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CONTENTS

Introductory remarks
Gy. Mózsik, A. Pár, G. Csomós, M. Kitajima, M. Kondo, C.J. Pfeiffer, K.D. Rainsdorf, P. Sikirić, S. Szabo 3

Basic, central, peripheral and cellular mechanisms of gastrointestinal cytoprotection

Pathways, mediators and mechanisms of gastroduodenal mucosal injury
S. Szabo, L. Nagy 9
Mechanisms of NSAID-induced ulcerogenesis: structural properties of drugs, focus on the microvascular factors, and novel approaches for gastro-intestinal protection
K.D. Rainsford 23
ATP breakdown and resynthesis in the development of gastrointestinal mucosal damage and its prevention in animals and human (An overview of 25 years ulcer research studies)
Gy. Mózsik, Ágnes Király, G. Sütő, Á. Vincze 39
Gastric anti-ulcerogenic drug effect. A possible mechanism of its molecular basis
G.A. Bálint 81
The significance of the gastroprotective effect of body protection compound (BPC): modulation by differenct procedures
P. Sikirić, M. Petek, R. Ručman, S. Seiwerth, Z. Grabarević, I. Rotkvić, V. Jagić, B. Turković, B. Mildner, M. Duvnjak, A. Cviko, M. Kolega, M. Dodig, A. Sallmani, S. Djačić, D. Lucinger, S. Mikulandra, D. Erceg 89
Ethanol and the antioxidant defense in the gastrointestinal tract
C.H. Cho, C.J. Pfeiffer, H.P. Misra 99
Brain-gut relationships: gastric mucosal defense is also important
G.B. Glavin, A.M. Hall 107
Stress ulcer modulation by limbic system structures
P.G. Henke, A.Ray 117
Individual behavioral characteristics and extent of stress-induced gastric ulceration in rats
R. Murison, A. Skjerve 127

Esophageal protection

Esophageal mucosal protection – Why do we need a special approach?
L. Simon 135

Gastric protection

A review of spontaneous ulcer disease in domestic animals: chickens, cattle, horses, and swine
C. J. Pfeiffer 149
Subcellular localization of prostaglandin E_2 receptors in the gastric mucosa
M. Beinborn, Susanne Netz, U. Staar, K.Fr. Sewing 159
Adenosine: A novel ulcer modulator in stomachs
C.H. Cho 175
5-hydroxytryptamine$_3$-receptor blockade protects against gastric mucosal damage in rats
C.W. Ogle, S-C.G. Hui, B.S. Qiu, K.M. Li 181
Role of mucus in mucosal protection through ethanol and pepsin damage models
Judit Matúz 189
Is acute surgical vagotomy an aggressor to gastric mucosa in pylorus ligated rats with and without indomethacin treatment?
Á. Vincze, Ágnes Király, G. Sütő, Gy. Mózsik 195
Effect of acute surgical vagotomy on the mucosal content of 6-keto-$PGF_{1\ alpha}$, PGE_2 and glutathione after intragastric 96% ethanol treatment in rats
G. Sütő, Ágnes Király, Á. Vincze, Gy. Mózsik 205
Correlation between the cytoprotective effect of beta-carotene and its gastric mucosal level in indomethacin (IND) treated rats with or without acute surgical vagotomy
Ágnes Király, G. Sütő, Á. Vincze, Gy. Tóth, Z. Matus, Gy. Mózsik 213
Acute and chronic surgical vagotomy (SV) and gastric mucosal vascular permeability in ethanol treated rats
Ágnes Király, G. Sütő, Á. Vincze, Gy. Mózsik 219
Synthesis and gastroprotective activity of 4*H*-pyrido[1,2-*a*]pyrimidin-4-ones
I. Hermecz, Judit Sipos, Lelle Vasvári-Debreczy, Klára Gyires, Z. Kapui 225
The mechanism of cytoprotective effect of CHINOIN-127
Z. Kapui, K. Boér, I. Hermecz, Klára Gyires 237
Are all "cytoprotective" drugs gastroprotective?
Klára Gyires 247
The antineoplastic drugs dose dependently inhibit the healing of subacute gastric ulcer produced by punching in rats
E. Ezer 257
Resistance to medical ulcer therapy – possible reasons and management
M. Jablonská 265
Selenium in the blood of patients with colorectal cancer and neoplastic polyp
Gy. Rumi, I. Imre, Cs. Sülle, I. Sarudi, J. Kelemen, Zs. Lassú 275
Measurement of non-steroid antiinflammatory drugs induced gastric microbleeding
T. Beró, L. Nagy, T. Jávor, Á. Vincze 281
Influence of sex hormones on ethanol-induced gastric haemorrhagic erosions in rats
F. László, E. Amani, Cs. Varga, F.A. László 289

The effects of local hyperthermia on the morphology of the small bowel
T. Tóth, A. Fenyvesi, I. Klemm 293
Histamine release and SOD, allopurinol and ranitidine pretreatment in haemorrhagic shock in the rat
I. Zöllei, H. Asakawa, S. Karácsonyi 303
Cholinergic neurons are involved in the effect of substance P on the circular muscle of the guinea pig small intestine
L. Barthó, G. Pethő 311

Large bowel injury and protection

The oxygen-centered radicals scavenging activity of sulfasalazine and its metabolites. A direct protection of the bowel
L. Prónai, Ichikawa Yukinobu, I. Láng, J. Fehér 317

Liver injury and protection

Pathogenesis and management of alcoholic liver injury
A. Pár 325
Role of free-radical fractions in liver diseases
J. Fehér, A. Vereckei, G. Lengyel 351
Cytoprotection in the nineties: experience with ursodeoxycholic acid and silymarin in chronic liver disease
F. Lirussi, L. Okolicsanyi 363
Clinical evidence of hepatoprotection induced by ursodeoxycholic acid
Á. Vincze, I. Patty, T. Jávor, A. Pár 369
The effect of silibinin (Legalon^{R}) on the free radical scavenger mechanisms of human erythrocytes *in vitro*
I. Altorjay, L. Dalmi, S. Sári, S. Imre, Gy. Balla 375
Endogenous scavanger levels (vitamin E and A) of patients with chronic alcoholic liver disease under different environmental conditions
Tünde Horváth, L. Szabó, Z. Nagy, Anna Blázovics, Klára Gergely, T. Schwarcz 381
Post-cholecystectomy syndrome and magnesium deficit
J. Szántay, Doina Varga, P.J. Porr 391

Pancreatic injury and protection

Pancreatic cytoprotection: new approaches
M. Papp 399
The effect of somatostatin, gabexate mesilate and dextran 40 on the microcirculation in sodium taurocholate-induced pancreatitis
K. Kusterer, M. Enghofer, T. Poschmann, K.H. Usadel 407
Adequate enzymatic substitution in treating exocrine pancreatic insufficiency
I. Šimek, K. Kubias, M. Duda 417
Antiinflammatory effect of a protein kinase C inhibitor (K-252a) on the development of the dextran-induced paw edema in the rat (Preliminary results)
M. Papp, H. Kase, G. Gigler 423

The Take Home Message
Gy. Mózsik, A. Pár 427

CELL INJURY AND PROTECTION IN THE GASTROINTESTINAL TRACT: FROM BASIC SCIENCES TO CLINICAL PERSPECTIVES

Proceedings of the Third International Symposium on Gastrointestinal Cytoprotection held at Pécs, Hungary, October 7–8, 1991

Edited by

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This International Symposium was dedicated
to the memory of professor

ANDRE ROBERT (1926—1991)

who discovered the existence of *gastric cytoprotection*
phenomenon and extremely stimulated the basic
and clinical research in this field.
The results of his research work entered
into the experimental and clinical medicine

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INTRODUCTORY REMARKS

The phenomenon of gastric cytoprotection was firstly observed by Chaudhury and Jacobson [1] and its existence was widely and internationally accepted by the works of Robert and co-workers [10]. The main point of "gastric cytoprotection" was that small doses of prostaglandins (PGs) prevented the development of gastric mucosal damage produced by different necrotizing agents, such as 0.2 M NaOH, 0.6 M HCl, 25% NaCl, 96% ethanol or thermal injury. Later the existence of "gastric cytoprotection" was proved in case of some other chemicals, such as atropine [5], cimetidine [9], pirenzepine [5], SH-containing compounds [2] and other scavengers [4, 6, 8, 11]. The conclusion of these observations was that the gastric cytoprotection is an unspecific phenomenon to PGs.

A series of International Symposia dealing with the problems of Gastrointestinal Cytoprotection was established by the Hungarian Academy of Sciences in collaboration with the Hungarian Society of Gastroenterology (Research Forum, Section of Nutrition and Metabolism). The First International Symposium on Gastrointestinal Cytoprotection was held at Pécs, Hungary, September 30-October 1, 1983. The organization of this symposium had two aims:

a. to give an overview on the research of common defense mechanisms in the gastrointestinal tract (GI);

b. to realize an international forum for the basic and clinical researchers to discuss the mutually interesting questions in this field.

The success of the First International Symposium encouraged us to organize similar type meetings in every four-year period.

The Second International Symposium on Gastrointestinal Cytoprotection was organized in October 15–16, 1987, Pécs, Hungary.

The Third Symposium was held in October 7–8, 1991, Pécs, Hungary, under the auspices of

Alimentaria Ltd., Co., Pécs,
Hungarian Academy of Sciences
Local Committee of Pécs,
Hungarian Society of Gastroenterology
Research Forum
Section of Nutrition and Metabolism,

Akadémiai Kiadó, Budapest

Liver Foundation,
Standing Committee of International Conferences
for Experimental Ulcer,
University Medical School of Pécs
First Department of Medicine
Secondary High School of Human Nutrition and Dietetics.

The scientific topics changed from time to time: the First Symposium dealt with the general problems of gastrointestinal protection [5], while the Second one covered the different details of gastric cytoprotection (new mechanisms, pharmacology of mucosal injury and protection, NOSAC-induced gastric mucosal damage, and as well as acute, subacute and chronic experimental models), main problems of liver, pancreas protection, oxygen free radicals and scavengers, and computer approach to cytoprotective agents [7].

The total series of organoprotection (esophageal, gastric, small intestinal, large bowel, liver and pancreatic) of the gastrointestinal tract was overviewed during the Third International Symposium on Gastrointestinal Cytoprotection, including the general aspects of cell protection, such as physico-chemical properties, peripherally and centrally involved mechanisms. The basic and clinical aspects of these problems were covered by this Symposium (Tables I and II).

Recently different arguments were obtained against the further use of the term "cytoprotection":

1. in case of the stomach, different groups of gastric cytoprotective agents produce mucosal damage in other models, or they have no protective effects in the general meaning of mucosal prevention (see Gyires [3]). So regarding the stomach, we have no possibility to speak about the general cytoprotective effects of different compounds;

Table I

Main topics of the Third International Symposium on Gastrointestinal Cytoprotection (held in October 7–8, 1991, Pécs, Hungary)

General aspects of gastrointestinal protection: central vs. peripheral mechanisms of cell injury and protection in the gastrointestinal (GI) tract:

Central, peripheral and cellular mechanisms of gastrointestinal injury and protection,
Esophageal injury and protection,
Gastric injury and protection,
Small intestinal injury and protection,
Large bowel injury and protection,
Liver injury and protection,
Pancreatic injury and protection.

Table II

Main etiological approaches to development of cell injury and protection in the cells of gastrointestinal tract by the Third International Symposium on Gastrointestinal Cytoprotection (held in October 7–8, 1991, Pécs, Hungary)

Central mechanisms:
Brain-stomach axis vs. brain-gut axis vs. brain-pancreas axis,
Stress vs. limbic systems,
Receptors in the central nervous systems,
Possible role of vagal nerve between the central vs. peripheral mechanisms.
Peripheral mechanisms:
Physico-chemical properties of cell injury and protective compounds,
Mediators, pathways and biphasic effects of different compounds,
Intracellular signal molecules,
Cellular energy systems,
Growth factors,
Isolation of prostaglandin receptors,
Blood flow vs. tissue hypoxia.
Special mechanisms:
General nutrition (glucose),
Micronutrients,
Oxygen free radicals,
Natural and other antioxidant compounds,
Hydro- and lipophil behaviour of compounds.

2. the compounds having cytoprotective effects *in vivo* systems are without practically significant preventing effect on isolated cell systems;

3. the drugs have protecting influence on the small and large bowel, liver and pancreas, they probably have no detectable impact of the exocrine (liver, pancreas) or secretory (small intestine, large bowel) functions of the target organs. If we have no data in the unchanged secretory or excretory functions of different organs, we cannot speak about their "true cytoprotective" effects exerted on different organs of the GI tract;

4. the dosage of gastric cytoprotective drugs is ineffective at the same dose level intestine or stomach in case of scavengers, etc.;

5. although some common mechanisms can be found in the development of cell injury in GI tract, the details differ significantly.

These main points led us to change the term "cytoprotection" for "cell injury and protection". We believe that this new terminology can cover the main etiological and therapeutical aspects of GI tract at the level of basic sciences as well as clinical perspectives.

International experts gathered in the Scientific Committee were as follows:

A. Bertelli (Milan, Italy), M. Beinborn (Hanover, F.R.G.), T. Beró (Pécs, Hungary), G. Bommelaer (Clermount-Ferrand, France), Gy. Buzás (Budapest, Hungary), M. Diaz-Rubio (Madrid, Spain), M. Dell Tacca (Pisa, Italy), E. Ezer (Budapest, Hungary), J. Fehér (Budapest, Hungary), M. Garamszegi (Pécs, Hungary), F. Görgényi (Budapest, Hungary), K. Gyires (Budapest, Hungary), I. Hermecz (Budapest, Hungary), M. Impicciatore (Parma, Italy), M. Jablonská (Praha, Czecho-slovakia), T. Jávor (Pécs, Hungary), J. Lonovics (Szeged, Hungary), F. Morón (Habana, Cuba), L. Nagy (Boston, U.S.A.), A. Németh (Pécs, Hungary), S. Okabe (Kyoto, Japan), M. Papp (Budapest, Hungary), S. Popescu (Cluj-Napoca, Romania), I. Rotkvic (Zagreb, Croatia), I. Šimek (Olomouc, Czecho-slovakia) L. Simon (Szekszárd, Hungary), J. Stachura (Krakow, Poland), L. Szporny (Budapest, Hungary), J. Szántay (Cluj-Napoca, Romania).

Drs. Ágnes Király, Gábor Sütő and Áron Vincze helped us in the work of Symposium Secretarial. Thanks are due to Mrs. Margaret Jermás and Susanne Kadinger for their skillful technical assistance.

The Symposium was sponsored by MADAUS AG, Cologne, F.R.G. and BIOGAL Pharmaceuticals, Debrecen, Hungary.

We are very grateful to the journal *Acta Physiologica Hungarica* for publishing the proceedings materials as regular papers. A special thank is due to Jenő BARTHA (Managing Editor) for his valuable help in the publication.

We are looking forward to meeting you in 1995 at Pécs, Hungary.

Pécs, March 18, 1992

GY. MÓZSIK	*(Pécs, Hungary)*
A. PÁR	*(Pécs, Hungary)*
G. CSOMÓS	*(Hamburg, F.R.G.)*
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PATHWAYS, MEDIATORS AND MECHANISMS OF GASTRODUODENAL MUCOSAL INJURY*

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This review provides evidence that gastroduodenal mucosal injury is a complex process because of the heterogenous structure and multiple functions of the gut. The action of exogenous etiologic agents is usually mediated in part, or amplified by endogenous mediators which very often exert biphasic, i.e., damaging and protective effects. The pathogenetic pathways involve direct/indirect chemical injury, vascular damage and its consequences, and acute or chronic inflammatory processes following infectious, chemical or ischemic injury. The role of oxygen, free radicals, calcium and proteases as well as the components and forms of gastroduodenal injury, e.g., reversible and irreversible cell injury, tissue necrosis, acute and chronic inflammation are also briefly discussed. Only a slight or moderate direct cytoprotection was demonstrated *in vitro* using isolated, mixed rat gastric mucosal cells by the known gastroprotective drugs including PG and SH compounds. Thus, the terms of organo or gastroprotection are more descriptive then the misleading "gastric cytoprotection".

Keywords: gastroduodenal mucosal injury, gut, endogenous mediators, protective, free radicals, calcium, proteases, prostaglandius, sulfhydryls

Before reviewing the role of etiologic agents, mediators and pathways of gastroduodenal mucosal injury, the distinction between cell and tissue injury [8] should be briefly discussed. Namely, cell injury and protection can be investigated both *in vivo* and *in vitro*, but the results of cell protection *in vitro* do not always translate into organ injury and organ protection. As results on gastroprotection revealed, the gastric surface epithelial cells may be killed in large numbers. Nevertheless the integrity and the function of the gastric mucosa is still preserved. Furthermore, critical injury to certain cells is more important than to others, i.e., the early microvascular endothelial injury in the gastric and duodenal mucosa sensitizes the mucosa to low concentrations of damaging agents such as 25–50% ethanol or

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*This is updated and shortened version of a review initially published in J. Clin. Gastroenterol., 1991.

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0.2–0.3 N HCl which alone would not create massive damage [9, 10]. Studies with leukotrienes and endothelins recently revealed that intra-arterial infusion of these vasoactive compounds sensitized the gastric mucosa to hemorrhagic erosions [9, 11–14]. Furthermore, the rapidly developing ethanol-induced endothelial damage as revealed by the vascular tracer monastral blue, precedes the extensive tissue destruction (Fig. 1), i.e. hemorrhagic erosions [15, 16]. Prevention or reduction by PG or SH derivatives of microvascular injury results in protection for most of the mucosa [15–19]. Because of this cascade of events (Fig. 1) and interaction between various cell types, it is thus appropriate to speak about cell and tissue injury, both from the pathogenetic [8, 15, 16] and from the protective [15, 17–20] points of view.

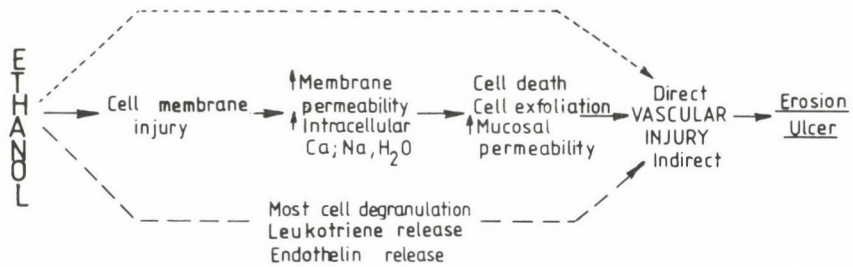

Fig. 1. Pathogenesis of ethanol-induced gastric mucosal injury. (Modified with permission from Szabo and Goldberg, Scand. J. Gastroenterol., 1990)

Etiology

Most of the cell and tissue injury in the gastroduodenal mucosa is produced by hypoxia, especially ischemia when hypoxia is due to inadequate blood flow. Chemicals such as exogenous drugs and endogenous mediators, and biologic agents including bacteria, viruses, inflammatory and immune reactions are also etiologic factors. In other tissues the causes of cell and tissue injury also include physical agents and genetic factors [21] which are of minor importance in the etiology of gastroduodenal mucosal injury (Table I).

The first etiologic agent in the stomach includes hypoxia which in most cases is due to a low blood flow or ischemia. Hypoxia might cause reversible or irreversible cell injury (see below under "Pathogenesis"). In addition to ischemia, the re-established blood flow very often aggravates the damage, probably because of the enhanced generation of free radicals.

Among the chemicals which cause cell and tissue injury in the stomach and duodenum, one has to distinguish between the exogenous chemicals *and* endogenous mediators, lipid-soluble *and* water-soluble compounds, enzymes *and* non-enzymatic derivatives, short (e.g., free radicals) *or* long (e.g., HCl, ammonia) half lives, directly

or indirectly acting chemicals (Table I). Studies with aspirin revealed that it is mostly in the non-ionized lipid-soluble damaging form, while the water-soluble components of aspirin in the gastric lumen are less damaging [2, 20].

Table I

Etiologic agents and mediators of gastroduodenal mucosal injury

Etiologic agents	Endogenous mediators
1. Hypoxia and ischemia	1. Endothelins
2. Chemicals, e.g., exogenous drugs and endogenous mediators	2. Eicosanoids, e.g., LT, TX, PG
3. Biologic agents, e.g., viruses, bacteria, inflammatory and	3. Platelet-activating factor
4. Immune reactions	4. Activated oxygen species, e.g. free radicals
5. Genetic factors, e.g., pernicious anemia	5. Monoamines
	6. Proteases
	7. Ammonia
	8. HCl and bile acids

The biologic agents as etiologic factors include bacteria among which the recently discovered Helicobacter pylori should be specifically mentioned because of its wide implication as an etiologic agent of gastritis. The bacterium may still exert its effect via the release of mediators such as ammonia and enzymes (e.g., proteases, lipases), but products of inflammation and immune reactions have been identified also as contributory etiologic factors.

Mediators

The mediators of gastric mucosal injury may act alone or with any of the primary etiologic agents, and aggravate or decrease the action of exogenous damaging compounds (Table I). The most investigated mediators are listed in Table I in order of decreasing potency. In this list, endothelins, because of their vasoactive potency are probably the most damaging agent to the gastric mucosa, e.g., ET-1 and ET-3 are 10-100 times more potent than leukotrienes C4 or D4 in causing microvascular injury in the stomach and enhancing the damaging action of exogenous

low concentrations of ethanol, HCl or aspirin [9, 11 – 14]. Other eicosanoid derivates are also potent mediators of injury such as thromboxanes (TX) and PG as mediators of inflammation. The unexpected and unusual cytoprotection or gastroprotection exerted by very low doses of PG in the stomach should not confound the well established principle that PGs are mediators of inflammation in all the tissues of the body. PGs are probably the best example of endogenous and exogenous chemicals which exert biphasic (i.e., damaging and protective) effects on the gastroduodenal mucosa (Table II).

Table II

Endogenous and exogenous chemicals which exert biphasic (protective and damaging) effect on the gastroduodenal mucosa

Endogenous	Exogenous
Prostaglandins	Ethanol*
Sulfhydryls (SH)	NaOH*
Histamine	Capsaicin
Epinephrine	SH alkylators
HCl* and bile acids*	
Glucocorticoids	
TRH	
CRF	
CGRP, Interleukin-1	
pH and O_2	

*refers to "adaptive cytoprotection"

The platelet-activating factor (PAF), like other potent endogenous mediators of mucosal injury, is also a potent vascular agent in causing congestion and sensitization to HCl-induced damage [12].

The activated oxygen species, e.g., free radicals, probably play a less important role than it was believed 5 – 10 years ago. Furthermore, although they are very potent toxic species, their very short half life seems to limit their overall contribution only to certain forms of mucosal injury (e.g., caused by ischemia and reflow) [22]. Namely, four biochemical methods to detect products of lipid peroxidation were virtually negative in the rat gastric mucosa after intragastric administration of ethanol, HCl, NaOH or aspirin [23, 24]. Nevertheless, catalase and superoxide dismutase (SOD) injected intravenously dose-dependently decreased the ethanol-induced gastric mucosal lesions implicating that free radicals, if they are involved in gastric mucosal damage, they do not act through enhanced lipid peroxidation, but probably affect soluble or membrane, structural or enzymic proteins.

Monoamines, especially histamine [25], serotonin [26] and epinephrine (Mózsik, personal communication) are also mediators of gastric mucosal damage but also offer gastroprotection (Table II). Among the protease mediators, the most studied is the aspartic protease pepsin. Because of its optimal pH, the action of pepsin is limited to the gastric lumen where the normal pH is 1–2. On the other hand, cysteine proteases such as cathepsin B have an optimal pH 5.6 and they degrade basement membrane, proteoglycans and activate procollagenase. By this virtue proteases markedly contribute to the damaging action in the gastric mucosa where they might propagate early direct chemical damage. Our recent studies show that ethanol-induced release of the cysteine protease cathepsin B might contribute to the development of hemorrhagic erosions, and inhibition of these enzymes by intragastric administration of SH alkylating agents such as N-ethylmaleimide or iodoacetate offered dose-dependent gastroprotection of long duration [27, 28]. Cysteine proteases are hence very powerful damaging agents, because in addition to wide substrate specificity in proteolytic activity and on optimal pH of 4.0–5.6, they are active in gastric tissue and lumen. Furthermore, they initiate a biochemical cascade of damaging action such as the activation of procollagenase.

Ammonia is placed before HCl in the list of etiologic agents (Table II) because on the molar basis, as our recent studies indicate, ammonia is more damaging than ethanol or acid [29]. Namely, *100%* ethanol, *0.6 N* HCl, *0.2 N* NaOH and *1%* ammonia, which is about *0.2 N* by molar concentration, produced hemorrhagic mucosal lesions of equal size in the stomach of fasted rats. Thus, on the molar basis, it appears that the stomach possesses much less protection against damage induced by bases such as NaOH and ammonia than against acid.

Hydrochloric acid and bile acids are listed mainly on historical basis, but as it turns out, the gastroduodenal mucosa has an efficient natural defense such as bicarbonate and mucus secretion, which normally maintains resistance against these potentially dangerous endogenous chemicals.

The additive or potentiating interaction among many of these etiologic factors should also be pointed out, e.g., it is well known that alcohol aggravates the damaging action of aspirin in the stomach, and acid predisposes to the action of pepsin by providing the optimal pH for intraluminal action of pepsin (pH 1–2). The stomach is indeed an active battleground where interaction of several endogenous and exogenous damaging agents might initiate, propagate or aggravate a damage.

Pathogenesis

In the pathogenesis of gastroduodenal mucosal injury, pathways, time elements as well as chemical cellular and tissue components (Table III) should be analyzed. Pathogenesis, i.e., in the mechanistic sequence of events leading to injury, involves

chemical injury, either by contact or indirectly involving enzymes such as proteases, or cyclo-oxygenase (e.g., aspirin-like drugs). Multiple pathways are involved in the indirect vascular damage (Fig. 1), e.g., release of vasoactive mediators, ischemia, reflow. Cellular inflammatory processes (e.g., acute and chronic inflammation, immune reactions) following infection, chemical and ischemic injury also play a role in the pathogenesis of mucosal injury.

Table III

Pathogenesis of cell and tissue injury in the gastroduodenal mucosa

Pathways
– *Direct chemical* injury – by contact (e.g., HCl, ethanol, ammonia)
– *Indirect chemical* injury by enzyme activation or release (e.g., proteases) or inhibition (e.g., aspirin)
– *Indirect vascular* damage (e.g., leukotrienes, endothelins, ischemia, reflow)
– *Cellular-inflammatory* process (e.g., acute and chronic inflammation, immune reactions)
– *Aggressive* factors vs. lack of *defense*, repair or regeneration
Components
– Common chemical entities (e.g., O_2, Ca, ATP, proteases, cell adhesion molecules)
– Reversible and irreversible cell injury
– Tissue necrosis
– Inflammation
– Dysplastic and neoplastic changes

The outcome of interactions of chemical and cellular elements in the pathways of gastroduodenal mucosal injury is very often determined by the balance between luminal or tissue aggressive factors and/or insufficient defense, repair or regeneration. The stomach and duodenum normally contain several luminal, e.g., bicarbonate, mucus, surface-active phospholipids, as well as tissue protective factors, e.g., PG, SH such as glutathione and the recently identified antiproteases. Most of these elements of chemical defense of the mucosa have been extensively investigated [29–32]. The presence of endogenous protease inhibitors in rat gastric mucosa and juice, on the other hand, has been recently identified [33] and awaits further elaboration both from pathophysiologic and pharmacologic points of view. Pathways of gastroduodenal mucosal injury resulting from each of the protective factors are already known, e.g., depletion of endogenous PG by NSAID (although factors other than low PG levels also contribute to the development of damage) [6, 7, 34] and the induction of duodenal ulcer by the dopaminergic neurotoxin MPTP is associated with slightly decreased gastric acid output and markedly reduced pancreatic and duodenal bicarbonate secretion [35]. In addition to the imbalance between chemical aggressive

and protective factors, inappropriate cellular defense such as rapid epithelial restitution as a form of early repair [36] or insufficient regeneration (i.e., cell multiplication) as a component of chronic ulcer healing also represent pathways of mucosal injury.

Among the components of pathogenesis (Table III) initially certain common chemical entities should be mentioned (Table IV). Namely, although there is no common final pathway of cell injury and death; however, a few shared chemicals should be considered (Table IV) [8, 21]. Oxygen is of central importance in the mechanisms of cell injury. The lack of oxygen causes cell injury, e.g., via ischemia. Excess oxygen may be directly toxic to certain organs, especially lungs and brain, or it might generate free radicals, which are the highly reactive and toxic activated species of oxygen and/or hydrogen peroxide (H_2O_2). In the presence of ferrous ion (Fe^{++}) hydrogen peroxide also yields hydroxyl radicals (Fenton reaction). Hydroxyl radicals alone, but especially in the presence of ferric ion (Fe^{+++}), initiate lipid peroxidation or disulfide (-S-S-) formation of membrane proteins, leading to membrane injury and increased permeability. Until recently, lipid peroxidation was regarded as almost the only major mechanism of irreversible cell injury, but now it is accepted as one of many pathways in which lipid peroxidation is often a secondary event or paraphenomenon.

Table IV

Common chemical entities in the pathogenesis of cell and tissue injury

Oxygen:	Insufficient amount of O_2 (hypoxia, ischemia) or excess; activated oxygen species, e.g., free radicals
Calcium:	Intracellular gradient; activation of phospholipases and cysteine proteases
ATP:	Effect on electrolyte pumps, e.g., Na^+, K^+, Ca^{++}; glycolysis, intracellular pH
Proteases:	From lysosomes and cytosol (intracellular and extracellular actions); role of endogenous inhibitors
Cell adhesion molecules:	Cell-to-cell adhesion (N-CAM, L-CAM, cadherin, integrins, lectins); cell-to-substrate adhesion (fibronectin, laminin, collagens, proteoglycans)
Antioxidants:	Glutathione, vitamin C, E, A, and other endogenous protective chemicals

Adapted from Szabo S, Kovacs K: Causes and mechanisms of cell and tissue injury in endocrine glands. In: Functional Endocrine Pathology, Kovacs K & Asa SL eds., Blackwell, Boston, 1991.

Some of the activated oxygen species, especially superoxide, cause or contribute to tissue damage indirectly; e.g., by causing vascular smooth muscle contraction directly or through interaction with nitric oxide (NO) [37–39]. The resultant ischemia may then be one of the major causes of tissue injury and necrosis in the gastroduodenal mucosa. It is thus not surprising that very often free radicals play a central role in pathogenesis of cell and tissue injury, and are frequently

considered as general chemical mediators of cell injury, irrespective of the fact that it may be caused by ischemia, chemicals or biologic agents.

Calcium also exerts a major regulatory role in cell damage. The viability of isolated hepatic or gastric mucosal cells incubated in the presence or absence of calcium in the medium, and of toxic concentrations of phalloidin or ethanol is much higher in the medium with little or no calcium [40, 41]. The elevated free calcium may activate enzymes such as phospholipases and initiate or accelerate membrane damage through calcium-dependent cysteine (thiol) proteases, which then attack the protein component of plasma membrane or other structural or enzymic proteins.

Adenosine triphosphate (ATP) production is related to the functional status of mitochondria. Selective depletion of ATP or prevention of its synthesis are usually not sufficient to cause severe cell injury. The decrease in ATP production, however, associated with other mitochondrial dysfunction in electron transport and membrane damage, will lead to loss of ion gradients, release of Ca^{++} from intracellular stores and influx from extracellular sources, and acceleration of loss of membrane integrity [42, 43]. This is aggravated by glycolysis and intracellular acidification, which are also the consequences of diminished ATP synthesis.

Proteases, released from lysosomes or activated from the cytosol, also have a major role in both cell and tissue injury, as has been recognized recently [44, 28, 33]. These enzymes, released by parenchymal cells or neutrophils which are attracted after initial cell damage, may then attack intracellular and extracellular structural proteins. Certain proteases are associated with endogenous inhibitors, the inactivation of which, e.g., during free radical generation, results in disinhibition of powerful proteases.

Cell adhesion molecules (Table IV) are also being recognized as important common chemical targets in various forms of tissue damage. Their crucial role underscores the importance of tissue vs. cell injury, and the complex interplay of several cellular and extracellular factors leading to extensive tissue injury.

The role of antioxidants and other protective factors in the pathogenesis of gastroduodenal mucosal injury was briefly mentioned above. It should be emphasized that next to the hepatocytes, gastric mucosal cells have the second highest concentration of reduced glutathione in the organism [45, 46] and non-SH antioxidants including certain vitamins exert considerable gastroprotection [47]. Decreased levels of endogenous antioxidants thus may indeed represent a common chemical component in the pathogenesis of gastroduodenal mucosal injury.

Of major emerging importance in the pathogenetic sequence is the distinction between or the transition from reversible to irreversible cell injury. The rate-limiting step between these two stages seems to be the extent of plasma membrane and mitochondrial damage (Fig. 2). The distinction is important because only cells in the reversible stage can be resuscitated by administration of certain chemicals, while at

the irreversible stage, cell death and limited or extensive tissue damage are inevitable. Necrosis is irreversible cell death in a tissue surrounded by normal and surviving cells. Necrosis usually triggers first acute inflammation which consists of vascular changes such as vasodilation, congestion, and infiltration of the damaged area by polymorphonuclear leukocytes. This is followed by repair or chronic inflammation when the inflammatory cells are mainly lymphocytes and plasma cells.

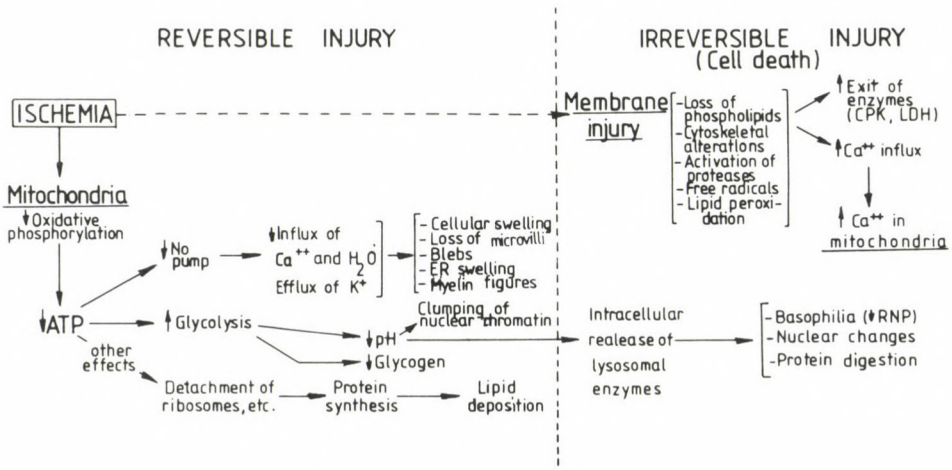

Fig. 2. Pathogenesis of ischemic cell injury: reversible and irreversible stages. (Reprinted with permission from Szabo & Kovacs: Causes and mechanisms of cell and tissue injury in endocrine glands. In: Functional Endocrine Pathology, Kovacs K & Asa SL eds., Blackwell, Boston, 1991)

Pathogenetically defined, the forms of gastroduodenal injury thus include diffuse mucosal necrosis and localized erosions or ulcers, with or without substantial hemorrhage, and acute or chronic inflammation. The body usually does not tolerate dead cells in the organism, and the irreversibly damaged cells are thus removed either by infiltrating inflammatory cells or by resident macrophages. However, leukocytes may also aggravate tissue damage [44, 48]. Thus, acute chemical injury is usually followed by infiltration by acute inflammatory cells such as polymorphonuclear leukocytes or eosinophils within minutes to hours after the initial chemical injury. If the lesion was excessive or persistent, chronic inflammatory cells such as lymphocytes, plasma cells and macrophages will be replacing the acute inflammatory cells. Gastroduodenal injury induced by infectious agents such as bacteria are notoriously known for involving both acute and chronic inflammatory cells.

Cell injury and protection in vivo and in vitro

Robert's original concept on gastric cytoprotection [7] has been criticized because of the incomplete protection by exogenous PG against chemically induced gastric mucosal damage [49, 50]. Terms of gastric histoprotection and organoprotection were suggested instead of "cytoprotection" [51] to imply maintenance of both the functions and morphologic features of rat stomach in presence of reversible or irreversible injury of superficial mucosal cells after pretreatment by PG, SH or other gastroprotective agents [46].

The best way to assess the phenomenon of direct gastric cytoprotection is to investigate *in vitro* the possible protection of isolated gastric mucosal cells by naturally occurring or exogenous compounds against chemically induced and measurable cell damage. Mixed population of gastric mucosal cells with long viability and preserved membrane receptor sensitivity can be isolated by low concentration of pronase and EGTA [52]. Selective examination of chemical injury and protection of plasma membrane, mitochondria and nuclei can be simultaneously determined by trypan blue exclusion and LDH release, succinic dehydrogenase activity and ethidium bromide-DNA fluorescence, respectively [53]. In these tests only slight or moderate (5–15%) concentration-dependent protection was detected by 9, 16,16-dmPGE_2, N-acetylcysteine, L-arginine and sucrose octasulfate against 15% ethanol-induced cytotoxicity [53, 54]. Consequently, minimal or no correlation was found between *in vitro* cytoprotection and *in vivo* gastroprotection by some drugs. The minimal or lack of *in vitro* direct cytoprotection by PG, SH or other compounds, on the other hand, is in accordance with the unprotected gastric superficial mucosal cells in animal experiments.

These data indicate that major part of the beneficial effects of gastroprotective agents seems to be mediated via complex tissue mechanisms (neurohumoral influences, mucosal blood flow, motility, etc.) instead of or in addition to limited direct cytoprotection.

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MECHANISMS OF NSAID-INDUCED ULCEROGENESIS: STRUCTURAL PROPERTIES OF DRUGS, FOCUS ON THE MICROVASCULAR FACTORS, AND NOVEL APPROACHES FOR GASTRO-INTESTINAL PROTECTION

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The multifactorial ulcer-producing actions of non-steroidal anti-inflammatory drugs (NSAIDs) are briefly reviewed and the main actions highlighted as the focus for potential strategies for reducing the ulcerogenic effects of these drugs. While some clinical benefits are evident from long-term clinical studies from application of PG analogues (misoprostol) and H^+/K^+-ATP- ase inhibitors (omeprazole) these are, ultimately, expensive approaches.

Chemical structural properties of the NSAIDs underlying differences in their ulcerogenicity are analyzed with the objective of establishing the reasons for the low ulcerogenicity of some of these drugs (e.g. azapropazone). These studies serve as a basis for developing less gastrotoxic drugs in the future.

In the studies we have undertaken analysis of the benefits of micronutrients and of modulating eicosanoid metabolism have been considered. The results of some clinical trials with micronutrients have proven encouraging. These and other approaches and pitfalls reported give further encouragement to explore the mechanisms of the protective effects of these latter agents and serve as a basis for future developments.

Keywords: anti-inflammatory drugs, non-steroidal, gastrointestinal mucosa, cytoprotection, prostaglandins, leukotrienes

To attain the goal of achieving protection of the gastrointestinal (GI) mucosa against the ulcerogenic effects of non-steroidal anti-inflammatory drugs (NSAIDs) is one of the most clinically-important developments in anti-arthritic therapy today. GI ulceration and haemorrhage are undoubtedly one of the most serious side-effects from NSAIDs [1, 2] and the case for GI protection against the ulcerogenic effects of these drugs has been well presented in a recent international meeting [2].

To satisfactorily achieve the goal of protection against NSAID-ulcerogenesis it is important to understand the mechanisms underlying the ulcerogenic effects of

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these drugs [3]. A summary of the principal actions of these drugs on the gastric and intestinal mucosa is outlined in Table I [3]. From this it can been seen that (1) NSAIDs have a multiplicity of actions on the GI mucosa – no single mechanism explains the effects of these drugs; (2) while there may be a considerable number of actions of NSAIDs on the stomach and intestinal mucosa which are common, there are important differences – two of these being the differing patterns of biodisposition of various NSAIDs in the GI tract and the actions of different endogenous bacteria in various regions of the GI tract; (3) the actions of NSAIDs involve a multiple attack on source in all mucosal defensive processes; and (4) individual NSAIDs differ markedly in their ulcerogenic effects in different regions of the GI tract. To obtain a single basis for protection against NSAID ulcerogenesis must be, at first sight, a virtual impossibility. Having said this it must be stated that there are already insights being made into the mechanisms of actions of NSAIDs on the mucosae which give some hope for more unifying attack on this problem than have hitherto been realized, some of these strategies will be explored in this paper.

Strategies for prevention of NSAID ulcerogenesis

Based on understanding of the mechanisms of ulcerogenic effects of NSAIDs it is now possible to speculate on what approaches might be employed to prevent these effects [3]. In essence two overall strategies can, and indeed, have been employed:

1. To prevent the drug having "contact" with the enzymes or biomolecules known to be affected by ulcerogenic NSAIDs.
2. To add anti-ulcer or more specific protective agents to the NSAIDs to either overcome their propensity to attack mucosal defence process, and/or to fortify the mucosa against the actions of their ulcerogenic drugs.

Each of these strategies has been extensively explored [2, 3] but to date none has achieved the ideal of *total* protection or prevention of NSAID ulceration. It is instructive to examine these various approaches and understand *why* each has its limitations because this highlights where other approaches can be employed. The approach in the future is, therefore, somewhat iterative since it is (1) going to have to take account of previous limited efforts to prevent/protect against NSAID ulceration, (2) benefit from knowledge of the mechanisms of actions of NSAIDs, and (3) consider new information on the mechanisms of mucosal defences.

Table I

Summary of physiopathological and biochemical changes involved in the pathogenesis of GI mucosal injury by NSAIDs

Factor	Principal Consequences
IMMEDIATE (PRIMARY ACTIONS):	
Physical effects of acid of drugs as (organic acid)	Reduced membrane integrity
Sloughing of surface mucus, decreased HCO_3^-, and altered phospholipid hydrophobicity	Impaired surface mucus and surface membrane protection
Back diffusion of acid from acidic drugs	Local decrease in cell pH (promotes drug uptake and local cellular autolysis)
Inhibition of cyclo-oxygenase leading to (a) decreased PGI_2 and PGE_2 synthesis, and (b)? diversion of arachidonate to lipoxygenase products	Altered blood flow – anoxia Platelet-vessel adhesion promotes microvascular injury Reduced 'cyto'-protection by: decreased mucus production decreased HCO_3^- secretion (probably PGE/I effect only) Promotion of leucocyte accumulation, adhesion (? from increased LTB_4 production and/or production of chemotactic peptides released from degraded proteins from local cell injury)
Release of lysosomal hydrolases	Local cellular autolysis
Cholinergic activation (CNS effects via PGE_2/PGI_2 reduction)	Acid/pepsin secretion enhanced in stomach
Histamine release from mast cells	Promotes acid secretion, vasodilation (stomach) Localized tissue destruction
Enhanced oxyradical production Reduced sulfhydryls	Possible loss of protection by mucosal biomolecules against oxyradical damage and pertubed eicosanoid metabolism
LONGER TIME EFFECTS:	
Enhanced motility (amplitude)	Altered GI transit ? relation to prostaglandin control of smooth muscle functions
Reduction in ATP production	Reduced capacity to resist cell injury from mucus and other synthetic reactions
Altered cAMP levels (? from phosphodiesterase inhibition)	Altered cell metabolism, including effects on acid and mucus secretion (stomach)
Inhibition of production of mucus layer and inhibition of mucus biosynthesis (at enzyme level)	Reduced mucus protection

Modified from ref. 3.

Strategy 1: Prevention of mucosal "contact"

These approaches essentially employ modification of the drug to:

(a) Slow its absorption in the stomach or intestine or spread its absorption to a broader range of sites in the GI tract (e.g. enteric-coating, sustained-release formulations). These are basically drug formulation approaches some of which have had limited success but none as striking as to manifest clinically significant reduction in ulcer incidence after long-term use in rheumatic patients.

(b) Develop pro-drugs, i.e. drugs which are "masked" in contact with biochemical and cellular sites of action, so therefore they are pharmacologically (or toxicologically) inactive in the GI tract but which are metabolized to their active forms after mucosal absorption.

From this it can be seen that few of these NSAID pro-drugs have really had any benefit in reducing GI ulceration after their long-term usage. It is possible that the reasons for this limited benefit are a combination of (a) relatively active or rapid mucosal metabolism and systemic conversion of the pro-drugs to their active forms, and (b) the potency of the active metabolites as ulcerogenic agents and inhibitors of PG biosynthesis. Thus, for example, sulindac (Clinoril®) has a relatively high rate of reporting in the USA and Sweden of severe adverse drug reaction (ADR's) in the GI tract [4, 5] although is has lower ulcerogenicity than its active metabolite sulindac sulphide in laboratory animal models [6]. However, administration of sulindac to pigs results in rapid conversion of this drug to its active metabolite which is taken up into the gastric mucosa from the circulation [7]. This rapid metabolism and fast kinetics of mucosal uptake favour accumulation of the drug in the mucosa, i.e. at a site where it is ideally not required. Another feature about sulindac is that its metabolite, sulindac sulphide is a potent PG synthesis inhibitor [8] and is markedly ulcerogenic [7]. Thus sulindac may represent an unfavourable example of where both pharmacokinetics and pharmacodynamics of the drug mitigate against ideal achievement of pro-drug design to prevent ulcerogenicity.

At the other extreme, nabumetone and fenbufen may represent examples of where pro-drug design has been more successful than with sulindac. It should be pointed out, however, that long-term data on the reports to drug regulatory authorities of GI ADR's from nabumetone and fenbufen are not available to the extent as from sulindac [9–12] so "the jury may yet be out" on this when assessments are made on the basis of such data sources. In any case fenbufen is only available in the UK and not in the USA and the latter is the major source of data on GI ADR's with sulindac. Furthermore, it is always possible that as a consequence of popularizing the belief that a drug is safer to the GI tract that it will be used more frequently in subjects with notable susceptibility to GI injury (e.g. the elderly frail

rheumatic patient) or on those individuals with apparent GI symptoms (e.g. heartburn, epigastric pain) symptoms of which may lead to promotion of the sensitivity to the ulcerogenic effects of NSAIDs.

Given these provisos there are indications that pro-drugs, such as fenbufen and nabumetone, whose active metabolites are not potent as PG synthesis inhibitors are not very ulcerogenic. Moreover, it is apparent that the kinetics of metabolic conversion of fenbufen and nabumetone to their respective major active metabolites are relatively slow. Thus the combination of slow biotransformation and moderate potency of metabolites as inhibitors of PG production and ulceration may favour the more favourable ulcerogenic profile of these drugs.

Another feature of the metabolism of NSAIDs which may be important in relation to their ulcerogenicity concerns the enantiomers of propionic acids. Aside from naproxen (which is the active S(+) isomer) other propionates have a chiral carbon atom attached to their carboxylic acid groups with the result that they exist as a 50/50 mixture of an active S(+) enantiomer and an inactive R(-) form. The former species which exerts anti-inflammatory effects and is the potent inhibitor of prostaglandin biosynthesis, while the R(-) antipode has appreciably lower activity. The question has been posed whether the presence of the inactive R(-) form in such abundance would result in lower ulcerogenicity of R/S mixtures as commercially available because of the weak PG synthesis inhibitory activity of the R(-) forms?

We have examined this aspect by determining the gastro-ulcerogenic effects of the R(-), S(+) and R/S enantiomers of a range of propionic acids using highly sensitive mouse cholinomimetic gastric ulcer assay previously described. The results of these studies are summarized in [14]. It was found that far from being less ulcerogenic the R(-) forms of all the propionic acid NSAIDs are almost equi-ulcerogenic with their S(+) antipodes and R/S mixtures. An obvious explanation for this is that the R(-) enantiomers are rapidly metabolized to their respective S(+) isomers and these are responsible for the ulcerogenic actions which have been observed. An important therapeutic consideration is, therefore, that the benefits of having R(-) in diastereoisomeric R/S mixtures of these drugs would seem to confer little or no benefit by way of reducing their likelihood of GI ulcerogenicity. However, the converse that their effectiveness as anti-inflammatory agents is not compromised assuming the appreciable metabolic conversion of R(-) to S(+) forms does occur as presumed. This aspect is now being explored further.

Strategy 2: Understand ulcerogenicity of low c.f. high ulcerogens

An NSAID with appreciably low ulcerogenicity, azapropazone = APZ (Rheumox® – Wyeth-Robins, UK; Prolixan – Siegfried, Switzerland) has been investigated by us to determine the features accounting for its low ulcerogenicity

observed in various laboratory animal models and in humans [3, 6, 15–17]. In relation to the previous discussion about propionates it was obviously important to determine if metabolites of the drug have more or less ulcerogenicity. The 8-hydroxymetabolite of APZ has been considered to have about the same low ulcerogenic properties with APZ itself (Jahn, *personal* communication). Other metabolites of APZ though, not formed in appreciable quantities have been analyzed for structure-activity effects. Even these in the mouse cholinomimetic assay have not revealed any notable ulcerogenicity above that of APZ itself [17].

A suggestion has been made by McCormack [18] based on a physico-chemical analysis of the properties of APZ by Dean [19] that the low ulcerogenicity of this drug may be due to its unique aquo-solubility at low pH's present in the stomach. This attractive suggestion is based on the interaction of a spare pair of electrons on the nitrogen atom the N-dimethyl amino group of APZ with the hydrogen of the hydroxyl moiety (Fig. 1). To test this hypothesis we prepared the des-dimethyl-amino derivative of the APZ (DDA) and compared its gastric ulcerogenicity with APZ itself in a highly-sensitive cholinomimetic-stimulated mouse assay [17, 20]. We reasoned that if the N-dimethyl-amino moiety has any role in modulating the intrinsic ulcerogenic activity of APZ then the ulcerogenicity of DDA should be greater than that of APZ [3]. Contrary to these expectations the ulcerogenic effects of these two compounds were identical [3, 17], thus ruling out any major effect of this group in (non)-ulcerogenicity of APZ.

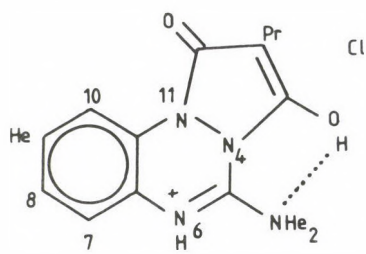

Fig. 1. Structure of the benzotriazine oxide NSAID, azapropazone (HCl salt)

This leaves open the question of why APZ should be less ulcerogenic than other NSAIDs, especially keto-enolates which have about the same acidity (pK_a) and lipophilic properties as APZ. Among these latter drugs phenylbutazone (PBZ) and oxyphenbutazone (OXB) have pK_a and log P (partition coefficients – a measure of lipophilicity) which are similar to those of azapropazone [20]. Thus, overall, the physico-chemical properties of APZ are similar to those of PBZ and OXB. The only conclusion which can be drawn is that there are intrinsic electronic and quantum

chemical differences in the molecular structure of the aromatic beings of the two classes of compounds.

APZ has a benzotriazine aromatic ring structure while that of PBZ and OXB are both pyrazolones (Fig. 1) [19]. Dean has carefully analyzed the properties of both classes of drugs and concluded that these two classes of drugs are entirely different [19]. While the exact components responsible for benzotriazine ring being different to that of the pyrazolone are not known it is cleat that there are obvious differences. The 1, 2, 4 nitrogens are likely candidates for contributing high electron-withdrawing activity of the benzotriazine ring and this may result in unique electron distribution over these nitrogens. How this may be important is not known as yet but further studies are in progress [17].

This information may be of practical significance since it might be possible to develop other less ulcerogenic yet more potent compounds based on the knowledge now available on their structure-activity relationships.

Strategy 3: Modulate perturbed eicosanoid metabolism

This in essence is the classical approach for it has been the basis for the use of prostaglandin E and I analogues to overcome the apparent deficiency caused by cyclo-oxygenase inhibition induced by NSAIDs. However, it is now emerging that prostaglandin E analogues have novel actions on their own and their co-administration with NSAIDs does not merely overcome a deficiency in prostaglandins but produces a totally different set of pharmacological reactions, some of which will be detailed shortly. Furthermore, interest has developed recently in the use of dietary precursors of the essential fatty (poly-unsaturated) acids (PUFAs) as means of manipulating the balance of eicosanoids and prevent ulceration from NSAIDs [21–25].

An aspect which is fundamental to this approach is the recognition that inhibition of cyclo-oxygenase [prostaglandin G/H synthetase] by the NSAIDs can cause diversion of arachidonate, released by phospholipase A_2, through the 5-lipoxygenase (5-LO) pathway and lead to excessive production of vasoconstrictor peptido leukotrienes (LTC_4, LTD_4, LTE_4), the leucoattractant LTB_4, and peroxidative derived oxyradicals (Ox [25]). A test of this hypothesis can, and has been, performed thanks to the availability of novel 5-LO inhibitors and selective LT antagonists. Unfortunately, the application of these by some authors (e.g. [26]) has resulted in negative results [25] because no account was taken of the pharmacokinetic and pharmacodynamic properties of these drugs. In the case of one such compound, MK-886 (formerly L-663, 536), the slow development of full inhibitory effects on 5-LO is now recognized as a key feature of this drug, possibly resulting from the time-dependent inhibition of the *F*ive *L*ipoxygenase *A*ctivating *P*rotein (FLAP) by this

drug, its principal site of action [27] as well as the slow kinetics of its GI absorption [28]. Using the cholinomimetic mouse [15] and adjuvant arthritic rat models we have established that prior oral treatment for 5 h with MK-886, significantly and appreciably reduces gastric ulcerogenicity induced by indomethacin (s.c. or p.o.) or aspirin (p.o.) [25]. However, if the 5-LO inhibitor is given simultaneously with that of the ulcerogenic NSAID then no protection is afforded [25]. This explains why Peskar et al. [26] were unable to obtain anti-ulcer activity with MK-886 since the authors obviously failed to take into consideration the long time it takes for 5-LO inhibition to manifest with this drug.

Earlier studies [24] have shown that concomitant administration of 5-LO inhibitors and LT antagonists, given either orally or parenterally, reduced the gastric ulcerogenicity of NSAIDs given by the alternative route. There are also patents claiming anti-ulcer activity of 5-LO inhibitors [29, 30]. While some of the earlier studies were performed with compounds that have antioxidant activity, a feature which confers gastric mucosal protection against NSAIDs [30], they nonetheless give some support to the hypothesis for the involvement of the 5-LO pathway in the pathogenesis of NSAID gastric ulcerogenesis.

Recent studies have shown that in addition to providing gastric mucosal protection against NSAIDs, prior oral administration of MK-886 (10–100 mg/kg) protects against intestinal injury induced over a 24 h period by s.c. and p.o. indomethacin (10 mg/kg) [25]. Thus potentially specific 5-LO inhibitors may be considered useful as anti-ulcer agents against NSAID induced mucosal injury in the entire GI tract.

A new slant to the actions of the PGE, analogue, misoprostol, involving the 5-LO pathways have also arisen from our studies in pigs [3, 32]. We found that oral administration of the NSAID, diclofenac sodium (5 mg/kg/d) to domestic pigs for 10 days resulted in a marked increase in all LTs in the gastric mucosa [32]. This effect was related in a dose-related fashion in those animals that received misoprostol. We infer that the effect of misoprostol is due to its stimulation of cyclic 3'-, 5'-AMP (cAMP) production following PGE-receptor mediated activation of adenylate cyclase, and that cAMP negatively regulates 5-LO activity. Thus a major part of the action of misoprostol could be attributed to its negative control of 5-LO activity not only causing reduction of peptidoleukotrienes and peroxyFA-derived oxyradicals, but also the leukoattract LTB_4. The effects of misoprostol in reducing leucocyte accumulation induced by NSAIDs [33] could be ascribed to its effects in inhibiting LTB_4 production.

These results show the important potential of 5-LO activity in the genesis of GI damage by NSAIDs and highlight important strategies for reducing the ulcerogenic effects of conventional co-inhibitory NSAIDs. Of course, the long-term strategy of developing safer drugs will evolve from combining LO inhibitory activity with that of

the CO-inhibitory effects both in one drug. Some examples of this approach have been developed [3, 20] and are under development.

Strategy 4: Nutrient induced mucosal fortification

(1) *Glucose + Tricarboxylate Cycle Precursors or Intermediates*

A well-known phenomena observed some years ago [33–36] that aspirin reduces gastric mucosal production of adenosine triphosphate (ATP) from adenine nucleotide precursors (ADP, AMP) or reduces the concentration of this high energy phosphate intermediate by catabolic events has recently received attention as a basis for reducing the ulcerogenic effects of this and other NSAIDs [3, 36–42]. The approach has been to employ critical proportions (concentrations) of glucose with an intermediate of precursor of the tricarboxylate (TCA) cycle to overcome the inhibitory effects of NSAIDs on enzymes of the Embden-Meyhoff glucose metabolic and TCA pathways as well as mitochondrial oxidative phosphorylation [36–42].

While it is possible that metabolic deficiency in ATP levels may occur as a *consequence* of ulcerogenesis, it is also likely to occur from the inhibition of dehydrogenases and mitochondrial oxidative phosphorylation by the NSAIDs. The end effect of reduced ATP should be, potentially, reversible by addition to the drugs dosed of selective nutrients. Accordingly, we tested this hypothesis by co-administering various ratios of glucose and TCA cycle intermediates with respect to molar or weight quantities of the NSAIDs to rats and mice exposed to certain mucosal sensitizing agents or procedures [36–40, 42]. Resulting from these studies we were able to critically optimize the amounts of required nutrients for a number of NSAIDs [36–42]. From these and other studies we established the specificity of effects of the added nutrients to overcome the GI ulcerogenic effects of selected NSAIDs. Also it was found that these effects were not due to osmotic effects of glucose or other nutrients and other non-specific effects. We developed formulations of these mixtures with aspirin, indomethacin and azapropazone for detailed clinical investigation [39, 40, 41]. As with the animal studies these formulations yielded impressive GI protection compared with that of the drugs above [30, 36–42]. The clinical benefits from the marked lowering of GI ulcerogenicity by addition of glucose and TCA cycle intermediates were in all cases not accompanied by reduction in bioavailability or anti-inflammatory and analgesic activities of the drugs [37, 40, 42]. While further aspects about the mechanisms of the protection afforded by one such formulation, the glucose + citrate in the indomethacin formulation ("INDO-RISE"), are underway the studies to date show that (a) the protective effects of glucose-citrate do not involve alteration in the normal PG synthesis inhibition observed in the gastric mucosa, (b) the glucose-citrate mixture does not alter the physico-chemical

properties of indomethacin, (c) indomethacin absorption from the GI tract is the same in the presence of glucose + citrate as from the drug alone, and (d) restoration of gastric mucosal ATP concentration depleted by indomethacin above is achieved by addition of glucose + citrate [38, 40].

(2) *Trace Metal Complexes*

Trace metals, especially Cu and Zn have received much interest in recent years for the role in protection or anti-inflammatory activity in various inflammatory conditions [43, 44]. We have been interested in the anti-inflammatory potential of zinc complexes, notably one which is a slow-release formulation, zinc monoglycerolate (ZMG; Glyzinc®, Glyzinc Pharmaceuticals Ltd., Janet Court, Highbury, SA, Australia) [46]. Such anti-inflammatory activity not involving inhibitory effects on PG production is of especial interest in certain GI inflammo-ulcerogenic states. Zinc sulphate and zinc acexamate have been shown to have anti-ulcer activity against a wide variety of ulcerogenic agents in laboratory animal models as well as in human peptic ulcer disease [46–57]. Unfortunately, $ZnSO_4$ has most unpleasant astringent effects in the gastric, buccal and oesophageal mucosae which may be related to the all-too-rapid absorption by the mucosa. The possibility exists that the astringent effects of free Zn^{++} ions may cause unspecific stimulation of PG production in the stomach and that this may underly the apparent protective effects of $ZnSO_4$.

Considering the slow-release properties of ZMG we reasoned these may be potentially useful in optimizing the delivery of free Zn^{++} ions in the stomach for achieving protective effects. The release of Zn^{++} is a pH-dependent process; greater dissociation of Zn^{++} being evident at low pH conditions such as present in the stomach [58]. We, therefore, examined the protective effects of ZMG in a variety of ulcer models induced in rats and mice including those induced by NSAIDs (Figs 2–5). We have shown that ulcer protection is achieved by ZMG in molar concentrations comparable with that of cimetidine and zinc acexamate and possible superior to that of $ZnSO_4$ [59].

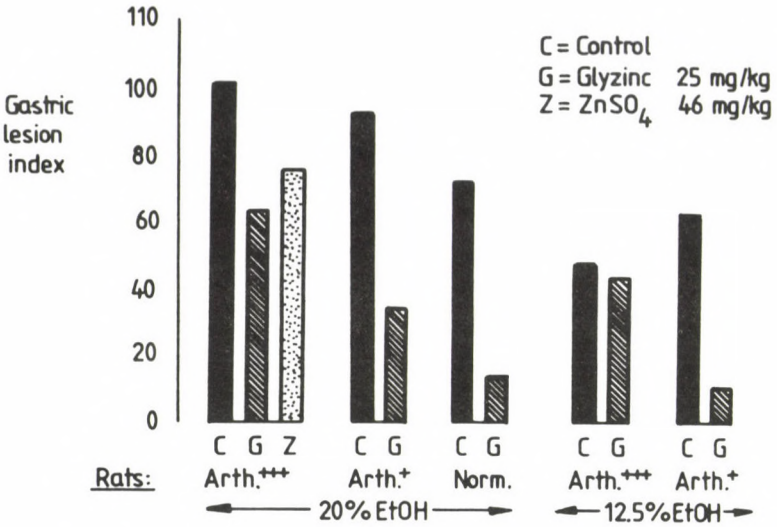

Fig. 2. Effects of zinc monoglycerolate (=ZMG; Glyzinc®) on gastric mucosal lesions induced by dilute ethanol solutions in adjuvant arthritic rats (these inflamed animals are highly sensitive to gastric ulcerogens). In this model the effects of ZMG were compared with zinc sulphate at equivalent doses of Zn (first group of data on left side). The number and severity of gastric lesions was determined in 24 h fasted rats given the ethanol solutions 10 min after oral dosing of ZMG; the animals being killed 2 h later. Statistically significant reduction (Student's 't' test; $p<0.05$) in the gastric lesion index was observed with all doses of ZMG. Based on data in ref. 59

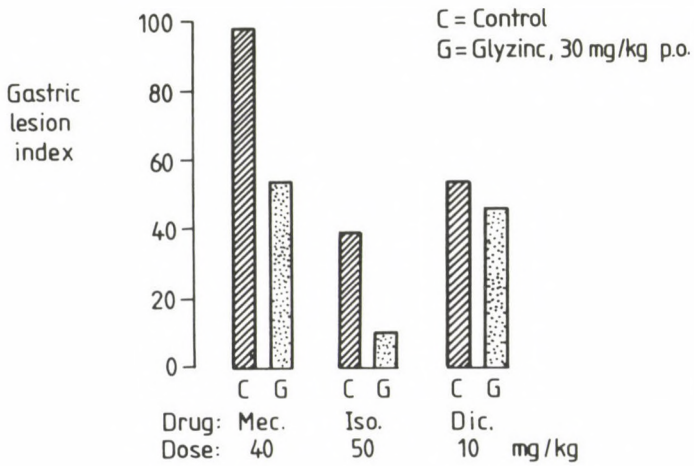

Fig. 3. Effects of ZMG on the gastric ulceration induced in arthritic rats by three non-steroidal anti-inflammatory drugs (NSAIDs). ZMG was given *p.o.* 10 min before *p.o.* dosing with the NSAIDs in 24 h fasted rats. The animals were killed 2 h later and the number and severity of gastric lesions determined and the gastric lesion index then calculated. Statistically-significant reduction in ulcers was observed in all ZMG rats (Student's 't' test; $p<0.05$). Based on data in ref. 59

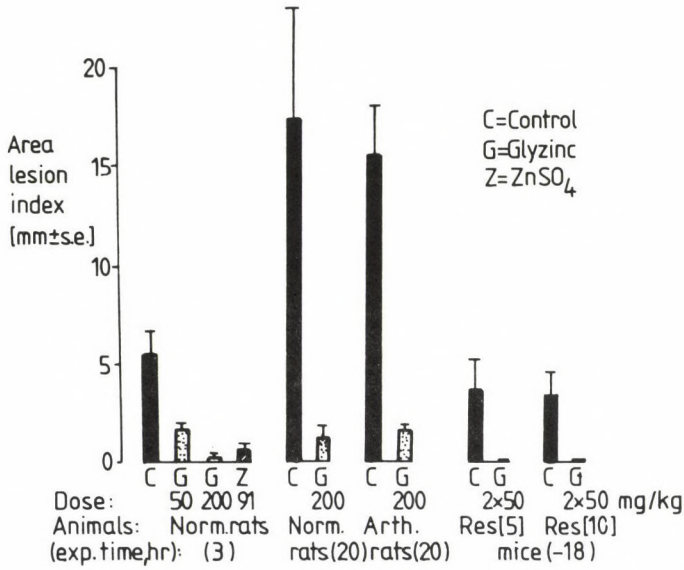

Fig. 4. Effects of oral administration of ZMG on ulcers induced by reserpine in normal and adjuvant-arthritic rats and mice (methods in ref. 59). Statistically significant reduction in area lesion index (mm^2) observed in all ZMG treated animals as well as in normal rats given zinc sulphate (left column). Based on data in ref. 59

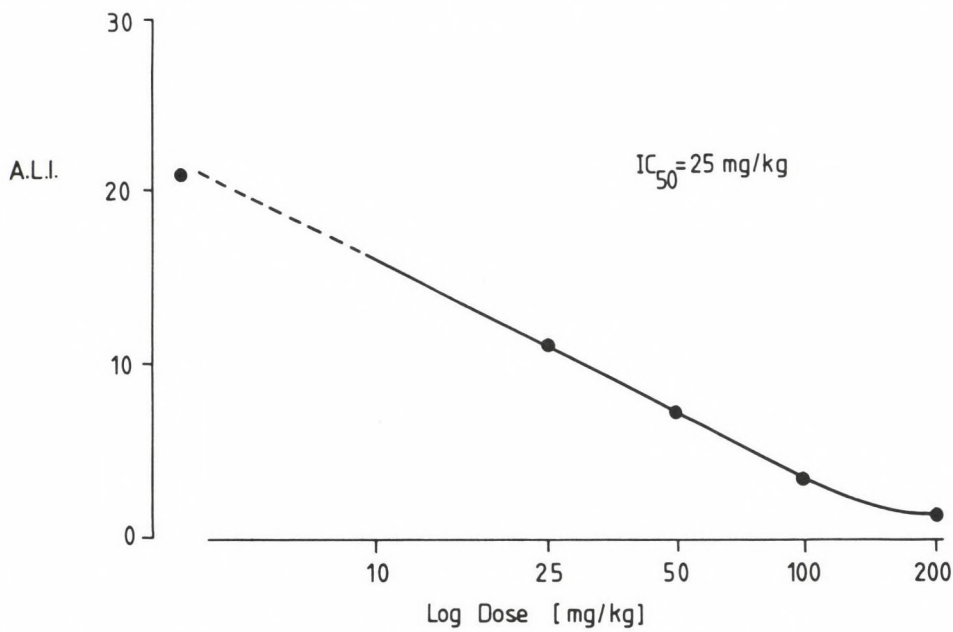

Fig. 5. Effects of varying oral doses of ZMG given −5 min before oral administration of 200 mg/kg aspirin together with bethanechol chloride (a cholinomimetic given to stimulate acid and pepsin production and so enhance the sensitivity of the gastric mucosa to the ulcerogenic effects of aspirin) [ref. 59]

The mechanisms of protective effects of Zn^{++} ions in preventing most cell degranulation, inhibiting gastric secretion, stabilizing lysosomal enzymes and the physico-chemical properties of mucus and sulfhydryl reactivity [46–56] underly the protection afforded by the Zn^{++} released from ZMG. Recently, it has been established that ZMG prevents the rise in 5-LO activity induced by aspirin in mice, thus implying that ZMG may inhibit the production of vasoactive peptido-leukotrienes and peroxy-FA derived oxyradicals [59]. ZMG has also been shown to exert protective effects without stimulating PG production and scanning electron microscopic observations confirm its lack of non-specific mucosal irritant actions [59]. There are no apparent effects of ZMG on mucosal ATP production [59].

These results show that ZMG has considerable potential as an ulcer preventative agents, especially against NSAID-induced GI ulceration. While further studies on the mechanisms of action of ZMG are awaited there is clearly impetus to examine the potential clinical benefits of this cheap and inexpensive anti-ulcer agent.

Conclusions

This review has focused attention on a range of approaches for preventing NSAID-induced GI ulceration and highlighted some of the more successful attempts. Clearly there is great potential for exploiting some of these approaches in the future. Furthermore, examination of the relative successes of these approaches gives valuable information on the protective mechanisms operating in different regions of the GI tract.

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ATP BREAKDOWN AND RESYNTHESIS IN THE DEVELOPMENT OF GASTROINTESTINAL MUCOSAL DAMAGE AND ITS PREVENTION IN ANIMALS AND HUMAN

(An overview of 25 years ulcer research studies)

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The changes in membrane-bound ATP systems (breakdown and resynthesis) were analyzed in different experimental ulcer models (such as ETOH, HCl, NaOH, 25% NaCl-induced, pyloric ligated + epinephrine treated, stress, reserpine treated, indomethacin treated rat models) and chronic antral, duodenal and jejunal ulcers in patients.

The energy system parameters (adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), cyclic AMP (cAMP), lactate) were measured from different sites of gastrointestinal mucosa, and values of ATP/ADP, adenylate pool (ATP + ADP + AMP) and energy charge ((ATP + 0.5 ADP)/(ATP + ADP + AMP)) were calculated. The biochemical measurements were done at different times during the development of gastrointestinal mucosal lesions, without and with application of different drugs (PGI_2, atropine, cimetidine) and bilateral surgical vagotomy.

The aims of our present paper were: 1.) To summarize the main directions of ATP breakdown during the development of gastrointestinal lesions or ulcers in different experimental models and human beings: 2.) To summarize the biochemical steps of defense of gastrointestinal mucosa against chemicals, drugs or unknown pathogenic factors; 3.) To analyze the importance of membrane-bound ATP-dependent energy systems in order to understand the mucosal lesions and their prevention; 4.) To evaluate the real values of changes in these parameters from the point of view of ulcerogenesis and its prevention; 5.) To find some correlation between the energy parameters during mucosal damage and its prevention: 6.) To understand better the types of tissue reactions (metabolic) due to development of mucosal lesions and prevention.

It has been concluded that; 1.) The development of gastrointestinal mucosal lesions is a consequence of very active metabolic processes (chemicals-induced mucosal lesions, pyloric ligation, stress ulcer, reserpine treatment, human chronic gastric, duodenal or jejunal ulcers) or of a negative metabolic adaptation (pylorus-ligated plus aspirin-treated, indomethacin-treated rats); 2.) The significant increase of ATP-ADP transformation gives the biochemical basis for the development of pyloric ligated rats and human chronic antral, duodenal and

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jejunal ulcers; 3.) The significant increase of ATP-cAMP transformation can be found in the development of stress- and reserpine treatment-induced mucosal lesions; 4.) The decreased extent of ATP-ADP transformation was obtained in pylorus-ligated plus aspirin-treated rats; 5.) The decreased extent of ATP-cAMP can be obtained in indomethacin treated rats; 6.) The decrease of increased extents of energy turnovers (both in ATP-ADP or ATP-cAMP systems) might represent a defense of GI mucosa (atropine, cimetidine, surgical vagotomy) which can be also present by their further energy liberation (PGI_2); 7.) The mucosal damage associated with a decreased energy turnover (ATP-ADP in pylorus ligated plus aspirin-treated ATP-cAMP in indomethacin-treated rats) can be prevented by the drugs increasing energy metabolism (PGs) or by further decrease of energy turnover (atropine, cimetidine, surgical vagotomy); 8.) A biochemically and pharmacologically well regulated feedback mechanism system exists between membrane-bound ATP-dependent energy systems both in case of development of mucosal damage and its prevention, which mainly depend on the functional and biochemical status of target organ; 9.) The starvation or hyperalimentation (with glucose) essentially changes the gastric mucosal biochemistry (ATP resynthesis, types of ATP breakdown) and mucosal resistance to chemicals.

Keywords: experimental gastric ulcer models, human gastric, duodenal, jejunal ulcers, exogenous and endogenous chemicals, irritants to gastric mucosa, cellular energy systems, ATP synthesis, ways of ATP breakdown, types of mucosal adaptations to chemicals, drug actions on cellular energy systems, surgical vagotomy, starvation, hyperalimentation with glucose, cellular (metabolic) adaptation of gastric mucosa after surgical vagotomy

The ulcer disease is a multifactorial process involving significant changes in vascular events [62–64], mucus secretion, gastric acid secretion, bicarbonate secretion [65], biochemism of GI mucosa. An equilibrium has been suggested between the aggressive (HCl, leukotrienes, pepsin etc.) and defensive (blood flow, prostaglandins, energy producing factors) which is impaired during the development of GI mucosal damage. The therapeutic tool was, for a long time, to reverse the impaired balance of ulcerated gastrointestinal mucosa to the normal state by decrease of aggressive factors. Chaudhury and Jacobson [5] indicated that prostaglandins prevent the gastrointestinal mucosa without inhibition of gastric acid secretion. The existence of "gastric cytoprotection" was generally accepted by the observations of Robert et al. [57]. These studies indicated a real possibility of treatment to separate the gastrointestinal defenses from the aggressive side, however, the real explanation for these phenomena remained unclear. The results of multiclinical, randomized and prospective studies indicated the equal efficacy of short treatments (4 or 6 weeks) with atropine, carbenoxolone and cimetidine on patients with duodenal ulcer [66].

The appearance of gastrointestinal mucosal damage is an unspecific reaction to gastric mucosa, however, different cells are involved in this process (epithelial cells, chief cells, parietal cells). Accepting this fact, it was suggested that both the mucosal damage and its prevention are involved unspecifically in the same tissue. On the other hand, many extracellular actions (drugs, hormones, vascular events, growth

factors, etc.) regulate the cell functions, which are basically necessary to translate the different extracellular influences to the language of cells.

One of the most common substrate of the cells is ATP, which is an energy storage molecule. Energy liberates by ATP breakdown. The tissue level of ATP does not represent any biochemical state for the tissue (cellular) response(s), because the ATP breakdown is basically necessary for energy liberation, which participates in the different physiological (or pathological) reactions of the cells. The ATP can be split into ADP (by membrane ATPase) and cAMP (by adenylate cyclase) in presence of Mg^{2+}. Both enzymes are located in the plasma membrane of cells [1, 2, 58, 59].

The change of ATP breakdown was studied during the acute mucosal damage of rats (produced by pyloric ligation, pyloric ligation plus aspirin, 96% ethanol, 0.6 M HCl, 25% NaCl, 0.2 M NaOH, stress, reserpine, indomethacin) and in chronic gastric, duodenal and jejunal ulcers [3].

The aims of this study were: 1.) To evaluate the ATP breakdown during physiological or pathological responses; 2.) To study the ATP breakdown in connection with the changes of vascular permeability; 3.) To study the connection between tissue hypoxia and changes in vascular permeability; 4.) To clear up the main biochemical changes in the gastric mucosa and in the development of mucosal damage; 5.) To evaluate the actual mucosal level of ATP modified by starvation or acute glucose hyperalimentation; 6.) To follow up the ATP metabolism after chemical or surgical vagotomy.

Materials and methods

Materials

The investigations were carried out on both sexes of Sprague-Dawley (CFY, LATI, Gödöllő, Hungary) strain rats, weighing 180 to 210 g body weight and on the resecates of stomach and small intestine of patients who underwent gastric surgery because of unhealed peptic ulcer.

Animal experiments

The animals were fasted 24 h before the experiments (except in case of nutritional experiments, see details later) before the experiments. The experiments began at 8.00 a.m.

Human studies

Human observations were carried out on both the resecates of stomach and small intestine of patients, who underwent gastric surgery. The patients were treated with different (dominantly with anticholinergic) drugs but they received only antacids before operation (the human experiments were carried out in years of 1970–1975) [20, 22–26, 28–30, 34–37, 40, 41].

Tissue specimens

The gastric mucosa of rats and gastric, duodenal and jejunal mucosa of patients were scraped. The tissue samples were put into liquid nitrogen, which were used for further biochemical examinations.

Mucosal lesions of ulcers

Animal experiments. The incidence, number of gastric mucosal lesions were calculated, and their extents were planimetrically determined using a computer assisted system (DIGICELL, ASK Ltd., Hungary). The results were expressed as means ± SEM.

Human observations. The gastric, duodenal and jejunal ulcers (after Billroth II resection) and their presence were endoscopically registrated.

Biochemical measurements

The tissue contents of adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP) and lactate were measured enzymatically (Boehringer Ingelheim, F.R.G.), while cyclic adenosine monophosphate (cAMP) by RIA (Becton Dickinson, Orangeburg, U.S.A.). The biochemical measurements were done a couple of years before, regarding to the patients, and their methods were published elsewhere [26, 28, 33–35, 40].

The protein content was assayed by the method of Lowry et al. [9]. The biochemical results were calculated in accordance to 1.0 mg protein (means ± SEM).

Determination of membrane (Mg^{2+}-dependent, Mg^{2+}-Na^{+}-K^{+}-dependent) ATPase was measured by the liberation of inorganic phosphate component [20, 26, 29, 30].

The results were expressed as means ± SEM. The unpaired Student's t-test was used for statistical analysis of the results, except of ulcer severity when the Mann-Whitney's test was applied.

Results

1. ATP breakdown vs. physiological or pathological responses

1.1. Biochemical changes in the gastric tissues, development of gastric hypersecretion and ulceration

The gastric acid secretion (by glandular portion) significantly increased until 7 hours after pyloric ligation, and thereafter it remained at the same level (Fig. 1).

The ATP breakdown was increased at 4 hr, thereafter ATP level returned back to 7 hr. After that time it decreased gradually. An opposite change was obtained in the tissue content of ADP, which was found at low level after 16 hr. On the other hand, tissue level of cAMP decreased significantly at the first time period (2 hr) and its level remained at low concentration for a time (from 7 to 24 hours) after pyloric ligation (Fig. 1). No ulceration was detected in the glandular portion of pylorus ligated rats.

The changes in the adenosine nucleotides, during the first 7 hours period, indicate that:

a.) The direction of ATP breakdown is gone to ADP,

b.) The transformation of ATP-cAMP is inhibited during that time,

c.) No tissue hypoxia can be detected in the glandular portion of the stomach (because against the increased extent of the ATP breakdown into ADP, its level elevated at 7 hr after pyloric ligation) [21, 31, 35, 38].

This is an active metabolic process, which results in an extremely increase of gastric acid secretion (by glandular stomach). The question is where the border (in the time or in gastric biochemistry) between the physiological and pathologic tissue reactions can be found. The correct answer cannot be given because no pathological reaction can be noticed from the side of glandular portion of the stomach.

Similar observations were done from the forestomach (rumen), which has no secretory property. Similar changes were obtained in the ATP, ADP, cAMP during the first seven hours after pyloric ligation. The ulceration appeared after 7 hours and its extent reached about 50 percent at 16 hours. The ulceration increased gradually in the rumen by time after pyloric ligation, however the gastric secretory response of glandular stomach remained unchanged after 7 hours of pyloric ligation (Fig. 2).

The extent of ATP-ADP transformation increased significantly in the forestomach, which was associated by a significant inhibition of ATP-cAMP transformation. It was also noticed that no tissue hypoxia can be found in the forestomach before macroscopic appearance of ulcer.

1.2. Chemicals-induced gastric mucosal damage

Robert et al. demonstrated that strong chemical agents, such as 96% ethanol, 0.6M HCl, 25% NaCl, 0.2M NaOH or thermal injury produce gastric mucosal damage in rats [57].

The biochemical background (namely cellular energy systems, lactate, and different ratios between cellular energy systems) was critically evaluated [13, 29]. The results of these studies indicated clearly that the cellular energy storage molecule, ATP, split without elevation of tissue lactate (Table I). By other words the ATP (vs. energy storage molecule) significantly decreased in the gastric mucosa during the development of gastric mucosal damage. Furthermore, the tissue levels of ADP were increased significantly in all cases, independently on the applied necrotizing agent [13].

Biochemically it is well proved that the transformation of ATP into ADP is associated with energy liberation.

When we analyzed the biochemistry of gastric mucosa at 0, 1, 5, 15, 30 or 60 min after giving of necrotizing agent (similar observations were carried out in rats treated with 96% ethanol and 0.6M HCl) the directions of ATP transformations into cAMP and ADP changed by time after applications of necrotizing agents [13, 47] (Fig. 3).

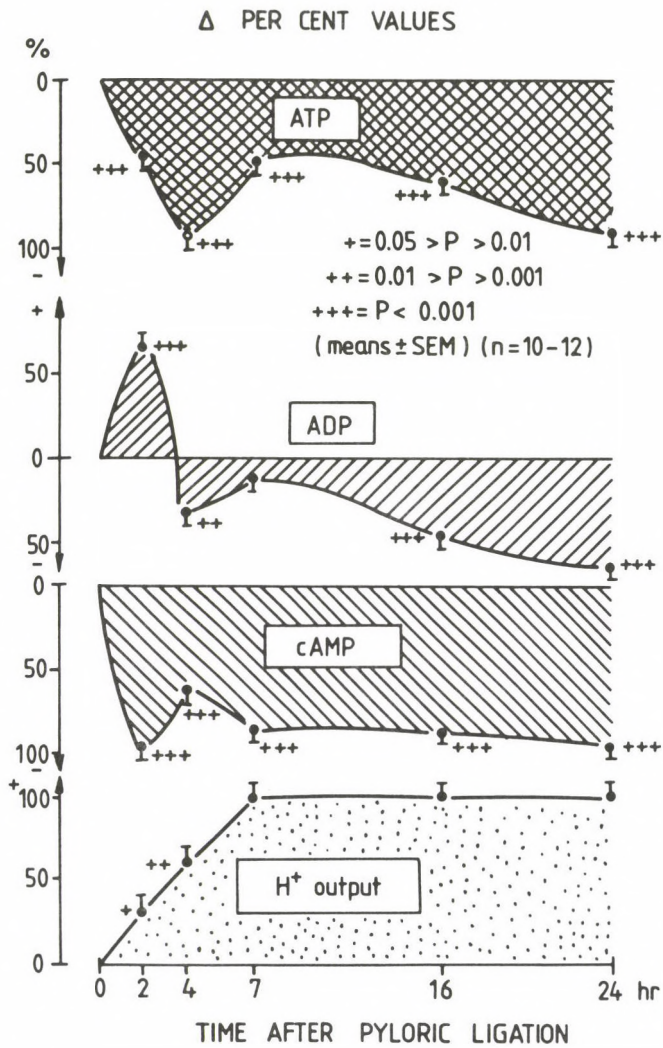


Fig. 1. Correlations between changes in the gastric mucosal ATP, ADP, cAMP in relation with gastric hypersecretion of fundic mucosa in 24-hr pylorus-ligated rats. Each point represents the average of the results obtained in 10–12 animals. P values were calculated between the results obtained at 0 hr vs. different times (hr) after pyloric ligation, except of H^+ output, when gastric secretory responses were compared to the results obtained at 24 hr vs. different times after pyloric ligation. The content of ATP and ADP was calculated in nmoles/mg protein, cAMP in pmoles/mg protein, H^+ output in μEq/rat stomach. The alterations in these parameters are expressed in percent values in this figure (means ± SEM)

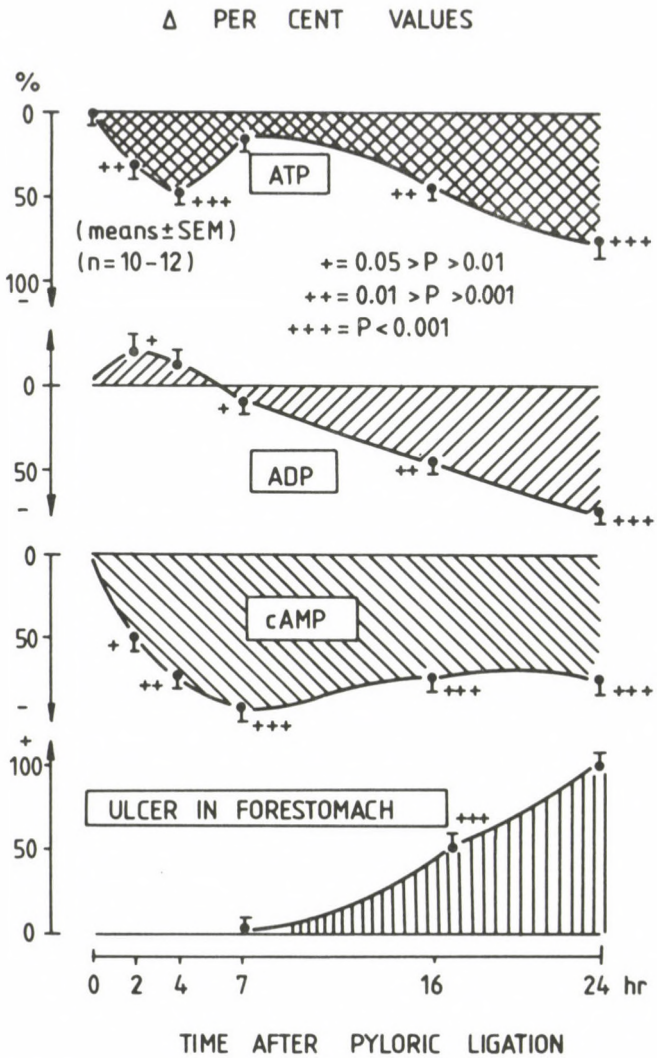


Fig. 2. Correlation between the changes in tissue levels of ATP, ADP, cAMP and ulcer development in the forestomach of 24 hr pylorus-ligated rats. The tissue levels of ATP and ADP were calculated in nmoles/mg protein, cAMP in pmoles/mg protein. The results were expressed in percent values of results obtained at 0 hr, except of ulcers, when the control values at 24 hr after pylorus ligation were measured. P values were calculated between the results obtained at 0 hr vs. different – except of ulceration, when the results were compared to the extent of forestomach ulceration obtained at 24 hr vs. at different times – after pyloric ligation

Table I

Changes in gastric mucosal ATP during development of gastric mucosal damage, produced by intragastric administration of 96% ethanol, 0.2M NaOH, 0.6M HCl or 25% NaCl. The results were expressed in nmoles/mg protein

Necrotizing agent	Mucosal level of ATP	ADP	ATP/ADP
Saline (1 ml i.g.)	12.2 ± 8	14.5 ± 1.6	0.84 ± 0.06
96% ethanol (1 ml i.g.)	8.2 ± 0.8**	15.2 ± 2.3	0.53 ± 0.04***
0.2M HCl (1 ml i.g.)	3.0 ± 0.3***	18.0 ± 1.0*	0.16 ± 0.01***
0.6M HCl (1 ml i.g.)	8.5 ± 1.0***	19.3 ± 2.0*	0.44 ± 0.03***
25% NaCl (1 ml i.g.)	5.0 ± 0.5***	18.0 ± 1.0*	0.27 ± 0.02***

The results based on data of paper published by Mózsik et al. (Prostagland. Leucotr. Med. 12; 423–436, 1983).
The number of gastric mucosal lesions was 13–16 ± 2 per rat stomach in each group except the saline treated one.
p values were calculated concerning the results obtained in saline vs. necrotizing agent treated groups. Abbreviations: NS: not significant, *: $p \leq 0.05$, **: $p \leq 0.01$. ***: $p \leq 0.001$

1.3. Reserpine- and stress-induced gastric mucosal damage

1.3.1. Reserpine ulcer

Reserpine (given it 5 mg/kg s.c.) produces gastric mucosal damage in the glandular portion (Fig. 4). Only few numbers of reserpine-induced gastric mucosal damage can be found at 6 hr after reserpine administration, and about 50% of results obtained at 12 hours can be noticed. However, the ATP-ADP transformation can be maximally inhibited, while cAMP level was found at its peak. It is interesting that the tissue level of ATP increased (against the increase of cAMP), which indicates that the ATP-ADP transformation has essential role in the regulation of tissue level of ATP (excluding the failure of the oxidative phosphorylation due to tissue hypoxia) [44] (Fig. 5).

