of melatonin and its precursor, L-tryptophan, against stress-induced and ischaemia-induced lesions is mediated by scavenge of oxygen radicals. Scandinavian Journal of Gastroenterology 32(5):433-438.

Lev, E. L., Eller, L. S., Kolassa, J., Gejerman, G., Colella, J., Lane, P., Scrofine, S., Esposito, M., Lanteri, V., Scheuch, J., Munver, R., Galli, B., Watson, R. A., Sawczuk, I., 2006. Exploratory factor analysis: strategies used by patients to promote health. World Journal of Urology Oct 26; [Epub ahead of print].

Lipton, A., 2006. Future treatment of bone metastases. Clinical Cancer Research 12(20 Pt2):6305s-6308s.

Ma, L., Chow, J. Y. C., Liu, E. S. L., Cho, C. H., 1999. Cigarette smoke and its extract delay ulcer healing and reduce nitric oxide synthase activity and angiogenesis in rat stomach. Clinical and Experimental Pharmacology and Physiology 26:828-829.

Martin, G. R. & Wallace, J. L., 2006. Gastrointestinal inflammation: a central component of mucosal defense and repair. Experimental biology & medicine (Maywood, N.J.) 231(2):130-137.

Mascarenhas, R., Mobarhan, S., 2004. New support for branched-chain amino acid supplementation in advanced hepatic failure. Nutrition Reviews 62(1):33-38.

Mayer, K. D., 1966. The Pathogenicity of the Ehrlich Ascites Tumor. British Journal of Experimental Pathology 47:537-544.

Melo, P. S., Justo, G. Z., Duran, N., Haun, M., 2004. Natural Killer cell activity and Anti-tumor effects of Dehydrocrotonin and its Synthetic Derivatives. European Journal of Pharmacology 487:47-54.

Miller Iii, E. R., Erlinger, T. P., Appel, L. J., 2006. The Effects of Macronutrients on Blood Pressure and Lipids: An Overview of the DASH and OmniHeart Trials. Current Atherosclerosis Reports 8(6):460-465.

Monks, A., Scudiero, D., Skehan, P., Shoemaker, R., Paull, K., Vistica, D., Hose, C., Langley, J., Cronise, P., Vaigro-Wolff, A., Gray-Goodrich, M., Campbell, H., Mayo, J., Boyd, M., 1991. Feasibility of a High-Flux Anticancer Drug Screen Using a Diverse Panel of Cultured Human Tumor Cell Lines. Journal of the National Cancer Institute, 83:757-766.

Monrad, C., Young, S.N., Ervin, F.R., 1983. Clinical response of aggressive schizophrenics to oral tryptophan. Biological Psychiatry 18:575-578.

Moore, P., Landolt, H. P., Seifritz, E., Clark, C., Bhatti, T., Kelsoe, J., Rappaport, M., Gillin, J. C., 2000. Clinical and physiological consequences of rapid tryptophan depletion. Neuropsychopharmacology 23:601–622.

Motilva, V., Alarcón de la Lastra, C., Bruseghini, L., Manuel Herrerias, J., Sanchez-Fidalgo, S., 2005. COX expression and PGE_2 and PGD_2 production in experimental acute and chronic gastric lesions. International Immunopharmacology 5:369-379.

Morton, D. B., Griffiths, P. H. M., 1985. Guidelines on the recognition of pain, distress and discomfort in experimental animals and a hypothesis for assessment. The Veterinary Record 116:431-436.

Moskowitz, D. S., Pinard, G., Zuroff, D. C., Annable, L., Young, S.N., 2001. The effect of tryptophan on social interaction in everyday life: a placebo-controlled study. Neuropsychopharmacology 25:277–289.

Mudway, I. S., Behndig, A. F., Helleday, R., Pourazar, J, Frew, A. J., Kelly, F. J., Blomberg, A., 2006. Vitamin supplementation does not protect against symptoms in ozone-responsive subjects. Free Radical Biology & Medicine 40(10):1702-1712.

Mundy, H. R., Williams, J. E., Cousins, A. J., Lee, P. J., 2006. The effect of L-alanine therapy in a patient with adult onset glycogen storage disease type II. Journal of Inherited Metabolic Disease 29(1):226-9.

Munro, H.N., 1970. Free amino pools and their role in regulation. In: Munro, H.N. (ed.) – Mammalian Protein Metabolism. Vol. IV. New York, Academic Press, p. 299-386.

National Cancer Institute, 1998. Psychological stress and cancer. Disponível em: <http://www.cancer.gov/cancertopics/factsheet/Risk/stress/print?page=&keyword=>. Acesso: em 29 jan. 2007.

Naghii, M. R., Fouladi, A. I., 2006. Correct assessment of iron depletion and iron deficiency anemia. Nutrition Health 18(2):133-139.

Nemeth, H., Toldi, J., Vecsei, L., 2006. Kynurenines, Parkinson's disease and other neurodegenerative disorders: preclinical and clinical studies. Journal of Neural Transmission (70):285-304.

Nishida, K., Ohta, Y., Ishiguro, I., 1998. Relation of inducible nitric oxide synthase activity to lipid peroxidation and nonprotein sulfhydryl oxidation in the development of stress-induced gastric mucosal lesions in rats. Nitric Oxide 2(4):215-23.

Ngoma, T. 2006. World Health Organization cancer priorities in developing countries. Annals of Oncology Jun;17 Suppl 8:viii9-viii14.

OECD (Organization for Economic Cooperation and Development). 2000. Guidance document on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluation. Paris, France: OECD.

OECD (Organization for Economic Cooperation and Development). 420 - Guideline for Testing of Chemicals – Acute Oral Toxicity – Fixed Dose Procedure. Adopted 17th December 2001.

OECD (Organization for Economic Cooperation and Development). 408 - Guideline for Testing of Chemicals – Repeated Dose 90-day Oral Toxicity Study in Rodents. Adopted 21st September 1998.

OECD (Organization for Economic Cooperation and Development). 409 - Guideline for Testing of Chemicals – Repeated Dose 90-day Oral Toxicity Study in Non-Rodents. Adopted 21st September 1998.

Ogilvie, G. K., Fettman, M. J., Mallinckrodt, C. H., Walton, J. A., Hansen, R. A., Davenport, D. J., Gross, K. L., Richardson, K. L., Rogers, Q., Hand, M. S., 2000. Effect of fish oil, arginine, and doxorubicin chemotherapy on remission and survival time for dogs with lymphoma: a double-blind, randomized placebo-controlled study. Cancer 88(8):1916-1928.

Oldendorf, W.H. & Szabo, J., 1976. Amino acids assignment to one of three blood-brain barrier amino acid carriers. American Journal of Physiology 230:94-98.

Ozer, C., Gonul, B., Ercan, Z. S., Take, G., Erdogan, D., 2006. The effect of tryptophan administration on ileum contractility and oxidant status in mice. Amino Acids May 26; [Epub ahead of print].

Pajares, J. M. & Gisbert, J. P., 2006. *Helicobacter pylori*: its discovery and relevance for medicine. Revista Española de Enfermidades Digestivas 98(10):770-785.

Paull, D. E., Thomas, M. L., Meade, G. E., Updyke, G. M., Arocho, M. A., Chin, H. W., Adebonojo, S. A., Little, A. G., 2006. Determinants of quality of life following pulmonary resection for lung cancer. American Journal of Surgery 192(5):565-571.

Pessina, A., Brambilla, P., Mocarelli, P., 1980. Surface antigen on Ehrlich ascites tumor cells. Biomedicine 33(4):105-109.

Poloni, M., Nappi, G., Arringo, A., Savoldi, F., 1974. Cerebrospinal fluid 5-hydroxyindoleacetic acid levels in migrainous patients during spontaneous attacks, during headache free periods, and following with L-tryptophan. Experimentia, 30:640-641.

Qiao, W. L., Wang, L., Zhang, J. F., Zhang, Y.M., 2006 Effects of gastric ischemia-reperfusion on gastric mucosal cellular apoptosis and proliferation in rats. Acta Physiologica Sinica 58(3):237-243.

Rajendram, R. & Preedy, V. R., 2005. Effect of alcohol consumption on the gut. Digestive Diseases 23(3-4):214-221.

Riemann, D., Feige, B., Hornyak, M., Koch, S., Hohagen, F., Voderholzer, U., 2002. The tryptophan depletion test: impact on sleep in primary insomnia – pilot study. Psychiatry Research 109(2):129-135.

Rubinstein, L. V., Shoemaker, R. H., Paull, K. D., Simon, R. M., Tosini, S., Skehan, P., Scudiero, D. A., Monks, A., Boyd, M. R., 1990. Comparison of in vitro anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. Journal of National Cancer Institute 82:1113-1118.

Sacoman, J. L., Monteiro, K. M., Possenti, A., Figueira, G. M., Foglio, M.A., Carvalho, J. E. Anticancer activity of *Pothomorphe umbellata* crude extract (enviado para Cancer Letters)

Schubert, M. L., 2000. Gastric secretion. Current Opinion in Gastroenterology 16:463-468.

Schrocksnadel, K., Wirleitner, B., Winkler, C., Fuchs, D., 2006. Monitoring tryptophan metabolism in chronic immune activation. Clinica Chimica Acta 364(1-2):82-90. Epub 2005 Sep 1.

Scopesi, F., Verkeste, C. M., Paola, D., Gazzolo, D., Pronzato, M. A., Bruschettini, P. L., Marinari, U. M., 1999. Dietary nucleotide supplementation raises erythrocyte 2, 3-diphosphoglycerate concentration in neonatal rats. Journal of Nutrition 129(3):662-665.