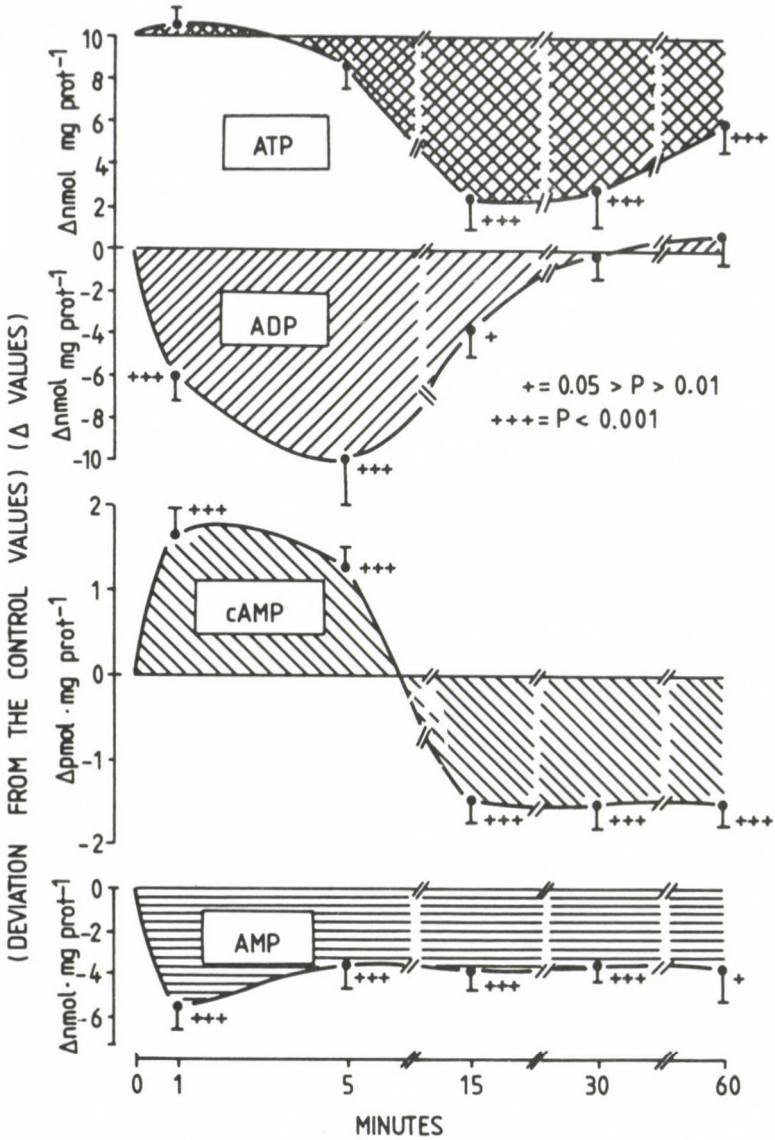


Fig. 3. Changes in gastric mucosal energy systems – obtained at different times – in the gastric mucosa of rats treated with ethanol (1 ml 96%, i.g.). The results were expressed as Δ values obtained at different times vs. 0 min after ethanol administration (means ± SEM) (n = 10). The results are based on results published by Mózsik and Jávor (Dig. Dis. Sci. 33; 92–105, 1988)

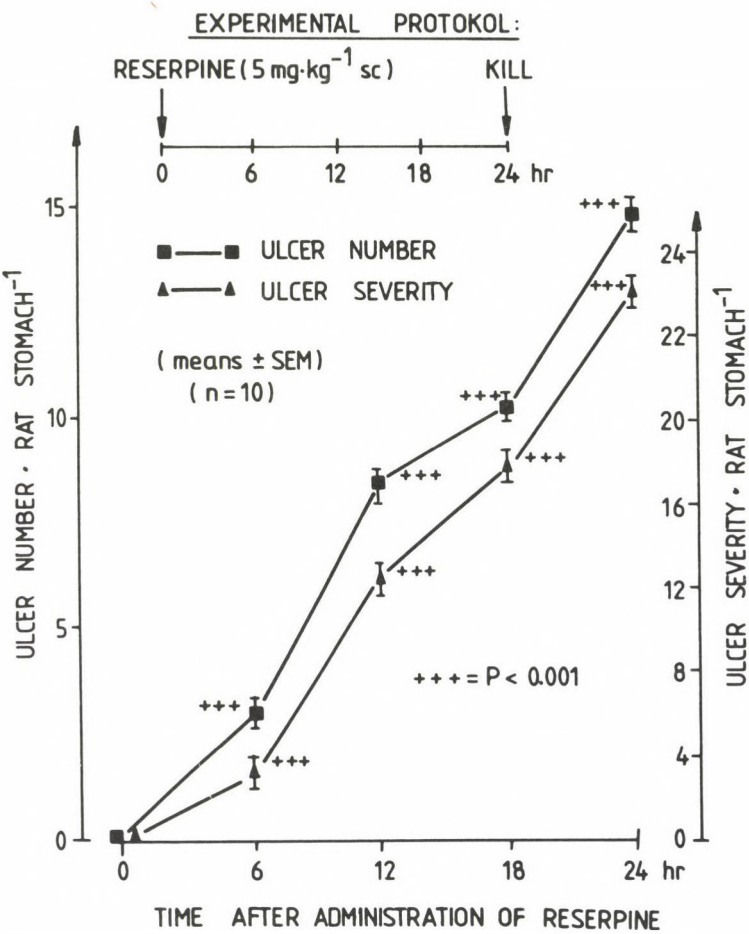


Fig. 4. Ulcer appearance in rat gastric fundic mucosa on time-dependence of time after reserpine (5 mg/kg, s.c.) administration (from results published by Mózsik et al., Acta Physiol. Hung. 62; 107–112, 1983)

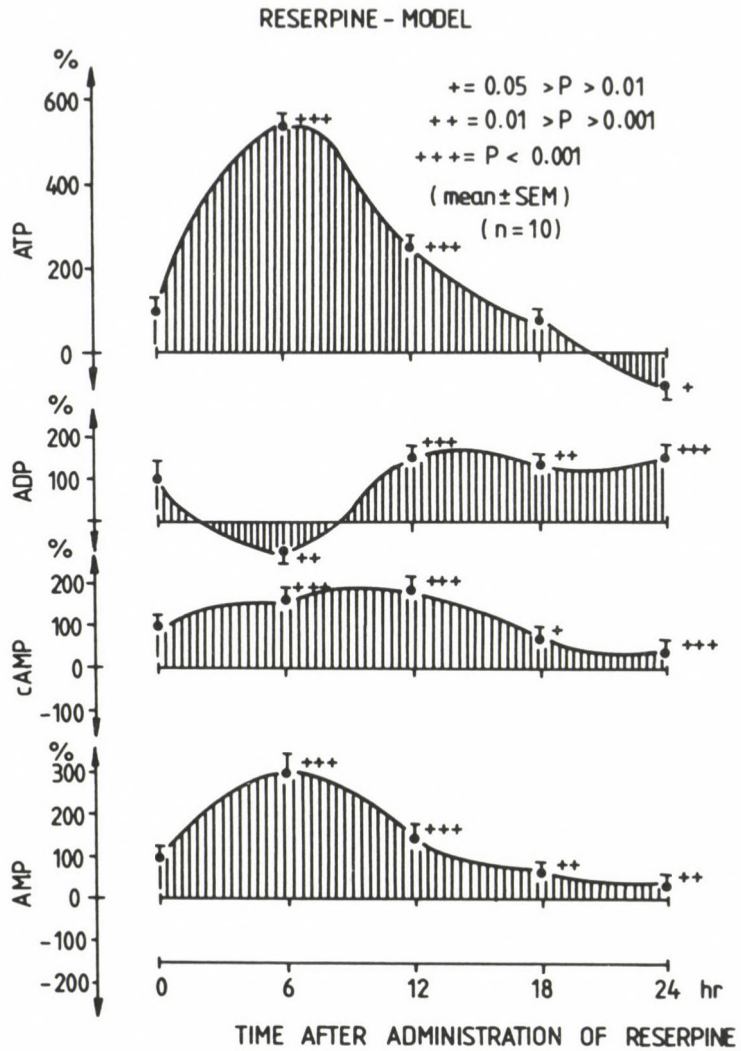


Fig. 5. Correlations between the gastric fundic mucosal levels of ATP, ADP, AMP (calculated in nmoles/mg protein) on time dependence after administration of reserpine (5 mg/kg, s.c.). The values are expressed in percent values of gastric mucosal levels of adenosine nucleotides (values at 0 hr = 100%). P values are between the results obtained at 0 hr vs. at other times after reserpine administration (from results published by Mózsik et. al., Acta Physiol. Hung. 62; 107–112, 1983)

1.3.2. Stress-induced gastric mucosal damage

Nagy and co-workers created a new experimental model to mimic the stress-induced mucosal damage in rats (Fig. 6).

The gastric mucosal lesions appeared at 2 and 3 hrs after stress introduction (Fig. 7), however, the gastric mucosal cAMP increased significantly, which associated with the decrease of gastric mucosal ATP and ADP (Fig. 8).

The changes in the mucosal energy systems indicate the increase of ATP-cAMP transformation, which associates with the total inhibition of ATP-ADP transformation. Furthermore, the extent of ATP-cAMP transformation increased so that the substrate level for the adenylate cyclase (ATP) decrease significantly [48, 53–56].

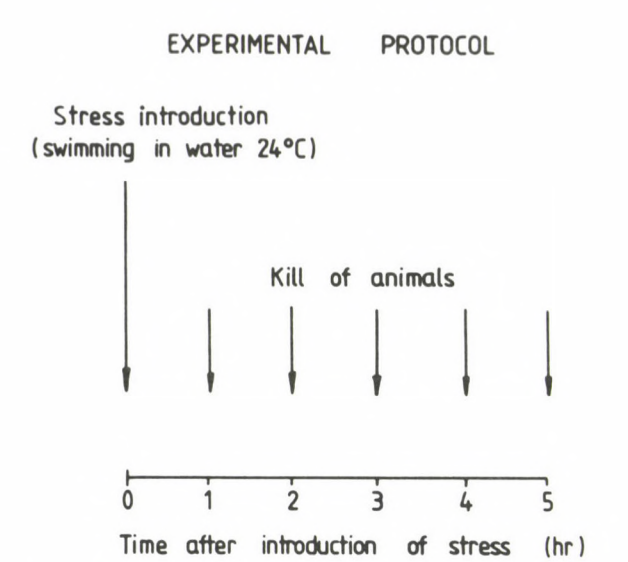
1.4.1. Prevention of the development of gastric hypersecretion and ulceration by acute surgical vagotomy or atropine treatment in pylorus-ligated rats

1.4.1.1. Surgical vagotomy

The acute surgical vagotomy inhibited the ATP-ADP transformation in association with the increase of tissue cAMP and decrease of gastric hypersecretion (by glandular stomach) and ulceration (by the forestomach) [32] (Table II).

1.4.1.2. Chemical (pharmacological) vagotomy

Atropine (given i.m. in doses of 0.1, 0.4, 1.0 mg/kg at the time of pyloric ligation) inhibited dose-dependently the gastric acid secretion, ulceration. The preventive effect of atropine is associated with the inhibition of ATP-ADP transformation and the increase of ATP-cAMP transformation (Table III & IV). It was interesting to note that atropine can inhibit the gastric mucosal biochemistry for 4–5 hours (after pyloric ligation), while its beneficial effects could be detected at 24 hours after pyloric ligation. This fact early indicates the importance of biochemical changes in gastric tissues before appearance of gastric ulceration [45, 46].



Kill of animals was carried out at 0, 1, 2, 3, 4 and 5 hr after introduction of stress

Measurements:

- number and severity of gastric mucosal lesions
- gastric mucosal level of ATP, ADP, AMP and lactate
- gastric mucosal level of cAMP by RIA

Calculations:

- ratio of ATP/ADP
- adenylate pool (ADP + ATP + AMP)
- „energy charge" = $\frac{ATP + 0.5\ ADP}{ATP + ADP + AMP}$

Fig. 6. Experimental protocol for study of stress-induced gastric mucosal lesions (from Mózsik et al., Ann. N.Y. Acad. Sci. 597; 264–281, 1990, with permission)

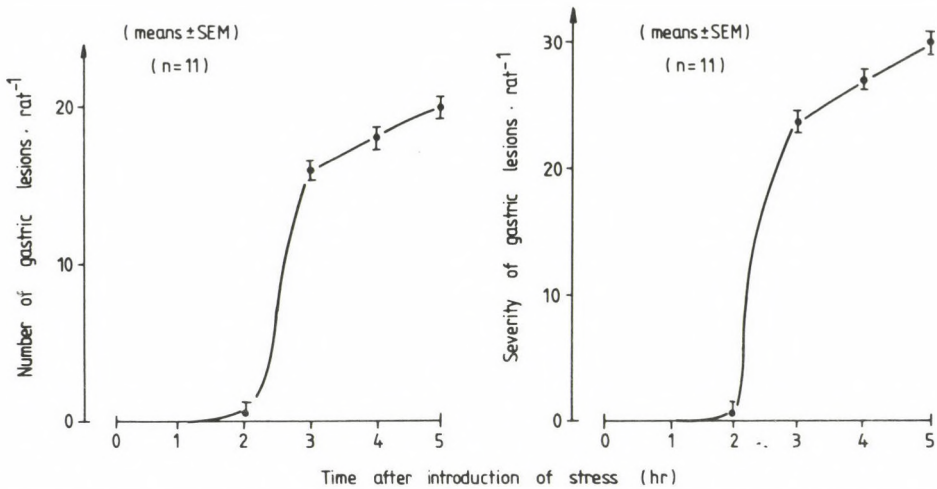


Fig. 7. Macroscopic appearance of gastric mucosal lesions (number and severity) after introduction of stress (means ± SEM) (from Mózsik et al., Ann. N.Y. Acad. Sci. 597; 264–281, 1990, with permission)

Table II

Correlations concerning the gastric acid secretion, ulceration and tissue levels of ATP, ADP in 24 hr pylorus-ligated rats, without and with acute surgical vagotomy. The results were expressed in per cent values of sham operated (tissue ATP, ADP) and 24 hr pylorus-ligated rats, without and with acute surgical vagotomy (gastric secretory responses, ulceration)

Examined parameters	Groups of animals: Sham-operated	Pylorus-ligated: without acute bilateral surgical vagotomy	Pylorus-ligated: with acute bilateral surgical vagotomy
Glandular stomach			
volume/24 hr		100 ± 13	13 ± 2***
H^+ concentration		100 ± 5	117 ± 18
H^+ output		100 ± 6	15 ± 2***
ATP	100 ± 4	21 ± 2***	67 ± 2***
ADP	100 ± 10	26 ± 2***	136 ± 15***
ATP/ADP	3.17	1.95***	1.55***
Forestomach (rumen)			
ulcers		100 ± 8	0 ± 0***
ATP	100 ± 9	36 ± 8***	177 ± 20***
ADP	100 ± 10	23 ± 6***	181 ± 20***
ATP/ADP	1.98	1.88	1.98

The results based on data of paper published by Mózsik and Vizi (Am. J. Dig. Dis. 22; 1072–1075, 1977). The biochemical results were calculated to the total glandular portion or forestomach (in nmoles) and expressed as means ± SEM.
P values were calculated concerning the results obtained in sham-operated vs. pylorus-ligated groups.
Abbreviations: ***: $p \leq 0.001$

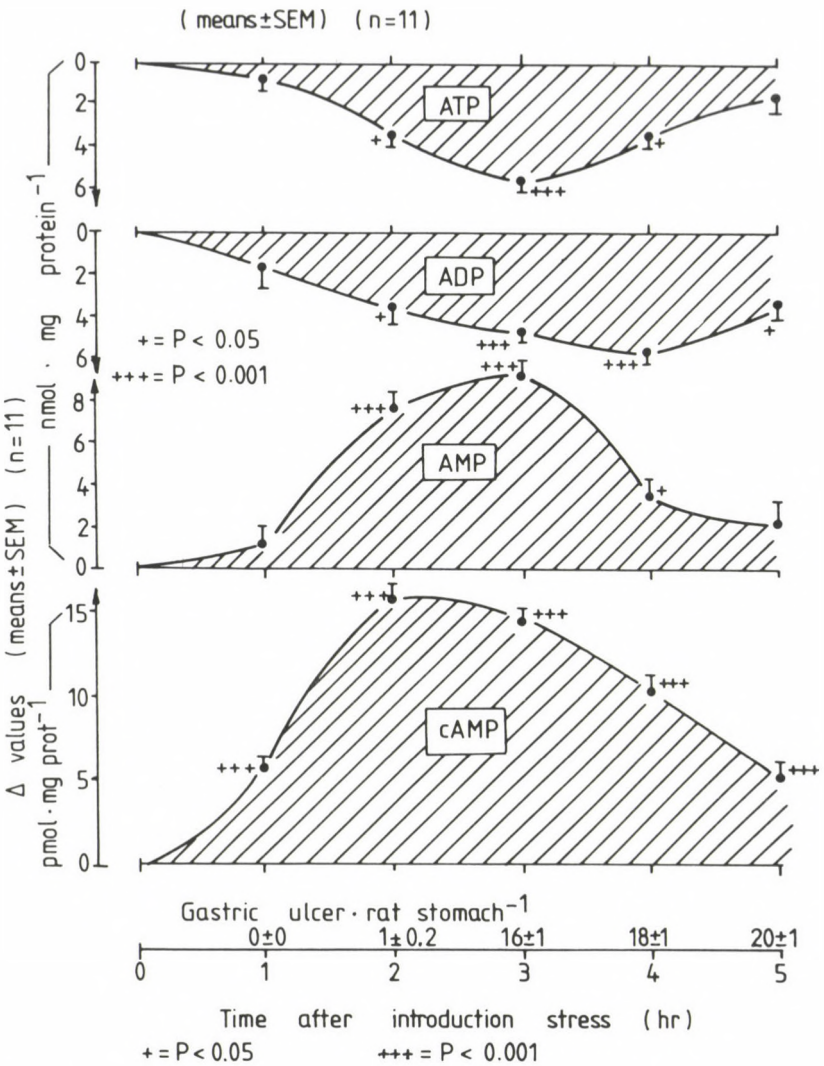


Fig. 8. Changes in gastric mucosal (fundic) levels of ATP, ADP, AMP (calculated in nmoles/mg protein) and cAMP (calculated in pmoles/mg protein) on time dependence after introduction of stress. The changes of adenosine nucleotides in the gastric fundic mucosa were expressed as Δ absolute values (means ± SEM) from the results obtained at 0 hr (from Mózsik et al., Ann. N.Y. Acad. Sci. 597; 264–281, 1990, with permission)

Table III

Correlations concerning the gastric acid secretion and tissue levels of gastric fundic mucosal ATP, ADP in 4 and 24 hr pylorus-ligated rats without and with atropine administration (atropine was s.c. given at the time of gastric surgery). The biochemical results were expressed in absolute values in nmol/mg mucosal protein (means ± SEM) (number of animals was 10-15 in each group)

Examined parameters	Groups of animals							
	Pylorus-ligated (control group)		Pylorus--ligated plus atropine-treated					
			0.1 mg/kg		1.0 mg/kg		5.0 mg/kg	
(Sham-operated group)	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h
	Glandular stomach							
Volume of gastric secretion (ml/100 g)	3 ± 0.5	11 ± 1	1.06 ± 0.1***	9 ± 1^{NS}	0.3 ± 0.1***	7 ± 1***	0.2 ± 0.1***	6 ± 1***
H^+ concentration (mEq/l)	66 ± 6	84 ± 4	66 ± 6^{NS}	80 ± 9^{NS}	68 ± 2^{NS}	80 ± 4^{NS}	69 ± 2^{NS}	81 ± 4^{NS}
H^+ output (mEq)	200 ± 30	924 ± 20	70 ± 10***	720 ± 90*	20 ± 10***	560 ± 40***	15 ± 5***	486 ± 50***
ATP (14 ± 1) (nmol/mg prot.)	7 ± 1***	2 ± 0.2***	9 ± 1**	6 ± 1***	11 ± 1**	8 ± 1***	12 ± 1**	10 ± 1*
ADP (10 ± 1) (nmmol/mg prot.)	17 ± 1***	4 ± 1***	8 ± 1***	15 ± 1*	9 ± 1^{NS}	13 ± 1*	10 ± 1^{NS}	10 ± 1^{NS}

The results were partially based on the results of paper published by Mózsik et al. (in Adv. Physiol. Sci. Vol. 29. Gastrointestinal Defence Mechanisms. Mózsik Gy., Hänninen O., Jávor T., eds, Pergamon Press, Oxford - Akadémiai Kiadó, Budapest, 1981, pp. 157—173).

p values were calculated concerning the results obtained in pylorus-ligated vs. pylorus-ligated plus atropine-treated groups at 4 and 24 hr, except of ATP and ADP when the p values were calculated concerning sham-operated vs. pylorus-ligated groups.

Abbreviations: NS: not significant, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$

Table IV

Correlations concerning the gastric ulceration and tissue levels of forestomach ATP, ADP in 4 and 24 hr pylorus-ligated rats, without and with atropine administration (atropine was given s.c. at the time of gastric surgery). The biochemical results were expressed in absolute values in nmol/mg mucosal protein (means ± SEM) (number of animals was 10-15 in each group)

Examined parameters	Groups of animals: Pylorus-ligated (control group)		Pylorus-ligated plus atropine-treated 0.1 mg/kg		1.0 mg/kg		5.0 mg/kg	
(Sham-operated group)	4 h	24 h	4 h	24 h	4 h	24 h	4 h	24 h
	Forestomach (rumen)							
Number of ulcers	0 ± 0	80 ± 2	0 ± 0	60 ± 6	0 ± 0	30 ± 2	0 ± 0	29 ± 2
ATP (9 ± 0.5) (nmol/mg prot.)	4.5 ± 0.5**	1 ± 0.3***	6 ± 1^{NS}	4 ± 0.5***	7 ± 1*	5 ± 0.5***	8 ± 1***	7 ± 1***
ADP (7 0.5) (nmol/mg prot.)	11 ± 0.5***	2 ± 0.3***	10 ± 1^{NS}	6 ± 0.5***	9 ± 1^{NS}	6 ± 0.5***	7 ± 1**	6 ± 1***

The results were partially based on the results of paper published by Mózsik et al. (in Adv. Physiol. Sci. Vol. 29. Gastrointestinal Defence Mechanisms. Mózsik Gy., Hänninen O., Jávor T., eds, Pergamon Press, Oxford - Akadémiai Kiadó, Budapest, 1981).

p values were calculated concerning the results obtained in pylorus-ligated vs. pylorus-ligated plus atropine-treated groups at 4 and 24 hr, except of ATP and ADP when the p values were calculated concerning sham-operated vs. pylorus-ligated groups.

Abbreviations: NS: not significant, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$

1.5. Biochemical changes in the gastric mucosa of pylorus-ligated plus aspirin-treated animals

1.5.1. Gastric mucosal biochemistry during development of gastric mucosal damage produced by intragastric aspirin in 4 hours pylorus-ligated rats

The gastric secretory responses were decreased significantly in pylorus-ligated plus aspirin-treated rats, however, the extent of ATP-ADP transformation was also decreased (Table V). On the other hand, the macroscopic appearance of gastric mucosal damage was detected in association with decreased gastric acid secretion and increased ATP-ADP transformation. Many of researchers are pointed out that this phenomenon is due to the increased H^+ backdiffusion [6, 40].

1.5.2. Biochemical background of atropine effect on gastric mucosal biochemistry in pylorus-ligated plus aspirin-treated rats

The gastric secretory responses and aspirin-induced mucosal damage could be inhibited dose-dependently by atropine, while the extents of ATP-ADP transformation could also be further decreased (Table VI).

1.6. Biochemical (energetical) background of human antral, duodenal and jejunal ulcers

In an earlier period (between 1970 and 1975) when it was possible to treat patients operated with gastric and small intestinal resection with anticholinergic agents. The resecates of stomach and small intestine were obtained (with and without ulcer) for biochemical measurements. The histology indicated a typical histology of peptic ulcer disease in all cases. The non-ulcerated parts (of the stomach and small intestine) were used as control tissue specimen for histologic and biochemical measurements. It was surprising to note that the tissue levels of ATP, ADP and AMP were significantly higher in the ulcerated tissues of antral and duodenal and jejunal mucosa than that in non-ulcerated part [23, 24, 28–30, 34, 39, 40, 46] (Table VII).

The therapeutic effect(s) of drug(s) can not be followed in these studies.

Table V

Biochemical background of aspirin-induced gastric mucosal injury in 4 hr pylorus-ligated rats

Experimental parameters	Sham-operated	4 hr pylorus-ligated		4 hr pylorus-ligated + aspirin (200 mg/kg in 150 mmol HCl, i.g.)
Volume (ml/100 g/4 hr)	-	1.9 ± 0.2		3.0 ± 0.8**
H^+ output (mEq/100 g/4 hr)	-	165 ± 15		110 ± 15*
Number of ulcers (1/rat stomach)	-	-		14 ± 2
ATP (nmol/mg prot.)	19 ± 2	4 ± 1***	xxx	11 ± 1***
ADP (nmol/mg prot.)	13 ± 1	18 ± 1.5**	xxx	6 ± 1***
ATP/ADP	1.46	0.22		1.83

The results based on data published by Fiegler et al. (Int. J. Tiss. Reac. 8; 15–22, 1986).

p values were calculated concerning the results obtained in sham-operated vs. pylorus-ligated groups.

Abbreviations: NS: not significant, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$

xxx: $p \leq 0.001$ between pylorus-ligated and pylorus-ligated plus aspirin-treated groups.

Table VI

Gastric mucosal preventive effect of atropine and its biochemical background in rats treated with aspirin (200 mg/kg dissolved in 2 ml of 150 mmol H^+, given i.g.) in 4 hr pylorus-ligated rats (n=10 - 12). The results were expressed as means ± SEM

Experimental parameters	Groups of animals			
	Aspirin-treated	Aspirin plus atropine-treated		
		0.1	0.5	1.0
		mg/kg given s.c. at the time of aspirin administration		
Volume (ml/100 g/4 hr)	3.1 ± 0.7	2.5 ± 0.1	2 ± 0.1	0.75 ± 0.1***
H^+ output (mEq/100 g/4 hr)	120 ± 20	100 ± 15	60 ± 7***	15 ± 5***
Number of ulcers (1/rat stomach)	14 ± 2	7 ± 1***	2 ± 1***	0 ± 0***
ATP (nmol/mg prot.)	11.5 ± 1.5	12 ± 1^{NS}	14 ± 1^{NS}	16 ± 1**
ADP (nmol/mg prot.)	6 ± 1	6 ± 1^{NS}	7 ± 1^{NS}	6 ± 1^{NS}
ATP/ADP	1.92	2.0	2.0	2.67

p values were obtained in aspirin-treated vs. aspirin plus atropine-treated groups.
Abbreviations: NS: not significant, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$

Table VII

Changes in gastrointestinal mucosal ATP, ADP metabolism and ATPase (Mg^{2+}-dependent, Mg^{2+}- Na^{+}- K^{+}-dependent and only Na^{+}- K^{+}-dependent parts) activity prepared from the non-ulcerated and ulcerated (in distance to 2.0 cm up to edge of ulcer) tissue specimens in patients suffering from "classical" peptic ulcer. The results are expressed in absolute values. The enzyme activity is expressed in mmoles/mg membrane protein/hr, liberated by the enzyme (n=number of patients)

Biochemical parameters	Gastric ulcer (n=12)		Duodenal ulcer (n=9)		Jejunal ulcer (n=6)	
	control	ulcerated	control	ulcerated	control	ulcerated
	mucosal specimens					
ATP (nmol/mg DNA)	25 ± 5	72 ± 18*	15 ± 5	39 ± 5***	12 ± 4	39 ± 5**
Mg^{2+}-dependent	1.5 ± 0.2	2.8 ± 0.5***	1.8 ± 0.2	2.4 ± 0.2*	1.2 ± 0.2	2.8 ± 0.5*
Mg^{2+}- Na^{+}- K^{+}-dependent	1.9 ± 0.2	4.3 ± 0.6***	2.9 ± 0.3	5.0 ± 1.0**	2.8 ± 0.7	5.2 ± 0.8**
Na^{+}- K^{+}- dependent	0.4 ± 0.1	1.5 ± 0.2***	1.1 ± 0.2	2.6 ± 0.3***	1.6 ± 0.2	2.4 ± 0.3**
ADP (nmol/mg DNA)	17 ± 3	54 ± 10*	21 ± 4	33 ± 4*	21 ± 8	33 ± 4^{NS}
ATP/ADP	1.47 ± 0.12	1.33 ± 0.11^{NS}	0.71 ± 0.06	1.18 ± 0.09***	0.57 ± 0.04	1.18 ± 0.20**

The results are based on data published by Mózsik et al. (Drugs Exptl. Clin. Res. 5; 27–42, 1979; Acta Med. Acad. Sci. Hung. 37; 39–49, 1980; Acta Med. Acad. Sci. Hung. 38; 129–134, 1981).

p values were obtained in control (non ulcerated) vs. ulcerated groups.

Abbreviations: NS: not significant, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$

2. ATP breakdown vs. vascular permeability

2.1. Pyloric ligation

No vascular permeability was obtained in the gastric mucosa at 4 hr after pyloric ligation, when the tissue level of ATP significantly decreased (without the presence of ulceration), but increased vascular permeability was not noticed either [24, 31, 35].

2.2. Chemical agents-induced gastric mucosal damage

Macroscopically it is well detected that the gastric mucosa indicates an edematous state, when the ulceration is appeared. This event is associated with ATP breakdown [43, 45].

In the observations the planimetric measurement is done by a computer-assisted system, but after formalin fixation. Generally we obtain about 20–25% ulcerated surface of gastric mucosa, however, this area is significantly higher without formalin fixation.

2.3. Pyloric ligated plus aspirin-treated rats

Independently on the suggested increased H^+ backdiffusion no visible changes can be seen in the gastric mucosa (without and surgical and chemical vagotomy) (Tables V and VI). The tissue level of ATP decreased, while its level was increased by chemical and surgical vagotomy.

2.4. Chronic antral, duodenal, jejunal ulcers in patients

Tissue edema can be noticed in all types of human chronic ulcer, the extent of ATP-ADP transformation increases significantly in the ulcerated antral, duodenal and jejunal mucosa (Table VII).

3. Tissue hypoxia vs. vascular permeability

There are different circumstances in which the significant decrease of tissue ATP and changed vascular permeability are together during the development of different experimental ulcers (chemicals-induced gastric mucosal damage). There is an other question whether the increased vascular permeability is obligately or facultatively associated with presence of tissue hypoxia. Biochemically the tissue

hypoxia can be characterized by the elevation of tissue level of lactate and significant decrease of tissue ATP due to insufficient oxidative phosphorylation [43].

3.1. Pylorus-ligated rats

No increase of tissue level of lactate was noticed in the gastric mucosa (including the glandular portion and rumen) in association with decrease of tissue level of ATP preceding the development of gastric mucosal damage (Figs 1 and 2).

3.2. Chemical agents-induced gastric mucosal damage

The vascular permeability was increased in the gastric mucosa of rats, after administration of necrotizing agents, however no any increase of tissue lactate was found in both before and after macroscopic appearance of gastric mucosal lesions [51].

3.3. Epinephrine-induced gastric ulcer

This model was created by Sethbakdi et al. in 1970. The epinephrine was s.c. given at 4 hours after pyloric ligation in rats, and the animals were killed 1 hour after epinephrine administration. Many mucosal lesions were found, however, the mucosal level of lactate did not elevate [52].

4. Main biochemical changes in the gastric mucosa and development of mucosal damage

The main questions are whether the measured changes in gastric mucosal biochemistry are causes of mucosal damage or the gastric mucosal injury produces changes in the gastric mucosal energy systems. We can answer to these questions only by the time-related analysis of the results (biochemical and pathological ones).

This type of analysis was done in case of pyloric ligated rats [21, 24, 35] 96% ethanol- [47], 0.6M HCl-, indomethacin- (unpublished results), reserpine- [44] or stress-induced [48] gastric mucosal damage, and the changes in tissue ATP metabolism preceded the development of gastric mucosal injury.

5. ATP metabolism vs. starvation

Earlier Nagy and co-workers [53–55] and Morón and co-workers [14] indicated that the epinephrine-, aspirin-, pyloric-ligation or 0.6M HCl-induced gastric

mucosal lesions are more serious if the animals were fasted for a longer time before starting the experiments, however, their gastric secretory responses decreased significantly depending on the starvation time. Naturally the starvation-induced changes in the gastric mucosa have been suggested to be in the background of this phenomenon (Tables VIII – X).

The animals were not fasted (they received 5% glucose solution for 24 hours) or fasted for 24 and/or 48 hours (but they received water ad libitum) before the experiments. The tissue levels of gastric mucosal ATP, ADP, AMP and cAMP were measured, while the ratio of ATP/ADP was calculated. Surprisingly the tissue level of ATP increased by the time of starvation, however the tissue levels of ADP and AMP decreased during this time. The ratio of ATP/ADP increased significantly, while the tissue level of cAMP remained unchanged. With other words, the adenosine compounds can be found in phosphorylated form in starved animals, meanwhile the breakdown of ATP (at least in direction to ADP) is inhibited (Fig. 9).

The number of 0.6M HCl-induced gastric mucosal injury increased by starvation.

6. ATP breakdown vs. acute hyperalimentation with glucose

The details of another part (side) of point 5 were analysed in rats with acute glucose hyperalimentation [14]. The animals received tap water, 5% or 20% glucose for 24 hours before the experiments.

The extent of 0.6M HCl-induced gastric mucosal injury can be prevented by acute glucose hyperalimentation (Fig. 10).

It was also interesting to note biochemically that: 1.) Tissue level of ATP was increased by hyperalimentation in association with increased level of ADP and cAMP and AMP. The conclusion is that the extents of ATP-ADP, ATP-cAMP and cAMP-AMP were increased in rats with acute glucose hyperalimentation (Fig. 11).

Table VIII

Starvation-induced changes in 17 hr pylorus-ligated rats (gastric secretory responses, ulcer development)

Experimental parameters	Time of starvation (hr)		
	0	24	48
	Glandular stomach		
Volume of gastric secretion (ml/100 g/17 hr)	11 ± 1	7 ±1***	7 ± 1***
H^+ concentration (mEq/l)	84 ± 4	90 ± 6^{NS}	90 ± 6^{NS}
H^+ output (mEq/100 g/17 hr)	924 ± 20	630 ± 30***	630 ± 30***
	Forestomach		
Incidence of ulcers (%)	70	85	98*
Number of ulcers	6 ± 1	9 ± 3	17 ± 2***
Number of perforations	0	2	4

The results were based on results of the paper published by Nagy et al. (in Advances in Physiol. Sci. Vol. 29. Gastrointestinal Defence Mechanisms. Mózsik Gy., Hänninen O., Jávor T. (eds) pp. 117—126. Pergamon Press, Oxford – Akadémiai Kiadó, Budapest, 1981). p values were obtained in 0- vs. 24- or 48-hr groups.

Abbreviations: NS: not significant, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$

Table IX

Starvation-induced changes in parameters of epinephrine-induced (0.4 mg/kg, given 4 hr after pylorus ligation, and the animals were killed 1 hr after administration of epinephrine)

Examined parameters	Time after starvation (hr)		
	0	24	48
Volume of gastric secretion (ml/100g/5 hr)	2.5 ± 0.4	2.6 ± 0.4^{NS}	2.3 ± 0.3^{NS}
H^+ concentration (mEq/l)	64 ± 3	69 ± 4^{NS}	80 ± 5*
H^+ output (mEq/100g/5 hr)	160 ± 10	179 ± 15^{NS}	184 ± 15^{NS}
Incidence of ulcer (%)	70	85	100
Number of ulcers	0.5 ± 0.1	0.8 ± 0.1*	1 ± 0.1***
Severity of ulcers	2.5 ± 0.2	3.9 ± 0.2***	4 ± 0.2***

The results were based on results of the paper published by Nagy et al. (in Advances in Physiol. Sci. Vol. 29. Gastrointestinal Defence Mechanisms. Mózsik Gy., Hänninen O., Jávor T. (eds) pp. 117–126. Pergamon Press, Oxford–Akadémiai Kiadó, Budapest, 1981).
p values were obtained in 0- vs. 24- or 48-hr groups.
Abbreviations: NS: not significant, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$

Table X

Starvation-induced changes in parameters of aspirin-induced gastric functions in 5 hr pylorus-ligated rats (the animals received 100 mg/kg aspirin i.g. at the time of pylorus ligation, and they were killed 5 hr after pyloric ligation)

Examined parameters	Time after starvation (hr)		
	0	24	48
	Glandular stomach		
Volume of gastric secretion (ml/100g/5 hr)	3.8 ± 0.3	4.0 ± 0.3	3.0 ± 0.3
H^+ concentration (mEq/l)	90 ± 5	94 ± 4	70 ± 9
H^+ output (mEq/100g/5 hr)	342 ± 20	376 ± 20	210 ± 15***
Incidence of ulcer (%)	2	80	90
Number of ulcers	0.1 ± 0.1	3.8 ± 0.5*	5 ± 1***
Severity of ulcers	0.2 ± 0.1	5.0 ± 0.4***	11 ± 1***

The results were based on results of the paper published by Nagy et al. (in Advances in Physiol. Sci. Vol. 29. Gastrointestinal Defence Mechanisms. Mózsik Gy., Hänninen O., Jávor T. (eds), pp. 117–126. Pergamon Press, Oxford–Akadémiai Kiadó, Budapest, 1981). p values were obtained in 0- vs. 24- or 48-hr groups.

Abbreviations: NS: not significant, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$

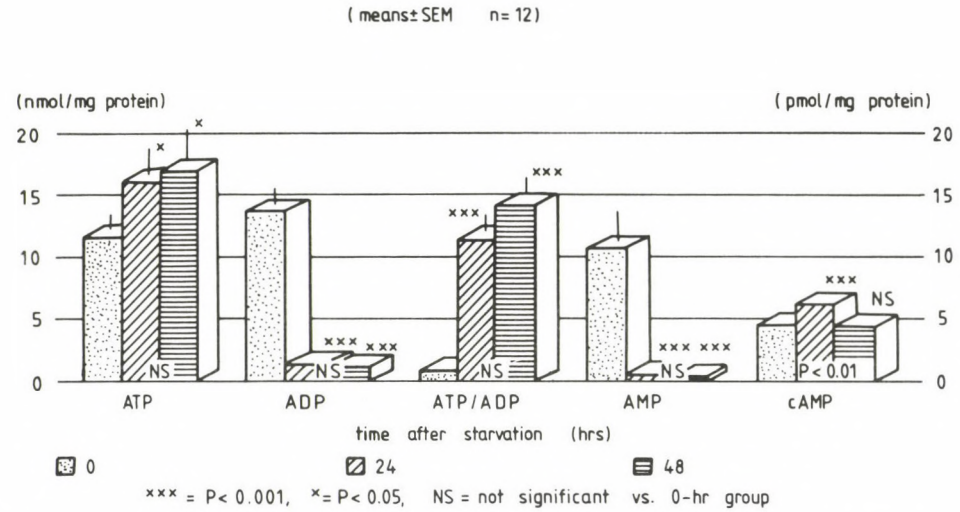


Fig. 9. Changes in gastric mucosal energy systems produced by 24 and 48 hr starvation. The results were expressed as means ± SEM. The results based on results published by Morón et al. (Acta Med. Hung. 41; 253–258, 1984)

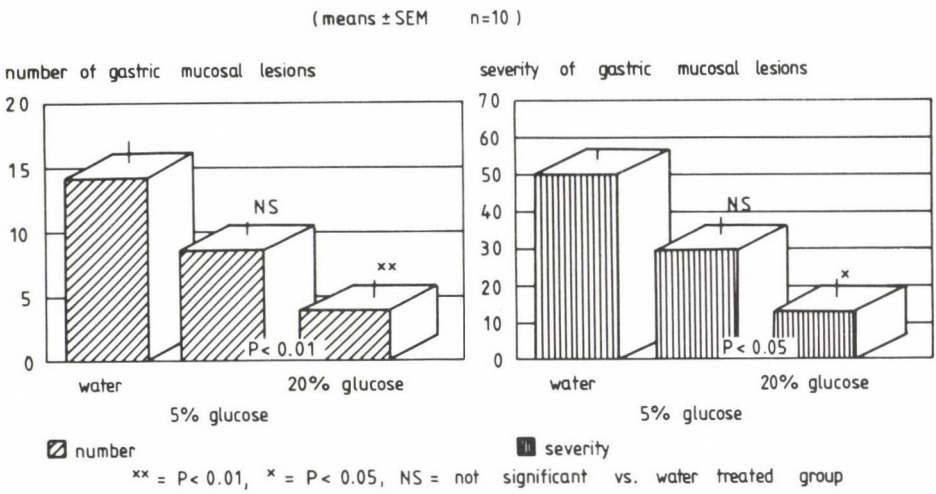


Fig. 10. Gastric mucosal prevention of rats produced by acute hyperalimentation with glucose. The results were expressed as means ± SEM. The results based on results published by Morón et al. (Acta Med. Hung. 41; 253–258, 1984)

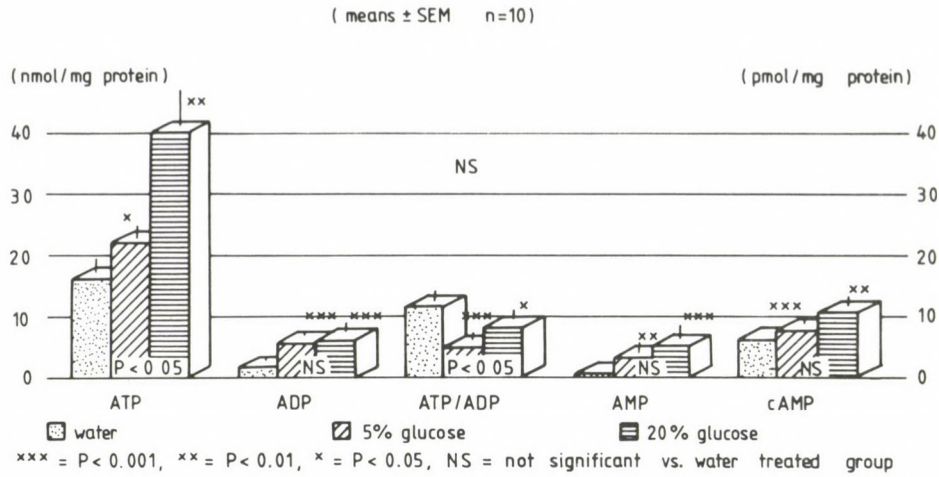


Fig. 11. Changes in gastric mucosal energy systems produced by acute hyperalimentation with glucose. The results were expressed as means ± SEM. The results based on results published by Morón et al. (Acta Med. Hung. 41; 253–258, 1984)

7. ATP metabolism vs. chemical or surgical vagotomy

The acute surgical vagotomy prevented the ATP breakdown in pylorus ligated rats and the gastric secretory responses decreased [28, 32].

No significant changes were obtained in the gastric mucosa at 1 1/2 hour after bilateral surgical vagotomy, without the application of any endogenous or exogenous necrotizing agents to the gastric mucosa [50], however, chemical vagotomy (atropine treatment) can decrease gastric mucosal level of ATP in association with a significant elevation of cAMP, while ADP level decrease [50]. It means that the ATP-cAMP transformation works, whereas the ATP-ADP transformation is inhibited by atropine administration without giving any necrotizing agents to gastric mucosa (Figs 12 and 13). These results suggest that an essential difference exists in the gastric mucosal biochemistry produced by surgical or chemical (atropine) vagotomy. Similar results were obtained earlier too [15, 17, 18, 27].

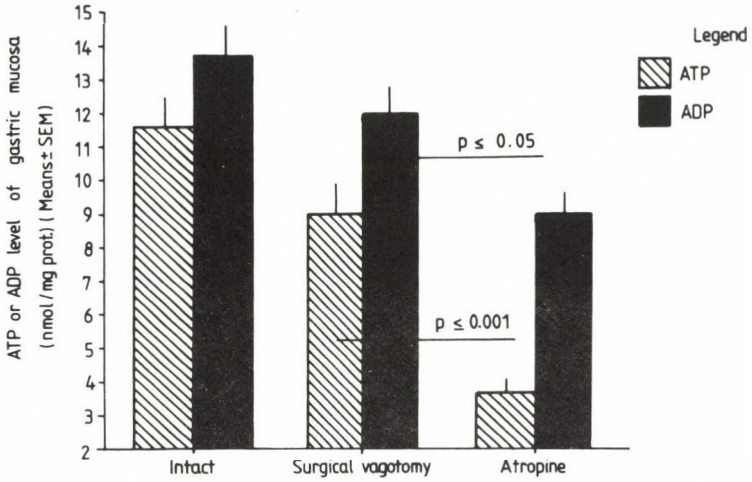


Fig. 12. Changes in the gastric mucosal ATP and ADP produced by acute bilateral surgical and chemical (atropine at a dose of 1.0 mg/kg sc.) vagotomy in intact animals. The results are based on results published by Mózsik et al. (J. Clin. Gastroenterol. 14 (Suppl. 1.); S135 – 139, 1992)

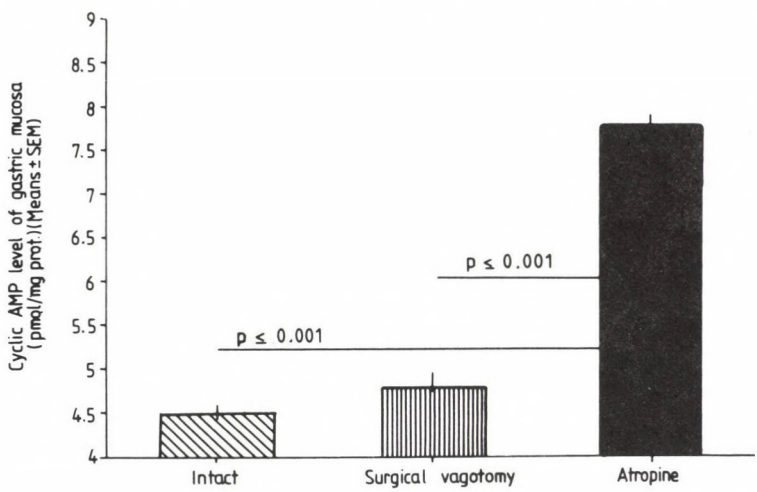


Fig. 13. Changes in gastric mucosal cAMP produced by acute bilateral surgical and chemical (atropine at a dose of 1.0 mg/kg sc.) vagotomy in intact animals. The results are based on results published by Mózsik et al. (J. Clin. Gastroenterol. 14 (Suppl. 1.); S135 – 139, 1992)

When ethanol or HCl, as gastric mucosal necrotizing agents, were i.g. given after surgical vagotomy, then the early vascular reaction became higher in association to the increased number (and severity) of agents-induced mucosal injury [50]. Our preliminary results indicate that the ethanol-induced biochemical changes in the gastric mucosa are more severe in vagotomized rats than in rats with intact vagus nerve. The decreased gastric mucosal adaptation to chemicals was also observed by Miller et al. [11, 12].

Discussion

The energy storing ATP molecule has a central role in the physiological and pathological regulation of cells (Fig. 14).

Many experiments clearly indicate that ATP breakdown is basically necessary for energy liberation. The gastric hypersecretion in pylorus ligated rats is an ATP energy liberation process, consequently the ATP level is very low. If the ATP level is high in the gastric mucosa of pylorus-ligated rats, no gastric secretory response occurs [31, 32].

In other case, in animals treated with 96% ethanol, 0.6M HCl, 0.2M NaOH or 25% HCl, the ATP level in the gastric mucosa is very low in association with increased level of ADP and clinical manifestation of gastric mucosal lesions. So, the tissue level of ATP decreased (because of the breakdown into ADP), representing an active metabolic event in the development of mucosal damage. If the rats underwent an acute surgical vagotomy, the acute metabolic response by the rat stomach disappeared (Mózsik et al. unpublished data).

Interestingly, the active metabolic adaptation of gastric mucosa (during the treatment of animals with mucosal damaging agents, that is in time of the development of mucosal lesions) can be further increased by prostacyclin [43, 45], and it can be decreased by atropine, cimetidine [45].

Surprisingly, the mucosal lesions can be prevented by both ways: a.) a further increased metabolic response of the stomach or b.) a decreased metabolic adaptation of gastric mucosa to chemicals (ethanol, HCl, NaOH, NaCl).

The essential role of ATP breakdown (into ADP) is emphasized in pylorus-ligated rats and in animals treated with ethanol, HCl, NaOH or NaCl [31, 43].

When the ATP breakdown is inhibited in pylorus-ligated plus aspirin-treated rats, a small gastric secretory response was observed. However, this fact can be explained only by the decreased ATP-ADP turnover, and not by the increased H^+ backdiffusion [6, 40]. Another interesting situation is that this small secretory response (in pylorus-ligated plus aspirin-treated animals) can be increased by atropine administration. So the decreased energy turnover can be further decreased by atropine, which results in a "prevention" for the gastric mucosa against the effect of aspirin plus endogenous HCl.

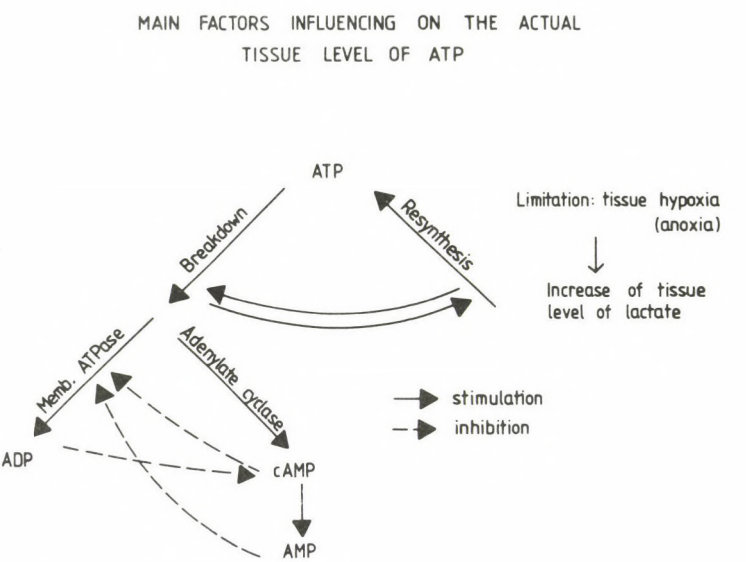


Fig. 14. Regulatory pathways of actual level of tissue ATP in the gastric mucosa (under different experimental circumstances) *(a schematic figure)*

Similar metabolic trends are present in the gastric mucosa of animals treated with reserpine and stress, during the development of mucosal damage, however, the energy liberation is done by ATP-cAMP transformation. The ATP-cAMP turnover associates with the complete blockade of ATP-ADP transformation and a significant decrease of tissue ATP. One major argument, against the presence of tissue hypoxia in the gastric mucosa, is that the tissue level of ATP increases in the later time, when gastric mucosal injury is detected. These results indicate a good equilibrium between the increased ATP breakdown and increased ATP resynthesis in the gastric mucosa, excluding the failure of oxidative phosphorylation.

There are a lot of pharmacological ways to inhibit (to prevent) the reserpine- and stress-induced gastric mucosal lesions, e.g. application of anticholinergic, β-blocking compounds. It was also interesting to note that the stress-induced gastric mucosal lesions could be prevented (or decreased) by pyloric ligation. The results of these experiments indicate that the ATP-ADP transformation represents the primary line of energy liberation in the gastric mucosa, whereas the ATP-cAMP transformation works only (or dominantly) after the blockade of ATP-ADP transformation.

Metabolic adaptation of gastric mucosa to acute mucosal injury:

I. Increased energy liberation (positive metabolic adaptation):

I.1. increased ATP-ADP transformation:

pylorus-ligated rats,

chemical-induced gastric mucosal injury,

I.2. increased ATP-cAMP transformation:

reserpine ulcer,

stress ulcer.

II. Decreased energy liberation (negative metabolic adaptation):

II.1. pylorus ligation plus aspirin treatment,

II.2. chemical-induced gastric mucosal lesions in surgically vagotomized animals.

Both ways of gastric mucosal adaptation represent a disequilibrium between the ATP breakdown and resynthesis. These pathways of metabolic responses of the gastric mucosa precede the macroscopic appearance of acute gastric mucosal injury. However, we can decrease the metabolic adaptations of tissues, than we can prevent of aggravate the development of acute mucosal damage. There is also an essential fact that when we try to modify the gastric mucosal metabolism, namely before or after application of necrotizing physical, chemical agents of stress. If the acute surgical vagotomy is done before administration of chemical agents, the metabolic adaptation is less (and the severity of macroscopic lesions is high). Biochemically, the extent of ATP-ADP transformation is inhibited by acute surgical vagotomy [32]. These days it is not known whether (at least in details) the surgical vagotomy has a beneficial effect on gastric mucosa in patients, however, the vagus nerve has an essential role in cyto- and general mucosal protection in animal experiments [8, 49].

It is important to emphasize that drugs modify the ATP-ADP and ATP-cAMP systems in different doses (the ATP-ADP system requires low doses and higher doses are needed to ATP-cAMP transformations), and these actions are reliable to intact stomach mucosa (when the drugs were given). The main steps of pharmacological modification of ATP breakdown are detailed in Table XI.

Table XI

The main steps of pharmacological modification of ATP breakdown in the gastrointestinal mucosa

Decrease of ATP-ADP transformation by atropine	Effects on ulcer development
pyloric ligated rats	prevention
aspirin-induced gastric mucosal injury	prevention
chemical-induced gastric mucosal injury	prevention
stress-induced gastric mucosal lesions	prevention
reserpine-induced gastric mucosal lesions	prevention
Decrease of ATP-cAMP transformation by H_2-receptor blockers	
pyloric ligated rats	aggravation
aspirin-induced gastric mucosal injury	aggravation
chemicals-induced gastric mucosal injury	aggravation
stress-induced gastric mucosal lesions	aggravation
reserpine-induced gastric mucosal lesions	aggravation
Increased ATP-ADP transformation by intact vagus nerve	
pyloric ligated rats	aggravation
Increase of ATP-cAMP transformation by PGI2	
chemicals-induced gastric mucosal injury	prevention

Recently, we have observed that the same doses of epinephrine produce significantly different changes in the gastric mucosa (in association with different macroscopic pathology) if the stomach is in different functional states [52].

There are a few questions, which were analysed in different experimental ulcer models:

1.) Speed of ATP breakdown vs. vascular reactions of the gastric mucosa

If the energy liberation is guided it is followed by an early vascular reaction (e.g. chemical-induced vascular permeability), however, it is prevented by PGI_2 (which produces a further ATP breakdown, without elevation of tissue lactate). This

sample supplies one more argument for the dissociation of ATP breakdown, changes in vascular permeability and tissue lactate.

Other observations indicate that the low rate of energy liberation associates with the late change in mucosal vascular permeability (pyloric ligated rats, reserpine- and stress-induced gastric mucosal injury).

2.) Correlations between ATP breakdown, changes in vascular permeability and tissue hypoxia

Biochemical pieces of evidence for tissue hypoxia in the gastric mucosa are as follows:

a.) Increased level of lactate,

b.) Failure of ATP resynthesis due to impaired oxidative phosphorylation.

It is important to note that the low level of ATP alone is not a biochemical signal for the existence of tissue hypoxia in the gastric mucosa.

The significant decrease of tissue ATP can be found in different experiments at early state of ulcer development, however, its level is elevated later (pyloric ligated rat, ethanol model, stress- or reserpine-induced mucosal damage) [47, 48].

The following two explanations exist for the absence of elevation in lactate levels in the gastrointestinal mucosa:

a.) The biochemical measurements are carried out from the total homogenate,

b.) The measurements from total homogenate can not exclude the focal existence of tissue hypoxia.

If the animals were hyperalimented with glucose then the numbers of chemical-induced gastric mucosal damage. The hyperalimentation with glucose produces significant changes in the ATP metabolism, which are described in Table XIII.

3.) Possible pathways for significant change of ATP metabolism in the gastric mucosal by alimentation

During short term (0 to 48 hr) starvation, the following metabolic adaptations can be noticed (See Table XII).

Table XII

Biochemical adaptation to starvation and its consequences in gastrointestinal mucosa

Adaptation
1. Total block of ATP-ADP transformation 2. Total block of cAMP-AMP transformation 3. Decrease of "adenylate pool" (ATP + ADP + AMP) 4. Adenosine compounds are in phosphorylated state
Consequences
1. Significant decrease of energy turnover 2. Increased vulnerability of gastric mucosa to chemicals

Table XIII

Biochemical adaptation to hyperalimentation and its consequences in gastrointestinal mucosa

Adaptation
1. Increase of ATP synthesis and turnover 2. Increased extent of ATP breakdown in direction to ADP and cAMP 3. Increase of adenylate pool (ATP + ADP + AMP) 4. Against increased energy turnover, the "energy charge" remained the same
Consequences
1. Increase of energy turnover 2. Significantly decreased vulnerability of gastric mucosa to chemicals

4.) Modification of ATP metabolism by chemical and surgical vagotomy

The modification of ATP metabolism by "surgical" and "chemical" vagotomy must be emphasized from two points of view:

a.) The decrease of aggressive factors (HCl, pepsin, etc.);

b.) Action on the defense of gastrointestinal mucosa to exogenous (chemicals, drugs) and endogenous (HCl, pepsin) mucosal damaging agents [42, 49].

The modification of ATP metabolism, produced by surgical vagotomy, can be connected with the decreased extent of aggressive factors (HCl, pepsin) in both animal experiments [42, 49] and clinical practice [8]. The situation is similar in the case of acute chemical vagotomy in animal experiments as well as clinical practice,

however, in the chronic anticholinergic treatment there are significant differences; namely that the phenomenon of pharmacological denervation and drug tolerance develop in patients [16, 17]. These phenomena (pharmacological denervation and tolerance to drug) disappear in 7–10 days after the cessation of anticholinergic drug [16]. The ATP metabolism, in background of acute and chronic surgical and chemical vagotomy, differs significantly in animal experiments [17, 18, 50].

Probably the changes in ATP metabolism, produced by surgical and chemical vagotomy, explain the separation (deviation) of chemical and surgical vagotomy to decrease the aggressive factors and to increase of defensive factors [49]. One of the most important recognitions is that both the aggressive and defensive sides depend on the intact vagal nerve, and these phenomena cannot be separated from each other [49]. By other words, after surgical vagotomy the extent of aggressive side significantly can be decreased, but gastric mucosal defense mechanisms – produced by prostaglandins, prostacyclin, β-carotine, small doses of atropine, and cimetidine – also disappear in association with the significant decrease of the aggressive side. On the other hand, the PGI_2-induced gastric cytoprotection can be enhanced by histamine and pentagastrin in animals with intact vagal nerve. These results suggest that the intact vagal nerve is basically necessary for energy liberation, which gives a biochemical basis of the metabolic adaptation of gastric mucosa to chemicals, drugs. If the cells are in surgically denervated status then the pharmacological regulation of gastric mucosal damage differs from the normal.

In case of pharmacological vagotomy, the pharmacological regulation of gastric mucosa cells in intact. That is an explanation for differences in biochemical changes in the gastric mucosa produced by surgical and chemical vagotomy, however, the details of these phenomena are unknown.

5.) The results obtained from the studies of ATP breakdown emphasize the prominent role of physiological, neural and pharmacological regulation in the development of metabolic adaptation of gastrointestinal mucosa to chemicals, drugs, or endogenous aggressive chemical (HCl). We could not prove the presence of tissue hypoxia (by elevation of tissue level of lactate) in the injured (damaged) gastrointestinal mucosa. The physiological examinations indicate the decrease of local blood flow in the gastric mucosa [4, 7]. The classical biochemical examinations clearly indicated the presence of tissue hypoxia in animals with hemorrhagic shock [10].

The difference of the results obtained in physiological and biochemical studies can be explained in two different ways:

a) Really no tissue hypoxia exists in the ulcerated gastrointestinal mucosa based on the biochemical studies, carried out systematically (we are in this opinion), except in hypovolemic or hemorrhagic shock);

b) Just only small local tissue hypoxia exists in the gastrointestinal mucosa, during mucosal injury, which cannot be detected biochemically (depending on the sensitivity of the method and of the small piece of this mucosa with a suggested hypoxia).

The changes in gastrointestinal mucosal ATP metabolism have been studied by our team, under very different experimental and clinical circumstances from 1967.

The results of our observations offer to conclude the following general laws:

1. Both positive and negative metabolic processes can be found in the development of gastrointestinal injury;
2. The tissue hypoxia can be biochemically proved by the increased tissue level of lactate and low level of ATP (produced by failure of ATP resynthesis). These biochemical phenomena indicate the tissue hypoxia, when they are present in the same time and tissue specimens;
3. The gastrointestinal mucosal biochemistry can be significantly modified by starvation or hyperalimentation with glucose, which associates with the decrease (starvation) and increase (hyperalimentation with glucose) of gastrointestinal mucosa to chemicals, drugs;
4. The acute "surgical" and "pharmacological" vagotomy produce different pathways of ATP regulation in the gastrointestinal mucosa;
5. The biochemical evaluation of possible role(s) of tissue ATP system can be done only by the simultaneous measurements of ATP, ADP, cAMP, AMP and lactate from the same tissue specimens, but used experimental circumstances.

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GASTRIC ANTI-ULCEROGENIC DRUG EFFECT. A POSSIBLE MECHANISM OF ITS MOLECULAR BASIS

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During experimental gastric ulceration in rats an elevation in the mucosal cAMP/cGMP ratio can be encountered. The cause of this significant elevation is mainly (but not entirely) the dramatic fall of the cGMP level.

Similar observations were obtained with prostacyclin application (100 μg/kg, p.o.), too. This prostaglandin derivative is well known, among others, because of its pronounced anti-ulcerogenic (cytoprotective) effect, too. Other substances of different molecular structure and properties may also exert such effect. The exact mechanism of action of this above-mentioned cytoprotection is still not completely understood.

H_2-receptor blocker drug cimetidine, given in such small dose (5 mg/kg, p.o.) which does not interfere with gastric acid secretion, also exerts very significant cytoprotective effect in stress (restraint)- and drug (indomethacin)-induced gastric ulcer models.

Under cimetidine effect – together with a noticeable endogenous prostacyclin mobilization – the gastric mucosal cAMP/cGMP ratio was also strongly elevated. We conclude that this elevation in the mucosal cAMP/cGMP ratio might be a possible molecular basis of the gastric cytoprotective (anti-ulcerogenic) drugs but it needs further investigations whether all substances exerting cytoprotective effect, e.g. atropine, somatostatin, sulfhydryl drugs, etc., have the same "shifting" property or not? Moreover the phenomenon of the so-called "adaptive cytoprotection" can not be ruled out completely either, therefore this problem needs attention, too.

Keywords: gastric ulceration, cAMP, cGMP, prostacyclin, cimetidine, cytoprotection, anti-ulcerogenic effect

Bálint and co-workers raised the possibility that during gastric ulceration a pronounced elevation in the mucosal cAMP/cGMP ratio might be one of the indicators of the anti-ulcerogenic, reparative processes in the rat.

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According to their investigations the cause of this significant elevation was mainly (but not entirely) the dramatic fall of the cGMP level [2, 4, 5].

Similar observations were obtained with PGI_2 application [7]. This prostaglandin derivative is well known, among others, of its pronounced antiulcerogenic (cytoprotective) effect [1, 14].

Cimetidine the other well-known anti-ulcerogenic, H_2-receptor blocker drug showed a similar, gastric mucosal cAMP/cGMP ratio "shifting" property, too, in due course of gastric ulceration elicited either by STR (restraint) or drug (indomethacin) [8].

Table I

Experimental design

Group	n	Intervention
CONTROL	5	Normal, untreated animals. cAMP, cGMP estimation.
IND-ULCER		
60 min	2x5	30 mg/kg IND i.p. and CIM *or* PGI_2 treatment at 0 min cAMP, cGMP estimation after 60 min of IND.
120 min	2x5	30 mg/kg IND i.p. and CIM *or* PGI_2 treatment at 0 min and 60^{th} min. cAMP, cGMP estimation after 120 min of IND.
240 min	2x5	30 mg/kg IND i.p. and CIM *or* PGI_2 treatment at 0 min, 60^{th} and 120^{th} min cAMP, cGMP estimation after 240 min of IND.
UNTREATED	6x5	30 mg/kg IND i.p. *No* treatment. cAMP, cGMP estimation after 60, 120 or 240 min of IND.
STR-ULCER		
8 hrs	2x5	cAMP, cGMP estimation after 8 hrs of immobilization. Meanwhile CIM *or* PGI_2 treatment at 0 min.
16 hrs	2x5	cAMP, cGMP estimation after 16 hrs of immobilization. Meanwhile CIM *or* PGI_2 treatment at 0 min and 8^{th} hour.
24 hrs	2x5	cAMP, cGMP estimation after 24 hrs of immobilization. Meanwhile CIM *or* PGI_2 treatment at 0 min, 8^{th} and 16^{th} hour.
UNTREATED	6x5	cAMP, cGMP estimation after 8, 16 or 24 hrs of immobilization. *No* treatment.

Adult female Wistar rats. Applied single doses: (p.o.)
PGI_2 – 100 μg/kg;
CIM – 5 mg/kg.

List of abbreviations used:
cAMP: 3'–5'-cyclic Adenosine Monophosphate;
CIM: Cimetidine;
cGMP: 3'–5'-cyclic Guanosine Monophosphate;
IND: Indomethacin;
PGI_2: Prostacyclin;
STR: Stress (restraint)

The experimental ulcer models used and the mode of evaluation were published earlier in details [1, 2, 3].

The mucosal levels of the cyclic nucleotides (cAMP, cGMP) have been measured by radioimmunoassay [2, 4, 7, 8].

Materials and methods

Our applied experimental design is presented in Table I.

Results and discussion

The experimental results are presented in Figs 1 – 4:

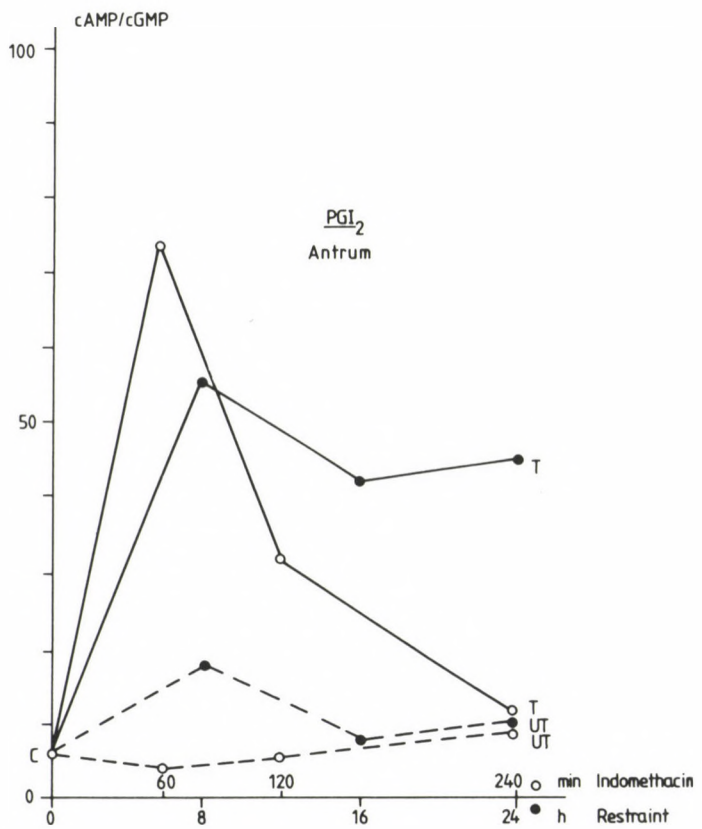

Fig. 1. The effect of PGI_2 on rat antral mucosal cAMP/cGMP ratios.
Dashed line (UT): Untreated animals; Solid line (T): Treated animals
C: Control (normal, physiological) value; o: Drug (Indomethacin)-induced ulceration; •: STR (restraint)-induced ulceration

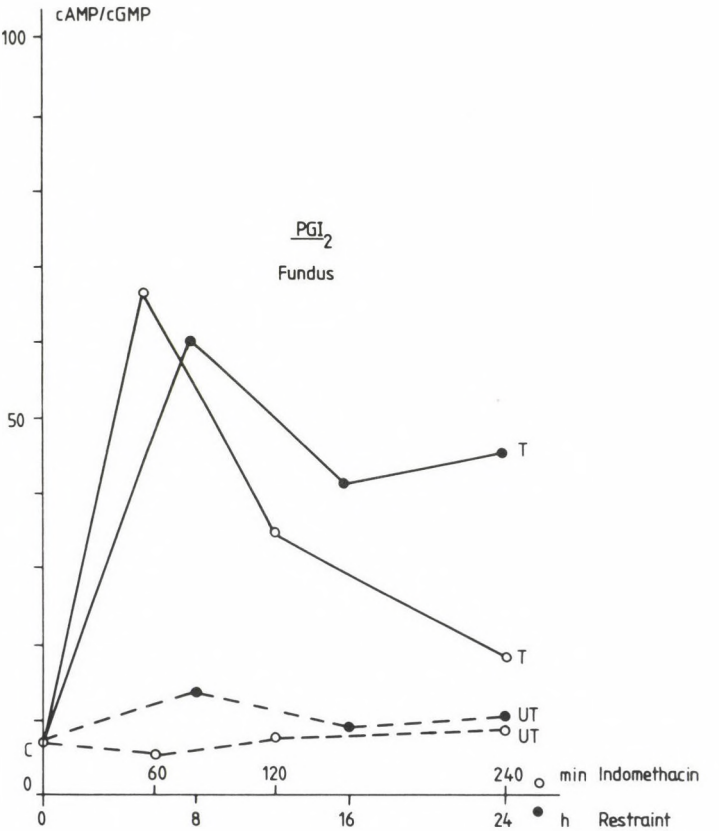

Fig. 2. The effect of PGI_2 on the rat fundic mucosal cAMP/cGMP ratios.
Dashed line (UT): Untreated animals;
Solid line (T): Treated animals;
C: Control (normal, physiological) value;
o: Drug (Indomethacin)-induced ulceration;
•: STR (restraint)-induced ulceration

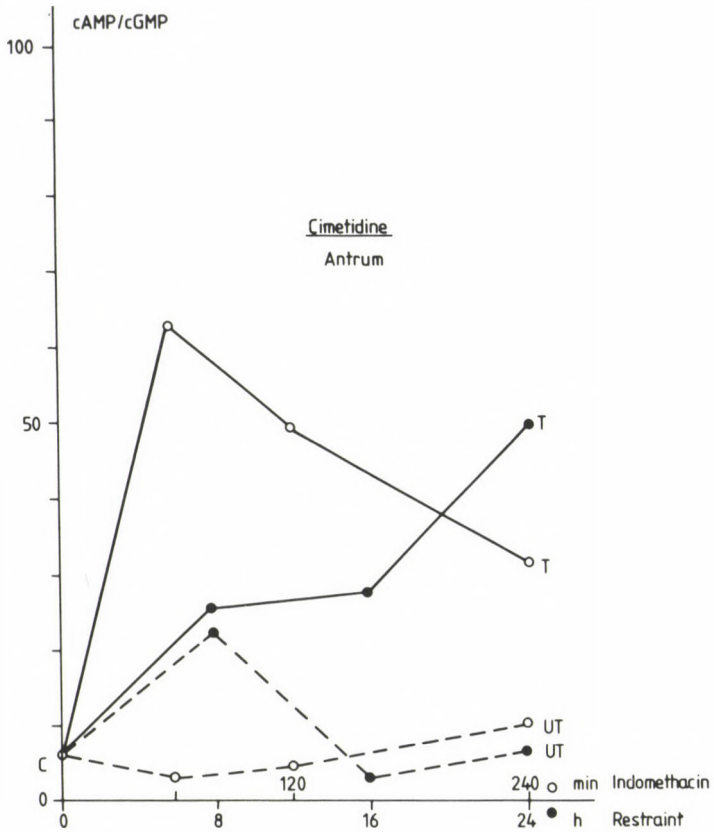

Fig. 3. The effect of CIM on rat antral mucosal cAMP/cGMP ratios.
Dashed line (UT): Untreated animals;
Solid line (T): Treated animals;
C: Control (normal, physiological) value;
o: Drug (Indomethacin)-induced ulceration;
•: STR (restraint)-induced ulceration

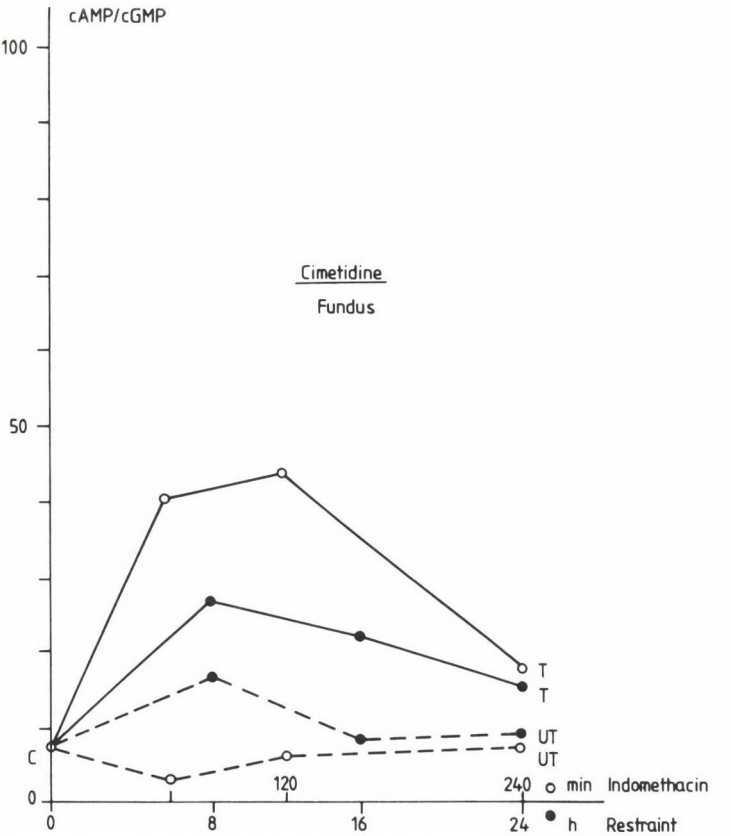

Fig. 4. The effect of CIM on rat fundic mucosal cAMP/cGMP ratios.
Dashed line (UT): Untreated animals;
Solid line (T): Treated animals;
C: Control (normal, physiological) value;
o: Drug (Indomethacin)-induced ulceration;
•: STR (restraint)-induced ulceration

The applied drugs evoked a very considerable elevation of the gastric antral and fundic mucosal cAMP/cGMP ratios.

On the basis of the data published in the literature, the cytoprotective, anti-ulcerogenic effect of prostaglandins is now indisputable but other substances of different molecular structure and properties (e.g. sulfhydryl drugs, atropine, H_2-receptor blockers, etc.) may exert also such effect [11, 12, 13].

The exact mechanism of action of this above-mentioned cytoprotection is still under debate and the cellular and/or molecular of the phenomenon is not completely understood.

It was also published that in due course of ulceration the gastric mucosal (antral and fundic) cyclic nucleotide metabolism and the so-called "intracellular second messenger system" are also involved together with a significant elevation in the cAMP/cGMP ratio [5, 8].

PGI_2 treatment had also the same effect [2, 4, 7]. As PGI_2 is one of the most potent anti-ulcerogenic (cytoprotective) drugs, it was concluded that a rise in the cAMP/cGMP ratio might be one of the indicators of the anti-ulcerogenic, reparative processes [5, 7, 8].

Hence H_2-receptor blocker drugs, as cimetidine, ranitidine, etc., given in such small doses which do not interfere with gastric acid secretion [10], also exert significant cytoprotective effect, the investigation of the possible changes of cyclic nucleotide turnover in the gastric mucosa under the effect of cimetidine, promised to be an intriguing problem.

It seems that cimetidine, similarly to PGI_2, also strongly elevates the gastric mucosal cAMP/cGMP ratio [8].

But on the other hand, the possibility which was published first by Bransky and co-workers [9] i.e. that cimetidine exerts its effect (at least partly) by the mobilization of different (mainly E-type) prostaglandins ("adaptive cytoprotection"), here at the discussion of our tenet, can not be ruled out completely either. Therefore it is a possibility that the encountered effects on the gastric mucosal cAMP/cGMP ratios are due (at least partly) to the cimetidine's adaptive cytoprotective effect.

Hence it needs further investigations whether all substances exerting cytoprotective effect, e.g. atropine, somatostatin, different sulfhydryl containing drugs, etc., have the same, cAMP/cGMP ratio elevating ("shifting") property or not?

From this point of view the sulfhydryl containing drugs are the most interesting ones, because it was established [6] that their cytoprotective, anti-ulcerogenic effect is not mediated via endogenous PGI_2, therefore the phenomenon of the "adaptive cytoprotection" most probably does not play a role in their anti-ulcerogenic effect.

Summing-up – the remaining questions are:

(a) Whether this (mainly) cGMP decreasing effect is a general property of all cytoprotective, anti-ulcerogenic substances?

(b) What is the physiological (or pathological) relevance (if there is any) of this change on cellular or molecular level?

Further investigations seem to be needed to clarify all details of this phenomenon, which could be the first attempt to give a molecular explanation of the gastric cytoprotective, anti-ulcerogenic drug effect.

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THE SIGNIFICANCE OF THE GASTROPROTECTIVE EFFECT OF BODY PROTECTION COMPOUND (BPC): MODULATION BY DIFFERENT PROCEDURES

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To elucidate any mechanism of a body protecting effect the observed events should be investigated also by the way of their modulations induced by ablation of particular organs. For Body Protection Compound (BPC), a newly partially characterized gastric juice peptide, the gastroprotection has largely been studied in basal and modified conditions of 48 h-restraint stress. Ablations or sham operations have been performed before restraint as follows: 60 min vagotomy, 24 h ovaria, testes, spleen, 48 h medulla of adrenal glands, 40 days thyroid + parathyroid glands. BPC (10 μg/kg) or saline (5 ml/kg) have been regularly applied (after surgery, 1 h before restraint) intraperitoneally. The group subjected to thyroparathyroidectomy received also once-daily BPC/saline treatment. A very strong gastroprotective effect in basal conditions has been modulated by ovariectomy and demedullation (abolishment), thyroparathyroidectomy (decrease), and no change occurred in case of vagotomy, splenectomy or orchidectomy. Sham operated rats did not differ from corresponding controls. Thus, seeing from point of view a wide range of organoprotective effects of BPC (intestinum, kidney, liver, pancreas, inflammation, diabetes mellitus, delayed type of hypersensitivity), the gastroprotection has been supposed a) to be of crucial pattern in the general concept of organo-protection and b) to be responsible for the mediation of the suggested "stomach stress organoprotective response". Therefore, the obtained modulations suggest a complex and specifical, sex-related action of the overall beneficial effects of BPC.

Keywords: Body Protection Compound (BPC), stomach stress coping response, organ ablations, modulations, gastroprotection, rats

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One of the most interesting concepts between the present concepts in pharmacology, pathology and pathophysiology is the concept of organoprotection (e.g. [37]). It is defined in terms of protection of different organs achieved after administration of organoprotective agents (e.g. [37]). So far this concept is very well defined for beneficial effects of prostaglandins, somatostatin and dopamine [2, 20, 21, 31–33, 37, 40, 43]. In this, in addition to previously described conventional concepts of organoprotection we defined a new organoprotective system, which is mediated by a new potent peptide [30]. It was isolated from human gastric juice, partially sequenced and because of its huge organoprotective activity called Body Protection Compound (BPC) by us [24–30]. Noteworthy, protection of G-I tract together with strong antiinflammatory activity, liver, pancreas (involving acute pancreatitis lesions as well as diabetes mellitus), kidney protection as well as delayed hypersensitivity lesions (e.g. DNFB-colitis) have been all reported by our group [24–30]. These data have been also independently confirmed [17].

In comparison with conventional organoprotections, BPC organoprotection seems to have particular advantages. Unlike practical limitations so far described for prostaglandins, somatostatine and dopamine related to short time duration, repeated application [2, 20, 21, 31–33, 37, 40, 43] using μg/ng/kg b.w. range, after either parenteral or peroral as well as local administration, providing a strong and long lasting beneficial effect has been obtained [24–30].

In this, the mechanism is also very important. To solve this, at least from gross point of view, the observed effects should be investigated by way of modulations, induced by ablation of particular organs. For this, BPC-gastroprotection has been investigated in both basal as well as modified conditions. 48 h-restraint stress has been used in all the investigations.

Materials and methods

Animals

Albino Wistar rats, stain of both sexes (80–200 g b.w.) have been used in all the experiments.

Ablation protocol and noxius procedure.

All the surgical procedures were applied in appropriate periods before 48 h-restraint stress (subdiaphragmal vagotomy 1 h, spleenectomy 24 h, thyroparathyroidectomy 24 days, ovariectomy 24 h, orchidectomy 24 h). Restraint stress has been applied as described before [27]. No food and water deprivation were introduced before restraint. Immediately after the end of the 48h-restraint period the animals have been sacrificed and stomach lesions assessed using the same procedure as before.

BPC application

BPC has been applied regularly only once after surgery, exactly 1 h before restraint stress 10.0 μg/kg b.w. i.p. In addition, the thyroparathyroidectomized rats received the same dosage once daily during 40 days. Simultaneously, saline (0.9% NaCl, 5.0 ml/kg b.w. i.p.) has been applied in control groups.

Statistical analysis

All data are expressed as means ± SEM. Mann-Whitney test has been used for statistical comparison. P values of 0.05 or less have been considered to be significant.

Results

A strong and complex influence has been demonstrated on BPC protection in restraint stress by the applied procedures. It should be noted that BPC effectiveness has been completely abolished by adrenomedullectomy and ovariectomy, partly reversed by removal of thyroid and parathyroid glands – and complete resistance has been observed after orchidectomy, vagotomy and spleenectomy. All these data are summarised in Figs 1–6.

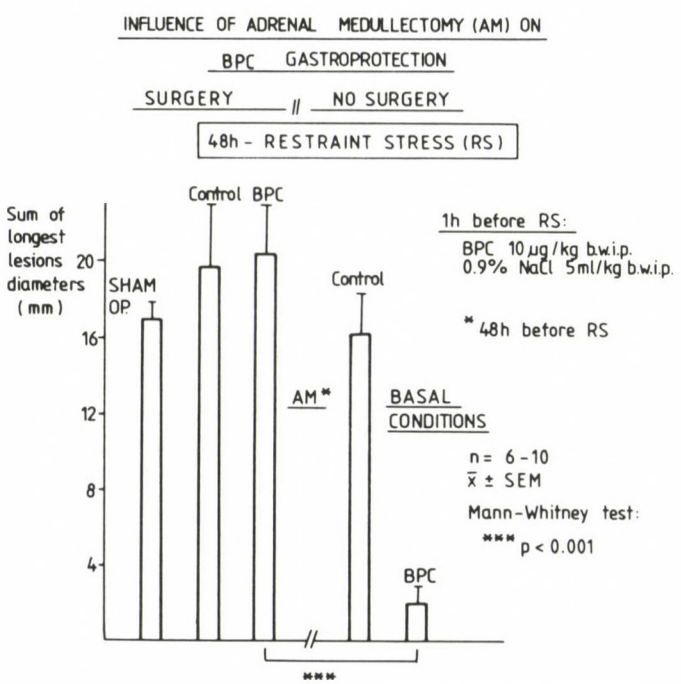

Fig. 1. Demedullation. Note strong protective effect in basal conditions of 48 h-restraint stress, and lack of protection after demedullation

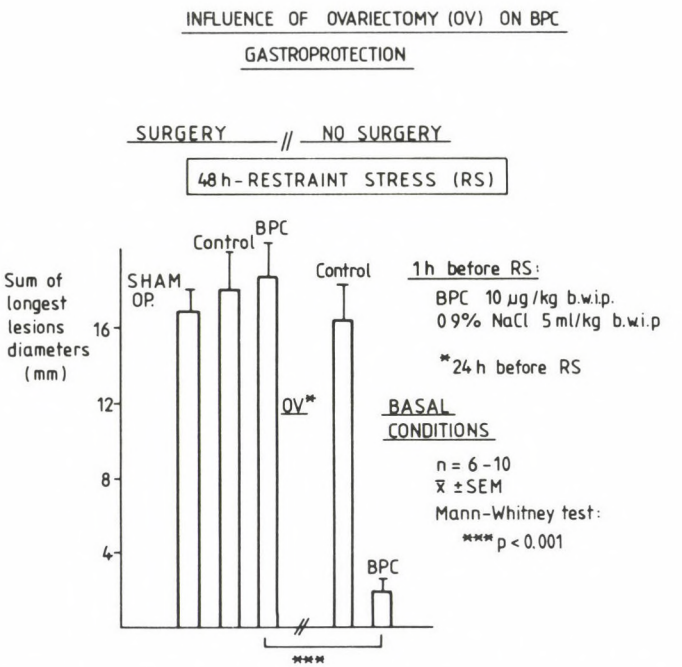

Fig. 2. Ovariectomy. Note strong protective effect in basal conditions of 48 h-restraint stress, and no protection after ovariectomy

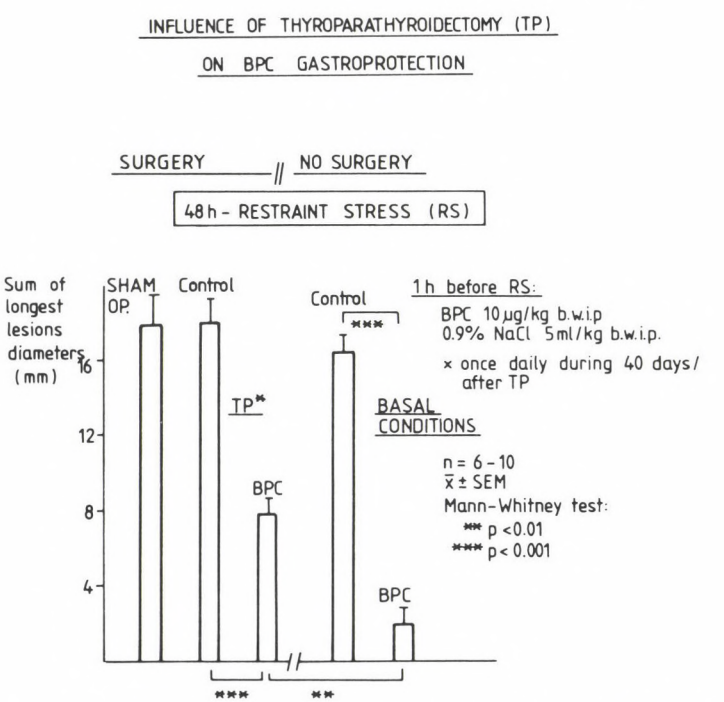

Fig. 3. Thyroparathyroidectomy. Note strong protective effect in basal conditions of 48 h-restraint stress. Relative to normal animals the protective effects seems to be lowered, but still significant comparing with control rats

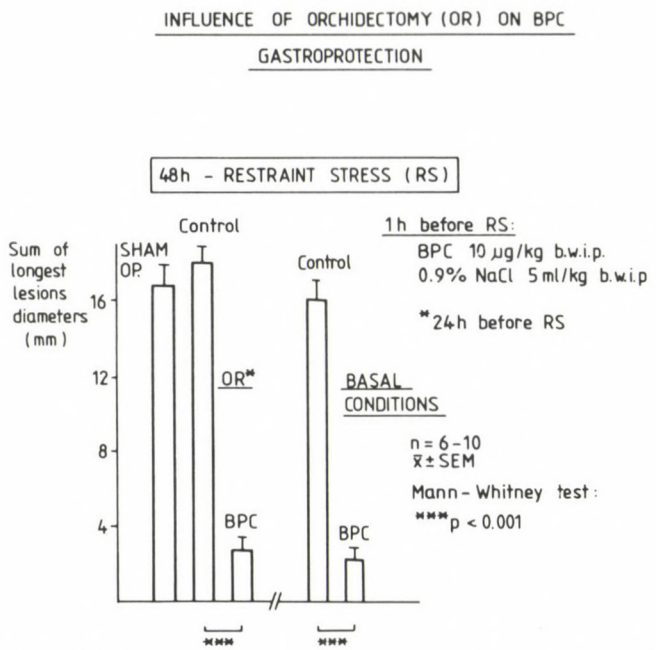

Fig. 4. Orchidectomy. Note strong protective effects in normal as in castrated male (unlike female) rats after 48 h-restraint stress

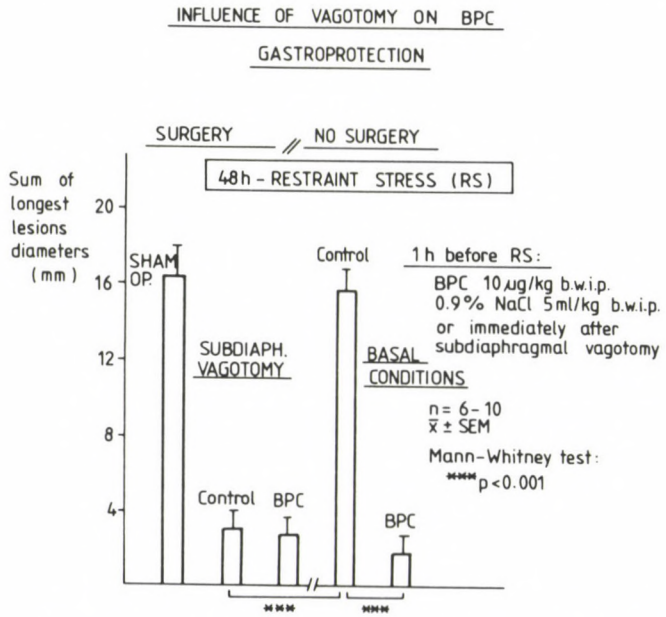

Fig. 5. Vagotomy. Note strong protective effect after vagotomy. The same effect has been obtained also in BPC post-treated rats

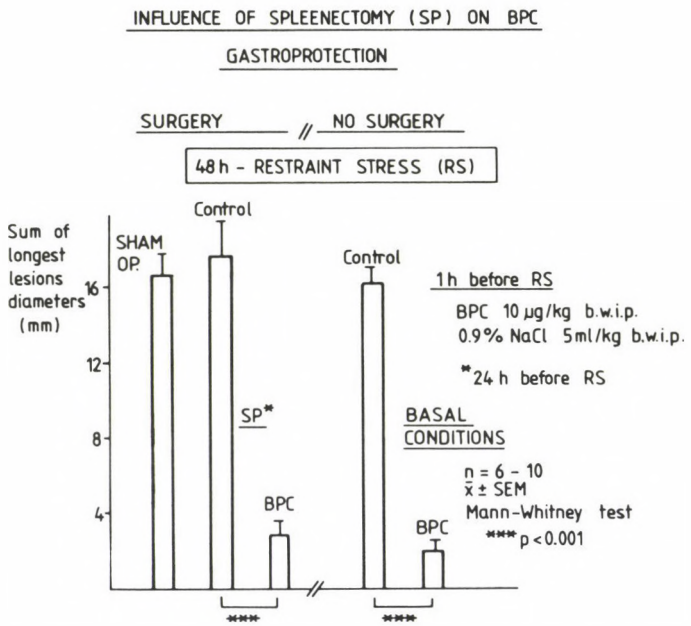

Fig. 6. Spleenectomy. Note no change in BPC protective effect against 48 h-restraint stress gastric lesions after spleenectomy

Discussion

1. The importance of gastroprotection as a target for the investigation of mechanism.

Before discussing the significance of the obtained data the importance of the gastroprotection itself should be briefly emphasized. This issue seems to be supported at least by two lines of evidence:

a) *Conventional organoprotection.* It should be noted that gastroprotection is present in each of the so far described organoprotective concepts [2, 20, 21, 31–33, 37, 40, 43] prostaglandins, somatostatin or dopamine, at the beginning of the investigations. Likewise, a large majority of the studies related to their beneficial effects seems to be related just to the gastroprotection (for review see e.g. [8, 23, 39]). Hence, it seems very likely to us, that just gastroprotection should be the crucial part of each of the described concepts [30].

b) *Stomach stress coping response.* This hypothesis has been defined very recently by our group [30]. Stomach is at present generally accepted to be affected by various stressors ("general pathology") leading to stress ulcer formation (for review see e.g. [7]). However, the stomach had been regarded only as a passive target, and stomach-stress response had been mostly considered locally, because of the assumed negative influence on gastrointestinal mucose integrity [7]. Contrasting with this, stomach-stress coping response hypothesis proposed the stomach to be able to initiate and to organize a feedback stress body response (so-called stomach-stress response) counteracting with it, *vice versa*, all noxious events leading to stress ulcer formation. The proposed central role of the stomach (and consequently gastroprotection) seems to provide both theoretical (experimental) support. Very early appearence of gastrointestinal hormones in evolution, the same neural crest origin for both organs, brain and gut-cells, secreting gastrointestinal hormones, are well defined [9, 18]. Noteworthy, their higher concentrations in G-I tract than in CNS are also known [9, 18]. All these data coupled with particular behaviour of dopamine system (otherwise accepted to be of crucial significance in stress) (e.g. [38]) specifically in stomach, with increase of dopamine receptor number in mucosa (unlike many "stress"-brain areas) [6] in life-threatening stress conditions and well defined axes connecting the stomach with a huge number of organs in the body [3, 4, 12, 14, 19, 36, 40, 42] could provide all together very intriguing theoretical background for proposed central role of the stomach.

In addition to these theoretical evidence, there is also a very simple, but clear practical support (since flowing at least partly from our experimental data [17, 24–30]). In general stress response terms, despite serious disagreements about mechanisms, there is very little doubt that the final result should be the body protection. Because of its huge organoprotective effects and its gastric origin, BPC could be the mediator of the stomach stress response, triggering there a purposeful interaction between CNS, endocrine and immune systems, leading to counteractions of all general pathology producing stress ulcer formations [17, 24–30].

2. The significance of gastroprotection and obtained data in light of the described hypothesis.

It is generally accepted that stomach stress lesions develop only when a particular organism cannot cope with the given stress(or). Consequently, the gastroprotection should be regarded as the best indicator of the body ability to cope with the given stress(or).

Therefore, taking together all the obtained results in this study in conjecture with the possible significance of the gastroprotection, a particular importance for understanding the mechanism of the overall beneficial effect of BPC (and/or stomach stress coping response) should be stressed. The evidence that the strong BPC gastroprotection in basal conditions of the 48 h-restraint stress has been influenced (abolished/decreased) just after adrenal medullectomy, overiectomy and thyroparathyroidectomy but not by other surgical procedures (orchidectomy, vagotomy, splenectomy) suggests that this response could be quite specific and complex, and most likely sex-related. Although this assumption is based mostly on grossly obtained data such as organ removal, the crucial involvement of the hormones of the adrenal, ovarium and thyroid and parathyroid glands [3, 4, 12, 14, 19, 36, 42] seems to be very likely. In view of the very well defined axes connecting the stomach (or G-I tract) besides other also with adrenal, gonadal, thyroid and parathyroid glands this seems to be not entirely unexpected. Consistently, the significance of catecholamines for stress response is generally accepted [1, 11, 22]. Likewise, the specific estradiol-binding proteins in the stomach of both female and male (castrated) rats controlled by the sex steroid hormones [34, 35] in conjecture with beneficial effects of pregnancy, estrogens and progesterone on gastrointestinal lesions [36] have been reported. Thyroid hormones have been reported to be of crucial significance for gastric mucosal cells development [42] hyperthyroidism to prevent gastric stress lesions [15] as well as calcitonin [5].

The role of the hormones of the testis, vagus and spleen could not be substantiated from these experiments. However, ethanol has been reported for instance to generate more severe lesions in male than in female rats [10]. Although vagus is generally accepted to be the final common pathway for pathologics changes in the gut as well as it is necessary for prostaglandin cytoprotection [16], it seems not to be important, for instance, for the release of CCK [13]. Therefore, their possible interactions with BPC protective effects, as well as the interactions with other organ systems are now under investigation.

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ETHANOL AND THE ANTIOXIDANT DEFENSE IN THE GASTROINTESTINAL TRACT

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Ethanol is known to have profound actions on the gastrointestinal tract. The present study was undertaken to examine the effects of ethanol on some of the natural antioxidant defensive enzymes in the gastrointestinal tract; the activities of these enzymes in the liver and the brain were also measured for comparison with those in the gastrointestinal tract. Oral administration of absolute ethanol induced severe gastric mucosal lesions and also damage in the small intestine, however the total superoxide dismutase was unaffected in the tissues measured. The glucose-6-phosphate dehydrogenase activity was reduced only in the stomach while the total glutathione was elevated in the small intestinal mucosa. The catalase activities were activated in the stomach, small and large intestines, and brain, but not in the liver which contained the highest concentration of the enzyme. The present findings indicate that endogenous hydrogen peroxide may be an important damaging agent towards biomolecules in different organs and the removal of this by catalase represents an important defensive mechanism against ethanol toxicity.

Keywords: ethanol, superoxide dismutase, glucose-6-phosphate dehydrogenase, catalase, glutathione, mucosal defense

It is known that the reduced form of glutathione is cyclically regenerated by glutathione reductase with concomitant oxidation of NADPH for the hexose monophosphate shunt. The role of this shunt is to produce five-carbon units required for nucleic acid synthesis, a process of major significance in epithelial cell regeneration [15]. It has been suggested that the depression of glucose-6-phosphate dehydrogenase and the reduced form of glutathione could relate to lesion formation in the gastrointestinel mucosa [12, 13, 16]. It has also been reported that oxygen-derived free radicals are demonstrated to be involved in hemorrhagic shock-evoked

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gastric lesions [5] and ischemic injury to the small intestine [11], and thus, the reactive oxygen scavenging enzymes (superoxide dismutase and catalase) could play an important role in antioxidant defense in the gastrointestinal tract. We, therefore, studied the activities and the distribution of these enzymes and the levels of glutathione in the gastrointestinal tract, and their response to the insult of ethanol. We also measured these parameters in the liver and brain for comparison with those in the stomach and intestines.

Materials and methods

Female Sprague-Dawley rats (160–180 g) were housed in a temperature- and humidity-controlled room. They were fed a standard laboratory pellet diet and given tap water *ad libitum*. Solid food was withheld 24 hr before experimentation. Animals were either given distilled water (5 ml/kg) or with equal volume of absolute ethanol (BDH). Animals were killed by cervical dislocation 1 hr after ethanol administration. The whole stomach and the intestinal tract were isolated. The severity of lesions in the glandular mucosa of the stomach and intestines was determined and scored from 0–3 (0: no lesion observed, 1: petechiae, 2: hemorrhagic lesions less than 5 mm in maximal length, and 3: hemorrhagic lesions larger than 5 mm in maximal length). After the lesions were graded, the gastric (corpus and fundus), small intestinal (the first 15 cm from the pylorus) and large intestinal (the whole colon) mucosae were scrapped off by a glass slide and weighed. The right upper lobe of the liver and the whole brain were also taken. Tissues were then homogenized in a polytron with a phosphate buffer solution (5 ml/g tissue wet weight) under ice-cold water bath. Homogenates were sonicated and centrifuged, and finally determined for enzyme activities and glutathione levels.

Glucose-6-phosphate dehydrogenase activity was determined by a Sigma Kit 345-B based on the Lohr and Walker method [7]. Superoxide dismutase was assayed by the procedure of Misra and Fridovich [9] and the catalase was spectrophotometrically measured by the method of Beers and Sizer [1]. All these enzymes were expressed as units/mg protein. The glutathione (moles/mg protein) and protein were determined by the methods of Sies and Akerboon [14] and Bradford [2], respectively. The bovine serum albumin was used as a standard for protein. The data were expressed as means ± SEM and were tested by the unpaired Student *t*-test.

Results

Oral administration of ethanol induced severe gastric mucosal damage. It also produced intestinal injury which was mostly in the form of petechiae, however, it did not adversely affect the large intestine (Table I). It was found that stomachs contained the highest concentration of glucose-6-phosphate dehydrogenase when compared with other tissues. Ethanol markedly depressed the enzyme in the stomach but not in other organs (Fig. 1).

Table I

Effects of oral administration of ethanol (100%, 5 ml/kg) on gastrointestinal mucosal damage (rats were killed 1 hr after administration)

Treatment P.O.	No. of Animals	Lesion score (0–3) Stomach	Intestine small	Intestine large
Distilled water 5 ml/kg	7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Ethanol 5 ml/kg	6	3.0 ± 0.0*	0.8 ± 0.2*	0.0 ± 0.0

Values indicate means ± SEM. *P < 0.05 when compared to the distilled water-treated control.

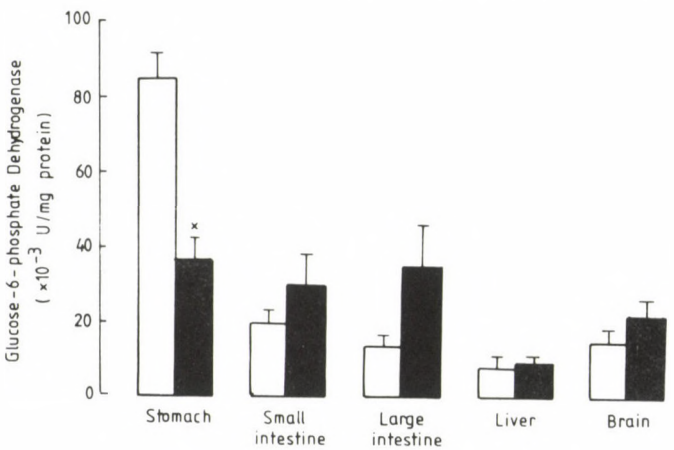

Fig. 1. Effects of oral administration of ethanol (■ : 100%, 5 ml/kg) or distilled water (□ : 5 ml/kg) on glucose-6-phosphate dehydrogenase activities in the gastrointestinal mucosae, liver and the brain. Rats were killed 1 hr after ethanol administration. Values indicate means ± SEM. Numbers of animals used in each group were the same as in Table I. *p < 0.05 when compared to the distilled water-treated control

The gastrointestinal mucosae and the brain contained similar levels of total superoxide dismutase but the liver possessed at least 4 times more than the amounts measured in these tissues. Ethanol administration did not affect the enzyme activities in these organs (Fig. 2). Liver also contained a very high concentration of catalase activity which was 150 times more than the levels in the gastrointestinal mucosae and the brain. Oral ingestion of ethanol significantly elevated the enzyme activities in all

the tissues measured except in the liver (Fig. 3). The gastrointestinal mucosae had higher total glutathione levels than the liver and brain. Ethanol significantly increased the glutathione content only in the small intestinal mucosa (Fig. 4).

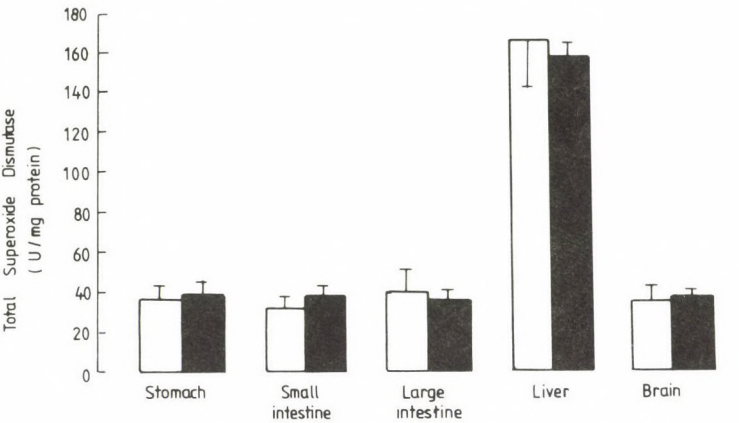

Fig. 2. Effects of oral administration of ethanol (■ : 100%, 5 ml/kg) or distilled water (□ : 5 ml/kg) on the total superoxide dismutase activities in the gastrointestinal mucosae, liver and the brain. Rats were killed 1 hr after ethanol administration. Values indicate means ± SEM. Numbers of animals used in each group were the same as in Table I

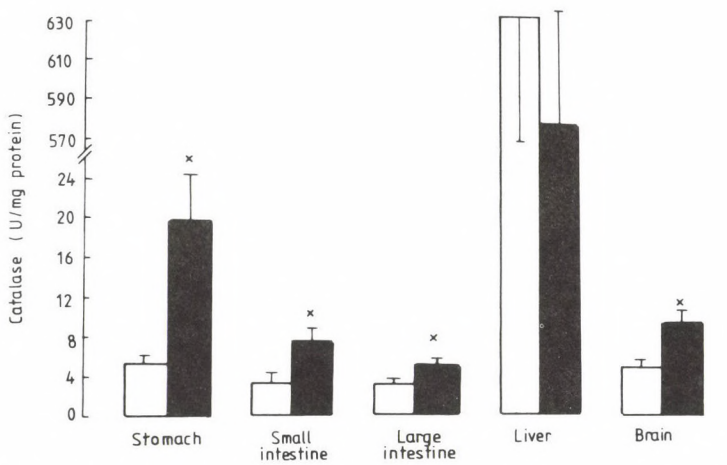

Fig. 3. Effects of oral administration of ethanol (■ : 100%, 5 ml/kg) or distilled water (□ : 5 ml/kg) on the catalase activities in the gastrointestinal mucosae, liver and the brain. Rats were killed 1 hr after ethanol administration. Values indicate means ± SEM. Numbers of animals used in each group were the same as in Table I. *$p < 0.05$ when compared to the distilled water-treated control

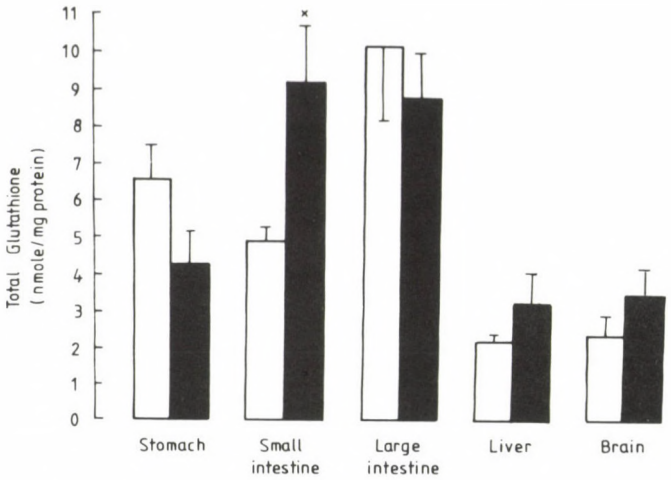

Fig. 4. Effects of oral administration of ethanol (■ : 100%, 5 ml/kg) or distilled water (□ : 5 ml/kg) on the total glutathione levels in the gastrointestinal mucosae, liver and the brain. Rats were killed 1 hr after ethanol administration. Values indicate means ± SEM. Numbers of animals used in each group were the same as in Table I. *$p < 0.05$ when compared to the distilled water-treated control

Discussion

The present findings demonstrated that oral administration of an ulcerogenic dose of ethanol did not decrease the glutathione levels in the tissues measured. Thus, it is likely that the depletion of the total glutathione level appears not to be involved in ethanol ulceration, however, it is noted that damage induced by free radicals to structural proteins is caused partly by the oxidation of essential sulfhydryl groups which scavenge some potentially harmful free radicals. This reaction was demonstrated to be closely related to the hexose monophosphate shunt which is important in tissue regeneration [15]. The capacity of tissues to regenerate enough glutathione to inactivate oxygen reactive species is still unknown because such dynamic changes cannot be detected in the present system, as we only measured the tissue glutathione levels at the end of experiments.

The glucose-6-phosphate dehydrogenase is very responsive to environmental stress. A depression of this enzyme has been associated with a decrease in hexose monophosphate shunt activity following the imposition of dietary, pharmacological, psychological or surgical stress [6, 12, 13]. The present study also demonstrated such a reaction with a marked decrease in gastric mucosal glucose-6-phosphate together with the formation of severe tissue damage. The enzyme activity in other tissues was not affected, with minimal injury or no lesions found. Thus, the importance of the hexose monophosphate shunt in the gastrointestinal tract is confirmed and it

augments the large energy demand of the mucosa and produces five-carbon units required for nucleic acid synthesis, a process required for epithelial tissue regeneration [15].

It is known that oxygen free radicals are likely one of the causative factors inducing tissue injury in the gastrointestinal tract, and superoxide dismutase, an O_2^- scavenging enzyme, has been demonstrated to be an antioxidant defensive enzyme during bowel injury [3, 4, 10, 11]. The present study did not confirm this notion because ethanol, which produced gastrointestinal damage, did not effect the enzyme activity (Fig. 2). These data suggest that neither the superoxide nor the enzyme scavenging it, play a significant role in the pathogenesis of ethanol-induced ulceration. It was found that ethanol markedly increased the catalase activity in the gastrointestinal mucosae, especially in the stomach where the organ was severely damaged. Surprisingly, the catalase level in the brain was significantly elevated, indicating that ethanol given in this concentration could also produce tissue alterations in this organ. Thus, the hydrogen peroxide produced during ethanol insult may be the damaging molecular species toward biomolecules, and removal of this by catalase would be self-defense mechanism of the tissues to prevent further development of lesion formation [8]. It was noted the catalase activity in the liver was not significantly affected, although the organ contained the highest concentration, which was 150 times more than the levels in other organs. This high concentration of enzyme in that tissue may be more than enough to detoxify any hydrogen peroxide formed after ethanol administration, and the liver may not need to induce further elevation in the amount of enzyme in order to inactive the reactive oxygen species.

It can be concluded that the ulcerogenic mechanism of ethanol is mediated at least in part by the depression of hexose-monophosphate shunt and the production of hydrogen peroxide. The latter oxidative reactive substance can be detoxified by the antioxidant defensive mechanism, i.e. by activating the catalase activity in tissues.

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BRAIN-GUT RELATIONSHIPS: GASTRIC MUCOSAL DEFENSE IS ALSO IMPORTANT*

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Growing recognition that there exists a functionally important brain-gut axis has prompted several research groups to examine more closely the role of central nervous system factors in gastric mucosal injury. Less attention has been directed toward brain regulation of *defensive* factors in the gut. Toward that end, we have been characterizing a growing role for dopamine as an important mediator of gastric defense. New data suggest that dopamine, and other substances including many peptides as well as interleukin, act not only to reduce aggressive elements which promote gastric mucosal injury (gastric acid, pepsin, gastrin, leukotrienes) but also to augment defensive factors which retard ulcerogenesis (mucus, bicarbonate, prostaglandins, free radical scavenging enzymes, vasodilators/relaxers). Increasing attention should be directed toward the often-neglected defensive aspect of gastric mucosal ulcerogenesis and protection.

Keywords: ulcer, gastric, duodenal, dopamine, brain-gut axis

Gastroduodenal ulcer disease is a frequently occurring, multi-faceted, pluricausal illness [36] the etiology of which is not fully understood [24]. Ulcer disease can be a life-threatening illness, particularly in otherwise compromised patients such as the elderly, very young infants, critically ill patients in the intensive care unit and in burn patients [1]. In the past two decades, there have been significant advances in the *management* of these diseases, including the development and widespread use of histamine H_2 receptor antagonists [2] and H^+-K^+-ATPase ("proton pump") inhibitors [22]. In spite of these advances, the *treatment* of ulcer disease remains palliative at best, relapse rates remain unacceptably high, and increasing effort must be directed toward new therapeutic strategies [2].

A frequently employed model used to characterize ulcer diseases is that of an interaction or balance/imbalance between aggressive and defensive factors in the gut [23]. Some aggressive factors include endogenous elements such as hydrochloric acid,

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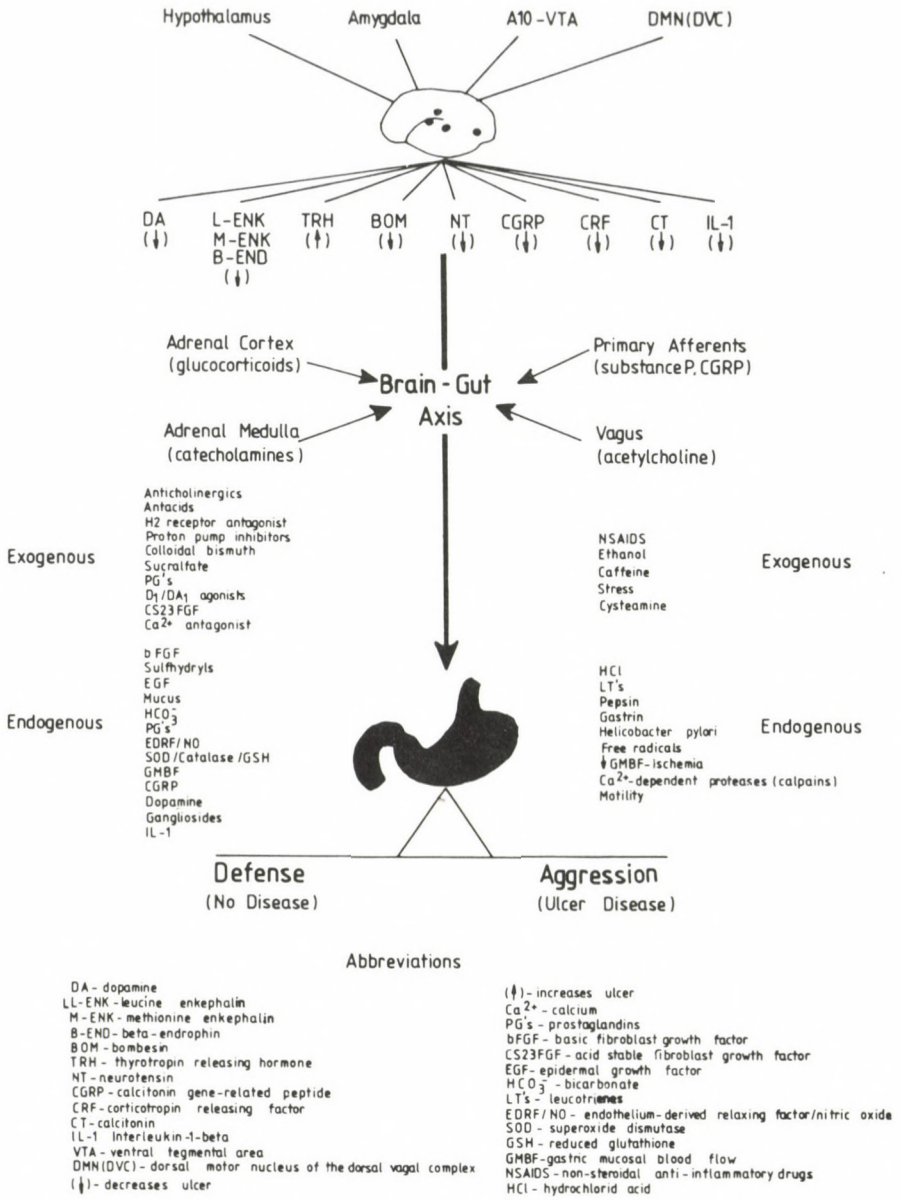
pepsin, gastrin, helicobacter pylori, proteases, free radicals, leukotrienes, dysmotility and impaired mucosal blood flow [12] as well as exogenous elements such as ingestion of non-steroidal and inflammatory drugs (NSAIDS), ethanol, nicotine, caffeine and stress [12]. Some defensive factors include gastric barrier mucus, bicarbonate, prostaglandins, certain polyamines, interleukin-1, superoxide dismutase and catalase as well as endothelium-derived relaxing factor/nitric oxide [41]. Most research investigating novel therapeutic agents for the treatment of ulcer disease has concentrated upon reducing aggressive factors, in particular, gastric acid secretion. This trend has persisted in the literature, in spite of many reports showing that a substantial number of both gastric ulcer patients and duodenal ulcer patients are normal or below normal secretors of hydrochloric acid [33]. Thus, there appears to be a need for treatments which operate on the other side of the ulcer "equation"; that is, compounds which augment endogenous protective or defensive elements in the gastrointestinal tract. Toward that end, this lab has been investigating a role for central and peripheral dopamine (DA) and its agonists and promoters, in terms of both reducing aggressive factors and enhancing defensive factors in the gut.

In the last several years, a growing body of evidence suggests the existence of a functionally important "brain-gut axis" [8, 26], originally designed to account for the fact that several peptides, including bombesin, neurotensin and calcitonin-gene-related peptide occur in both brain and gut and seem to exert opposite actions on gut function when administered centrally and peripherally. Over the years, a great deal of further evidence supporting a brain-gut axis has been forthcoming [8, 14] and is illustrated in Fig. 1.

The intimate relationship between the brain and the stomach is best exemplified by the long-realized clinical observations that affective or emotional states have profound influence on gut function [30]. For example, the production of experimental stress gastric lesions involves a "negative" or aversive component that is mediated centrally [27]. In patients with ulcer disease, stress and anxiety are associated with the active ulcer phase [3]. Evidence also suggests that certain anxiolytic [4] and antidepressant [17] drugs protect the stomach significantly against stress-induced gastric lesions in animals and provide significant amelioration of active disease symptoms in man [18]. While the apparent anti-ulcer effects of antidepressant drugs may be related to their ability to block histamine receptors peripherally [29], the fact that they also protect the gut when given centrally [17] argues for a more widespread involvement of the central nervous system in gastroduodenal ulcerogenesis than previously believed. In fact, Hernandez recently hypothesized that ulcer disease may be, in part, a "brain-derived" disorder [14].



Abbreviations

DA - dopamine
LL-ENK - leucine enkephalin
M-ENK - methionine enkephalin
B-END - beta-endrophin
BOM - bombesin
TRH - thyrotropin releasing hormone
NT - neurotensin
CGRP - calcitonin gene-related peptide
CRF - corticotropin releasing factor
CT - calcitonin
IL-1 Interleukin-1-beta
VTA - ventral tegmental area
DMN(DVC) - dorsal motor nucleus of the dorsal vagal complex
(↓) - decreases ulcer
(↑) - increases ulcer
Ca^{2+} - calcium
PG's - prostaglandins
bFGF - basic fibroblast growth factor
CS23FGF - acid stable fibroblast growth factor
EGF - epidermal growth factor
HCO_3^- - bicarbonate
LT's - leucotrienes
EDRF/NO - endothelium-derived relaxing factor/nitric oxide
SOD - superoxide dismutase
GSH - reduced glutathione
GMBF - gastric mucosal blood flow
NSAIDS - non-steroidal anti-inflammatory drugs
HCl - hydrochlorid acid

Fig. 1. Schematic representation of the brain-gut axis, using the balance-imbalance model of ulcerogenesis. The primary sites of central action, the known central modulators of gut function, the peripheral influences on the brain-gut axis, and a summary of known defensive and aggressive elements whose interaction may determine whether an ulcer develops, are illustrated

Growing recognition of important brain-gut interactions has prompted both basic and clinical scientists to search for additional mechanisms and explanations for gastroduodenal ulcer disease. One focus of this work has been on DA, a known central and enteric neurotransmitter [31] located peripherally in nerve terminals of the myenteric plexus as well as in amine precursor uptake and deamination cells and gastric mucosa and muscle [43]. Specific receptors for DA, likely of the DA_1 subtype, have been identified in the gastric mucosa of rats [13] and man [15]. Several additional lines of evidence which support a role for DA in gastroprotection will now be reviewed briefly. In this paper, the convention of referring to *central* DA receptors as D_1 and D_2 [19] and *peripheral* receptors as DA_1 and DA_2 [20] has been followed.

In 1965, Strang [34] reported a higher than expected incidence of gastroduodenal ulcer disease in Parkinsonian patients whose disease is related to a central DA deficiency. On the other hand, schizophrenic patients, whose brain disorder is typified by DA excess and/or overactivity, rarely exhibit gastric ulcer disease [35]. Thus, there appeared to be an inverse relationship between central DA level or activity and susceptibility to peripheral gastrointestinal illness.

Szabo began the systematic study of a role for DA in duodenal ulcer disease, with the observation that cysteamine treatment, a procedure known to induce gross and histologically verifiable proximal duodenal ulcers in rats, was associated with a marked reduction in DA content of *both* gastric and duodenal mucosa *and* whole brain [38]. Pretreatment of rats with the catecholamine precursor, l-tyrosine, obtunded both central and peripheral DA depletion as well as the duodenal ulcers consequent to cysteamine treatment [25]. Overall, a significant inverse correlation was obtained between central and duodenal DA level and duodenal ulcer severity, suggesting a significant pathogenetic role for DA depletion in the genesis of experimental duodenal ulcers. To explore this hypothesis more directly, Szabo administered DA agonists, including l-dopa, apomorphine, and bromocriptine as well as DA antagonists, such as haloperidol and pimozide, coincident with cysteamine treatment. DA agonists reduced, while DA antagonists exacerbated duodenal ulcerogenesis, supporting a role for DA in both the pathogenesis and possibly treatment of, duodenal ulcers [5]. Still further evidence of a role for DA in ulcer disease arises from the observations of Szabo's group showing that 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a selective DA neurotoxin and Parkinsonogenic drugs of abuse, induced massive nigrostriatal DA loss and duodenal ulcers in rats [37]. These lesions could be reduced by pretreatment with the selective monoamine $oxidase_B$ inhibitor, l-deprenyl, which also reduces the conversion of MPTP to its more toxic ionic form in brain nigrostriatal tracts [37].

Later, our laboratory showed that DA agonist and promoters (l-dopa, bromocriptine, l-deprenyl, apomorphine, methylphenidate, para-hydroxy-methylphenidate (a hydroxylated analogue of methylphenidate which does not

penetrate the brain) and d-amphetamine reduce, while DA antagonists (haloperidol, pimozide, domperidone) worsen, *gastric* lesions caused by restraint-cold stress [10]. Similar observations were seen in the case of ethanol-induced gastric mucosal damage [21] as well as in pylorus-ligated rats [40]. It also appears that ulcerogenic factors beyond DA receptor blockade may not be necessary to induce damage – Sikirič et al. [32] reported that haloperidol alone induced gastric lesions in mice, and Szabo and Neumeyer [39] noted that psychotic patients treated with high doses of haloperidol exhibit a high incidence of duodenal lesions.

Recently, Wallace and his colleagues [21] demonstrated a significant role for DA as an endogenous gastroprotectant. In experiment involving "adaptive cytoprotection" (the phenomenon wherein pretreatment of rats with a mild irritant protects the gastric mucosa against subsequent challenge with a necrotizing, ulcerogenic agent), Wallace showed that gastric protection conferred by pretreatment with hypertonic saline is significantly enhanced by coadministration of DA agonists, including bromocriptine and l-dopa. This phenomenon occurred even in the absence of endogenous prostaglandin synthesis (indomethacin pretreatment), and in the absence of other receptor activity (blockade of alpha-adrenergic, histaminergic and cholinergic receptors), suggesting significant DA involvement in endogenous gastric mucosal protection.

The issue of the DA receptor subtype which mediates the apparent gastroprotective effects of DA was addressed by Hernandez [16] and by Glavin [6]. Hernandez demonstrated the existence of DA receptors in both rat [13] and human [15] gastric mucosa. Based upon competition binding and receptor solubilization studies, Hernandez concluded that the gastric dopamine receptor is of the DA_1 subtype [22]. Glavin then examined selective DA_1 and DA_2 agonists and antagonists for their activity in several models of experimental ulcer [6]. The DA_1 agonist, SKF38393 protected significantly against stress- and ethanol-induced gastric mucosal lesions. The selective DA_1 antagonist, SCH23390, worsened the gastric damage observed in all models. All compounds (agonists or antagonists) selective for the DA_2 receptor, were inactive. We have summarized the dopaminergic compounds tested for gastrointestinal activity in our laboratory [8]. The overall conclusion based upon these data suggests the following rank order of potencies for dopaminergic activity in the gut [8]:

AGONISTS:

SKF38393 > > > DOPAMINE > NO437 > NO434 > QUINPIROLE (LY171555)
(DA1) (DA1-DA2) (DA2) (DA2) (DA2)

ANTAGONISTS:

SCH23390 > > DOMPERIDONE > > > ETICLOPRIDE = YM09151-2 = SULPIRIDE
(DA1) (DA1-DA2) (DA2) (DA2) (DA2)

The conclusion at this stage is that the gastroprotective effects of DA in the periphery, are mediated through DA_1 receptors.

A role for *central* DA in mediating gastric function and dysfunction has also been postulated by several groups [8, 14, 44]. In particular, the literature suggests that, of the three major central DA tracts (nigrostriatal, mesolimbic and mesocortical), the mesolimbic DA tract is preferentially activated by stress [42] and may be involved in the pathogenesis of stress-related gastric mucosal injury [45]. Glavin showed that non-selective central administration (icv) of l-deprenyl (DA promoter via MAO_B inhibition) was associated with virtually complete abolition of stress-induced gastric lesions [11]. However, *direct* evidence of a role for mesolimbic DA in mediating stress-induced gastric injury is not abundant. Henke [28] administered DA, as well as a DA agonist, apomorphine, into the central nucleus of the amygdala, and observed significant inhibition of stress ulcer formation. Kauffman's group [44] also noted a role for mesolimbic DA in ameliorating stress ulcerogenesis, particularly in that DA appears to be necessary for neurotensin-induced gastroprotection.

Recently [7], Glavin showed that microinjections of the selective D_1 agonist, SKF38393, into the cell body region of the mesolimbic DA tract was associated with a significant degree of protection against stress- and ethanol-induced gastric ulcers. Less striking effects were seen upon infusion into the cell body region of the nigrostriatal tract and no effects were seen upon administration of this compound into the cell body region of the mesocortical DA tract. Infusion of the DA_1 agonist into the mesolimbic cell body region also resulted in a significant reduction of gastric acid secretory volume and concentration as well as pepsin output in pylorus-ligated rats. A similar pattern was seen with exacerbation of experimental gastric lesions induced by stress or ethanol upon microinjection of the D_1 antagonist, SCH23390, into these same cell body regions, with the most prominent effects seen upon infusion into the mesolimbic cell body region.

When these same compounds were microinjected into a representative terminal field of the three major central DA tracts, the same results were observed – profound ulcer and secretory inhibition upon administration into the central nucleus of the amygdala (mesolimbic terminal field) with the D_1 agonist and significant exacerbation with the D_1 antagonist. Less striking effects were seen when the compounds were given into the caudate nucleus (nigrostriatal terminal field) or cingulate cortex (mesocortical terminal field). Based upon this work with selective dopaminergic compounds in selective central regions, it was concluded, in a manner similar to that based upon the *peripheral* data, that the central D_1 receptor is the functionally important dopamine receptor subtype mediating the gastrointestinal effects of DA and its agonists (Table I).

Recently, we obtained preliminary data which suggest that *peripheral* administration of a DA_1 antagonist obtunds the gastroprotection afforded by *central* treatment with a D_1 agonist. Similarly, *peripheral* administration of a DA_1 agonist

Table I

Summary of dopaminergic central-peripheral interactions in stress ulcerogenesis. In all cases, central administration (agonist or antagonist) exerts a larger effect than peripheral treatment

	Central (mesolimbic) Treatment		
	VEH	Agonist (SKF38393)	Antagonist (SCH23390)
VEH	↑	↓↓	↑↑
Agonist (SKF38393)	↓	↓↓	↑ to 0
Antagonist (SCH23390)	↑	↓ to 0	↑↑

Legend: ↑ increase
↓ decrease
0 no effect
↑↑ large increase
↓↓ large decrease

reduces the exacerbatory effect of *central* treatment with a D_1 antagonist. These data strongly support the existence of a link between mesolimbic DA and gastric function – a specific dopaminergic brain-gut axis.

Very recently, Glavin showed that intra-amygdalar SKF38393 reduces cysteamine-induced *duodenal* ulcers [9], which *may* be associated with an increase, or "preservation" of the capacity of the duodenum to secrete bicarbonate (in preparation). Intramesolimbic DA agonists also appear to be associated with increased gastric barrier mucus (in preparation). These latter findings suggest that DA plays a significant role in protecting the gastrointestinal tract against stress or toxin challenge and that at least part of the mechanism of action of these compounds may involve action on the *defensive* side of the ulcer "equation"; that is, they may not act solely by antagonizing aggressive elements, but also by enhancing defensive factors in the gut. Systematic exploration of this hypothesis should be the focus of future work.

Overall, the research summarized herein suggests a significant role for DA and its agonists in reducing experimental gastric and duodenal mucosal injury. Both published and preliminary data collected in this laboratory suggest that DA agonists have effects on *both* the aggressive *and* defensive sides of the ulcer "equation" and appear to exert these effects both centrally and peripherally (Fig. 1). We suggest that increasing attention should be directed toward an examination of actions of putative gastroprotective compounds on *defensive* factors in the gut, rather than almost

exclusive reliance on testing for anti-secretory (aggressive) activity. Systematic evaluation of a particular compound should include its effects on *both* sides of the ulcer equation and thereby generate a more complete "profile" of its actions. Thus, "gastroprotection" connotes *both* anti-aggressive *and* pro-defensive activity.

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STRESS ULCER MODULATION BY LIMBIC SYSTEM STRUCTURES

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A number of studies suggest that the telencephalic limbic system modulates stress ulcer development. The amygdala is assumed to connect sensory experiences, including stressful stimuli, with the emotional reactions and gastrointestinal effects these reactions normally produce. The hippocampal formation (entorhinal cortex, dentate gyrus, hippocampus) is part of a gating system, modulating the organism's coping ability. Changes in transmission in this temporal brain region are linked to individual differences in stress ulcer severity. Interactions among "classical" transmitters and several neuropeptides mediate these differences.

Keywords: limbic system, amygdala, hippocampal formation, stress ulcer, individual differences

For a number of years, limbic system structures have been linked to emotions, visceral reactions, and "psychosomatic" disorders. In fact, the notion of a "visceral brain", as applied to these brain regions, was suggested by MacLean in 1949, elaborating the theory of emotions which had been proposed by Papez in 1937 [23, 24]. In more recent years, several studies have supported the idea that the limbic system modulates the development of stress ulcers. In other words, one source of individual differences normally seen in the susceptibility to stress ulcers seemingly is related to changes in the neurophysiology of these limbic pathways. Specifically, a large body of data suggest that (a) the amygdala and the hippocampal formation connect stressful sensory stimuli with the appropriate adaptive behaviors and the gut reactions seen under such circumstances, and (b) the efficacy of synaptic transmission in the temporal region of the limbic system frequently reflects the organism's vulnerability to stress ulcer development.

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A large body of additional data also indicates that these structures are implicated in learning and memory mechanisms [3]. This suggests that the proposed "stress-gating" function of these limbic areas may be modifiable by experience. It seems, therefore, that the protection afforded by this gating mechanism is important in an evolutionary sense, that is, how learning experiences influence the organism's coping ability under stressful conditions.

Amygdala

Early studies with limbic lesions in monkeys showed that damage to the amygdala and surrounding temporal cortex produced a collection of symptoms, now known as the Klüver–Bucy syndrome [21]. It included indiscriminate dietary behaviors, stereotyped "orality" (mouthing everything presented to the animal), sexual abnormalities, emotional docility and fearlessness. In a sense, the brain lesions seemed to have disconnected sensory stimuli from normal adaptive behavioral responses. This was especially apparent in potentially dangerous situations for the monkeys.

These findings suggested that the sensory systems must have access to the amygdaloid complex, and this information is then integrated with emotional reactions and the associated visceral responses. It is now well-established that environmental stimuli reach the amygdala from sensory association cortex, sometimes multi-sensory inputs converging on single cells. Efferent fibers from the amygdala reach the hypothalamus and the dorsal vagal complex. In other words, the amygdala is in a strategic position to integrate environmental inputs with autonomic and endocrine activities [14, 18].

Early studies with electrical stimulation of amygdalar nuclei found that excitatory as well as inhibitory effects on gastrointestinal functions could be detected, depending upon the locus of stimulation. Activation of centromedial regions frequently produced increased acid secretions and motility effects, stimulation of more posterolateral regions produced opposite results. A reduction in mucosal blood flow has been reported after stimulations of the central amygdala. Anteromedial and central amygdalar stimulations also produced gastric erosions which, in fact, were indistinguishable from so-called stress ulcers [4, 20, 33, 34, 36, 38, 41].

Electrophysiological recordings of multiple-unit activity in the amygdaloid complex showed that restraint-stress affected neurons in the medial, central and lateral nuclei of rats. Stomach erosions were also seen after electrical stimulation of these neurons in the central nucleus of the amygdala (CEA) [6, 7].

In the CEA, distinct patterns of neural activity were found to be correlated with the eventual degree of stress ulceration of the rats. These neuronal response profiles were also associated with emotionality differences in an open-field situation,

i.e., high-emotional rats showed greater defecation and had more severe stomach ulcers [7]. Patterned electrical stimulation of the amygdala which then produced seizure activity, a procedure known as "kindling", made rats more susceptible to stress ulcer development [16]. It is interesting that kindling in this region also increased thyrotropin-releasing hormone (TRH) levels 3 to 4 times above normal [22]. Direct applications of TRH to the CEA have been found to aggravate stress ulcers, dose-dependently [17, 19, 30, 32].

Table I

Injections into the central nucleus of the amygdala and stress ulcer severity

Treatment	Pathology
Cholinergic	
Atropine	↓
Physostigmine	↑
Adrenergic	
Norepinephrine	↓
Propranolol	↑
Dopaminergic	
Dopamine	↓
Apomorphine	↓
Haloperidol	↑
Clozapine	↑
SKF 38393	↓
SCH 23290	↑
GABAergic	
GABA	↓
Chlordiazepoxide	↓
Midazolam	↓
RO 15 – 1788	↑
Peptidergic	
Neurotensin	↓
Beta-endorphin	↓
Met-enkephalin	↓
Leu-enkephalin	↓
Naloxone	↑
Thyrotropin-releasing hormone	↑
Bombesin	0
Vasoactive-intestinal peptide	0

Note: ↑ increase, ↓ decrease, 0 no effect

Indeed a number of "classical" transmitters, as well as, so-called brain-gut peptides seemingly interact in CEA under stressful conditions. Table I lists agonists and antagonists of putative transmitters tested in this nucleus. Table II presents

Table II

Transmitter interactions in the central amygdala and gastric ulcers

Treatment	Pathology
DA + TRH	↓
Cloz + DA + TRH	↑
Cloz + DAMEA	↑
TRH + DAMEA	↓
A + TRH	↓
CDP + TRH	↓
Midaz + TRH	↓
CDP + PHY	↓

Note: ↑ increase, ↓ decrease; DA, dopamine; TRH, thyrotropin-releasing hormone; Cloz, clozapine; DAMEA, [D-Ala^2] Met-enkephalinamide; Hal, haloperidol; A, atropine; CDP, chlordiazepoxide; Midaz, midazolam; PHY, physostigmine.

the results of interaction studies in CEA under cold-restraint stress. Dopamine (DA) transmission in CEA seems particularly important [8], and possibly acting through D1 receptors [1, 2], interacts with a number of neuropeptides (Table II). The results of these studies show that microinjections of DA into CEA reduced the stomach pathology, an effect that was blocked by prior treatment with 6-hydroxydopamine (6-OHDA). Destruction of DA neurons with 6-OHDA also reversed the attenuation of stress ulcers usually seen with intra-CEA neurotensin or met-enkephalin. Maybe more importantly, naloxone treatment in CEA produced an intrinsic aggravation of the pathology, implicating endogenous mechanisms under stress conditions [25, 26, 27, 28, 29]. On the other hand, injections of 6-OHDA into CEA by itself produced only weak and inconsistent effects on stress ulceration [25]. It is possible that this finding relates to other data showing that this damage to DA neurons in the amygdala apparently initiates compensatory increases in DA activity in the nucleus accumbens [35]. This latter structure has also been implicated in stress ulcer development [40].

DA release in CEA also inhibits the ulcer-aggravating effects of TRH injections in this nucleus of the amygdala. On the other hand, the DA antagonist clozapine severely aggravated the stomach pathology produced by TRH. Intra-CEA injections of neurotensin given prior to TRH, however, reduced the ulcer severity to control levels [17].

GABA (gamma aminobutyric acid) transmission is influenced by benzodiazepines (BZD) in CEA and modifies stress ulcer development in rats. Earlier data had shown that chlordiazepoxide, when injected intra-peritoneally,

changed the activity of neurons in CEA. Furthermore, electrical stimulation of these neurons produced stomach erosions [6]. But microinjections of GABA or BZD (chlordiazepoxide, midazolam) attenuated the stress ulcer severity. The BZD antagonist RO 15–1788 blocked these effects and also aggravated ulcer severity when injected by itself [30, 37].

Recent studies have also shown that cholinergic mechanisms in CEA interact with TRH and BZD (Table II). Atropine injections into CEA attenuated stress ulcers, whereas, physostigmine increased the pathology. Pretreatment with atropine also blocked the usual aggravation produced by either physostigmine or TRH. A similar reversal was found with BZD [30]. Further interaction tests revealed that met-enkephalin injections into CEA also neutralized the TRH-induced aggravation normally seen under stressful conditions [32].

Taken together, the CEA seems to be an important limbic site in which stressful experiences influence gut functions. This nucleus connects directly with hypothalamic and lower brainstem areas concerned with gastrointestinal control. It also receives input from other temporal regions concerned with stress ulcer modulation, i.e., the hippocampal formation.

Hippocampal Formation

Some time ago, a study reported that bilateral lesions of the posterolateral amygdala aggravated stress ulcers in rats, lesions in centromedial regions attenuated the pathology, and simultaneous damage in both of these areas also decreased the severity of stomach ulcers. It seemed reasonable, at that time, to interpret these findings as indicating that posterolateral areas directly influenced centromedial structures [5]. Recent data, however, suggest the involvement of more complex pathways.

Additional lesions studies showed that damage to the ventral hippocampus (including dentate gyrus) or the adjacent entorhinal cortex aggravated the stomach ulcers induced by restraint [12, 15]. The major pathway connecting these temporal regions is the perforant path projection from entorhinal cortex to dentate gyrus. Measurements of evoked potentials in this pathway showed that the efficacy of transmission correlated with the severity of stomach ulcers in rats. It was found that increased potentials at the granule cells of the dentate gyrus were associated with less ulceration, whereas, electrophysiological suppression was seen in vulnerable animals. Reduced coping ability in the so-called "learned-helplessness" situation was also correlated with suppression of evoked amplitudes at this entorhinal-dentate synapse [11]. Conversely, when transmission was facilitated at these granule cells, by inducing long-term potentiation (LTP), it buffered the rats against the impact of stressful conditions [10, 13]. In fact, further studies showed that N-methyl-D-aspartate

(NMDA) receptor blockade, using intra-ventricular (ICV) infusions of aminophosphonovaleric acid (AP5), eliminated the granule cell LTP, aggravated the stress ulceration, and increased the behavioral helplessness of the rats [9, 13].

It has been shown that LTP at dentate granule cells can also be produced by high-frequency electrical stimulation of the posterolateral amygdala. This treatment also reduced stress ulcers in rats and facilitated the habituation to restraint conditions (shown by a faster decrease in "struggling" activity) [13]. Recently, it was found that microinjections of DA or bromocryptine (a DA-agonist) into the posterolateral amygdala reduced the stress ulcer severity in rats. On the other hand, 6-OHDA or haloperidol injections into this brain region produced opposite effects [31]. Taken together, these findings suggest that a pathway linking the posterolateral amygdala, entorhinal cortex, and ventral dentate gyrus is involved in the modulation of the impact of environmental stressors on gastric functions.

Output fibers from the hippocampal formation apparently include caudally directed projections which reach the lateral edge of the central amygdala from the ventral CA1 region of the hippocampus. LTP in this pathway reduced stress ulcers in rats [12], in effect completing the circuitry shown in Fig. 1. The efficacy of transmission in the neural loop, connecting amygdala and hippocampal formation, apparently predicts the organism's relative resistance to stress ulcer formation.

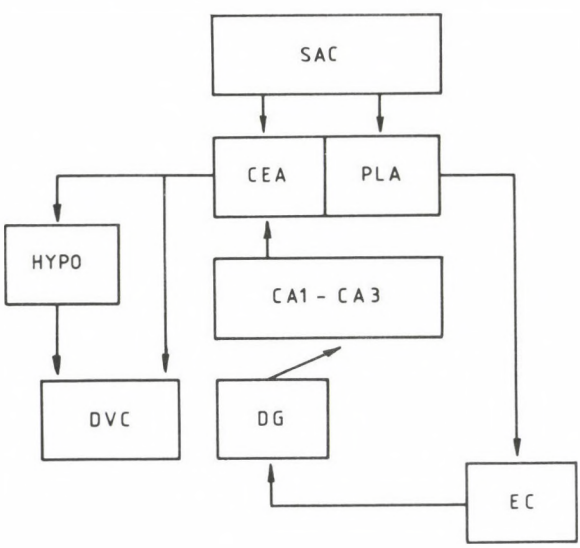

Fig. 1. Diagram of major limbic system connections involved in stress ulcer modulation

Abbreviations: SAC, sensory association contex; CEA, central amygdala; PLA, posterolateral amygdala; EC, entorhinal cortex; DG, dentate gyrus; CA1 – CA3, hippocampal fields; HYPO, hypothalamus, DVC, dorsal vagal complex.

Additional data indicate that LTP at ventral dentate granule cells may depend on the release of endogenous opiates. It is possible, therefore, that enkephalins released by perforant path terminals under stressful conditions suppress the inhibitory input on these granule cells and allow dendritic depolarization to reduce the presumed magnesium block of NMDA receptors [39].

Numerous data indicate that synaptic transmission at these dentate granule cells in modifiable through learning experiences [3]. The implication of these findings is, therefore, that the proposed stress-gating mechanism at these synapses might also be influenced by such experiences. One source of the individual differences seen in the ability to cope with stressful circumstances may, therefore, be a function of experience-based modifications at these synapses.

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INDIVIDUAL BEHAVIORAL CHARACTERISTICS AND EXTENT OF STRESS-INDUCED GASTRIC ULCERATION IN RATS

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Individual rats differ amongst themselves with respect to both behavior and the extent of stress-induced gastric ulceration, even though they have been treated identically, are from the same stock, age, etc. The relationship between behavior and ulcer susceptibility is of interest in its own right, and is reminiscent of the extensive body of literature on personality characteristics and disease risk in humans.

In the Sprague-Dawley rat, we have found that animals react differentially to the introduction of new stimuli in a previously learned Lashley-maze, and that the increase in latency is negatively related to attack frequency in a classic intruder test. Furthermore, we have found a negative correlation between attack latency in the intruder test and the amount of gastric ulceration induced by restraint-in-water stress.

We have further found highly significant relationships between the responses of otherwise untreated animals to a simple startle test and the extent of gastric ulceration induced by restraint-in water stress.

We believe that greater notice should be taken of the individual animal's behavioral profile for three reasons. First, prior behavioral screening may be a useful method for reducing error variance. Second, physiological and neuroendocrinological differences between high susceptible and low susceptible individuals are of interest in understanding the psychobiology of stress ulcerations both in animals and humans. Finally, an understanding of the etiology of these individual differences may cast light on links between behavior patterns and stress pathology.

Keywords: behavioral characteristics, stress-induced gastric ulceration, Sprague-Dawley rat

The pathophysiology of gastrointestinal ulcer remains an area of intense scientific endeavour in the 1990's despite the considerable advances made over the last decades in our understanding of the gut, and the numerous therapeutic measures

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now available for treating its pathology. However, there are many questions which remain to be answered, and asked, and there is little likelihood that those of us interested in the workings of the gut will find ourselves idle. The reason for this continued interest are two-fold. First, the stomach and the rest of the gut provides a fascinatingly complex target for research, and it represents an organ system demanding complex integration within itself and with the rest of the body. Secondly, despite advances in pharmacological treatments of gastrointestinal disorders, the malfunctioning gut remains a source of personal distress and, on the societal level, a drain on manpower and financial resources in terms of time off work etc.

Notwithstanding the advances made by our colleagues in pharmacology and experimental physiology, we do believe that one vitally important area has been undervalued, even ignored. It is our argument that however well we may understand the mechanisms involved in gastric cytoprotection and/or pathology at the organ, cellular, and even molecular levels, we ignore at our own peril the *inputs* to the system from the brain and other organs. It should not be necessary here to review the important contributions made by neuroscientists to our understanding of how the brain and endocrine systems influence the gut, both directly and indirectly [e.g., 5, 6, 7]. We should remember that brain – gut interactions do not happen only in the neuroscientist's laboratory, but also in each and every animal, including those in the physiologist's and pharmacologist's laboratory. The mysteries of gut physiology will essentially only be fully revealed in the whole organism. To this end, the psychobiological approach will make an important contribution.

We have for some years studied how the experimental animal's prior experience with one stressor modulates the extent of gastric pathology arising during exposure to a later, dissimilar, stressor. Our results indicate that rats prior exposed to one stressor, uncontrollable footshock, exhibit enhanced amounts of gastric ulceration on later exposure to later restraint-in-water stress. Furthermore, this increased sensitivity appears to be at least partially *psychobiological* in nature, because the presence, absence, or power of this effect is dependent on *psychological* factors: whether the animal is able to escape the footshock stressor [9], whether the shock is associated with safety signals [12], and whether the second stressor, restraint-in-water, is presented to the animal under similar contextual conditions as the prior shock experience [11]. It appears that prior experience with escapable shock provides a true cytoprotection, although the possible mechanisms remain unexplored. At the risk of being repetitious, the amount of gastric pathology found in the stressed animal is at least partly determined by the animal's prior history, and a good deal of that is what we might call psychological [see 10]. Although work with rats in this area has focused on gastric pathology, recent research also indicates that the cysteamine-induced duodenal ulcer in rats is also modulated by a prior stressful experience (Paré and Bakke, in preparation).

From an interactionist point of view, the somatic stress response of the organism will depend on how the organism perceives the stressor, what kind of expectancies the organism has concerning its abilities to do something with that stressor, and what kind of expectancies the animal has concerning the duration of the stressor [16]. All these are a function of two factors – the genetic "personality" of the animal and the learning afforded by previous experience.

Despite our relatively clear cut picture of what may or may not effect the extent of gastric pathology under stress, one issue remains bothersome – that of individual differences, or error variance, in animals which have, *as far as we know*, been raised under identical conditions and have been exposed to identical life experiences. In any number of studies, we and others have watched in frustration as the ulcer data, despite all sorts of statistical transformations, appear to be spread all over the place within the same treatment group. In fact, we sometimes seem to have a bimodal distribution of ulcer scores in animals exposed to identical stress conditions and with the same prior histories. Of course, we can conveniently deal with the issue by sophisticated statistical methods, but this to some extent is avoiding a very interesting issue in itself. Why do animals exhibit such marked individual differences in the amount of pathology? Is it all genetic? Or are there differences arising from differences in life history which we have overlooked? From the experimentalist's viewpoint, are there markers which enable us to identify in advance which animal is more or less sensitive to development of gastric pathology than others?

Taking the last point first, there is a history of studies indicating that we can indeed identify ulcer-susceptible animals using *behavioral* measures. To digress one moment, there is an alarming tendency for researchers to cite and take into consideration only the latest research papers. Of course, this is partly related to a desire to be (and to be seen to be) up-to-date, and partly because younger researchers may not have had the opportunity to read earlier literature on the subject. However, science does have a history, and studies of stress ulcerations in animal models go back a long way. Research data published in the 1950's or earlier have as much validity and relevance today as data published more recently. One may even argue that some of the experimental work performed in the past might have been more stringently performed than is the case today, what with the increasing pressure to publish new material, and diminished emphasis on the value of observation. Whatever, the point is that old data are not necessarily bad data, and may cast light on some of the issues we study today. This is the case here.

A number of studies have indicated that individual differences in stress ulcer susceptibility may be predicted. For example, the work by Ader with animals [1], and by Weiner [17] with enlisted military personnel showed that levels of pepsinogen were a reasonable predictor of the pathological consequences of stress for the

individual. However, it is both costly and in itself stressful to the animal to obtain material for analysis of pepsinogen or other implicated biological materials. Perhaps the most sensitive measure of an animal's state or trait is behavior. Various very simple noninvasive, behavioral measures which involve minimal stress are extremely sensitive to the animal's state of health and, for want of a better word, "emotionality." And indeed, early work demonstrated that animals judged to be more "emotional" in a number of simple tests are more likely to develop greater amounts of stress ulcer compared to their less emotional cousins or brothers [2, 14].

In an attempt to convince ourselves that this truly is the case, we have employed a very simple behavioral test for emotionality, derived from studies by Koolhaas and his colleagues [8]. Briefly, the test consisted of first exposing animals in a new environment to a 75-dB white noise for 5 minutes. Twenty-four hours later, the animals were returned to the test environment with the same 75dB background noise. After 2 minutes, the noise was abruptly turned off. The reaction of the animal was to exhibit freezing behavior – immobility. Measurements can be made of freezing behavior, exploratory movements, etc. What is interesting here is that it was the *removal* of the auditory stimulus which elicited the response. Using previously unstressed (as far as we know[1]) animals, we observed a significant positive correlation between the amount of time freezing in response to the withdrawal of the habituated auditory stimulus, and the extent of gastric ulceration developing under two hours of restraint in room temperature water at a later time (for details of this method, see ref. [11]). In two separate tests, we have found that whatever underlies the freezing behavior accounts for between 40% and 48% of the variance of ulceration length.

We have earlier reported that prior shock experience of the type known to induce the behavioral deficit known as "learned helplessness" exacerbates the extent of gastric ulceration under later restraint-in-water stress. We have also observed that the same prior shock treatment increases freezing duration in the test described above, and again that shock increased ulcer in these animals (see Fig. 1). The implication is that a common underlying variable influences both the animal's reactivity in our simple behavioral test and the extent of stress ulceration and it is tempting to call that underlying variable "emotionality."

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[1]It is never possible to say that a rat is unstressed prior to our experimental manipulations. The behavioral interactions involved in the formation of dominance hierarchies within groups of young animals will inevitably result in a greater or lesser degree of stress, and the degree of perceived stressfulness of the situation will vary amongst individuals.

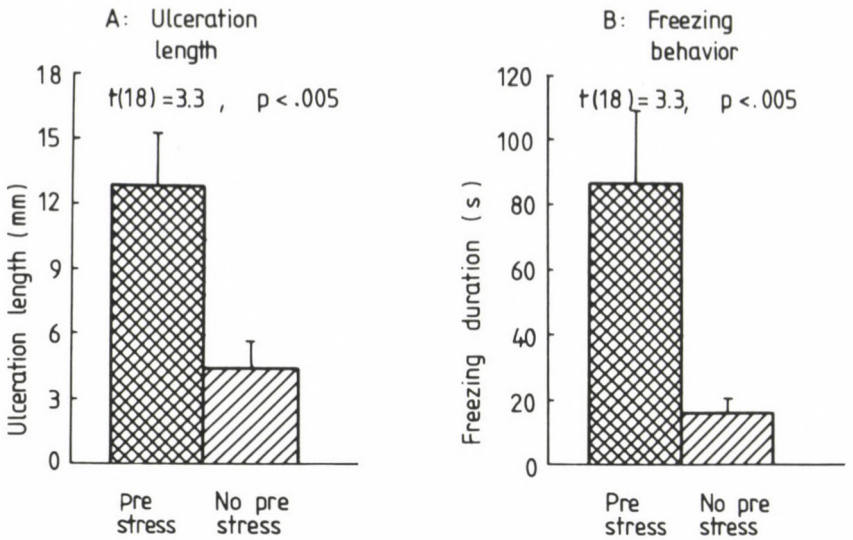

Fig. 1. Duration of freezing behavior (B) induced by removal of an auditory stimulus and (A) amount of gastric ulceration induced by water restraint stress in animals previously subjected to uncontrollable foot-shock

A large number of studies have examined the phenomenon of individual differences in intraspecies aggression. We have become interested in how the animal's position or status within the social hierarchy might be related to its ulcerogenic response to stress. Any group of colony housed animals will form a hierarchy over time, and Benus [3] has reported that the animal's position in this hierarchy is reflected both in social and nonsocial behaviors. For example, more aggressive animals appear to be less flexible in their behaviors in a maze task when the "rules" of the maze task are altered.

Our original thinking on how ulcer susceptibility might be related to social position was that the psychological characteristics associated with ulcer disease in humans seemed on the surface to resemble those of subdominants [13], whereas the characteristics associated with cardiovascular risk (type A) resembled more those of dominant animals. Of course, such speculation is most unscientific, but it does generate hypotheses which can be tested. Our hypothesis was therefore that animals exhibiting relatively high levels of aggression towards conspecifics would develop less ulcer under later stress than animals who were less aggressive.

We tested male animals for aggressive behavior in a classic intruder test and scored them for a number of aggressive and defensive behaviors towards the intruder. The resident animals had previously been colony housed in the presence of a female. The latter is important to increase levels of aggression. On the introduction

of an intruder animal there was in our test generally a low level of aggression in our animals, who had not been specifically bred for aggression studies as so often is the case. However, we were able to differentiate between degree of aggressive behaviors. Some weeks later, the animals were exposed to ninety minutes of restraint-in-water stress and examined for the extent of ulceration. To our surprise, we found a significant negative correlation between the latency to attack the intruder animal and the amount of ulceration developing. That is, the more aggressive animals appear to be more ulcer susceptible than the nonaggressive animals. The interpretation of this is fraught with problems. For example, is attack behavior in the intruder test a good measure of the animals social position? To what extent does an intruder test cause the animal to be stressed, and therefore proactively effect later responses to water restraint stress? Anyway, the point is that there is a relationship between behavior in the intruder test and the amount of ulceration developing. Our findings are in agreement with earlier work by Sines and Eagleton [15] who reported that, in competition for water, animals bred for ulcer susceptibility were dominant over standard Sprague-Dawley animals.

Interestingly, we have also found a significant relationship between our animals' responses to alterations in a well-learned maze task and behavior in the intruder test, which confirm the finding by Benus and his colleagues [3]. Briefly, those animals which are classified as most aggressive towards an intruder show least increase in running latency when a distracting stimulus is placed in the maze. This has earlier been interpreted as indicating that the behavior of the aggressive animals is based more on inner, previously learned factors, and are less dependent on outer stimulus factors.

There are two points which we believe should be made as a consequence of our results. First, the amount of ulcer developing under stress is largely determined by inputs from the brain and endocrine system, and these are related to behavior in both social and nonsocial settings. It is therefore possible to screen experimental animals for susceptibility to stress ulcer prior to stress exposure. By choosing animals carefully, it should be possible to reduce error variance, and thus reduce the number of animals required in any given experiment. Given the increasing concern over the often unnecessarily large number of animals in biomedical research, this is not an insignificant consideration.

Secondly, the phenomenon of individual differences provides us with a number of interesting questions. What underlies these differences, both at a behavioral/psychological level, and at a physiological level. The important studies by Cools and his colleagues [4] into brain dopaminergic function in aggressive and nonaggressive animals in an outbred strain provide us with specific hypotheses concerning the underlying physiological differences between our animals. Basic neuropharmacological and neurochemical differences between ulcer susceptible and

ulcer unsusceptible animals will in turn guide us towards the underlying differences in the physiology of brain-gut interactions.

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ESOPHAGEAL MUCOSAL PROTECTION – WHY DO WE NEED A SPECIAL APPROACH?

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The epidemiology and natural history of reflux induced peptic esophageal diseases remain incompletely understood. That is why it is easy to explain that the traditional therapeutic efforts were mostly restricted to the use of acid-reducing or neutralizing drogs. The author tries to survey – mainly on theoretical bases – a new approach of the maintenance treatment of peptic esophagitis and consequential columnar metaplasia.

The mechanism of the esophageal antireflux barrier is composed by the (a) lower esophageal sphincter tone, (b) upper esophageal sphincter tone, (c) esophageal acid clearance and (d) *esophageal epithelial resistance*.

The data of a 100-patient-group of gastroesophageal reflux disease cases were retrospectively evaluated principally considering the efficacy of antisecretory treatment relating to the accompanying diseases, recurrence of symptoms and prevention the development of Barrett's columnar lined esophagus and Barrett's ulceration. The decrease of exposure by damaging factors is an essential criterion of antisecretory therapy, having several disadvantages. Based only to logically well established arguments the author believes that gastroesophageal reflux disease and consecutive conditions might be an ideal model for studying and introducing esophageal cyto (-mucosal, -tissue) protection, considering that in the esophagus – in contradiction to the stomach – the cell and tissue injury, induced by several pathogenic agents, does not develop rapidly, and when the organ damage develops gradually, interventions may be possible to protect esophageal cell and the mucosa directly.

Keywords: mucosal protection, gastroesophageal reflux disease, Barrett-esophagus, sucralfate

The introduction of the concept of "cytoprotection" has been strictly related to the experiences of Robert and co-workers [1, 2]. Studies on cell injury and protection were primarily performed in the stomach, the multifactorial nature of these damaging and protective process has not been always acknowledged.

Considering the heterogeneity and complex structure of the gastrointestinal mucosa and organs [3], and included them into the theory the principles of the

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"tissue"- and "organoprotection", the mechanism of the gastrointestinal protection conceptually can be divided into two parts:

(1) *Preservation of existing cells* by enhanced resistance of cells or by decreased exposure to damaging agents [4],

(2) *replacement of lost tissues* by original cells or connective tissue repair [4].

Although it has been estimated that 10% of a general population may have symptoms of heartburn and regurgitation on a daily basis, and 30% to 50% less frequently [5], moreover it is generally accepted that the columnar metaplasia (Barrett) lesions occur predominantly through chronic injury to squamous epithelium by gastroesophageal reflux [6], the epidemiology and natural history of reflux-induced peptic esophageal diseases remain incompletely understood. That is why it is easy to explain that the traditional therapeutic efforts were mostly restricted to the use of acid-reducing or neutralizing drugs.

The lack of pharmacologically more convenient short-term and maintenance treatment modalities led me to survey the theoretical background and possibilities.

Gastroesophageal reflux disease (GERD)

There are three principal physiological defense mechanisms against esophageal injury due to the reflux of gastric contents [5, 7].

These are:

(1) the lower esophageal sphincter (LES),

(2) esophageal motor function with acid clearance,

(3) esophageal mucosal (tissue) resistance.

The GERD encompasses *reflux esophagitis*, which implies unequivocal histologic changes of the lining mucosa [8]. The reflux itself is not an "all-or-none" phenomenon [9, 10], but a physiological one, which often occurs in healthy asymptomatic subjects.

To establish the correct diagnosis of GERD – considering the above mentioned defense mechanism criteria – several questions should be clarified:

(1) Is abnormal gastroesophageal reflux present? Is the LES relaxation the prevalent mechanism causing reflux episodes?

(2) Is the acid reflux responsible for the patient's symptoms? Does the esophageal acid clearance play a determinant role in the development of mucosal injuries?

(3) Is the decreased esophageal epithelial resistance a relevant factor of GERD?

(Ad 1.) The primary functions of the LES are relaxation from the usual tonic state during swallowing to allow the free passage, and maintenance of an effective high-pressure zone to prevent reflux of the gastric contents [11]. LES competence and LES pressure are determined by anatomic, neural, hormonal and life-style related factors [8, 11]. Anatomic factors include the role of diaphragm, the "cardiac

angle" and the intra-abdominal segment of the esophagus. Moreover, regional differences exist in the exposure of the esophageal mucosa to the refluxed gastric acid. The acid exposure time in the distal esophagus is greater than in the remainder of esophagus, the differences are more pronounced in supine position [12].

Significant diurnal variation has been demonstrated in basal LES pressure [13], and the list of foods, drugs, substances and hormones that alter lower esophageal sphincter pressure grows every year [8]. These are shown in Table I.

Table I

Factors altering lower esophageal sphincter pressure

Decrease		Increase
	A. Foods	
Fat		Proteine
Ethanol, cola		
Cholate		
	B. Drugs	
Caffeine		Cholinergic agents
Tobacco		Metoclopramide
Theophylline		Cisapride
Anticholinergics		Anticholinesterases
Dopamine		Indomethacine
Morphine		Histamine
Lidocaine		Antacids
Diazepam		
Ca-channel blockers		
Birth control pills		
	C. Hormones	
Secretin		Gastrin
Cholecystokinin		Pitressin
Glucagon		Angiotensin
VIP		Bombesin
Progesterone		Substance P
Hypothyroidism		Motilin

The basal tone and changes of LES pressure can be exactly measured by invasive esophageal manometry only [14], and it has been demonstrated that reflux events rarely occur unless the LES pressure falls below 3 mmHg [15]. However, there are very few patients with a resting LES pressure of 3 mmHg or below [14].

On the other hand, to answer the question whether abnormal gastroesophageal reflux was present or not, well-defined quantitative evaluation as continuous esophageal pH-monitoring are required, which define normal control values, derived from a reasonably large sample of healthy asymptomatic subjects [16].

(Ad 2.) The role of abnormal esophageal acid clearance is an important factor in the pathogenesis of GERD. The normal clearance itself is probably a two-step process [17], composed by a single swallow peristaltic volume clearance (first step), and by the neutralization of intraluminal residual acid by salivary bicarbonate (second step). Delayed esophageal emptying may be the predominant component of abnormal acid clearance, regardless of its primary or secondary origin. It is still unclear, whether esophageal motor disturbances are the major cause of GERD, or GER causes secondary esophageal dismotility, but several studies proved, that reflux may contribute to a vicious cycle on which reflux causes abnormalities in LES pressure and esophageal clearance that lead to further reflux [8]. In a well-controlled manometry-acid perfusion study [18] the majority of GERD patients were shown to have esophageal motor abnormalities, which consisted of three types: nonprogressive (tertiary) esophageal contraction; increased amplitude and duration of contractions; and increased esophageal tone. However, it seems to be remarkable, that a great part of these motor disorders proved to be reversible after antireflux surgery [19, 20]. It is not easy to tell whether acid reflux was responsible for the complaints of the patient. The acid perfusion test was introduced by Bernstein in 1958 and it seemed to be promising, but – unfortunately – its sensitivity does not exceed the 54–80 per cent and specificity the 59 per cent, either.

(Ad 3.) The next determinant of esophageal antireflux barrier, the esophageal epithelial (tissue) resistance is not a single factor, but represents a number of layered structures and functions that interact to form a dynamic barrier [22]. The components of this structure are listed on Table II.

Table II

Esophageal epithelial resistance

1. Preepithelial
   a. Mucus
   b. Unstirred water layer
   c. Surface bicarbonate
2. Epithelial (cell membrane)
3. Postepithelial (metabolic buffering)
4. Gastroduodenal influences
   a. Potency of the refluxate
   b. volume of the refluxate

Besides these very important factors (mucus, high bicarbonate-containing "unstirred water layer", etc. [22]), vascular factors are relatively new elements in investigations on mechanisms of gastrointestinal protection [23] and they were very rarely evaluated in the development of peptic esophageal lesions. In the stomach [24] and small intestine it has been demonstrated [25] that ethanol-induced mucosal injury is associated with an early increase in microvascular permeability. Recent studies suggest that this type of increased microvascular permeability occurs very early in the course of acid-induced esophageal lesions, too [26].

The Barrett Domain

In 1950 Barrett [27] described a case with an esophageal peptic ulcer probably developed on the ground of columnar metaplasia. Although Barrett's original conception of anatomical interpretation proved to be incorrect, the columnar metaplastic esophagus and esophageal peptic ulcer was labelled with his name.

The natural history, epidemiology, clinical development and endoscopical aspects of columnar-lined esophagus shows two distinct types [28]. *Type I* shows a regular columnar lining, with a sharply defined proximal limit resembles the normal Z-line. In the *Barrett type II* the columnar and squamous epithelium is separated irregularly, the proximal margin is asymmetric with several islands of squamous epithelium scattered throughout the columnar cell replacement. This type II is far more frequent than type I, and while type I may be sometimes found in younger age, and type II occurs more frequently in adults with long-term history of esophagitis, therefore it was concluded that type II. columnar metaplasia could be an acquired lesion attributable to ulcerative peptic esophagitis, whereas type I cases could be of congenital origin.

Compared of population-based clinical and autopsy findings [29], it was concluded that the majority of Barrett's esophagus cases remains unrecognized in the general population. Findings of unselected autopsies suggest a prevalence of 376 cases per a population of 100 000, in 5 of 7 cases Barrett's esophagus was first detected at the time of autopsy [29]. Several studies were designed to determine the prevalence of columnar-lined esophagus in population of patients with symptoms of gastroesophageal reflux, and generally the results suggest that this lesion complicates the course of patients with persistent reflux symptoms significantly more often than it was previously thought. Savary [28] in a group of 3681 esophagoscopes for gastroesophageal reflux disease found 340 cases (9,2%) of columnar lined esophagus, Winters [30] in 12,4% of 97 patients, and our own results (12/100 cases, 12%) also correlate with the data of world literature (8 to 20%) [31].

In the diagnosis the sensitivity of endoscopy was significantly more higher (92%) than that of radiology (24%) and performing well orientated biopsies taken at

2 cm intervals along the esophagus proved to be able to recognize columnar epithelium – even in expert hands – only in 90% of the patients (30, 31%). Endoscopic picture and complications may show extremely various experiences: for a superficial survey only, some literature data and our own results are demonstrated on Table III.

Table III

Endoscopic findings in columnar-lined esophagus

	Esophagus	Ulcer	Stenosis	Dysplasia (D) Carcinoma (C)
Stadelmann 1981	10/14	3/14	7/14	N/A
Herlihy 1984	12/20	2/20	8/20	N/A
Hameeteman 1989	50/50 (100%)	12/50 (24%)	8/50 (16%)	D:8/50 (36%) C:5/50 (10%)
Savary 1985	N/A	(15%)	(36%)	C:30/179 (16%)
Collins 1991	(60%)	(37%)	(14%)	C:10%
Own	11/12	2/12	2/12	D:1/12 C:1/12

The functional characteristics of Barrett's esophagus are featured by the presence of oxyntic cells, producing acid, demonstrable pepsinogen and pepsin secretion and increased tissue gastrin level [32]. In the acidic area, colonisation of *Helicobacter pylori* may occur [33], as we have also demonstrated in two cases of treatment-resistant Barrett-ulceration [34].

The association of Barrett's metaplasia and esophageal adenocarcinoma has long been recognised. A well controlled prospective study showed an incidence of Barrett's cancer of 1 in 52 patient-years, a 125-fold increase compared with the general (Dutch) population [35]. Other studies showed a prevalence between 5 and 46.5%, with a certain significance of minor risk factors (male sex, white race, older age, tobacco), and an average 32-fold risk of the development of esophageal carcinoma in high-grade dysplasia Barrett's esophagus cases [36, 37].

The phenomenon of Barrett's columnar metaplasia may be of extreme interest reviewing the pathophysiological factors [31, 34, 36, 38], which provoke the development of the lesion (Table IV):

Table IV

Pathophysiological factors inducing the development of columnar metaplasia

(1) Severe gastroesophageal reflux - abnormally frequent reflux episodes - defective esophageal acid clearance
(2) Gastric acid hypersecretion
(3) Hyposensitivity of esophagus to acid reflux - decreased epithelial resistance
(4) Agressiveness of refluxed material (not only that of low pH!)
(5) Adverse effects of antineoplastic chemotherapy

On the one hand, it seems to be obvious, that the Barrett patients, in whom the diagnosis has been made serendipitously, may be the top of large iceberg of asymptomatic patients [31]. On the other hand, although it is now evident that one of our principal treatment possibilities is an effective acid suppression, that is theoretically and practically not enough to neutralize the other pathogenetic agents, and it is illogical to believe, that this symptomatical treatment for only a limited period might prevent the development or extension of columnar metaplasia and late complications. I tried to evaluate our GERD material considering these standpoints.