Shansis, F. M., Busnello, J. V., Quevedo, J., Forster, L., Young, S., Izquierdo, I., Kapczinski, F., 2000. Behavioural effects of acute tryptophan depletion in healthy male volunteers. Journal of Psychopharmacology 14(2):157-163.

Simat, T., van Wickern, B., Eulitz, K., Steinhart, E. H., 1996. Contaminants in biotechnologically manufactured L-tryptophan. Journal of Chromatography B: Biomedical Sciences and Applications 685(1):41-51.

Skehan, P., Storeng, R., Scudiero, D., 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. Journal of National Cancer Institute, 82(13):1107-1118.

Smith, Q.R., 2000. Transport of glutamate and other aminoacids at the blood-brain-barrier. Journal of Nutrition 130:1016S-1022.

Sobre seu estômago: gastrite e dispepsia funcional. Disponível em <http://www.gastronet.com.br/images/mucosagastr.jpg>. Acesso em: 10 dez. 2006.

Sorbye, H. & Svanes, K., 1994. The role of blood flow in gastric mucosal defense, damage and healing. Digestive Diseases 12(5):305-317.

Stein, C. J. & Colditz, G. A., 2004. Modifiable risk factors for cancer. British Journal of Cancer 90(2):299-303.

Stephen, A. I., Avenell, A., 2006. A systematic review of multivitamin and multimineral supplementation for infection. Journal of Human Nutrition & Dietetics 19(3):179-190.

Stokes W. S., 2000. Reducing Unrelieved Pain and distress in Laboratory Animals Using Humane Endpoints. ILAR Journal 41:59-61.

Stone, T. W., Mackay, G. M., Forrest, C. M., Clark, C. J., Darlington, L. G., 2003. Tryptophan metabolites and brain disorders. Clinical Chemistry and Laboratory Medicine 41(7):852-859.

Suzuki, S., Tourkina, E., Ludwicka, A., Hampton, M., Bolster, M., Maize, J., Silver, R., 1996. A contaminant of L-tryptophan enhances expression of dermal collagen in a murine model of eosinophilia myalgia syndrome. Proceedings of the Association of American Physicians 108(4):315-322.

Syrtsova, L. A., Nadtochenko, V. A., Denisov, N. N., Timofeeva, E. A., Shkondina, N.I., Gak, V. Y., 2000. Kinetics of elementary steps of electron transfer in nitrogenase in the presence of a photodonor Biochemistry (Mosc) 65(10):1145-1152.

Szathmary, E., 2003. Why are there four letters in the genetic alphabet? Nature Reviews Genetics 4(12):995-1001.

Takeuchi, K., Aihara, E., Hayashi, M., Sasaki, Y., 2005. Role of prostaglandin E receptor subtypes in gastroduodenal HCO_3-secretion. Medicinal Chemistry 1(4):395-403.

Tam, M., Gomez, S., Gonzalez-Gross, M., Marcos, A., 2003. Possible roles of magnesium on the immune system. European Journal of Clinical Nutrition 57(10):1193-1197.

Taniguchi, E., Kawaguchi, T., Shimada, M., Kuwahara, R., Nagao, Y., Otsuka, M., Iwasaki, S., Matsuda, T., Ibi, R., Shiraishi, S., Itou, M., Oriishi, T., Kumashiro, R., Tanaka, S., Saruwatari, Y., Sata, M., 2006. Branched-chain amino acid supplementation complements conventional treatment for spontaneous bacterial peritonitis. Digestive Diseases Sciences 51(6):1057-1060.

Tannenbaum, J., 1999. Ethics and pain research in animals. ILAR Journal 40:97-110.

Taylor, T., 1949. Final Report to the Secretary of the Army on the Nuremberg War Crimes Trials under Control Council Law N^{o}. 10. Washington, D.C.: Government Printing Office.

Toth L. A., 2000. Defining the moribund condition as an experimental endpoint for animal research. ILAR Journal 41:72-79.

Travis, L. B., 2006. The Epidemiology of Second Primary Cancers. Cancer Epidemiology Biomarkers & Prevention Oct 20; [Epub ahead of print].

Ullman-Culleré, M. H. & Foltz, C. J., 1998. Body Condition Scoring: A Rapid and Accurate Method for Assessing Health Status in Mice. Laboratory Animal Science 49(3):319-323.

UKCCCR (United Kingdom Coordinating Committee on Cancer Research). 1997. Guidelines for the Welfare of Animals in Experimental Neoplasia. London: UKCCCR.

van der Stelt, H. M., Broersen, L. M., Olivier, B., Westenberg, H. G., 2004. Effects of dietary tryptophan variations on extracellular serotonin in the dorsal hippocampus of rats. Psychopharmacology (Berl). 172(2):137-144.

Verrey, F., 2003. System L: heteromeric exchangers of large, neutral amino acids involved in directional transport. Pfluegers Archives European Journal of Physiology 445:529-533.

Villamor, E., Mbise, R., Spiegelman, D., Hertzmark, E., Fataki, M., Peterson, K. E., Ndossi, G., Fawzi, W. W., 2002. Vitamin A supplements ameliorate the adverse effect of HIV-1, malaria, and diarrheal infections on child growth. Pediatrics 109(1):E6.

Vincent, P. C. & Nicholls, A., 1967. Comparison of the Growth of the Ehrlich Ascites Tumor in Male and Female Mice. Cancer Research 27:1058-1065.

Wallace J., 2000. Humane endpoints and cancer research. ILAR Journal 41:87-93.

Wallace, J. L., 2006. Nitric oxide, aspirin-triggered lipoxins and NO-aspirin in gastric protection. Inflammation & allergy drug targets 5(2):133-137.

Wallace, J. L. & Tigley, A. W., 1995. Review article: new insights into prostaglandins and mucosal defense. Alimentary pharmacology & therapeutics 9(3):227-235.

Widner, B., Laich, A., Sperner-Unterweger, B., Ledochowski, M., Fuchs, D., 2002. Neopterin production, tryptophan degradation, and mental depression--what is the link? Brain, Behavior and Immunity 16(5):590-595.

World Health Organization, 1983. Safety Requirements for the First Use of New Drugs and Diagnostic Agents in Man. C.D. Dollery & Z. Bankowski, Eds. Geneva: WHO/FAO;1-61.

World Health Organization, 2002. The Scientific basis for diet, nutrition and prevention of type 2 diabetes. Geneva: WHO/FAO;1-53.

World Health Organization, 2002. Handbook for good clinical research practice (GCP): Guidance for implementation ISBN. Disponível em: <http://www.who.int/medicines/areas/quality_safety/safety_efficacy/gcp1.pdf>. Acesso em: 11 nov. 2006.

World Medical Organization, 1996. Declaration of Helsinki. British Medical Journal (7 December) 313(7070):1448-1449.

Wright, M. E., Mayne, S. T., Stolzenberg-Solomon, R. Z., Li, Z., Pietinen, P., Taylor, P. R., Virtamo, J., Albanes, D., 2004. Development of a comprehensive dietary antioxidant index and application to lung cancer risk in a cohort of male smokers. American Journal of Epidemiology 160(1):68-76.

Young, S. N. & Oravec, M., 1979. The effect of growth hormone on the metabolism of a tryptophan load in liver and brain of hypophysectomized rats. Canadian Journal of Biochemistry 57:517-522.

Yoshida, S., Kaibara, A., Ishibashi, N., Shirouzu, K., 2001. Glutamine supplementation in cancer patients. Nutrition 17(9):766-768.

APÊNDICES

I. Teste de Citotoxicidade em Células Tumorais e Normais – Detalhamento Metodológico

1. Cultivo: São utilizadas linhagens de células tumorais humanas cultivadas em frascos T25 (área para confluência celular de 25 cm^2) com 5mL de meio de cultivo RPMI 1640, suplementado com 5% de soro fetal bovino inativado (SFB), e células normais humanas cultivadas em frascos T75 (área para confluência celular de 75 cm^2) com 10 mL de meio de cultivo DMEM, suplementado com 10% de soro fetal bovino inativado (SFB). Todas as células são incubadas a 37ºC em atmosfera úmida com 5% CO_2. Quando a monocamada celular atinge cerca de 80% de confluência, as garrafas são repicadas em condições estéreis em fluxo laminar vertical classe BII;

2. Repique: o sobrenadante dos frascos com o tapete celular é aspirado sob vácuo com pipeta Pasteur estéril e adicionado 500 µL de tampão Hank´s a cada frasco, sendo vertido 10 vezes para lavagem do tapete celular. Essa solução é descartada e 500 µL de tripsina são adicionados a cada garrafa, de modo a ressuspender o tapete celular aderido a garrafa. A cada garrafa são adicionados 5 mL de meio RPMI com 5% SFB (ou 10 mL de DMEM com 10% de SFB, para as células normais) para que as células, fiquem em suspensão e alíquotas posam ser repassadas a 2 frascos de manutenção e outros frascos adicionais para a realização do teste de citotoxicidade;

3. Inoculação das células: As células previamente cultivadas em frascos T25 (ou T75) são utilizadas para o ensaio de citotoxicidade. Todo o procedimento de lavagem, desprendimento das células e ressuspensão das mesmas utilizados no repique celular (item 2) são repetidos novamente. As células, em suspensão, são contadas em câmara de Neubauer para a quantificação do número de células. Com a densidade encontrada é realizada uma regra de três com a densidade de inoculação previamente padronizada para a linhagem celular. Dessa forma, as proporções de célula e meio a serem utilizados e adicionados a placa são ajustadas na respectiva densidade de inoculação da linhagem celular. Da solução celular com densidade de inoculação ajustada, 100 µL/compartimento são inoculados em placas de 96 compartimentos e essas são incubadas por 24 horas a 37ºC em atmosfera de 5% de CO_2 e ambiente úmido.