Patient material, methods, results

A 100-patient-group of gastroesophageal reflux disease cases was evaluated specially from the points of view of therapeutic requirements based on before mentioned theoretical considerations. The diagnostic possibilities are indicated on Table V.

Table V

Diagnostic methods

(Barium esophagography)
Esophagoscopy and biopsy
Acid perfusion (Berstein) test
Radionuclide scanning
Prolonged pH monitoring
(Esophageal manometry)
Laryngotracheal investigations

Obviously the greater part of data was evaluated retrospectively, therefore the specification of rate and efficacy of single methods seems to be purposeless. On Table VII have distributed our patient material

Table VI

Patient material

No. of patients with gastroesophageal reflux: 100	
GER-related conditions:	
- hiatal hernia	21
- duodenal ulcer	34
- Barrett esophagus	12
Histologically proved esophagitis	64
"Pure" GERD	33
with esophagitis	20/33
without esophagitis	13/33
Esophageal stenosis	
related to DU	1/34
related to Barrett	2/12
related to GERD	6/20/33

by special respects, continuing the appraisal on Table VII according to therapeutic efficacy of antisecretory drugs

Table VII

Therapeutic results with antisecretory drugs

	Fair	Good
Related to Du	4/33	29/34
Related to HH	17/21	4/21
"Pure" GERD	15/27	12/27

used in a 6 week–8 month period, but regardless the specification and dosages of the drugs. (I have to emphasize the greater therapeutic efficacy of omeprazole, compared with H2-blockers, but deeper analysis of these results might not be the aim of this review.)

General overview of short-term therapeutic results seems to support that

(1) antisecretory treatment is mainly effective in cases of duodenal ulcer disease (gastric hypersecretion, delayed gastric emptying):
(2) withdrawal of antisecretory treatment is followed promptly by recurrence of esophagitis and symptoms to their pretreatment severities;
(3) reviewing the history of Barrett's esophagus and peptic stricture cases, repeated courses of antisecretory treatment did not prevent the long-term mucosal injury and consecutive alterations.

Cytoprotection, histoprotection, organoprotection in the esophagus

The facilities of medical treatment of GERD are summarized in Table VIII.

Concerning the field of non-surgical antireflux therapy there is only anecdotical information on the benefit of alimentary habit and life-style modifications. Prokinetic agents may have – in selected cases – an additional beneficial effect, but their use – principally in monotherapy – seems to be more than doubtful. The long-term efficacy of antireflux surgery and the technical factors that determine the durability of the antireflux effect need further study in patients with Barrett's esophagus more than ever before, now that long-term medical treatment is an effective option [31]. The decrease of exposure by damaging factors (shortened exposure, decreased absorption, diluted concentrations) is an essential criterion of antisecretory therapy. Nevertheless, antisecretory drugs – being not causal treatment – have several disadvantages:

Table VIII

Antireflux therapy

(A) Dietary modification
(B) Life-style modification
(C) Avoidance of refluxogenic agents
(D) Prokinetic agents
. bethanecol
. metoclopramide
. cisapride
"Anti-damaging-factor" therapy
(A) Antacids
(B) H-2 blockers
(C) Omeprazole
Prevention: Mucosal cytoprotectants

- they do not prevent mucosal injury,
- their long-term use may result in non-desirable adverse effects,
- they have discordant treatment effects according to the special types of diseases,
- withdrawal of treatment is followed by immediate renewal of damaging effects,
- gastric acid is not alone responsible for mucosal damage,
- this treatment modality is generally introduced in already developed irreversible conditions, e.g. histologically proved esophagitis or columnar metaplasia.

The fundamental aim of this review was to criticize the actual treatment possibilities in GERD and related lesions. Hereupon, it would be obvious to offer an alternative treatment possibility, involving the hitherto reached results of gastrointestinal cytoprotection. Unfortunately, in lack of real results, theoretical conclusions could only be drawn:

(1) All of the before detailed logically well established arguments seem to support, that gastroesophageal reflux disease and consecutive conditions may be an ideal model for studying and introducing esophageal cyto- (-mucosal, -tissue -) protection, regarding the following reasons:

(1.1) the decreased exposure for exogenous and endogenous damaging agents was already criticized before

(1.2) theoretically it seems probable, that in the esophagus – in contradiction to the stomach – the cell and tissue injury, induced by several different pathogenetic agents, does not develop rapidly, and when the organ damage develops

gradually, interventions may be possible to protect the esophageal cell and mucosa directly.

(2) Sucralfate is one of the few drugs that has both acute cytoprotective and ulcer healing effect, as well [4] or preventive effect which was recently proved in experimental animals [39, 40]. The pharmacological actions of sucralfate – under both acute and chronic conditions [4]:

(2.1) absorption of bile acids and pepsin stimulation of mucus and bicarbonate secretion

(2.3) probable binding to endogenous sulfhydrils and increasing of prostacyclin and prostaglandin synthesis

may act either in acute mucosal protection or in healing of chronic – (ulcerative?) – lesions. On the other hand,

(2.4) protection of vascular integrity,

(2.5) protection of proliferative zone may result in acute prevention of repeated or continuous short-term injuries caused by reflux or alimentary factors [4], while

(2.6) epidermal growth factor binding [4] and

(2.7) barrier-production may facilitate the healing and re-epithelization of the damaged mucosa.

The averting of the greatest difficulty, namely the rapid clearing of the drug from the esophagus, and the practical verification of these theoretical ideas need further extensive studies.

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A REVIEW OF SPONTANEOUS ULCER DISEASE IN DOMESTIC ANIMALS: CHICKENS, CATTLE, HORSES, AND SWINE

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The human species is perhaps unique for its high incidence of spontaneous, chronic ulcer of the glandular mucosa of the stomach and duodenum. Nevertheless, spontaneous ulcers, usually of the stomach, commonly occur in many domestic animals. Some of these lesions are chronic and they may occur in either the glandular or squamous-lined regions of the stomach. As with the human disease(s) the pathogenesis in domestic animals is multifactorial, poorly understood, and variable between and within species. Some parallelisms exist in aggressive and defensive factors, but parasitic factors, via gastrinemia, and a histaminic factor via diet may occur in some animal ulcers. Underlying environmental stresses, of debated importance with the human disease but of proven importance in several rat ulcer models, may play a key role in some spontaneous gastric ulcer situations in swine and cattle. This is manifest in crowding and transporting situations. Seasonal, age, and weaning factors also alter the incidence of ulcer in cattle. Psychologic/environmental stress-related factors, as well as drug and physiologic stress factors appear to upset the balance in the horse between resistance and aggressive mucosal factors. Dietary factors which are highly important in ulcer disease in swine and chickens, have not yet been incriminated in spontaneous, equine ulcer disease. More investigation of the pathogenesis of domestic animal ulcers will prove useful for both human and veterinary medicine in terms of a) elucidating pathogenetic mechanisms for all species, b) may provide new animal models for study, and c) may enhance prevention of such lesions in domestic animals for economic and humanitarian reasons.

Keywords: spontaneous ulcer, cattle, swine, chicken, bovine, equine, porcine

Since peptic ulcer disease is a common human malady, it has been subjected for many decades to experimental investigation in which humans and laboratory animals have been the main subjects of study. Thus, much has been learned about ulcer pathophysiology in small mammals such as rats and mice, other rodents, dogs

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and cats, and laboratory ferrets [60, 61, 72]. However, ulcer disease also occurs in large domestic animals, domesticated birds, various forms of wildlife including marine mammals, etc. The pathogenesis of ulcer disease has been much less studied in the non-laboratory types of animals which are generally less suitable for experimental manipulations, and spontaneous ulcer disease usually goes undetected in such animals. Further, the cross-over of information between veterinary scientists and medical scientists has traditionally been slight, with few veterinary scientists participating in peptic ulcer congresses and most medical or surgical ulcer specialists being poorly informed about ulcer disease in farm animals or wildlife.

In spite of the above dilemma, there is great and sometimes preventable morbidity attributable to spontaneous ulcer disease in non-laboratory animals. As will be described below, the incidence of gastric ulcers in species such as cattle, horses, chickens and swine may in high risk conditions occur in large percentages of animals. Due to economic factors in veterinary medicine and farm management, it often is considered not feasible to diagnose or medically treat ulcer disease in cattle, chickens and swine, frequently mortality is low, and weight gain, which is of high importance to farm managers, is only temporarily reduced. However, investigation of ulcer disease in some of these non-laboratory species is of great value since elucidation of their pathogenesis may a) make prevention of ulcer disease more attainable in farm and field conditions, and b) by demonstration of both unique and similar factors in ulcer development and healing in these natural and non-experimental situations, the understanding of ulcer disease in man may be enhanced. As will be delineated in the brief review below, much of the information on non-laboratory animal ulcers is based upon epidemiologic and post-mortem pathology reports, although in the case of chicken and swine ulcers, some manipulative experimental studies have also been undertaken.

Ulcers in chickens

Spontaneous gastric ulcers in chickens are one of the most intriguing diseases from a pathophysiologic standpoint even though avian species are remote from humans. The upper digestive tract of chickens has, adjacent to the lower esophagus, a specialized storage sac, the crop. This squamous-lined pouch has some limited transport and protein secretory functions but is usually not subject to hemorrhagic ulceration. More distally there are essentially two stomachs including a muscular stomach, the gizzard (ventriculus), associated with mastication of food particles. It is commonly a site of hemorrhagic erosions on its cuticular (koilin layer) surface due to acid-peptic secretion from the glandular stomach, proventriculus, situated adjacent to the gizzard. The proventriculus is histologically like the glandular stomach (corpus and antrum) of mammals and its mucosa contains parietal, chief, and endocrine cells.

The earliest accounts of gizzard erosions in chickens were published in 1929 [18] and in the early 1930's, when a scurvy-like disease was seen in chicks. A study of this condition, correctable by feeding cabbage (vitamin C) supplements, showed that 95 per cent of chicks had hemorrhagic erosions on the gizzard lining [36]. Later workers, however, found that ascorbic acid did not always prevent these erosions, but that an anti-hemorrhagic vitamin, vitamin K, prevented the lesions [19, 20]. This was later disputed. Interestingly, gizzard erosions were also observed in unhatched chicks and day-old chicks [6]. A series of early investigations attempted to identify some nutritional factor or deficiency responsible for these ulcerations. Cheney [16] working not in a poultry science department but at Stanford University Department of Medicine, was the first to associate gastric hyperacidity in chicks on a deficient diet and with gizzard ulcers. Other historically important early reports showed that bile paradoxically prevented gizzard erosions [5], that the lesions histologically originated from submucosal capillary hemorrhage [45] and could be reduced by grit in the diet [4] or liquid milk [3], or enhanced by adrenalin in pre-hatched chicks [73]. The pathogenesis of chick gizzard lesions was then ignored until 1968, when Good et al. [29] reported strain differences in susceptibility. The gizzard lesions were not only reported as erosions, but some were seen to perforate [17]. Perhaps the first indication of their true cause was published in reports from Peru and the Netherlands in 1971, when investigators observed that the erosions were associated with high intake of fish meal in the diet [37, 42]. It had been considered that fish meal contained an unknown growth factor which promoted maximal chick growth, [9] but it had also earlier been reported that there was a general toxicity to chicks by histamine produced by microbial spoilage of tuna in their diet [70]. Subsequently, it was shown that addition of histamine to the diet of chicks produced typical gizzard erosions, and that the type of fish meal used and extent of bacterial spoilage altered the coarse of fish meal toxicity [32, 33]. Miyazaki and co-workers [49, 51] have more recently studied the gizzard erosion problem and a casein-histidine mixture, found in fish meal, which gives rise to histamine. Recently, Swedish workers have noted *Clostridium perfringens* infection commonly present at the site of gizzard erosions [25]. Also, it was recently demonstrated that anticholinergic and H_2 blocking agents, but not an H_1 blocking agent, prevented gizzard erosions induced by a heated casein-histidine mixture in the diet [50].

Although much remains to be clarified about the pathogenesis of gizzard erosions in chickens, especially those formed prior to hatching or not caused by histidine in the diet, there are important analogies to gastric ulcers in man and other mammals. These include features such as the acid component, the role of histamine and preventive actions of H_2 blocking agents, and the role of early vascular changes in the pathogenesis.

Ulcers in cattle

Inflammatory processes, including ulceration of the bovine stomach are complicated by the fact that cattle have a compound stomach with four main compartments, the rumen, reticulum, omasum, and abomasum. Only the most distal compartment, the glandular abomasum, is comparable to the human stomach. All other compartments are lined by a stratified squamous epithelium, are associated with fermentation of foodstuffs to absorbable volatile free fatty acids, and have an elaborated macroscopic architecture. Thus, mucosal damage might occur in any of these four compartments and the pathogenetic basis for ulceration might be region-specific. Early accounts of spontaneous gastric ulceration of the stomach in cows (and sheep), based upon observations of dissected stomach at the abattoir, showed seasonal variation of active lesions [67]. Late winter and early spring were the high risk times. Large, irregular ulcers were observed in the first three gastric compartments, and smaller, round lesions were seen in the abomasum. Acute ulcers, chronic ulcers, and healed ulcers were all found in the same cow. In this early, classic report, Rosenow isolated streptococci from the ulcers and erroneously concluded that the ulcers were caused by such bacteria. Other early reports also discussed human ulcer-like lesions in the abomasum of cattle [27], bacteria in rumenal ulcers, [71] and trauma-induced gastritis in cows [39]. Rumenitis was early recognized as a problem associated with liver abscess and was also experimentally produced by dietary means, such as feeding excessive grain to cattle [38]. Experimental induction of gastritis by intra-gastric foreign bodies, perforation of spontaneous abomasal ulcer, and presence of fungi in spontaneous abomasal ulcers were also reported in early days [28, 43, 78]. In subsequent years many case reports of abomasal ulcer in cattle appeared, some of which described perforation or surgical therapy of the ulcers. French veterinary workers concluded that ulcerative lesions of the first three compartments of the bovine stomach resulted from dietary factors (inadequate amounts of roughage and too rapidly fermentable feeds), whereas ulcers of the abomasum resulted from central or peripheral neural factors or stress [46, 74]. Petechial hemorrhage, erosion and ulceration of the bovine abomasum may also result from infection of cattle by *Chlamydiae, Ostertagia osterlagi*, or *Cooperia oncophora* parasitic infections [22, 48].

One interesting feature of some of the parasitic infections of the alimentary tract in large animals is that they may induce hypergastrinemia in the host animal, thus aggravating an ulcer problem by chronically stimulating gastric acid secretion.

There have been a few large-scale epidemiologic investigations on ulcer disease in cattle, some of which attest to the importance of this disease(s). Hemmingsen studied 63 cows with perforating abomasal ulcers presenting from a population of 300 000 cattle [34, 35]. The highest frequency was in cows greater than two years of

age at their time of calving, one month post partum. Frequently, a single perforation on the ventral wall of the fundus was found. The frequency of ulcer decreased with age, and little correlation between erosion and ulceration was observed. Erosions and ulcers occurred in different parts of the abomasum; ulcers were more common than erosions in the pyloric region. Thus, as in humans there may be a complex of different ulcer diseases in cattle targeting different regions of the stomach. Aukema and Breukink [8] studied 1370 normal cattle, 1200 cows brought to the Utrecht Veterinary faculty for emergency slaughter, and 141 cows with abomasal hemorrhage. Bleeding ulcers, as well as ulcer scars, were usually found on the greater curvature on the most ventral part of the fundus. Active ulcers were associated with grass feeding in the summer, not with the stress of calving. Jensen and co-workers [41] also undertook a large study of 407 000 yearling feedlot cattle. Of 3943 that died, 1988 were necropsied, and abomasal ulcers were seen in 1.6 per cent. These were fatal perforations, and a similar per cent had innocuous ulcers. Ulcers were seen during all seasons but were most prevalent during the first 45 days of winter-initiated fattening.

Few pathophysiologic studies have been done on the problem of ulcers in cattle, and for economic reasons, severely afflicted animals are sacrificed rather than treated. It will be challenging to learn more about the pathogenesis of ulcers in cattle. If the risk factors for abomasal ulcers can be more precisely identified it will be useful both for preventing lesions in cattle and may even help clarify human ulcer pathogenesis.

Ulcers in horses

Although the horse is a herbivorous animal with a distinctive digestive tract, most of its digestion and absorption of carbohydrate is in the specialized large intestines, and its stomach is a small, single compartment mostly lined by a glandular mucosa like that of the human. There is also a squamous-lined proximal region of the equine stomach somewhat similar to the rat forestomach.

The recognition of gastric and duodenal ulcers in horses has increased in recent years, partly owing to the use of fiberoptic endoscopes designed for large animals. Unlike the problem of gastric ulcers in cattle, the diagnosis and treatment of equine ulcers has taken on greater importance since many horses have high personal of economic value, particularly in the racing industry. The cause of gastric ulcers in foals and adult horses is generally not known, although in selected cases it is highly likely that the therapeutic use of phenylbutazone or flunixin or co-existing maladies in the horse contributed to gastric or duodenal ulcerations [75–77]. There are also reported instances in which erosions, ulcers, or perforations of the glandular gastric mucosa have been attributed to *Candida* colonization [30], the toxic response to

cantharidin present in ingested blister beetles, *Epicauta* spp. [68] or due to attached larvae of the bot fly, *Gasterophilus intestinalis* [69, 79, 81] on the mucosa. Nevertheless, most gastric glandular mucosal ulcers or duodenal ulcers have an unknown cause, or are associated with stress conditions. Prevalence studies on gastric ulcers in foals and older horses undertaken at our College revealed that a high percentage (51 per cent of 72 foals) of young foals have ulcers, usually (60 per cent) in the squamous portion of the stomach. This included foals on farms and in the hospital, and horses that did not present with clinical symptoms (colic, diarrhea, etc.) or ulcer. Of 50 older horses in the hospital because of various medical problems, 80 per cent showed ulcers by endoscopy [55]. Numerous case reports have described gastric ulcers in foals in which the cause was not known [11, 15, 62, 66, 80]. In some instances, horses presented with pyloric obstruction or gastric retention which was likely secondary to duodenal ulcer stricture, or chronic gastric ulcer [47, 59, 82]. Also, ulcerative duodenitis in foals and perforating duodenal ulcer in horses have been reported [1, 10].

Few pathophysiologic studies have been done to enlighten the veterinarian on the problem of equine ulcers. Many of the lesions occur under obvious conditions of stress and post-operatively, but many have no known adverse condition. Mucosal resistance factors have not been studied in this species, but H_2 blocking agents and sucralfate are effective therapeutic agents in the horse.

Ulcers in swine

The pig has an alimentary tract partially similar to that of man, although its single compartment stomach has a proximal portion lined by squamous epithelium, referred to the "pars esophagea." Ulcers occur most commonly in the pars esophagea, but also in the glandular mucosal region. If appearing in the squamous zone, they are referred to as "esophagogastric ulcers." The pig has been a popular animal for study of the ulcer problem, partly because esophagogastric ulcers occur frequently in farm animals worldwide. Esophagogastric ulcers in swine were first reported in 1939 by Jensen and Frederick at the University of Illinois [39] but have now been reported in 21 different countries. The per cent of pigs affected depends upon the country of origin, diet, handling characteristics, strain, etc., but has ranged from 4.7 per cent (Australia) to 100 per cent (Japan). In larger scale studies, e.g., in the U.S.A., 5.0 per cent of 20 000 pigs had ulcers and in Hungary 12.7 per cent of 13 400 pigs had ulcers [58].

Esophagogastric ulcers can be lethal due to massive intragastric hemorrhage. They occur in all strains of pigs of either sex and at any age. However, there is a genetic influence on susceptibility, and Berruecos and Robinson [12] have shown that strains bred for rapid growth and lean back fat have a higher risk for esophagogastric

ulcers. Ulcers in swine, as with ulcers in humans, are more prevalent in winter than in summer [14]. Swine with esophagogastric ulcers may have concomitant and multiple ulcers in the glandular mucosa (cardia, fundus, and pylorus), and they may have ulcers in a peracute stage and appear in excellent health [44]. Many studies have been conducted to search for etiologic factors of ulcers in swine, and a number of these were directed at infectious agents since fungi and bacteria were frequently isolated from ulcerative lesions. However, none of those agents have been implicated as a primary cause of esophagogastric ulcers. Also, many studies have been directed at possible dietary aspects of the pathogenesis [56–58, 63, 64] and details of these findings are reviewed elsewhere [44]. The principal finding was that finely ground feed was the highest risk factor predisposing swine to esophagogastric ulcers [52], especially in confined pigs. This type of diet was also found to be associated with greater acidity in the esophageal area of pigs [64]. The relationship between stress and esophagogastric ulcers was also proven in various laboratories, including restraint stress, transport stress, parturition stress, and crowding stress [44, 52]. Thus, from these findings there is likely a multifactorial cause to esophagogastric ulcers involving the environmental factors as well as non-natural dietary factors.

Ulcers of the glandular region of the pig stomach can also be induced experimentally by chronic gastric stasis resulting from vagotomy [23, 24]. Esophagogastric ulcers have been induced experimentally in swine by intramuscular reserpine [53] and by intramuscular histamine [54], per os copper administration [2] and experimental extrahepatic biliary obstruction [13]. Deep or penetrating ulcers of the glandular stomach have been induced by parasitic infections, including the stomach worm, *Hyostrongylus rubidus* [21], and blow-fly larvae, *Sarcophaga bullata* [7]. Esophagogastric ulcers in swine were also caused by *Ascaris suum* [26, 31, 65].

Because of some similarities of the pig and human digestive tracts, the great veterinary importance of spontaneous esophagogastric ulcers in swine, and popularity of pigs as research animals, continued study of ulcer disease in pigs will be most fruitful.

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SUBCELLULAR LOCALIZATION OF PROSTAGLANDIN E_2 RECEPTORS IN THE GASTRIC MUCOSA

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Gastric mucosal PG E_2 receptors are the common antisecretory working point of all prostanoid types and may also be involved in "protective" effects. We investigated the subcellular localization of these receptors, as measured by displaceable ^{3}H-PG E_2 binding, and identified different organelles by monitoring the activities of specific marker enzymes. Porcine mucosal homogenates were subdivided by differential centrifugation into fractions P1 ($1000 \times g$), P2 ($20\,000 \times g$), P3 ($300\,000 \times g$) and the supernatant S1. P3 was further fractionated over a series of sucrose step gradients.

Mitochondria and lysosomes were enriched in P2 (maximum specific activities of cytochrome-c-oxidase of β-glucosidase, β-glucuronidase, β-galactosidase, respectively). Plasma membranes (alkaline phosphatase, γ-glutamyl-transpeptidase, 5-nucleotidase), tubulovesicles (H^+/K^+-ATPase) and rough endoplasmic reticulum (NADPH-cytochrome-c-reductase) were mainly found in P3, which also contained the majority of ^{3}H-PG E_2 binding sites. In contrast, prostanoid binding was barely detectable in S1.

Density fractionation of P3 revealed that ^{3}H-PG E_2 binding sites shared a similar sedimentation profile with plasma membranes and tubulovesicular markers. No or negative correlation was found with lysosomes, rough endoplasmic reticulum and mitochondria.

We conclude that mucosal PG E_2 receptors are predominantly located at the cell surface. This supports the view that prostanoids inhibit gastric secretion through membrane receptors, but gives no clue for intracellular "protective" working points.

Keywords: PGE_2 receptors, gastric mucosa, marker enzymes, plasma membranes, lysosomes

Prostaglandin (PG) E_2 receptors mediate inhibition of acid and pepsinogen secretion in the gastric mucosa [6, 31]. Initial evidence suggests that these receptors are coupled to inhibitory G_i proteins, which in turn control membrane bound adenylate cyclase activity [11]. In addition to their antisecretory effects prostanoids can also induce mucosal "protection", but the potential involvement of PG receptors is uncertain [4].

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It has been suggested as one of the mechanisms to enhance mucosal resistance that prostanoids stabilize gastric lysosomes, thereby preventing the release of aggressive enzymes and the formation of acute erosions [19]. However, little is known whether this is a direct effect of PGs on lysosomal membranes in the stomach or whether enzyme release is regulated through intracellular cyclic nucleotides, as shown for granulocytes [20, 40].

Our goal was to further clarify the role of gastric PG E_2 receptors in antisecretory or "protective" effects by characterizing their subcellular distribution in the gastric mucosa. Previous investigations in other tissues support a predominant receptor localization on plasma membranes [8, 9, 18, 21, 23, 27], but evidence was also found for lysosomal PG E receptors [25, 28]. Initial experiments in the stomach gave no final clues since ^{3}H-PG E_2 binding shared the same sedimentation profile with the plasma membrane marker 5'-nucleotidase and also with the lysosomal enzyme acid phosphatase [34]; more recently PG E_2 receptors were additionally postulated in the cytosolic fraction [41].

Materials and methods

^{3}H-PG E_2 (specific activity 160–200 Ci/mmol) was purchased from Amersham-Buchler, Braunschweig. All chemicals used in this study had analytical grade and were obtained from standard commercial sources, mainly from Sigma, Munich.

Subcellular fractionation

Since the pig has been established as the most appropriate model for specific studies on lysosomal involvement in human gastric ulceration [13, 38], all receptor binding experiments were performed with porcine mucosa. Stomachs were collected at the local slaughterhouse. The fundic mucosa was bluntly separated from muscle and connective tissue, rinsed, and further processed at 4 °C in 10 mmol/l Tris/HCl, pH 7.4, with 10 μmol/l indometacin. The tissue was homogenized in a chaff cutter and with 20 strokes of a Potter-Elvehjem homogenizer. Unbroken pieces and contaminating mucus were removed by filtration and lowspeed centrifugation [5]; the resulting initial homogenate PO was then subdivided by differential centrifugation (Fig. 1). All subcellular fractions were resuspended in 10 mmol/l Tris/HCl and adjusted to a protein concentration of 2 mg/ml, as monitored after precipitation with trichloroacetic acid and lipid extraction with ether/ethanol [26]. The samples were stored at −80 °C for less than 2 months until used for enzyme or ^{3}H-PG E_2 binding assays.

For further fractionation some of the 300 000 × *g* homogenate P3 was loaded on gradients consisting of 4 layers with different sucrose densities. After centrifugation at 190 000 × *g* for 60 min, protein banding at the sucrose step interfaces was collected with Pasteur pipets and the bottom sediment was also recovered (Fig. 1). All fractions were washed free of sucrose and resuspended in 10 mmol/l Tris/HCl.

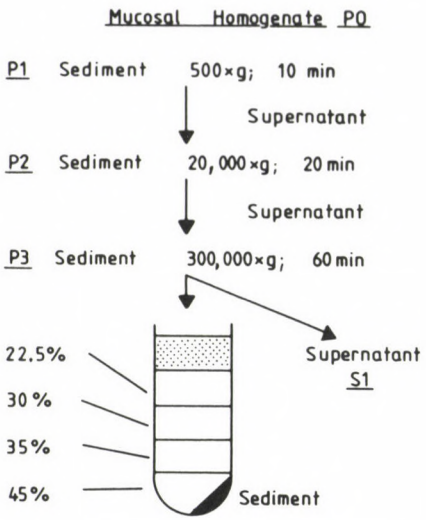


Fig. 1. Flow scheme for subcellular fractionation of the porcine fundic mucosa

In other experiments aliquots of P3 were layered on top of a series of two-step gradients. These gradients (Fig. 5, inset) had a constant upper layer (12.5% sucrose) and a variable cushion at the bottom (22.5–65% sucrose). After centrifugation at 190 000 × *g* for 60 min, increasing amounts of protein were recovered from the interface with growing density of the bottom layer. Subtraction of protein, enzyme, or binding recoveries between every pair of gradient tubes with consecutive sucrose density steps allowed to attribute specific activities to the corresponding floating density (Fig. 5a–b).

Enzyme assays

The conditions for all marker enzyme assays were standardized with respect to incubation time and tested protein concentrations, so that linear reaction rates were obtained. Activities were calculated in U/mg protein, where 1 enzyme unit catalyzes the turnover of 1 μmol substrate/min.

The activity of *γ-glutamyl-transpeptidase*, E.C. 2.3.2.13, was determined according to Szasz [33] with some modifications. The reaction took place in 185 mmol/l Tris buffer, pH 8.25, with 40 mmol/l glycyl-glycine and 4 mmol/l γ-glutamyl-p-nitranilide. The generation of 5-amino-2-nitrobenzoate after addition of 10–100 μg protein/ml was continuously monitored at room temperature and 405 nm.

H^+-K^+-ATPase activity, E.C. 3.6.1.36, was measured as previously described [3], with correction for HCO_3^--stimulated enzyme activity.

The assay of *5'-AMPase* activity, E.C. 3.1.3.5, was modified from [16] and performed in 100 mmol/l Tris buffer, pH 7.7, with 6 mmol/l $MgCl_2$. After addition of subcellular fraction protein, the reaction mixture was preincubated for 10 min at 37 °C. 2 mmol/l AMP were then added and after another 15 min the reaction was terminated with HCl (0.5 mol/l final concentration). Inorganic phosphate was determined in a 500 μl aliquot, which was mixed with 1.2 ml 0.4 mol/l HCl/0.175% molybdate and 300 μl 0.042% malachite green. After 3 min at room temperature, 3.9% H_2SO_4 was added and the extinction increase was read 30 min later at 625 nm.

The activity of *alkaline phosphatase*, E.C. 3.1.3.1, was measured in 1 mol/l diethanolamine buffer at pH 9.8. The assay mixture contained also 5 mmol/l $MgCl_2$ and 10 mmol/l 4-nitrophenyl-phosphate [15]. After incubation for 30 min at 37 °C and stopping on ice, the extinction was determined at 420 nm.

Acid phosphatase, E.C. 3.1.3.2., was assessed in 200 mmol/l sodium acetate buffer, pH 5.0, with 0.25% Triton X-100 [36]. Subcellular fraction protein was preincubated for 15 min at 37 °C in order to release all lysosomal enzymes and to inactivate most nonspecific phosphatases. The reaction was then started by addition of 8 mmol/l 4-nitrophenyl-phosphate and terminated 30 min later with 0.6 mol/l Tris/0.2 mol/l potassium phosphate, pH 8.5. Extinction readings were done as for alkaline phosphatase.

The lysosomal markers *β-glucuronidase*, E.C. 3.2.1.31, *β-glucosidase*, E.C. 3.2.1.21, and *β-galactosidase*, E.C. 3.2.1.23, were determined in 100 mmol/l sodium citrate buffer [2]. Again, 4-nitrophenyl-phosphate served as substrate and enzyme activities were released by 0.25% Trition X-100. Incubations were performed at 37 °C for 30 min at the respective pH optima (pH 4.5 for β-glucuronidase, pH 5.5 for β-glucosidase and pH 4.0 for β-galactosidase). The reactions were stopped on ice by addition of 0.25 mol/l sodium carbonate/sodium hydrogen carbonate.

Determination of *cytochrome-c-reductase* activity, E.C. 1.6.99.2, was done in 20 mmol/l potassium phosphate, pH 8.0. The assay mixture for this endoplasmic reticulum marker [32] contained 0.7 mg/ml cytochrome-c, 1 mmol/l NADPH, and 2 mmol/l NaN_3. The extinction decrease induced by subcellular fraction protein was continuously recorded at 550 nm.

Cytochrome-c-oxidase activity, E.C. 1.9.3.1, was also assessed by continuous recording and graphical evaluation of the reaction rate, but using dithionite-reduced cytochrome-c as substrate [10].

Binding assay

^{3}H-PG E_2 binding was measured as earlier established in our laboratory [5]. Briefly, subcellular fractions were adjusted to a protein concentration of 1 mg/ml in 25 mmol/l succinate/Tris buffer, pH 5.5. Quadruplicates of each sample were incubated with 0.9 nmol/l radioligand for 150 min at 30 °C. Free radioligand was then removed by glass-fibre filtration (Whatman GF/B) and protein-bound ^{3}H-PG E_2 retained in the filters was determined by liquid scintillation counting. Displaceable binding was calculated after correction for radioligand binding obtained in the presence of a 1000-fold excess of unlabelled PG E_2. Saturation analyses of ^{3}H-PG E_2 binding were performed in experiments where displaceable binding was tested over a radioligand range from 0.2 to 6 nmol/l.

Electron microscopy

Having localized ^{3}H-PG E_2 binding sites and plasma membranes in the light portion of P3 (see below), we prepared a membrane-rich fraction for control electron microscopy. Particles were collected from the interface of a two-step sucrose gradient as described above, with a 37.5% and a 12.5% sucrose layer. The sample was incubated for two hours at room temperature in 0.1 mol/l sodium cacodylate/HCl, pH 7.2, with 2.5% glutaraldehyde/2% formaldehyde (modified from [22]). Further fixation was done in the same sodium cacodylate/HCl buffer with 2% OsO_4 for 90 min at 4 °C. The samples were then dehydrated in ethanol/toluol and imbedded in Epon 812. Thin sections thereof were stained with uranyl acetate [37] and lead nitrate [29] for electron microscopic photography.

Calculations

All results are shown as mean ± SEM, where N indicates the number of independent experiments from different mucosal preparations. Scatchard plots of ^{3}H-PG E_2 saturation experiments were calculated with the EBDA/Ligand computer program [24].

Results

97 ± 5% of the protein in P0 was recovered during differential centrifugation (N=5; not shown). 22 ± 1% were found in P1, 15 ± 2% in P2, 12 ± 1% in P3 and 49 ± 4% in S1.

The distribution of ^{3}H-PG E_2 binding sites resembled that of the membrane markers γ-glutamyl-transpeptidase, 5'-AMPase and alkaline phosphatase (Fig. 2a). There was relatively much ^{3}H-PG E_2 binding in P2, whereas enzyme activities were higher in S1, than in P_2, particularly 5'-AMPase. The tubulovesicular marker H^+/K^+-ATPase also had its maximum in P3, but almost no activity in P2.

Surprisingly, the lysosomal marker acid phosphatase had also a similar subcellular distribution as ^{3}H-PG E_2 binding with maximum activity in P3 (Fig. 2a). The other lysosomal enzymes β-glucuronidase, β-glucosidase and β-galactosidase had a different maximum in P2 (Fig. 2b). Mitochondria, as represented by cytochrome-c-oxidase, sedimented mainly in P2 and P1 and were clearly separated from ^{3}H-PG E_2 binding sites. P3 had also the highest specific activity of cytochrome-c-reductase and contained considerable amounts of endoplasmic reticulum.

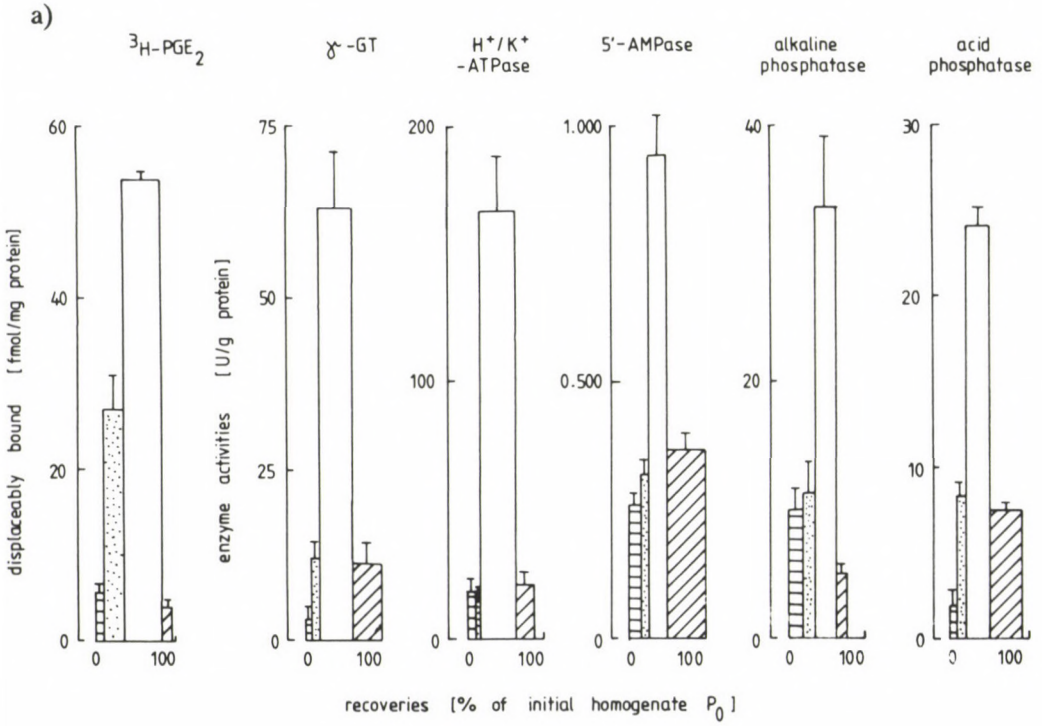


Fig. 2a

b)

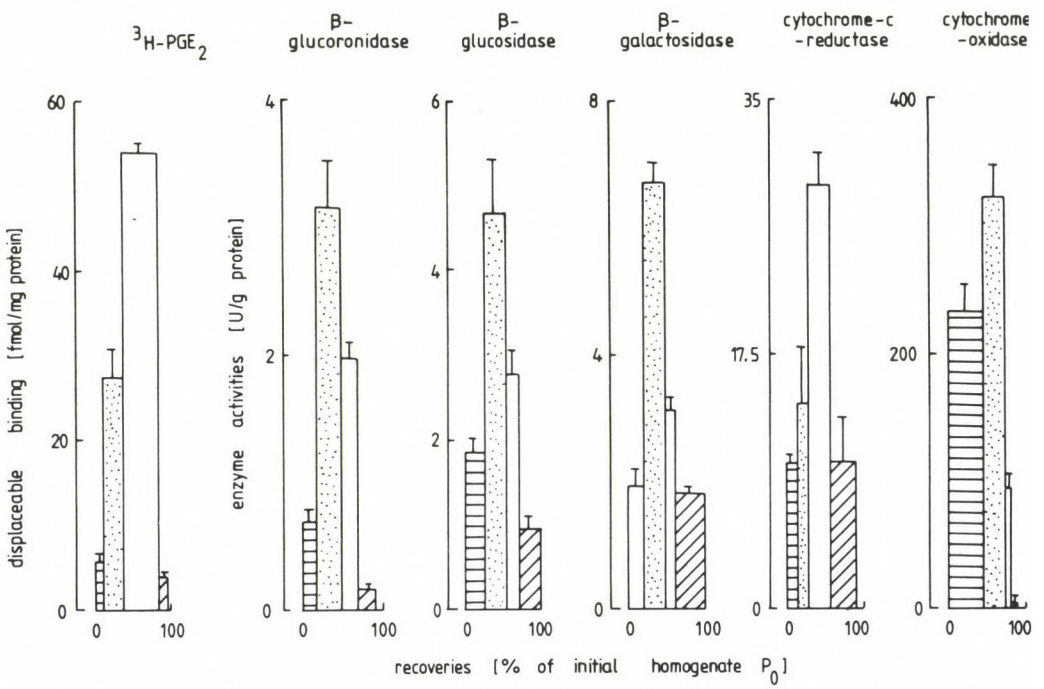


Fig. 2a–b. Distribution of ^{3}H-PG E_2 binding sites and specific marker enzymes after differential centrifugation. (N=4–9)

- ☐ 1,000 × g sediment P1
- ☐ 20,000 × g sediment P2
- ☐ 300,000 × g sediment P3
- ☐ supernatant S1

Figure 3 reveals that there was only one class of ^{3}H-PG E_2 binding sites in P0, which was truly enriched in P3 during differential centrifugation. This suggests that potential binding sites on different cellular organelles, if existent at all, must be very homogeneous and have similar binding affinities.

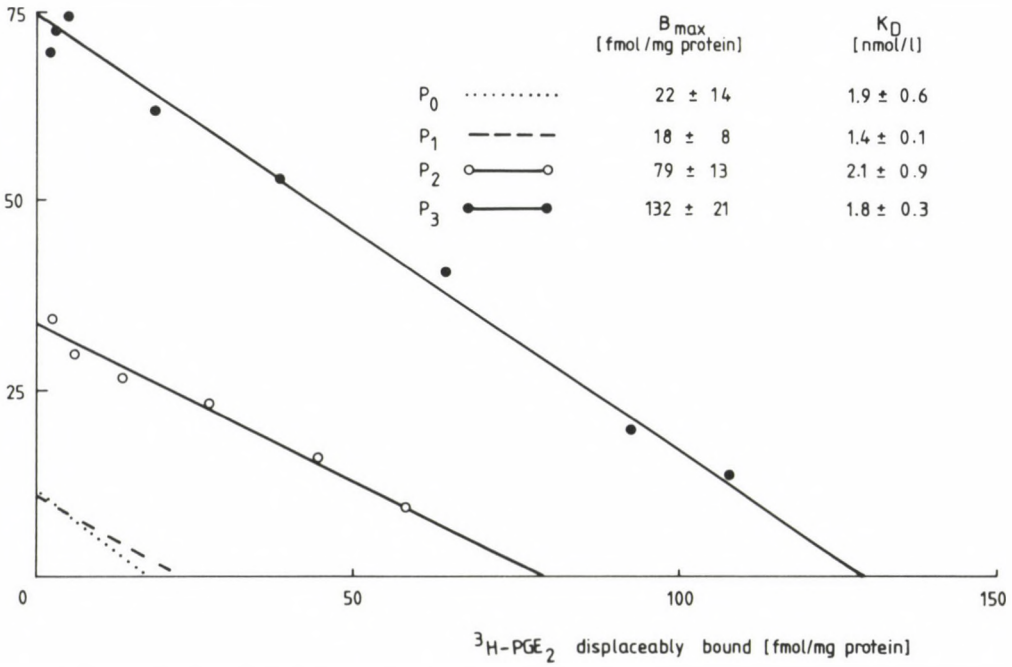


Fig. 3. Scatchard plots of ^{3}H-PG E_2 binding in the mucosal homogenate P0 and in particulate fractions generated by differential centrifugation. Curve fitting was not improved when a two-site model was applied

a)

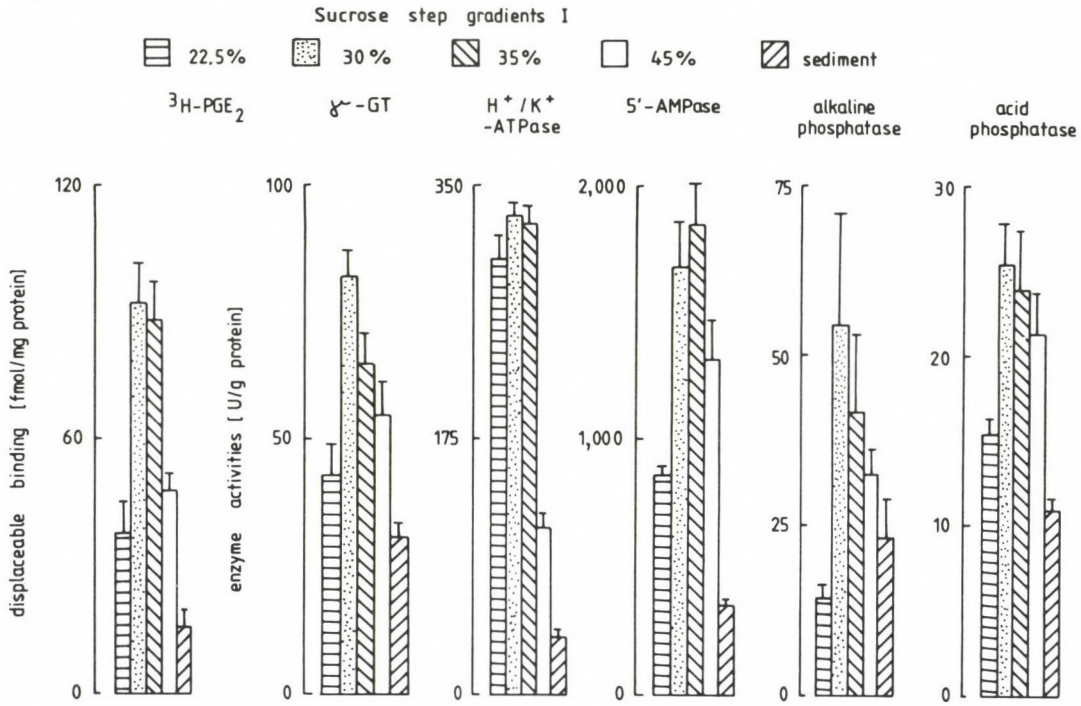

Sucrose step gradients I
22.5%
30%
35%
45%
sediment
^{3}H-PGE$_2$
γ-GT
H$^+$/K$^+$ -ATPase
5'-AMPase
alkaline phosphatase
acid phosphatase
displaceable binding [fmol/mg protein]
enzyme activities [U/g protein]
120
60
0
100
50
0
350
175
0
2,000
1,000
0
75
50
25
0
30
20
10
0

Fig. 4a

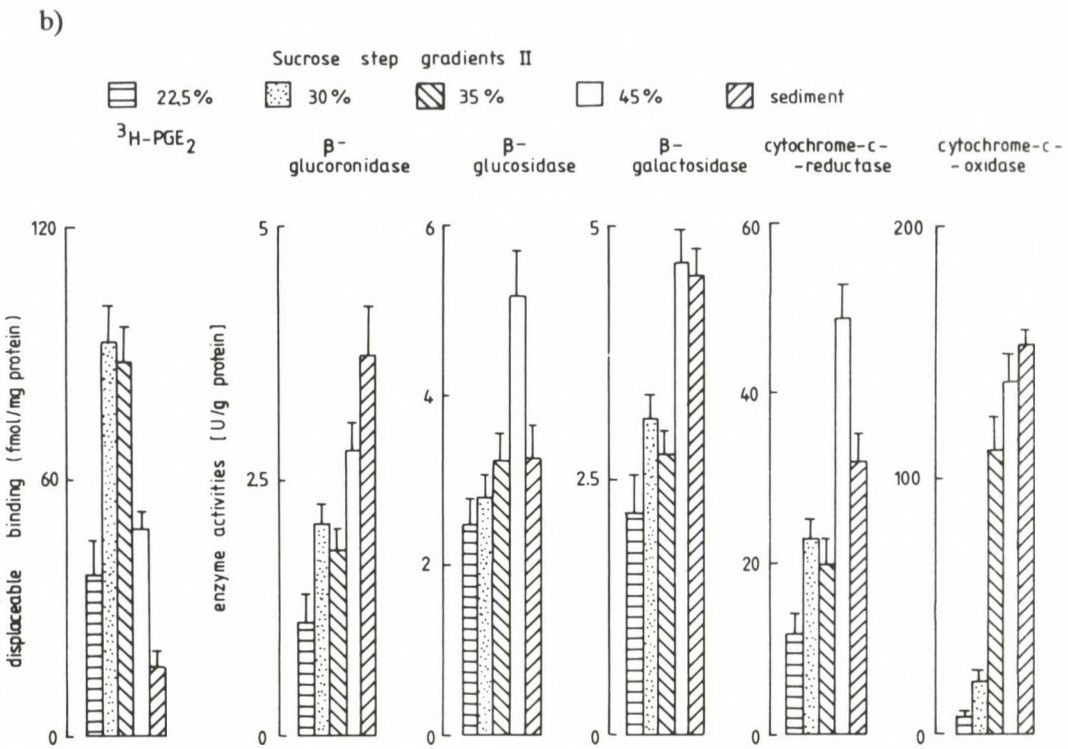
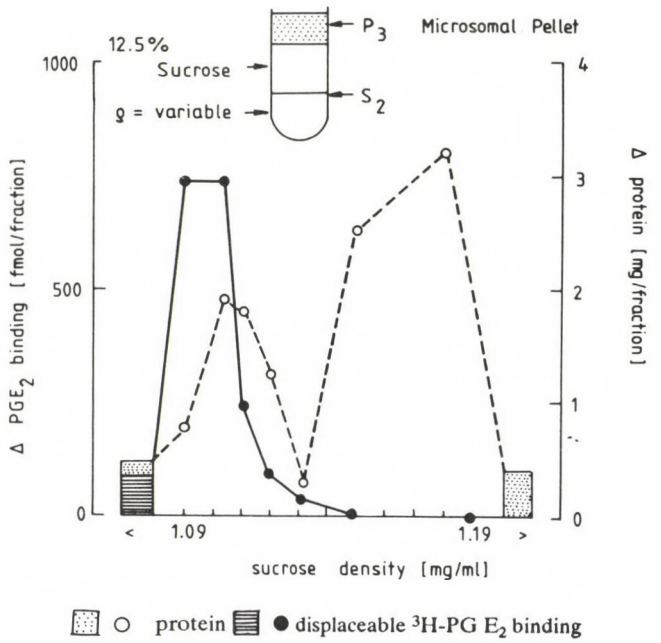


Fig. 4a–b. Subcellular distribution of ^{3}H-PG E_2 binding sites and specific marker enzymes in fractions of the 300 000 × *g* sediment P3. (N = 3–8)

Figure 4 gives a finer resolution of the particle composition in fraction P3. The plasma membrane markers γ-glutamyl-transpeptidase, 5'-AMPase and alkaline phosphatase sedimented similarly as ^{3}H-PG E_2 binding. H^+/K^+-ATPase, acid phosphatase and cytochrome-c-reductase had overlapping, but evidently different sedimentation profiles. Other lysosomal markers and mitochondria are enriched, as expected, in the heavy portion of P3.

a)



b)

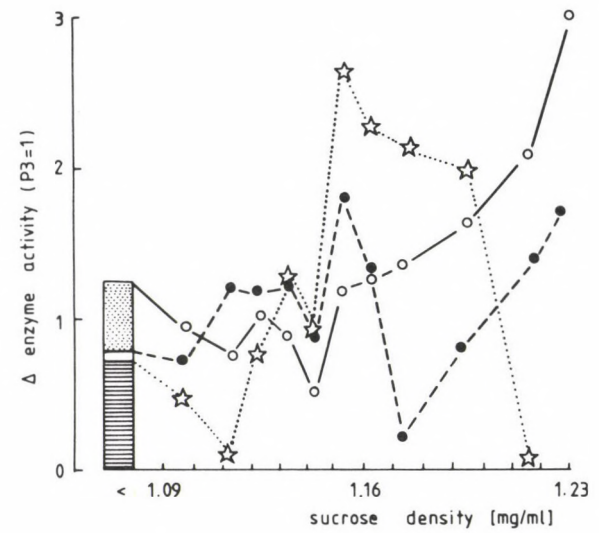


Fig. 5 a–b. Density distribution of protein, ^{3}H-PG E_2 binding and selected intracellular organelle markers (N=3; SEM ≤ 20%)

NADPH-cytochrome-c-reductase ☆ acid phosphatase ● cytochrome-c-oxidase

Figure 5 confirms this finding and shows that acid phosphatase and cytochrome-c-reductase had their maxima at sucrose densities around 1.16 mg/ml, whereas ^{3}H-PG E_2 binding sites floated closer to 1.10 mg/ml. A considerable portion of particles cosedimenting at this density consisted of double membrane vesicles, which is again in agreement with their plasma membrane origin (Fig. 6).

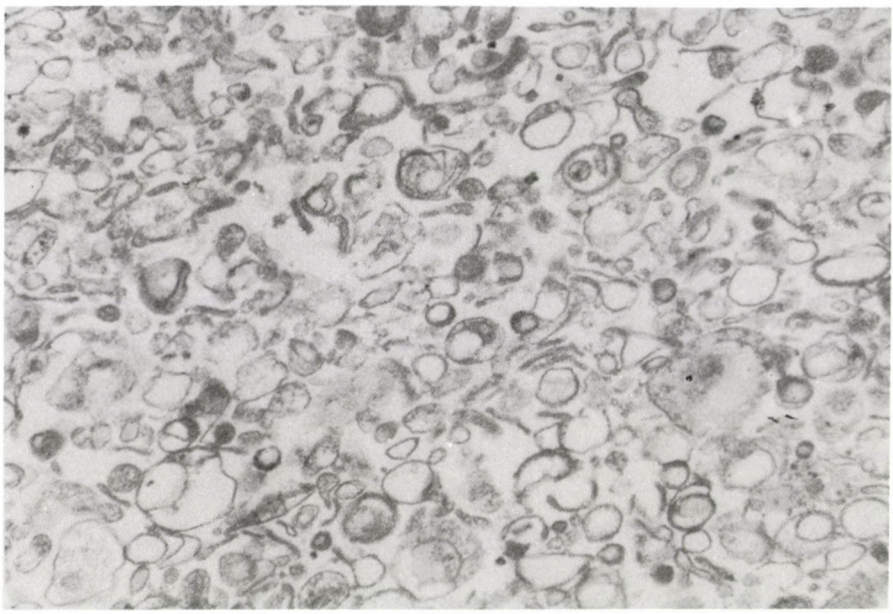


Fig. 6. Electron micrograph of a thin-sectioned preparation of mucosal plasma membranes (12.5%/37.5% sucrose interface as in Fig. 5). Magnification × 40 000

Discussion

Our experiments revealed a good correlation of plasma membrane markers with ^{3}H-PG E_2 binding sites, but no such link was seen with any of the intracellular marker enzymes. There was no indication for cytosolic PG E_2 receptors in the porcine stomach either, which was supported by the absence of additional low-affinity binding sites, as recently postulated for the rat [41].

The complex results with lysosomal marker enzymes indicate inherent problems with biochemical localization of such organelles:

– The assay for acid phosphatase may cross-react with microsomal glucose-6-phosphatase activity [2].

– There are several types of lysosomes with different densities [7]. For example, acid phosphatase is predominantly located in the lighter subpopulation and may also have partial plasma membrane localization [14].

– All tested marker enzymes are linked to the lysosomal membrane, but they do exist in the soluble matrix, too. When lysosomes are disrupted during vigorous homogenization or centrifugation, their membranes may show a similar sedimentation pattern as plasma membranes and their soluble matrix can secondarily stick to other subcellular fragments [30].

In our hands, all 4 lysosomal marker enzymes had a different sedimentation profile when separated over multiple two-step gradients, which is shown in Fig. 5 only for acid phosphatase. Interestingly, all lysosomal enzymes shared a common main peak at 1.16 mg/ml (not shown) which may thus represent the true density of most of these organelles. We believe therefore that it is essential to investigate different lysosomal markers simultaneously in order to get reliable information about their association with receptors and other structures. Although we cannot finally exclude selective ^{3}H-PG E_2 binding to one of the lysosomal subpopulations, it appears safe to conclude that lysosomes in general are no major site for gastric PG E_2 receptors. This is also in line with the functional finding that prostaglandins have no direct effect of the stability of isolated gastric lysosomes [1].

The tubulovesicular enzyme H^+/K^+-ATPase could not be separated as clearly as other intracellular markers from plasma membranes and ^{3}H-PG E_2 binding sites. This problem is well known from previous studies and stems from specific difficulties when working with gastric tissue:

– Parietal cell tubulovesicles are in continuous exchange with the apical plasma membrane pool [17]. It will be necessary to block stimulation of these cells as a prerequisite to further improve the separation of different membrane types.

– Tubulovesicles, together with the apical plasma membrane, have far more mass than the basolateral plasma membrane. The latter is expected to have higher density and to be the predominant location of most receptors, which may be reflected by relatively high ^{3}H-PG E_2 binding in P2 [12].

– The use of additional marker enzymes, more specific for the basolateral cell pole, could help to get a sharper separation from tubulovesicles. Alkaline phosphatase and γ-glutamyl-transpeptidase are apical marker enzymes [30]. 5'-AMPase, the most commonly used plasma membrane marker, has multiple subcellular locations in certain cells and is thus not reliable enough for refined separations [35, 39].

At this stage, we have sufficient evidence that gastric PG E_2 receptors are predominantly connected to plasma membranes and do not occur in significant quantities in the endoplasmic reticulum, in mitochondria or in the cytosol. Furthermore, we could not confirm the existence of lysosomal PG E_2 receptors in the

stomach, notwithstanding a possible heterogeneity within this types of organelles. The close functional association of plasma membrane PG E_2 receptors with the histamine-H_2 receptor and the adenylate cyclase system suggests a basolateral orientation [11], which is presently further investigated.

Acknowledgements

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ADENOSINE: A NOVEL ULCER MODULATOR IN STOMACHS

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Adenosine has been demonstrated for its actions on gastric secretion and stress-induced gastric ulceration in animals. We examined the pharmacological actions of adenosine on ethanol-evoked gastric lesions and gastric mucosal blood flow (GMBF) in rats, because both of them are closely related. Adenosine pretreatment, in dose of 7.5 mg/kg increased GMBF and protected against ethanol-evoked gastric lesion formation. However, this antiulcer action was followed by an aggravation of gastric lesions and reduction in GMBF. We further investigated whether these actions could act through the adenosine A_1 or A_2 receptors, therefore L-phenylisopropyladenosine (L-PIA) or N-ethylcarboxamidoadenosine (NECA), the adenosine A_1 or A_2 receptor agonists, respectively, were used. The drugs given in doses of 10 or 50 μg/kg for L-PIA and 1 or 5 μg/kg for NECA, dose-dependently inhibited GMBF and potentiated ethanol-induced gastric damage. When the two drugs were given together to animals, they did not further aggravate the severity of ulceration and reduction of GMBF. These findings indicate that the antiulcer action of adenosine is not mediated via the adenosine A_1 and A_2 receptors but if acts through different adenosine receptor subtypes. It was because the lesion worsening effects of adenosine at the second stage of the biphasic responses were similar to the actions of L-PIA and NECA, the ulcer potentiating effect is probably acting through adenosine A_1 and A_2 receptors in anaesthetised rats.

Keywords: adenosine, L-phenylisopropyladenosine, N-ethylcarboxamidoadenosine, ethanol, gastric damage, gastric mucosal blood flow, rats

Adenosine is a purine nucleoside and is recognized as an endogenous substance responsible for modulating cell functions e.g. blood flow, seizure, airway resistance and neuronal activity. Recently, there is experimental evidence that adenosine is also regarded as a regulation for gastric secretion and lesion formation in animals [1–5]. Gerber and Guth [6] have reported that adenosine A_2 receptors are located in the submucosal artery. The stimulation of which could be responsible in part, for the increase in gastric mucosal blood flow (GMBF) during pentagastrin-

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stimulated gastric acid secretion. The mechanism as how adenosine affects GMBF and prevents gastric is still unknown. Furthermore, the role of different adenosine receptors in these parameters is still not clear. We therefore, used the adenosine A_1 and A_2 receptor agonists to differentiate the actions of adenosine on GMBF and on ethanol-induced gastric mucosal damage in rats.

Materials and methods

Male Sprague-Dawley rats, weighing 200–210 g, were used. They were kept in an air-conditioned room in which the temperature was maintained at 22 ± 1°C and humidity at 65–70%. They were fed a standard laboratory diet and given tap water to drink *ad libitum*. They were fasted for 24 h before experiments.

The rats were anaesthetized with sodium pentobarbital (Abbott; 50 mg/kg, i.p.) and they were kept warm with a heating lamp. The trachea was cannulated and a midline laparotomy was carried out. The stomach was isolated and an *ex vivo* chamber was prepared [7]. Each experiment consisted of six sequential 15-min periods. In the first period, the luminal bathing solution was 1.5 distilled water, which was replaced by the same solution for the second and third periods. At the third period, the luminal solution was either changed to the same solution throughout the rest of the experiment or to 1.5 ml absolute ethanol (BDH) which was subsequently used in the remaining 30 min. At the end of each 15-min period, the GMBF in the gastric glandular mucosa was recorded [8], by means of a laser Doppler flowmeter (Periflux, Sweden), in which the laser detector was placed about 0.5 mm above and perpendicular to the mucosal surface. The GMBF was recorded as arbitrary units and expressed as per cent of GMBF at zero time. The severity of gastric mucosal lesions was determined by measuring the areas of haemorrhagic lesions 45 min after ethanol administration.

Adenosine (Sigma) was dissolved in saline (0.9% w/v, NaCl) and given in the dose of 7.5 mg/kg. The drug was injected subcutaneously (s.c.) 15, 30 or 45 min before ethanol administration. L-phenylisopropyladenosine (L-PIA) and N-ethylcarboxamidoadenosine (NECA) were purchased from Sigma. They were prepared in 0.25% w/v dimethylsulfoxide (DMSO, Sigma) and given in doses of 10, 50 μg/kg for L-PIA or 1, 5 μg/kg for NECA. Both drugs were injected s.c. at zero time when the experiment was started.

Statistical significance was analyzed by two-tailed unpaired Student's *t*-test and also by analysis of variance (ANOVA) when appropriate.

Results

Ethanol incubation given at different time intervals produced severe haemorrhagic lesions in the glandular mucosa. Adenosine pretreatment, injected at 15 or 30 min beforehand, significantly reduced gastric lesions but it markedly increased the injury when the drug was given 45 min before ethanol administration (Fig. 1). The nucleoside increased the basal GMBF at 15 min but reduced it at 45 min after injection (Fig. 2).

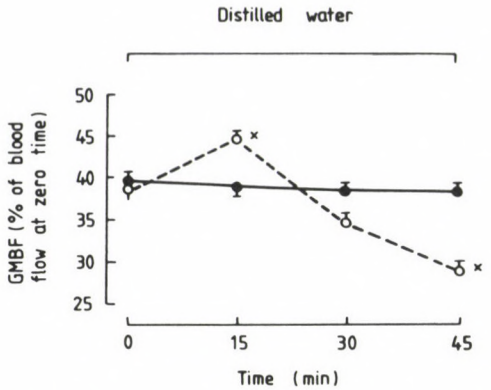

Fig. 1. Effects of adenosine pretreatment (o, 7.5 mg/kg) given s.c. at different time before ethanol administration on gastric mucosal damage. Values are means ± S.E.M. of 10 rats. $^{x}p < 0.05$ when compared with the saline (•, 1 ml/kg)-injected control

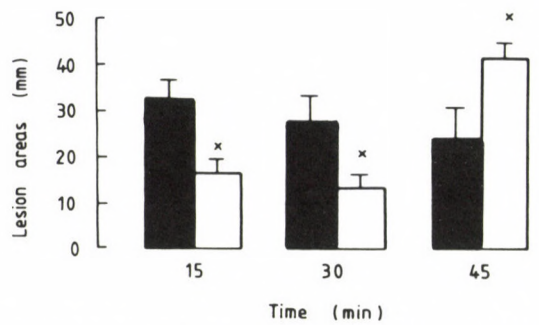

Fig. 2. Effects of adenosine treatment (□, 7.5 mg/kg) given s.c. at zero min, on gastric mucosal blood flow (GMBF). Values are means ± S.E.M. of 10 rats. $^{x}p < 0.05$ when compared with the corresponding saline (■, 1 ml/kg)-injected control.

L-PIA or NECA given s.c. 30 min before ethanol administration, dose-dependently potentiated the gastric damage (Fig. 3) and the effect was significant (ANOVA: $p<0.001$ for both L-PIA and NECA). Ethanol time-dependently decreased the GMBF and this was further depressed by L-PIA and NECA; the two drugs also markedly reduced the basal GMBF (Fig. 4). When L-PIA was given together with NECA to animals, they produced the similar effects as the individual drug (Figs 3, 5).

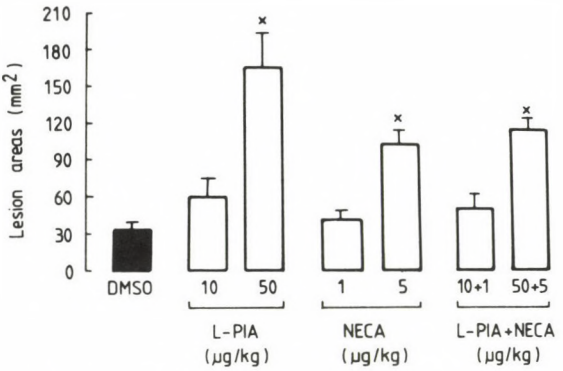

Fig. 3. Effects of L-phenylisopropyladenosine (L-PIA) and/or N-ethylcarboxamidoadenosine (NECA) pretreatment given s.c. 30 min before ethanol administration on gastric mucosal damage. Values are means ± S.E.M. of 6 rats. $^{x}p < 0.05$ when compared with the dimethylsulfoxide (DMSO, 0.25% 1 ml/kg)-injected control

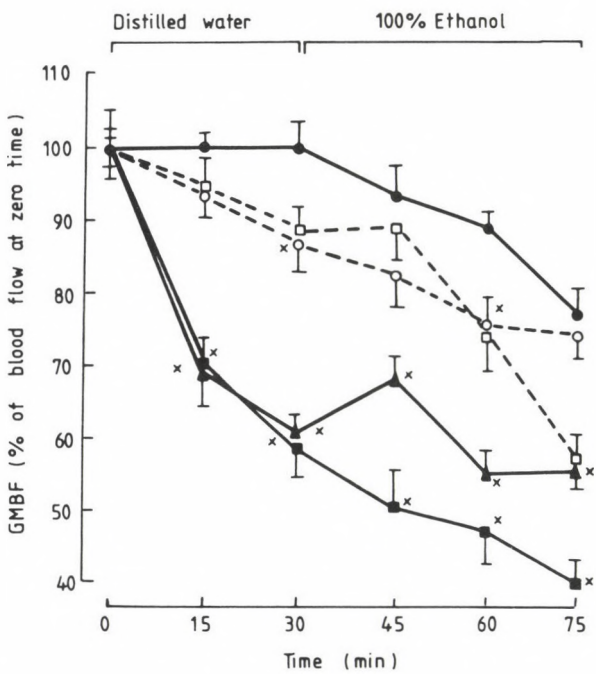

Fig. 4. Effects of L-PIA (o, 10 mg/kg; ■, 50 mg/kg) or NECA (□, 1 mg/kg; ▲, 5 mg/kg) pretreatment given s.c. at zero min, on ethanol-induced changes of gastric mucosal blood flow (GMBF). Values are means ± S.E.M. of 6 rats. $^{x}p < 0.05$ when compared with the corresponding DMSO (•, 0.25% 1 ml/kg)-injected control

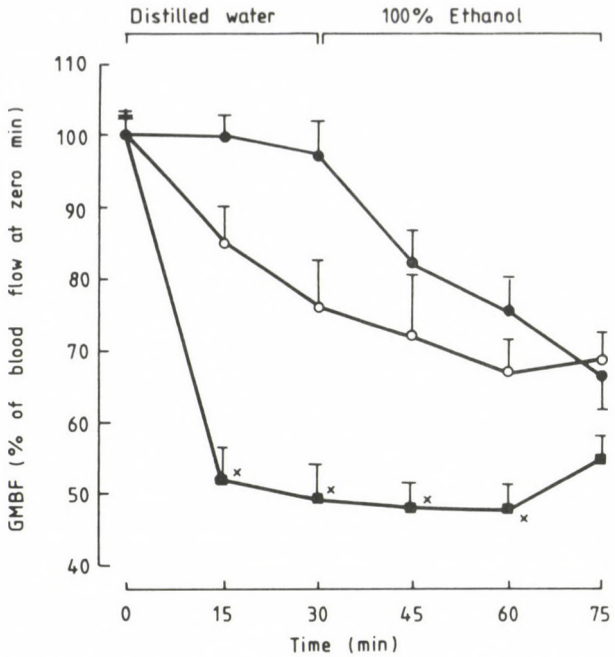

Fig. 5. Effects of L-PIA and NECA (o, L-PIA 10 μg/kg + NECA 1 μg/kg; ■, L-PIA 50 μg/kg + NECA 5 μg/kg) pretreatment given s.c. at zero min, on ethanol-induced changes of gastric mucosal blood flow (GMBF). Values are means ± S.E.M. of 6 rats. $^{x}p < 0.05$ when compared with the corresponding DMSO (●, 0.25% 1 ml/kg)-injected control

Discussion

Adenosine has been demonstrated to prevent ethanol-evoked gastric damage in rats [1]. This protection is likely to be due to the maintenance of GMBF and increase in gastric secretory volume. The present study not only confirms the findings of GMBF and the antiulcer action of adenosine but also extends the understanding the actions of the nucleoside. It was shown that adenosine produces biphasic responses both in lesions formation, and GMBF, and they were closely related. It has been found that adenosine is a local modulator of GMBF during acid secretion because it is a gastric mucosal vasodilator demonstrated by an *in vivo* microscopic study [9]. This acute effect was substantiated by the present experiment in which the GMBF was increased by adenosine given 15 min before blood flow measurement. However, this stimulatory action was followed by a depression when the compound was injected 45 min beforehand. These findings are interesting because it is the first time to demonstrate that adenosine possesses an additional action which is opposite to the action reported previously [1].

The mechanism as how these biphasic responses could occur is still unclear. It is known that there are two types of receptors for adenosine. PIA is a potent A_1 receptor agonist associated with decrease in adenylate cyclase, whereas NECA is more potent at A_2 receptors, stimulation of which is generally accompanied with an increase in adenylate cyclase activity [9, 10]. It is interesting to differentiate which type of adenosine receptors is involved in the biphasic responses produced by adenosine. We therefore, also investigated the pharmacological effects of a specific adenosine A_1 agonist L-PIA [9] and an adenosine A_2 stimulator NECA [9] on gastric mucosal damage and GMBF.

L-PIA and NECA not only did not protect against ethanol-evoked gastric mucosal injury, but it also worsened ulceration. They also decreased the GMBF instead of elevation in blood flow in the gastric mucosa (Fig. 3). These findings suggest that neither the protective action of nor the increase in GMBF produced by adenosine are likely to be due to activation of the A_1 and A_2 receptors, but could act through other receptors of adenosine, which remains to be defined. It is noted that the adverse effects produced by L-PIA and NECA either on gastric damage or reduction in GMBF are very much similar to the findings at the second stage of the biphasic responses evoked by adenosine. Thus, it is suggested that the detrimental actions of adenosine are probably mediated via the adenosine A_1 and A_2 receptors. However, it is still unclear why the two drugs, when given together did not have any additive effect on lesion formation and reduction in GMBF. It is difficult to explain this drug interaction at the present stage, further study is needed.

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5-HYDROXYTRYPTAMINE$_3$-RECEPTOR BLOCKADE PROTECTS AGAINST GASTRIC MUCOSAL DAMAGE IN RATS

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Ondansetron, a specific 5-hydroxytryptamine$_3$ (5-HT$_3$)-blocker, injected s.c. (0.038, 0.075, 0.15 or 0.3 mg/kg) every 12 h with the fourth dose given 0.5 h before restraint at 4°C (stress) or oral administration (p.o.) of 1 ml 80% ethanol, dose-dependently prevented gastric mucosal damage in female Sprague-Dawley rats (160–180 g); the animals were killed 2 or 1 h after stress or ethanol p.o., respectively. A similar pretreatment regimen with cyproheptadine (0.1, 0.25 or 0.5 mg/kg) or ketanserin (15, 30, or 75 μg/kg), both being 5HT$_2$-receptor antagonists, also dose-dependently lowered the severity of stress- or ethanol-induced mucosal lesions. Only the higher doses of phenobarbitone (25 or 50 mg/kg given s.c. in a single dose 0.5 h beforehand) inhibited stress-induced gastric ulcers; however, even the lowest non-antiulcer dose (12.5 mg/kg), effectively produced CNS depression. These preliminary findings suggest that 5HT$_3$-receptor blockade not only can antagonise stress- or ethanol-evoked gastric mucosal damage, but also may act through a peripheral mechanism.

Keywords: ondansetron, cyproheptadine, gastric lesions, rats

5-hydroxytrypyamine (5-HT) has been shown to contribute to gastric mucosal damage by cold-restraint stress [3] or by oral administration (p.o.) of ethanol [9]. 5-HT$_2$-receptor antagonism by methysergide protects against both types of lesion formation [3, 9], but this action appears more effective against ethanol-induced mucosal damage [9].

Specific 5-HT$_3$-receptor blockade by ondansetron has been shown to produce antiemetic [7] and anxiolytic [6] actions. Its strong antiemetic property is thought to result from 5HT$_3$-receptor antagonism by a peripheral action of the vagus nerve endings as well as a central effect on the chemoreceptor trigger zone; both these sites play a vital role in the emetic pathway [1].

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The influence of specific 5-HT_3-receptor antagonism on experimentally-induced gastric mucosal lesions is not known. This communication reports some preliminary findings on the effects of ondansetron on cold-restraint stress- or ethanol-provoked stomach damage in rats.

Materials and methods

Female Sprague-Dawley rats, weighing 160–180 g, were housed in an air-conditioned room in which the temperature was maintained at 22 ± 1 °C and humidity at 65–70%. The animals were fed a standard pellet (Ralston Purina Company) diet and drank tap water. Solid food was withdrawn 24 h before experiments, but free access to a solution of 8% sucrose in 0.2% NaCl w/v was permitted; this drinking solution was removed 1 h before experimentation [4].

Cyproheptadine (Sigma) 0.1, 0.25 or 0.5 mg/kg, ketanserin (Sigma) 15, 30 or 75 μg/kg, or ondansetron (Glaxo) 0.08, 0.15 or 0.3 mg/kg, was injected s.c. every 12 h with the fourth dose given 0.5 h before starting experiments. The antagonists were freshly dissolved in absolute ethanol (BDH) (ketanserin) or in an 0.9% NaCl (saline) solution (cyproheptadine and ondansetron); all doses are expressed as the base. Phenobarbitone (Bayer) 12.5, 25 or 50 mg/kg, freshly dissolved in saline, was injected s.c. in a single dose 0.5 h before experimentation.

Rats were stressed by restraint in individual close-fitting cylindrical cages at 4 °C [8]. Nonstressed controls were returned to their starvation cages in the room where they were normally housed. All animals were killed by a sharp blow on the head 2 h later. In the case of experiments with ethanol-induced gastric mucosal damage, 80% ethanol (BDH), in a volume of 1 ml, was given orally (p.o.) by means of a plastic intragastric tube; the animals were killed by a blow on the head 1 h after alcohol administration [9]. Stomach were removed immediately after killing the rats, opened along their greater curvatures and the mucosal surfaces of their glandular segments examined under an illuminated magnifier (×3). Lesions produced by stress were measured along their greatest lengths; in the case of petechiae, five of these were taken as the equivalent of a 1 mm ulcer [2]. The sum of the lesion lengths in each group of animals was divided by its number and expressed as the mean ulcer index. Ethanol-induced gastric mucosal damage was graded by measuring the area of the lesions with a transparent grid (each grid was 1 mm^2) placed on the mucosal surface [9]; five petechiae were equated with an area of 1 mm^2. A single-blind method was used for all lesion measurements, where the treatment regimen was unknown to the investigator. Following mucosal lesion measurements, the glandular segment of each stomach was separately fixed in 4% w/v lead acetate (E. Merck) for 48 h before processing the tissue and standing the mast cells with toluidine blue (E. Gurr Ltd). The number of metachromatically stained mast cells in the upper one third of the glandular mucosa was counted in 42 oil immersion fields (×1000) [2].

Locomotor activity was measured over a 2-h period using a computerized animal activity monitoring system. A Watchman I animal activity monitor (designed by the Electronic Services Unit of the University of Hong Kong), utilizing video cameras, infrared lights and a computer (IBM PC/AT), recorded the vertical (rearing of jumping) and horizontal movements of rats placed individually in plastic-walled chambers (length 350 mm; width 245 mm; depth 430 mm). Measurements were carried out at a fixed time of day (0900–1100 h) in a quiet fluorescent-light illuminated (of similar intensity and quality as that in the air-conditioned room, with 12-h light/dark cycles, where the animals were normally housed) room.

All values were expressed as means ± S.E.M. and the data analyzed for statistical significance by Student's unpaired two-tailed *t*-test.

Results

The influence of 5-HT_2- or 5-HT_3-receptor block on the gastric effects of stress

Nonstressed rats given vehicles or largest doses of the 5-HT_2- or 5-HT_3-receptor blockers showed comparable glandular ulcer indices due to occasional petechiae, as well as mucosal mast cell counts (Table I/A).

Table I

The effects of 5-HT_2 or 5-HT_3 blockade on gastric glandular ulceration and mast cell degranulation in rats stressed for 2 h

Treatment (s.c.)			Glandular ulcer index (mm)	Glandular mucosal mast cell count /42 o.i.f.
A. *Nonstressed rats*				
Saline vehicle	2	ml/kg	0.02 ± 0.02	68 ± 3.5
Ethanol vehicle	2	ml/kg	0.03 ± 0.02	61 ± 3.3
Cyproheptadine	0.5	mg/kg	0.03 ± 0.03	62 ± 6.1
Ketanserin	75	μg/kg	0.02 ± 0.01	60 ± 2.8
Ondansetron	0.3	mg/kg	0.02 ± 0.01	62 ± 4.1
B. *Stressed rats*				
Saline vehicle (C)	2	ml/kg	5.50 ± 0.40*	37 ± 4.4*
Saline vehicle (O)	2	ml/kg	5.26 ± 0.48*	40 ± 3.0*
Ethanol vehicle	2	ml/kg	5.67 ± 0.95*	38 ± 3.1*
Cyproheptadine (C)	0.1	mg/kg	2.97 ± 0.60*+++	41 ± 2.9*
Cyproheptadine	0.25	mg/kg	2.14 ± 0.54*+++	41 ± 4.8*
Cyproheptadine	0.5	mg/kg	2.04 ± 0.69*++++	37 ± 2.0*
Ketanserin	15	μg/kg	2.93 ± 0.41*#	42 ± 2.3*
Ketanserin	30	μg/kg	2.26 ± 0.74*##	39 ± 2.8*
Ketanserin	75	μg/kg	2.43 ± 0.42*###	40 ± 3.0*
Ondansetron (O)	0.08	mg/kg	3.56 ± 0.56*+	40 ± 3.5*
Ondansetron	0.15	mg/kg	3.73 ± 0.36*+	41 ± 3.6*
Ondansetron	0.3	mg/kg	3.50 ± 0.31*+++	42 ± 2.8*

Means ± S.E.M. of 6–8 rats per group, o.i.f. = oil immersion field (×1000).
*$p < 0.001$ when compared to the corresponding nonstressed control.
+$p < 0.05$; ++$p < 0.02$; +++$p < 0.01$ ++++$p < 0.001$ when compared to its own saline-injected control.
#$p < 0.05$; ##$p < 0.02$; ###$p < 0.01$ when compared to its own ethanol-injected control

Stress by restraint at 4 °C for 2 h produced severe haemorrhagic ulcers in the glandular mucosa and reduced the mast cell counts in the glandular mucosal layer in saline or ethanol vehicle-treated animals (Table I/B). $5HT_2$- or $5HT_3$-receptor antagonism significantly lowered stress-evoked ulceration, but did not influence mast cell degranulation.

The influence of 5-HT_2- or 5-HT_3-receptor block on the gastric effects of ethanol p.o.

Animals treated with saline vehicle, cyproheptadine or ondansetron showed occasional petechiae in the glandular mucosa when given distilled water p.o.; similarly, the mast cell counts were comparable in all three groups (Table II/A).

Table II

The effects of 5-HT_2 or 5-HT_3 blockade on gastric glandular lesions and mast cell counts in rats given ethanol p.o. and killed 1 h later

Treatment (s.c.)			Glandular lesion index (mm^2)	Glandular mucosal mast cell count /42 o.i.f.
A. *Distilled water 1 ml p.o.*				
Saline vehicle	2	ml/kg	0.02 ± 0.01	61 ± 3.6
Cyproheptadine	0.5	mg/kg	0.02 ± 0.01	64 ± 3.9
Ondansetron	0.3	mg/kg	0.02 ± 0.01	67 ± 2.5
B. *Ethanol 80% 1 ml p.o.*				
Saline vehicle (C)	2	ml/kg	107.0 ± 10.9*	35 ± 2.1*
Saline vehicle (O)	2	ml/kg	127.0 ± 11.9*	32 ± 2.7*
Cyproheptadine (C)	0.1	mg/kg	80.0 ± 12.5*	37 ± 3.4*
Cyproheptadine	0.25	mg/kg	54.3 ± 10.7*++	29 ± 3.1*
Cyproheptadine	0.5	mg/kg	39.3 ± 6.4*+++	30 ± 3.2*
Ondansetron (O)	0.04	mg/kg	77.6 ± 8.3*++	35 ± 2.2*
Ondansetron	0.08	mg/kg	76.0 ± 12.8*+	35 ± 2.9*
Ondansetron	0.15	mg/kg	74.9 ± 3.4*+++	34 ± 2.1*
Ondansetron	0.30	mg/kg	68.3 ± 8.8*++	36 ± 1.9*

Means ± S.E.M. of 6–10 rats per group, o.i.f. = oil immersion field (×1000).
*$p < 0.001$ when compared to the corresponding distilled-water treated control.
+$p < 0.02$; ++$p < 0.01$; +++$p < 0.001$ when compared to its own saline-injected control

When given 80% ethanol p.o., saline vehicle-treated rats had highly significant increases in glandular lesion size and decreases in mast cell counts 1 h later (Table II/B). Four-dose treatment with cyproheptadine or ondansetron markedly lowered glandular lesion size, but did not influence mast cell degranulation by ethanol.

The influence of phenobarbitone on the gastric effects of stress

Nonstressed rats given saline vehicle or phenobarbitone 0.5 h beforehand showed a low glandular ulcer index, due to occasional petechiae, and similar mast cell counts (Table III/A).

Table III

The effects of phenobarbitone on gastric glandular ulceration and mast cell degranulation in rats stressed for 2 h

Treatment 0.5 h beforehand (s.c.)			Glandular ulcer index (mm)	Glandular mucosal mast cell count /42 o.i.f.
A. *Nonstressed rats*				
Saline vehicle	2	ml/kg	0.09 ± 0.04	65 ± 6.5
Phenobarbitone	12.5	mg/kg	0.06 ± 0.04	64 ± 5.9
Phenobarbitone	25	mg/kg	0.06 ± 0.03	62 ± 6.3
Phenobarbitone	50	mg/kg	0.04 ± 0.02	67 ± 6.7
B. *Stressed rats*				
Saline vehicle	2	ml/kg	6.19 ± 1.28****	44 ± 4.5**
Phenobarbitone	12.5	mg/kg	5.12 ± 1.36****	47 ± 5.1*
Phenobarbitone	25	mg/kg	3.19 ± 0.63****+	51 ± 5.3
Phenobarbitone	50	mg/kg	2.02 ± 0.67***+++	63 ± 5.9++

Means ± S.E.M. of 12–14 rats per group, o.i.f. = oil immersion field (×1000).
*$p < 0.05$; **$p < 0.02$; ***$p < 0.01$; ****$p < 0.001$ when compared to its corresponding nonstressed control.
+$p < 0.05$; ++$p < 0.02$; +++$p < 0.01$ when compared to its own saline-injected control

Stress produced a high ulcer index due to grossly haemorrhagic ulcers and marked mast cell degranulation in the vehicle-treated controls (Table III/B). Only the two higher pretreatment doses of phenobarbitone reduced ulceration significantly, mast cell degranulation was significantly reduced by the highest dose of the barbiturate.

The influence of phenobarbitone, cyproheptadine or ondansetron on CNS activity

All three pretreatment doses of phenobarbitone similar to those used in the stress experiments, reduced significantly only the vertical rearing movements (Table IV). 5-HT_2-receptor blockade by cyproheptadine or 5-HT_3-receptor antagonism by ondansetron, in doses which effectively lowered lesion formation in stressed rats, did not influence any of the parameters used to evaluate locomotor behaviour which reflected CNS activity.

Table IV

CNS activity in rats observed for 2 h after s.c. injection of phenobarbitone (or the last dose of cyproheptadine or ondansetron) given 0.5 h beforehand

Treatment (s.c.)		Horizontal movement (mm/sec)	Vertical rearing (count/ 15 min)	Jumping activity (count/ 15 min)
Saline vehicle	2 ml/kg	4.6 ± 0.5	3.1 ± 0.7	0.9 ± 0.5
Phenobarbitone	12.5mg/kg	4.5 ± 0.6	1.4 ± 0.4*	0.4 ± 0.3
Phenobarbitone	25 mg/kg	4.0 ± 0.4	1.3 ± 0.5*	0.1 ± 0.1
Phenobarbitone	50 mg/kg	4.4 ± 0.6	1.2 ± 0.5*	0.0 ± 0.0
Saline vehicle	2 ml/kg	4.1 ± 0.5	2.8 ± 0.7	0.8 ± 0.5
Cyproheptadine	0.1mg/kg	3.2 ± 0.7	2.9 ± 0.8	0.8 ± 0.8
Cyproheptadine	0.25mg/kg	3.0 ± 0.4	2.8 ± 0.8	0.5 ± 0.5
Cyproheptadine	0.50mg/kg	3.4 ± 0.5	2.4 ± 0.6	0.7 ± 0.7
Saline vehicle	2 ml/kg	3.9 ± 0.4	3.1 ± 0.6	1.0 ± 1.0
Ondansetron	0.08mg/kg	4.2 ± 0.6	2.2 ± 1.0	0.8 ± 0.8
Ondansetron	0.15mg/kg	4.0 ± 0.6	2.8 ± 0.9	0.7 ± 0.4
Ondansetron	0.30mg/kg	4.1 ± 1.0	3.0 ± 0.5	0.8 ± 0.4

Means ± S.E.M. of 6–8 rats per group.
*$p < 0.05$ when compared to its own saline-injected control

Discussion

This preliminary study demonstrates that 5-HT_3-receptor antagonism by ondansetron can effectively reduce gastric mucosal damage produced by either cold-restraint stress or intragastric instillation of 80% ethanol. It was previously found that 5-HT_2-receptor block by methysergide was much less effective against stress-induced ulcers [3], unlike the strong antagonism shown against ethanol-provoked gastric mucosal damage [9]. The current findings, using cyproheptadine and ketanserin,

suggest that blockade of these 5-HT_2 receptors can also significantly prevent lesions caused by stress, although to a lesser degree in intensity than against those produced by ethanol; both 5-HT_2-receptor blockers had similar antiulcer potencies in the dose ranges used in the stress experiments. Ondansetron, tended to have a slightly weaker antistress-ulcer action in the doses employed. This tendency for 5-HT_3-receptor blockade to have a weaker antilesion effect, when compared to 5-HT_2-receptor antagonism, was also seen with ethanol-induced lesions.

The effectiveness of 5-HT_3-receptor blockade suggests that ondansetron could be acting on the peripheral and/or central receptors, if it is assumed that its antagonistic actions to stress- or ethanol-evoked gastric lesions are similar to those which give rise to its established antiemetic [7] and anxiolytic [6] properties. In the case of stress-induced ulcers, it is already known that an efferent pathway, via the vagus nerve, plays a predominant role in ulceration; impulses originating from the central effects of stress lead to vagal overactivity which in turn releases histamine and 5-HT from the mast cells and other sources of these ulcerogenic factors in the stomach [3]. Thus, an anxiolytic action must be considered. The findings with graded doses of phenobarbitone indicate that, in the dose range used, only the two higher doses antagonized stress-induced ulceration whereas the smallest dose was without an antiulcer effect. When these pretreatment doses of the barbiturate were studied for their influence on CNS activity, it was found that all three dose levels were effective in inhibiting vertical rearing movements which seem to be a voluntary manifestation of CNS activity [unpublished findings]. On the other hand, 5-HT_2- or 5-HT_3-receptor block did not appear to influence this parameter. One interpretation of these observations is that an anxiolytic influence by antagonism of the 5-HT_3-receptors, if this occurred within the dose range used, did not lessen the gastric effects of stress, at least to a significant extent. Thus, the possibility of anxiolysis accounting for the antiulcer action of ondansetron is minimal, if any; the findings point more to a dominant peripheral mechanism. Ethanol-provoked stomach lesions are considered to be due to the localized action of intragastrically-instilled alcohol [9]; thus, the effects of alcohol are peripheral in origin.

The ability of 5-HT_2-receptor blockade by cyproheptadine to antagonise effectively ethanol-induced gastric lesions confirms past findings and conclusions [9]. The possibility of ethanol possessing a central element to its lesion-producing action, in contrast to stress, is less likely because evidence for this effect of ethanol is equivocal [Ogle, unpublished findings]. As ondansetron produced similar antagonistic actions, it would appear that 5-HT_3-receptor stimulation can produce the same result as 5-HT_2-receptor activation in the gastric microvessels and mural smooth muscle, which contribute to the aetiology of these lesions [3, 9]; the ulcerogenic effects of histamine release by 5-HT [5] must also be considered. These peripheral actions of 5-HT_3-receptor blockade, as exemplified by past findings with 5-HT_2-receptor

involvement, also apply to the present observation with stress-induced ulceration. However, the question of 5-HT_3-receptor block at the vagal nerve endings, to interrupt the peripheral afferent pathway of the emetic reflex [7] and consequently lessen stress-evoked ulceration, remains to be clarified because these lesions are caused by vagal-transmitted impulses of central origin.

The current findings are preliminary and do not permit extended speculation. They, however, indicate that 5-HT_3-receptor blockade by ondansetron can lessen stress- as well as ethanol-provoked gastric lesion formation. Further studies are indeed required to elucidate these interesting findings which, if confirmed, could have additional clinical potentials for this drug.

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ROLE OF MUCUS IN MUCOSAL PROTECTION THROUGH ETHANOL AND PEPSIN DAMAGE MODELS

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Gastrointestinal mucus is considered an important part of the mucosal defence mechanism against endogenous aggressors such as acid and pepsin. The mucus gel layer, adherent to the mucosal surface creates a diffusion barrier to luminal pepsin, thus protecting the underlying epithelium from the digestion by pepsin. The mucolytic pepsin will, however, digest the mucus at its luminal surface, but that lost is normally balanced by secretion of new mucus. This dynamic balance is disrupted when the mucus is exposed to excess pepsin, which causes focal haemorrhagic damage by progressively hydrolyzing the adherent mucus. The adherent mucus gel layer cannot contribute to the protection against exogen damaging agents such as ethanol and nonsteroidal anti-inflammatory drugs, as these compounds easily penetrate the mucus barrier causing, at high concentration, epithelial exfoliation.

This study describes the basic properties and characteristics of gastric mucus and compares the pepsin-induced damage with the ethanol damage model.

Keywords: adherent mucus, mucus barrier, mucus thickness, pepsin, ethanol

The gastrointestinal mucus

Properties

Gastrointestinal mucus occurs as a stable, water-insoluble gel adherent to the mucosal surface together with its two other phases namely the viscous soluble luminal mucus and the presecreted mucus stored in intracellular vesicle [5].

Soluble luminal mucus which contains degraded mucin produced by peptic erosion of the adherent mucus layer, together with some soluble polymeric mucin secreted directly by the mucosa may lubricate the surface of the adherent gel barrier and minimizes its erosion by mechanical forces. In contrast to the adherent mucus

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gel layer soluble mucus is freely mobile and unlikely to be effective in protection against the gastric juices with which it is mixed [4].

It is the adherent mucus gel layer that provides the stable protective barrier over the mucosal surface [5]. It can be observed on unfixed mucosa as a thin, gelatinous layer easily distinguishable from the mucosa and luminal bathing solution. As the prime function of the adherent mucus is to protect the epithelial cells it is important that it forms a continuous cover over the mucosa. Unfixed mucosal preparations now unambiguously favour that the mucus gel is continuous [4, 10]. However, the continuity of the adherent gastric mucus layer *in vivo* has been strongly disputed [15] mainly on the basis of the discontinuous layer seen in histologically fixed sections. Standard processings for electron microscopy studies are likely to be responsible for the discrepancies since routine preparations for histological observations dehydrates and shrinks the mucus gel [14]. If the mucus gel is first stabilized by mucus antibodies or snap freezing a continuous layer is observed [24].

Measurement

The mucus dynamics can be assessed by the quantitation of its three phases. Intracellular mucin biosynthesis can be measured by incorporation of radioactive precursors. Results can depend on whether appropriate separation technique has been used to distinguish between the radioactive mucin and the similarly labelled lower sized non-mucin glycoprotein [9]. Luminal mucus is estimated by a variety of chemical or, more sensitive immunological methods [17]. But the amount of mucin in luminal juice cannot be taken as a direct measure of mucus secretion or as an index of changes in the functional mucus gel barrier because gastric luminal mucin content, for example, has been demonstrated to vary quite independently of changes in the thickness of the adherent gastric mucus barrier [13]. The adherent mucus gel is frequently characterized by measuring its thickness. Two techniques have been developed to assess the mucus gel thickness on unfixed fresh mucosa. One is an indirect method using a slit lamp and pachymeter [6] while the other is a direct observation using light microscopy [10]. The latter is rapid and simple to perform and allows many measurements from one sample, clearly distinguishes between mucus and the unstirred water layer above the gel and it shows the wide variatons in gel thickness across the mucosa [2]. Although there are views questioning the precision of this technique – mainly its validity is disputed for the demonstration of the gel continuity [16].

Using the Kerss's method, the adherent mucus gel of gastric mucosa is observed of variable thickness, about 50–450 μm (median, 180 μm) in man and 10–230 μm (median, 80 μm) in the rat [10].

Adherent mucus and protection against acid and pepsin

The most important function of the mucus in the stomach and duodenum is the protection of the mucosa. This protective effect is attributed to the adherent mucus gel layer which protects the epithelial surface from acid by providing a stable, unstirred layer at the mucosal surface. This unstirred layer supports the neutralization of H^+ by mucosal HCO_3^-, thus establishing a pH gradient, from an acid pH in the lumen to a near neutral pH at the mucosal surface [7, 20].

The importance of this mucus: bicarbonate barrier is strongly questioned by Wallace [26] who experimentally demonstrated that the gastric mucosa of the rat can resist damage induced by acid under circumstances in which a mucus: bicarbonate based pH gradient would be ineffective, if at all present and concluded that the existing pH gradient on the surface of an undamaged gastric mucosa does not seem to serve an important function as a protective barrier against acid and pepsin.

True, that even the proponents of the mucus: bicarbonate barrier face the unresolved problem of how the gastric glands, secreting acid and pepsin, can be protected against their own secretion since there is no mucus: bicarbonate barrier in these glands [4].

The adherent mucus gel protects the mucosa from proteolytic attack by providing a permeability barrier to pepsin, as it is impermeable to protein (Mr 17 000) [1]. Pepsin in the gastric lumen will, however, progressively digest the mucus layer at its luminal aspect to produce soluble degraded mucin. If the adherent mucus layer is accepted to be continuous over the undamaged mucosa *in vivo*, that lost by erosion must be replaced by secretion of new mucus. This dynamic balance can be damaged following exposure to excess pepsin and acid but not acid (pH 2.2) alone [11]. Instillation of pepsin into the rat stomach at two or three times the excess of maximal secretion results in disruption of the adherent mucus layer, increases release of proteolytically degraded mucin into the lumen, focal epithelial damage and mucosal haemorrhage while acid alone does not cause any observable damage to the adherent mucus or epithelium.

On the other hand, the adherent mucus gel layer is not a diffusion barrier to damaging agents such as bile salts, hypertonic saline, ethanol and nonsteroidal anti-inflammatory drugs, which readily penetrate the gel resulting in the destruction of the underlying epithelium [4].

Mucosal damage by ethanol and epithelial repair

In the last decade much attention has been given to understanding the so-called phenomenon of cytoprotection which according to Robert et al. refers to

protection against chemically (e.g. concentrated ethanol, acid or base) or physically induced haemorrhagic acut gastric erosions and ulcers [21].

After the discovery of the cytoprotective property of prostaglandins much effort has been undertaken to investigate anti-ulcer drugs from cytoprotective point of view. Cimetidine and probanthine were the first drugs described to exert cytoprotective-like effects [8]. However, Cimetidine by Robert [21] and Puurunen [19] failed to protect against ethanol-induced gastric damage. Later, the first "cytoprotective" compounds were followed by several others and nowadays it is easier to list those drugs which are not "cytoprotective".

To examine the "cytoprotective" – recently proposed gastroprotective – effects of antiulcer drugs mainly the ethanol-provoked damage model is used. In this model adherent gastric mucus does not protect the mucosa. Ethanol in high concentration rapidly penetrates the mucus barrier resulting in destruction of the underlying epithelial cells and in more severe cases vascular damage and/or visible lesions [22].

After such damage the epithelium (not at the sites of haemorrhage) is rapidly re-epithelialized. A near neutral and pepsin-free environment is believed to be the prerequisite for this process to occur. This could be achieved by plasma exudation providing the surface with neutralising HCO_3^- and by formation of a thick gelatinous coat in association with plasma exudation from the damaged mucosa [4]. This mucoid coat would prevent the subsequent repair from toxic agents by providing a microenvironment for the epithelial restitution in the lumen. This statement was supported experimentally since removal of the mucoid cap resulted in significant inhibition of epithelial repair [25]. According to Sellers et al. [23] the mucoid coat is quite different in composition and properties from the original adherent mucus layer. It is composed of fibrin and exfoliated necrotic epithelium. That mucoid coat is much thicker (median thickness 700 μm compared to 80 μm in the rat), more granular and much less viscous in appearance than the adherent mucus gel. In the repair process, the adherent mucus may act as a template for the fibrinogen-fibrin conversion.

Pepsin damage model

The pepsin-induced damage model described by Leonard et al. [11] shows a different mucosal response than that seen with ethanol. Changes in the mucosa produced by the endogenous damaging agent, pepsin, might be more relevant to the changes taking place in ulcer disease. There are data about increased degradation by pepsin of the adherent gastric mucus barrier in peptic ulcer [18]. Thus, the pepsin induced damage model can provide a different and probably a more appropriate approach to the question of whether antiulcer drugs have mucosal protective properties in addition to their other activities.

The pepsin induced damage model, using anaesthetized animals, involves the cannulation of the stomach in order to get access to the gastric juice. The animal experiment consists of a series of gastric instillations with 1 to 4 ml of 0.1 M HCl or pepsin (0.5–5 mg/ml)/0.1 M HCl for five successive 30-min periods. The instillates are assayed for soluble degraded mucin (measured by the method of Mantle et al. [12]) bleeding (by measuring the haemoglobin bound iron using atomic absorption) and the mucus thickness is checked by Kerss et al. [10].

Leonard [11] reported after the instillation of pepsin an increase in mucus glycoprotein and iron content and the adherent mucus was disrupted and discontinuous. While using buffer (pH 2.2) these effects were absent.

Allen et al. [3] compared the damage caused by ethanol and pepsin by measuring the transmucosal potential difference, haemoglobin bound iron in the lumen (for assessment of mucosal bleeding) and carried out histological observations.

The study highlights the basic differences of the two models: excess pepsin causes mucosal damage by progressively hydrolysing the adherent mucus and causing focal haemorrhagic damage after reaching the underlying epithelium. In contrast, excess ethanol penetrates rapidly and causes total epithelial exfoliation. Potential difference is unaffected in pepsin-damage model but ethanol causes a collapse of transmucosal potential difference.

Some H-2 blockers have already been examined (unpublished data from A. Allen) by using the pepsin damage model. H-2 antagonists significantly decreased the pepsin induced bleeding over 2 h, reduced the discontinuities in the adherent gel present after 2 h pepsin instillation and increased the mucus thickness. They had no effect on the amount of degraded mucin in the instillates after 2 h exposure to pepsin.

Methods, like the previously quoted pepsin damage model can contribute to the resolution of the conflict on whether H-2 antagonists have direct mucosal protective actions and furthermore may provide a suitable, alternative experimental model to ulcer research.

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IS ACUTE SURGICAL VAGOTOMY AN AGGRESSOR TO GASTRIC MUCOSA IN PYLORUS LIGATED RATS WITH AND WITHOUT INDOMETHACIN TREATMENT?

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Previously it was proved that intact vagal nerve is basically necessary for the development of gastric cytoprotection. The aims of this study were to receive further data about the role of vagal nerve in the development of gastric mucosal damage. The observations were carried out on Sprague-Dawley rats. Acute bilateral surgical vagotomy was done with pylorus ligation and/or indomethacin (IND) treatment (20 mg/kg, sc.) at the time of operation. The animals were sacrificed 4 h after the operation. The number, the severity (semiquantitative method), the mean size and summed surface (computer assisted quantitative method) of gastric mucosal damage, the H^+ output and the mucosal PGE_2 level were determined.

It has been found that 1) the ASV itself (without IND or pylorus ligation) provoked gastric mucosal damage, which was more severe than in the pylorus ligated animals at 4 h; 2) IND was able to reduce the summed surface of mucosal damage after ASV; 3) ASV aggravated the gastric mucosal damage in pylorus ligated animals in spite of the decreased H^+ output; 4) the PGE_2 level was lower in vagotomized and vagotomized + pylorus ligated animals then in the control group, and the IND did not cause further decrease in its level after ASV.

It has been concluded that the balance between aggressive and defensive factors of gastric mucosa was shifted to the aggressive side in surgically vagotomized animals.

Keywords: acute surgical vagotomy, gastric mucosal damage, pylorus ligation, prostaglandins, indomethacin

The upper gastrointestinal tract is innervated parasympathetically by vagal nerve, which has important regulatory role in gastric acid secretory and motility responses. The existence, physiologic and pathophysiologic role of brain-gut (including brain-stomach) axis has been investigated widely, and it was reviewed contemporary from the point of view of neuropeptides [19] as well as that of the autonomic nervous system [3].

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The role of vagal nerve was investigated recently in the maintenance of gastric mucosal integrity. It was found that surgical truncal vagotomy markedly lessened the protective effect of 16,16-dimethyl-PGE_2 and that of the mild irritant (30% ethanol) against topically administered 100% ethanol-induced gastric mucosal damage [4]. There are also some contradictory data about the effect of vagotomy on gastric mucosal lesions [2, 13]. In our experiments, acute bilateral surgical vagotomy (ASV) was able to enhance the gastric mucosal lesions induced by different necrotizing agents [10], which was partly explained by the increased vascular permeability [5]. Moreover the vagal nerve has an essential role in the cytoprotection induced by different chemicals, such as PGI_2, β-carotene, and small doses of atropine and cimetidine, which do not affect the gastric acid secretion [11, 12]. These earlier tested compounds have different mechanisms of action in respect to time periods [15, 18], gastric mucosal biochemistry [9], prevention of early vascular events and repair mechanisms in the late phase [17, 20] after administration of a necrotizing agent (e.g. 96% ethanol, intragastrically) in animal experiments. As it was proved, the cytoprotection did not appear after ASV. The biochemical and physiological background of the failure of cytoprotection in vagotomized animals is unknown.

In spite of the huge number of experimental data the exact role of vagal nerve in the development of gastric mucosal damage and during the mucosal protection has not been cleared up yet. The aims of present study were to gather further data about the importance of vagal nerve in the maintenance of gastric mucosal integrity and to understand its mechanism of action.

Materials and methods

The observations were carried out on Sprague-Dawley CFY (Gödöllő, Hungary) strain rats after 24 h starvation. The animals were anaesthetized with ether slightly and an upper median laparotomy was done. Pylorus ligation was carried out in the first group. The gastric acid secretory responses were measured in 4-h pylorus ligated Shay rats. This gastric hypersecretion is only a vagus-dependent secretory response, the 4-h time seems optimal to study the gastric secretory responses [8]. In the second group of animals about 2–4 mm pieces of nerve were cut, removed from both side of vagus nerve located on the lower, intraabdominal part of esophagus. Surgical vagotomy and pylorus ligation together were done in the third group of animals. Each group was divided into two parts, the first part received indomethacin at a dose of 20 mg/body weight kg, the second part of groups received saline instead of indomethacin in 1 ml volume.

The animals were sacrificed 4 h after the operation. The number, the severity (semiquantitative method [7]), the mean size and summed surface (computer assisted quantitative method, DIGICELL) of gastric mucosal damage, the H^+ output and the volume of gastric content were determined, the mucosal PGE_2 level was assayed by RIA kit (Institute of Isotopes, Budapest, Hungary). The protein content was measured by the method of Lowry et al. [6]. The prostaglandin levels were calculated to 1 mg protein, all results were expressed as means ± SEM. For the statistical evaluations Student's *t*-test was used excepting the severity of lesions, which was evaluated by Mann-Whitney test.

The grounds of DIGICELL system is the following: the black and white CCD camera is connected to the computer through an analog/digital converter, which converts the original color picture

into a high resolution black and white picture with 256 grade gray scale. The points with different color are well distinguishable by using this gray scale. The evaluation of the damaged surface is relatively simple and fast by the applied planimetric software. We have also possibility to determine even the size of each separate lesion and the mean size of those. The equipment and software were developed and shipped by ASK Ltd., Hungary. The comparison of our earlier used semiquantitative method [7] and this planimetric method was previously demonstrated [16].

Results

The IND caused significant increase in the number of mucosal lesions in the pylorus ligated groups. Surprisingly, gastric mucosal damage could be detected 4 h after surgical bilateral vagotomy, the number of lesions was higher than in the pylorus ligated groups and the IND did not cause further increase. No significant differences between the vagotomized and pylorus ligated plus vagotomized groups was detectable. Similar results were found in the case of the semiquantitatively measured severity (Fig. 1).

During the planimetric evaluation the mean size of damaged areas of the gastric mucosa was the highest when pylorus ligation and vagotomy was carried out simultaneously. The summed surface of mucosal lesions was the highest in the pylorus ligated plus vagotomized group. The lesions were more severe after ASV than after pylorus ligation. Interestingly, IND decreased the summed surface of mucosal damage in vagotomized rats (Fig. 2).

There was not significant difference between the pylorus ligated and pylorus ligated plus vagotomized groups in respect of gastric secretory volume. The ASV caused only a slight but not significant decrease in the gastric volume, and the differences between the saline and IND treated groups were not statistically significant. The IND diminished slightly the acid secretion in the group with intact vagal nerve. After ASV the hydrogen ion concentration was at a very low level and there was not any difference between the saline and IND treated animals (Fig. 3).

IND decreased the gastric mucosal PGE_2 level approximately to 40% of the baseline level after 1 h. The inhibitory effect of IND was not detectable 4 h after the application. The gastric mucosal PGE_2 level did not change after pylorus ligation, but its level was lower when ASV and pylorus ligation were carried out together. The IND caused only a slight decrease in mucosal PGE_2 level 4 h after pylorus ligation. It was interesting that in the vagotomized groups the IND was not able to decrease the mucosal PGE_2 level. The significantly lower level of PGE_2 in the third group seemed a consequence of the mucosal damage, because the summed surface of lesions was the largest in this group (Fig. 4).

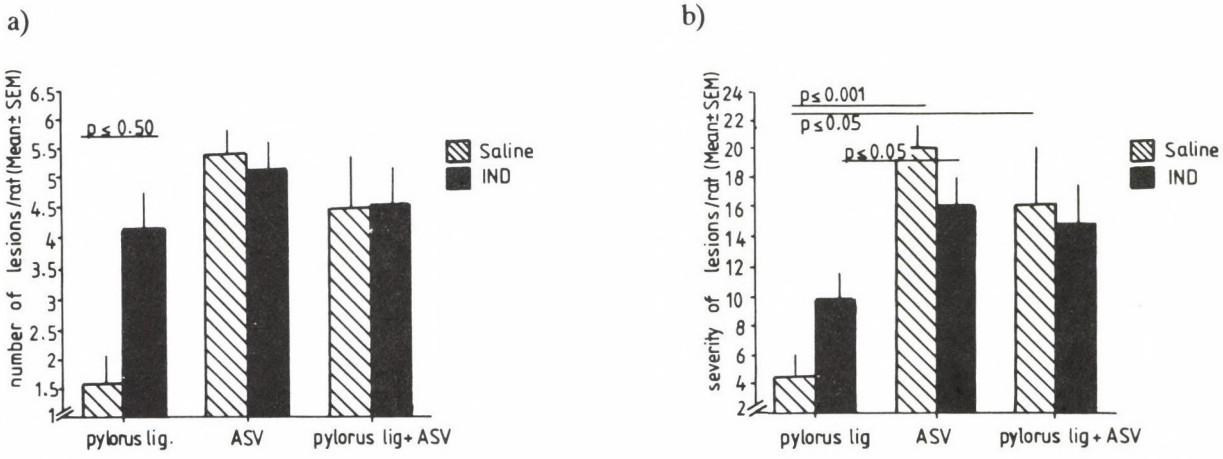

Fig. 1. The effect of (indomethacin) IND on the number (left part) and severity (right part) of gastric mucosal lesions in pylorus ligated, surgically vagotomized and pylorus ligated plus surgically vagotomized rats (n = 8 in each group).

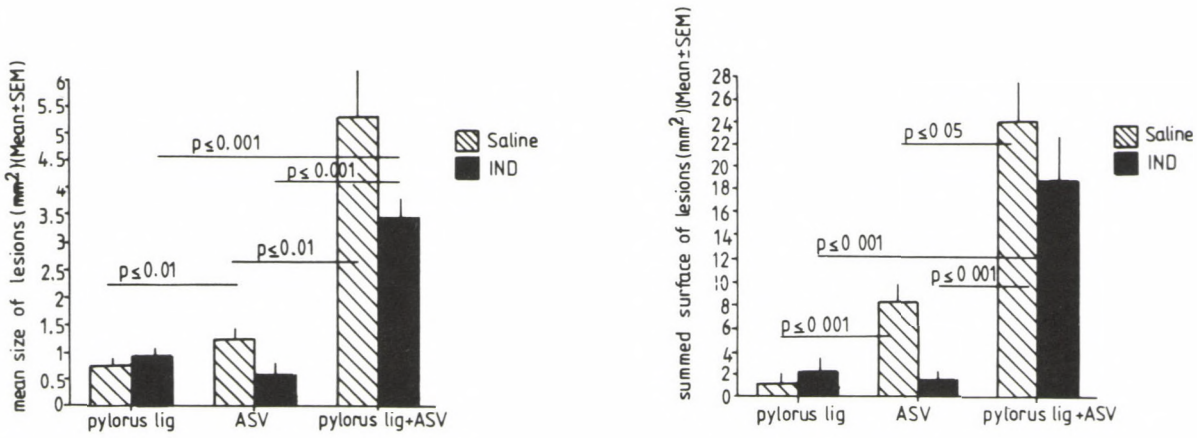

Fig. 2. The effect of IND on the mean size (left part) and summed surface (right part) of gastric mucosal lesions in pylorus ligated plus surgically vagotomized rats (n=8 in each group)

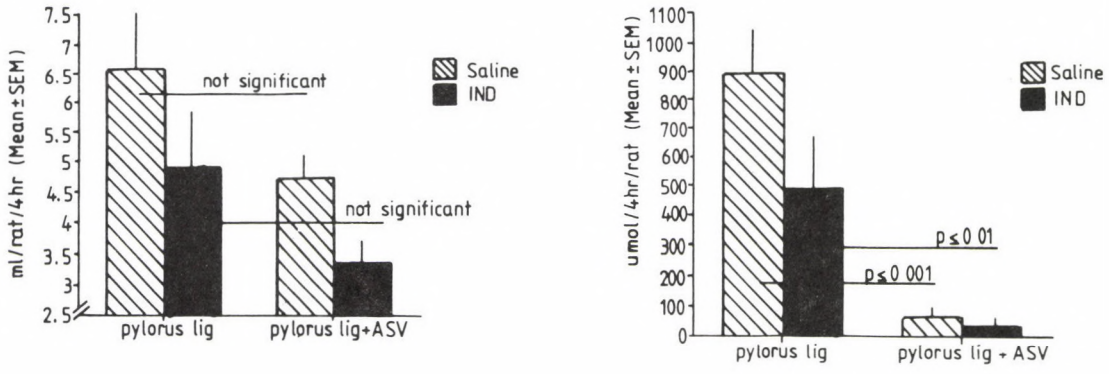

Fig. 3. The effect of IND on the gastric secretory volume (left part) and acid output (right part) in pylorus ligated, surgically vagotomized and pylorus ligated plus surgically vagotomized rats (n=8 in each group)

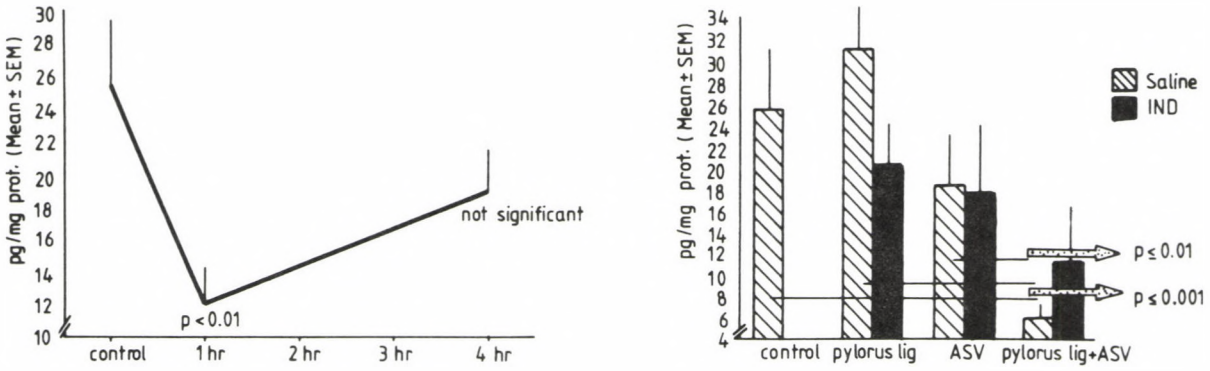

Fig. 4. The effect of IND on the gastric mucosal PGE_2 content in intact (left part), pylorus ligated, surgically vagotomized and pylorus ligated plus surgically vagotomized animals (right part) (n = 8 in each group)

Discussion

Based on these results it could be concluded that the balance between aggressive and defensive factors of gastric mucosa was shifted to the aggressive side after surgical vagotomy.

ASV itself was able to provoke gastric mucosal damage, 4 hours after the operation, which was more severe than in pylorus-ligated rats. The role of stress situation could not be ruled out under these experimental circumstances. This stress may have exerted an aggressive effect to the gastric mucosa after ASV, when the susceptibility of mucosa was higher. Previously it has been shown that vagotomy was able to diminish gastric mucosal blood flow [1], that might be an interpretation for the increased susceptibility of gastric mucosa.

IND was able to reduce the mean size and summed surface of mucosal damage after ASV comparing to the control (saline-treated) group. From these data it cannot be answered directly whether a competition between the two aggressors (namely the IND treatment and the vagotomy) existed. This competition may be realized at the level of prostaglandin synthesis or inhibition of that, but further evidence is needed to support this hypothesis.

ASV itself did not influence the mucosal PGE_2 level significantly, but a slight reduction of its level was observed. It was found by others that the stimulation of vagal nerve yielded endogenous prostaglandin production in the gastric mucosa [14]. Any kind of stimuli through vagal nerve is absent after ASV, which can be an explanation for the above-mentioned results.

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EFFECT OF ACUTE SURGICAL VAGOTOMY ON THE MUCOSAL CONTENT OF 6-KETO-PGF_{1alpha}, PGE_2 AND GLUTATHIONE AFTER INTRAGASTRIC 96% ETHANOL TREATMENT IN RATS

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It has been observed earlier that gastric cytoprotection produced by PGI_2, beta-carotene, small doses of atropine or cimetidine has failed in surgically vagotomized rats. This phenomenon may be in connection with endogenous prostaglandins (PGs) and glutathione (GSH) level of the gastric mucosa. The aims of the study were to evaluate the effect of vagus nerve on the gastric mucosal 6-keto-PGF_{1alpha}, PGE_2 and glutathione after intragastric 96% ethanol (ETOH) treatment.

The observations were carried out on CFY rats. The gastric mucosal damage was produced by intragastric administration of 1 ml 96% ETOH. Acute bilateral surgical vagotomy (ASV) was carried out 30 min prior to ETOH application. The animals were sacrificed 1, 5, 15 or 60 min after ETOH installation. The number and the severity of gastric mucosal lesions were noted and 6-keto-PGF_{1alpha}, PGE_2 an GSH contents of gastric mucosa were measured.

It has been found that: 1. the number and the severity of gastric mucosal lesions were increased after ASV compared to those with intact vagal nerve, 2. 96% ETOH treatment increased both the gastric mucosal PGs and GSH levels, 3. 6-keto-PGF_{1alpha} peaked at 5 min PGE_2 and GSH peaked at 15 min after ETOH treatment, 4. ASV decreased the gastric mucosal PGs content and delayed the peaks of PGE_2 and GSH.

It has been concluded that the decreased content of PGs and the delayed GSH increase may play a pathological role in the failure of gastric cytoprotection of rats after ASV.

Keywords: gastric mucosal damage and protection, acut surgical bilateral truncal vagotomy, 6-keto-PGF_{1alpha}, PGE_2, GSH

The phenomenon of gastric cytoprotection induced by prostaglandins has been proved by Robert et al. [14] in 1979. Since than many compounds such as prostacyclin [11], sulfhydryls (17), beta-carotene and other carotenoids [5], atropine [10] and cimetidine [8] have been found which are able to inhibit the development of necrotizing agent-induced gastric mucosal lesions.

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It has been observed that gastric mucosal protection induced by PGI_2, beta-carotene, low dose of atropine of cimetidine failed after acute bilateral truncal vagotomy [9]. Adaptive cytoprotection to ethanol-induced gastric mucosal injury was also inhibited by vagotomy [15, 4]. The stimulation of vagus nerves enhanced the tissue formation and release of gastric mucosal PGs [1, 16].

The gastric mucosa contains usually high concentrations of GSH [2]. There are many conflicting results on the role of GSH in mucosal injury and prevention. Depletion of endogenous gastric glutathione can induce mucosal protection which is directly related to the degree of glutathione depletion [13]. A decreased mucosal GSH content during the development of chemical-induced gastric mucosal lesions and a protective role of exogenous sulfhydryls were also demonstrated [17]. An increased gastric mucosal GSH content was found after intragastric 96% ETOH or 0.6N HCl instillation [12].

The aims of the present study were to evaluate the effect of acute surgical vagotomy on the gastric mucosal 6-keto-PGF_{1alpha} PGE_2, GSH contents during the development of gastric mucosal lesions induced by 96% ethanol (non-acid dependent model).

Materials and methods

The investigations were carried out on both sexes of CFY rats (LATI, Gödöllő, Hungary), which originate from the Sprague-Dawley strain (USA). The animals were fasted for 24 h before the experiments, although they received water *ad libitum*. The animals were kept in wire cages to prevent coprophagy. All of the observations were carried out in the morning. The gastric mucosal lesions were provoked by intragastric 96% ETOH (1 ml) instillation. 30 min prior to the administration of ETOH under ether anaesthesia midline laparotomy was carried out. The trunks of the vagus nerves were isolated just below the diphragm and 2–4 mm pieces were cut. Sham operation (only laparotomy) also was carried out in other group of animals. The animals were sacrificed 0, 1, 5, 15 or 60 min after ETOH administration. Number and severity of gastric mucosal lesions were noted. A semiquantitative scale was used to evaluate the severity of gastric mucosal lesions [11]. The gastric mucosa was scraped off. For biochemical evaluations, 1.5 ml physiological saline was added to the mucosa of one rat and the specimens were homogenized (Branson Sonic) for 2 min in ice-cold water bath. The homogenate was centrifuged (16 000 rpm, 30 min, 4C). The GSH content was measured from the supernatant by Ellman's method [3]. PGE_2 and 6-keto-PGF_{1alpha} content was determined by RIA (Institute of Isotopes, Budapest, Hungary). The protein content was assayed by the method described by Lowry et al. [7]. The biochemical results were expressed in accordance with 1 mg mucosal protein. The results were expressed as means ± SEM. The experimental data were analyzed by Student's '*t*'-test, except of ulcer severity which was analyzed by Mann-Whitney's test.

Results

The ethanol-induced gross gastric mucosal lesions appeared 1 min after ETOH administration. ASV caused an increase in both the number (Fig. 1) and the severity (Fig. 2) of gastric mucosal lesions.

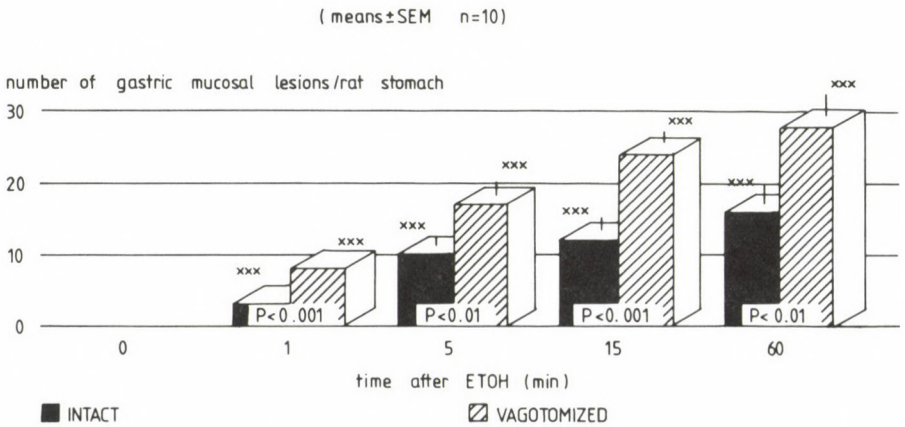

Fig. 1. Effect of acute surgical vagotomy on the number of gastric mucosal lesions induced by intragastric 96% ethanol instillation in rats. *** = $p < 0.001$ vs. control (0 min)

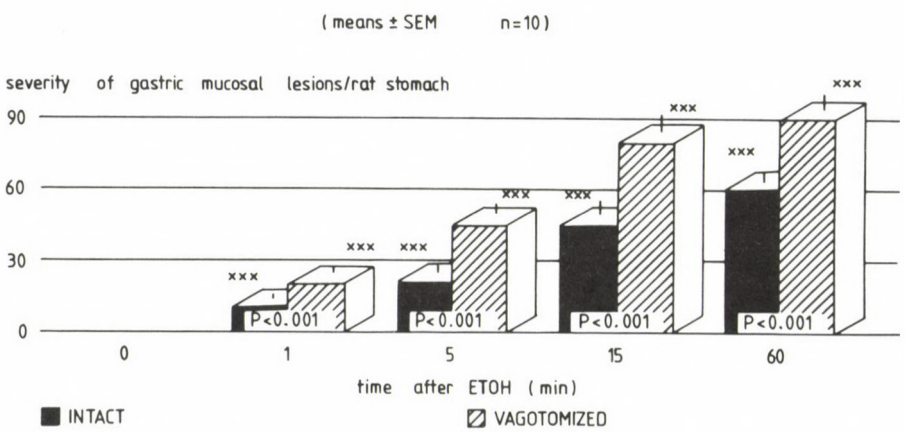

Fig. 2. Effect of acute surgical vagotomy on the severity of gastric mucosal lesions induced by intragastric 96% ethanol instillation in rats. *** = $p < 0.001$ vs. control (0 min)

The gastric mucosal content of PGE_2 (Fig. 3) and 6-keto-PGF_{1alpha} (Fig. 4) increased 5 min and 60 min after the administration of ETOH. ASV decreased gastric mucosal PG formation 5 min and 60 min after ETOH application.

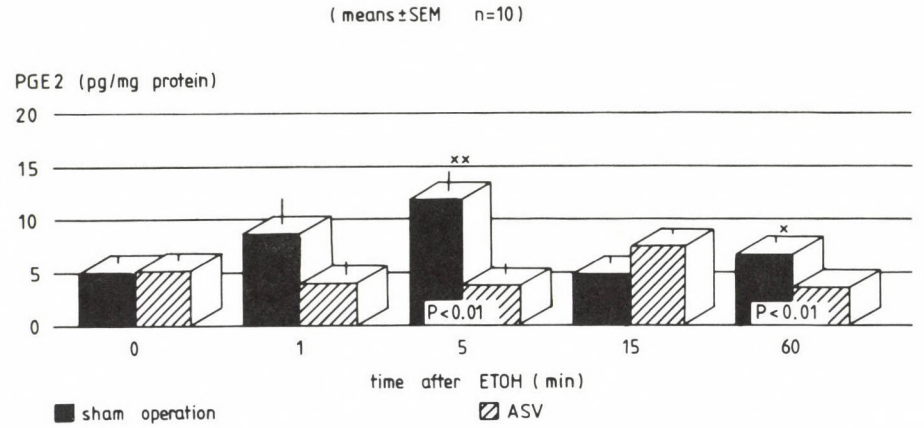

Fig. 3. Effect of acute surgical vagotomy on gastric mucosal 6-keto-PGF1alpha content after intergastric 96% ethanol administration in rats. ** = $p < 0.01$, * = $p < 0.05$ vs. control (0 min)

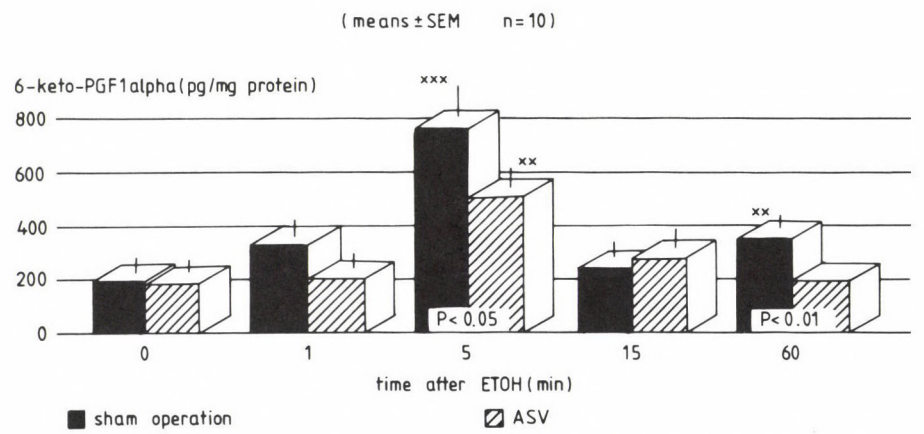

Fig. 4. Effects of acute surgical vagotomy on gastric mucosal PGE_2 content after intragastric 96% ethanol administration in rats. *** = $p < 0.001$, ** = $p < 0.01$ vs. control (0 min)

GSH content of the gastric mucosa was enhanced by 5 min after ETOH application. ASV delayed the increase of GSH to 15 and 60 min after ETOH instillation (Fig. 5).

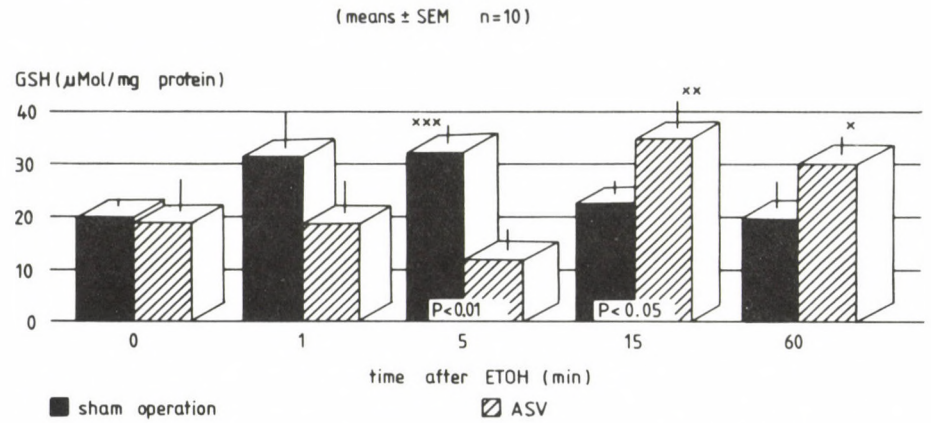

Fig. 5. Effect of acute surgical vagotomy on gastric mucosal glutathione content after intragastric 96% ethanol administration in rats. *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$ vs. control (0 min)

Discussion

The time dependent effect of acut bilateral surgical truncal vagotomy was studied on the development of gastric mucosal lesions induced by 96% ethanol and gastric mucosal contents of 6-keto-PGF_{1alpha}, PGE_2 and glutathione.

96% ETOH treatment increased both PG and GSH contents of gastric mucosa, the development of gastric mucosal lesions is accompanied by active metabolic processes (e.g. PG and GSH production). Konturek et al. also reported an increased PGE_2 formation of gastric mucosa 60 min after absolute ETOH application [6]. The role of GSH in the development of gastric mucosal damage and protection is not yet clear. Both GSH depletion [13] and exogenous sulfhydryls [17] were proved to produce mucosal protection and GSH depletion was observed during the development of ETOH-induced gastric mucosal lesions [17]. In the present study the gastric mucosal damage was accompanied by elevated GSH content of gastric mucosa. Our previous results were the same and the changes of GSH levels in the gastric mucosa were found to be the result but not the cause of gastric mucosal damage [12].

ASV decreased the mucosal PG contents and delayed the peak of GSH in association of more severe damage of gastric mucosa. These changes of gastric mucosal biochemistry suggest that mucosal PG formation is mediated by vagus

nerves during the development of gastric mucosal lesions induced by 96% ETOH. This hypothesis is supported by Singh and Bennett's finding: an increased PG formation and release after vagal stimulation was observed in rats [1, 16].

Gastric mucosal damage could be macroscopically detected in a short time (within 5 min) after ETOH administration. This appearance was accompanied by changes of PG and GSH formation of the gastric mucosa. ETOH caused more severe damage after ASV, the gastric mucosal content of PGs was decreased during that time while that of GSH was delayed after 15 min. The final conclusion is that these biochemical changes may play a pathological role not only in the more severe damage of the gastric mucosa after intragastric ETOH administration but in the failure of gastric cytoprotection of rats after acute surgical vagotomy.

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CORRELATION BETWEEN THE CYTOPROTECTIVE EFFECT OF BETA-CAROTENE AND ITS GASTRIC MUCOSAL LEVEL IN INDOMETHACIN (IND) TREATED RATS WITH OR WITHOUT ACUTE SURGICAL VAGOTOMY

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As to earlier observations that beta-carotene prevents the development of gastric mucosal injury produced by different noxious agent, however, its cytoprotective effect can be abolished by acute surgical vagotomy.

The aim of this study was to evaluate the possible correlation between the gastric mucosal cytoprotective effect of beta-carotene and its gastric mucosal level in rats treated with IND.

The gastric mucosal damage was produced by the administration of IND (20 mg/kg s.c.). The instillation of beta-carotene and acute surgical vagotomy (ASV) or SHAM operation were carried out 30 min before IND treatment. The rats were sacrificed 4 h after IND application, and the number and severity of gastric mucosal erosions were noted. The blood rats was collected quantitatively, the liver and the gastric mucosa were removed, and the beta-carotene and vitamin A level of the gastric mucosa, serum and liver were measured with HPLC.

It was found that: 1. Beta-carotene induced gastric cytoprotection in SHAM-operated rats treated with IND but its effect disappeared after ASV. 2. Although the beta-carotene level of the gastric mucosa increased its concentration was not elevated in the serum of intact and vagotomized animals either. 3. Vitamin A Formation was not detected in the liver of animals with or without ASV.

It was concluded that the lack of intake of beta-carotene into the gastric mucosa can not play etiologic role in the failure of gastric cytoprotection of rats with acute bilateral surgical vagotomy.

Keywords: beta-carotene absorption, gastric mucosal damage, indomethacin, gastric cytoprotection, acute bilateral surgical vagotomy

The original description of gastric cytoprotection was interpreted by Chaudhury et al. [2] and Robert et al. [8]. Small doses of prostaglandins [8], vitamin

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A and other carotenoids [5], PGI_2 [7], were found to be able to induce this defence mechanism without the inhibition of gastric acid secretion. Acute bilateral surgical vagotomy (ASV) was proved to abolish the cytoprotective effect of beta-carotene, PGI_2, small doses of atropine and cimetidine [6].

The aim of this study was to evaluate the possible correlation between the gastric cytoprotective effect of beta-carotene and its absorption and gastric mucosal concentration in the rats treated with indomethacin (IND).

In most of other species dietary beta-carotene is largely converted to vitamin A before absorption [1]. The conversion of beta-carotene to vitamin A takes place largely in the intestinal mucosa [4] and the efficiency of conversion varies in different species. Very little or none intact beta-carotene is taken up into the circulation by rats [10] pigs and chickens [3].

Materials and methods

Both sexes of Sprague-Dawley strain rats, weighing 180–200 g, were used in the experiment. The rats were kept in stainless steel cages and fasted for 24 h before the experiment. Coprophagy was not possible in the cages.

The gastric mucosal injury was produced by subcutaneous administration of IND (20 mg/kg). Acute bilateral surgical vagotomy or SHAM-operation and the beta-carotene application (1 mg/kg i.g.) were carried out 30 min prior to the injection of IND. The rats were killed four h after the treatment of IND, the gastric mucosa was scraped off, the blood was collected, the liver was dissected out. The number and severity of gastric mucosal lesions were noted using a semiquantitative scale [11], their liver and serum were frozen at −80 °C until samples were analyzed for vitamin A or carotenoids.

Preparation of samples: the collected tissue was homogenized in 96% ethanol, 1 ml of the sample was shaken with 2 ml of ETOH for 3 min, then it was extracted with 2 ml of hexane being shaked for 3 min again. The mixture was centrifuged for 5 min. As an internal standard 1 ml 4.5×10^{-6} M solution of canthaxantin in hexane was added to the removed homogenous organic phase. Then it was evaporated to dryness in vacuum, and the residue was dissolved in 0.2 ml 1:4 mixture of dichloromethane and methanol. 25 µl of this solution was injected.

The chromatographic system consisted of a gradient former Model 250B (Gynkotec, Germany), HPLC pump Model 300B Glenco injector (Gynkotec, Germany), and a time-programmable UV-vis detector Model 166–2, equipped with Gold chromatography software (Beckman, USA). The column was 150×4.6 mm i.d. packed with Chromsil-C18 6 µm not endcapped reversed phase packing (Labor RT., Hungary).

The eluent was 3% (v/v) water in methanol (A), methanol (B) and 20% (v/v) dichloromethane in methanol (C). The flow rate was 1.5 ml/min. The gradient program was 100% A 30 s; to 100% B in 3 min; 100% B 1.5 min; to 100% C in 4 min; 100% C 5 min (linear steps). The time program of wavelenght was 325 nm for 3.5 min (detecting vitamin A), then 450 nm (detecting carotenoids).

Quantification: The chromatograms were evaluated quantitatively by relating the peak areas of the individual compounds to that of canthaxantin used as an internal standard. The rations of the mole extinctions of the authentic samples to that of canthaxantin were employed as correction factor the detector signals. The results were given in µmol/l or in nmol. The coefficients of variation (CV) were 0.54% for the chromatographic procedure and 4.03% for the preparation of the samples.

Results

The macroscopic gastric mucosal injury developed by 4 h after IND administration (Fig. 1).

Beta-carotene prevented the development of gastric mucosal erosions (Fig. 1), but its protective effect was abolished by ASV.

The beta-carotene level of the gastric mucosa increased significantly after beta-carotene pretreatment both in SHAM-operated and in vagotomized rats (Fig. 2).

No vitamin A was detected in the gastric mucosa 4 h after beta-carotene application in any group of animals. No beta-carotene was found in the serum, or in the liver of experimental animals.

The vitamin A level of the liver was $0.18 \pm 0.002 \times 10^4$ (μmol/liver) (Fig. 3). No significant difference was obtained between the SHAM-operated, IND-treated + SHAM, and ASV + IND treated groups (Fig. 3).

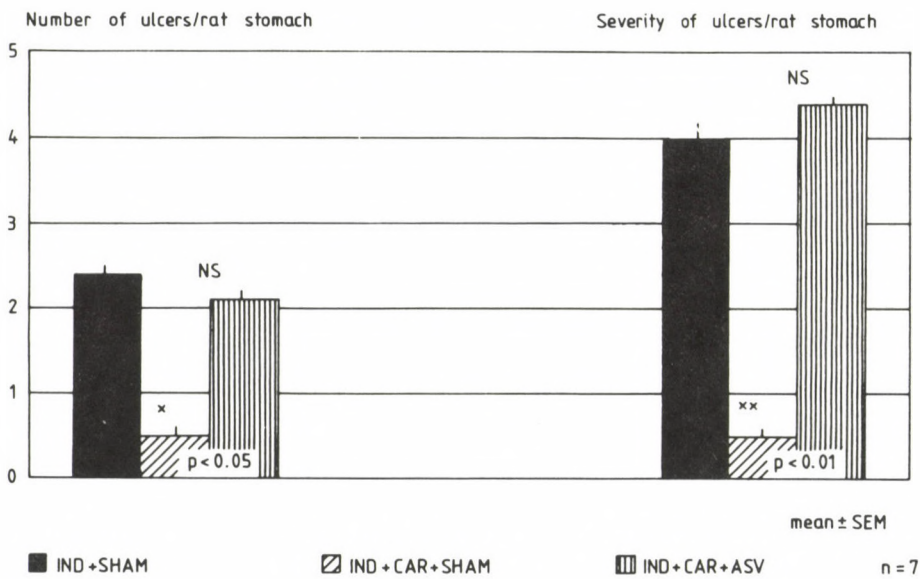

Fig. 1. Effect of acute surgical vagotomy on the development of gastric cytoprotection induced by beta-carotene in rats treatment with IND

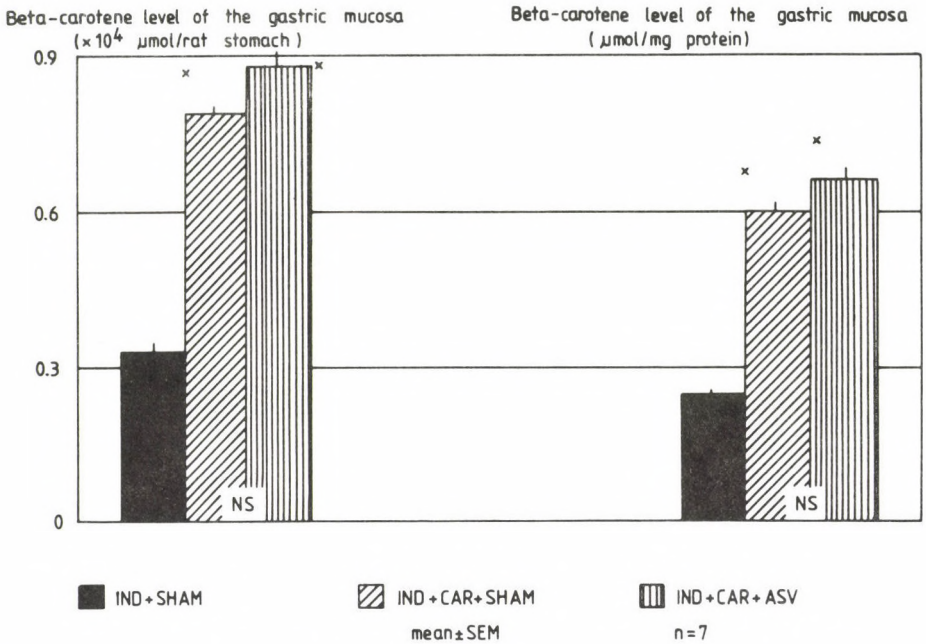

Fig. 2. Correlation between the gastric mucosal concentration of beta-carotene and mucosal protection in rats treated with IND, without or with ASV. No vitamin A could be detected in the gastric mucosa with simultaneous measurement

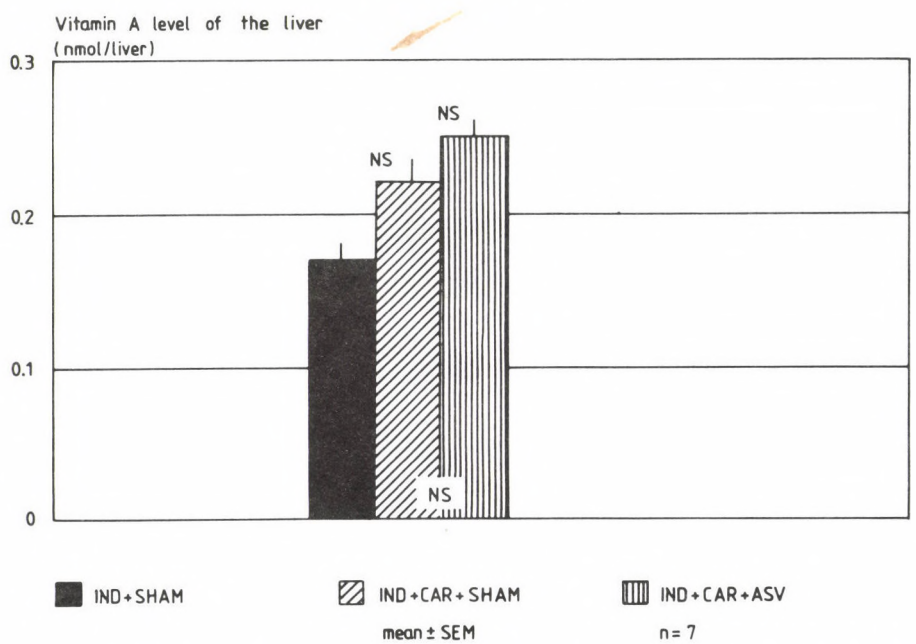

Fig. 3. Correlations between the vitamin A quantity of the liver 4 h after i.g. treatment of beta-carotene (1 mg/kg) in IND-treated rats with and without ASV. No beta-carotene was detected in the liver of rats with simultaneous chemical determination

Discussion

The gastric mucosal level of beta-carotene and its cytoprotective effect was examined in the present study. It was earlier proved that ASV diminished the cytoprotective property of beta-carotene during the development of ethanol-induced gastric mucosal damage (6). The mechanism of action of ASV is not yet cleared. The present results are in good correlation with the previous findings [6].

In rats beta-carotene is largely converted into vitamin A before absorption [6], thus no intact beta-carotene is taken up into the circulation. The conversion of beta-carotene into vitamin A takes place in the intestines. The most of absorbed carotenoids are stored in the liver of rats [1]. Our results have demonstrated that beta-carotene was not taken up into the circulation of rats, thus the rats have no capacity to absorb significant amount of beta-carotene. The vitamin A storage was not increased in the liver either in SHAM-operated or in vagotomized rats during 4 h experimental period. The mucosal level of beta-carotene was increased in carotene treated rats but this increased level was not modified by ASV. Thus the failure of cytoprotection of beta-carotene after ASV is not due to the lack of mucosal intake of carotenoids. There are other mechanisms (e.g. decreased prostaglandin formation and delayed glutathione synthesis of the gastric mucosa) which are responsible for the dimished effect of beta-carotene after ASV [9].

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ACUTE AND CHRONIC SURGICAL VAGOTOMY (SV) AND GASTRIC MUCOSAL VASCULAR PERMEABILITY IN ETHANOL TREATED RATS

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The role of vagus nerve was studied in the development of gastric mucosal damage induced by ethanol (ETOH).

The investigations were carried out on Sprague-Dawley rats. The gastric mucosal damage was produced by i.g. administration of 1 ml 96% ETOH. Acute surgical vagotomy (ASV) was carried out 30 min, chronic surgical vagotomy (CSV) 14 days before the ETOH application. The animals were sacrificed at 0, 1, 5, 15, 60 min after ETOH. Evans blue (EB) (1 mg/100 g) was given i.v. 15 min before autopsy. The number and severity of lesions the EB accumulation of the gastric juice and gastric mucosa were noted.

It was found, that: 1. The vascular permeability increased after ETOH treatment at an early state (within 1–5 min) in association to the macroscopic appearance of erosions. 2. The number and extension of lesions, the EB concentrations in gastric juice and gastric mucosa were significantly higher both after ASV and CSV. 3. Surgical vagotomy alone did not increase the vascular permeability. 4. No significant ulcer formation was observed in vagotomized rats without ETOH treatment.

It was concluded, that 1. Both ASV and CSV enhanced the development of gastric mucosal injury induced by ethanol. 2. Neither acute nor chronic surgical vagotomy exerted an effect of the development of mucosal injury and vascular permeability without the application of the noxious agent. 3. The further increase of enhanced vascular permeability by vagotomy probably has an etiologic role in the aggravating effect of ASV and CSV on the development of chemical-induced lesions.

Keywords: gastric mucosal damage, acute and chronic surgical vagotomy, vascular permeability, ethanol

The effect of autonomic nervous system on the defense mechanisms of the gastric mucosa is still not well known. Acute surgical vagotomy (ASV) was proved to be able worsen the ethanol-induced gastric mucosal damage [3], to abolish the adaptive cytoprotection [1], to inhibit the cytoprotection induced by small doses of

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beta-carotene, PGI_2, atropine and cimetidine [2]. These results suggested that the vagal nerve is necessary in the defense of the gastric mucosa against necrotizing agents. Neither the way of action of ASV nor the influence of chronic surgical vagotomy (CSV) on the development of gastric mucosal injury has been studied sofar. The etiologic role of increased vascular permeability in the development of hemorrhagic erosions was found by Szabo et al. [5]. The aim of this study was to clear the effect of ASV and CSV on the changes of gastric mucosal lesion formation and on the changes of vascular permeability after intragastric ETOH treatment in rats with ASV or CSV.

Materials and methods

Both sexes of Sprague-Dawley (LATI, Gödöllő, Hungary) strain rats, weighing 180–200 g, were used. All the animals were fasted 24 h before the experiments with water *ad libitum*. Coprophagy was not possible in the cages of animals. Under ether anaesthesia bilateral surgical vagotomy or sham operation was performed 30 min or 2 weeks prior to the application of the noxious agent, respectively. In the case of CSV pyloroplasty was also done to maintain the emptying of the stomach. The gastric mucosal lesions were produced by intragastric administration of 1 ml 96% ETOH. The animals were sacrificed 0, 1, 5, 15, or 60 min after ETOH application. The number and severity of the gastric mucosal lesions were noted using a semiquantitative scale [4]. To determine the changes of the vascular permeability of the gastric mucosa Evans blue was used (1 mg/100 g b.w. intravenously) 15 min before the autopsy of the animals as it was described by Szabó et al. [5]. The gastric juice was quantitatively collected and the gastric mucosa was scraped off weighed and digested in concentrated (32%) HCl. After 1 h digestion Evans blue was extracted in chloroform and its concentration was measured spectrophotometrically at a wavelenght of 610 nm and compared to standards of known concentration of Evans blue. The unpaired Student's *t*-test was used for statistical analysis of the results except of ulcer severity when Mann-Whitney's test was applied.

Results

The grossly visible mucosal lesions appeared at 5 min in intact and at 1 min in vagotomized rats (Fig. 1). Significant gastric erosion formation was not provoked by vagotomy (Figs 1 and 2). The number and severity of erosions increased as long as 15 min after ETOH administration (Figs 1 and 2). Both ASV and CSV increased the development of gastric mucosal damage. Although the extension of lesions were higher in rats with CSV, we could not find significant difference in the number of ulcers between the chronically vagotomized and sham operated groups at 60 min (Fig. 2). No significant difference was found in either the number or the severity of erosions after acute or chronic surgical vagotomy (Figs 1 and 2).

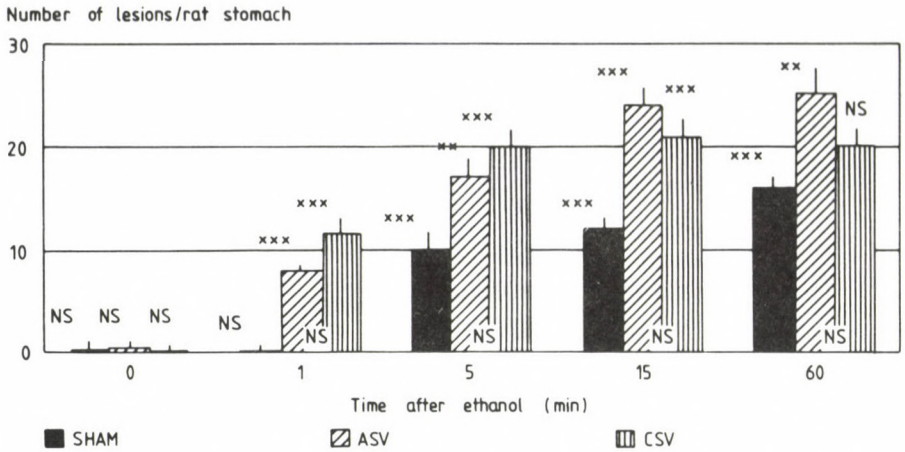

Fig. 1. Effect of acute and chronic bilateral surgical vagotomy on the development of gastric mucosal ulceration induced by ethanol (number of ulcers)
The results were expressed as means ± SEM. NS: not significant, **: $p < 0.01$, ***: $p < 0.001$ vs SHAM animals n = 10

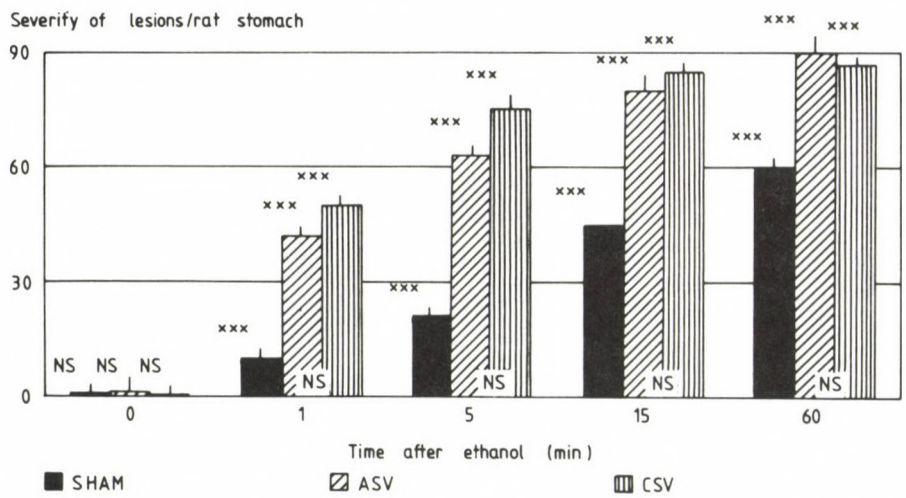

Fig. 2. Effect of acute and chronic bilateral surgical vagotomy on the development of gastric mucosal ulceration induced by ethanol (severity of ulcers)
The results were expressed as means ± SEM. NS: not significant, ***: $p < 0.001$ vs SHAM animals n = 10

EB was penetrated into the gastric juice at a very early phase of the development of mucosal injury (within 1 min) and its concentration was elevated till 5 min (Fig. 3). ASV and CSV alone did not influence the vascular permeability (Figs 3 and 4). Both ASV and CSV induced a further increase in the dye concentration of

the gastric juice after ethanol treatment. The EB level of gastric juice was significantly higher in rats with ASV than that with CSV and this difference remained constant over the first 15 min of the observation. However no significant difference was detected at 60 min after ETOH instillation (Fig. 3).

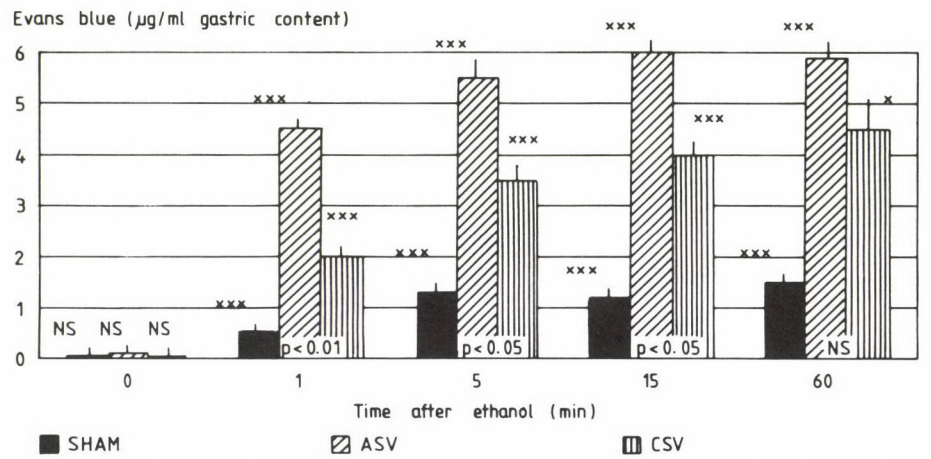

Fig. 3. Effect of acute and chronic bilateral surgical vagotomy of the changes of vascular permeability of the gastric mucosa (Evans blue content of the gastric juice)
The results were expressed as means ± SEM. NS: not significant, *: $p < 0.05$, ***: $p < 0.001$ vs. SHAM animals n = 10

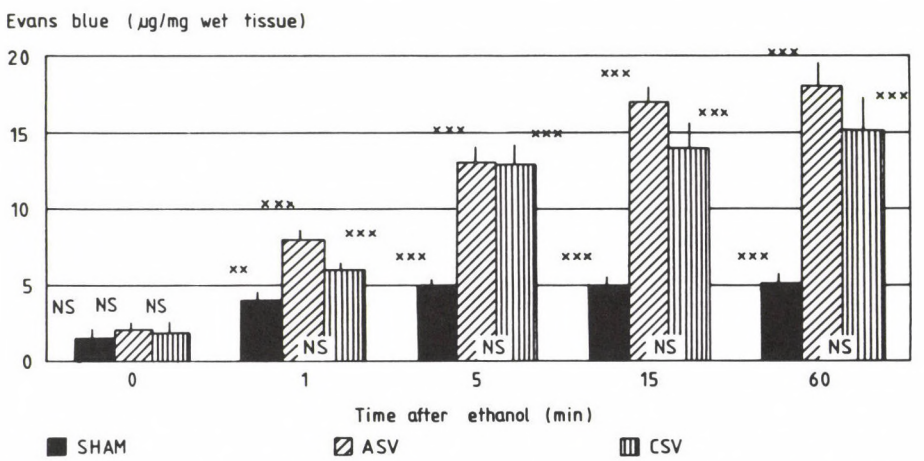

Fig. 4. Effect of acute and chronic bilateral surgical vagotomy on the changes of vascular permeability of the gastric mucosa (Evans blue content of the gastric mucosa)
The results were expressed as means ± SEM. NS: not significant, **: $p < 0.01$, ***: $p < 0.001$ vs. SHAM animals n = 10

EB accumulation was observed in the gastric mucosa within 1 min after ethanol in sham-operated animals (Fig. 4). The dye concentration doubled at 5 min and remained constant over the next 55 min. The level of EB in the glandular stomach of rats with ASV or CSV was two or three times higher than that in rats with intact vagus (Fig. 4). The difference between the EB contents of the gastric mucosa of rats with ASV and CSV was not significant (Fig. 4).

Discussion

The development of gastric mucosal injury and the changes of vascular permeability were studied after ethanol treatment in rats with intact vagus or after ASV or CSV. Our study demonstrated the aggravating effect of both acute and chronic surgical vagotomy on the development of gastric mucosal lesions induced by ethanol. These results are in good correlation with our previous findings [2, 3]. Since the Evans blue is linked to the albumin fraction of the serum it cannot be found in the extravascular tissue. Its penetration through the capillary wall and appearance in the extravascular tissue is the result of increased vascular permeability. An enhanced extravasation of Evans blue was found after ETOH instillation (within 1 min) that preceeded the development of macroscopic erosions. ASV and CSV increased further the vascular permeability in association with the formation of grossly visible injury. Vagotomy alone did not induce either ulcer formation or further increased in the vascular permeability.

As the time between the enhanced accumulation of EB and the ethanol treatment is very short (within 1–5 min) the failure of gastric motility can be probably excluded in the development of ETOH-induced gastric mucosal damage in chronically vagotomized rats.

As to these results one may conclude that: 1. Intact vagal nerve takes place in the regulation of the vascular permeability of the gastric mucosa. 2. ASV or CSV alone can not induce a detectable in the gastric mucosal vascular permeability. 3. Acute and chronic bilateral surgical vagotomy causes a more vulnerable state of the gastric mucosa to chemicals.

Acknowledgement

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SYNTHESIS AND GASTROPROTECTIVE ACTIVITY OF 4H-PYRIDO[1,2-*a*]PYRIMIDIN-4-ONES

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The cytoprotective effect of different types of 4*h*-pyrido[1,2-*a*]pyrimidin-4-one derivatives was investigated. Short synthesis of the investigated compounds was depicted. The gastroprotective effect was determined against acidified ethanol induced mucosal lesions in rats. The most effective compounds belong to unsaturated 4-oxo-4*h*-pyrido[1,2-*a*]pyrimidine-3-carboxamide derivatives, and the most active one contains a methyl group in position 6 and a cyclopentyl group on the nitrogen of the carboxamide group. Further pharmacological, biochemical and clinical studies may justify, that the representative of this type of compounds may be useful as profilactic agents against gastric damage caused by non-steroidal antiinflammatory agents.

Keywords: cytoprotective agents, pyrido[1,2-*a*]pyrimidine

In the last decade we analyzed the pharmacological activities of 4H-pyrido[1, 2-*a*]pyrimidin-4-one derivatives, first of all their analgetic, anti-inflammatory and antithrombotic activities [12]. The first table shows some representatives of this ring system together with some pharmacological activities. Compound 1, Rimazolium® [16, 17], was introduced into the market in Hungary and in some other European countries as an analgetic agent. The two other derivatives, the tetrahydro- and hexahydrocarboxamides, compounds 2 and 3, exhibit similar analgetic activities, but the tetrahydro derivative 2, CHINOIN-105 [1], shows strong antithrombotic activity, while hexahydropyridopyrimidinecarboxamine 3, CHINOIN-127 [15], exhibits significant anti-inflammatory properties, too.

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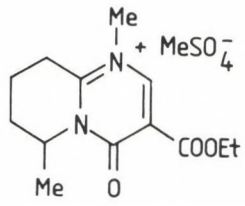
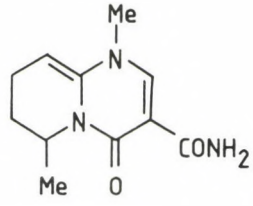
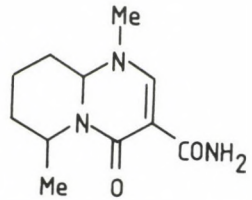
Table I

Some pharmacological data of pyrido[1,2-a]pyrimidin-4-ones (1-3) on rats by p.o. route







Acute toxicity ID_{50} mmoles/kg	4.42	2.26	1.66
Hot plate test ED_{50} mmoles/kg	0.62	0.61	0.52
Acetic acid induced writing test ED_{50} mmoles/kg	0.23	0.32	0.25
Antithrombotic activity IC_{50} mmoles/kg on Human PRP		0.045 ADP 0.170 collagene	
Carragenin rat-paw oedema ED_{50} mmoles/kg	0.83	0.33	0.20

PRP: Platelet-rich plasma

When the side-effects of CHINOIN-127 3, were investigated, it was surprising that this compound did not cause ulcer, but it exerted a protective effect against indomethacin-induced ulceration [13]. Similarly, the toxicity and gastrointestinal side effects of indomethacin also decreased in rats when it was combined with either Rimazolium® 1 [14, 24] or CHINOIN-105 2 [6].

Table II shows the effect of Rimazolium® 1, CHINOIN-105 2 and CHINOIN-127 3, cimetidine and atropine on intestinal ulceration induced by indomethacin in rats [4, 6, 13]. At these experiments the test compounds (Rimazolium®, CHINOIN-127, CHINOIN-105, cimetidine, atropine) were given four consecutive days either per os, or subcutaneously and indomethacin was administered orally on the second day treatment together with the test compounds. Seventy two hours later the rats were sacrificed and the total small intestine (from the pylorus to the caecum) was carefully removed, and investigated.

Table II shows that pyridopyrimidine derivatives exert a protection while cimetidine and atropine were ineffective in this model. Among pyridopyrimidine the hexahydrocarboxamide, CHINOIN-127 3 was the most effective.

The above and other experiments [5, 7–9, 23] suggested that gastroprotective effect is a general feature of 4H-pyrido[1,2-*a*]pyrimidin-4-ones, therefore we decided to investigate this activity more in details.

In the present work we give account of the investigation carried out conserning the gastro-protective effect of different 4-oxo-4H-pyrido[1,2-*a*]pyrimidine-3-carboxamides in a modified Robert's cytoprotective test, using acidified ethanol in rats.

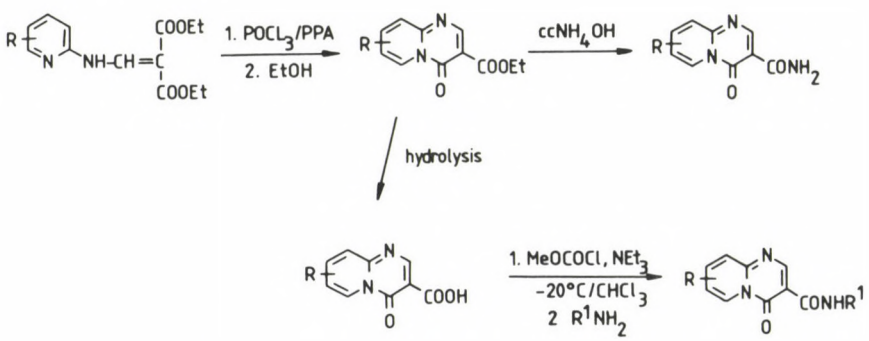


Fig. 1. Synthesis of 4-oxo-4H-pyrido[1,2-*a*]pyrimidine-3-carboxamides

Table II

The effect of Rimazolium®(1), CHINOIN-105 (2), CHINOIN-127 (3), cimetidine and atropine on intestinal ulceration induced by indomethacin on rats (n=10)

Compound	Dose (mg)	Ulcer incidence %	Lethality %	Ulcer index %	Intestinal length (cm)	Body weight (+)gain; (-)loss (g)	Appearence of ascites %
Control	0	0	0	0	118.8 ± 4.2	+19 ± 1.0	0
Indomethacin	15 p.o.	100	30	3.4 ± 0.34	80.7 ± 3.74	-18 ± 20	80
Indomethacin +Rimazolium®	15 p.o. 4*200 s.c.	56	10^{x}	1.7 ± 0.4	110.0 ± 4.2^{xx}	+2 ± 0.4	26^{x}
Indomethacin +CHINOIN-105	15 p.o. 4*100 s.c.	70	20	1.18 ± 0.3	101.0 ± 3.3	-20.0 ± 2.7	30^{x}
Indomethacin +CHINOIN-127	15 p.o. 4*50 s.c.	56	10^{x}	1.6 ± 0.2^{x}	101.0 ± 8^{x}	-6.7 ± 0.9	25^{x}
Indomethacin +Cimetidine	15 p.o. 4*50 s.c.	100	20	3.3 ± 0.4^{xxx}	76.0 ± 8	-16.5 ± 2.1	90
Indomethacin +Atropine	150 p.o. 4*1 s.c.	70	10^{x}	2.1 ± 0.4^{xxx}	96.0 ± 10	-8.0 ± 2.0	50

$^{x}p < 0.05$

$^{xx}p < 0.01$

^{xxx}Earlier this data was given wrongly in Ref.14.

The next two schemes depict the syntheses of the investigated 4-oxo-4H-pyrido[1,2-*a*]pyrimidine-3-carboxamides. The unsaturated derivatives were obtained either directly from an ester by amidation [19], or the ester was first hydrolyzed to carboxylic acid, then the carboxylic acid was converted to a mixed anhydride which was then reacted with amines [11] (Fig. 1). The unsaturated ester 4 was hydrogenated over palladium catalysts. The formed tetrahydroester 5 was reacted with aqueous ammonia, then the carboxamide 6 was converted to quaternary salt 7 by reacting with dimethyl sulfate. From the quaternary sulfate 1, 6, 7, 8-tetrahydrocarboxamide 2 was obtained by the treatment with an aqueous solution of sodium bicarbonate, while hexahydrocarboxamide 3 was prepared by reduction of quaternary salt with sodium borohydride [10, 18] (Fig. 2).

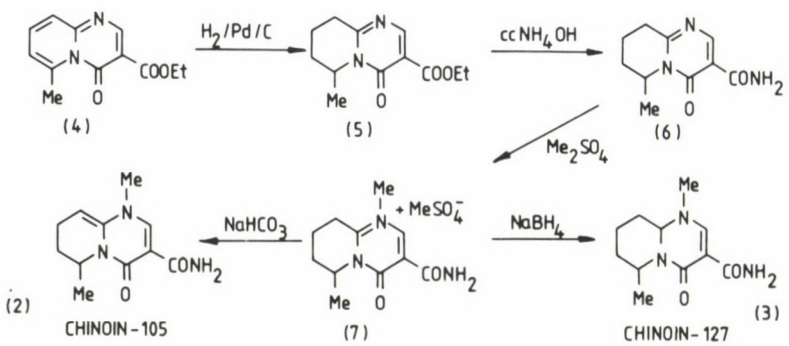


Fig. 2. Syntheses of CHINOIN-105 (2) and CHINOIN-127 (3)

The protocol of the cytoprotective test is depicted on Fig. 3. Robert's original test [22] used only absolute ethanol, but at these experiments we applied absolute ethanol which contained 2 per cent of concentrated hydrochloric acid, too. So, this way we obtained more characteristic gastric mucosal lesions. A half millilitre of acidified absolute ethanol was given orally to rats after a 24-h starvation. Test compound was given orally 40 min before the alcohol challenge, and the animals were sacrificed 2 hours later. The stomach was removed and the number and severity (0–4) of gastric damage were determined. ID_{50} values were also calculated.

First we investigated the effect of the saturation of the bicycle, therefore we examined the activity of hexahydro-, 1,6,7,8-tetrahydro-, and unsaturated 4-oxo-4*h*-pyrido[1,2-*a*]pyrimidine-3-carboxamides 3, 2 and 8. The 1,6,7,8-tetrahydropyridopyrimidinecarboxamide 2 was about four time less active, and the unsaturated derivative 8 proved to be about three times more active then the hexahydro derivative 3 (Fig. 4).

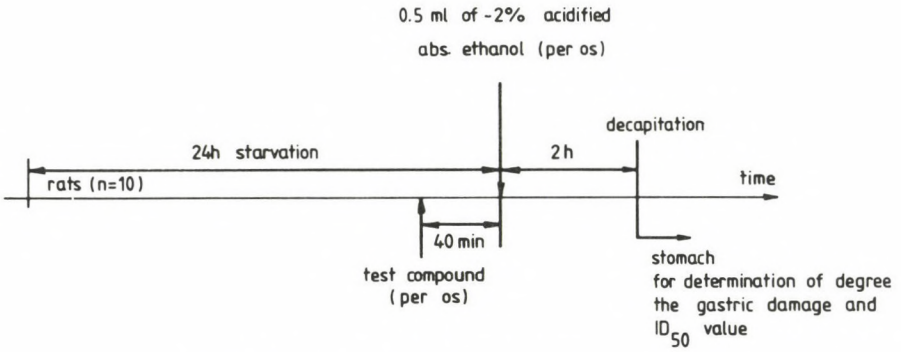


Fig. 3. Protocol of cytoprotective test in rats

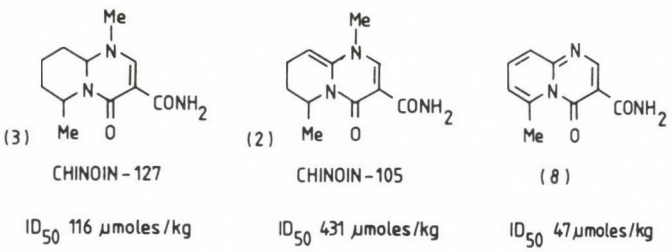


Fig. 4. The effect of the saturation of the bicycle on the cytoprotective activity

Earlier we experienced that the unsaturated 4-oxo-4H-pyrido[1,2-*a*]pyramidine-3-carboxylates 9 were sensitive to hydrolytic ring opening [18, 19] to give highly toxic 2-pyridylaminomethylenemalonic acid derivative 10 (Fig. 5). In our case, however, the substitution of the ester group by a carboxamide group, which has much weaker electron withdrawing effect, increased the stability of the bicyclic ring system.

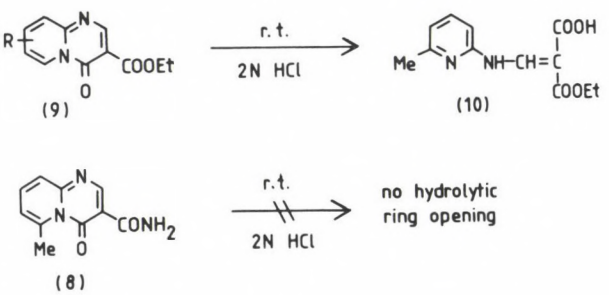


Fig. 5. Hydrolytic ring opening gives highly toxic 2-pyridylaminomethylenemalonic acid derivative

No hydrolytic products were obtained when the unsaturated carboxamide was stirred in an acidic or basic aqueous solution for 24 h at room temperature. However, the very poor solubility of the carboxamide 8 restricted its further pharmacological and biochemical screening. We assumed that the low solubility of this compound was the consequence of the formation on intermolecular hydrogen bonds involving the carboxamide group. The solubility could be increased when one of the hydrogens of the carboxamide group was substituted by an alkyl group (Fig. 6).