4. Tratamento das células com as substâncias-teste: após 24 horas da inoculação das células, 100 μL/compartimento da amostra são adicionados em triplicata e em quatro concentrações diferentes. No momento da adição das drogas uma placa de controle celular, ou seja, as linhagens celulares sem tratamento (To), é fixada com ácido tricloroacético 50% para a determinação do conteúdo protéico. Inicialmente, para obtenção de soluções estoque, as amostras são diluídas em dimetilsulfóxido de sódio (DMSO) na concentração de 0,1g/mL. Para adição nas placas de 96 compartimentos, a solução estoque é diluída no mínimo 400 vezes em RPMI/ SFB/ Gentamicina. As concentrações das amostras aplicadas, portanto, são de 0,25; 2,5; 25 e 250 μg/ mL.

5. Após a adição dos tratamentos, as placas são incubadas por 48 horas para a reação entre célula e amostra.

6. Fixação das células: após as 48 horas, as células são fixadas com 50 μL de ácido tricloroacético (TCA) a 50% a 4ºC por 1 hora. Após esse período, as placas são submetidas a quatro lavagens consecutivas com água destilada para a remoção dos resíduos de TCA, meio, SFB e metabólitos secundários. Estas placas são mantidas à temperatura ambiente até a secagem completa.

7. Coloração com Sulforrodamina B: após secagem das placas, essas são coradas pela adição de 50 μL de sulforrodamina B (SRB) a 0,4% (peso/volume) dissolvido em ácido acético a 1% e incubadas a 4ºC por 30 minutos. Posteriormente, as placas são lavadas por 3 vezes consecutivas com uma solução de ácido acético 1%. O resíduo da solução de lavagem é removido e as placas são novamente secas à temperatura ambiente.

8. Solubilização da coloração: corante ligado às proteínas celulares é solubilizado em 150 μL de uma solução de Trizma Base na concentração de 10 μM, pH 10,5. A leitura espectrofotométrica é realizada em 540 nm em um leitor de microplacas (ELISA).

9. Análise dos resultados: A atividade antiproliferativa será analisada pelo cálculo das médias das absorbâncias descontadas de seus respectivos brancos e através da fórmula abaixo, será determinada a inibição de crescimento (IC) de cada amostra testada, sendo que T é a média da absorbância da célula tratada, C é o controle de célula, To é o controle das células no dia da adição das drogas.

Se T > C a droga estimulou o crescimento, não apresenta IC.

Se T >= To mas < C, a droga será citostática e a fórmula é 100 X [(T-To)/(C- To)].

Se T.< To a droga é citocida e a fórmula utilizada é 100 X [(T- To)/(To)]

10. O resultado obtido será subtraído de 100% obtendo-se então a porcentagem de inibição de crescimento. As amostras serão consideradas ativas quando apresentaram inibição de crescimento maior que 50% e ainda de forma dose dependente (Skehan et al., 1990).

II. Teste de Atividade Antitumoral *in vivo* em modelo ascítico de Ehrlich – Detalhamento Metodológico

Uma das atividades desenvolvidas no mestrado incluiu a implantação de um modelo de atividade antitumoral "in vivo" na Divisão de Farmacologia e Toxicologia (CPQBA/Unicamp). Para tanto, elegemos o Tumor Ascítico de Ehrlich por apresentar fácil reprodutibilidade e permitir a avaliação de atividade antitumoral de substâncias que possam ter apresentado atividade antiproliferativa no painel de cultura de células tumorais humanas.

II. a) Material e Métodos

- Animais: Camundongos (*Mus musculus*) *SwissWebster* machos adultos pesando entre 30-40g (8 a 12 semanas de idade) provenientes do Centro Multidisciplinar de Investigação Biológica (CEMIB) da UNICAMP e mantidos em gaiolas de policarbonato, sob condições controladas (ciclo claro/escuro 12/12; temperatura 20 ± 2ºC), com fornecimento de água e ração comercial pelitizada *ad libitum*. Os animais foram aclimatados às condições do laboratório pelo período mínimo de 7 (sete) dias, antes de serem submetidos aos procedimentos experimentais. Todos os ensaios foram conduzidos de acordo com as diretrizes éticas aceitas pelo Colégio Brasileiro de Experimentação Animal (COBEA), seguindo o Manual sobre Cuidado e Uso de

Animais de Laboratório, *National Research Council* (2003), que atende as recomendações nacionais e internacionais, bem como do *United Kingdom Coordinating Committee on Cancer Research (UKCCCR)* e normas éticas para experimentos com dor (OECD, 2000; Morton & Griffiths, 1985);

- Células: O tumor de Ehrlich é um modelo de neoplasia maligna transplantável, pobremente diferenciado que se originou como um adenocarcinoma espontâneo de camundongo (Pessina et al., 1980) que permite modelos sólidos e ascíticos. No presente estudo, o modelo ascítico de Ehrlich (EAC) foi mantido na cavidade abdominal de camundongos e semanalmente transplantados através de aspiração e inoculação intraperitoneal no animal receptor.

- Preparação da suspensão celular: após a eutanásia do animal doador, o líquido ascitico é gentilmente transferido para tubo de ensaio contendo o Ficoll-Paque® e centrifugado, seguindo-se as instruções do fabricante. Em seguida, será desprezado o sobrenadante com o auxílio de pipeta Pasteur, deixando a camada de células tumorais na interface. Deverá ser realizada nova centrifugação, desta vez após a adição de solução de Hank's a fim de retirar todo o resíduo de Ficoll-Paque®. A viabilidade celular após o procedimento de obtenção da suspensão celular e imediatamente anterior à inoculação será obtida por exclusão em azul tripan e contada em câmara de Neubauer. Em cada animal receptor, serão inoculadas 1×10^4 células de tumor ascítico de Ehrlich.

- Período experimental: Durante todo o período experimental, os animais serão diariamente submetidos à avaliação clínica individual e receberão uma pontuação correspondente à seu estado naquele determinado dia. Por motivos éticos, a eleição do estado moribundo como ponto final do experimento em lugar do óbito tem por objetivo minimizar o estresse e o desconforto, bem como evitar a perda do material biológico para análise (Toth, 2000; Tannenbaum, 1999). É importante salientar que, à medida que os grupos experimentais vão se aproximando dos valores máximos na tabela de pontuação (Avaliação Clínica Individual), a inspeção, antes diária, é realizada duas vezes ao dia, a fim de acessar a morbidade e optar pela necessidade da eutanásia (Stokes, 2000; Wallace, 2000; Ullman-Culleré & Foltz, 1998). A inoculação de 10^4 EAC células começa a induzir sintomas graves por volta do décimo primeiro dias pós-

inóculo. Estes sintomas se manifestam como acentuada distensão abdominal produzida pelo acúmulo de líquido ascítico, dispnéia, postura encurvada e pelagem eriçada e sem brilho chegando até um estado pré-comatoso (pontuação máxima no *body codition score*). Em nosso estudo, ao atingir esta pontuação, os animais deverão ser sacrificados por deslocamento cervical e necropsiados. Os valores referentes à avaliação clínica diária foram registrados em Fichas de Avaliação Clínica Individual (Figura 6).

- Tratamento: quatro dias após a inoculação das células, o desenvolvimento tumoral foi certificado pelo aumento significativo no ganho de peso dos animais e distensão abdominal. Os animais foram aleatoriamente distribuídos em grupos a fim de serem submetidos aos respectivos tratamentos, a saber: controle negativo (solução salina 0,09% estéril 10 mL/Kg; p.o., diariamente), controle positivo (Doxorrubicina 5 mg/kg; i.p., 4° e 11° dia pós-inóculo), Timina (400 mg/kg; p.o., diariamente), Triptofano (400 mg/kg; p.o., diariamente) e "TK3" (1000 mg/kg; p.o., diariamente).

II. b) Resultados

- A análise dos dados referentes à data de óbito/eutanásia dos animais dos diversos grupos revelou que, ao 16º dia pós-inóculo, a totalidade dos animais pertencentes ao grupo controle negativo (salina) foi a óbito. Em contrapartida, nesta mesma data, a maioria dos animais do grupo controle positivo (Doxorrubicina) apresentava excelente estado geral. Estes dados validam o ensaio experimental e revelam uma resposta positiva do tumor experimental frente ao tratamento proposto. Com relação às amostras testadas, nesta mesma data (16º dia pós-inóculo), metade dos animais do grupo tratado com o suplemento nutricional "TK3" foi a óbito. Nos grupos tratados com timina e triptofano (componentes da fórmula original do suplemento), o resultado foi semelhante revelando uma discreta melhora quando comparado ao grupo salina. Os dados referentes à taxa de sobrevida/n experimental dos animais encontram-se na tabela 4.

TABELA 4. Taxa de sobrevida / n experimental dos diferentes grupos tratados.

GRUPO	7 dias*	10 dias*	13 dias*	16 dias*	19 dias*	22 dias*	25 dias*	28 dias*	31 dias*
Salina	10 / 10	10 / 10	7 / 10	0 / 10	0 / 10	0 / 10	0 / 10	0 / 10	0 / 10
Dox	9 / 9	9 / 9	9 / 9	8 / 9	8 / 9	8 / 9	7 / 9	6 / 9	6 / 9
TK3	8 / 8	8 / 8	6 / 8	4 / 8	3 / 8	1 / 8	1 / 8	1 / 8	1 / 8
Timina	7 / 7	7 / 7	4 / 7	2 / 7	1 / 7	0 / 7	0 / 7	0 / 7	0 / 7
Triptofano	9 / 9	9 / 9	5 / 9	2 / 9	1 / 9	0 / 9	0 / 9	0 / 9	0 / 9

- As diferenças relativas ao n experimental de cada grupo devem-se à perda de animais durante os procedimentos de coleta de sangue anterior ao inóculo. Na tabela 5 encontram-se os dados referentes à avaliação clínica individual dos animais.