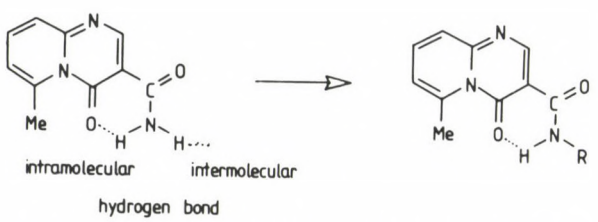


Fig. 6. The solubility could be increased when one of the hydrogens of the carboxamide group was substituted by an alkyl group

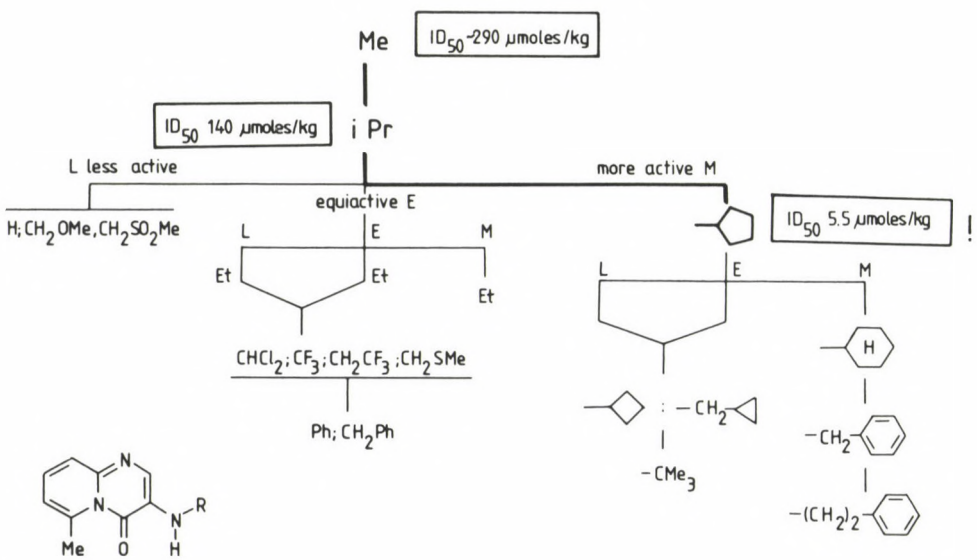
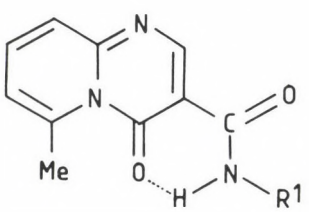


Fig. 7. Optimalization of substituent R of carboxamide moiety of 4H-pyrido[1,2-*a*]pyrimidin-4-one according to Topliss's "decision tree"

When the substituent R of carboxamide group was optimized we followed the "decision tree" of Topliss [25]. This "decision tree" suggests that after the *h*-methyl derivative, the isopropyl derivative has to be investigated (Fig. 7). Depending upon whether the isopropyl derivative is less active, equactive or more active compound containing methoxymethyl, ethyl or cyclopentyl substituent, respectively, are suggested to be investigated in the next step. As the isopropyl derivative was more active than the methyl derivative, the cyclopentyl derivative was selected for investigation. The cyclopentyl compound exhibited high activity. The activity decreased dramatically when cyclopentyl group was substituted by more lipophilic cyclohexyl, or benzyl groups. The n-propyl, cyclopropyl and terc-butyl derivatives were also less active than the cyclopentyl derivative (Table III).

Table III

Optimalization of group R^1 of carboxamide moiety

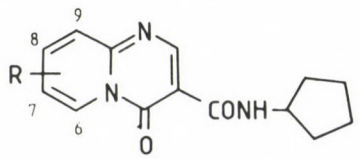


R^1	ID_{50} μmoles/kg	π	δ•	E_S
Me	~290	0.50	0.00	0.00
iPr	140	1.53	-0.19	-0.66
	5.5	2.14	-0.20	-0.51
	~210	2.51	-0.15	-0.79
nPr	37	1.55	-0.12	0.56
	90			
tBu	99	1.98	-0.30	-1.54
CH_2Ph	>400	2.63	-0.22	-0.38
H	47	0.0	0.49	1.24

Finally in the N-cyclopentyl series we changed the positon of the ring methyl group, and we also investigated the desmethyl analogue (Table IV).

Table IV

Optimalization of the position of methyl group
(R = Me)



R	ID_{50} μmoles/kg
H	50
9-Me	66
8-Me	294
7-Me	>370
6-Me	5.5

The 7-methyl derivative was practically ineffective, while the desmethyl and 9-methyl derivative exhibited nearly similar activities. The 6-methyl derivative was about 10 times more potent then the previous two compounds.

Further biochemical, pharmacological investigations of this type of compounds may prove whether that one of their representatives is worth applying as a cytoprotective substance [20, 21] to prevent the ulcerogenic effects [2] of non-steroidal anti-inflammatory agents [4]. Recently the cytoprotective misoprostol was successfully introduced into the therapy with similar indications [26].

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THE MECHANISM OF CYTOPROTECTIVE EFFECT OF CHINOIN-127

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According earlier investigations nitrogen bridgehead compounds make a representative group of non-prostaglandin type gastroprotective agents. One member of this group is CHINOIN-127 (1,6-dimethyl-4-oxo-1, 6, 7, 8, 9, 9a-hexahydro-4H-pyrido-(1, 2a)-pyrimidine-3-carbox-amide). CHINOIN-127 is a potent non-narcotic analgesic and anti-inflammatory agent and has a remarkable protective effect on indomethacin induced ulcer (ED_{50}=25 mg/kg p.o.) and on acidified ethanol induced ulcer (ED_{50}=26 mg/kg p.o.). In this study we examined the mechanism of action of cytoprotective effect of this drug and we made a comparison between the cytoprotective effect of 20% ethanol and 25 mg/kg CHINOIN-127.

In the gastric mucosa of control rats we observed a balance between TxA_2 and PGI_2 (PGI_2/TxA_2=3.8) and between the cytoprotective prostaglandins (PGI_2 and PGE_2) and ulcerogen eicosanoids (TxA_2 and leukotrienes) (PGI_2+PGE_2/TxA_2+LTs=3.9). 100% ethanol treatment causes disintegration of this balance, shifting the synthesis towards the ulcerogen eicosanoids production. CHINOIN-127 and 20% ethanol pretreatment improves the deranged balance between cytoprotective prostaglandins and ulcerogen eicosanoids. Our results demonstrate that CHINOIN-127 and 20% ethanol have a similar mechanism of cytoprotective action on ethanol induced ulcer in rats.

Keywords: peptic ulcer, cytoprotection, CHINOIN-127, prostacyclin, thromboxane

The term "cytoprotection" is used in "gastric mucosa" circles to refer to the property of exogenous prostanoids of the A, E and F types, given at non-antisecretory doses, to prevent macroscopic gastric mucosal injury produced by alcohol, HCl, NaOH, hypertonic NaCl and thermal injury [1]. The term was initially coined by Andre Robert in collaboration with Eugene Jacobson in the context of protection of the distal ileum by exogenous prostanoids against injury produced by orally administered indomethacin. In this situation, the protective effect of prostanoids is independent of their antisecretory effect. Nowadays a number of investigators attempt to define the mechanisms of cytoprotection such as increasing mucosal blood flow, stimulated gastric mucus and biocarbonate secretion and

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stimulated epithelial proliferation, but no unified theory emerged. Lacy and Ito [2] studied cytoprotection histologically, demonstrating that surface epithelial cells were not protected, however, the depth of mucosal injury is clearly reduced. Although surface epithelial cells are not "protected", cytoprotection as a concept continues to be studied and referred to as "direct cytoprotection".

In 1980, Chaundry and Robert [3] reported that low doses (which themselves were non-injurious) of known damaging agents, termed "mild irritants", when topically applied to rat gastric mucosa could significantly inhibit or totally ablate the production of experimental lesions produced by higher concentrations of the same agents or even different agents. They called this intriguing phenomenon "adaptive cytoprotection". Subsequent work by a number of other investigators has confirmed the existence of such a protective response in a variety of experimental models, including rat [4, 5], dog [6] and human [7]. Although the precise mechanism responsible for eliciting adaptive cytoprotection remains unknown, a potential role for endogenous prostaglandins (PGs) has been suggested.

Robert described cytoprotection by sodium salicylate against alcohol and ASA-induced mucosal lesion [8]. Szabo et al. [9] reported that sulphydryl compounds are cytoprotective. Atropine, cimetidine [10, 11, 12] and pirenzepine, a selective anticholinergic agent [13] were reported to exert cytoprotective action. Similar cytoprotective action was produced by carbenoxolon [14].

Our results suggest that some members of pyrido-pyrimidines inhibit gastric mucosal damage induced by PG synthesis inhibitors [15, 16]. Pyrido-pyrimidines were also effective against alcohol-induced injury [17].

In an earlier study we examined the mechanism of gastroprotective effect of CHINOIN-127 on indomethacin induced ulcer in rats. We pointed out, that CHINOIN-127 pretreatment restores the changed TxA_2/PGI_2 ratio and the changed activity of TxA_2 and PGI_2 synthetases induced by indomethacin [16, 17].

In this study we evaluated the effects of 20% ethanol, CHINOIN-127 and 100% ethanol on endogenous prostanoid and leukotriene levels in rat gastric mucosa, and we also examined the gastroprotective effect of CHINOIN-127 and 20% ethanol.

Materials and methods

Wistar rats, weighing 180 g in average, were used in all studies. The animals were fasted for 36 h in wide mesh wire bottomed cages to prevent ingestion of hair and faeces, but they were allowed free access to water. On the day of experimentation, all animals were randomly assigned to one of the groups. Initially their stomach were gavaged with 1 ml of 20% ethanol (vol/vol) administered via an oroesophageal tube. Fifteen minutes later, some of these animals were sacrified and their stomach were removed to determine the effect on mucosal prostanoid level. All remaining animals, which received a 1 ml oral bolus of necrotizing agent of 100% ethanol, were sacrified 1 h later, and the perturbations in tissue prostanoid and leukotriene levels were determined again. In further experiments, additional groups of animals were studied in similar fashion with the only exception that they received pretreatment

with CHINOIN-127 (25 mg/kg given orally), 40 min prior to the administration of 100% ethanol. This dose of CHINOIN-127 was chosen because it has previously been shown to inhibit the ethanol induced gastric lesions by about 50%. In a separation experiment we determined the mucosal prostanoid and leukotriene levels 1, 2, 4 and 6 h after 100% ethanol treatment. Each experimental group consisted of 10 and 15 animals.

The stomach mucosal layers, were separated from the muscle layers by gentle scraping with a thin glass slide. The mucosal layers were weighed, homogenized in a Potter homogenizer at 4 °C and diluted to a final concentration of 25 mg wet weight per ml. 10 mM phosphate buffer containing 100 μM indomethacin was used at pH 6.5 for the homogenization and for the assay. From this homogenate, samples of 0.5 ml were drawn and placed rapidly on dry ice. These samples served for the determination of the eicosanoid content of the mucosal layer.

The samples were stored in a deep freezer at -40°C. 6-oxo-PGF_1 (the stable hydration produce of PGI_2) and TxB_2 (the stable metabolite of TxA_2), PGE_2 and leukotrienes were subsequently measured by radioimmunoassay. PGE_2 and leukotrienes radioimmunoassay kits were purchased from Amersham, and 6-oxo-PGF_1 and TxB_2 RIA kits were obtained from the Institute of Isotopes of the Hungarian Academy of Sciences.

Results

First of all, we determined the eicosanoid level (thromboxane, PGs and leukotrienes) in the control mucosa (Fig. 1). The figure demonstrates well that in the control mucosa the protective prostaglandins are predominate considering ulcerogen eicosanoids (thromboxane, leukotrienes). The balance between the protective prostaglandins and ulcerogen eicosanoids is shifted towards the direction of protective prostaglandins (Fig. 1).

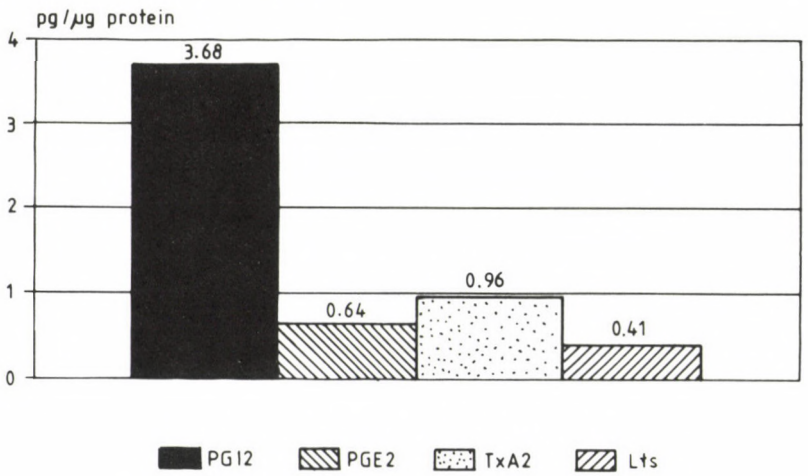

Fig. 1. Eicosanoids levels in control gastric mucosa

The ulcer was induced by 1 ml 100% ethanol treatment. The animals were sacrificed 1, 2, 4 and 6 h after ethanol treatment, and the mucosal level of 6-keto-

PGF_1, TxB_2, PGE_2 and leukotrienes were determined. The mucosal level of TxB_2 shows a significant increase 1 h after ethanol treatment. After this time the TxB_2 content decreases, but not significantly (Fig. 2).

One hour after treatment the mucosal level of 6-keto-PGF_1 shows a slight increase, and after this time this level decreases continously. 6 h after ethanol treatment the mucosal level of 6-keto-PGF_1 is significantly lower than that in control mucosa (Fig. 2).

Since the level of TxB_2 increases more rapidly than that of 6-keto-PGF_1 mucosa (6-keto-PGF_1/TxB_2 = 3.8) shifts towards the direction of ulcerogen thromboxane 1 h after ethanol treatment (6-keto-PGF_1/TxB_2 = 2.83), and this overturned balance was maintained during the time of the study.

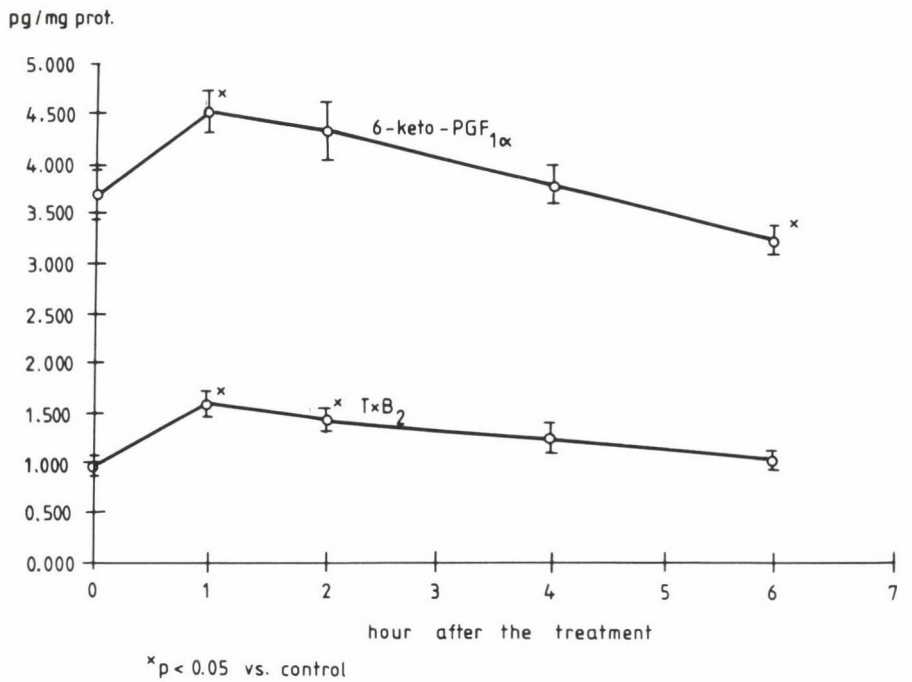

Fig. 2. Concentration of 6-keto-PGF_{1alfa} and TxB_2 in gastric mucosa following oral administration of 100% ethanol

Figure 3 shows, that the mucosal level of leukotrienes shows a dramatic increase 1 h after ethanol treatment. After this time it decreases, but 6 h after treatment the mucosal level of leukotrienes is significantly higher than in the control mucosa.

The mucosa level of PGE_2 shows a dramatic rise 1 h after treatment. After this time it shows a dramatic drop. 6 h after the treatment the mucosal level of PGE_2 is

significantly lower than in the control mucosa. These results demonstrate that 1 h after treatment the balance between gastroprotective prostaglandins (PGI_2 and PGE_2) and ulcerogen eicosanoids (leukotrienes and thromboxane) is shifted towards the direction of ulcerogen eicosanoids. This perturbed balance was maintained during the time of the examination.

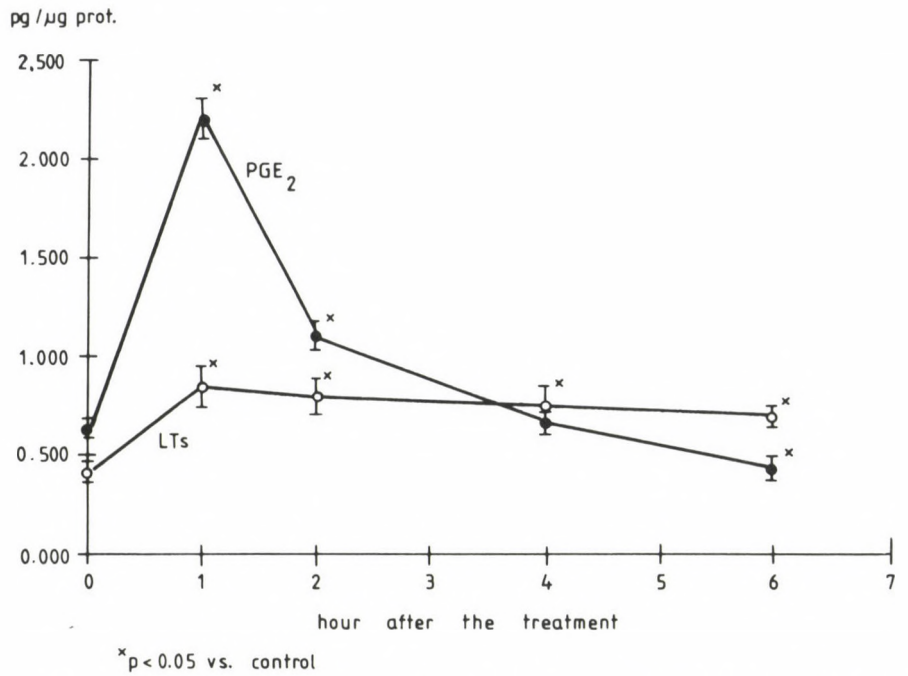

Fig. 3. Concentration of PGE_2 and LTs in gastric mucosa following administration of 100% ethanol

Figures 2 and 3 show that great part of the changes in arachidonic acid metabolism takes place during the first hour after treatment. For this reason we determined the mucosal level of eicosanoids 1 h after ethanol treatment only.

20% ethanol and 25 mg/kg CHINOIN-127 do not influence the eicosanoid level of gastric mucosa significantly (Figs 4 and 5).

Figure 4 shows, that 1 h after the administration of the strong irritant the mucosal level of thromboxane and leukotrienes are lower but the difference is not significant in the case of mild irritant pretreatment compared to that without pretreatment. The protective PG (PGI_2 and PGE_2) levels show significant changes 1 h after strong irritant treatment, the mucosal level of these prostanoids are significantly higher in the group with mild irritant pretreatment than in the group without pretreatment.

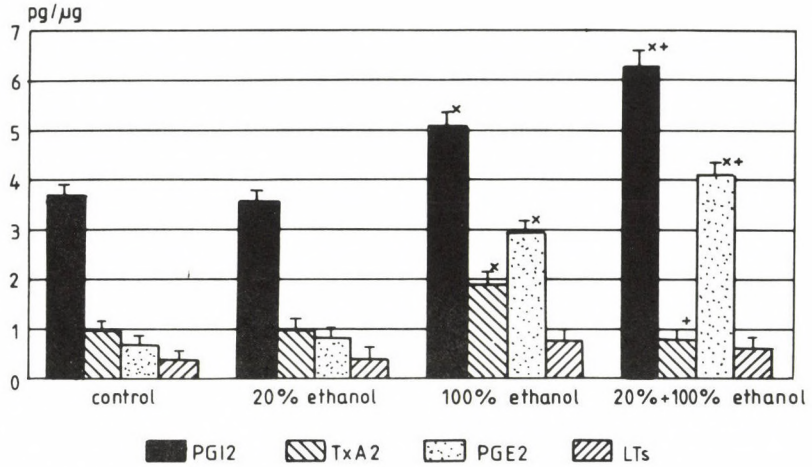

Fig. 4. Adaptive cytoprotection at 20% + 100% ethanol

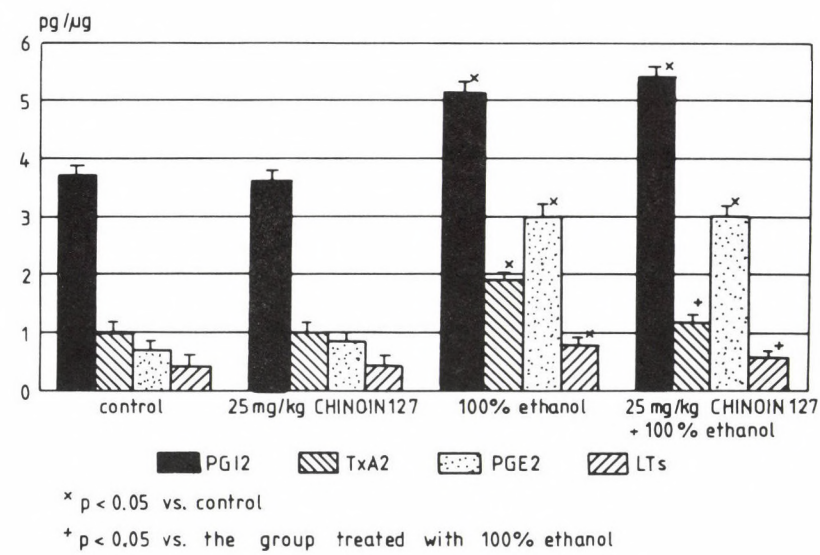

Fig. 5. Cytoprotective effect of CH-127

Figure 5 demonstrates the changes in mucosal eicosanoids levels 1 hour after ethanol treatment in the case of CHINOIN-127 pretreatment, and without

pretreatment. There are no significant changes in the mucosal levels of protective PGs between the both cases. CHINOIN-127 pretreatment influences significantly the mucosal leukotrienes and thromboxane levels. One hour after 100% ethanol treatment the mucosal level of these eicosanoids are significantly lower in the group with CHINOIN-127 pretreatment than in the group without pretreatment. These results suggest, that CHINOIN-127 pretreatment prevents the increase of mucosal ulcerogen eicosanoid contents induced by 100% ethanol.

Discussion

Although the precise mechanisms responsible for eliciting cytoprotection remain unknown, a potential role of endogenous prostaglandins has been suggested. Two lines of evidence support this hypothesis. This first relates to the increased tissue levels of one or more endogenous prostaglandins after pretreatment with certain mild irritants [4, 18], while the second involves the reversal of this mild irritant that induces protective response either completely or partially by the cyclo-oxygenase inhibitor, indomethacin [3, 4, 5].

In contrast with Konturek and Robert [4, 18], but parallel with Miller [19] we have found, that 20% ethanol treatment did not cause significant changes in the mucosal eicosanoid levels. We pointed out, that the amount of PGs having protective activity (PGI_2 and PGE_2) shows significant changes, 1 h after strong irritant treatment the mucosal concentration of these prostanoids are significantly higher in the group with mild irritant pretreatment than in the group without pretreatment. This effect of 20% ethanol possibly can be partially prevented by indomethacin. This result may support, the second evidence of the hypothesis above.

In the gastric mucosa of control rats we observed a balance between TxA_2 and PGI_2 (PGI_2/TxA_2 = 3.8) and between the cytoprotective prostaglandins (PGI_2 and PGE_2) and ulcerogen eicosanoids (TxA_2 and leukotrienes) (PGI_2+PGE_2/TxA_2+LTs = 3.9). Ethanol treatment causes disintegration of this balance, shifting the synthesis towards the production of ulcerogen eicosanoids. CHINOIN-127 and 20% ethanol pretreatment restore the perturbed balance between cytoprotective prostaglandins and ulcerogen eicosanoids (Table I).

Table I

Changes of the ratio of protective and ulcerogenic factors in rat mucosa after treating with 20% ethanol of CHINOIN-127 followed by 100% ethanol

	Control	1 h after ethanol treatment	20% ethanol pretreatment	CHINOIN-127 pretreatment
PGI_2/TxA_2	3.8	2.83	7.7	4.6
PGI_2+PGE_2	3.9	3.00	9.2	5.1
TxA_2+LTs				

Table I demonstrates, that CHINOIN-127 and 20% ethanol have a similar mechanism of cytoprotective action on ethanol induced ulcer in rats. The cytoprotective action of CHINOIN-127 and 20% ethanol is apparently similar, both compounds improve the perturbed balance between cytoprotective prostaglandins and ulcerogen eicosanoids, but they do it in a different way. CHINOIN-127 prevents the increase of mucosal thromboxane and leukotriene content, still 20% ethanol strengthens the endogen cytoprotective mechanism of gastric mucosa, since the 20% ethanol pretreatment increases the mucosal endogen gastroprotective prostanoid level in comparison to giving 100% ethanol alone.

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ARE ALL "CYTOPROTECTIVE" DRUGS GASTROPROTECTIVE?

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The effect of three – structurally different – groups of compounds was compared against gastric mucosal damages induced by ethanol or prostaglandin (PG) synthesis inhibitors, as well as against carrageenan-induced inflammation. All the compounds studied – SH-compounds (cysteamine, 2,3-dimercaptosuccinic acid, D,L-penicillamine), SH-blocking N-ethylmaleimide (NEM) and morphine-exerted dose-dependent inhibition on carrageenan edema test and against ethanol-induced gastric damage. Mucosal lesions induced by PG synthesis inhibitors (indomethacin 20 mg/kg, naproxen 75 mg/kg, piroxicam 60 mg/kg) were inhibited by drugs studied when the compounds were given before the damaging agents. However, when the drugs were injected 1 h after the inhibitors of PG synthesis opposite actions were observed; SH-compounds exerted protective, while NEM (2 mg/kg p.o.) and morphine (5 mg/kg p.o.) aggravating action. The results suggest that: 1. SH-compounds might have therapeutic importance in the treatment of gastric damage induced by prostaglandin synthesis inhibitors. 2. Reconsideration of the use of the term "cytoprotection" is necessary, since "cytoprotective" agents may aggravate mucosal damage in other ulcer model.

Keywords: sulfhydryls, N-ethylmaleimide, morphine, gastroprotection

The definition of the term "cytoprotection" has undergone changes in the last 10 years. According to the original definition of Robert [11] several prostaglandins (PG) protect the gastric mucosa against absolute ethanol, strong acid and base, hypertonic solution and boiling water. 'This property is called "gastric cytoprotection" ... Cytoprotection by prostaglandins is unrelated to the inhibition of gastric acid secretion.' The term "cytoprotection" has been criticized since Lacy and Ito [9] reported that only the deep hemorrhagic necrosis is prevented while the surface cell injury is not decreased by prostaglandins, that is not all the mucosal cells are protected. Recently, Robert et al. [12] emphasized in their response to an editorial in Gastroenterology [13] that since most of the gastric cells are protected (about 80%) to suggest that the term of cytoprotection should not be used is unwarranted.

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Similarly to prostaglandins sulfhydryls [14], sulfhydryl blocking agents [5, 16] and morphine [4] were reported to inhibit the ethanol-induced gastric mucosal necrosis. However, there are less information about their effect on mucosal damage induced by PG synthesis inhibitors. In the present experimental series we have studied whether these structurally different agents exert mucosal protection – similarly to prostaglandins – also against gastric mucosal damage induced by PG synthesis inhibitors or their gastroprotective profile differs from the prostaglandin-type gastroprotection.

Methods

1. Carrageenan edema test

Sprague-Dawley CFY rats of both sexes (130–150 g) were used. The method described by Winter et al. [17] was applied. 0.1 ml of 1% carrageenan was injected intraplantarly to the rats. The volume of edema was measured by means of plethysmograph 3 h after carrageenan injection. The test compounds were given either orally (p.o.) or intraperitoneally (i.p.) 60 or 30 min respectively before carrageenan injection.

2. Gastric mucosal damage

Sprague-Dawley CFY rats of both sexes (160–180 g) were used. The rats were fasted for 24 h before the experiment, but they were allowed free access to water. They were kept in cages with a metal grid to avoid coprophagy. The degree of gastric mucosal damage was estimated using a binocular magnifier (2X) by the observer unaware of the treatment the rats received. The lesions were scored as follows: 1: petechiae, erosions less than 1 mm; 2: erosions of 1–2 mm; 3: erosions of 3–4 mm; 4: erosions of 5–6 mm. Ulcer index was expressed as a sum of partial scores in each group of rats divided by the number of animals.

a. Acidified ethanol-induced gastric damage.

After a 24 h starvation the rats were given 0.5 ml of acidified ethanol (98% ethanol in 200 mmol/l HCl) by gavage and killed 2 h later. The stomachs were removed and examined for mucosal lesions described above. The test compounds were given orally using a glass gastric probe 40 min prior to ethanol administration.

b. Gastric damage induced by different inhibitors of PG synthesis

After a 24 h starvation the rats were given 20 mg/kg of indomethacin, 75 mg/kg of naproxen or 60 mg/kg piroxicam orally and were killed 4 h later. The stomachs were removed and examined for lesions described above. The test compounds were injected orally 1 h either before or after the administration of damaging agents.

Drugs used: cysteamine (Sigma, USA), 2,3-dimercaptosuccinic acid (Sigma, USA), D,L-penicillamine (Sigma, USA), indomethacin (Chinoin, Hungary), morphine HCl (Alkaloida, Hungary), N-ethylmaleimide (Sigma, USA), naproxen (Alkaloida, Hungary) piroxicam (Sigma, USA).

Statistical analysis: The data are presented as the means ± SEM. The non-parametric Mann-Whitney U test was used to evaluate the statistical significance.

Results

1. The effect of cysteamine, 2,3-dimercaptosuccinic acid, D,L-penicillamine, N-ethylmaleimide and morphine on gastric lesions induced by acidified ethanol and on acute inflammation induced by carrageenan in the rat (Table I).

Table I

The effect of cysteamine, 2,3-dimercaptosuccinic acid, DL-penicillamine, N-ethylmaleimide (NEM) and morphine on acidified ethanol-induced lesions and carrageenan-induced edema (rat, n = 7)

Compound	ID_{50} p.o. acidified ethanol-lesions		ID_{30} carrageenan edema	
	μmoles/kg	mg/kg	μmoles/kg	mg/kg
Cysteamine	163.0 (133.6 – 195.6)	20.0 (16.3 – 24.0)	176.9 (140.8 – 220)	21.0 p.o. (16.0 – 24.8)
2,3-dimercapto-succinic acid	1043.0 (869 – 1251)	190.0 (158 – 228)	131.8 (106.5 – 161.1)	24.0 i.p. (19.8 – 29.04)
D,L-penicill-amine	1228.0 (1006 – 1498)	180.0 (147 – 219)	863.2 (707.3 – 1052.8)	101.0 p.o. (82.7 – 123.2)
NEM	4.4 (3.5 – 5.4)	0.55 (0.43 – 0.67)	2.0 (1.5 – 2.6)	0.25 p.o. (0.18 – 0.32)
Morphine	2.9 (2.4 – 3.1)	0.1 (0.08 – 0.12)	0.26 (0.22 – 0.37)	1.1 i.p. (0.83 – 1.2)

The ID_{50} values of sulfhydryls, sulfhydryl blocking N-ethylmaleimide (NEM) and morphine against acidified ethanol-induced gastric damage was compared with their antiphlogistic action on carrageenan-induced edema test. As the data show all the compounds studied exerted gastroprotective and anti-inflammatory effect. NEM and especially 2,3-dimercaptosuccinic acid were more effective against inflammation than against mucosal damage.

2. The effect of sulfhydryls, N-ethylmaleimide and morphine on indomethacin-induced gastric damage given either before or after indomethacin (Table II).

All the test compounds decreased the indomethacin-induced gastric lesions, however, while the sulfhydryls exerted dose-dependent inhibition, the protective effect of morphine and NEM was less pronounced and not dose-dependent. Even more marked difference in the effectiveness of the drugs was observed when they were given after indomethacin; while the sulfhydryls exerted protective, NEM and morphine had an aggravating action on indomethacin-induced gastric damage.

Table II

The effect of cysteamine, 2,3-dimercaptosuccinic acid, D,L-penicillamine, N-ethylmaleimide (NEM) and morphine on indomethacin-induced gastric mucosal damage in the rat (n = 7)

Compound	Dose mg/kg p.o.	% increase (+) decrease (−) of gastric lesions given before indomethacin (20 mg/kg p.o.)	% increase (+) decrease (−) of gastric lesions given after indomethacin (20 mg/kg p.o.)
Cysteamine	25	−62 (± 11)	−68 (± 9)
	50	−83 (± 21)	−87 (± 13)
2,3-dimercapto-succinic acid	100	−68 (± 13)	−64 (± 8)
	200	−85 (± 17)	−88 (± 15)
D,L-penicill-amine	100	−86 (± 14)	−90 (± 17)
	150	−91 (± 21)	−92 (± 18)
NEM	2	−59 (± 8)	+92
	4	−48 (± 7)	
Morphine	2.5	−59 (± 10)	+67 (± 9)
	5.0	−49 (± 8)	+72 (± 14)

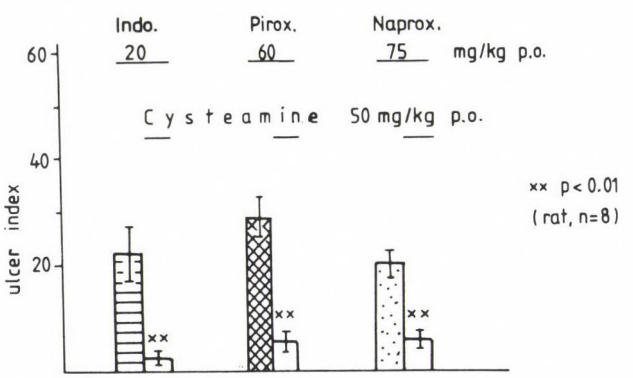

Fig. 1. The effect of cysteamine (50 mg/kg p.o.) on gastric mucosal damage induced by indomethacin (20 mg/kg) piroxicam (60 mg/kg p.o.) and naproxen (75 mg/kg p.o.) given one hour after the damaging substances. indo.: indomethacin, pirox.: piroxicam, naprox.: naproxen. Mann-Whitney U test was used for statistical comparison

3. The effect of cysteamine on piroxicam and naproxen-induced gastric mucosal damage (Fig. 1).

Cysteamine in the dose of 50 mg/kg p.o. exerted a pronounced inhibition on piroxicam- and naproxen-induced gastric lesions given 1 h after the administration of the damaging agents. For comparison the protective effect of cysteamine on indomethacin-induced damage is also shown.

4. The effect of D,L-penicillamine and 2,3-dimercaptosuccinic acid on indomethacin- and piroxicam-induced gastric mucosal damage (Fig. 2).

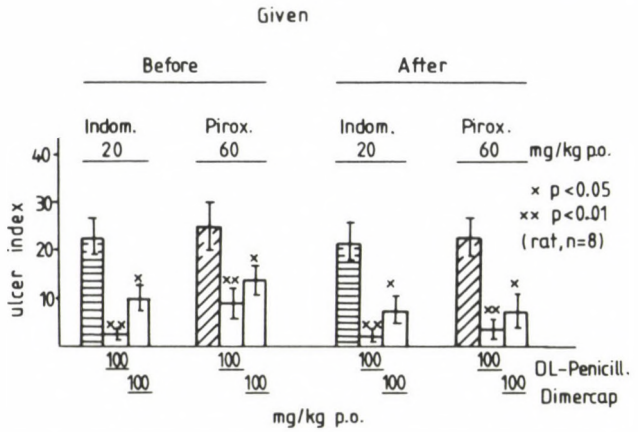

Fig. 2. The effect of D,L-penicillamine (100 mg/kg p.o.) and 2,3-dimercaptosuccinic acid (100 mg/kg p.o.) on mucosal damage induced by indomethacin (20 mg/kg p.o.) or piroxicam (60 mg/kg p.o.) given one hour either before or after the damaging substances. indo.: indomethacin, pirox.: piroxicam, D,L-penicill.: D,L-penicillamine, dimercap.: 2,3-dimercaptosuccinic acid.
Mann-Whitney U test was used for statistical comparison

Both of the sulfhydryls – in the dose of 100 mg/kg p.o. – exerted similar inhibitory action on piroxicam and indomethacin-induced mucosal lesions given both before and after the PG synthesis inhibitors.

5. The effect of N-ethylmaleimide on indomethacin, piroxicam, naproxen and phenylbutazone-induced gastric damage given after the ulcerogens (Fig. 3).

NEM – in the dose of 2 mg/kg – aggravated the lesions produced by different PG synthesis inhibitors, especially the phenylbutazone-induced mucosal damage was aggravated by NEM.

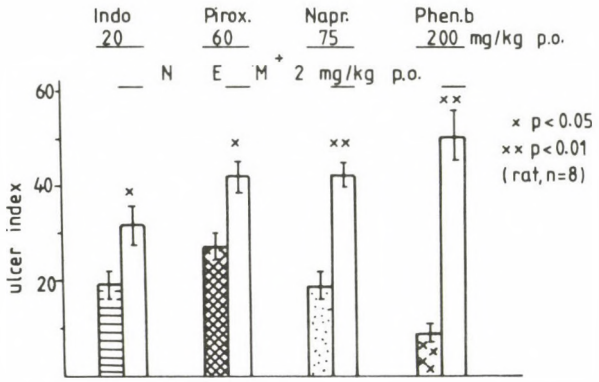

Fig. 3. The effect of NEM on indomethacin (20 mg/kg p.o.), piroxicam (60 mg/kg p.o.), naproxen (75 mg/kg p.o.) and phenylbutazone (200 mg/kg p.o.) induced gastric damage given one hour after the damaging substances.
indo.: indomethacin, pirox.: piroxicam, napr.: naproxen, phen.b.: phenylbutazone.
Mann-Whitney U test was used for statistical comparison

Discussion

The protective action of sulfhydryls was described first by Szabó et al. [14]. Recently, it was found that not only sulfhydryls but the sulfhydryl blocking N-ethylmaleimide exerted also protective action given orally in low doses [5, 16]. Endogenous sulfhydryls are supposed to mediate – at least partly – the gastroprotection induced by prostaglandins [14] and morphine [4]. On the other hand, according to our previous data prostaglandins are supposed to play role in the gastroprotective action of morphine and NEM [4, 7] but prostaglandins have less importance in the mucosal protective action of cysteamine [8]. We have wondered and analyzed whether these substances show similar gastroprotective profile using different ulcer models or there are qualitative differences in their mucosal protective spectrum.

Comparing the effect of drugs studied against acidified ethanol the rank order of gastroprotective potency was: morphine > NEM >cysteamine > D,L-penicillamine > 2,3-dimercaptosuccinic acid. Cysteamine was shown to inhibit the enhanced vascular permeability of gastric mucosa in the early phase of ethanol-induced gastric damage [15] and recently we demonstrated that both NEM and morphine decreased the enhanced vascular permeability of gastric mucosa due to ethanol [4, 6]. Since enhanced vascular permeability and vasodilatation are common symptoms both in acute inflammation and mucosal damage due to ethanol we examined the effect of drugs against acute inflammation induced by carrageenan. All

the compounds studied showed antiinflammatory activity; the rank order of potency was: NEM > morphine > cysteamine > 2,3-dimercaptosuccinic acid > D,L-penicillamine. These data suggest that the antiinflammatory property of sulfhydryls, NEM and morphine might be involved in their gastroprotective effect or the vasoprotection induced by these agents might contribute to their antiinflammatory activity. All the three groups of compounds inhibited the mucosal lesions induced by indomethacin (given 1 h before challenge), though morphine and NEM exerted less pronounced inhibition against indomethacin than against ethanol-induced damage.

The difference became even more evident when the test substances were injected one hour after indomethacin; morphine – as we reported previously [3] – and NEM aggravated, while the sulfhydryls inhibited the lesions formation.

At this stage of experiments the following questions were raised: 1. Can sulfhydryls reduce the mucosal damage induced by other PG-synthesis inhibitors?

2. Is the aggravating action of NEM and morphine restricted to indomethacin-induced gastric damage, or do they aggravate the lesions due to other PG-synthesis inhibitors?

As our data suggest the sulfhydryls inhibited the lesions due to piroxicam and naproxen, too. On the other hand, NEM exerted a very pronounced aggravating action also on piroxicam-, naproxen- and phenylbutazone-induced gastric mucosal lesions. (The experiments on the effect of morphine on PG synthesis inhibitors – given 1 h after the PG-synthesis inhibitors – is in progress.)

On the basis of these results it can be concluded:

a.) since SH compounds were effective against mucosal damage induced by PG-synthesis inhibitors given both before and after the damaging agents, they might have therapeutic importance in the treatment of gastric mucosal lesions caused by PG-synthesis inhibitors.

b.) Studying the mucosal protective profile of a potential anti-ulcer agent it can be useful to determine its activity against PG-synthesis inhibitor-induced gastric damage – given 1 h after the challenge.

How can the dual – protective and aggravating – action of morphine and NEM be explained. Previously it was raised that enhanced leukotriene formation might play role in the aggravating action of morphine and NEM [4, 6, 10]. However, FPL 55712 – a leukotriene antagonist – failed to prevent the aggravating action of morphine and NEM [6], though Parmar et al. [10] reported that FPL 55712 prevented the aggravating action of morphine on indomethacin-induced mucosal lesions. On the basis of the present data the question is raised again when the term "cytoprotection" can be used. Namely, both sulfhydryls, sulfhydryl blocking substance and morphine can be considered as cytoprotective substances, because they were effective against acidified ethanol induced lesions and as we previously demonstrated they failed to affect the gastric acid secretion in the protective doses [7]. However,

given NEM and morphine after PG synthesis inhibitors severe potentiation of mucosal damage was observed. Another example for the dual action of a "cytoprotective" drug: 2-cyano-3(ethylthio-3-methylthio)-2-propeonic acid methyl ester, a partial proton pump inhibitor with cytoprotective activity aggravated the indomethacin-induced gastric damage [1].

Since it seems to be a paradox that a cytoprotective substance aggravates mucosal lesions, the following, preliminary suggestion is raised for the classification of cytoprotective agents.

1 Agents, with *general* cytoprotective property are effective practically against all types of experimental ulcer models (e.g. prostaglandins).

2 Agents, with *partial* "cytoprotective" property are effective in several models, but not in all (e.g. morphine, NEM).

3 Agents with *single* "cytoprotective" property are effective against one type of mucosal damage.

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THE ANTINEOPLASTIC DRUGS DOSE DEPENDENTLY INHIBIT THE HEALING OF SUBACUTE GASTRIC ULCER PRODUCED BY PUNCHING IN RATS

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The aim of present study was to investigate the effects of some antineoplastic drugs on the healing process of subacute gastric ulcer, produced by punching, in rats. We determined the doses which inhibit the healing rate by 50%. Our results confirm the general observation that present applied anticancer drugs cannot distinguish between normal and tumor cell poliferation. The ID_{50} values of tested drugs strongly correlate with human doses.

Keywords: healing, subacute ulcer, antineoplastic drugs

In our laboratory a simple new method has been development for producing subacute gastric ulcer (by punching) in rats, and it has been combined with a novel method for the quantitative evaluation of the healing process. We have studied the effects of several nonsteroidal anti-inflammatory drugs and antiulcer agents [1].

The idea for studying antineoplastic drugs with new methods is based on two general observations.

1. proliferating cells are much more sensitive to antineoplastic drugs than nonproliferating cells,
2. the most common side effect of antineoplastic drugs is located in the gastrointestinal tract [2, 4].

The aim of present study was to investigate the effects of some anticancer drugs on the healing process of subacute gastric ulcer.

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Materials and methods

The following drugs were used in the experiments:

Vincristine (Richter, Budapest)
Vinblastine (Richter, Budapest)
Navelbine (Richter, Budapest)
Adriblastina /adriamycin/ (Farmitalia, Milano)
Rubomycin (Medexport, Moscow)
Phthoruracil /5-fluoruracil/ (Medexport, Moscow)
Cyclophosphamide (Germed, Dresden)
Methotrexate (La Chema, Brno)
Platidiam /Cisplatin/ (La Chema, Brno)
Prednisolon (Richter, Budapest)

Induction of subacute gastric ulcers in rats

Female, RG-Wistar rats, weighing 120–150 g, were fasted for 24 h in cages with wire sieves at the bottom. Water was provided *ad libitum*. Rats were anaesthetized by ether and a polyethylene catheter inserted in the stomach orally. The catheter included a fine steel wire with a needle tip (1.2 mm diameter) at the lower end. After the canula had reached the gastric wall, the upper end of the steel wire was pressed in a definite manner, so as to puncture the gastric wall. Each rat was held in the same position during the intervention. Therefore, the punctures occurred at nearly the same region of the glandular part of the stomach.

Substances under study were administered subcutaneously or intraperitoneally, 30 min and 24 h after the puncture. Free access to food and water was provided from 2 h after the intervention up to the end of the experiment. Each group of animals consisted of 8–15 rats.

The statistical analysis were carried out by Student's *t*-test.

Evaluation of the healing by measurement of the tensile strength of the ulcerous gastric wall

The healing of chronic ulcers is evaluated by the measurement of their diameter. It is obvious, however, that "real" ulcers extend to the deeper layers of the gastric wall as well, thus weakening the tissue. Taking this fact into account we developed a new inflating technique by which the extent of ulceration can be evaluated in a more quantitative way. Our method makes the determination of ulcerous alterations even in deeper layers of the gastric wall possible.

Rats were killed by an overdose of ether after 96 h of the puncture. The stomach was dissected and opened along the lesser curvature, and extensively rinsed in tap water. Then it was fixed by thread to the end of a polyethylene tube of 10 mm diameter (plastic tip for an automatic pipette) in a position with the punched ulcer in the center. The end of the tube with the fixed gastric wall was suspended in a beaker containing physiological saline, and the pressure in the tube was gradually increased by a valved rubber ball connected to the other end of the tube. The third part of the system was a tonometer calibrated up to 1 bar. The value of tension at which bubbles appeared at the ulcerous gastric wall was noted. This value is termed as tensile strength and can be expressed in mm Hg. The extent of the healing of gastric ulcers can be characterized by a so-called healing rate (HR), as shown below:

$$HR = \frac{A-B}{C} \ (\text{mm Hg} \cdot \text{h}^{-1})$$

where A is the tensile strength value (in mm Hg) at C time point after the puncture, respectively, and B is the tensile strength value at 30 min time point after the puncture; this value is 143 mm Hg (n = 52). C is the time course (in h) of experiment.

Results

The natural repair of the subacute ulcer proceeds in a relatively rapid manner. The time course of this reparative process is shown in Figure 1. Tables I–IV show that vinca alkaloids dose dependently inhibit the natural healing process of subacute gastric ulcer. The ID_{50} values (doses which inhibit the natural healing rate by 50%) are as follows: vincristine 0.5 mg/kg (s.c.), 0.4 mg/kg (i.p.), vinblastine 0.7 mg/kg (s.c.), navelbine 2.0 mg/kg (i.p.). Table V shows that methotrexate ID_{50} is 0.4 mg/kg s.c. The results of cyclophosphamide are in Table VI. The ID_{50} is 54 mg/kg s.c. The ID_{50} value of 5-fluoruracil is 60 mg/kg i.p. (Table VII). The results of rubomycin and adriamycin are in Tables VIII–IX. The ID_{50} values of cis-platin and prednisolon are in Tables X–XI. In Table XII we summarized our results compared with human therapeutic doses. The ID_{50} values of tested drugs surprisingly correlate with human doses.

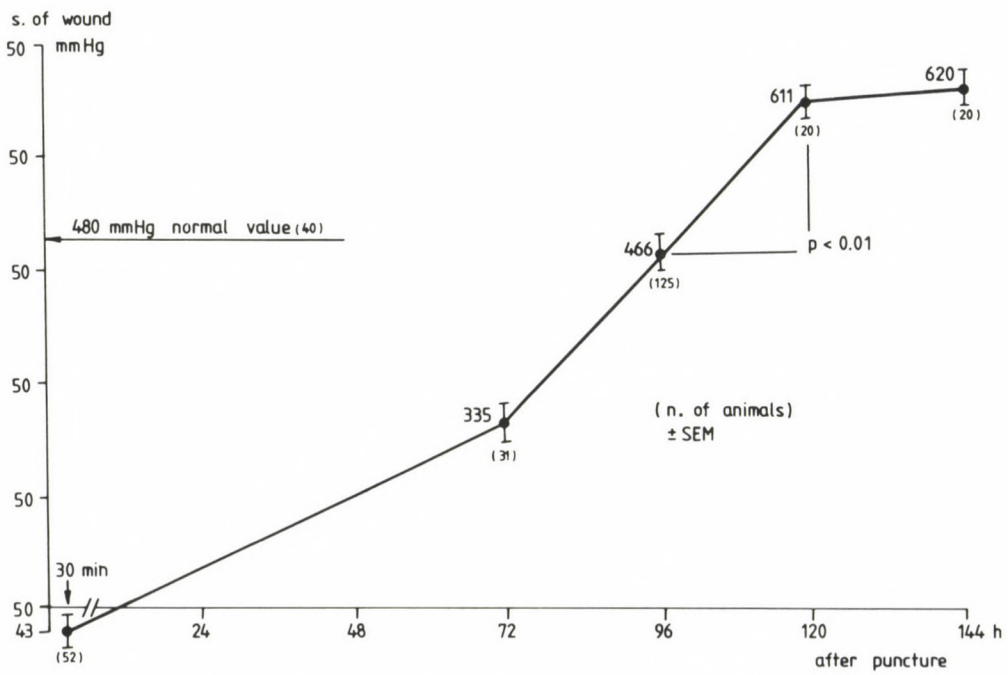

Fig. 1. Spontaneous healing process of punched rat gastric wound

Table I

Vincristine inhibits the healing of the subacute ulcer produced by gastric puncture in the rat

Treatment	N	Dose mg/kg s.c.	Healing rate mm Hg/h	change %
Control	146	vehicle x 2	2.84 ± 0.12	–
Vincristine	10	0.2 x 2	2.36 ± 0.27	−17
Vincristine	10	0.5 x 2	1.81^{x} ± 0.31	−37
Vincristine	10	1.0 x 2	0.75^{x} ± 0.5	−74
Vincristine	10	2.0 x 2	–	–

ID_{50}: 0.57 mg/kg
$^{x}p < 0.01$ vs control
± S.E.M.

Table II

Vincristine inhibits the healing of subacute ulcer produced by gastric puncture in the rat

Treatment	N	Dose mg/kg i.p.	Healing rate mm Hg/h	change %
Control	126	vehicle x 2	2.72 ± 0.14	–
Vincristine	9	0.2 x 2	2.63 ± 0.44	−4
Vincristine	10	0.5 x 2	1.14^{x} ± 0.29	−52
Vincristine	10	1.0 x 2	0.2^{xx} ± 0.28	−92

ID_{50}: 0.3 mg/kg i.p.
x : $p < 0.05$ vs control
xx : $p < 0.01$

Table III

Vinblastine inhibits the healing of subacute gastric ulcer produced by gastric puncture in the rat

Treatment	N	Dose mg/kg s.c.	Healing rate mm Hg/h	change %
Control	146	vehicle x 2	2.84 ± 0.12	–
Vinblastine	8	0.1 x 2	3.18 ± 0.33	+10
Vinblastine	19	0.5 x 2	2.05 ± 0.28	−28
Vinblastine	10	1.0 x 2	0.95^{xx} ± 0.4	−67
Vinblastine	9	10 x 2	–	–

ID_{50}: 0.72 mg/kg
x : $p < 0.01$ vs control

Table IV

Navelbin inhibits the healing of the subacute gastric ulcer produced by gastric puncture in the rat

Treatment	N	Dose mg/kg i.p.	Healing rate mm Hg/h	change %
Control	126	vehicle x 2	2.72 ± 0.14	–
Navelbin	10	0.5 x 2	2.70 ± 0.46	−1
Navelbin	9	1.0 x 2	1.17^{xx} ± 0.41	−57
Navelbin	10	5.0 x 2	0	−100

ID_{50}: 2.0 mg/kg i.p.
xx : $p < 0.01$ vs control

Table V

Methotrexate inhibits the healing of the subacute gastric ulcer produced by gastric puncture in the rat

Treatment	N	Dose mg/kg s.c.	Healing rate mm Hg/h	change %
Control	146	vehicle x 2	2.84 ± 0.12	–
Methotrexate	10	0.1 x 2	2.91 ± 0.28	+2
Methotrexate	18	1.0 x 2	0.81^{x} ± 0.31	−72
Methotrexate	10	5.0 x 2	0.11^{x} ± 0.10	−94

ID_{50}: 0.44 mg/kg
$^{x}p < 0.01$ vs control

Table VI

Cyclophosphamide inhibits the healing of the subacute ulcer produced by gastric puncture in the rat

Treatment	N	Dose mg/kg s.c.	Healing rate mm Hg/h	change %
Control	146	vehicle x 2	2.84 ± 0.12	–
Cyclophosphamide	14	5 x 2	1.54^{x} ± 0.31	−46
Cyclophosphamide	8	25 x 2	2.10 ± 0.28	−26
Cyclophosphamide	10	50 x 2	1.57^{x} ± 0.21	−45
Cyclophosphamide	6	100 x 2	1.03^{x} ± 0.18	−64

ID_{50}: 54 mg/kg
x : $p < 0.05$ vs control

Table VII

Phthoruracil /5-fluoruracil/ inhibits the healing of subacute ulcer produced by gastric puncture in rat

Treatment	N	Dose mg/kg i.p.	Healing rate mm Hg/h	change %
Control	126	vehicle x 2	2.72 ± 0.14	–
Phthoruracil	9	5 x 2	3.40 ± 0.47	+25
Phthoruracil	11	25 x 2	2.65 ± 0.39	−3
Phthoruracil	10	50 x 2	1.38^{x} ± 0.53	−49
Phthoruracil	8	100 x 2	0.27^{xx} ± 0.17	−90

ID_{50}: 60 mg/kg i.p.
x : $p < 0.05$ vs control
xx : $p < 0.01$

Table VIII

Rubomycin inhibits the healing of subacute ulcer produced by gastric puncture in the rat

Treatment	N	Dose mg/kg i.p.	Healing rate mm Hg/h	change %
Control	126	vehicle x 2	2.72 ± 0.14	–
Rubomycin	8	0.2 x 2	3.19 ± 0.75	+17
Rubomycin	10	1.0 x 2	2.10 ± 0.46	−23
Rubomycin	8	2.0 x 2	2.0 ± 0.54	−27
Rubomycin	10	10 x 2	1.49^{x} ± 0.26	−46

ID_{50}: 11 mg/kg i.p.
x : $p < 0.05$ vs control

Table IX

Adriblastine (adriamycin) inhibits the healing of subacute ulcer produced by gastric puncture in rat

Treatment	N	Dose mg/kg i.p.	Healing rate mm Hg/h	change %
Control	126	vehicle x 2	2.72 ± 0.14	–
Adriblastina	8	0.2 x 2	2.51 ± 0.45	−8
Adriblastina	10	1.0 x 2	1.97 ± 0.36	−28
Adriblastina	9	2.0 x 2	1.64^{x} ± 0.33	−40
Adriblastina	10	5.0 x 2	0.29^{xx} ± 0.21	−90

ID_{50}: 2.5 mg/kg i.p.
x : $p < 0.05$ vs control
xx : $p < 0.01$

Table X

Platidiam (cis-platin) inhibits the healing of subacute ulcer produced by gastric puncture in rat

Treatment	N	Dose mg/kg i.p.	Healing rate mm Hg/h	change %
Control	126	vehicle x 2	2.72 ± 0.14	–
Platidiam	6	0.5 x 2	3.00 ± 0.50	+10
Platidiam	9	1.0 x 2	1.09^{xx} ± 0.31	−60
Platidiam	8	5.0 x 2	1.03^{xx} ± 0.29	−63
Platidiam	8	10.0 x 2	0.2^{xx} ± 0.33	−93

ID_{50}: 0.6 mg/kg i.p.
xx : $p < 0.01$ vs control

Table XI

Prednisolon inhibits the healing of the subacute gastric ulcer produced by gastric puncture in the rat

Treatment	N	Dose mg/kg s.c.	Healing rate mm Hg/h	change %
Control	146	vehicle x 2	2.84 ± 0.12	–
Prednisolon	10	0.5 x 2	2.19 ± 0.29	−23
Prednisolon	10	5 x 2	1.79^{x} ± 0.28	−37
Prednisolon	10	50 x 2	0.58^{x} ± 0.33	−80

ID_{50}: 6.5 mg/kg
x = $p < 0.01$ vs control

Discussion

The cell proliferation is a common basic step of the natural healing and that of the tumor growth. The cell proliferation is well controlled in the healing process, while in the tumor growth it is uncontrolled. The idealized anticancer drug should distinguish between the proliferative processes of normal and tumor cells. These results confirm the general observation that present applied anticancer drugs cannot distinguish between normal and tumor cell proliferation [1]. The ID_{50} values of tested anticancer drugs, calculated according to new method, strongly correlate with human doses. This inhibitory effect of drugs on the natural healing is independent from the mechanisms of action. The investigated anticancer drugs belong to the following classes: cyclophosphamide as alkylating agent, methotrexate, 5-fluoruracil

Table XII

Efficacy of some antineoplastic drugs on the rat gastric healing model: ID_{50} values strongly correlate with human doses

Treatment, route		ID_{50}[a] mg/kg	Human therapeutic dose range (mg/kg)
Vincristine	s.c.	0.5	0.3 – 0.6
Vincristine	i.p.	0.3	
Vinblastine	s.c.	0.7	0.3 – 0.6
Navelbine	i.p.	2.0	0.5 – 2
Adriamycin	i.p.	2.5	2 – 4
Rubomycin	i.p.	11	0.5 – 2
Cis-platin	i.p.	0.6	1 – 3
Methodrexate	s.c.	0.4	0.4 – 0.6
Cyclophosphamide	s.c.	54	40 – 60
5-Fluoruracil	i.p.	60	10 – 20
Prednisolon	s.c.	6.5	1 – 3

[a]: ID_{50} dose which inhibits the healing rate with 50%

as antimetabolite, adriamycin, rubomycin as antibiotics, vincristine, vinblastine, navelbine as plant alkaloid, prednisolon as adrenocorticoid, cis-platin as synthetic agent.

It is likely that this new simple model can be useful as a complementary method in evaluating anticancer drugs.

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RESISTANCE TO MEDICAL ULCER THERAPY – POSSIBLE REASONS AND MANAGEMENT

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In 112 patients with duodenal ulcer (males, mean age 29, range 18–35) with a history of at least 3 years, and never being treated with H_2-antagonists, maintenance therapy with Tagament was started. All of them had ulcer recurrence when entering the study. The first two months a full dose was applied, after this bedtime doses up to 24 months. Endoscopy was performed at the beginning, after the first and second months and then every two-month period. Gastric secretion was measured every 2–3 months. A resistant ulcer was defined as one 1/not healing within the two initial months, 2/recurring on maintenance treatment. Results: in 78% the ulcer was healed within one month, in 96% within two months, in the rest within the following 3–4 weeks. Relapses occurred in 21 patients, altogether 27 times. Factors likely to contribute to recurrence included a large ulcer size, longer duration, an inflamed mucosa and – most frequently – heavy smoking. Patients with relapses had higher initial secretory values and smaller decrease during maintenance treatment. Helicobacter pylori (examined in a subgroup) was not clearly associated with ulcer recurrence.

Keywords: ulcer therapy, duodenal ulcer, H_2-antagonits, gastric secretion

There is a group of patients with peptic ulcer who appear to be resistant to therapy with H_2-receptor antagonists [1] which in trials may represent 5–15%. However, true resistance may be rare with latest more potent agents like omeprazole or with extended treatment with H_2-blockers. So the definition of resistant (or refractory) ulcer is arbitrary and should be clearly established in trials dealing with the problem (including diagnostic procedures, definition of the type of ulcer, complications, possible association with other important diseases, possibility of gastrinoma). Our present study is based on a long-term prospective follow-up including both the outcome of short- and long-term treatment.

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Materials and methods

112 duodenal ulcer patients (males, mean age 29, range 18–35) were selected for maintenance therapy with H_2-antagonists. Each of them had a history of duodenal ulcer for at least 3 years relapsing every year (mostly twice) under "conventional" therapy with antacids and/or anticholinergics. None of them had been treated with H_2-blockers previously. In 23 of them surgery had been recommended by other physicians, but was not performed until entry into our study. All the patients when entering had endoscopically proven ulcer recurrence. No patients with severe complications (massive frank upper gastrointestinal bleeding, penetration or perforation, development of pyloric stenosis) were included.

Definition of "resistant" ulcer: A resistant ulcer was defined as one not healing up to 8 weeks of full-dose treatment with Tagamet and/or one that relapsed under maintenance therapy with Tagamet extended in all patients after the initial period for a long-term period of 24 months. The daily dose of Tagamet was 800–1000 mg for the first two months and 400–600 mg at bedtime for maintenance treatment. This treatment was often combined with antacids at day-time and in a few cases with Pirenzepine.

Upper gastrointestinal endoscopy was performed initially-before starting the trial, after the first and second months from onset, and then every two months up to two years (if necessary these intervals were shortened). Serum gastrin levels (basal, postprandial and after secretin) were measured in a limited number of patients with high basal secretion (repeatedly more than 10 m mol/h) where the possibility of a Zollinger-Ellison syndrome was considered. – Gastric secretion (basal and after pentagastrin stimulation) was measured before starting the trial and then every two or three months. – Parietal cell sensitivity to pentagastrin and to the effects of Cimetidine, Glucagon and TRH (thyreotropine releasing hormone) on pentagastrin stimulated secretion was measured (with the construction of dose-response curves) in 28 patients before starting and after 6–8 months of maintenance treatment.

In all the patients sonographic examination performed initially showed a normal picture of the liver, bile ducts and pancreas. Biochemical examinations showed normal values of liver function tests. Patients with reflux oesophagitis were not included. There were no clinical or other signs of any important associated disease.

After finishing maintenance therapy 108 patients were followed-up two more years and 98 of them for another period of two years (altogether four years).

The factors evaluated which could possibly affected healing rates included: ulcer size, morphology of the duodenal mucosa (bulb), gastric secretion – basal and pentagastrin stimulation – initially and during maintenance treatment, pariental cell sensitivity (28 patients) heavy smoking (more than 20 cigarettes daily), duration of the disease prior to the start of the trial, stressful life events, response to treatment of the initial attack, a history of bleeding (patients with a history of massive upper gastrointestinal bleeding were not included), age of onset, a family history of ulcer, helicobacter pylori found in the antral mucosa (35 patients examined). Patients showing non-compliance and/or taking frequently or systematically NSAIDs (non steroidal anti-inflammatory drugs) were not included.

Gastric ulcer: here the situation is more complex since gastric ulceration that does not heal adequately should be considered from the aspect of possible malignancy. In our study there were originally 45 patients with gastric ulcer (31 males, 14 females, mean age 37, range 28–51), most carefully examined endoscopically and particularly histologically to exclude malignancy. All of them had a trial of colloidal bismuth subcitrade (DE NOL); all the ulcers healed (proven by endoscopy) within 4–5 weeks. The results of the further follow-up of these patients and more clinical data are presented in a separate study.

Results

1. *Initial attack:* in 78% of the patients the duodenal ulcer was completely healed on endoscopy performed after 4 weeks. After 8 weeks the ulcers were completely healed in altogether 96%. In the remaining cases the ulcer size was considerably smaller. In these few cases full-dose treatment was extended for 3–4 additional weeks and endoscopy performed after this showed complete healing.

2. *Relapse rates during maintenance treatment.* In 21 patients (19%) relapse occurred during the two-year period (altogether 27 endoscopically proven relapses), in 7 patients during the first and in 14 during the second year. The frequency of relapses was highest at the end of the first and beginning of the second year (18 patients), in 3 patients relapses occurred in the second half of the second year. More than one relapse (two relapses) occurred in six patients (in three of them with a one year interval, in the remaining three with an interval of 8–10 months. Eight of the 27 relapses confirmed on endoscopy were asymptomatic (in four of them it was the second relapse in the same patient).

3. *Factors likely to contribute to ulcer recurrence* during maintenance Tagamet treatment (Table I) and also to a certain delay in healing of the initial attack.

Table I

Delay in healing and relapse-contributing factors

Ulcer size:	in 17/21 > 20 mm
Inflamed duodenal mucosa:	in 15/21
Delayed healing of initial attack:	in 15/21
Longer duration of disease:	in 18/21 > 5 years[x] mean 9.5 range 5–17
Heavy smoking	in 20/21

x vs. group without relapses $p < 0.05$

Ulcer size. In 17 of the 21 relapsing patients the initial size of the ulcer was more than 20 mm. In these cases healing of the initial attack occurred mostly in the second month or even later and also healing of the recurrent ulcer was delayed in comparison with others, however, the recurring ulcers seemed to be smaller than they were initially.

Duodenal mucosa. An inflammed mucosa of the duodenal bulb persisting to a certain degree after initial healing was associated with 15 of the 21 relapsing cases. A more pronounced degree of scarring (without stenosis), if not associated with an

inflamed mucosa seemed to be less important for the relapse rate (in 4 of the 21 patients).

Initial attack. In 15 of the relapsing cases complete healing of the initial attack occurred later than after one month and all these ulcers were considerably large (as mentioned above).

Duration of disease. In 18 of the 21 relapsing patients the disease had lasted more than 5 years at the time of the start of the trial. The longest duration was 17 years, the shortest 5 years (mean 9.5 years), whereas in the group without relapses the mean duration when entering the study was 4.5 years ($p < 0.05$).

Heavy smoking. 20 of the 21 patients with relapses were heavy smokers and did not reduce the daily number of cigarettes below 20 (or even 25). All of the 6 patients with two relapses during maintenance therapy were among them, as well as all those showing some delay in healing during the first attack.

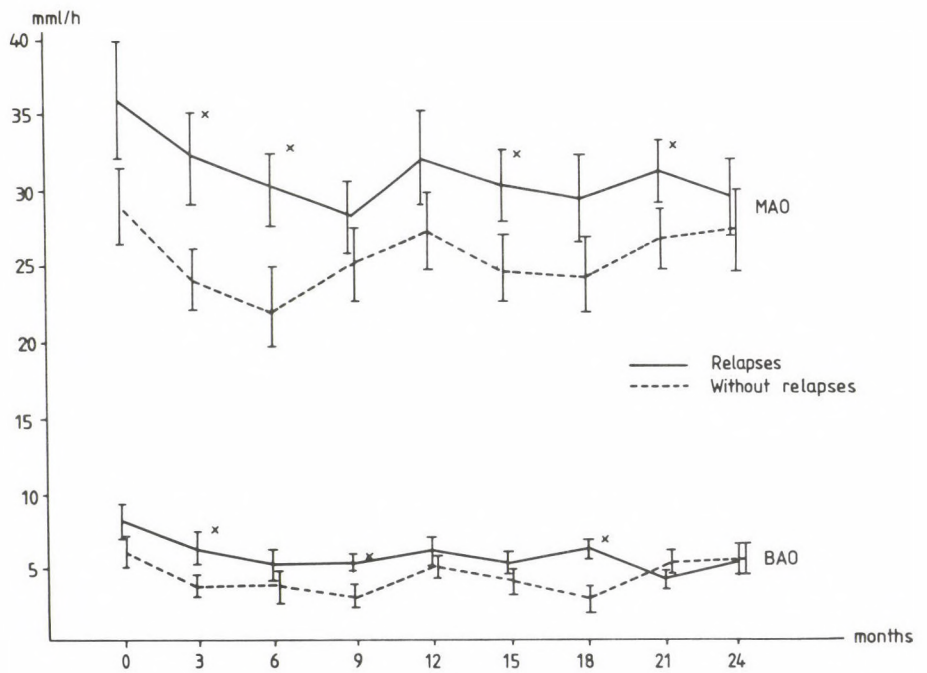

Fig. 1. Acid secretion in patients with relapses (×: $p < 0.05$)

4. *Gastric secretion.* In 10 of the 21 patients with relapses (altogether in 12 of the 112 of the whole group) basal acid output was initially higher than 10 mmol/h (in all these patients). Serum gastrin was measured and it showed normal values. Pentagastrin stimulated secretion was initially higher than 35 mmol/h in 13 of the 21

relapsing patients. Basal acid output decreased during maintenance treatment in the relapsing group, although quite unfrequently below 5 mmol/h. Pentagastrin stimulated secretion (MAO) also decreased, however not more than by 20% and on the whole the difference – compared with values in the non-relapsing group – is just on the level of significance. Figure 1 shows the 2-year course of basal and pentagastrin stimulated secretion in both groups for the whole 2-year period. The values in the non-relapsing group are lower than in the relapsing group, sometimes at the level of significance, but these more important differences occurred in a part of the patients (in 10 out of 21) and were only in 6 cases associated with ulcer recurrence (Fig. 1).

Parietal cell sensitivity. Sensitivity to the inhibition of pentagastrin stimulated secretion by a 200 mg dose of Tagamet i.v. did not change during maintenance treatment with Tagamet (neither after 6 nor 12 months) and was not different in the patients with relapses (15 out of 28 examinations). However, there was a significant decrease in sensitivity to pentagastrin stimulation after four and eight months of treatment, D_{50} being significantly higher ($p < 0.05$) than before treatment in all of the 28 investigated patients. In the 14 relapsing patients among them the increase in D_{50} was less pronounced but not enough to cause a significant change in sensitivity. There was no change in the moderate, although significant inhibition of pentagastrin stimulated secretion by either Glucagon or TRH during maintenance therapy in the whole group of 28 patients; D_{50} was not significantly different in the group with relapses.

5. *Heliobacter pylori (H.P.)*. This investigation was performed in 35 of the patients of the whole group, mostly during the first year. Positivity of H.P. in the antral mucosa was found altogether in 11 patients, in two of them with relapses (2 among 9 patients investigated with relapses) and in 9 of the 26 investigated subjects of the non-relapsing group.

6. *Lack of convincing evidence* for an association with ulcer relapses was found in this study in a history of bleeding (melaena) and in the age of onset and a familial aggregation of ulcer. This seems to demonstrate a favourable effect of Tagamet maintenance treatment in the subset of patients with early onset and a positive family history of ulcer where – according to our previous experience – under "conventional" therapy 75% (36 out of 48 individuals with familial occurrence, 41 of them with onset before age 18) presented constantly with frequent relapses. However, there is still a subset of ulcer patients of young age with familial occurrence of ulcer and early complications (perforation, severe bleeding), frequently just as almost the first clinical manifestation of the disease.

7. *Stressful life events*. Special attention was given to this problem since a majority of our young patients had stressful situations in their professional and/or family life which doubtlessly might have contributed to the development of their

ulcers. In our young patients the relatively favourable outcome after finishing maintenance therapy (see later) seems to be often associated with an improvement of their social and often also private conditions. During maintenance therapy a stressful event seemed to be immediately associated with ulcer recurrence in six cases, however, also with other risk factors (particularly smoking), in most other cases stressful life events emerging during maintenance therapy appeared to cause transitory impairment of symptoms without endoscopically proven ulcer recurrence.

8. *Management and course of relapses*. In all cases with proved H.P. treatment with DE NOL was started (both in the 2 patients relapsing and in the 9 of the non-relapsing group. H.P. was eradicated on repeated investigation after this trial (about 5 weeks) and Tagamet treatment continued.

In 6 cases the first step after the recognition of a recurrent ulcer was to reinforce Tagamet treatment (again a full dose therapy). This treatment continued for 2 months. In 10 cases of relapse Ranitidine treatment was started instead of Tagamet (Ranisan) and applied for 1 or 2 months (according to availability), after this Tagamet treatment continued in 9 cases of relapse Tagamet treatment was combined with Pirenzepine for 1 or 2 months, all the recurring ulcers were healed on repeated endoscopy performed in these cases already after 1 month; 19 of the total of recurring ulcers were completely healed after one month (70%), all of the others after the second month.

9. *Further courses*. 29 out of 108 patients followed-up after finishing maintenance therapy relapsed during 2 years and 21 out of 98 during another period of 2 years. This represents 34 individuals with altogether 50 relapses (16 having each two endoscopically proven relapses). During the whole period (4 years) 21 of them (62%) constituted the group of relapsing cases also during the period of maintenance therapy, 15 of them still smoking more than 20 cigarettes daily.

Discussion

The basic events of failure of ulcer healing have been recently the subject of profound studies resulting in a new insight into these processes [13, 16]. It is not quite clear whether the failure of normal healing of ulcers is due to defective regulation of the healing processes or to interference with these processes. Healing processes include a variety of factors, cellular migration and replication regulated by humoral mechanisms including both inhibitory and stimulatory peptides.

Migration also depends on the chemical constitution of the ulcer base [17]. Cellular migration may be stimulated by EGF in low serum concentrations; this migration – the promoting effect of EGF – may be involved in mucosal repair [3, 6].

Equally important for the healing of ulcer is the stimulation of proliferation of cells in the edge of the ulcer due to the activity of growth promoting factors [6, 10].

An abnormal mucosa might be associated with some abnormality of cellular proliferation [13] – duodenitis in duodenal ulcer with persisting histological alterations even when the ulcer is healed [4]. In gastric ulcer – on the other hand – healing seems to be associated with the abnormally increased proliferation of gastric mucosal cells in atrophic gastritis [14, 15].

Profound abnormalities of the processes involved in the restitution of the mucosal architecture of the gastric and duodenal mucosa are indicated by cell differentiation as a result of alteration of the precursor cells involved in repair [16].

Interference with healing may be associated with abnormalities of production or action of mitogens and defective epithelization – defective cellular adherence [7, 11]. Defective vascularization may be the cause of the failure to heal; failure of the reparative processes may be due to defective angiogenesis [13, 16]. A definite effect of intraluminal gastric juice has not been convincingly established [16]. Histamin stimulated gastric secretion probably delays healing through various direct noxious effects on the mucosa and healing processes, whereas pentagastrin, also stimulating gastric secretion, actually promotes healing of gastric ulcers in rats.

Summarizing it is not yet known whether the tendency to form chronic mucosal wounds and their tendency to recur are due to interference with the processes including normal mucosal repair or whether there is a failure of the repair processes since none of the factors mentioned above has been convincingly proven to be the cause of delay of prevention of healing of peptic ulcer – it appears that these problems also indicate the great complexity of clinical non-responsiveness in human peptic ulcer. So it is difficult to explain the mode of action of the clinical factors which seem to contribute to unresponsiveness in clinical practice in terms of the mechanisms of ulcer healing and interference with healing described above; moreover, the term "unresponsiveness" or "resistance" is probably rather a relative one depending on the criteria used for the individual situation.

According to clinical experience as shown by endoscopy it seems just likely that severe morphological alterations of the mucosa of the duodenal bulb may delay healing and favour recurrence in spite of potent medical treatment, it is not clear whether such alterations are associated with excessive acid secretion. In our experience most patients with very high secretory values did have a pathological mucosa of the duodenal bulb and a tendency to relapse; it is not clear – on the other hand – whether this pathological picture is rather a sequel of an ulcer of larger size as very often observed in such cases or a condition preceding the ulcer.

The effect of smoking – a broad chapter – has not been clarified. In previous studies we were not able to prove a direct significant effect of smoking on either basal or stimulated secretion. However, a positive correlation was found between maximal acid output and the number of cigarettes smoked daily for many years. In our trial most patients did not stop smoking completely and their ulcers healed under

adequate treatment. However, the unfavourable effect of "heavy" smoking appears to be striking; the mechanisms of this effect – perhaps interference with basic defense processes – remains to be established.

Stress situations may act through disturbances of central regulatory factors (brain peptides). Alterations in parietal cell sensitivity may – perhaps – account for a delay in healing and a tendency to relapse in some cases (in our study a slightly less pronounced decline in sensitivity to pentagastrin during maintenance therapy). Heliobacter pylori – a subject of many contraversies – was studied only in a minority of patients in our trial where its effect did not seem convincing; however, there is some strong evidence that it might play an important role in recurrent ulcers also in a subset of patients.

At about the same a smaller group of duodenal ulcer patients [52] with a shorter history and less severe morphology was treated "conventionally" with antacids and/or anticholinergics over a period of 2 years and followed-up in a similar way. The recurrence rate during this period was relatively almost three times higher than in the Tagamet group. However, compliance in this group seemed to be less satisfying and in most cases the treatment was not continuous.

The question whether in cases of delayed healing and/or recurrence under maintenance therapy an increased dose of H_2-blockers should be used, whether H_2-blockers should be combined with other agents or wether one should switch treatment to a drug acting predominantly on mucosal defense has not been answered definitely. Clinical trials have reported mixed results [1, 8, 9, 5] – the very potent suppression of acid by Omeprazole has shown to be very effective in the treatment of resistant duodenal ulcers – not responding to any of the modalities mentioned above, whereas gastric ulcers did not seem to heal more rapidly with Omeprazole than with Tagamet [4]. So – since the role of non-antisecretory agents in maintenance therapy is as yet unclear – their use in slowly healing gastric ulcers with low acidity should be considered. Sucralfate appears to be effective in such eases [9] and recent evidence even suggests that also colloidal bismuth may be used safely for longer periods in a small dose [2].

Conclusions

True resistance of peptic ulceration to medical treatment seems to be rather unfrequent and definitions are different. In this study a resistant ulcer was defined as one not healing on endoscopy within 8 weeks of full-dose treatment with H_2-blockers and/or recurring during maintenance therapy, always confirmed by endoscopy. – The basic reasons for a marked delay in ulcer healing are very complex including a variety of factors. Clinical factors involved in a delay in ulcer healing and recurrence during maintenance treatment with H_2-blockers include – apart from non-

compliance – a large size of the ulcer, a pathological mucosa, heavy smoking, a longer duration of the disease, stress situations, a high secretory activity probably not sufficiently inhibited during treatment. These observations are based on the courses of duodenal ulcer; in gastric ulcer the situation although similar-seems to be different, probably – at least in part – due to an approach to exclude malignancy and may be due also – in part – to a different response to acid – suppressing drugs. After finishing maintenance treatment in duodenal ulcer patients a further follow-up showed relapses more frequently in patients relapsing also during maintenance treatment. It appears that the definition of an ulcer as one definitely resistant to medical therapy should be evaluated carefully with an attempt to eliminate unfavourable factors.

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SELENIUM IN THE BLOOD OF PATIENTS WITH COLORECTAL CANCER AND NEOPLASTIC POLYP

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Samples of whole blood were obtained from 51 patients with newly diagnosed colorectal cancer as well as from 76 patients with neoplastic colorectal polyp, and from 30 healthy blood bank donors. Selenium was determined by the fluorimetric method. Significantly decreased selenium concentrations of blood samples from patients with colorectal cancer and villous adenoma were found. There was not any correlation between the blood selenium levels of patients with adenomatous polyp and the severity of dysplasia in removed polyps. The lowest mean selenium level in patients with villous adenoma indicates that selenium deficiency may be an important factor in the development of colorectal cancer arising from villous adenomas.

Keywords: selenium, protective effect, colorectal cancer, neoplastic polyps

Prospective human epidemiological studies have shown an increased risk of cancer in populations with low selenium levels [7, 13]. Blood selenium concentrations of cancer patients including the ones with colorectal cancer, have been reported to be lower than in healthy controls [5, 7, 9, 10, 13]. Whether selenium deficiency is a primary etiological factor or a paraneoplastic phenomenon is a widely discussed problem [1, 8, 9, 11]. In patients with early, cancers and/or precancerous lesions paraneoplastic effects are considered to be negligible. Colorectal cancer is generally regarded to arise from neoplastic polyps.

The aim of this study was to select patients with colorectal cancer and colorectal polyps comparing their blood selenium concentrations, to examine the correlation between the blood selenium levels and histological types including the severity of dysplasia of polyps removed.

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Patients and methods

Subjects were examined colonoscopically and the definitive diagnosis was based on histology. All of the polyps were removed endoscopically.

Fifty-one patients with colorectal cancer and seventy-six with colorectal polyp were examined. According to the histology of removed polyps the patients with polyps were divided into four groups.

The first group comprised 18 patients with adenomatous polyps without any evidence of dysplasia of with moderate dysplasia. The second group comprised 20 patients with adenomatous polyps with medium grade of dysplasia. The third group comprised 21 patients with adenomatous polyps with severe dysplasia or carcinoma *in situ*. The fourth group comprised 17 patients with villous adenoma.

The control group consisted of 30 healthy donors of the local blood bank.

A 10 ml sample of blood was requested to be taken and to be allowed to clot without any additives. All samples were kept frozen in the laboratory until analysis was performed. The diagnoses were not known for staff of the laboratory. All samples were analyzed by fluorometric method.

Statistical analysis of differences among group means was performed using the Student's *t*-test.

Results

Whole blood selenium concentrations of healthy blood donors were significantly higher than those of patients with newly diagnosed colorectal cancer and colorectal polyps (Table I). There wasn't any correlation between the blood selenium concentrations of patients with adenomatous polyp and the severity of displasia in removed polyps.

Table I

Blood selenium concentrations of patients with colorectal cancer and polyp

Sample	n	Mean (μg/l ± SD)
Controls	30	64 ± 19
Colorectal cancer	51	49 ± 18^{x}
Colorectal polyp	76	54 ± 19^{xx}

x: Mean different from control group with $P < 0.001$
xx: Mean different from control group with $P < 0.02$

The blood selenium levels of patients with villous adenoma proved to be significantly lower than those of controls (Table II).

Table II

Blood selenium concentrations of patients with different types of colorectal polyp

Sample	n	Mean (μg/l ± SD)
Controls	30	64 ± 19
Adenomatous polyp without any dysplasia or with moderate dysplasia	18	56 ± 18
Adenomatous polyp with medium grade dysplasia	20	56 ± 19
Adenomatous polyp with severe dysplasia or carcinoma *in situ*	21	57 ± 22
Villous adenoma	17	45 ± 19[x]

[x]: Mean different from control group with $P < 0.001$

In spite of different degrees of dysplasia in adenomatous polyps the patients suffering from them proved to make up a homogeneous group regarding their blood selenium levels, so drawing them together might be reasonable.

Whole blood selenium concentrations of patients with villous adenoma were significantly lower than those of patients with adenomatous polyp (Table III).

Table III

Blood selenium concentration of patients with adenomatous polyp and villous adenoma

Sample	n	Mean (μg/l ± SD)
Controls	30	64 ± 18
Adenomatous polyp	59	56 ± 20[xx]
Villous adenoma	17	45 ± 19[x]

[x]: Mean different from both control and adenomatous polyp with $P < 0.001$

[xx]: Mean not significantly different from control groups

Discussion

The selenium concentrations observed in whole blood samples of healthy blood donors involved in our study are somewhat lower than it could have been expected according to European data [12]. Further studies are needed to examine whether selenium deficiency exists in our region.

Both epidemiological and laboratory studies indicate that selenium may offer some protection against the risk of cancer. While the exact mechanism of action of selenium in protection remains unclear, it may be related to the antioxidant effect of glutathion peroxidase which detoxifies organic peroxides within the cell. In our patients with colorectal cancer significantly lower blood selenium concentrations were found. Robinson et al. [6] in their study of the inhabitants of a low selenium region conclude that the low selenium levels in patients with cancer were more likely the consequence of their illness than the cause of cancer.

Few data have been published about the blood selenium in patients with precancerous lesions. Brock et al. [2] found no correlation between serum selenium levels and the risk of *in situ* cervical cancer. According to Jaskiewicz et al. [4] mean selenium levels of subjects with premalignant and malignant esophageal cytological changes were significantly lower than those of subjects without such lesions.

No correlation has been found between the blood selenium levels of our patients with adenomatous polyp and the severity of dysplasia. Dworkin et al. [3] concluded that selenium stores cannot play an important role in the *de novo* formation of benign neoplastic colonic polyps, but it is possible that patients with polyps and the lowest selenium levels are at higher risk for malignant transformation. Our data showing that the average selenium level proved to be lowest among patients with villous adenoma indicate that selenium defiency may be an important factor in the development of colorectal cancer arising from villous adenomas.

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MEASUREMENT OF NON-STEROID ANTIINFLAMMATORY DRUGS INDUCED GASTRIC MICROBLEEDING

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The damage of the mucous membranes in the gastrointestinal tract caused by non-steroid antiinflammatory drugs are well known. The gastrointestinal microbleeding was measured by the method of Fischer and Hunt before and after the intake of indomethacin (4×25 mg), naproxen-sodium (4×275 mg), diclofenac (3×50 mg) and azapropazone (2×600 mg). In the indomethacin group microbleeding increased from 0.91 ± 0.12 ml/24 h to 7.30 ± 1.20 ml/h. In the naproxen-sodium group from 1.22 ± 0.16 ml/24 h to 3.56 ± 0.40 ml/24 h, in the diclofenac group from 0.86 ± 0.14 ml/24 h to 3.18 ± 0.28 ml/24 h, in azapropazone group from 0.92 ± 0.18 ml/24 h to 2.50 ± 0.20 ml/24 h, respectively.

All non-steroid antiinflammatory drugs increased the gastric microbleeding, however, there were considerable differences in the degree of enhancement. This can be explained by the different inhibitory activities of the drugs on the cyclooxygenase enzyme activity.

Keywords: non-steroid antiinflammatory drugs, gastric microbleeding, cytoprotective prostaglandins, erosive gastropathy

Rheumatic disease are among the most common medical disorders. The majority of patients with rheumatic diseases use NSAIDs to releive pain and inflammation, and thus remain functional. The introduction of NSAIDs made it possible to improve the quality of life of these patients. The use of these agents has increased in the last ten years. Besides the advantageous effects of these drugs, the side effects also have become known with a leading role of the damage of the gastrointestinal mucosa [3, 5, 9, 10, 13, 15, 16, 25]. The prevalence of active peptic ulcer is higher in patients using non-steroid antiinflammatory drugs, and in the proven cases of peptic ulcer in 25% non-steroid antiinflammatory drug therapy was found. There are many data in literature on the severe side effects of non-steroid antiinflammatory drugs (perforation, bleeding ulcer) [13, 15, 16].

The publications on the "micro" side effects are, however, rather rare [12, 20]. Sometimes these microlesions cannot be seen never endoscopically they are only

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detectable by the search for gastrointestinal microbleeding, or by the increase of intestinal permeability [4, 6, 7].

The development in the pharmaceutical research provided new non-steroid antiinflammatory drugs with the same clinical effects but decreased side effects [1, 23, 24]. The aim of our study was to determine and compare the intensity of microbleedings in the stomach – the unwanted effect of NSAIDs – after the administration of Indomethacin, Diclofenac, Naproxen-sodium and Azapropazone.

Patients and method

19 patients in the Indomethacin group and 15 patients in the other groups were included in the study (age 20–60). They were free of any gastrointestinal diseases, they were properly informed and gave written consent on the conduct of the study. Gastric microbleeding was provoked by the administration of Indomethacin (4×25 mg orally) (Capsula Indomethacin-Chinoin), Diclofenac (3×50 mg orally) (Voltaren-Biogal), Naproxen-sodium (4×275 mg orally) (Apranax, Synthex Pharm AG Allschwill/Basel), and Azapropazone (2×600 mg orally) (Siegfried Pharma LTD, Zofingen). Following a 12-h period of starving a plastic tube was led to the stomach of each patients 55 cm deep from the teeth. After the suction of the content of the empty stomach, 250 ml distilled water was injected in the stomach, then it was sucked. The stomach was washed this way and then the test solution was injected into the stomach. The content of this was: 10 g D-glucose, 40 mg cc. phenol-red (as non-absorbable volumen indicator) dissolved in 100 ml distilled water. After 10 min the stomach content was emptied by continous suction (Fig. 1).

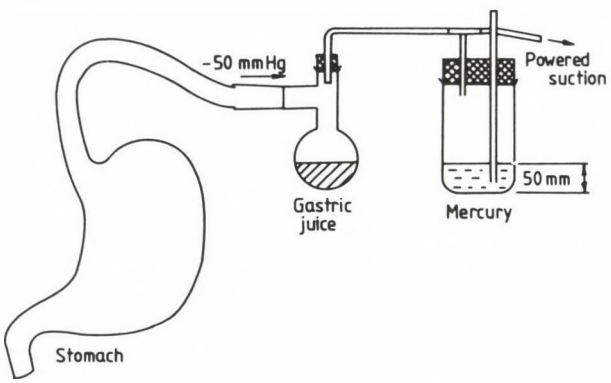

Fig. 1. Apparatus for the continuous aspiration of gastric juice in man

This procedure was repeated twice. The volumes from the stomach were homogenized for 10 min then the hemoglobin content was determined by the method of Fischer and Hunt [12]. The blueish colour is produced by the reaction of hemoglobin, orthotulidin and H_2O_2, and this can be photometered at 640 μm. The concentration of phenol-red was determined by spectrophotometrically. The volumes sucked after the 10-min periods were corrected to the original amount by using the volume-indicator correction. The bleeding during the 10-min exposure time was calculated on unit and multiplied to a 24-h period. On figures 2, 3, 4 and 5 the mean blood-loss values are indicated (± S.E.M.).

The tests were carried out before and following the one-day treatment period.

Results

The "basal" blood-loss in the Indomethacine group was 0.91 ± 0.12 ml/24 h. Following a one-day of 4x25 mg therapy with indomethacine the 24-h blood-loss increased significantly to 7.30 ± 1.20 ml/24 h (Fig. 2).

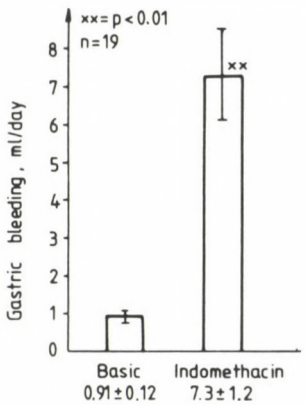

Fig. 2. Gastric microbleeding caused by the administration of Indomethacin (4×25 mg orally) for one day. Mean ± S.E.M.

The basal blood-loss in the group treated with Diclofenac was 0.86 ± 0.14 ml/24 h, which increased after the one-day treatment to 3.18 ± 0.28 ml/h (Fig. 3).

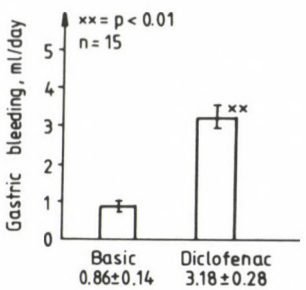

Fig. 3. Gastric microbleeding caused by the administration of Diclofenac (3×50 mg orally) for one day. Mean ± S.E.M.

In the Apranax group the basal microbleeding was 1.22 ± 0.16 ml/24 h, following the one-day therapy of 4x275 mg Naproxen-sodium the blood-loss increased to 3.56 ± 0.40 ml/24 h (Fig. 4).

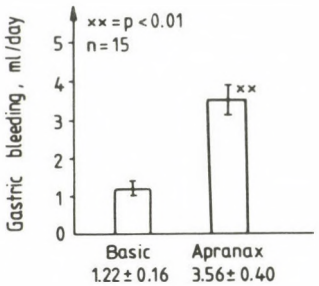

Fig. 4. Gastric microbleeding caused by the administration of Apranax (4×275 mg orally) for one day. Mean ± S.E.M.

The basal blood-loss in the Azapropazone group was 0.92 ± 0.18 ml/24 h. After the administration of 2×600 mg Azapropazone the rate of gastric microbleeding increased to 2.5 ± 0.2 ml/24 h.

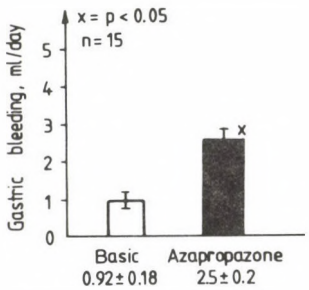

Fig. 5. Gastric microbleeding caused by the administration of Azapropazone (2×600 mg orally) for one day Mean ± S.E.M.

Discussion

Most NSAIDs used in current medical practice are molecules with acidic properties, with the exception of nabumetone. Most are derivates of salicylic acid and may be classified as carboxylic acid derivates. Phenylbutazone and Azapropazone differ in structure from the majority (Fig. 6).

Theories concerning the pathogenesis of NSAIDs-induced gastropathy are as follows: simple topical injury and systemic effect. Drug induced topical injury to the gastric mucosa is though to be due to the solubility of an intrinsically corrosive drug in acid contents, thereby permitting entry to cells. In the stomach, at acid pH, NSAIDs of pKa values of 4 to 5 will be non-ionized. Entry of the drug into gastric mucosal cells in the non-ionized state will enhanced, but cytoplasmic pH being neutral, these drugs then became ionized, less lipid soluble, unable to leave the cell and thus accumulate within gastric mucosal cells.

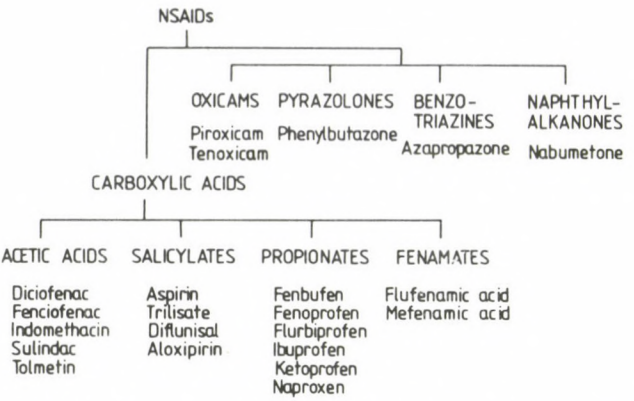

Fig. 6. Non-steroid antiinflammatory drugs classified by chemical type

Many of the toxic effects of NSAIDs arise from the same mechanism as their therapeutic effect, namely, inhibition of cyclooxygenase, the enzyme that catalyzes the synthesis of cyclic endoperoxydases from arachidonic acid to form prostaglandins. NSAIDs reduce the secretion of bicarbonate and the synthesis and secretion of mucus in both the stomach and duodenum, and the reduction may impair mucosal defense. NSAIDs reduce gastric mucosal blood flow. Although it is not known which, if any, of the above-mentioned factors are important in preventing chronic NSAID-associated ulcers. We found during the study, that the gastric microbleeding caused by certain non-steroid antiinflammatory drugs was of different grade. Local and systemic changes in the barrier function of the mucous membranes of the gastrointestinal tract lead to microbleedings. The main function of the barrier is the protection of the gastric mucosa from the damaging effect of pepsin and hidrogen ion. This defence consists of many factors: *the mucous layer, the bicarbonate secretion,* which gives a pH gradient between the lumen of the stomach (pH: 2.0) and the inside of the mucous membrane cell (pH: 7.0). These mechanism are further complicated by the *regeneration rate* of the mucous membranes and the *blood supply* from the submucosa. These four main elements of the defence system are influenced by the synthesis of prostaglandins. NSAIDs can inhibit the activity of cyclooxygenase enzyme at different rate [4, 19, 20]. In our observations the basal gastric bleeding was practically the same in the four groups. Contrary to this, the increases of gastric bleeding were found to be considerably different after a one day administration of these drugs. Indomethacin is a strong inhibitor of cyclooxygenase pathway. Due to its effect the concentration of PGE_2 at the local site of the mucous membrane is decreased which leads to the development of erosions and ulcers. The inhibitory effect of naproxen-sodium, diclofenac and azapropazone on the cyclooxygenase enzyme activity is weaker, this is why the side effects are also milder compared to the same effect of indomethacin. The least gastric microbleeding was found in case of azapropazone administration. The lower gastrointestinal ulcerogenity of azapropazone is due to:

– The slow rate of gastric absorption of the drug and unique physicochemical properties, which combine to minimize the irritation to the surface mucosal cells.

– Its weak inhibitory actions on the synthesis of mucosal protective prostaglandins.

– The drug may inhibit the production of 5-lipoxygenase products which appear to contribute to vascular injury.

– The lack of inhibitory effects on the biosynthesis of the protective mucus layer in the gastrointestinal tract.

– Its propensity to stabilize the lysosomal membrane.

– The drug exhibits anti-oxydant actions and inhibitory effects on the generation of oxygen free radicals.

The primary duty of clinicians is the prevention of the adverse drug reactions. In this sense it is most important to give them in proper indications. Antacids may influence the complaints, but will not prevent lesions. The H_2 inhibitors (cimetidin, ranitidin, famotidin), the proton-pump inhibitor omeprazole given concomitantly with the NSAIDs will not inhibit the development of gastric lesions but reduce the duodenal disorders [2, 11, 21, 22]. Sucralfate has similar effects. Misoprostol seemed to be effective in preventing gastric and duodenal lesions [8, 14, 18, 19].

Two methods can be used to measure gastrointestinal bleeding. An indirect method is the ^{51}Cr labelled red blood cells i.v. injection, where the activity in the stool gives an estimated value. A direct method is the measurement of hemoglobin from the gastric juice. This sensitive method is very useful in clinico-pharmacological studies. The determination of hemoglobin in the gastric juice is a simple, high-sensitive, rapid, cheep method, and the advantage of it is the omission of radiolabelled materials.

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INFLUENCE OF SEX HORMONES ON ETHANOL-INDUCED GASTRIC HAEMORRHAGIC EROSIONS IN RATS

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The role of sex hormones in the pathogenesis of ethanol-induced gastric erosions was investigated following the recent observation that ethanol generates more severe gastric damage in male rats.

Female and male Wistar rats aged 110±6 days were used. Intact female, ovariectomized female, intact male, orchidectomized male and cyproterone acetate-pretreated (this compound a testosterone antagonist) male rats were investigated. 1 ml of 75% ethanol was used to induce gastric lesions. The extent of the erosions was determined planimetrically 60 min after ethanol administration. The plasma testosterone and 17-beta-oestradiol levels were checked by radioimmunoassay (RIA) in gonadectomized rats.

Ethanol generates more severe lesions in male rats. Orchidectomy and cyproterone acetate treatment each reduced the extent of ethanol-induced gastric erosions in male rats. Ovariectomy had no effect in this model. The plasma testosterone and 17-beta-oestradiol levels were significantly reduced after gonadectomy.

It is concluded that endogenous testosterone plays an aggressive role in the pathogenesis of ethanol-induced gastric erosions in rats.

Keywords: ethanol, gastric mucosa, gastric ulcer, sex hormones

Several observations suggest that sex hormones possibly play a role in the generation of various experimental ulcers. Restraint and activity stress induce more severe lesions in the female rat stomach [6, 7]. Pregnancy and lactation in rats markedly reduce steroid-induced gastric lesions [2]. We have recently demonstrated that ethanol generates more severe lesions in the stomach of male rats [3].

The aim of the present study was an investigation of the effect of sex hormones in the development of ethanol-induced gastric haemorrhagic erosions.

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Materials and methods

Female and male Wistar rats (aged 110±6 days) were used. Five groups of rats were investigated: 1. intact males, 2. intact females, 3. orchidectomized males, 4. cyproterone acetate-pretreated male (this compound, supplied by Schering AG, Berlin, Germany, is a testosterone antagonist; it was administered orally in a dose of 1 mg/100 g/day for 2 weeks), 5. ovariectomized females. Surgical interventions were performed under ether anaesthesia 2 weeks before the experiments. Each group contained 12–15 rats.

The rats were fasted for 24 h before the experiments. One ml of 75% ethanol was then injected orally via a gastric tube in each group. Sixty minutes after ethanol administration, the rats were killed by cervical dislocation, the stomach was removed, and the lesions were analyzed planimetrically. The ratio of the lesioned to the total mucosa was established, and expressed as a percentage to describe the extent of ethanol-induced lesions.

Plasma testosterone and 17-beta-oestradiol levels were checked after orchidectomy and ovariectomy by RIA methods described previously [9, 10].

Statistical comparisons were performed by the non-parallel Mann-Whitney U-test.

Results

75% ethanol generated more severe gastric mucosal damage in male than in female rats. Orchidectomy and cyproterone acetate pretreatment significantly reduced the extent of ethanol-induced gastric haemorrhagic erosions in male rats, while ovariectomy caused no change vs. intact females in this model (Fig. 1).

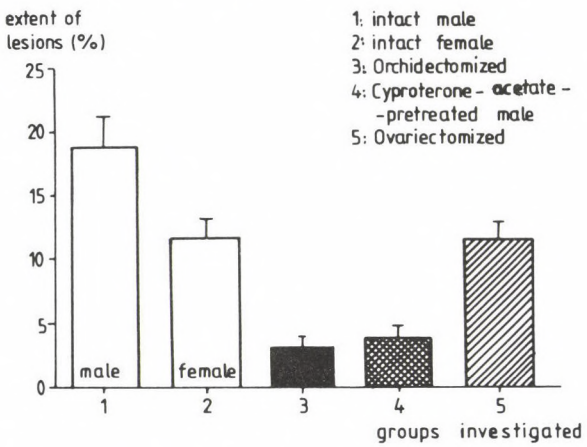

Fig. 1. The comparison of the extents of gastric mucosal lesions induced by the oral administration of 1 ml of 75% ethanol in intact male, intact female, orchidectomized male, cyproterone acetate-pretreated male and ovariectomized female rats. Each group consisted of 12–15 animals. Mean ± SEM; $p < 0.01$ between groups 1 and 2, 1 and 3, 1 and 4, 2 and 3, and 2 and 4

The plasma testosterone level reduced from 11.4 ± 1.6 nmol/l to 4.6 ± 0.5 nmol/l ($p < 0.001$) after orchidectomy, and the plasma 17-beta-oestradiol level decreased from 176.1 ± 10.7 pmol/l to 97.9 ± 4.1 pmol/l ($p < 0.001$) by ovariectomy.

Discussion

Orchidectomy and cyproterone acetate pretreatment were found to reduce ethanol-induced gastric haemorrhagic erosions in this model, whereas ovariectomy had no effect in accordance with the observation of Szabó et al. [8]. These results suggest that endogenous testosterone is of pathophysiologic importance in the generation of ethanol-induced lesions.

The recent study by Adeniyi [1] demonstrated that gastric acid secretion is reduced in orchidectomized rats relative to intact males. The decreased gastric acid secretion in castrated males is a possible explanation why ethanol generates less severe damage in this group.

Endogenous vasopressin plays a significant aggressive role in the generation of ethanol-induced gastric lesions [4]. Castration blocks vasopressin release and downregulates the number of vasopressin binding sites in the rat aorta [5]. It appears possible that orchidectomy protects the rat gastric mucosa via the blockade of the endogenous aggressor vasopressin.

It is concluded that endogenous testosterone plays an aggressive role in the generation of gastric mucosal erosions induced by ethanol.

Acknowledgements

This work was supported by the Hungarian Research Foundation (No. OTKA T 1406) and the Hungarian Academy of Sciences (No. AKA 88-0-556). The authors acknowledge the help of Dr. Gy. Falkay, Department of Obstetrics and Gynaecology, Szent-Györgyi Albert Medical University, for the determination of plasma testosterone and 17-beta-oestradiol levels.

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THE EFFECTS OF LOCAL HYPERTHERMIA ON THE MORPHOLOGY OF THE SMALL BOWEL

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Intestinal lesions were produced experimentally by heating the eventrated loop of the small intestine of Wistar rats in 44 °C 0.9% saline 20 minutes. Luminal haemorrhage and elevation of the potassium concentration in the wash-out fluid were registered from the fifth minute of the experiment. In the sediment of the wash-out fluid erythrocytes, degenerated and necrotic epithelial cell clusters were found. Histologically the mildest lesions were subnuclear vacuolization of the epithelial cells and stromal oedema of the villi. In the medium-degree lesion subepithelial cleft formation, desquamation of the epithelial cells and denudation of the villi occurred. The fully developed lesion was characterized by the destruction of the villi intestinales and haemorrhage. This lesion produced by local heating differs from the intestinal lesions of the rats exposed to high environmental temperature basically in the haemorrhagical nature of the former lesion.

Keywords: rat, small intestine, hyperthermia, necrosis

High environmental temperature induces intestinal lesions in the majority of the exposed rats [9, 10]. The rectal temperature of the animals in these experiments is highly elevated. The pathogenesis of this injury is not clear but the role of the elevated intestinal temperature has to be considered. By the modification of the model described by Shimizu, Maeta and Koga [7] we designed an experimental model for locally produced intestinal thermal injury. The local effects of the high temperature are in some aspects comparable with the lesions produced by high environmental temperature but in other aspects there are important differences between them.

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Materials and methods

Wistar rats, weighing 245 g in average were operated after 24 h food deprivation in general anaesthesia (Ketamin^R Parker – Davis Zdravlje was given in a single dose of 4 mg/kg intraperitoneally). The operation was carried out as longitudinal medial laparotomy (wound about 1 cm) and eventration of a loop of the small intestine in length of 4–5 cm. The loop was washed through by 0.9% NaCl (the temperature of the solution was 37 °C) and immersed in the same solution preheated to 44 °C. The exposure time was 20 minutes during which the temperature of the solution was thermostatically controlled. The loop was washed through again with 5 ml 44 °C saline in the fifth, the tenth and the twentieth minute of the experiment and the wash-out fluid was analyzed macroscopically, cytologically (smears from the sediment after centrifugation) as well as chemically on the potassium ion concentration (by flame photometer). Immediately after the experiment autopsy was carried out. The heated part of the intestine was taken, fixed in formaline for 18 h and routinely processed. Sections of 3–5 micrometers were stained by hematoxylin – eosin, Mallory and modified Giemsa methods. The control group of the rats was anaesthetized, operated and examined in the same way without exposure to heat of the intestinal loops.

Results

All the heat-exposed bowel loops were macroscopically and histologically changed. The loops got a dark red colour for 5 minutes which gradually increased later. The lumen of the loops was filled with bloody material.

The wash-out fluid was watery clear before the experiment but it became haemorrhagically red in the fifth minute of the heating. In the tenth minute the wash-out material was paler red and at the end of the experiment it became clear again.

In the cytological smears of the sediment of the wash-out fluid before the experimental heating elements of the chyme and rare epithelial cells could be identified. In the smears after the heating red blood cells, some neutrophiles and many degenerated and necrotic epithelial cells, singularly or in clusters were found (Figure 1).

The concentration of the potassium ions in the wash-out fluid before the heat exposure was 0.05 mmol/l (range 0.02–0.11 mmol/l), in the fifth minute 0.38 mmol/l (range 0.23–0.53 mmol/l), in the tenth minute 0.54 mmol/l (range 0.17–0.75 mmol/l).

The histological changes were evident but not of the same degree in the whole exposed intestinal loop. The mildest change consisted of subnuclear vacuolization of the epithelial cells of the villi intestinales and oedema of the stroma of the proximal half of the villi (Figures 2 and 3). Subepithelial cleft formation, desquamation of the epithelial cells and denudation of the villi are the characteristics of the medium-degree injury (Figure 4). The lumen of the bowel is filled with blood and epithelial cells with the signs of degeneration and necrosis. The fully developed lesion shows destruction of the villi and vasodilatation. Rupture of the small vessels with luminal

haemorrhage is a constant phenomenon but small regions of the extravasation of the erythrocytes in the stroma of the villi or in the muscle coat of the intestine can also be present. In some regions of the intestinal loop prominent vacuolization of the cytoplasm of the muscle cells and ganglion cells occur (Figure 5).

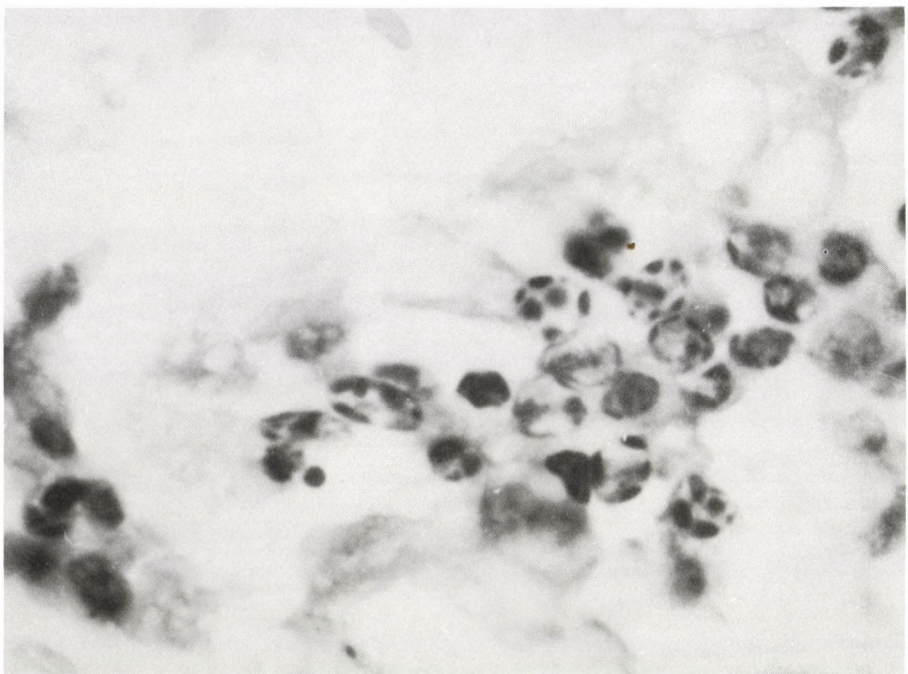

Fig. 1. Cytological smear of the sediment of the wash-out fluid in the tenth minute of the heat exposure: a cluster of epithelial cells showing cytoplasmic vacuolization, kariorhexis and lost cell borders. H-E 1000×

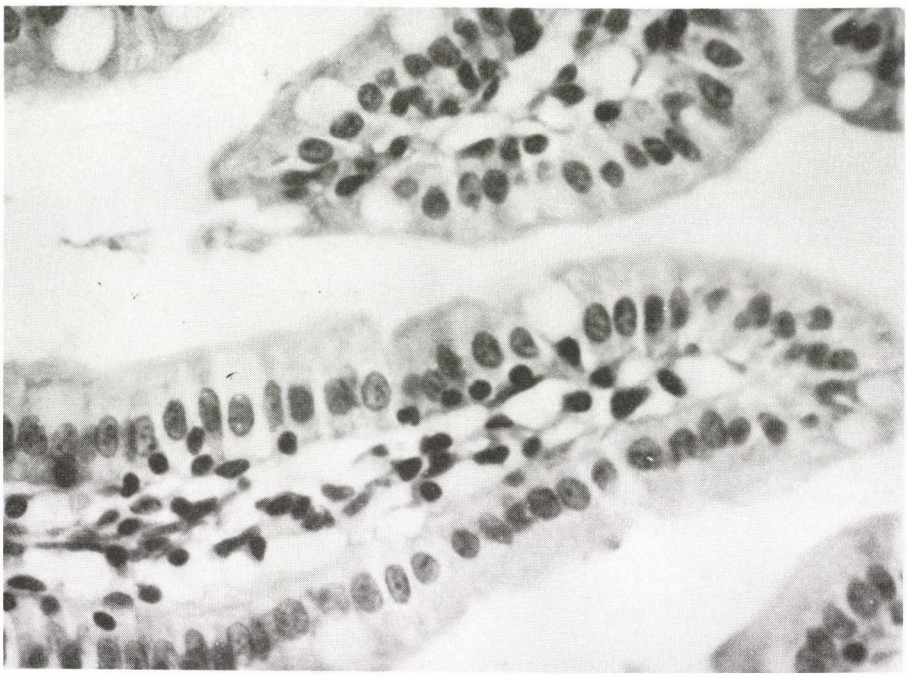

Fig. 2. Normal morphology of a villus intestinalis from a control animal (for comparison with Figure 3). H-E 400×

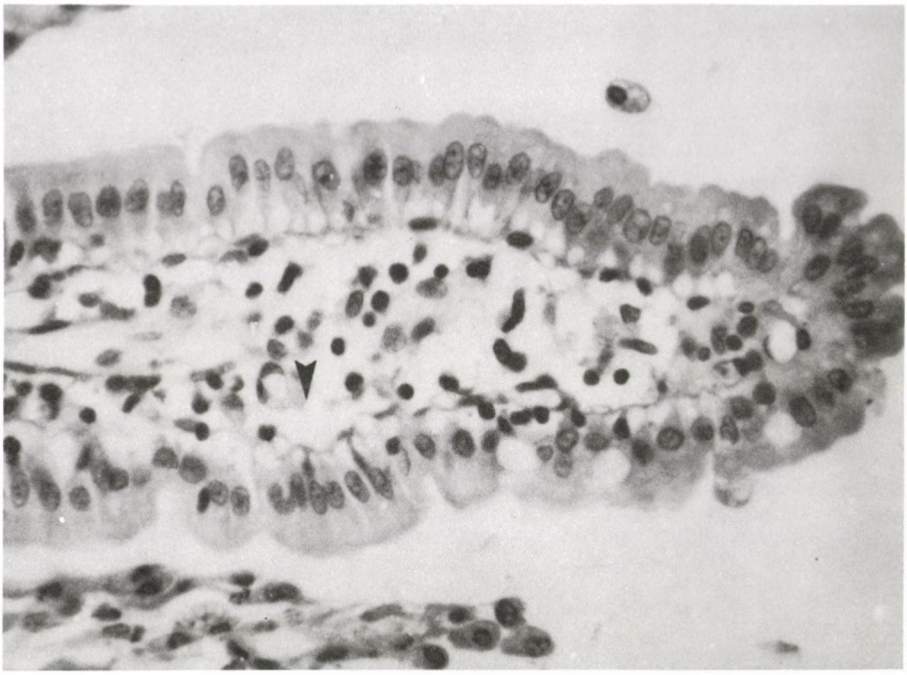

Fig. 3. Mild thermal injury: a villus intestinalis showing subnuclear vacuolization of the epithelial cells, a small subepithelial cleft (arrowhead) and stromal oedema. H-E 400×

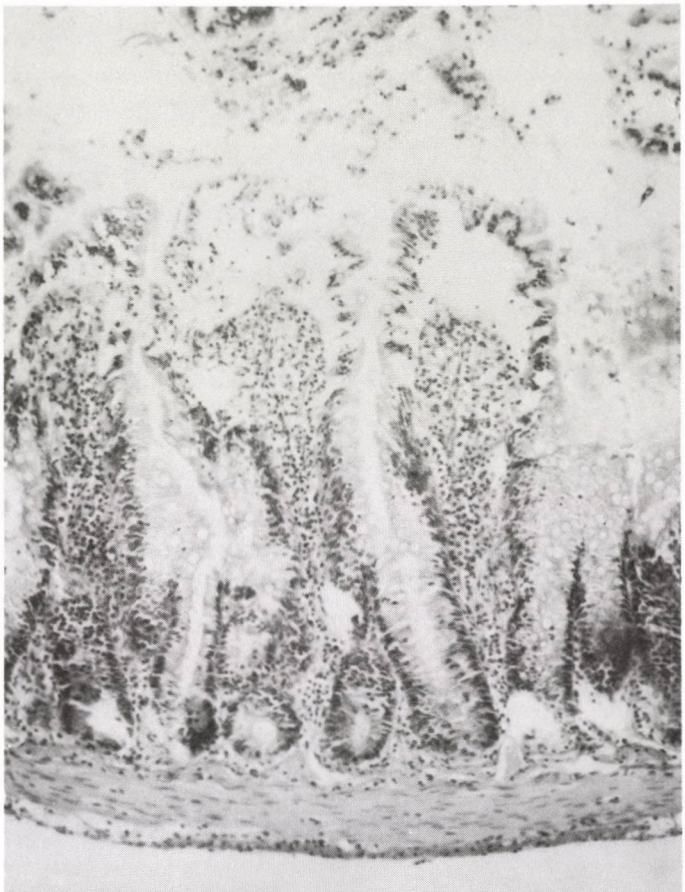

Fig. 4. Wide subepithelial clefts, desquamation of the epithelial cells and partially lost villous structure. H-E 200×

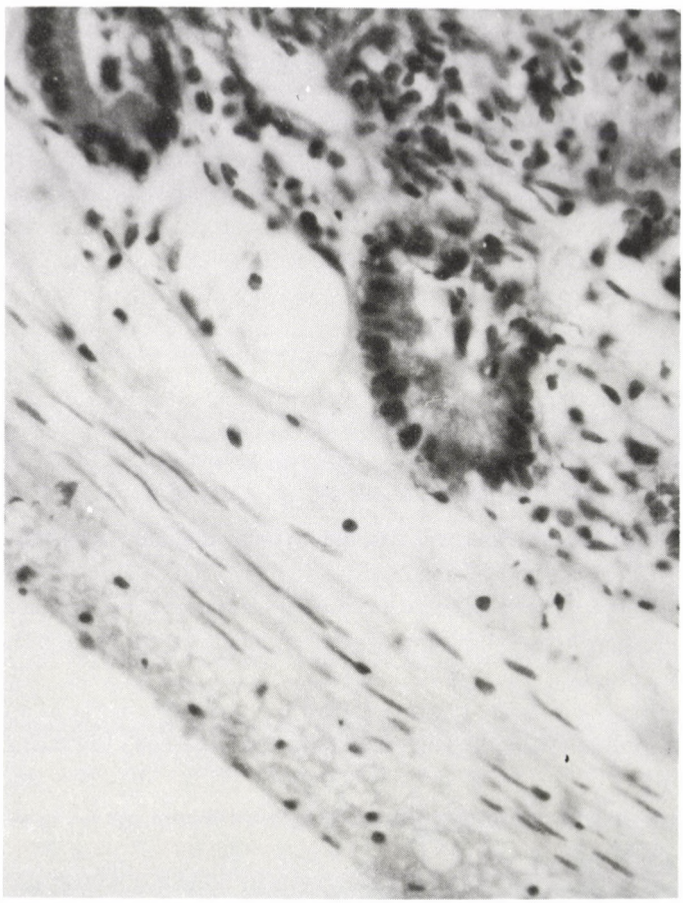

Fig. 5. Vacuolized smooth muscle cells in the outer layer of the muscle coat of the small intestine. H-E 400×

Discussion

Exposure of the small intestinal loop to local heat of 44 °C in duration up to 30 minutes is not lethal for the rats [7]. In agreement with this in our experiments with reduced exposure time all the animals survived the procedure. Although some authors referred about the protective effect of intraluminal fluid application [4] in the case of mesenteric occlusion, in our model this phenomenon was not present. It must be said that we used saline preheated on the temperature of the bowel loop.

Morphologically very similar lesions to our findings were produced by experimental shock [3], temporary ligation of the superior mesenteric blood vessels [4], reduction of the intestinal blood flow by a special pump [2] and by deep hypothermia [1]. Although the histological changes in the model of environmental heat exposure and the model of local heating of the bowel are very similar, there are some macroscopical differences. The bowel loop is pale yellow, dilated and filled with milkish white debris in the first model [9], it is dark red, non-dilated and filled with bloody material in the latter model. The basic difference is in haemorrhagic nature of the lesion in the case of local heat application. The histologically identified sequence of events in all the mentioned experiments as well as in our models is: subnuclear vacuolization of the epithelial cells, subepithelial cleft formation by coalescence of the vacuoles, epithelial shedding and autolysis of the denuded villi. In agreement with Wagner and other authors [11] we think that these lesions are nonspecific changes of the small intestine related to low flow state in the parts of the mesenteric vascular bed with consequent ischemia. Early microvascular changes in the first minutes of the heat exposure probably play the crucial role in the genesis of the ischemia [6, 8]. The role of the heat-shock proteins has also to be considered [5].

Some authors pointed out that the initial changes in the ischemic injury of the intestinal epithelium in the mentioned experiments, especially the occurrence of the subnuclear vacuoles of the epithelial cells, seems to be related to the hypoxic inhibition of the sodium pump of the absorptive cells with consequent "osmotic explosion" and influx of sodium ions and water into the cells [2]. Simultaneous eflux of potassium ions can be suspected. Our findings, evident elevation of the potassium ion concentration in the wash-out fluid after the fifth minute of the experiment, prove this hypothesis. Inspection of the collected wash-out fluid, the determination of the potassium concentration in it and the sediment cytology are rapid, reliable methods allowing the follow up of the intestinal injury and protection without resection of the intestinal loop.

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HISTAMINE RELEASE AND SOD, ALLOPURINOL AND RANITIDINE PRETREATMENT IN HAEMORRHAGIC SHOCK IN THE RAT

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Histamine release have been demonstrated in haemorrhagic shock. There are some observations that oxygen free radicals can cause histamine release. Oxygen free radicals play a role in the pathogenesis of gastric mucosal lesions. The goal of this study was to determine whether ranitinide or SOD and allopurinol pretreatment modify the histamine release during and after the haemorrhagic shock in the rat.

In the anaesthetized rat 0.1 N HCl was instilled into the stomach and the rat was bled to reduce the blood pressure to 30 mmHg for 20 min. The shed blood was reinfused. Twenty min later the stomach was removed. The area of gastric mucosal lesions were measured, histological grading was made. Blood samples taken from the carotid artery were examined by radioimmunoassay (IMMUNOTECH) to determine the plasma histamine level.

Plasma histamine level did not change significantly during the preparative surgery, but there was a significant increase of histamine level by the end of shock period. After the reinfusion of the blood the plasma histamine remained essentially at the same level for five min. Oxygen free radicals did not cause an important histamine release. By the end of the experiment the histamine level decreased dramatically.

Ranitidine, allopurinol and SOD pretreatment provided significant protection against the gastric mucosal lesions. Allopurinol and SOD did not influence significantly the histamine level. Ranitidine caused significant histamine release immediately after the injection and every histamine value was significantly higher in this group except for the final value which was lower than the control one.

The oxygen free radicals were not found as endogenous histamine releasers in this study.

Keywords: haemorrhagic shock, oxygen free radicals, histamine release, allopurinol, SOD, ranitidine

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Histamine release has been demonstrated in haemorrhagic shock [10]. Several experimental studies proved the association between haemorrhagic hypotension-reperfusion and the formation of gastric lesions [7, 11, 13, 15]. Oxygen derived free radicals arising from the increased peroxidation of lipid components of cellular and mitochondrial membranes play an important role in the pathogenesis of this phenomenon [8, 15]. The link between oxygen free radicals and histamine release [8, 9] has been established by *in vitro* system. A local histamine release [5] was found *in vivo* after intestinal ischaemia and reperfusion, and antioxidant pretreatment was effective against this histamine release. These data suggest that oxygen free radicals may play an important role of histamine release during and after the shock period.

The goal of this study has been to investigate in our shock model whether there is any histamine release after the reperfusion of the shed blood when the oxygen free radicals damage the membranes.

The next purpose of this study was to investigate whether the increased histamine level in the end of shock period and the possible new histamine release after the reperfusion can be modified or not with allopurinol, xanthine oxidase inhibitor (Ziloric, Wellcome Comp.), and superoxide dysmutase (SOD, from bovine liver, S 4761, Sigma Chem. Comp.) and a H2-receptor antagonist, ranitidine (Zantac, Glaxo).

Materials and methods

Animal preparation

Male Wistar rats (300–400 g) kept on normal laboratory diet were used. The animals were anaesthetized intraperitoneally with 40 mg/kg of pentobarbital sodium (Nembutal). A catheter was inserted into the left femoral artery for monitoring the blood pressure. Another catheter was placed into the right carotid artery to withdraw and reinfuse blood. The abdomen was opened and the cardia was ligated. Through the duodenum a tube was inserted into the stomach to do a proper lavage with physiological saline. 1 ml of 0.1 N HCl per 100 g body wt. was instilled into the stomach via the gastric tube. Subsequently the tube was removed and the pylorus was ligated.

Experimental procedure

In five minutes after the intragastric instillation of HCl blood was withdrawn from the right carotid artery over a 2 min period of time into a syringe containing 0.4 ml Heparin. The mean systemic blood pressure was reduced to 20 to 30 mmHg and it was maintained at the same level for 20 min by additional withdrawal of appropriate volumes of blood as required. Then the shed blood was reinfused. 20 min later the rat was killed by thoracotomy and the stomach was removed. One minute before killing 1 ml of 1% Evans blue was injected via the right carotid artery to enhance the contrast of gastric lesions. The stomach was opened along the greater curvature, pinned out on a cardboard and fixed in 10% formalin.

Blood samples for histamine measurements were taken from the carotid artery seven times: the first after the cannulation of carotid artery, the second after the preparative surgery, the third when the

systemic blood pressure was reduced to 25 mmHg, the fourth at the final second of shock-period, the fifth, sixth and seventh at the 1st, 5th, 19th min in the reperfusion period.

Measurement of gastric lesions

Photos were made after the formalin fixation from each stomach. After 3× magnification an independent person measured the areas of gastric lesions and the total gastric mucosal surface with grid (in mm^2). Statistical analysis (multiple variance + Scheffe test) was made by the Computing Centre.

Histological grading

The formalin fixed specimens stained with hematoxilin-eosin were used for grading of the lesions according to Arvidsson [3]. The mucosal lesions were graded 0–4.

Grade 0 = normal mucosa
Grade I = oedema just beneath the superficial epithelium
Grade II = disappearance of the surface epithelial cells
Grade III = damage to the upper half of the glandular cells of gastric crypts
Grade IV = disappearance of the glands

The gastric mucosal damage index was calculated as the sum of the maximum damage in the three fundic areas.

Drug studies

The animals were divided into seven groups.
Control group: no pretreatment
Ranitidine groups:
1 mg/kg
2 mg/kg pretreatment
4 mg/kg
8 mg/kg
Allopurinol group: 50 mg/kg pretreatment
SOD group: 20000 U/kg pretreatment

The different drugs were given at the same time when animals received the pentobarbital sodium intraperitoneally.

Measurements of plasma histamine levels

Histamine levels were determined in the control group and allopurinol, SOD, Ranitidine (8 mg/kg) pretreated groups. Arterial blood from carotid artery was taken into cooled centrifuge tubes and immediately centrifuged at 4 °C for 10 min with 4000 g (Kokusan H-500 R centrifugal instrument). The plasma was separated and stored below −20 °C a maximum of 3 weeks before analysis. Histamine plasma levels were determined by radioimmunoassay (IMMUNOTECH S.A., France). The detection was carried out by 1282 COMPUGAMMA LKB WALLAC instrument. Wilcoxon's rank sum test was carried out for estimation of stochastic probability of the intergroup comparison.

Results

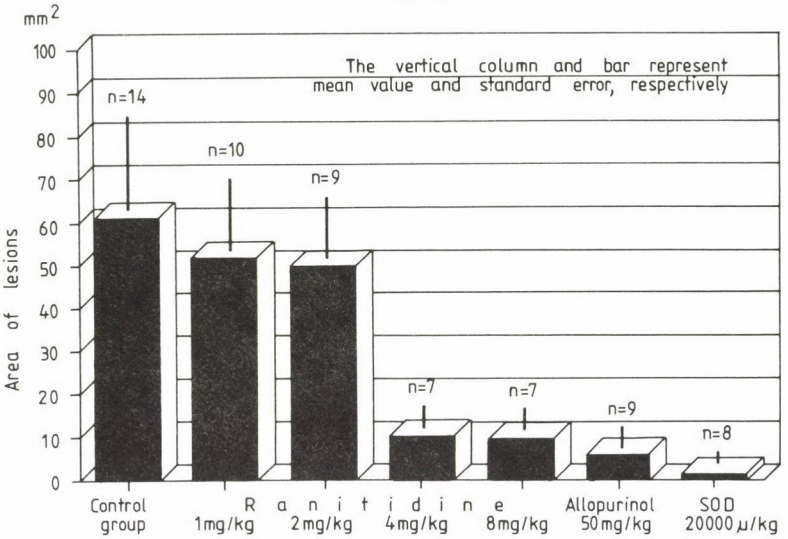

Fig. 1

Figure 1 shows the effect of different drugs on area of gastric lesions induced by haemorrhagic shock. The allopurinol, SOD and ranitidine (4 mg/kg and 8 mg/kg) pretreatment were effective against these lesions and the damaged areae were significantly smaller in these groups compared to the control group.

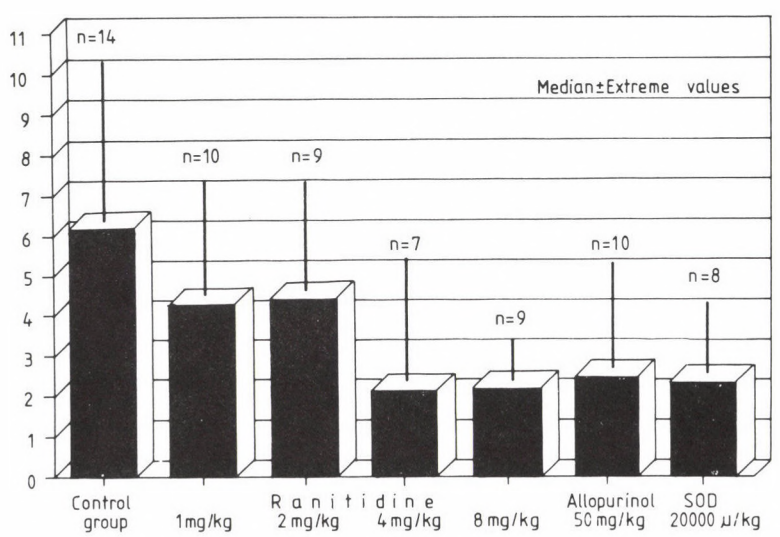

Fig. 2

Figure 2 shows the result of histological examination. The median values of mucosal damage index are as follows: control group: 6, ranitidine (1 mg/kg): 4, ranitidine (2 mg/kg): 4, ranitidine (4 mg/kg): 2, ranitidine (8 mg/kg): 2, allopurinol group: 2, SOD group: 2. These values also proved the protective effect of allopurinol, SOD and the high dose ranitidine in this shock model.

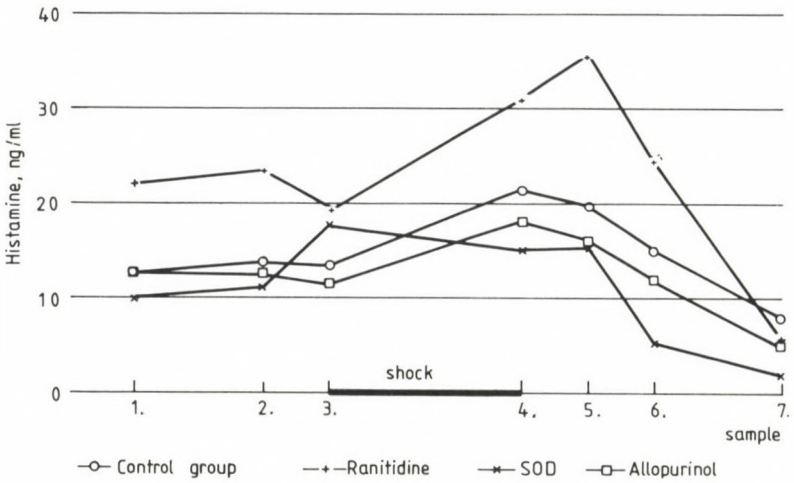

Fig. 3

Figure 3 shows the results of histamine measurements. Histamine levels were determined in the control group, and the allopurinol, SOD and ranitidine (8 mg/kg) pretreated groups.

In the control group the histamine level did not change significantly during the preparative surgery, but there was a significant increase of histamine level by the end of shock period. The histamine concentration in the plasma after the reinfusion of the shed blood remained essentially at the same level for five minutes, and later it decreased dramatically.

Allopurinol and SOD pretreatment did not influence significantly the values of histamine. The histamine plasma level in these groups were slightly smaller compared to the control but the difference was not significant. After the reinfusion of shed blood no histamine release was detected in these three groups. In the ranitidine pretreated group the plasma histamine values were different compared to the values of the control group. The ranitidine bolus injection caused significant histamine release immediately and every histamine level was significantly higher in this group compared to the control except for the final value which was lower than the control one. Beside the initial histamine release another release of histamine could be

observed by the end of shock period. After the reinfusion of shed blood a small increase of histamine level was found, but it was not significant.

Discussion

Oxygen free radicals play an important role in the pathogenesis of gastric mucosal lesions in hypotension-reperfusion model [7, 15]. These oxygen free radicals can cause histamine release in vitro system [8, 9]. Local histamine release [5] was found after intestinal ischaemia and reperfusion, and antioxidant pretreatment was effective against this histamine release in canine. The plasma histamine level of rats is high [1, 4] because of the low activity of histamine-methyltransferase and histamino-diamineoxidase of microvascular endothelial cells. In human normal level of plasma histamine is 0.2 – 1.4 ng/ml, but the level is 17 ng/ml in rats [1, 4].

The role of histamine and the regulation of histamine release [14] has been studied for many years and further investigation is necessary. It was published that there is histamine cytoprotection against gastric lesions induced by HCl in rats [12]. The mucosal protective action of histamine may be mediated by endogenous prostaglandins through stimulation of H2-receptors.

Histamine release has been demonstrated in haemorrhagic shock [10] in canine. The histamine curve is similar in this experiment. Significant histamine release is proved by the end of shock period. The reinfusion of shed blood was not followed by a further histamine release as it must have been anticipated when the oxygen free radicals damage the cell membranes. The oxygen free radicals were not found as endogenous releasers.

Allopurinol (xanthine oxidase inhibitor) and (superoxide dysmutase) SOD pretreatment were effective against the formation of gastric lesions, but they did not modify significantly the histamine curve.

Ranitidine, H2-receptor blocker was effective against the formation of gastric lesions induced by haemorrhagic shock at the doses 4 mg/kg and 8 mg/kg. The smaller doses, 1 and 2 mg/kg were ineffective. Ranitidine, H2-receptor blocker given intraperitoneally as a bolus (8 mg/kg) caused a histamine release immediately after the injection. This phenomenon was published earlier [6]. A new histamine release was observed by the end of the shock period, but there was no significant histamine release after the reperfusion of the shed blood. It was published [2] that in shock mortality rate of rats in the ranitidine pretreated groups were higher compared to the other groups. We observed a similar fact in the ranitidine group (4 mg/kg). The correct pathomechanism is unclear.

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CHOLINERGIC NEURONS ARE INVOLVED IN THE EFFECT OF SUBSTANCE P ON THE CIRCULAR MUSCLE OF THE GUINEA PIG SMALL INTESTINE

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The mode of action of the excitatory neuropeptide substance P was studied on the circular muscle of the guinea pig ileum *in vitro*. Atropine or tetrodotoxin strongly inhibited substance P-induced phasic contractions. The atropine-resistant part of the circular response was blocked by tetrodotoxin. A newly-developed method for quantitative evaluation revealed a rightward displacement of the substance P concentration-response curve, as well as a strong depression of the maximum effect, in the presence of atropine. These results indicate that cholinergic (and probably also non-cholinergic) excitatory neurons mediate phasic contractions due to substance P. The tonic component of the substance P-induced contraction was slightly reduced by atropine.

Keywords: substance P, guinea pig ileum, myenteric neurons, acetylcholine

The neuropeptide substance P (SP) occurs both in intrinsic and extrinsic neurons of the guinea pig small intestine (for reviews see [2, 6]). This peptide is likely to play a role in the peristaltic reflex, especially if cholinergic transmission is inhibited [2, 3, 5].

The circular muscle (CM) plays an important role in the enteric motor reflexes. This is the layer of the gut wall whose movements propel the contents in the aboral direction. Hence, the effects of SP on the CM deserve attention. The available data of the literature are contradictory, as far as the involvement of cholinergic neurons is concerned. SP has been reported to contract the CM of intact intestinal segments predominantly by activating cholinergic neurons of the myenteric plexus [4], whereas in a previous report SP-induced rhythmic CM contractions could not be influenced by tropicamide, a synthetic atropine-like drug [7], although a strong

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inhibition has been observed with tetrodotoxin (TTX). In these studies, qualitative or semiquantitative estimates of CM activity have been made.

The present study aimed at investigating the possible role of endogenous acetylcholine in the contractile effect of SP on the guinea pig ileal CM. To reach firm conclusions, we developed a method of quantifying drug-induced phasic CM contractions. An attempt has also been made to clarify the nature of the tonic component of the SP-induced response.

Materials and methods

Guinea pigs of either sex were stunned and bled. Isolated ileal preparations were fitted in a similar way as described earlier [7]. Approximately 2-cm segments were pulled over a rod of 1 mm diameter. For recording CM activity a cotton string was fixed to the mesenteric attachment in the middle of the segments. The preparations were placed, in a horizontal position, into organ baths containing 10 ml of oxygenated Tyrode solution of 37 °C. CM contractions were recorded by means of low-friction isotonic transducers on a compensograph (MTA-Kutesz, Budapest, Hungary), under a tension of 5 mN (0.5 g). At most four preparations were taken from each animal. After 30 min of incubation the amplitude of the maximal CM spasm (totally obstructing the lumen) was determined with the aid of cholecystokinin octapeptide (CCK-8, 100 nM), added for 1 min to the bath. Twenty minutes of rest was then allowed to the preparations. Gut segments showing spontaneous CM activity were not used in these experiments.

Two experimental protocols were employed. In the first one, preparations were exposed to increasing concentrations of SP (each for 3 min), at appropriate time intervals to avoid desensitization (15 min). This procedure was then repeated in the presence of atropine (0.5 μM).

The other protocol was used for avoiding the possible error resulting from the long duration of experiments. Pairs of preparations (obtained from adjacent segments of intestine) were used, one of them being continuously incubated with atropine (0.5 μM) from the point of time when CCK-8 was washed out. These preparations were then exposed to increasing concentrations of SP; 3–4 exposures were carried out at intervals of 15 min. A similar procedure was also performed with TTX (1 μM).

For quantitative evaluation, each phasic contraction was compared to the possible maximal spasm (determined with the aid of CCK-8). The possible maximal spasm (referred to hereafter as one "circular contraction unit", CU) was measured from the actual baseline; this was necessary because tonic contraction was also present (see Fig. 1). Thus, the maximum value for each phasic contraction was 1 CU. The amplitudes of the phasic contractions were then summed up for the whole 3-min exposure to SP (contractions not exceeding 0.1 CU were not considered). Tonic contractions were expressed as % of the maximal circular spasm.

Drugs used were: atropine sulfate (Merck); cholecystokinin octapeptide (Sigma); substance P (Peninsula); tetrodotoxin (Sigma).

Quantitative results are expressed as mean ± SEM. Statistical significance of differences was assessed by using Student's *t*-test for independent samples.

Results

In agreement with earlier data [4, 7] substance P caused phasic contractions, superimposed on a tonic contraction (Fig. 1). Atropine (0.5 μM) markedly reduced, but usually did not abolish phasic contractions due to SP. In the presence of atropine, the amplitude of the phasic contractions consistently decreased and only exceptionally reached the maximal spasm. In 2 out of 7 preparations atropine practically abolished phasic contractions to SP. Increasing the concentration of atropine from 0.5 to 1.5 μM did not increase its effects, indicating that the lower concentration was sufficient to block the available muscarinic receptors (n=3). Atropine-resistant phasic contractions were abolished by TTX (1 μM; n=5). The tonic contraction to SP was apparently less affected by atropine or atropine plus TTX.

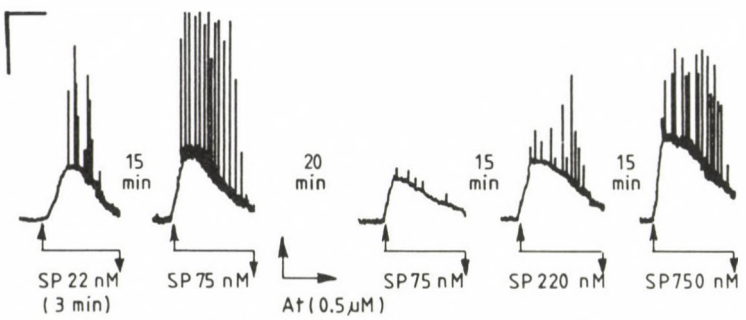

Fig. 1. Circular contractions the of guinea pig ileum due to substance P (SP; concentrations as indicated), before and after the application of atropine (At). Calibrations (top left); horizontal, 1 min; vertical, 10% of the maximal circular spasm obstructing the lumen (note that many contractions in response to 75 nM of substance P in the control period reached this height)

Since the duration of these experiments was too long, we used the second experimental protocol described in the Methods for quantitative evaluation.

Concentration-response curves of SP on adjacent gut segments clearly show a significant inhibitory effect of atropine on phasic contractions due to SP, which manifests itself as both a rightward displacement in the curve and a depression of the maximum effect (Fig. 2). In the presence of atropine, at least a 10-times higher concentration of SP was needed to obtain similar CU value as in the absence of the drug, if it was possible at all. In control segments, the concentration producing half-maximal effect was about 50 nM. The effect seemed to reach its maximum at 220 nM both in the absence and in the presence of atropine. Tonic contractions were slightly smaller in atropine-treated segments than in control ones; the difference was statistically significant at two out of three concentrations of SP (Fig. 2).

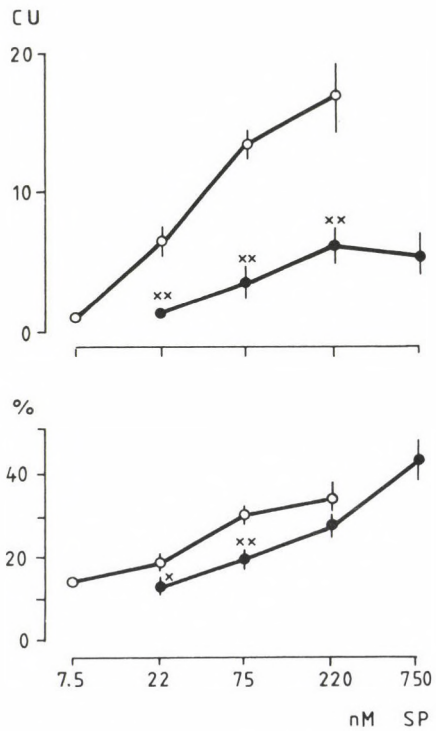

Fig. 2. Phasic (upper panel) and tonic (lower panel) circular contractions of the isolated guinea pig ileum in response to substance P (SP). Phasic responses are expressed in circular contraction units (CU) for the whole 3-min exposure to substance P, tonic responses as % of the maximal circular spasm (obstruction of the lumen), determined by means of cholecystokinin octapeptide. Open symbols, control gut segments. Closed symbols, preparations treated with atropine (0.5 μM). Mean ± SEM are given. Asterisks mark statistically significant differences; * – $p < 0.05$, ** – $p < 0.001$ (Student's *t*-test for independent samples). Number of experiments n = 8–15 (control segments); n = 9–14 (atropine-treated segments)

Tetrodotoxin very strongly inhibited phasic contractions evoked by SP (Table I). Tonic contractions were slightly smaller in the TTX-treated preparations but the difference did not reach statistical significance.

Table I

Effects of tetrodotoxin on phasic and tonic circular muscle responses to substance P

Substance P	Tetrodotoxin	Phasic contractions (CU)	Tonic contractions (% of maximum)
22 nM	– –	6.9 ± 1.3	21.1 ± 2.8
75 nM	– –	15.1 ± 1.9	31.8 ± 3.7
75 nM	1 μM	1.0 ± 0.7**	23.8 ± 2.1
220 nM	1 μM	3.4 ± 1.8	29.2 ± 3.1
750 nM	1 μM	6.6 ± 1.1	33.8 ± 4.8

Number of experiments n = 6 in all groups. ** $p < 0.001$ compared to the control group.

Discussion

Our findings with atropine suggest that cholinergic neurons play an important role in the action of SP on the CM of the small intestine. However, a substantial atropine-resistant component of the effect of SP was also noted (cf. [4, 7]). The strong inhibitory action of TTX, found in the present study, confirms neuronal contribution to the SP-induced response [4, 7], even in the presence of an antimuscarinic drug [7]. Inhibition by TTX of the atropine-resistant contractile effect of SP probably indicates activation of intrinsic tachykinin neurons of the myenteric plexus. Only a small component of the SP-induced phasic contractions seems to take place on the smooth muscle directly. It should be noted that the contractile action of SP appeared in higher concentrations than those necessary to cause longitudinal muscle spasm. However, CM strips of which the mucosa and submucosa have been removed were reported to be more sensitive to SP than whole segments [4], moreover, the site of action of the peptide appears to be on the CM directly in mucosa-free preparations. Thus, the direction from which SP reaches the CM may be decisive as to its mode of action.

The tonic component of the SP-induced response was invariably present in our recordings and was more resistant to the effect of atropine or TTX. This component received little attention in previous studies because it cannot be excluded that longitudinal muscle activity is also partly involved. However, concentration-response relationships, together with the significant inhibitory action of atropine seem to indicate a predominant role of the CM in this response. Tonic contractions increased with the concentration of SP (see Figs 1 and 2) in a concentration range where SP causes maximal longitudinal spasm, irrespective of whether atropine is present or not, in the bath (cf. for example [2], where 0.7–3 nM SP evoked half-maximal longitudinal contractions).

While this study was in progress, another type of CM preparation was described and characterized as to tachykinin sensitivity [8]. In narrow strips of guinea pig ileum (corresponding to only one opened ring of the gut) the presence of all three neurokinin receptor types (NK_1, NK_2 and NK_3) has been demonstrated by using receptor selective agonists and antagonists. Only activation of NK_3 receptors led to neuronally-mediated response that partly involved acetylcholine.

In conclusion, the present findings suggest multiple sites of action of SP in the CM of the guinea pig ileum, involving stimulation of myenteric cholinergic and, probably, non-cholinergic neurons, as well as direct excitation of the smooth muscle. The quantitative method of evaluating phasic CM activity described in this study seems to be more accurate than previously-reported semiquantitative approaches, which allows deeper insight into neurotransmitter mechanisms involved in phasic CM responses.

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THE OXYGEN-CENTERED RADICALS SCAVENGING ACTIVITY OF SULFASALAZINE AND ITS METABOLITES. A DIRECT PROTECTION OF THE BOWEL

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Oxygen-centered radicals, such as superoxide (O_2^-) and hydroxyl radicals (·OH) generated by phagocytes have been suggested to be involved in the pathogenesis of chronic inflammations of the bowel, such as Crohn's disease and colitis ulcerosa. Recently, sulfasalazine (SASP) and its metabolites have been reported to exert their effects as a direct scavenger of oxygen-centered radicals in the bowel. To scavenge oxygen-centered radicals *in vivo*, however, SASP and its metabolites have to react with O_2^- and/or ·OH *in vitro* very rapidly, furthermore they have to reach an appropriate (possible millimolar) concentration range at the site of inflammation. To test this possibility, we investigated the direct O_2^- and ·OH scavenging activity of SASP and its metabolites using the specific electron paramagnetic resonance/spin trapping method, and we compared the 50% inhibition rates of SASP and its metabolites with their known concentrations in the bowel and in the human plasma. It was found that SASP and its metabolites, such as 5-amino-salicylic acid (5-ASA), and acetyl-5-amino-salicylic acid (AC-5-ASA), but not sulfapyridine (SP) and acetyl-sulfapyridine (Ac-SP) have a direct O_2^- and ·OH scavenging activity *in vitro* systems. Among the compounds, SASP and 5-ASA can reach a concentration which is appropriate to scavenge oxygen-centered radicals in the bowel but not in the human plasma. It was concluded that the *in vivo* anti-inflammatory effects of SASP and its metabolites are, at least partly, due to the direct oxygen-centered scavenging activity of these drugs.

Keywords: hydroxyl radicals, oxygen centered radicals, superoxide, sulfasalazine, inflammatory bowel diseases

Sulfasalazine (SASP) is the first drug of choice in the treatment of chronic inflammatory bowel diseases such as Crohn's disease and colitis ulcerosa [6]. The

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orally taken SASP reaches the colon almost intact where it is converted by bacteria into sulfapyridine (SP) and 5-aminosalicylic acid (5-ASA) (Fig. 1) [11]. The mode of action of SASP and its metabolite has been a matter of dispute. 5-ASA, the active metabolite of SASP, was shown to exert its effect by diminishing the epithelial cell loss in the colon [3]. SASP and its metabolites have been shown to reduce the production of prostaglandins and/or leukotrienes [4, 16], to block the release of deoxyribonucleic acid from epithelial cells and to suppress the generation of oxygen-centered radicals (OCR) by polymorphonuclear leukocytes (PMN) [8, 9]. However, none of these effects alone is unique to SASP and 5-ASA. The major argument against the hypotheses listed above was that neither the other inhibitors of prostaglandin synthesis [2] nor the other anti-inflammatory drugs having a blocking effect on OCR generation of PMN [7, 10], could be effectively used in the treatment of inflammatory bowel diseases.

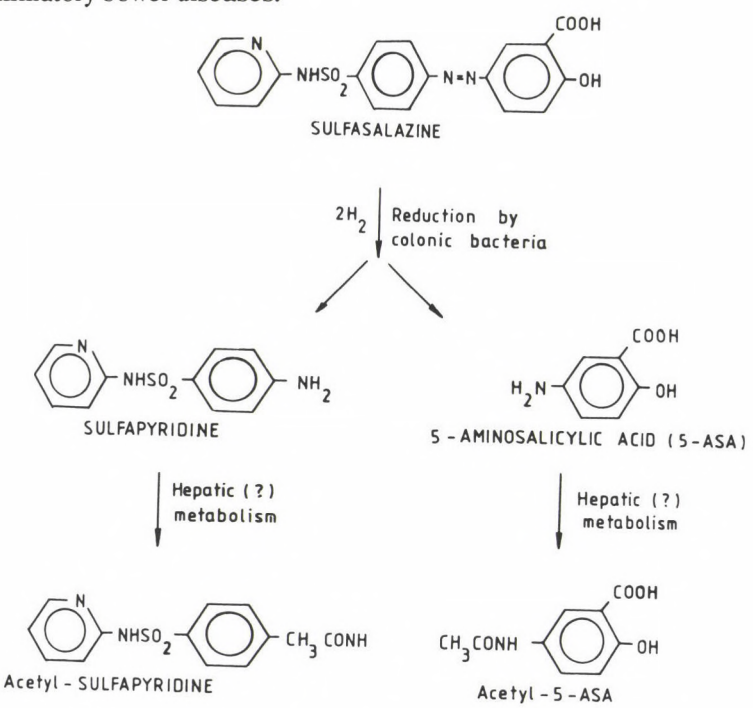

Fig. 1. The fate of orally administered sulfasalazine in the human body

As a further way of action of SASP and metabolites, a direct OCR scavenging activity of these agents has been suggested to play an important role in the action mechanism of the drug [3, 8, 15]. To effectively scavenge OCR, however, any drug has to reach an appropriate concentration at the site of inflammation and it has to

have a high reactivity with OCR. On the other hand, because of the high reactivity and short half-life of OCR, it is necessary to use specific and sensitive methods for correct evaluation of these reactions. Since the methods used in the previous studies were indirect and the results were based on spectrophotometric assays where the possible direct effects of SASP and its metabolites on the OCR generating systems themselves could not be studied [3, 8, 15], we re-evaluated the OCR scavenging activity of SASP and its four metabolites such as 5-ASA, SP, acetyl-5-aminosalicylic acid (Ac-5-ASA) and acetyl-sulfapyridin (Ac-SP), using the specific, electron paramagnetic resonance/spin trapping method. In addition, the direct effect of SASP and its metabolites on the OCR generating systems themselves was also studied.

Methods

Chemicals

SASP and its metabolites were obtained from Seikagaku Kogyo Co. Ltd., Tokyo, Japan. They were dissolved in 1 N NaOH and then adjusted to pH 7.4 with 1 N HCl. Xanthine oxidase (XO) was obtained from Boehringer-Mannheim GmbH (Mannheim); hypoxanthine (HT) and desferrioxamine (DFO) from Sigma Chem Co. (St. Luis); 5,5-dimethyl-pyrroline-N-oxide (DMPO) from Shonan Analytic Center (Tokyo).

Measurement of superoxide and hydroxyl radical scavenging activities (SSA and OH-SA)

Superoxide and hydroxyl radical scavenging activities (SSA and OH-SA) were measured as described by Prónai et al. [12–14]. Briefly, O_2^- or ·OH generated in the HT/XO or Fe/H_2O_2 systems can be trapped by DMPO and forms DMPO-OOH or DMPO-OH spin adducts. The heights of DMPO-OOH of DMPO-OH spin adducts correlate with the amount of O_2^- or ·OH generated, and can be decreased by any scavenger in a competitive manner. The samples contained hypoxanthine (0.5 mM), or $Fe(NH_4)_2(SO_4)$ (2.5 mM), DFO (0.5 mM or 0.1 mM), and DMPO (0.67 and 1.34 M or 10 and 100 mM) in phosphate buffer (pH 7.4, final volume 0.2 ml). The reaction started by the addition of XO (final 0.05 U/ml) or H_2O_2 (1 mM) to the systems, respectively. For the measurement of SSA or OH-SA of the compounds, a 0.05 ml sample (diluted in PBS) was used instead of 0.05 ml of phosphate buffer. The reaction mixture then was stirred for 3 seconds and transferred into a flat quartz cell (JEOL Co. Ltd., Tokyo, Japan; 0.18 ml). All spectra were recorded 40 sec after the addition of XO or H_2O_2. The height of DMPO-OOH or DMPO-OH was measured and compared with that of the internal standard, manganese peak. EPR spectra were recorded on a JES-FE 2XG (JEOL Co. Ltd., Tokyo) with field set at 355 ± 5 mT, modulation frequency of 9.42 GHz, modulation of 0.125 mT, amplitude of 1000, response of 0.3 sec, sweep time of 2 min and microwave power at 8 mV. Experiments were performed in triplicate and results were expressed as mean ± SD.

The principle of true SSA and OH-SA

The background to the method described by Prónai et al. [14] is as follows: When an OCR scavenger (Scav) and DMPO are present in an OCR generating system, Scav and DMPO scavenge OCR competitively according to Eqs (1) and (2):

$$\text{DMPO} + \text{OCR} + \text{H}^+ \xrightarrow{k_D} \text{DMPO-spin trap adduct} \quad (1)$$

$$\text{Scav} + \text{OCR} \xrightarrow{k_S} \text{Product} \quad (2)$$

In case: the concentrations of DMPO and Scav are much higher than that of OCR, pseudo-first-order rate constants, k_D and k_S in Eqs (1) and (2), can be defined and the following equation can be easily derived:

$$I_0/I = 1 + (k_S/k_D)([\text{Scav}]/[\text{DMPO}]) , \quad (3)$$

where I_0 and I are the signal intensity of DMPO spin adduct without and with Scav, respectively, and [Scav] and [DMPO] are the initial concentrations of Scav and DMPO, respectively. Thus, the OCR scavenging activity of Scav can be assayed by utilizing a linear correlation between I_0/I and [Scav].

Since any Scav may also directly affect the OCR generating system, it is important to separate this effect of Scav from its true OCR scavenging effect. For this purpose, it is necessary to use at least two different concentrations of DMPO (10 and 100 mM) in this system. The compound with true OCR scavenging activity (SSA and/or OH-SA) should have a similar slope of regression line between I_0/I and S/D. Therefore, the OCR scavenging activity of Scav can be estimated by the following modified equation:

$$I_0/I = (1 + rS)(1 + k/k_0\, S/D) , \quad (4)$$

where "r" is the inhibition coefficient of Scav against the OCR generating system. The intensity ratio (I_0/I) is proportional to S/D and k/k_0. Scav can be estimated from the slope of regression line between I_0/I and S/D. If the Scav, however, also inhibits the OCR generation in proportion to the concentration of the substrate, the linearity between I_0/I and S/D is disrupted.

To make it simple, if the I_{50} concentration of Scav is proportional to the two different concentrations of DMPO, the compound has a true OCR scavenging activity (SSA and/or OH-SA).

Results

The 50% inhibition OCR scavenging rates (I_{50} SSA and I_{50} OH-SA) and the reported *in vivo* concentrations of SASP and its metabolites in the bowel and in the plasma are listed in Table 1. Both SASP and its metabolite, 5-ASA had a true SSA in cell free system, and SASP and its metabolite, Ac-5-ASA had a true OH-SA in cell free system.

Table I

Oxygen-centered radical scavenging activity of SASP and its metabolites determined by EPR/spin trapping method

SASP and metabolites (mM)	50% inhibition rate (I_{50})				Reported concentrations (mM)		Direct effect on the system	
	SSA		OH-SA		bowel [11]	plasma [5]		
	Concentration of DMPO (mM)							
	670	1340	10	100			SSA	OH-SA
SASP	34	70	9	80	0.1–2*	0.05	–	–
5-ASA	6	13	2	6	2–30*	0.006	–	+
Ac-5-ASA	0.6	26	2	17	–	0.04	+	–
SP	–	–	–	–	1–5	0.02	–	–
Ac-SP	20	120	–	–	–	0.05	+	–

* The reported concentration is in the range of *in vitro* OCR scavenging activity

We also separated the true SSA and/or OH-SA from the direct effects of SASP and its metabolites on the OCR generating systems. Ac-5-ASA and Ac-SP had a SSA, but they also affected the O_2^- generating system itself, whereas 5-ASA had an OH-SA but it affected the ·OH generating system. The effects of these metabolites on the systems themselves would not mean that these compounds could not scavenge OCR, but the results obtained should not be directly compared with that of compounds without such a direct effect.

The other metabolite, SP had no SSA in cell free system. Neither SP nor Ac-SP had OH-SA in cell free system (data not shown).

Discussion

We have shown with specific methods for O_2^- and ·OH radicals that SASP and most of its metabolites have a remarkable OCR scavenging activity in cell free systems. Our studies in general reconfirm the findings of Ronne et al. [11] and Aruoma et al. [1] who have shown the free radical scavenging effect of the drug. However, we also could distinguish in between the clear (true) scavengers and those affecting the OCR generating systems. In addition, we could clearly separate the SSA of SASP and its metabolites from that of the OH-SA of the drugs. The fact that the

acetylated metabolites of SASP, Ac-5-ASA and Ac-SP affect the O_2^- generating system does not necessary mean that these compounds would not scavenge O_2^- effectively, but makes the quantitative comparison between the different metabolites impossible. Since the previous reports used a similar OCR generating system, a cautious evaluation of the results presented is necessary.

As about the specificity of SASP and its metabolites for the different OCR, 5-ASA has the strongest SSA, whereas Ac-5-ASA has the strongest OH-SA. The other metabolites, SP and Ac-SP would not account for the *in vivo* OCR scavenging activity of SASP. The observation that 5-ASA and Ac-5-ASA are stronger OCR scavengers than that of SASP has its particular interest, since these metabolites were shown to mediate the *in vivo* effect of SASP [1–3].

Since OCR-mediated reactions are chain-reactions and proceed very rapidly, in order to effectively block these reactions, it is very important for any OCR scavenger to be present at the time and site of OCR generation in an appropriate concentration. In other words, from a clinical point of view it is important whether the drug reaches the *in vivo* effective concentration in human fluids and tissues. At present only a few drugs are known having a direct *in vivo* SSA and/or OH-SA. Particular drugs with *in vivo* OH-SA had to reach a millimolar concentration range in human fluids and tissues [1]. Based on earlier reports showing the exceptional high, millimolar local concentration of SASP and 5-ASA in the distal part of the bowel [11], it seems reasonable that these compounds act as direct OCR scavengers in the bowel. On the other hand, neither SASP nor its metabolites reached an appropriate concentration in human plasma to scavenge OCR effectively [5].

Whether the direct OCR scavenging ability of SASP and its metabolites and/or the fact that only SASP but not the other anti-inflammatory drugs can reach a millimolar concentration in the bowel what is the extra effect needed for the effectiveness in the treatment of inflammatory bowel diseases, remains to be determined.

Acknowledgement

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PATHOGENESIS AND MANAGEMENT OF ALCOHOLIC LIVER INJURY

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The present paper is devoted to overview the basic concepts of ethanol-induced hepatic injury and therapeutic modalities by which alcoholic liver disease can be alleviated.

The role of alcohol dehydrogenase of both hepatic and gastric origin as well as the importance of the number one metabolite acetaldehyde are discussed, furthermore the effects of microsomal ethanol oxidizing system are also described. The features of the major clinicopathological consequences of alcohol abuse fatty liver, alcoholic hepatitis are briefly outlined, and the basic pathogenetic mechanisms that lead to cirrhosis – cell necrosis, regeneration and fibroplasia – are shown.

The understanding of the pathophysiology of alcohol-induced liver injury may improve the therapy with drugs and nutritional factors, and allow successful prevention through the early recognition of heavy drinkers before their social or medical disintegration. In the management of alcoholic liver diseases, among the true hepatoprotective agents a naturally occurring flavonoid silymarin and an active methyl-donor metabolite S-adenosyl-L-methionine seem to be promising. An antifibrotic treatment with colchicine might also be of importance. Further prospective, well-designed, controlled clinical trials are still warranted to evaluate real efficacy of these drugs.

The hepatic consequences of alcohol abuse may be treatable, however, prevention would be the true resolution of the major global health problem of alcoholism.

Keywords: alcohol, metabolism, toxicity, cirrhosis, hepatoprotection

There are two major global health problems regarding the severe liver injury: while in the Southeast Asia, China, Africa and Mediterranean Europe about 300 million hepatitis B virus carriers live at risk for cirrhosis and hepatocellular carcinoma, in the Western world and Europe, including Hungary, alcohol abuse is responsible for most of the liver diseases.

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Alcohol abuse costs the U.S. more than 116 billion dollars, per year of which about 12 per cent is for direct costs of medical care. Alcohol accounts for about 100.000 deaths per year in the U.S. In Sweden, the premature deaths caused by alcoholism were as frequent as deaths resulted from cancer or coronary artery disease [45]. In Hungary, six thousand persons die annually due to liver cirrhosis and most of them have alcoholic liver disease.

This paper is aimed to review the pathogenesis and management of alcoholic liver injury.

I. Metabolism and hepatotoxicity of ethanol

Until 25 years ago it was believed that liver disease of alcohol variety is due to *malnutrition* and not to direct toxic effects of alcohol itself. Alcoholic cirrhosis was called "fatty nutritional cirrhosis". Charles Best wrote: "there is no more evidence of a specific toxic effect of pure ethyl alcohol upon liver cells than there is for one due to sugar" [5].

During the last three decades however, many experimental studies proved that ethanol can also affect the liver independently of malnutrition, that is the view of *Best* was displaced by the concept of *direct* toxicity of ethanol. Thus now the ethanol-induced hepatic, nutritional and metabolic abnormalities are well known, and malnutrition whether primary and secondary, has been differentiated from direct toxicity, attributed to redox changes, acetaldehyde, hypoxia, direct membrane alteration of effects on microsomal induction [20] (Fig. 1).

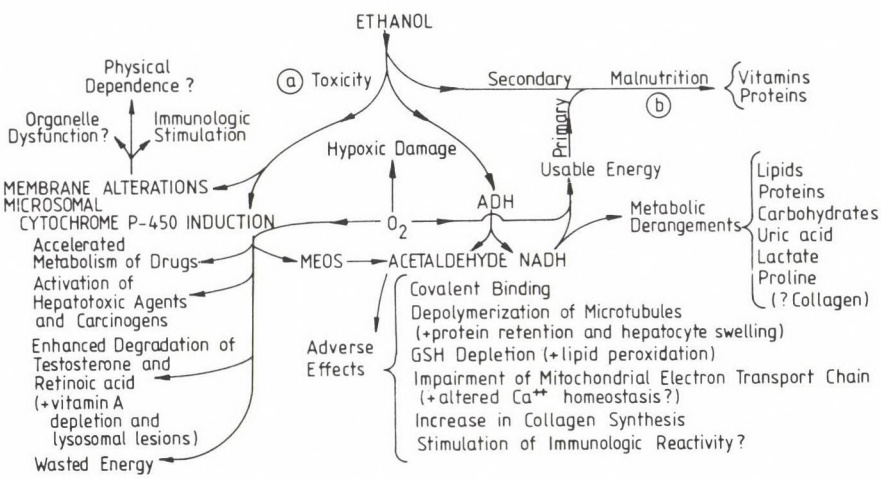

Fig. 1. Hepatic, nutritional and metabolic abnormalities after ethanol abuse. Malnutrition whether primary or secondary has been differentiated from direct toxicity (Lieber, 1984. Ref. 20)

The hepatocyte contains more *pathways for ethanol metabolism,* each is located in different subcellular compartment:

1. *alcohol dehydrogenase* (ADH) functions in the cytosol, while

2. *microsomal ethanol oxidizing system* (MEOS) and ethanol-inducible cytochrome P450IIE1 in the smooth endoplasmic reticulum, and

3. *catalase* in peroxisomes.

The oxidation of ethanol results in production of acetaldehyde which in turn is metabolized by aldehyde dehydrogenase to acetate (Fig. 2).

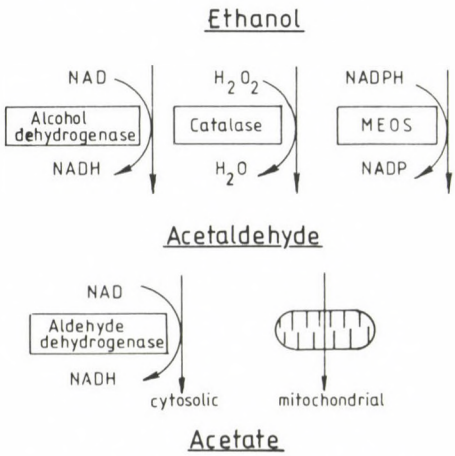

Fig. 2. Pathways of ethanol metabolism

1. *Alcohol dehydrogenase* in the hepatic cytosol is responsible for the 80% of alcohol oxidation and *acetaldehyde* formation, mostly at low level of ethanol and during short-term administration. This enzyme catalyzes conversion of ethanol to acetaldehyde in a reaction which requires NAD, thus ethanol-oxidation generates *reducing equivalents* such as free NADH in hepatic cytosol. These *redox changes* are exacerbated mostly in perivenular areas, where a normally existing low oxygen tension may aggravate ethanol toxicity [20].

At the same time, *lactate*/pyruvate ratio is elevated which will be the one of the key factors in the initiating of fibrotic processes, dominantly in the mentioned perivenular (centrilobular) zone 3 areas [36].

Gastric alcohol dehydrogenase may also be of importance in the pathogenesis of alcoholic liver injury. As ADH in gastric mucosa plays a role in the *bioavailability* of ethanol, gastric ADH represents a "first line barrier" to the penetration of ethanol: it can modulate ethanol toxicity.

The gastric barrier of ADH may be physiologically *low in females* as compared with males, this increased bioavailability can explain the increased susceptibility to

ethanol toxicity in females, a well-known phenomenon [12]. On the other hand, the gastric barrier of ADH can be lost during a long-lasting ethanol-consumption, that will further enhance the toxicity. Thirdly, gastric ADH can also be decreased due to H_2-receptor antagonists, that is *cimetidine* treatment may result in increased bioavailability of ethanol [22].

Acetaldehyde, as the main toxic product of ethanol metabolism has a central role in the alcoholic liver injury. (Its level can be elevated due to either an increased production or diminished elimination by aldehyde dehydrogenase.)

The *main effects of ethanol and acetaldehyde* can be listed as follows:

a) induction of membrane lipid peroxidation
b) impairment of hepatic glutathione synthesis
c) immune reaction to cell membrane proteins
d) impairment of microtubules and secretory function
e) abnormal mitochondrial oxidation of fatty acids
f) alteration of fluidity of plasma membranes.

a) Lipid peroxidation

Lipid peroxidation is discussed in details by Fehér's paper, presented elsewhere in this issue. Briefly, metabolism of ethanol results in various biologically active *free*

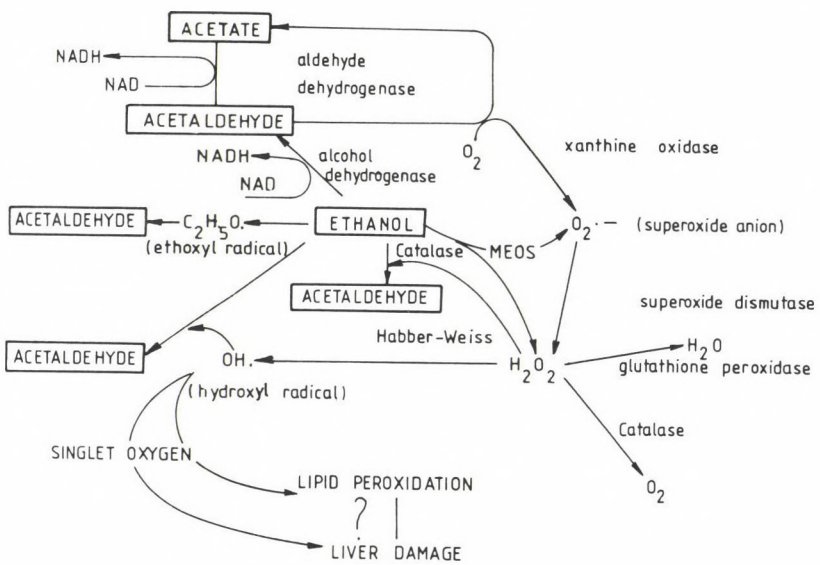

Fig. 3. Metabolism of ethanol, free radicals and scavengers in liver injury

radicals, which leading to a chain-reaction can play a basic role in tissue damage. Acetaldehyde can serve as a substrate of the superoxide-producing enzyme xanthine oxidase, which reduces molecular oxygen to superoxide. Oxygen free radicals mainly the *hydroxyl radicals* attack polyunsaturated lipids, forming conjugated diene hyperoxides. Lipid peroxidation affect biological membranes causing loss of membrane integrity, thus cell injury and finally cell deaths [22] (Fig. 3).

As an end-product of lipid peroxidation, thiobarbituric acid reactive substance (malondialdehyde) was found to be elevated in the sera of patients with chronic active (agressive) hepatitis and those with alcoholic hepatitis, but not in patients with inactive (persistent) hepatitis. When patients with chronic alcoholic hepatitis were treated with an antioxidant, radical scavenging flavonoid hepato-protective agent cianidanol, the elevated serum TBA-reactive substance level was gradually normalised [29] (Fig. 4).