TABELA 5. Médias dos scores obtidos a partir da avaliação clínica dos animais dos diferentes grupos tratados.

GRUPO	7 dias*	10 dias*	13 dias*	16 dias*	19 dias*	22 dias*	25 dias*	28 dias*	31 dias*
Salina	2,1	9,0	14,0	-	-	-	-	-	-
Dox	0	1,6	4,2	5,2	6,1	6,7	7,6	8,2	8,7
TK3	0,3	5,4	11,8	12,6	8,0	8,0	8,0	8,0	8,0
Timina	1,2	6,8	13,0	14,0	15,0	-	-	-	-
Triptofano	0,9	8,5	14,4	15,0	15,0	-	-	-	-

- A análise do hemograma dos animais não revelou alterações importantes que pudessem ser relacionadas aos tratamentos, além da leucopenia (decréscimo do número de leucócitos circulantes) 24 horas após o tratamento com quimioterápico. Este fato está em concordância com os dados de literatura como efeito adverso da droga utilizada como controle positivo do experimento.

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Ficha de Avaliação Clínica Individual – Teste: _____

Identificação do animal	***score***	**___/__ M/T**	**___/__ M/T**	**__/__ M/T**	**__/__ M/T**	**___/__ M/T**	**__/__ M/T**
APARÊNCIA							
Normal	0						
Pêlos sem brilho, orelhas normocoradas	1						
Ligeira piloereção, pequenas áreas de alopécia, corrimento nasal e/ou ocular	2						
Piloereção, postura encurvada, orelhas pálidas, extensas áreas de alopécia	3						
SINAIS CLÍNICOS							
Temperatura, freq. cardíaca e respiratória normais	0						
Discretas alterações	1						
T ± 1°C, freq. cardíaca / respirat. ↑ ou ↓ 30%	2						
T ± 2°C, freq. cardíaca / respirat. ↑ ou ↓ 50%	3						
COMPORTAMENTO ESPONTÂNEO							
Normal	0						
Ligeiras alterações	1						
Menos móvel e alerta, isolado	2						
Vocalização, auto-mutilação, hiperatividade / imobilidade, letargia	3						
COMPORTAMENTO PROVOCADO							
Normal	0						
Ligeira depressão ou resposta exacerbada	1						
Moderadas alterações no comportamento esperado	2						
Reações violentas / muito fracas e precomatosas	3						
AJUSTE DE PONTUAÇÃO							
Se for registrado 3 mais de uma vez, marque um ponto adicional para cada 3	2-4						
TOTAL	0-16						

Critérios para pontuação (0-3):

Aparência (representa o estado geral do animal):
0 – normal, pelagem brilhante, olhos claros e brilhantes, mucosas normocoradas;
1 – leve alteração no aspecto geral, pêlos sem brilho, com resíduos de fezes e/ou urina, mucosas normocoradas;
2 – piloereção moderada, pêlos sem brilho, olhos semicerrados, orelhas pálidas, corrimento nasal e/ou ocular;
3 – piloereção acentuada, postura anormal, encurvada, olhos cerrados, orelhas pálidas, intenso corrimento nasal e/ou ocular, caquexia.

Sinais clínicos:
0 – temperatura corporal normal, freqüências cardíaca e respiratória normais;
1 – discretas alterações nos parâmetros fisiológicos (temperatura, freqüências cardíaca e respiratória);
2 – discreta hipo ou hipertermia (± 1°C), freqüências cardíaca e/ou respiratória ↑ ou ↓ 30%;
3 – acentuada hipo ou hipertermia (± 2°C), freqüências cardíaca e respiratória intensamente alteradas (aumento ou redução superior a 50%).

Comportamento espontâneo:
0 – comportamento normal, exploratório;
1 – discretas alterações de comportamento;
2 – comportamento anormal: menos ativo e alerta, isolado;
3 – vocalização, automutilação, hiperatividade / imobilidade, letargia.

Resposta a estímulos:
0 – normal;
1 – ligeira diminuição ou exacerbação dos reflexos frente a estímulos;
2 – apático, reage tardiamente ao toque, movimenta-se somente após estímulo ou de forma exagerada;
3 – reações violentas ou muito fracas e pré-comatosas (permanece imóvel mesmo quando colocado fora da gaiola).

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Antiulcerative properties of bovine α-lactalbumin

L.F.H. Mezzaroba, J.E. Carvalho, A.N. Ponezi, M.A. Antônio, K.M. Monteiro, A. Possenti, V.C. Sgarbieri*

State University of Campinas (Unicamp), Dept. Foods and Nutrition, Rua Monteiro Lobato, 80, CEP 13083-862, Campinas, SP, Brazil

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Abstract

The capacity of α-lactalbumin to protect the rat stomach mucosa against ulcerative lesions caused by indomethacin or absolute ethanol was investigated. An α-lactalbumin prepared in the laboratory by anion exchange and molecular exclusion chromatography (Lab.α-La) and a commercial α-lactalbumin (Comm.α-La) were compared with a saline control. Comm.α-La offered 44% protection versus Lab.α-La 12%, in the indomethacin model. For absolute ethanol, no statistical difference was found in the inhibition of the development of ulcerative lesions for the two α-lactalbumins, ranging from 32% to 50%. The content of prostaglandin E_2 (PGE_2) and mucus increased in the mucosa after α-La intubation, suggesting that the protein protects the mucosa via stimulation of PGE_2 and mucus production. Blocking free sulfhydryl groups by in vivo *N*-ethylmaleimide alkylation caused an increase in mucosal ulcerative lesions, suggesting the importance of sulfhydryl compounds in the gastric mucosa protection.

Keywords: α-Lactalbumin; Ulcerogenesis; Indomethacin; Ethanol

1. Introduction

α-Lactalbumin (α-La) has recently been associated with reduction of stress (Markus et al., 2000), immunomodulation (Montagne, Cuiliere, Mole, Bene, & Faure, 2000), antimicrobial activity (Pelligrini, Thomas, Bramaz, Hunziker, & Fellenberg, 1999; Pihlanto-Leppälä et al., 1999), bactericidal and apoptotic activity (Hakansson et al., 2000; Svensson, Hakansson, Mossberg, Linse, & Svanborg, 2000), and antiulcer activity (Matsumoto, Shimokawa, Yshida, Toida, & Hayasawa, 2001; Ushida, Shimokawa, Matsumoto, Toida, & Hayasawa, 2003). In addition to its well known activity as enzymatic cofactor in mammary gland synthesis of lactose (Walstra, Geurts, Noomen, Jellema, & van Boekel, 1999).

The gastric mucosa is among the most important tissues in the organism on account of its function, structure and pathological processes that can take place in it (Melo, Castro, Lanna, Guimarães, & Sobrinho, 1993). It is accepted that the peptic ulcerogenesis (gastric and duodenal) results from an imbalance between infectious agent like the bacteria *Helicobacter pylori* or aggressive chemical agents versus protective substances such as mucus, bicarbonate, prostaglandins (PGE_2 and PGI_2), sulfhydryl compounds such as proteins, glutathione and others, as well as the blood flux to the mucosa cells (Abdel-Salam, Czimmer, Debreceni, Szolesanyi, & Mózsik, 2001; Allen, Flemström, Garner, & Kivilaasko, 1993; Kontereck, 1993; Robert, 1979; Szabo, Nagy, & Pevebani, 1992). The mucus and bicarbonates are responsible for the formation of an immobile viscous layer that protects the mucosa from the action of hydrochloric acid (Allen et al., 1993). The bicarbonate ions bind to the mucus creating a pH gradient (pH 1–7) from the stomach interior to the mucosa surface. The regulation of acid secretion by the HCl secreting cells is very important in the control of peptic ulcers. The acid secretion control is normally done by using H_2 receptor antagonists, inhibitors of proton pump and/or neutralization of the secreted acid using antacid substances (Rang, Dale, & Ritter, 1997).

Sulfhydryl substances such as cysteine, glutathione, and others can protect the mucosa by acting as antioxidants, free-radical scavengers and as regulators of the integrity

*Corresponding author. Tel./fax: +55 19 32877400.
E-mail address: sgarb@fea.unicamp.br (V.C. Sgarbieri).

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and functioning of the cell membranes (Szabo et al., 1992). The glutathione in conjunction with some prostaglandins (PGE_2 and PGI_2) seem to be the main protecting substances against free radicals and other aggressive agents to the gastric mucosa. Depletion of these substances aggravates gastric erosion and ulcerative lesions caused by chemical agents and stresses. Mucosal protection may occur even at low concentrations of glutathione and prostaglandins, suggesting that other protective agents such as polyamines, growth factors, neurotransmitters and steroids, may be present (Szabo et al., 1992). When the content of prostaglandins is reduced by the treatment with non steroidal anti-inflammatory drugs or corticosteroids the mucosa cells become vulnerable to the effect of stomach acid, pepsin and a series of substances ingested with our daily diet (Robert, 1979; Vane, 1971).

PGE_2 has a number of pro-inflammatory effects including induction of fever and erythema, increased vascular permeability and vasodilation. Acting on T cells, it enhances their production of some interleukines (IL-4, IL-5, IL-10) but inhibits their production of IL-2 and interferon-γ. Acting on B cells, PGE_2 stimulates the production of immunoglobulins G1 and E. PGE_2 also stimulates macrophages and dentritic cells to induce IL-10 and inhibit the expression of IL-12, IL-12 receptor and IL-1β. The overall balance is an enhancement of T Helper 2 (Th2) immune responses and inhibition of Th1 responses, favoring activation of B cells and inhibition of macrophages (Calder, 1998; Harris, Padilla, Kourmas, Ray, & Phipps, 2002).

Two models of ulcerative lesions were employed in this investigation, the indomethacin and the absolute ethanol models. Indomethacin induces gastric mucosa lesions (Robert, 1979) mainly based on the inhibition of prostaglandin synthesis (Morimoto, Shomohara, Oshima, & Kakayuri, 1991; Vane, 1971) and by interference with gastric acid secretion, consequently decreasing the gastric mucosal cell protection. Cimetidine is a reversible competitive antagonist of the histamine action exerted on the H_2 receptors. Cimetidine inhibits gastric secretion promoted by histamine and strongly inhibits the secretion promoted by gastrin. The reduction of gastric secretions by cimetidine protects against gastric ulceration caused by stress, gastric acid production, or treatment with drugs that block the cyclooxygenase enzymes (Bernardi, 1999; Ito, Segami, Tsukahara, Kojima, & Suzuki, 1994).

Absolute ethanol acts by destroying mucosal cells by direct contact (Robert, 1979), independently of the gastric acidity. It destroys initially the stomach protecting layers and finally reaches the superficial mucosa causing cells necrosis and liberation of vasoactive mediators leading to vasoconstriction, edema and hemorrhage (Oates & Hakkinen, 1988).

The main objectives of the present research were to study the antiulcerative properties of α-La prepared in the authors laboratory and to compare it with a commercial preparation. Also, the possible mechanisms or metabolic pathways through which α-La would protect the stomach mucosa from ulcerative lesions caused by absolute ethanol or indomethacin, were investigated in a rat model.

2. Materials and methods

2.1. Preparation of α-La samples

Whey protein concentrate (WPC), 80% protein on dry basis, was produced in a pilot plant by microfiltration, ultrafiltration/diafiltration (Zinsly, Sgarbieri, Jacobucci, Pacheco, & Baldini, 2001) and used as starting material for the preparation of α-La and β-lactoglobulin (β-Lg). α-La and β-Lg were prepared by ion exchange chromatography (Q-Sepharose fast flow) in a Pharmacia Fast Flow Liquid Chromatography System (Pharmacia, São Paulo, Brazil), using a 50×100 mm column equilibrated with 0.02 M Tris–HCl, pH 7.0 (buffer A), and eluted with buffer A gradually substituted by buffer A containing 1 M NaCl (0–100% substitution). β-Lg was eluted in homogeneous state at 30% substitution with a recovery of 82%. The fraction with α-La was eluted at 10% substitution and contained various protein contaminants. Therefore, it was submitted to molecular exclusion chromatography (Pharmacia Sephacryl S-200) equilibrated in 0.02 M, Tris–HCl, pH 7.0. Total α-La recovery was only 32% and the methodology applied in the purification was basically the same as used by Gerbeding and Byers (1998) and as that of Yoshida (1990). After each chromatographic step the samples were dialysed and freeze-dried. The purified α-La contained 98% protein ($N \times 6.38$) on a dry basis. Homogeneity of prepared α-La, β-Lg and the commercial α-La (Comm.α-La; Davisco Foods International, Greenville, SC, USA) was investigated by SDS–PAGE electrophoresis (Laemmli, 1970) using a vertical Pharmacia apparatus (Pharmacia). β-Lg was essentially pure revealing only one protein band in the gel and both α-La (Lab.α-La and Comm.α-La) showed a main protein band (α-La) plus a much weaker band identified as β-Lg contaminant. Hydrolysate was prepared from Comm.α-La by using pancreatin (Sigma P1750, St. Louis, MO, USA) in a pH-stat (Methrom, Le Uleis, France), according to Schmidt and Poll (1991). Degree of hydrolysis (20%) was determined by the method of Adler-Nissen (1979). The α-La hydrolysate was fractionated by tangential membrane filtration (Prep/Scale^{TM}-TFF Cartridges1ft^2, molecular mass cut off 1 kDa; Millipore, São Paulo, Brazil). Permeate and retentate were frozen and freeze-dried and then stored at 4 °C prior to use.

2.2. Experimental ulcerogenesis

Two models of ulcerogenesis were applied to Wistar male rats, 250–300 g body weight, by using indomethacin or absolute ethanol. In the indomethacin model (Morimoto et al., 1991) ulcerative lesions were induced in the rat stomach mucosa by subcutaneous injection of 60 mg kg^{-1}

body weight (bw). Physiological saline (10 mL kg^{-1} bw) and cimetidine (100 mg kg^{-1} bw), dissolved in physiological saline were given by intragastric intubation, as negative and positive control, respectively (Robert, 1979). In the absolute ethanol model the rats received gastric intubations of 1 mL ethanol as ulcerogenic agent, 10 mL kg^{-1} bw saline as negative control, and carbenoxolone (200 mg kg^{-1} bw) in physiological saline as positive control. The testing samples (Lab.α-La, Comm.α-La, Comm.α-La hydrolysates) dissolved in saline were given by gastric intubation at 200 mg kg^{-1} bw. For the determination of the DE_{50}, the effective dose for 50% lowering of the ulcerative lesion index (ULI), increasing single doses of 100, 200, 350 and 500 mg kg^{-1} bw were used. The use of a negative and a positive control (an anticulcer drug) permitted a better evaluation of the range of percentual decrease of the ULI by the various samples tested. Cimetidine shows stronger inhibitory specificity against mucosa lesion development caused by indomethacin whereas carbenoxolone shows more protection against ethanol induced lesions. For most experiments five to six rats were used in each group treated. The general protocol followed in the various experiments is illustrated in Fig. 1. Tests using single dose rather than consecutive daily doses were performed by omitting step 2 of Fig. 1.

2.3. Ulcerative lesion analysis

The ULI was estimated by the summation of parameters (Gamberini, Skorupa, Souccar, & Lapa, 1991) as follows: loss of normal mucosa discoloration, 1 point; mucosa edema, 1 point; hemorrhage, 1 point; petechial points (until 9), 2 points; petechial points (>10), 3 points; ulcers (up to 1 mm), $n^* \times 2$ points; ulcers (>1 mm), $n^* \times 3$ points; perforated ulcers, $n^* \times 4$ points; (n^* = number of ulcers found). Percent decrease of the ULI was calculated by the expression: %decrease ULI = {(mean ULI negative control−mean ULI testing sample)/mean ULI negative control} × 100. The number of rats used in each test for the calculation of the ULI is specified in the results presented. For evaluating the participation of sulfhydryl compounds in the gastric mucosa protection, groups of 8 rats were submitted to fasting of 24 h

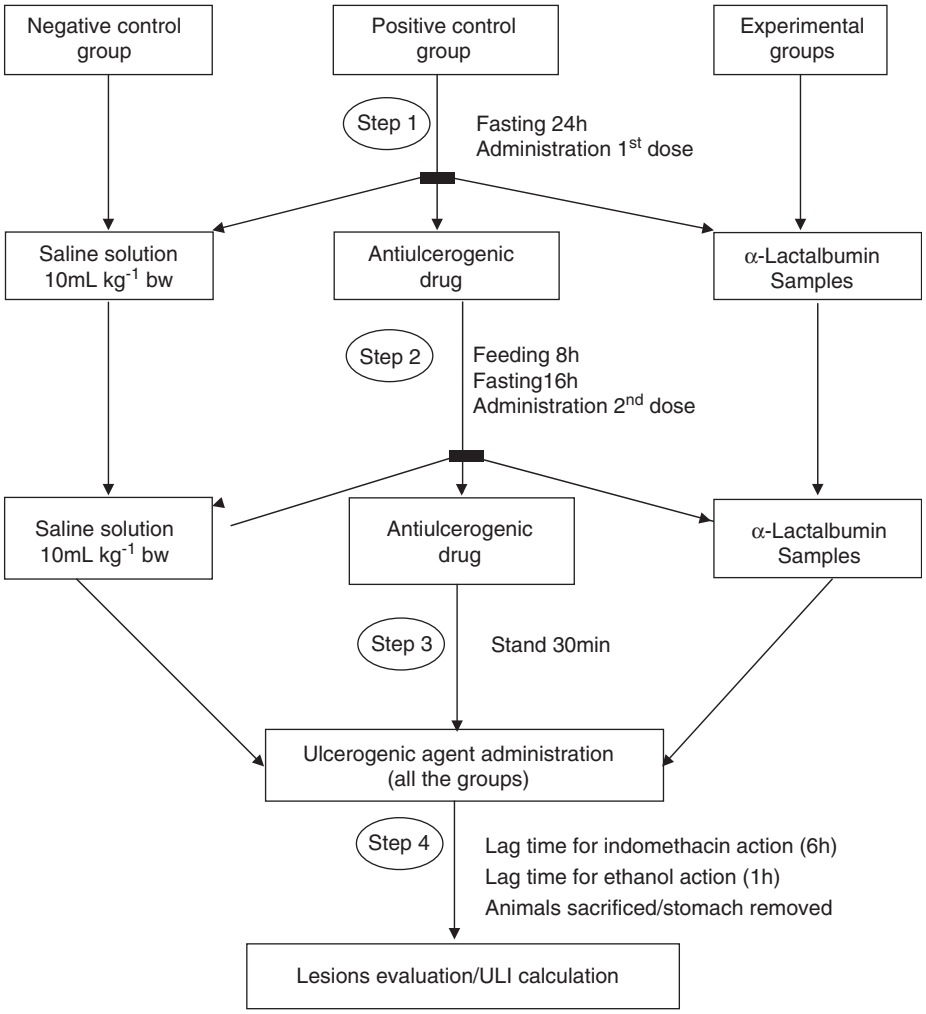

Fig. 1. General protocol for the indomethacin and absolute ethanol induction of gastric ulcer (after intubation of two consecutive daily doses of α-La samples or antiulcerogenic drugs) including the estimation of the ulcerative lesion index (ULI). Wistar male rats (250–300 g bw) were used, and dosages were in mg kg^{-1} body weight, except for the saline control, given in mL kg^{-1} bw.

(Fig. 1) with free access to water. This was a single dose experiment with two controls: one control group received only saline (10 mL kg^{-1} bw), the second control group and the experimental groups received a subcutaneous injection (10 mg kg^{-1} bw) of *N*-ethylmaleimide (NEM) 30 min prior to the dose administration (Fig. 1). The second control group was intubated with physiological saline (10 mL kg^{-1} bw) and the experimental groups were intubated with the testing samples (200 mg kg^{-1} bw) dissolved in saline. From this point on the protocol of Fig. 1 was followed to the end, omitting step 2.

2.4. PGE_2 determination in the gastric mucosa

The ethanol model was followed, according to Fig. 1. The number of rats used in each test is specified in Table 3. At the end of the experimental protocol (Fig. 1) the rats were sacrificed by cervical dislocation, the stomach mucosa removed and cut into small pieces for prostaglandin E_2 extraction with 6 mL ethanol (−20 °C) containing 100 μM indomethacin to block PGE_2 synthesis. The extract was vigorously agitated (vortex) for about 1 min and then centrifuged (500 × *g*). One milliliter of the supernatant was transferred to a polypropylene tube containing 10 μL glacial acetic acid. After a second centrifugation (500 × *g*), the supernatant was purified in a chromatographic minicolumn (C_{18} Amprep Amersham, Pharmacia). The minicolumn (1 mL vol) was washed with 2 mL 10% ethanol, following the addition of 0.8 mL of PGE_2 extract and subsequently the column was washed first with 1 mL distilled water and then with 1 mL hexane. The fraction eluted was dried under vacuum and dissolved in the appropriate buffer (Amersham Pharmacia Biotech, Kit RPM 222) for enzyme linked immunosorbent assay (Horton, Williams, Smith-Phillips, & Martin, 1998).

2.5. Determination of mucus linked to the gastric mucosa

One hour after administration of ethanol (Step 3, Fig. 1) the mucus glandular region of the stomach mucosa was removed and immersed into 10 mL 0.1% alcian blue solution for 2 h, then washed (2 ×) with 10 mL 0.25 M sucrose solution for 15 and 45 min. The dye complexed with the gastric mucus was extracted (2 h) with 10 mL 0.5 M $MgCl_2$. The extracted material was emulsified with 10 mL ethyl ether and then centrifuged (1300 × *g*, 15 min). The ether phase was discarded and the aqueous phase was read in a spectrophotometer at 598 nm (Corne, Morrisey, & Woods, 1974). Linked alcian blue was quantified in gram dye g^{-1} original tissue, based on a dye standard curve.

2.6. Statistic analysis

All experimental results were submitted to analysis of variance (ANOVA), and the differences among means were determined by the Duncan test ($p<0.05$). A logarithmic regression analysis was applied to evaluate the dose–response relationship.

3. Results and discussion

The effect of increasing doses of the Comm.α-La on the ULI, due to subcutaneous injection (60 mg kg^{-1} bw) of indomethacin is shown in Table 1. No statistical difference was detected in the ULI for the doses of 200, 350 and 500 mg kg^{-1} bw. Changes in ULI ranged from 48.3% to 51.5% and no dose–response correlation could be demonstrated, therefore, the effective dose (DE_{50}) for 50% decrease in the ULI could not be calculated for Comm.α-La in the indomethacin model.

In Fig. 2 the effectiveness of the Lab.α-La, Comm.α-La, and cimetidine on decreasing the ULI, caused in the rat

Table 1
Dose-effect of commercial α-lactalbumin (Comm.α-La) on indomethacin induced stomach ulcer in rat[a]

Intragastric intubation	Dose	ULI (mean ± sdm)	% Change of ULI
Saline (control)	10 mL	108.3 ± 21.4[a]	–
Comm.α-La	100 mg	110.0 ± 9.5[a]	0
Comm.α-La	200 mg	56.0 ± 12.3[b]	48.3
Comm.α-La	350 mg	52.7 ± 14.1[b]	51.3
Comm.α-La	500 mg	52.3 ± 16.3[b]	51.7

[a]ANOVA $F_{(4,18)} = 15.56$ $p<0.001$. Different superscript letters (column) indicate statistical differences ($p<0.05$), related to saline. Dosage was per kg body weight. Results obtained by administration of two consecutive daily doses; mean ± sdm = mean plus or minus standard deviation of mean.

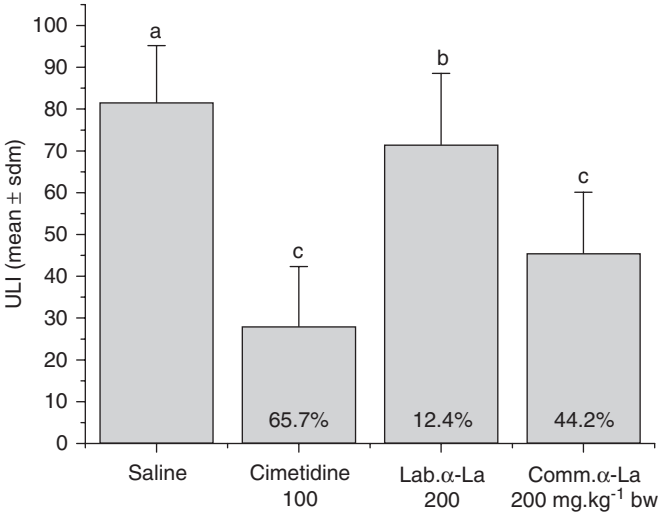

Fig. 2. Effectiveness of the laboratory produced α-lactalbumin (Lab.α-La), a commercial α-lactalbumin (Comm.α-La), saline (negative control) and cimetidine (antiulcerogenic drug) on decreasing of the ulcerative lesion index (ULI) produced by indomethacin (subcutaneous injection of 60 mg kg^{-1} bw). ANOVA $F_{(4,23)} = 8.80$ $p<0.001$. Different superscript letters indicate statistical differences ($p<0.05$) among results, referred to the saline control.

stomach by subcutaneous injection of indomethacin is compared with the saline control. Cimetidine lowered the ULI by 65.7%, as compared to the control. Comm.α-La lowered the ULI by 44.2% while Lab.α-La decreased it by only 12.4%. Therefore, Comm.α-La was 3.6 fold more effective than Lab.α-La. The hydrolysate of Lab.α-La did not show any protection against ulcerative lesions by indomethacin (data not shown).

The much lower inhibitory activity of the Lab.α-La for indomethacin lesion formation cannot be readily explained. It may be attributed to differences in the methodology of preparation of each of the two α-La products. Nevertheless, the two preparations were equally efficient against ethanol induced lesions (Fig. 4). A peculiar behavior of the Comm.α-La was a lack of dose-effect relationship (Table 1) in the indomethacin-induced ulcerative lesion.

Studies reported recently (Rosaneli, Bighetti, Antônio, Carvalho, & Sgarbieri, 2002, 2004) with a WPC produced in a pilot plant (Zinsly et al., 2001) and with a Comm.α-La (Matsumoto et al., 2001) demonstrated that whey proteins were capable of inhibiting the development of ulcerative lesions in the rat stomach mucosa by various ulcerogenic agents. Rosaneli et al. (2004) used the same WPC, which was used in the present work to isolate α-La, and reported a 50% decrease of the ULI when two consecutive daily doses of 1000 mg kg^{-1} bw were administered to the rat, in the indomethacin model. If one considers α-La as the only active compound in WPC against ulcerative lesions caused by indomethacin and an average concentration of 20% α-La in the WPC, then the 50% lowering in ULI by a 1000 mg dosage of WPC is perfectly coherent with the 12% decrease by 200 mg kg^{-1} bw of α-La used in the present study.

The effect of increasing doses of Comm.α-La on gastric ULI caused by ethanol, in the rat, is illustrated in Fig. 3. Contrary to the results obtained with indomethacin, the α-La inhibition of ulcerative lesion development by oral ingestion of absolute ethanol (1 mL per rat) showed a dose-response relationship given by the equation ($y = 31.198 \ln x - 112.14$, $r = 0.98$) used for the calculation of a DE_{50} of 181.3 mg α-La kg^{-1} bw.

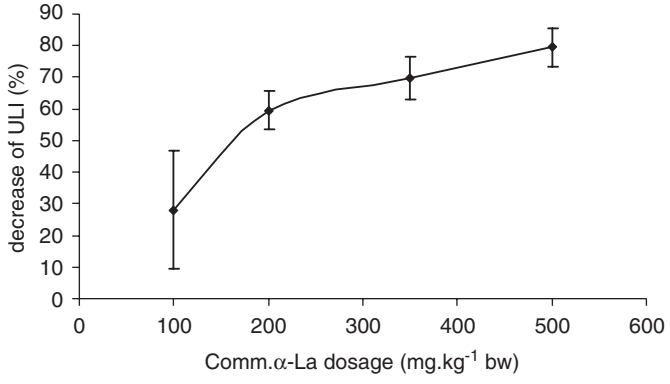

Fig. 3. Dose response curve for estimation of the effective dose for 50% decrease of ULI (DE_{50}) using commercial α-lactalbumin in the ethanol model. Regression equation ($y = 31.198 \ln x - 112.14$, $r = 0.98$, $DE_{50} = 181.3$ mg kg^{-1} bw.

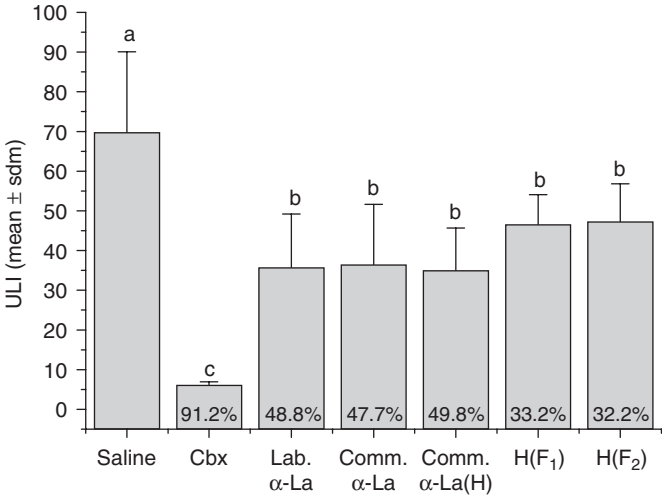

Fig. 4. Effect of two consecutive daily doses of saline (10 mL kg^{-1} bw), carbenoxolone (cbx), α-lactalbumin produced in the laboratory (Lab.α-La), a commercial α-lactalbumin (Comm.α-La), a hydrolysate (H) of Comm.α-La and two hydrolysate fractions, F_1 (<1 kDa) and F_2 (>1 kDa), all in dosage of 200 mg kg^{-1} bw, on decreasing ulcerative lesions index (ULI) caused by the oral administration of 1 mL absolute ethanol. ANOVA $F_{(6,36)} = 17.63$ $p < 0.001$. Different superscript letters indicate statistical differences ($p < 0.05$) among samples, referred to saline.

Fig. 4 presents the percent decrease of the ULI promoted by intragastric intubation of 1 mL absolute ethanol. Lab.α-La, Comm.α-La, a pancreatin hydrolysate (~20% DH) of Comm.α-La and two hydrolysate fractions, F_1 (<1 kDa) and F_2 (>1 kDa), decreased the ULI in the range of 32.2–49.8% as compared with the control, without statistical differences ($p > 0.05$) among them while the drug carbenoxolone lowered the ULI by 91%. It has been verified (Rosaneli et al., 2002) that the administration of two consecutive daily doses of 1000 mg kg^{-1} bw WPC reduced the ULI caused by 1 mL absolute ethanol by 73% while carbenoxolone (200 mg kg^{-1} bw) reduced it by 86.5%, based on physiological saline as the control.

In a previous investigation (Matsumoto et al., 2001) using the ethanol model and a dosage of 200 mg kg^{-1} bw of a Comm.α-La reported 82% reduction of the ULI, a value practically double of the one found in the present work, for both the Lab.α-La and Comm.α-La. Matsumoto also tested a commercial whey protein isolate (WPI) with 25% α-La and concluded that on the basis of percent reduction of the ULI and concentration of α-La in the WPI, α-La seemed to be the only protein responsible for the antiulcerative property of the WPI. Since in the present work both different starting material for α-La preparation and different methodology to calculate the ulcerative lesion index were used, it becomes difficult to explain the substantial difference in decrease of ULI caused by α-La reported in this paper as compared with one reported by Matsumoto.

Table 2 shows the data of bound mucus recovered from the rat stomach mucosa after two consecutive daily doses of the various compounds (Protocol Fig. 1). Carbenoxolone showed an increase in mucus production of 65% referred to saline (negative control). The most stimulating compound was Lab.α-La with an increase of 74%, compared with 53.8% for Comm.α-La, 34% for F_1 (<1 kDa) and only 9.8% for F_2 (>1 kDa) hydrolysate fractions. The latter increase differed statistically ($p<0.05$) from that observed for the other samples, which did not show mutual statistical differences. Mucus accumulation in the stomach mucosa is related to prostaglandin production. Increase in PGE_2 and consequent mucus production seems to be the main mechanism of gastric mucosa protection against the ethanol ulcerative lesions.

Table 3 presents data on inhibition or stimulation of PGE_2 production in the mucosa by the various compounds tested. Carbenoxolone showed the highest difference compared to saline, followed by the F_1 (<1 kDa) hydrolysate fraction. Lab.α-La, Comm.α-La, and the F_2 (>1 kDa) hydrolysate fraction showed the equal stimulation in PGE_2 production. Indomethacin, a specific inhibitor of cyclooxygenase, a key enzyme in the prostaglandin biosynthesis, caused a decrease of PGE_2 content in the mucosa, all the results referred to the saline control.

The pro-inflammatory property of PGE_2 may have been partly neutralized by the administration of the protective agents (α-La samples and carbenoxolone) prior to gastric intubation of ethanol (Fig. 1). On the other hand the vascular permeabilization and vasodilation property of PGE_2 should be beneficial for a faster elimination of ethanol from the mucosa. Previously reported work (Ushida et al., 2003) showed an increase of 158% PGE_2 in the gastric mucosa, 30 min after administration of an oral dose (200 mg kg^{-1} bw) of α-La.

The protection of sulfhydryl compounds against development of ulcerative lesion by ethanol is shown in Table 4. Blocking the sulfhydryl compounds by in vivo alkylation of SH groups with NEM caused an increase in the ULI ($p<0.05$) of the order of 30%, when saline was taken as reference. The protective effect of the α-La compounds in the absence of NEM in the single dose experiments was expressed as an ULI decrease of 12–20%. In the presence of NEM this protective effect was negligible or disappeared entirely. This is a strong indication of the protective function of sulfhydryl groups, which are inactivated after reaction with NEM. Gastric cytoprotection by sulfhydryl compounds has been reported previously (Szabo, Trier, & Frankel, 1981).

Our group (Rosaneli et al., 2002, 2004) showed the importance of sulfhydryl substances in the protection of

Table 2
Effect of α-lactalbumin produced in the laboratory (Lab.α-La), commercial α-lactalbumin (Comm.α-La) and two Comm.α-La hydrolysates (F_1 and F_2), and carbenoxolone on the gastric production of mucus[a]

Intragastric intubation	Doses	Number of animals	Quantity of mucus (μg Alcian Blue per g of tissue)	% Increase compared to control
Saline (control)	10 mL	4	73.4 ± 9.4^{b}	–
Carbenoxolone	200 mg	6	121.4 ± 11.3^{a}	65.3
Lab.α-La	200 mg	5	127.9 ± 14.3^{a}	74.1
Comm.α-La	200 mg	6	113.0 ± 6.5^{a}	53.8
F_1 (< 1 kDa)	200 mg	5	98.4 ± 6.3^{a}	34.0
F_2 (>1 kDa)	200 mg	6	80.6 ± 12.6^{b}	9.8

[a]ANOVA $F_{(5,26)} = 22.15$ $p<0.001$. Different superscript letters (column) indicate statistical differences ($p<0.05$), referred to saline. Dosage was per kg body weight. Results from two consecutive daily doses.

Table 3
Effect of α-lactalbumin produced in the laboratory (Lab.α-La), a commercial α-lactalbumin (Comm.α-La) and two Comm.α-La hydrolysates (F_1 and F_2), indomethacin, and carbenoxolone on the biosynthesis of prostaglandin (PGE_2) in the rat gastric mucosa

Intragastric intubation	Dose	Number of animals	PGE_2 in the mucosa (pg mg^{-1} of tissue)	% Difference referred to saline
Saline (control)	10 mL	5	25.8 ± 9.7^{d}	–
Indomethacin[b]	30 mg	5	7.8 ± 4.7^{d}	−69.6
Carbenoxolone	200 mg	3	298.6 ± 25.7^{a}	1057.1
Lab.α-La	200 mg	4	68.3 ± 14.2^{c}	164.9
Comm.α-La	200 mg	3	62.5 ± 21.0^{c}	142.3
F_1 (<1 kDa)	200 mg	4	164.1 ± 15.6^{b}	535.7
F_2 (>1 kDa)	200 mg	4	70.4 ± 15.2^{c}	172.9

[a]ANOVA $F_{(6,21)} = 15.79$ $p<0.001$. Different superscript letters (column) indicate statistical differences ($p<0.05$), referred to saline. Dosage was per kg body weight. Results of two consecutive daily doses.
[b]Indomethacin given by subcutaneous injection.

Table 4
Protection of gastric mucosa by sulfhydryl compounds from ethanol ulcerative lesion by intragastric intubation of a commercial α-lactalbumin (Comm.α-La) and two Comm.α-La hydrolysate fractions, F_1 and F_2[a]

Intragastric intubation	ULI (mean ± sdm)	% decrease of ULI	
		Saline (control)	Saline + NEM (control)
Saline	59.5 ± 15.4[b]	–	–
Saline + NEM[b]	79.3 ± 4.5[a]	−33.2	–
Comm.α-La	47.3 ± 16.5[b]	20.5	–
Comm.α-La + NEM	94.2 ± 21.92[a]	–	−18.8
F_1(MW < 1 kDa)	52.4 ± 14.9[b]	11.9	–
F_1 + NEM	77.5 ± 10.1[a]	–	2.2
F_2 (MW > 1 kDa)	47.2 ± 17.1[b]	20.6	–
F_2 + NEM	76.2 ± 20.3[a]	–	3.9

[a]ANOVA $F_{(7,33)} = 5.85$ $p < 0.002$. Different superscript letters indicate statistical differences between groups ($p < 0.05$). Calculation of % decrease of ULI based on saline or saline + NEM as control. Gastric intubations were single dosages of 200 mg kg^{-1} body weight. Results are average of 8 rats per group.

[b]NEM = N-ethylmaleimide, 10 mg kg^{-1}bw given by subcutaneous injection; mean ± sdm = mean plus or minus standard deviation of mean.

gastric mucosa against indomethacin and absolute ethanol. The combination of saline plus NEM permitted to estimate the protection offered to the gastric mucosa by naturally occurring sulfhydryl substances. Mucosa protection offered by the experimental samples can be attributed to SH-compounds present in the α-La plus the stimulation of glutathione synthesis which is known to occur upon administration of whey proteins to the rat (Sgarbieri, 1999).

4. Conclusions

From the results reported in the present work, supported by results from recent literature it can be concluded that whey protein and whey protein hydrolysates protect the rat stomach mucosa against ulcerative lesions caused by indomethacin or ethanol. Evidences have accumulated that the main, if not the only, whey protein that protects the mucosa against ulcerative lesions is the α-La. Investigations on the mode of action of whey protein in the gastric mucosa protection suggest that stimulus to prostaglandin synthesis and mucus production, and the presence of naturally occurring sulfhydryl substances, as well as sulfhydryl groups in the α-La, may all participate in the protection of the stomach mucosa from ulcerative lesions. The results reported in this paper are scientifically significant leading to possibilities of future product development in the areas of special foods and food supplements capable of helping people preventing or treating peptic ulcer.

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References

Abdel-Salam, D. M. E., Czimmer, J., Debreceni, A., Szolesanyi, J., & Mózsik, G. (2001). Gastric mucosal integrity: Gastric mucosal blood flow and microcirculation. An overview. *Journal of Physiology*, *95*, 105–127.

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzene sulphonic acid. *Journal of Agricultural and Food Chemistry*, *27*, 1256–1262.

Allen, A., Flemström, G., Garner, A., & Kivilaasko, E. (1993). Gastroduodenal mucosal protection. *Physiological Reviews*, *73*, 823–857.

Bernardi, R. R. (1999). Peptic ulcer disease. In J. T. Dipiro, R. L. Talbert, G. C. Yee, G. R. Matzke, B. G. Wells, & L.M. Pose (Eds.), *Pharmacotherapy: A pathophysiologic approach*. (Vol. 1, 4th ed., pp. 548–570). Stamford, CT, USA: Appleton & Lange.

Calder, P. C. (1998). Immunoregulatory and antinflammatory effects of n-3 polyunsaturated fatty acids. *Brazilian Journal of Medical and Biological Research*, *31*, 467–490.

Corne, S. J., Morrisey, S. M., & Woods, R. J. (1974). A method for the quantitative estimation of gastric barrier mucus. *Journal of Physiology*, *242*, 116–117.

Gamberini, M. T., Skorupa, L. A., Souccar, C., & Lapa, A. J. (1991). Inhibition of gastric secretion by a water extract from *Baccharis triptera* Mart. *Memórias do Instituto Oswaldo Cruz*, *86*(Suppl.), 137–139.

Gerbeding, S. J., & Byers, C. H. (1998). Preparative ion-exchange chromatography of proteins from dairy whey. *Journal of Chromatography*, *808*, 141–151.

Hakansson, A., Svensson, M., Mossberg, A. K., Sabharwal, H., Linse, S., Lazon, I., et al. (2000). A folding variant of α-lactalbumin with bactericidal activity on *Streptococcus pneumoniae*. *Molecular Microbiology*, *35*, 589–600.

Harris, S. G., Padilla, J., Kourmas, L., Ray, D., & Phipps, R. P. (2002). Prostaglandins as modulators of immunity. *Trends in Immunology*, *23*, 144–150.

Horton, J. K., Williams, A. S., Smith-Phillips, Z., & Martin, R. (1998). New direct EIA for quick and simple measurement of intracellular, secreted and total cellular PGE_2. *Life Science News I*, 1–2.

Ito, M., Segami, T., Tsukahara, T., Kojima, R., & Suzuki, Y. (1994). Effect of cimetidine and omeprazole on gastric ulcer healing of rats with limited food intake time. *European Journal of Pharmacology*, *263*, 245–251.

Kontereck, S. J. (1993). Mechanisms of gastroprotection. *Scandinavian Journal of Gastroenterology*, *174*(Suppl.), 15–28.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature*, *227*, 680–685.

Markus, C. R., Olivier, B., Panhuysen, G. E. M., Gugten, J., van der Alles, M. S., Truiten, A., et al. (2000). The bovine alpha-lactalbumin increases the plasma ratio of triptophan to the other large neutral amino acids, and in vulnerable subjects raises brain serotonin activity, reduces cortisol concentration, and improve mood under stress. *American Journal of Clinical Nutrition*, *71*, 1536–1544.

Matsumoto, H., Shimokawa, Y., Yshida, Y., Toida, T., & Hayasawa, H. (2001). New biological function of bovine alpha-lactalbumin: protective effect against ethanol- and stress-induced gastric mucosal injury in rats. *Bioscience Biotechnology and Biochemistry*, *65*, 1104–1111.

Melo, J. R. C., Castro, J. P., Lanna, J. C. B. D., Guimarães, J. T., & Sobrinho, J. M. D. L. (1993). Anatomia do estômago e anatomia do desenvolvimento. In R. Dani, & L. P. Castro (Eds.). *Gastroenterologia clinica*, (Vol. 1. 3rd ed., pp. 449-459), Rio de Janeiro Brazil: Guanabara Koogan, S.A.

Montagne, P. M., Cuiliere, M. L., Mole, C. M., Bene, M. C., & Faure, G. C. (2000). Dynamics of the main immunologically and nutritionally available proteins of human milk during lactation. *Journal of Food Composition and Analysis*, *13*, 127–137.

Morimoto, Y., Shomohara, K., Oshima, S., & Kakayuri, S. (1991). Effects of the new antiulcer agent KB-5492 on experimental gastric mucosal lesions and gastric mucosal defensive factors, as compared to those of teprenone and cimetidine. *Japanese Journal of Pharmacology*, *57*, 495–505.

Oates, P. J., & Hakkinen, J. P. (1988). Studies on the mechanism of ethanol-induced gastric damage in rats. *Gastroenterology*, *94*, 10–21.

Pelligrini, A., Thomas, U., Bramaz, N., Hunziker, P., & Fellenberg, R. (1999). Isolation and identification of three batericidal domains in the bovine alpha-lactalbumin molecule. *Biochemistry Biophysics Acta*, *1426*, 439–448.

Pihlanto-Leppälä, A., Marnila, P., Hubert, L., Rokka, T., Korhonen, H. J., & Karp, M. (1999). The effect of alpha-lactalbumin and beta-lactoglobulin hydrolysates on the metabolic activity of *Escherichia coli* JM 103. *Journal of Biological Chemistry*, *87*, 540–545.

Rang, H. P., Dale, M. M., & Ritter, J. M. (1997). *Farmacologia* (Vol. 1, 4th ed.). Rio de Janeiro, Brazil: Guanabara Koogan S.A.

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

Rosaneli, C. F., Bighetti, A. E., Antônio, M. A., Carvalho, J. E., & Sgarbieri, V. C. (2002). Efficacy of a whey protein concentrate on the inhibition of stomach ulcerative lesions caused by ethanol ingestion. *Journal of Medicinal Food*, *5*, 221–228.

Rosaneli, C. F., Bighetti, A. E., Antônio, M. A., Carvalho, J. E., & Sgarbieri, V. C. (2004). Protective effect of bovine whey protein concentrate on the ulcerative lesions caused by subcutaneous administration of indomethacin. *Journal of Medicinal Food*, *7*, 309–314.

Schmidt, D. G., & Poll, J. K. (1991). Enzymatic hydrolysis of whey proteins Hydrolysis of α-lactalbumin and β-latoglobulin in buffer solutions by proteolytic enzymes. *Netherlands Milk and Dairy Journal*, *45*, 225–240.

Sgarbieri, V. C. (1999). Food protein and peptides presenting specific protection to human health (a review). In J. R. Whitaker, N. F. Haard, C. F. Shoemaker, & R. P. Singh (Eds.), *Food for Health in the Pacific Rim* (Vol. 1, 1st ed. pp. 335–352). Thrumbull, CT, USA: Food and Nutrition Press Inc.

Svensson, M., Hakansson, A., Mossberg, A. K., Linse, S., & Svanborg, C. (2000). Conversion of α-lactalbumin to a protein inducing apoptosis. *Proceedings of the National Academy of Science USA*, *97*, 4221–4226.

Szabo, S., Nagy, L., & Pevebani, M. (1992). Glutathione, protein sulfhydryls and cysteine proteinases in gastric mucosal injury and protection. *Clinica Chimica Acta*, *206*, 95–105.

Szabo, S., Trier, J. S., & Frankel, P. W. (1981). Sulfhydryl compounds may mediate gastric cytoprotection. *Science*, *214*, 201–202.

Ushida, Y., Shimokawa, Y., Matsumoto, H., Toida, T., & Hayasawa, H. (2003). Effects of bovine alpha-lactalbumin on gastric mechanisms in naive rats. *Bioscience Biotechnology Biochemistry*, *67*, 577–583.

Vane, J. R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action of aspirin-like drugs. *Nature*, *231*, 323–325.

Walstra, P., Geurts, T. J., Noomen, A., Jellema, A., & van Boekel, M. A. J. S. (1999). *Dairy Technology Principles of milk properties and processes*. New York, USA: Marcel Dekker Inc.

Yoshida, S. (1990). Isolation of β-lactoglobulin and α-lactalbumin by gel filtration using Sephacryl S-200 and purification by diethylaminoethyl ion-exchange chromatography. *Journal Dairy Science*, *75*, 2292–2298.

Zinsly, P. F., Sgarbieri, V. C., Jacobucci, H. B., Pacheco, M. T. B., & Baldini, V. L. S. (2001). Produção piloto de proteínas de leite bovino: composição e valor nutritivo. *Brazilian Journal of Food Technology*, *4*, 1–8.