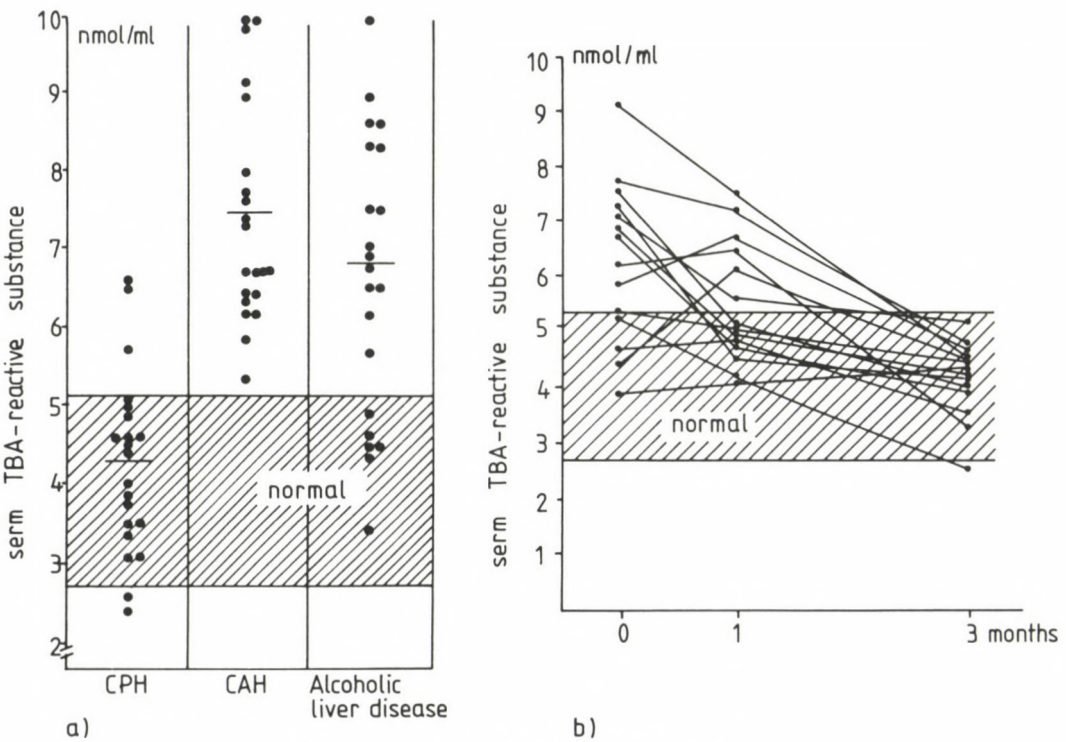

Fig. 4. Lipid peroxidation in chronic liver disease
a) serum thiobarbituric acid (TBA)-reactive substance (malondialdehyde) levels in different forms of chronic hepatitis: CPH = chronic persistent hepatitis. CAH = chronic active hepatitis.
b) Changes in serum TBA level during the course of chronic active/alcoholic hepatitis

b. Glutathione depletion

Acetaldehyde binding cysteine and glutathione results in a depression of reduced glutathione (GSH) thus diminishing the free radical scavenging effect of GSH, and further enhancing lipid peroxidation. During alcohol consumption an enhanced GSH turnover leads to a decrease in scavenging enzyme GSH-peroxidase as well [22].

In the blood of patients with chronic alcoholic hepatitis a decreased activity of GSH-peroxidase and catalase has been found at the same time, an elevated serum TBA-reactive substance (malondialdehyde) content was shown [25] (Table I).

Table I

Lipid peroxidation (malondialdehyde = MDA) and "scavenger enzymes" in the blood of patients with chronic hepatitis

	Serum		RBC hemolysate	
	MDA nmol/ml	GSH-Px U/g prot	GHS-Px U/g prot	catalase B.U./g
Control (n = 30)	3.9 ± 1.2	1.3 ± 0.6	1.7 ± 0.4	108.2 ± 33.2
Chr. active hepatitis (n = 10)	5.9 ± 1.3	0.6 ± 0.4	1.8 ± 0.8	82.5 ± 16.4
Chr. alcoholic hepatitis (n=6)	7.5 ± 2.2	0.4 ± 0.2	1.0 ± 0.3	75.7 ± 5.0

(RBC = red blood cell)

c. Altered immune reactions

Acetaldehyde protein adducts form and can evoke immune response, and altered cytoskeleton structures can also function as *neoantigens*.

Immune reaction develops when the release of cytokines promotes inflammation and fibrogenesis (see later).

The hepatocyte membrane damage – as non-specific activation – may stimulate neutrophil leukocytes to produce further super-oxide anions.

All these processes contribute to the aggravation or perpetuation of alcoholic liver injury [22, 28].

The following indices can reflect the *derangement of the immune system* in patient with chronic alcoholic liver disease.

a) humoral factors increased serum immunoglobulins specially IgA elevation, antinuclear, anti-smooth muscle antibodies, antibodies to liver specific protein (LSP), antibodies to acetaldehyde-related "neoantigens" or to alcoholic hyaline.

b) Cell-mediated immune reactions: lymphocyte transformation to alcoholic hyaline, circulating cytotoxic cells, enhanced expression of MHC class I molecule due to alcohol.

We have found *liver-specific autoantibody anti-LSP* in 58 per cent of patients with alcoholic hepatitis, the same was found in 81 per cent of patients with autoimmune hepatitis. Anti-LSP is regarded as a key factor in the liver-specific antibody dependent K-cell cytotoxicity reaction, an accepted tissue-damaging immune mechanism [6]. Non organ-specific autoantibodies, such as antinuclear, anti-smooth muscle and anti-gammaglobulin antibodies also occurred in our patients with alcoholic hepatitis and cirrhosis (Fig. 5).

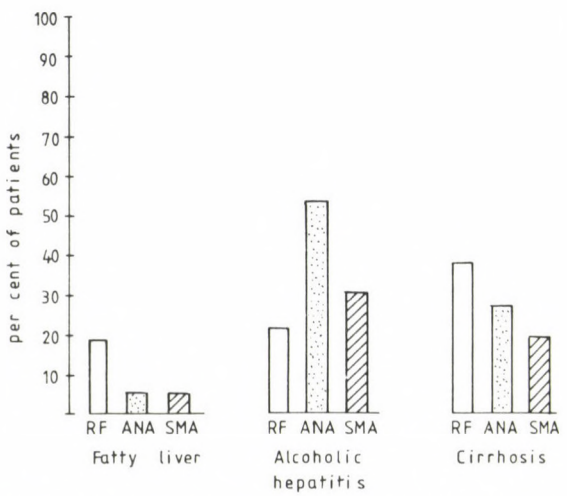

Fig. 5. Autoantibodies in alcoholic liver diseases ANA = antinuclear antibody, SMA = anti-smooth muscle antibody RF = rheumatoid factor (anti-gammaglobulin antibody)

d) Impairment of microtubules and secretory function

Acetaldehyde binding cysteine can cause a decrease in the amount of tubulin, resulting in a diminished polymerization of microtubules leading to the *impairment of the secretory function* of the cell, that is to "the constipation of the cell" (Popper). An accumulation of proteins (albumin, transferrin, fatty-acid binding protein), lipids, electrolytes and water in the cell causes swelling ("ballooning") of hepatocytes, an increase in their volume of more than 10-fold, that reduces intercellular space ending in a (reversible !) portal hypertension (ascites !) even in this early *fatty liver* stage of alcoholic liver disease [4].

Acetaldehyde binding DNA/RNA causes a failure of cell replication, while in interaction with arachidonates or essential fatty acids, it can alter the prostaglandin synthesis as well.

e) Ethanol and acetaldehyde impair mitochondrial functions

The hepatic oxygen uptake decreases, abnormal cristae develop and the swelling of mitochondriae (megamitochondriae) can also be seen. An impairment fatty acid oxidation occurs.

f) A decreased fluidity of plasma membranes

develops as an adaptation to the initial fluidizing effect of ethanol [22].

Microsomal ethanol-oxidizing system (MEOS) and ethanol-inducible cytochrome P450IIE1 play a key role in the metabolism of ethanol, after high dose, long-term consumption of alcohol, when the proliferation of microsomal membranes (smooth endoplasmic reticulum), the activation of this adaptive system of oxidation ("enzyme induction") occur. – In such circumstances, this system is responsible for the increased metabolism of ethanol. The *specific fraction* of MEOS activity is due to the ethanol-inducible P450IIE1 [22, 26]. The ethanol-inducible cytochrome P450IIE1 when activated, enhances not only the metabolism of alcohol, but results in the *"metabolic activation"* of other *xenobiotics* as well, leading to toxic metabolites and free radicals.

The increased metabolism of various steroids and vitamins can occur, thus e.g. *vitamin A stores* may be diminished by means of this way [22]. In hepatic tissue of patients with alcoholic liver disease decreased vitamin A and vitamin E contents were found, while malondialdehyde level was elevated (Table II).

Table II

Lipid peroxidation (malondialdehyde = MDA) and "scavenger vitamins" in the hepatic tissue of patients with alcoholic liver disease

	MDA nmol/g	Vitamin A U/g	Vitamin E U/g
control (n = 6)	13.3 ± 7.9	101.9 ± 53	0.112 ± 0.05
alcoholic liver disease (n = 4)	22.6 ± 17.8	39.8 ± 31	0.083 ± 0.05

The *most important xenobiotics* metabolized through P450IIE1 are the following: *industrial solvents* such as carbontetrachloride, benzene, *drugs* like INH, Rifampicin, paracetamol, anaesthetic agent Halothane, warfarin, propranolol, *carcinogens*, e.g. aflatoxin and nitrosodimethylamine.

During the *acute ethanol intoxication*, however, at the *presence of alcohol*, by a direct competition for a common metabolic process, ethanol will inhibit the metabolism of xenobiotics. Their blood level may remain unchanged, and the toxicity related to the parent drug increases, while toxicity due to metabolites may decrease [19] (Fig. 6).

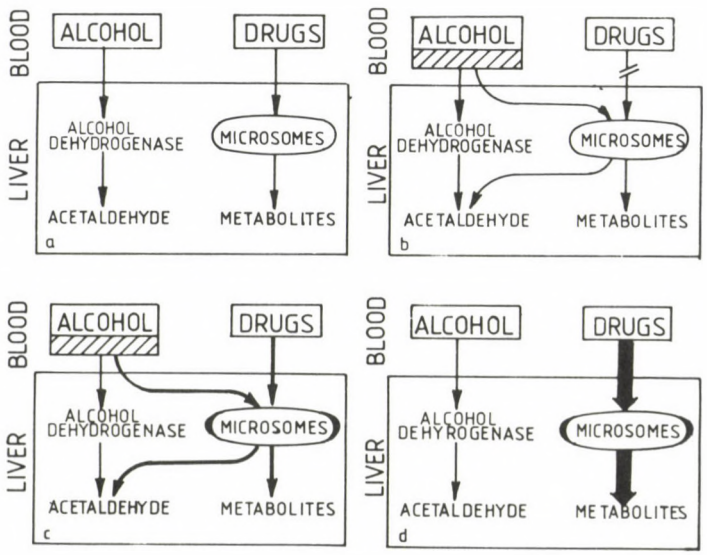

Fig. 6. The role of microsomal ethanol oxidizing system (MEOS) in the metabolism of ethanol and xenobiotics
a) alcohol is metabolized by alcohol dehydrogenase and drugs by microsomes
b) microsomal drug metabolism in inhibited in the presence of high concentrations of ethanol, in part through competition for a common microsomal detoxification process
c) microsomal induction after long-term alcohol consumption contributes to accelerated ethanol and drug metabolism at high blood ethanol levels
d) increased drug metabolism and activation of xenobiotics (due to microsomal induction) persists after cessation of long term alcohol consumption. ▨ = high alcohol levels
(Lieber, 1988, Ref. 22)

In conclusion, the various influences of alcohol on liver metabolism and its pathophysiological consequences are shown in Fig. 7.

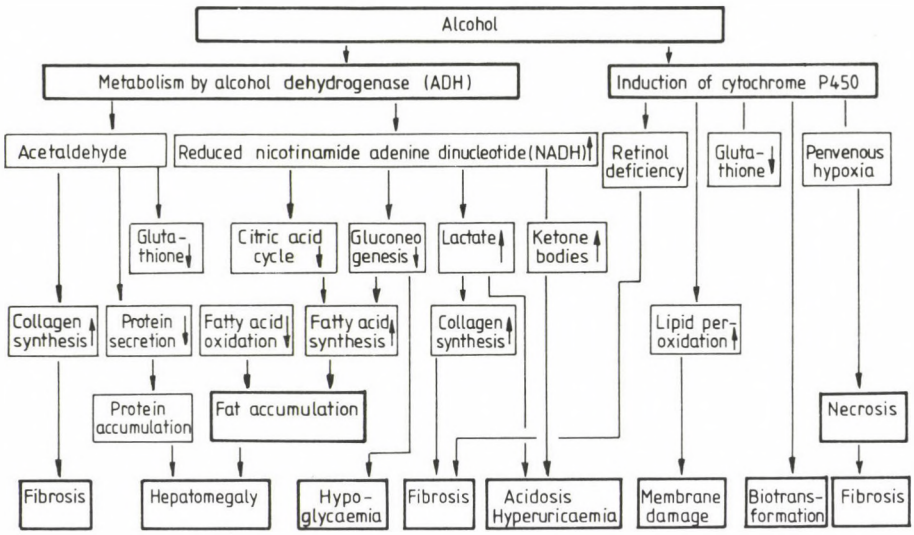

Fig. 7. Influence of alcohol on liver metabolism and its pathophysiological consequences (Schölmerich, Holstege, 1990. Ref. 37)

II. Clinical forms of alcoholic liver injury and the pathogenetic mechanisms that lead to cirrhosis

Alcohol abuse leads to three forms of liver disease: fatty liver (steatosis), alcoholic hepatitis and cirrhosis. Each of them can be regarded as distinct clinical entity, although patients frequently have combinations of these three lesions, so that clinical features tend to overlap.

The signs and symptoms of liver injury are generally result of hepatocellular necrosis and/or complications of portal hypertension, often both of them.

Fatty liver is the most common abnormality of hepatic morphology in the alcoholic populations. In one series of asymptomatic alcoholics biopsy showed that 33 per cent had fatty livers, but this value presumably may be much higher, even 50–60 per cent.

Fatty liver means macrovesicular fat accumulation, triglyceride containing vacuoles in liver tissue, as a result of the impaired mitochondrial fatty acid oxidation and defective lipoprotein secretion from the hepatocyte.

Although the commonly accepted upper limit of non-toxic daily dose of alcohol is 60–80 g in men and 20–30 g in women, our findings suggested that fatty liver developed in males after about 5 years of 100–120 g/day and in females after 2–3 years of 60 g/day alcohol consumption respectively. Fatty liver patients frequently have no clinical or biochemical evidence of liver injury, hepatomegaly

however, as only sign may be present in about 70% of patients. Seldom jaundice, fluid retention, splenomegaly can be found, serum bilirubin, AST/GOT and gamma-GT may be elevated in one third of patients with fatty liver.

Fatty liver can be considered as a *reversible* condition, and it is assumed not to be the precursor of cirrhosis. Yet, if *perivenular fibrosis* is present, a progression may occur, as this histological alteration may be a reliable marker of the increased risk for developing cirrhosis [43].

Alcoholic hepatitis or acute sclerosing hyaline necrosis is a histopathologic diagnosis, consisting of fatty changes, hepatocyte necrosis, infiltration with polymorphonuclear leukocytes, fibrosis and aggregates of cytosceletal intermediate filaments. The process is a true *precursor of cirrhosis*. Patients may be asymptomatic or may be jaundiced and critically ill with fever, leukocytosis and hepatic encephalopathy. Five-year survival varies between 50–70%. Alcoholic hepatitis can be seen in men after a 5–10-year ethanol consumption of 100–120 g/day, and in women after 2–3 years of 60–80 g/day, respectively.

Cirrhosis is the third stage of alcoholic liver injury. The micronodular or portal cirrhosis consists of the triad of cell necrosis + regeneration + fibroplasia. It may coexist also with fatty changes and/or alcoholic hepatitis. Alcoholic cirrhosis may develop in males after 15–20 years of 100–120 g/day, and in women after 8–10 years of 60–80 g/day alcohol consumption. Interestingly, only 60% of cirrhotic patients have symptoms referring liver injury, that is about 40% of cases are discovered accidentally or at autopsy. Hepatomegaly in advanced disease may even be absent (small liver!), while the splenomegaly is common as indicator of portal hypertension. Hypoalbuminaemia and low protrombin level as well as high serum bilirubin well reflect the severity of the condition. Five-year survival may vary between 34–70% depending on the presence of portal hypertension and on abstinence. The terminating complication of alcoholic liver disease may be the hepatocellular carcinoma.

Basic pathogenetic mechanisms in alcoholic cirrhosis

The hepatocellular *necrosis*, the *regeneration*, and the *fibroplasia* are the main characteristic features of cirrhosis which account for the final functional aberrations, disturbance of hepatic microcirculation and portal hypertension.

1. Hepatocellular necrosis

The necrosis is the basic factor, which is responsible for the initiation of cirrhosis events. (Necrosis may be absent in the developed stage, but occurs at least sometime during development.) It has significance for understanding evolution of

cirrhosis, morphological indication of etiology, reflection of activity and explanation of consequences [31]. *Cell necrosis is a continuous process*, progressing in several steps to cell death. The process of necrosis really is not a simple continuous cascade, but rather it consists of a *network of multiple pathways* and feedback loops.

The mechanisms of hepatic necrosis

Initiating factors can there be as follows: *free radicals*, toxic *oxygen species*. (Active hydroxyl radicals, superoxide and peroxides are resulted from many metabolic processes, including the action of the cytochrome P−450 system). Furthermore, formation of some *eicosanoids*, cellular *iron* mobilization or the activation of xanthine oxidase in ischemia may also be of importance in the initiation. An excess of toxic products may be generated either with in hepatocytes, or in macrophages or neutrophil leukocytes.

There are multiple *targets of toxic radicals* or oxygen species in hepatocytes: macromolecules, proteins, nucleic acids and phospholipids may be peroxidated by chain reactions.

The increase of cytosolic ionized calcium is an important signal for many metabolic processes. *Calcium excess* caused by increased hepatocyte uptake from the extracellular space, or by release from organelle depots is associated with hepatocellular degeneration and cell death.

Depletion of the reduced tripeptide *glutathione* is also of significance in hepatic necrosis.

Each of the various pathogenetic factors react with each other in a complex network of interacting key events, that undergo both amplifications and feedback inhibition. *The injury of the plasma membrane is the common terminal pathway* initiated by any of the factors listed above. The membrane may also be a primary target either by abnormal activation of enzymes (phospholipases) or by immunological or other cell-induced reactions. Neutrophils and macrophages can "burn" the membrane by oxidative bursts, lymphocytes may poison it by secretion of cytokines such as tumor necrosis factor (TNF), while eosinophils may plug a hole as do various toxins and complement. Lipopolysaccharides from endotoxins can stimulate phagocytes to release potent mediators, particularly prostanoids.

Consequences of necrosis are the following: collapse of hepatic parenchyma, regeneration that is formation of nodules, which by exerting pressure may interfere with drainage of hepatic blood which results in postsinusoidal hypertension, furthermore fibroplasia, which can be stimulated by peptides released from injured hepatocytes or from lymphocytes or monocytes (activated by hepatocyte injury). Fibroplasia may induce necrosis by fibrocompression: a vicious circle develops.

2. Regeneration

As was mentioned above, the loss of hepatocytes results in sequence of events leading to regeneration [16]. There are some *experimental models* for liver cell regeneration: e.g. galactosamine hepatitis and the *partial hepatectomy in rats*. This latter operation can induce a synchronized proliferation of hepatocytes and later Kupffer cells and endothelial cells. This compensatory hyperplasia of the remaining liver leads to restoration of the original liver mass within 1–2 weeks. Proliferation of the liver cells is also induced by acute liver injuries associated with disseminated necrosis such as galactosamine hepatitis, carbon tetrachloride injury or viral hepatitis.

Partial hepatectomy is followed by regeneration only, whereas viral or chemical injuries may lead to partial regeneration as well as to fibrosis and cirrhosis.

Humoral factors as initiating signals for hepatocyte proliferation are:

1. epidermal growth factor, inzulin, glucagon – all *initiate DNS synthesis* in hepatocyte.

2. hepatopoietin, vasopressin and triiodothyronine *stimulate proliferation of the liver cells*. The platelet-derived beta-type transforming growth factor means an *inhibitory* signal. There must be a balance between factors promoting or inhibiting hepatocyte proliferation.

When a massive loss of hepatocytes, that is a sudden decrease in functional parenchyma occurs in man (e.g. due to alcoholic damage !) similar events start and a disseminated necrosis will stimulate regeneration. However in man, not only partial nodular regeneration but fibrosis and finally cirrhosis will follow the necrosis. During an active regeneration of hepatocytes, collagen synthesis and degradation are also occurring. Both of these processes are coordinated and return to a normal state upon completion of regeneration. When the injury is small but sustained, the stimulus will not suffice to induce regeneration rather production of connective tissue and scar formation.

Common features of regenerative process

The interaction of before mentioned growth factors with plasma membrane receptors will be followed by activation of a signal transduction pathway and it leads to an enhanced expression of genes with products enabling cell proliferation [40], (Fig. 8).

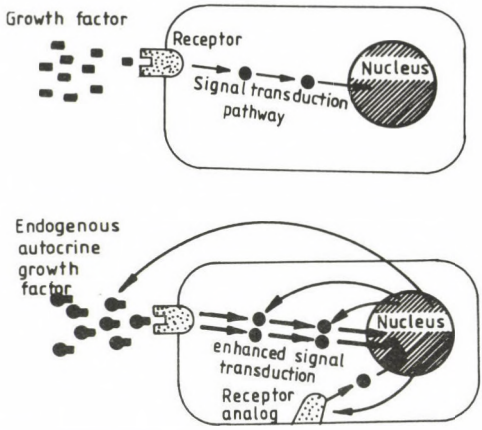

Fig. 8. Growth factor-induced mitogenesis. Action of a growth on its receptor (upper scheme) may lead to endogenous production of growth factors (lower scheme) stimulating the cell further by interaction with growth factor receptors on the cell surface, and/or leading to the synthesis or activation of receptor analogues and/or enhancing the components of the signal transduction pathway. (Keppler, 1987, Ref. 16)

The products of these growth-related genes include:

1. substance which enhance the signal transduction pathway
2. endogenous growth factors acting in an autocrine fashion
3. receptor structures responsible for the transduction of signals to the nucleus [14].

Proto-oncogene expression is a basic feature. Liver regeneration can be defined as a "reprogramming of gene expression" with transient quantitative changes in the expression of relative few genes. The changes are reversible and gene expression is only quantitatively altered [40, 42].

Concentrations of messenger RNAs for the proto-oncogenes c-fos, c-myc, p^{53} increase after partial hepatectomy. The first wave of cell division is preceded by a programme associated with quantitative changes in gene expression.

There are changes in regulatory metabolites and enzyme activities.

Diadenosine tetraphosphate (Ap_4A) is a signal molecule which binds to RNA-polymerase and acts as primer for DNA synthesis after the loss of liver cell [44].

Polyamine metabolism also shows major changes [15, 42]. The polyamines play a role in cell proliferation, their synthesis precedes the onset of DNA synthesis. In this process ornithine-decarboxylase (DOC) is a key enzyme, as it converts ornithine to putrescine, a polyamine, which stabilizes RNA.

Changes in nucleoside and nucleotide metabolism also occur during regeneration. Increased uptake of the pyrimidine nucleotide precursors, orotate and thymidine, and uridine uptake into the liver was seen after partial hepatectomy.

In carbohydrate metabolism an increase in gluconeogenic capacity may occur in the regenerating liver [15].

Table III summarizes the sequence of events during regeneration of rat liver after partial hepatectomy.

Table III

Sequence of events during regeneration of rat liver after partial hepatectomy

	Partial hepatectomy or hepatocellular necrosis
	↓
	Increase in circulating factors promoting liver growth
	↓
< 15 min	Entry of orotate, uridine, thymidine increased
	↓
< 30 min	Expression of growth-related proto-oncogenes (c-fos, c-myc)
	↓
2 h	Increase in activity of anabolic enzymes (e.g. ODC)
	↓
4 h	Increased influx through biosynthetic pathways (e.g. synthesis of nucleotides)
	↓
18 h	DNA synthesis, ras gene expression, histone synthesis
	↓
24 h	End of S-phase
	↓
29 h	Mitosis

(Keppler, D. 1987, Ref. 16)

3. Fibroplasia

A third basic process in the pathogenesis of alcoholic cirrhosis is the increased deposition of connective tissue components, i.e. the fibroplasia.

The prolonged *hepatocyte injury* may cause release of fibroplastic *peptides* and of antigen-like materials, which stimulate macrophages and the regulatory limb of the lymphoid system. The *effector cells* like the mononuclear inflammatory and immunologic cells then will produce fibroplasia-promoting *cytokines* that attract fibroblast as *target cells*, induce their multiplication and stimulate the production of collagens or matrix substances that form scar. The products of the inflammatory cells also recruit other target cells, like fat-storing Ito cells, myofibroblasts, endothelial cells, in support of fibroblast in the production of the extracellular matrix (Fig. 9) [38].

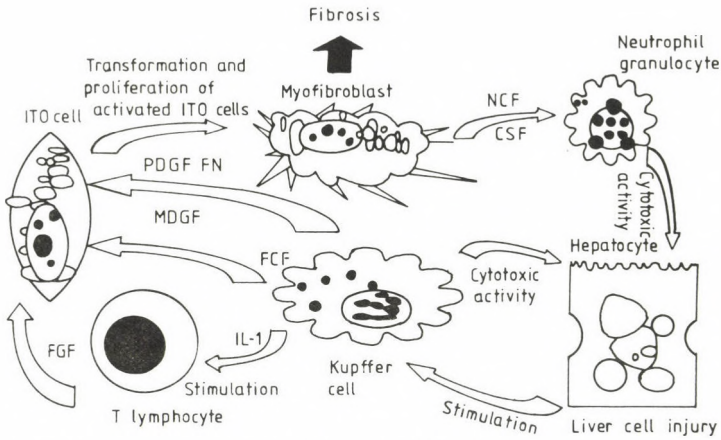

Fig. 9. Fibroplasia: cytokine-mediated interactions between white blood cells, non-parenchymal and parenchymal liver cells

Abbreviations:
ITO cell = fat storing cell
PDGF = platelet-derived growth factor
FN = fibronectin
MDGF = macrophage-derived growth factor
FCF = fibroblast-chemoattractant factor
IL-1 = interleukin-1
NCF = neutrophil-chemoattractant factor
CSF = colony stimulating factor
LFGF = lymphocyte-derived fibroblast growth factor
(Schuppan et al. 1988, Ref. 38)

Myofibroblasts are special smooth muscle cells, which are responsible for wound-contraction in the skin. (It seems that the scar formation in the liver is similar to scar formation in the skin, there is a common mechanism regardless of where it occurs.) Myofibroblast is the common cell around the terminal hepatic venules in the connective tissues or immediately under the endothelial cells. Alcohol consumption increases the number of myofibroblasts and other mesenchymal cells, these will produce collagen filaments, fibrills (type III) and (type I collagen) and basal laminas (type IV collagen), localized between endothelial cells and myofibroblasts or around to myofibroblasts. Myofibroblast processes also extend into Disse space, where the commonest mesenchymal cell is the *lipocyte* (fat storing Ito cell). Lipocytes contain lipid droplets, microfilament bundles, dense bodies and pinocytic vesicles electronmicroscopically examined.

After alcohol consumption, about the half of the lipocytes is replaced by *transitional cells* between lipocytes and fibroblasts. Their lipid content decreases, while the rough endoplasmic reticulum amount increases and they lack of surrounding basal laminae. There is a good correlation between hepatic fibrosis and the per cent of transitional cells, which are presumably derived from Ito cells, after inflammatory stimulation in conjuction with collagen deposition [30, 32]. The alcohol-induced elevated *acetate level* in liver tissue may play a role in the fibrogenesis, possibly by means of the increased proline hydroxylase activity and inhibition of proline oxidase [36].

Acetaldehyde can stimulate collagen synthesis. After a previous increase in activity, collagenase activity may decrease, resulting in a diminished degradation, thus contributing to collagen accumulation.

The earliest deposition of fibrous tissue in alcoholic liver damage is generally around the central veins named as *perivenular fibrosis*. This may be associated with necrotizing process called sclerosing hyaline necrosis, seen mostly in alcoholic hepatitis. All these processes may lead to obliteration of central veins and cause postsinusoidal *portal hypertension*. Collagen deposition, associated with reduction in the fenestrations between sinusoids and Disse space, may isolate the hepatocyte from its blood supply, altering exchanges between sinusoidal blood stream and the parenchyma. Thus the process may further enhance the tissue injury: the excess matrix – in addition to *altering phenotypic expressions of hepatocytes* – will have a constricting effect on liver cells, inducing additional injury in a *vicious circle* [30] (Fig. 10). The events finally will progress into a real micronodular cirrhosis, the end-stage of alcoholic liver disease.

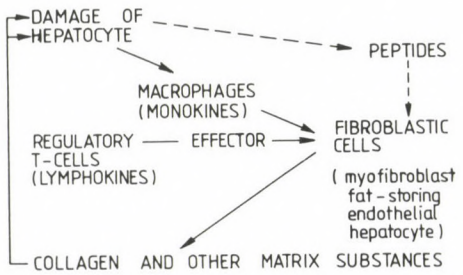

Fig. 10. Feedback loop in fibroplasia (Popper 1987, Ref. 37)

It can be stressed, that the *perivenular of the perisinusoidal fibrosis* may provide a valuable index of progression toward cirrhosis (Fig. 11). The fibrotic activity and inflammation, on the other hand, can be followed-up and checked by means of determining the serum level of *type III procollagen peptides*. These peptides during collagen extrusion from the cell are cleaved from the procollagen and released into the blood stream [35].

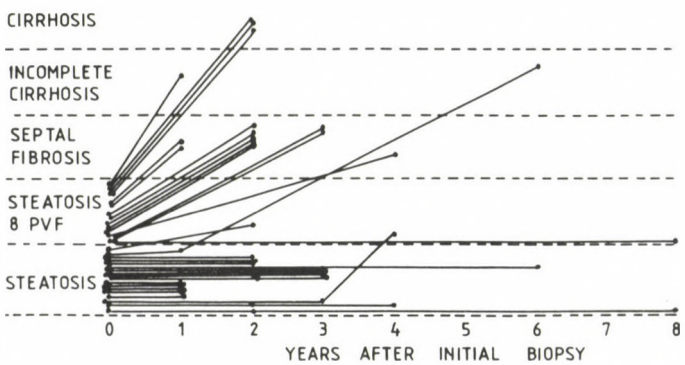

Fig. 11. Significance of perivenular fibrosis in the progression to cirrhosis in alcoholics (Worner and Lieber, 1985, Ref. 43)

The significance of fibrosis in the development of cirrhosis was emphasized by Hans Popper: "The one who will prevent or reduce hepatic fibrosis will cure most of the chronic liver diseases" [30].

III. The management of alcoholic liver injury

The goals of the treatment in alcoholic liver disease are to scavenge toxic free radicals, torestore endogenous scavenger capacity (e.g. GHS), to reduce the severity of necrotic processes and inflammatory reactions, to support regeneration, to stop fibroplasia and prevent cirrhosis. To treat the complications of cirrhosis is also of importance. Major tools for the therapy are "hepatoprotective" as well as antifibrotic agents. In the end-stage liver failure – in selected cases – only transplantation may be the resolution.

a) Hepatoprotective agents

Steroids

– *Corticosteroids.* There have been many uncontrolled and at least ten controlled trials concerning the evaluation of the efficacy of glucocorticosteroids – as antiinflammatory agents – in alcoholic liver disease. Only a few studies, however, stated benefical effects. Maddrey and colleagues administered either prednisolone or placebo in severe alcoholic hepatitis: the short-term mortality was 32 per cent in the latter group, whereas it was only 6 per cent in the group that received prednisolone ($p < 0.001$) [23]. In other studies the short-term mortality was uneffected by the administration of prednisolone and there was no effect on long-term survival either. Thus, most of the authors have come to the same conclusion, though corticosteroids may be benefical in selected cases, commonly they are of no benefit in the treatment of alcoholic hepatitis, and routine prednisolone treatment is not recommended in the management of alcoholic liver disease.

– *Anabolic androgenic steroids.* As certain hormones – and among them androgenic steroids – seemed to support regenerative processes, they have been investigated as therapeutic tools in alcoholic liver disease. Some trials have shown encouraging results. A cooperative study was conducted to determine the efficacy of 30 days of treatment with either a glucocorticoid (prednisolone) or an anabolic androgen (oxandrolone) in moderate or severe alcoholic hepatitis. Although neither prednisolone nor oxandrolone improved short-term survival, *oxandrolone* therapy showed a beneficial effect on long-term survival. Among those who survived for one-two months after the start of the treatment, a conditional six-month death rate was 3.5 per cent after oxandrolone and 19–20 per cent after placebo ($p < 0.02$). No consistent long-term effect was associated with prednisolone therapy [24].

Insulin plus glucagon

To stimulate hepatic regeneration, the use of insulin and glucagon has also been studied in alcoholic hepatitis. In the first trial, although there was some improvement in liver function tests of treated patients, no change in survival was found [2]. A multicentre study was performed in Hungary [8], where 33 patients with alcoholic hepatitis received daily infusions of insulin and glucagon plus glucose, and an equal number of patients served as controls. The mortality was 14 of 33 in control and 5 of 33 in treated groups, in addition liver tests improved more rapidly in those who received hormonal therapy. These results may be encouraging, but not confirmed yet, thus more pieces of evidence are warranted to support the findings. On the other hand, the use of insulin may also be dangerous as patients already are at risk for hypoglycemic episodes.

The use of *other hepatocyte growth factors* such as epidermal growth factor, would be safer and may now be possible with the successful cloning and preparation of the human forms of these agents [45].

Propylthiouracil

As it has been suggested that a hypermetabolic state and hypoxia play major roles in the pathogenesis of alcoholic liver injury, the effect of propylthiouracil was investigated in both animal experiments and clinical studies. Although the drug did not alter short-term survival, administering propylthiouracil of 300 mg/day for two years resulted in a cumulative mortality rate of 13 per cent, significantly less, than in the placebo group of 25 per cent [27]. The patients who benefited most from the propylthiouracil therapy were those who had the lowest consumption of alcohol, whereas the heavy drinkers were not protected by the drug. As serious side effects may be associated with the use of propylthiouracil, caution is recommended in the use of this agent [45].

Flavonoids

Cianidanol a naturally occurring flavonoid was shown to have antioxidant and radical scavenging, membrane stabilizing and immunomodulatory capacity. Its efficacy on liver tests was reported in alcoholic liver disease, but no effect on survival was shown [7], and the drug was even withdrawn worldwide following the report of 54 cases of haemolytic anaemia resulting 5 deaths internationally.

Silymarin another flavolignan group, the active principle of the milk thistle, Silybum marianum, was also proved to protect experimental animals and man against various hepatotoxins, to inhibit lipid peroxidation, to stabilize membranes and to promote liver cell regeneration [34]. In Hungary, Fehér and colleagues performed a

randomized, placebo controlled trial, that – based on biochemical, histological and immunological findings – showed beneficial effects of the drug in alcoholic liver disease [9]. Ferenci et al. carried out a double-blind, prospective, long-term clinical study with silymarin in cirrhotic patients. The treatment has been lasted at least for 2 years, the mean observation period was 41 months. The 4-year survival rate was 58 ± 9% in silymarin patients and 39 ± 9% in the placebo group ($p = 0.036$).

The difference in survival was most significant in alcoholic cirrhosis and in patients with liver disease of Child A severity [11] (Fig. 12).

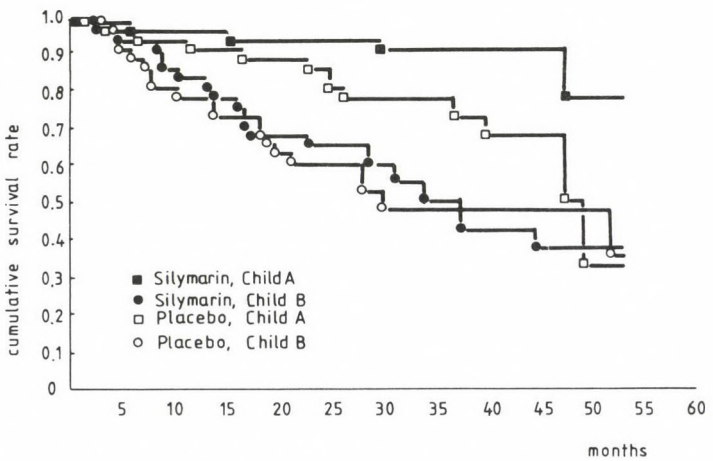

Fig. 12. Survival rate in cirrhosis and the effect of silymarin treatment (Ferenci, 1989, Ref. 11)

A new possibility: S-adenosyl-L-methionine

S-adenosyl-L-methionine (SAMe), an active metabolite of methionine, a principal methyl-donor is an essential compound in the synthesis of glutathione. SAMe is formed from methionine by an enzyme SAMe-synthetase, which transfers an adenosyl moiety from ATP to methionine [13] (Fig. 13).

SAMe is of fundamental importance in a number of biochemical reactions involved enzymatic *transmethylation*, contributing to the synthesis, activation of metabolism of hormones, neurotransmitters, nucleic acids, proteins. SAMe regulates liver membrane lipid composition and fluidity, activates *trans-sulfuration* pathway, favoures endogenous detoxifying processes. It has antioxidant properties allowing methylation of cellular components of phospholipids, and can increase biliary membrane fluidity, thus reducing *cholestasis* [1] (Fig. 14).

SAMe could prevent the depletion of reduced glutathione in the liver, normalize mitochondrial enzyme activity, inhibit acetaldehyde production and fat accumulation in alcoholic liver injury [10, 41].

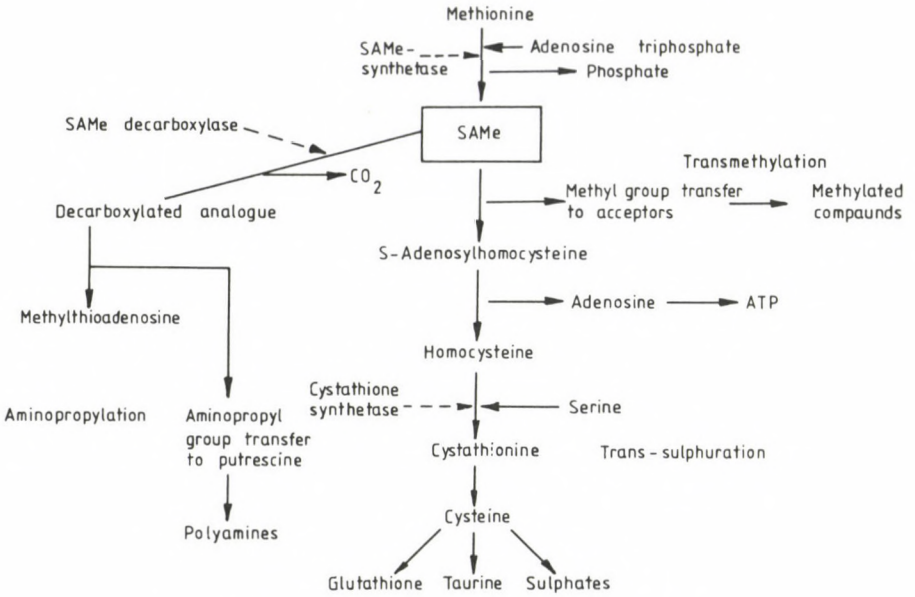

Fig. 13. Metabolic fate of S-adenosyl-L-methionine (SAMe)

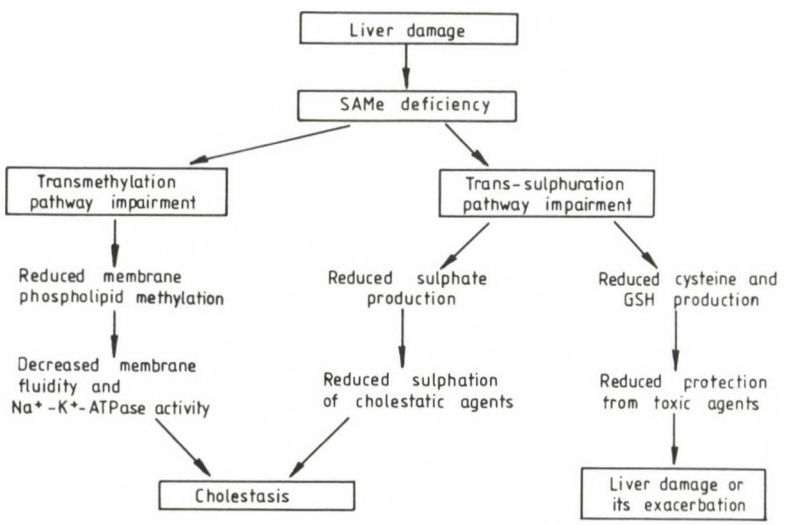

Fig. 14. Liver damage can cause SAMe-synthetase deficiency and hence low SAMe content, which in turn leads to further liver injury. Exogenous SAMe will be of benefit

SAMe is already a well-known drug for the treatment of patients with affective disorders: participating in the synthesis and metabolism of neurotransmitters, it can stimulate activity of dopamine and serotonine pathways, thus may affect the mood as an *antidepressant* agent [3].

SAMe can be a new potent therapeutic modality in the future for the patients with alcoholic liver disease, but its real value remains to be determined in well-designed, prospective, randomized comparative trials.

Nutritional support in "hepatoprotection"

Malnutrition is a common feature of patients with alcoholic liver disease, and the severity of protein-caloric malnutrition correlates with mortality and with severity of liver disease [45]. A number of studies have been performed, that attempted to improve the nutritional state of alcoholics, in order to improve liver function and decrease mortality. Now it seems that a *3000 Kcal, 100 g protein diet* as well as administration of parenteral *thiamine*, furthermore *folate* (*possible* vitamin E as well as Zn supplementation!) – may improve both the patient's nutritional and general immunologic states and therefore enhance recovery. *Vitamin A* supplementation in alcoholics might improve liver dysfunction, such therapy, however, is complicated by the fact, that excessive amounts of vitamin A are hepatotoxic. It may be due to a toxic metabolite of vitamin A and the production of this toxin is enhanced by the ethanol-related microsomal induction. Consequently, in heavy drinkers vitamin A might hasten rather than alleviate the liver disease [22].

The efficacy of *intravenous* amino acid infusion is debated. Although the initial studies suggested encouraging results, the lack of a clear definite effect in later trials, and the risk of sepsis from indwelling catheters – should limit the long-lasting intravenous amino acid treatment to control studies. Oral supplements as mentioned above seem to be satisfactorily useful [45].

b) Antifibrotic treatment

– *Colchicine*

Colchicine interferes with the assembly of microtubules and the transcellular movement of collagen, inhibits the synthesis and secretion of collagen, furthermore increases collagenase function as well. Thus it can play a role in the treatment of hepatic fibroplasia and cirrhosis.

In a study in Mexico, cirrhotic patients received either placebo or colchicine (1 mg five days a week) for as long as 48 months. The study was continued with some patients followed-up to 14 years. The cumulative five-year survival in the colchicine

and placebo groups was 75 per cent and 34 per cent, respectively ($p < 0.001$). Thirty months of therapy were required before any significant effect of colchicine could be seen [17]. These results are promising, but additional large, long-term studies with well-matched patients are needed to exactly determine the role of colchicine in the treatment of liver cirrhosis.

– *D-penicillamine*

D-penicillamine is known to retard the cross-linking of collagen molecules thus rendering newly formed fibers susceptible to collagenolytic activity. In a double blind trial in alcoholic hepatitis without cirrhosis. Resnick et al. have seen no improvement in survival or in liver function tests in the experimental group. Although a reduction in the degree of hepatocellular necrosis and in the deposition of collagen after 8 weeks of therapy appeared to be greater in the penicillamine-treated patients, statistically significant conclusions were not allowed [33].

c) Therapy of end-stage cirrhosis: liver transplantation

The use of *orthotopic liver transplantation* (OLT) to treat patients with end-stage alcoholic liver disease has commonly been criticized, saying that provision of a new liver is a futile gesture as well as the waste of an organ in the case of alcoholics, because they seem too physically and emotionally frail to withstand the rigors of such a large operation and subsequent immunosuppression. Yet, Starzl and colleagues, regarding alcoholism as a treatable disorder, performed 56 OLT in alcoholic patients between 1963 and July 1987.

During the cyclosporin era, from 1980, they found no significant difference between the survival of the 41 patients who received OLT for alcoholic cirrhosis when compared with the survival of 562 adults treated with OLT for other causes [39]. Recently Krom from the Mayo Clinic reported a mean 2-year survival of 70 per cent in alcoholic cirrhosis and 95 per cent in other cirrhotic patients after OLT, while the ciclosporin neurotoxicity occurred in 39 per cent vs 9 per cent, respectively. Furthermore, 17 per cent of alcoholic cirrhosis patients returned to drinking. The findings suggested that at least 6 months abstinence might be required before the OLT in alcoholics [18].

Conclusions

The present paper is devoted to overview the basic concepts of ethanol-included hepatic injury and therapeutic modalities by which alcoholic liver disease can be alleviated.

The role of alcohol dehydrogenase of both hepatic and gastric origin as well as the importance of the number one metabolite acetaldehyde are discussed, furthermore the effects of microsomal ethanol oxidizing system is also described. The features of the major clinicopathological consequences of alcohol abuse fatty liver, alcoholic hepatitis are briefly outlined, and the basic pathogenetic mechanisms that lead to cirrhosis – cell necrosis, regeneration and fibroplasia – are shown.

The understanding of the pathophysiology of alcohol-induced liver injury may improve the therapy with drugs and nutritional factors, and allow successful prevention through the early recognition of heavy drinkers before their social or medical disintegration. In the management of alcoholic liver diseases, among the true hepatoprotective agents a naturally occurring flavonoid silymarin and an active methyl-donor metabolite S-adenosyl-L-methionine seem to be promising. An antifibrotic treatment with colchicine might also be of importance. Further prospective, well-designed, controlled clinical trials are still warranted to evaluate real efficacy of these drugs.

The hepatic consequences of alcohol abuse may be treatable, the prevention, however, would be the true resolution of the major global health problem of alcoholism.

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ROLE OF FREE-RADICAL REACTIONS IN LIVER DISEASES

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Role of free-radical reactions is most significant in toxic liver injuries. Two traditional groups of liver injuries induced by drugs and chemicals are distinguished, 1. direct toxic type and 2. idiosyncratic type. Liver injury of direct toxic type is generally developed following toxin exposure, it is dose dependent, incubation period is short, and the injury often affects other organs (e.g. kidney). Direct toxins frequently cause typical zonal necrosis usually without concomitant signs of hypersensitivity. It is typical of idiosyncratic reaction that it appears only in a shorter period of exposure, it cannot be predicted, it is not dose-dependent, its incubation period varies and sometimes (in one-fourth of cases) it is accompanied by extrahepatic symptoms of hypersensitivity (fever, leukocytosis, eosinophilia, rashes), its morphologic picture shows great variety. A part of direct toxins is toxic itself, in the other part the basic compound is not toxic but it changes into toxic metabolites in the liver.

Liver is well-protected against free-radicals developing in the organism: it is one of our best antioxidant supplied organs. It is probably due to the one of the important tasks of liver, namely detoxication of drugs, chemicals and toxic materials, with subsequent release of free-radicals. It is proved by the fact that in normal bile peroxidized lipids produced by free-radical chain reactions can also be detected.

The pathologic free-radical reactions and one of their sequelae, peroxidation of lipids (LPO) do not necessarily cause cell and tissue damage. Antioxidant protection of cells and tissues is able to prevent free-radical injury and it enables, that the already developed damages become reversible. According to recent investigations, the lipid peroxidation, caused by free-radical reactions, or covalent binding of radical products to biomolecules does not lead directly to cellular destruction, only via further reactions. Such intermediary steps can be the phospholipase A2 activation, accumulation of lysophosphatides, poly-ADP-ribose polymerase repair enzyme activation, following oxidative damage of DNA, with subsequent NAD and ATP depletion. Its significance may be that the irreversible cellular and tissue damage can be prevented perhaps not only by administration of antioxidants, but also by compounds (e.g. phospholipase A2 inhibitors) affecting the above-mentioned biochemical mechanisms.

Keywords: free-radical reactions, liver disease, toxin exposure, zonal necrosis, detoxication of drugs, peroxidized lipids, antioxidant protection of cells

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The oxygen free-radicals, which play an important role in the living organism, are generated from oxygen by excitation or reduction. The primary free-radicals generated are univalent and divalent products of oxygen, O_2^-, and H_2O_2.

Recently it has been found that most of toxic metabolites originating from drugs and chemicals are very reactive free-radicals, having unpaired electron in their external electron shell (it corresponds to free valence) which can damage tissues and cells reacting with surrounding macromolecules, membranes, forming new free-radicals in chain-reactions [1, 2, 3, 4].

It should be emphasized that free-radical reactions are not solely of damaging character but occur in the organism in a controlled way and can be the basis of several physiological processes (e.g. phagocytosis, arachidonic acid metabolism, regulation of certain immune processes) [5, 6].

Free-radicals may have a part in the pathogenesis of liver diseases. The damage caused by the free-radicals can be prevented by scavenger molecules, which are natural or synthetic antioxidants; thus complementary treatment with drugs of this group can be suggested in diseases where free-radicals play a leading role.

Discussion

1. Alcoholic liver damage

In order of severity, three forms of alcoholic liver damage are distinguished; 1. steatosis, 2. alcoholic hepatitis, 3. alcoholic cirrhosis. Probably, the lipid peroxidation (LPO) plays a causal role in all three forms, however, experimental evidence proves it mainly in steatosis. Decreased hepatic GSH content and increased conjugate dien content were found in liver biopsy sample of patients with alcoholic liver disease [7, 8]. Following ethanol inhalation in rats decreased hepatic CAT and CuZnSOD activities were found with normal MnSOD activity. The observed differences are probably explained by reactive oxygen intermediates (ROI) released during microsomal metabolism of ethanol [9]. Also in rats and baboons, both acute and chronic alcohol administration caused an increase of conjugate dien and significant decrease of GSH level in the liver [10]. LPO mechanism induced by alcohol is not exactly known. It can be possibly explained with Lewis and Paton's hypothesis which presumes the role of cytoplasmatic xantine oxidase [6].

First metabolite of alcohol, the acetic aldehyde, can be metabolized not only by acetic aldehyde dehydrogenase (though this enzyme metabolizes it mainly) but also by xantine oxidase.

In healthy cells xanthine oxidase acts as NAD^+ reducing dehydrogenase, not as oxidase. As a result of unfavourable metabolic circumstances, e.g. proteolysis, ischemia, however, it changes into oxidase. The enzyme may use acetic aldehyde

instead of hypoxanthine as substrate, and while it oxidizes it into acetate, O_2 is produced from molecular oxygen. As acetic aldehyde is present in large scale at chronic alcoholists, excessive O_2^- production is developed because the acetic aldehyde supply (contrary to purin metabolites) is unlimited.

Hypothesis of Lewis and Paton is supported by experimental results; if tolbutamid, inhibiting aldehyde dehydrogenase, was given to rats, it increased the decrease of reduced glutation (GSH) level induced by alcohol, so it increased the level of circulating acetic aldehyde, while the allopurinol had a reverse effect. *In vitro* bovine xanthin dehydrogenase/oxidase incubated with acetic aldehyde, selective inactivated xanthine dehydrogenase, which was somewhat different – from the effect expected on the basis Lewis–Paton theory. At the same time the acetic aldehyde did not influence the oxidase activity of the enzyme. Consequently, the ROIs produced by acetic aldehyde do not originate from the metabolisation of acetic aldehyde by xanthine oxidase, but they are the sequelae of dehydrogenase → oxidase conversion [11, 12].

Another possible way is that the consumption of ethanol increases the activity of microsomal enzymes of liver, including also the system oxidizing microsomal ethanol which participates in microsomal metabolism of ethanol. ROIs released during microsomal enzyme activity may have a role in the effect of alcohol producing hepatic LPO [9, 10, 13]. Besides, acetic aldehyde produces ROIs, not only in the course of xanthine oxidase-dependent metabolism presumed in Lewis–Paton hypothesis, but also in that regulated by aldehyde oxidase enzyme [9, 14].

The binding of acetic aldehyde to SH-containing GSH precursors results in a decrease of GSH concentration and is a third alternative mechanism [15].

Decreased CuZnSOD activity was found in erythrocytes of chronic alcoholists suffering from liver damage. So besides other known tests, this examination seems to be suitable for detection of alcohol consumption [16].

The free-radical reactions play a role in the pathogenesis of some hematological divergences associated with alcoholic liver diseases (macrocytosis, hemolytic anemia, formation of target cell and spur cell). In patients with Zieve syndrome (alcoholic liver disease associated with hyperlipidemia and hemolytic anemia) decreased number of erythrocytes, polyunsatured fatty acid, GSH and Vitamin E content, decreased pyruvate kinase activity and lower serum Vitamin E level were found. The erythrocytes of patients were more sensitive to H_2O_2 stress in vitro. Thus besides other known factors (folic acid deficiency, decreased prothrombin level), the disbalance of oxidant–antioxidant system also plays an important role in the induction of hematological complications of alcoholic liver disease [17].

Our own investigations also support the role of free-radical reactions in the pathogenesis of chronic liver diseases. Lysosomal enzyme activity was studied in serum and granulocytes of patients with chronic liver disease. It is known that LPO

can damage the lysosomal membranes, causing lysosomal enzyme-release. In hepatic steatosis (SH), chronic active hepatitis (CAH) and hepatic cirrhosis serum beta-glucuronidase activity was significantly increased. Serum acid-phosphatase activity was increased in all three diseases, but in CH this increase was not significant. Finally, the serum-cathepsin-D activity was not significantly increased, while the activity of beta-glucuronidase in granulocytes of hepatopatic patients was depleted compared to control group. Calculating the release rate of beta-glucuronidase from the granulocytes, this parameter was higher in patients with chronic liver disease. Higher serum beta-glucuronidase activity partly originates from the granulocytes, but it cannot be totally responsible for the greater activity of serum. It confirmed our suggestion that the enhanced enzyme release from different tissues including liver, is a result of decreased stability of lysosomal membranes. Damage of lysosomes in CH was confirmed by electronmicroscopic examination. It is interesting that the level of serum beta-glucuronidase was the highest in patients suffering from CH, a lower increase was found in CAH and SH. Accordingly, the beta-glucuronidase activity of granulocytes definitely decreased in CH – while there was a smaller decrease in CAH and SH. Similarly to mitochondrial and microsomal liver enzyme examinations routinely applied in diagnostics, the serum and granulocyte lysosomal enzyme test, respectively, can be used in diagnostics of liver diseases; moreover, their determination may have differential diagnostic significance in chronic liver diseases [18, 19, 20, 21]. Administration of antioxidant, Catergen settled the elevated acidphosphatase level of hepatic patients with chronic disease within 3 months, significantly decreased the beta-glucuronidase activity and normalized the enzyme release from granulocytes [22].

Inorganic free-radicals (N_3, $(SCN)^{2-}$, OH, TrP, CO_2^-, O_2^-) produced by pulse radiolysis readily react with the compounds, which transform into exceptionally long-lived, unreactive transients. Time evolution of the UV and visible spectra indicate that oxidizing radicals form a phenoxyl type radical from silibinin, while ·OH forms an adduct by attacking, simultaneously, at various sites of the molecule. Superoxide radicals reduce silibinin and oxidize CH 402 and MTDQ-DA. It is concluded, that the drugs might exhibit antioxidant behavior in living systems [23].

At our Department the efficiency of Legalon treatment, containing silymarin and having antioxidative properties besides other important pharmacological effects, was investigated in a double blind study, in patients with chronic alcoholic liver disease. 36 patients with chronic alcoholic liver disease participated in this study (27 men, 9 women, average age: 46 ± 7 years). Daily alcohol consumption exceeded 60 g in men and 30 g in women. Period of chronic alcohol consumption was 8 ± 4 years. The patients were vascularly compensated, symptom of encephalopathy were not observed, malnutrition did not occur and they had no other associated disease. The virus and immunological (antinuclear antibody, anti-smooth muscle antibody)

markers were negative. Silymarin-placebo randomization was done by the pharmaceutic company (Madaus, Cologne). The placebo contained the vehicle of silymarin in the same form. The treatment lasted for 6 months. The code was disclosed by the factory after the end of treatment. 17 patients (15 men and 2 women, average: 38 ± 7 years/took 3×140 mg silymarin per day/3×1 Legalon R/capsule) and 19 patients (12 men, 7 women, average age; 44 ± 6 years/took 3×1 capsules of placebo. Histological examination of liver in silymarin group verified micronodular cirrhosis showing signs of chronic inflammatory activity in 6 cases (associated with steatosis in four patients, hemosiderosis at 3 patients). In 10 cases steatosis with reactive fibrosis and round-cell infiltration (associated with hemosiderosis at 1 patient) and in one case septal fibrosis showing chronic inflammatory activity were observed. In the placebo group the histological diagnosis was micronodular cirrhosis with chronic inflammatory activity in 5 cases (associated with steatosis at 1 patient). In 12 patients steatosis with reactive fibrosis and round-cell infiltration (associated with hemosiderosis in 1 case), in 1 patient acute alcoholic hepatitis associated with septal fibrosis and in 1 case septal fibrosis showing chronic inflammatory activity was found. The activity of serum glutation peroxidase (GPX E.C. 1.11.1.9.) serum free – SH group, superoxide dismutase (SOD, E.C. 1.15.1.1.) of erythrocytes and lymphocytes, as well as SOD expression in lymphocytes were determined.

Initial values and values following treatment of the two groups of patients (treated by placebo and silymarin, respectively) are summarized in Table I. It can be established that there was no considerable difference in the examined parameters (I vs. III) in the initial data of groups. The results did not show significant changes following the administration of placebo (III vs. IV).

Level of malondialdehide (MDA) marker of serum LPO, decreased significantly during the silymarin treatment ($p < 0.02$). There was significant difference between the values of the two groups following the treatment ($p < 0.02$).

Following silymarin administration the serum glutation peroxidase (GPX) activity significantly increased ($p < 0.05$), and the values of groups following treatment showed significant difference ($p < 0.02$). Silymarin treatment significantly increased the SH group level ($p < 0.05$). The values of the two groups following treatment showed significant difference, too ($p < 0.05$).

During silymarin treatment superoxid dismutase (SOD) activity of erythrocytes and lymphocytes increased distinctly regarding both types of cells (erythrocyte SOD: $p < 0.001$, lymphocyte SOD: $p < 0.01$). The values of groups following the treatment were significantly different (erythrocyte SOD: $p < 0.01$, lymphocyte SOD: $p < 0.01$).

Table I

Effect of silymarin treatment on lipid peroxidation and antioxidant system in chronic alcoholic liver diseases, in six-month double blind examination (average + SEM)

Groups (n)	MDA (nM/ml)	Serum GPX (U/g plasma protein)	Free SH (μM/Ml)	Erythrocyte SOD (U/ml)	Lymphocyte SOD
Silymarin [17]					
I: 0 month	15.1 ± 2.5	0.65 ± 0.28	0.44 ± 0.20	72.9 ± 14.5	32.6 ± 10.3
II: 6 month	10.2 ± 1.0	0.94 ± 0.25	0.63 ± 0.17	130.8 ± 19.6	74.9 ± 19.3
Placebo [19]					
III: 0 month	14.7 ± 2.3	0.67 ± 0.21	0.45 ± 0.12	76.5 ± 20.1	29.4 ± 14.2
IV: 6 month	15.9 ± 2.1	0.54 ± 0.26	0.43 ± 0.15	85.7 ± 21.7	27.7 ± 16.1
Significance:					
I vs. II	$p < 0.02$	$p < 0.05$	$p < 0.05$	$p < 0.001$	$p < 0.01$
II vs. IV	$p < 0.02$	$p < 0.02$	$p < 0.05$	$p < 0.01$	$p < 0.01$

During a 6-month administration of placebo considerable changes in SOD expression of lymphocytes were not observed, whereas, the SOD expression of lymphocytes increased significantly in the group treated with silymarin.

During the six months of treatment side-effects described to silymarin were not observed. Our studies show that in patients with chronic alcoholic liver disease the extrahepatically detectable oxidative stress state – its typical biochemical parameters – were favourably influenced by Legalon treatment. The therapy applied decreased lipid peroxidation and improved the patient's antioxidant protection. Our studies also showed immune modulating effect of Legalon, though details are not given here. Following the six-month treatment, all these favourable effects resulted in the settling or significant improvement of liver function values of patients, proving the liver protective effect of the drug [24, 25].

2. Liver injury caused by drugs and chemicals

The best-known liver injury is caused by carbon tetrachloride (CCl_4) and by galactose amine. Prerequisite for their damaging effect is an actively metabolizing liver. CCl_4 is reduced by cytochrome P450 a terminal oxidase of a heterogeneous oxidase system in the endoplasmatic reticulum of liver. Following the splitting of

Cl_3C-Cl bond, the compound is metabolized to CCl_3 then it forms a more electrofil and reactive radical CCl_3O_2. The latter produces LPO of endoplasmatic reticulum, so in liver injuries caused by CCl_4, LPO developed mainly in microsomes. Liver injury caused by CCl_4 is known as the model of liver injury caused by xenobiotics [26, 27, 28]. Contrary to CCl_4, the galactose amine is water-soluble, so the radical reactions induced by it take place is the watery compartment. Although similarly to CCl_4. LPO plays some role in eliciting liver injury. It is not the primary cause of pathology in contrast to CCl_4. Liver injury caused by galactose amine can be considered as a model of inflammatory liver injury (hepatitis caused by virus) [29]. It should be mentioned here that chronic administration of CCl_4, inducing injury by the ROIs production and resulting in the development of hepatomas in experimental animals, is another evidence of the role of free-radical reactions in carcinogenesis [26]. In clinical treatment of acute CCl_4 intoxication the antioxidant therapy has proved useful, the intravenously administered N-acetylcysteine substantially decreased the secondary hepatorenal injury and its occurrence [30].

Our own investigations also proved the participation of free-radical reaction in the hepatotoxic effect of CCl_4, and galactose amine. Following the administration of toxins, markedly elevated MDA-levels and increased beta-glucuronidase release were determined, which are the indicators of LPO injury. MTDQ could significantly decrease the hepatotoxic effect of CCl_4; as indicated by laboratory parameters and morphologic changes.

The water-soluble MTDQ-DS was also able to decrease significantly the hepatotoxic effect of galactose amine (MTDQ-DS, MTDQ) [31, 32, 33, 34, 35]. Similar protective effect of Catergen on the increased lysosomal enzyme release in chronic liver disease was detected *in vitro* [18, 36]. Furthermore, the liver-damaging effects of halothane of similar structure ($CF_3CHClBr$) hydrazine (iproniazid, INH), hydralazine (Depressan), trichloroethylene, carbon disulphide, alpha-methyldopa (Dopegyt), acetaminophen (Phenacetin), Nitrofurantoin, paraquat are probably due to free-radical reactions. They can be presumed in liver injury caused by DDT, aflatoxin and Hibernal [6, 37, 38]. Mechanism of hepatotoxicity of halothane will be given in more details due to its side-effects in anaesthesia. Halothane also requires metabolic activation for development of hepatotoxicity. Its biotransformation takes place at the site of cytochrome P_{450}. Two metabolic pathways depending on oxygen tension exist. One of them is the oxidative degradation, which results in non-toxic, stable end-product excreted in urine.

The other way is a reductive way (inhibited by the rise of oxygen tension), which is highly activated at low oxygen tension or under anaerobic conditions. During this process a radical ($CF_3\dot{C}HCl$) is generated which is able either to initiate LPO, or to bind covalenty to microsomal membrane compounds, including cytochrome P_{450}. LPO and covalent binding explain decrease of cytochrome P_{450} level observed

following halothane-exposure in hypoxic atmosphere. Hypoxic conditions may develop, during anaesthesia, which increase the hepatotoxicity of halothane. According to epidemiological data, liver injury induced by halothane is more frequent in obese patients. It might be due to the decreased oxygen supply of hepatocytes in obese patients, compared to those with normal weight. Hence in obese patients the damaging reductive metabolic way is dominant under hypoxic conditions [27, 39, 40]. N-acetylcysteine in treatment of acetaminophen intoxication, and Legalon treatment in lethal Amanita poisoning proved to be clinically effective and save the life of such patients [1, 6].

3. Metabolic liver diseases

Wilson's disease

In Wilson's disease the O_2^-concentration in cells and extracellular space increases.

a) Coeruloplasmin is an extracellular radical scavenger with mild SOD activity. Low coeruloplasmin levels typical of the disease decrease the antioxidant protection of extracellular space.

b) Due to ferroxidase character of coeruloplasmin, it catalyses the ferro – ferri(iron) oxidation, its absence leads to ferroiron autooxidation with production of O_2^-.

c) Finally, in coeruloplasmin deficiency free copper is deposited in tissues. SH groups can reduce Cu^{2+} to Cu^+, which autooxidaze and induce O_2^- production. Cirrhosis and extrapyramidal damage developed in Wilson's disease support the possible free-radical origin of cirrhosis and extrapyramidal clinical pictures of other origin. Moreover, apart from the above mechanisms hemolytic episodes occasionally observed in Wilson's disease can be caused partly by the occurrence of copper in the circulation [41].

Hemochromatosis

The is evidence that the damaging effect in secondary hemosiderosis (transfusion, beta-Cooley's anemia) is caused by LPO, hence the free-radical reactions might play a part in the pathogenesis of organ damages developed in hemochromatosis. It is verified by the presence of lipofuscin and ceroid found both in Wilson's disease and hemochromatosis, which prove the *in vivo* LPO. D-penicillamine has a favourable effect not only as a metal chelator in Wilson's disease and hemochromatosis, but also probably due to the SOD activity of its copper complex [41, 42, 43, 44].

Many liver protective drugs used in clinical practice have antioxidant effect: e.g. Vitamin E, Lipoic acid, Legalon, D-penicillamine, the previously used, but due to its toxic side-effects Catergen not anymore proposed in treatment of toxic liver diseases. The use of antioxidant compounds provides new opportunities; it is clearly demonstrated by the successful clinical use of N-acetylcysteine in CCl_4 and acetaminophen intoxication, and that of Legalon in lethal Amaenita poisoning. Our results and data of literature seem to be promising in the use of Legalon in treatment of widespread chronic alcoholic liver disease.

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CYTOPROTECTION IN THE NINETIES: EXPERIENCE WITH URSODEOXYCHOLIC ACID AND SILYMARIN IN CHRONIC LIVER DISEASE

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The authors report their experience in the use of ursodeoxycholic acid and silymarin in patients with active cirrhosis of different aetiology. Both drugs seemed safe and ameliorated the biochemical indices of cytolysis; however, the former did not appear to be effective when hepatic dysfunction was associated to hepatitis C infection. The residual functional liver mass, as assessed by quantitative liver function tests, was not affected by either cytoprotective agent.

Keywords: chronic liver disease, ursodeoxycholic acid, silymarin

In the last few years, we gained a considerable experience in the treatment of chronic liver disease of different severity and aetiology using two cytoprotective agents: ursodeoxycholic acid (UDCA) and silymarin.

UDCA is a well-known litholytic agent for cholesterol gallstones which decreases biliary cholesterol secretion. Recently UDCA has been shown to improve some indices of liver function especially in chronic cholestatic liver disease [12, 6, 14], but also in chronic non-cholestatic liver disease [7, 11]. Its mechanisms of action include: i) displacement of endogenous "toxic" bile acids from the bile acid pool; ii) displacement of hydrophobic – and therefore more detergent – bile acids from hepatocyte membranes; iii) stimulation of bicarbonate rich hypercholeresis and, iv) reduction of the immunological injury, possibly leading to a decrease in piecemeal necrosis [3, 1, 5].

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Silymarin is a natural flavonoid. Its active component, silibinin, has been demonstrated to protect the liver of experimental animals against different hepatotoxins [15, 4] and to improve liver function in patients with chronic liver disease, especially of alcoholic origin [2, 3]. Silibinin is known to "stabilize" hepatocyte membranes mainly by decreasing the turnover rate of membrane phospholipids [9].

We have recently shown that silymarin decreases biliary cholesterol concentration and secretion both in rats and in men, probably by decreasing hepatic cholesterol synthesis [10].

Thus, both UDCA and silymarin act not only as membrane stabilizers but also as anti-lithogenic agents. Based on these properties, we carried out a prospective study on the effect of UDCA and silymarin, given alone or in combination, on liver function in patients with active cirrhosis of different aetiology.

Method

Twenty-one such patients (13 males, 8 females) with a mean age of 60 ys ± 6 SD were enrolled in the study. The selection criteria are shown in Table I. They received either UDCA (600 mg/day) or silymarin (420 mg/day) for 6 months according to a randomized controlled cross-over study. The results of the routine liver function tests were analysed by means of the analysis of variance according to a complete randomized block design, whereas changes in the mean values of the galactose elimination capacity (GEC) test and antipyrine clearance (APCL) were assessed using the paired Student's t test.

Table I

Criteria for patient selection in the cross-over trial of UDCA versus silymarin

1) Laparoscopy-biopsy proven chronic active hepatitis *plus* compensated liver cirrhosis
2) Serum transaminase levels 2 – 10 times the upper limit of normal
3) Absence of clinical, endoscopic or ultrasonographic signs of portal hypertension
4) No autoimmune disease and no HBsAg and/or HBeAg positivity

Results and discussion

During UDCA treatment mean serum AST and ALT levels decreased by 30% and 22% respectively and y-GT dropped from 142 U/L ± 30 to 67 ± 13 ($p < 0.05$), thus nearly returning to normal values. No significant changes were observed in the other common markers of liver function such as serum total bilirubin, alkaline phosphatase and lactate dehydrogenase (Table II).

Table II

Percent changes in routine liver function tests in respect to pre-treatment values during the cross-over trial of UDCA versus silymarin in 21 patients with active cirrhosis

	ALT	AST	y-GT	ALP	BIL	LDH
UDCA	−22	−30	−53	+9	+2	−11
Silymarin	−23	−15	−9	−1	−12	−5

ALT = alanine amino transferase; AST = aspartate amino transferase; y-GT = gamma-glutamyltranspeptidase; ALP = alkaline phosphatase; BIL = total bilirubin; LDH = lactate dehydrogenase.

Similarly, silymarin treatment caused a 15% and 23% decrease of serum AST and ALT and had no effect on the other routine liver function tests, including y-GT (Table II).

GEC and APCL, which were nearly half the normal values in the 21 patients at entry, showed virtually no changes after 6 and 13 months of treatment in both groups, suggesting that the residual functional liver mass was maintained over this time period.

Twenty of the twenty-one patients participating in the cross-over study, took part in a further study looking at the effect of the combined administration of UDCA and silymarin, or their withdrawal, on liver function. When no cytoprotective therapy was given for 6 months, mean levels of serum transaminase and y-GT returned to pretreatment values, but decreased again after another six-month course of UDCA. Combination therapy caused a progressive and further reduction of these enzymes over a 12 month period. Again, no significant changes of the quantitative liver function tests were observed either during combination therapy or when no cytoprotective therapy was given.

Thus, looking at the entire period of 25 months of treatment (13 mo. of the cross-over study plus 12 mo. of the open study) serum transaminases were reduced by an overall 31−43% and y-GT by 41−52% with no deterioration of liver function as assessed by the "dynamic" liver function tests.

No side-effects, which could be attributed to the administration of either drug, were reported. However, four patients experienced one episode of ascites (which responded to diuretics) and another patient developed hepatocellular carcinoma and was treated with chemoembolization.

Finally, we specifically looked at the role of hepatitis C virus (HCV) infection in relation to UDCA treatment [8]. A total of 29 patients with active cirrhosis were divided into two groups according to the presence (n = 17) or absence (n = 12) of

the anti-HCV antibodies. Positivity was assessed by four different tests (including the 2nd generation RIBA-4test), thus minimizing the possibility of false positives and false negatives.

The two groups were well matched in terms of male to female ratio, mean age and body weight, Child-Pugh score and duration of therapy (10–13 mo). The anti-HCV positive patients appeared somewhat "resistant" to UDCA treatment with a 12–14% decrease of serum transaminase, as compared to a 35–43% reduction of serum ALT and AST in the anti-HCV negative patients.

By contrast, the effect on y-GT was of the same magnitude as that described before and was independent from the anti-HCV status.

In conclusion, both UDCA and silymarin seem safe and beneficial in active cirrhosis of the liver. In fact, they reduce the degree of cytolysis in the liver, without affecting the residual functional liver mass. Combination therapy does not seem to be superior to either treatment alone which, as regards UDCA, should be restricted to anti-HCV negative patients with chronic liver disease.

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CLINICAL EVIDENCE OF HEPATOPROTECTION INDUCED BY URSODEOXYCHOLIC ACID

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Ursodeoxycholic acid (UDCA) was introduced to the clinical practice as an effective agent for the dissolution of gallstones. The efficacy of UDCA was proved recently in the treatment of patients with chronic cholestatic liver disease. We demonstrate the hepatoprotective effect of UDCA in a patient with chronic cholestatic liver disease.

A sixty-nine years old male patient was admitted to our department with severe jaundice. The laboratory and radiologic examinations revealed significant cholestasis without any morphological alterations. Among the serological tests the anti-HCV antibody was positive. Based on these findings and anamnestic data (no blood transfusion and/or operation), sporadic chronic C virus hepatitis was assumed with dominant cholestasis. The corticosteroid therapy even in high doses was ineffective, the liver function parameters worsened. Later UDCA (Ursofalk, Falk Pharma) was given at a dose of 250 mg three times daily. Clinical improvement was seen after the first week of UDCA treatment. The patient's complaints relieved parallel with decrease of serum bilirubin, γ-glutamil transferase and transaminase levels. These parameters showed further decrease during the treatment.

Keywords: cholestasis, ursodeoxycholic acid, hepatoprotection

Ursodeoxycholic acid (UDCA) was introduced to the clinical practice as an effective agent for the dissolution of gallstones. The efficacy of UDCA was proved recently in the treatment of patients with chronic cholestatic liver disease [1–4]. We demonstrate the hepatoprotective effect of UDCA in a patient with chronic cholestatic liver disease.

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Case report

A sixty-nine years old male patient was admitted to our department with severe jaundice slight abdominal pain and pruritus. The jaundice became gradually more severe, the patient did not have stronger cramping pain before the admittance. During the physical examination only icterus, 3 cm larger liver and slight right hypochondriac tenderness were found. The laboratory tests revealed significant cholestasis. The serum bilirubin levels before the admittance was about 300 μmol/l, dominantly in conjugated form. The laboratory test showed the levels as follows: GOT 95, GPT 82, ALP 370, GGT 180, LDH 566 LU/l. Viral and autoimmunological serological tests were negative. No morphological alterations were found during the radiological examinations. Liver biopsy was carried out, the portal spaces were inflamed and a slight intra-hepatic cholestasis was seen, with beginning peace-meal necrosis.

The patient received Prednisolon at a dose of 50 mg/day in the first week and afterward the dose was gradually decreased to 10 mg/day, which was given continuously during a few weeks. The patient's complaints vanished, the bilirubin level decreased (Fig. 1), but the GGT level significantly increased during this period (Fig. 3).

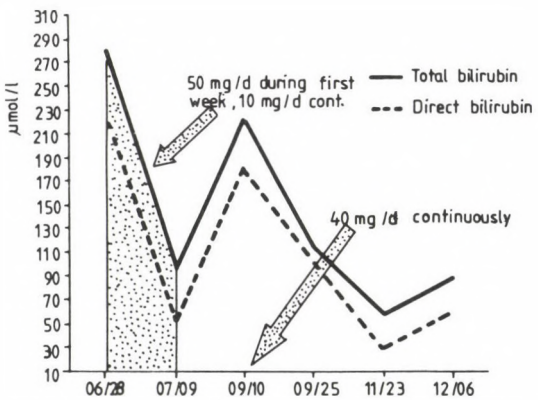

Fig. 1. The effect of Prednisolon treatment on liver functions. The changes of total and direct serum bilirubin levels. The horizontal axis shows the date of laboratory examination (month, day in 1990)

A few months later the jaundice became again more severe, the serum bilirubin level enhanced above 200 μmol/l, the transaminase levels were about 100 IU/l, the GGT level was higher than 200 IU/l. Corticosteroid therapy at a daily dose of 40 mg Prednisolon was started again. The next three months the bilirubin level decreased (Fig. 1), the GOT level decreased slightly the GPT level increased slightly, (Fig. 2), and the GGT level got three fold higher till the end of this period (Fig. 3).

Viral serological tests were repeated, the anti-HCV antibody was positive by using different "first generation" kits (Ortho and Abbott). Based on these findings and anamnestic data (no blood transfusion and/or operation), a "sporadic" chronic C virus hepatitis was suspected with dominant cholestasis. May be that was the cause of the ineffective steroid therapy even in high doses.

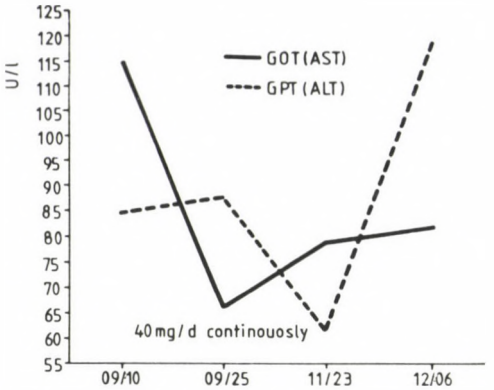

Fig. 2. The effect of Prednisolon treatment on liver functions. The changes of GOT and GPT levels. The horizontal axis shows the date of laboratory examination (month, day in 1990)

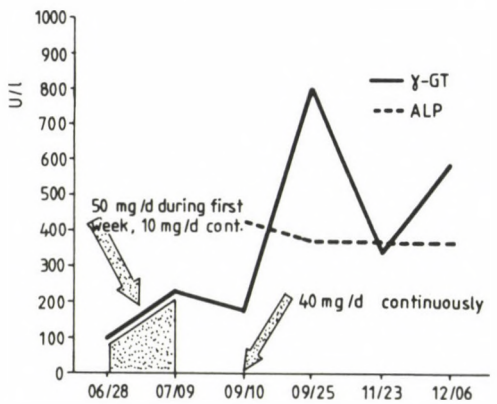

Fig. 3. The effect of Prednisolon treatment on liver functions. The changes of γ-GT and ALP levels. The horizontal axis shows the date of laboratory examination (month, day in 1990)

At that time we started to give UDCA (Ursofalk, Falk Pharma), 250 mg three times daily. The clinical improvement was demonstrable after the first week. The patient's complaints relieved parallel with the decrease of serum bilirubin level (Fig. 4).

The GOT and GPT levels also decreased after the first week and remained at that lower level during the whole period of UDCA therapy (Fig. 5).

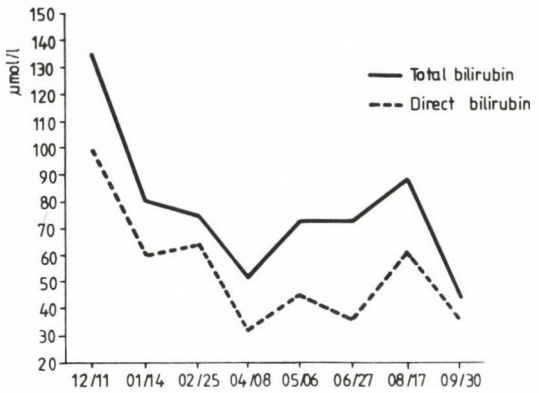

Fig. 4. The effect of Ursofalk treatment (3×250 mg/day) on liver functions. The changes of total and direct serum bilirubin levels. The horizontal axis shows the date of laboratory examination (month, day in 1990 – 1991)

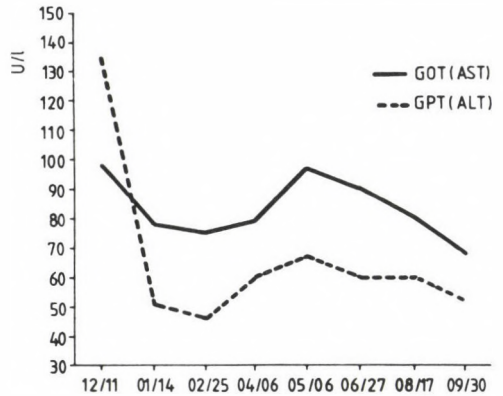

Fig. 5. The effect of Ursofalk treatment (3×250 mg/day) on liver functions. The changes of GOT and GPT levels. The horizontal axis shows the date of laboratory examination (month, day in 1990 – 1991)

The largest improvement was found in the γ-glutamil transferase level, which decreased from 600 IU/l below 100 IU/i. The ALP level was not significantly altered during this period, its level was a little bit higher than the normal level (Fig. 6).

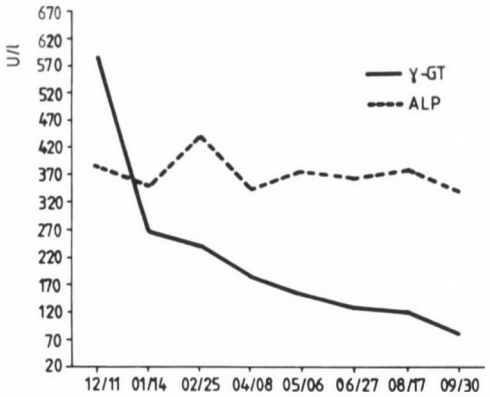

Fig. 6. The effect of Ursofalk treatment (3×250 mg/day) on liver functions. The changes of γ-GT and ALP levels. The horizontal axis shows the date of laboratory examination (month, day in 1990–1991)

Discussion

Ursodeoxycholic acid treatment seemed effective also, and we did not observe any kind of undesirable side effect during the long period (about 9 months) of treatment. The possible mechanisms of UDCA can be summarized as follows:

The favorite effect of this hydrophilic bile acid can be the consequence of the decrease of the amount of hydrophobic bile acids in the bile. It has a great importance, because it is well known that the hydrophobic chenodeoxycholic and deoxycholic acid are capable to induce cholestasis and liver cell injury [5]. The amount of hydrophobic bile acids increases in chronic hepatitis because of the impaired secretion of bile [3].

It was proved also earlier that during UDCA therapy the HLA I type antigen expression is inhibited on the liver surface [6], and the liver cell injury, partly caused by immunological mechanisms is lessened. The immunomodulatory effect of UDCA was described by others, too [4, 7].

Finally, there are some most recent data about a direct protective effect of UDCA in animal models not only in liver cell injury [8], but a protection was found for example against water immersion restraint stress ulcer of rats [9]. These data suggest a general cell protective effect of UDCA.

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THE EFFECT OF SILIBININ (LEGALON^R) ON THE FREE RADICAL SCAVENGER MECHANISMS OF HUMAN ERYTHROCYTES *IN VITRO*

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The effect of Legalon^R was investigated parallel with that of Adriblastina^R (doxorubicin) and paracetamol on some parameters characterizing the free radical scavenger mechanisms of human erythrocytes *in vitro* and on the time of acid haemolysis performed in aggregometer. Observations suggest that Adriblastina enhances the lipid peroxidation of the membrane of red blood cells, while paracetamol causes significant depletion of intracellular glutathione level, thus decreasing the free radical eliminating capacity of the glutathione peroxidase system. Legalon^R on the other hand, is able to increase the activity of both superoxide dismutase and glutathione peroxidase, which may explain the protective effect of the drug against free radicals and also the stabilizing effect on the red blood cell membrane, shown by the increase of the time of full haemolysis.

Keywords: Legalon^R, free radical scavenger mechanisms, dynamic haemolysis curve, erythrocytes

Silymarin (Legalon^R) is a well-known cytoprotective agent, which proved to be effective in several investigations. Though it was isolated from Silibum Marianum only in 1968 [19], the hepatoprotective effect of the plant-extract was already discovered in 1949 [4]. During the past two decades the protective effect of silibinin was demonstrated among others in carbon tetrachloride poisoning [11] and in liver damage caused by galactosamine, alcohol, phalloidin and heavy metal salts as well [18]. The registered parameters were usually different hepatic enzymes (ASAT, ALAT, gamma-GT, LDH, etc.) and changes of serum bilirubin. Morphologically the alteration of the triglyceride and glycogen content of hepatocytes and degenerative changes of mitochondria were tested [17]. In connection with other cells, silibinin was

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found to decrease the excretion of different lysosomal enzymes (acid phosphatase, beta-glucuronidase, etc.) from isolated macrophages, while the phagocytosis-capacity was enhanced [9]. On the other hand, incubation of erythrocytes with silibinin resulted in increased osmotic resistance [14].

The way of action of silibinin is multiple: due to a membrane stabilizing effect water-uptake and potassium excretion of the cells is decreased. Phospholipid turnover of the membrane is also reduced, leading to the preservation of the normal triglyceride content [3]. On the other hand, the rate of mitoses of the hepatocytes is enhanced, probably due to the stimulation of the synthesis of ribosomal RNA, which also results in an increased protein synthesis that helps regeneration [6]. Finally, the protective effect against carbon tetrachloride and alcohol poisoning suggests a free radical scavenger effect, though plenty of data also support the possibility of lipoxigenase and prostaglandin synthetase inhibition, both leading to decreased lipid peroxidation [5]. Since there are still some unanswered questions in this field, it was reasonable to investigate the effect of Legalon^R on free radical elimination.

According to our previous experiences erythrocytes seem to be a practical model for this purpose, since the free radical scavenger mechanisms of erythrocytes can easily be investigated in vitro. On the other hand, we found certain correlation between the time of full haemolysis and lipid peroxidation [1], therefore we decided to investigate the influence of Legalon^R on the time of acid haemolysis, too. To get more pronounced differences, in a comparative study we examined Legalon^R parallel with Adriblastina^R (doxorubicin) and paracetamol (acetaminophen). According to literary data, adriamycin induced cardiotoxicity is an oxidative pathology arising from intracellular generation of relatively high levels of free radicals [16]. On the other hand the phase-I metabolism of paracetamol causes dose-dependent lipid peroxidation in the mouse [20].

Materials and methods

Erythrocytes taken from healthy adults with heparin were incubated at 37 °C for two hours with the different drugs. The applied drug concentrations for silibilin, adriamycin, and paracetamol were 10 μg/ml, 0.1 mg/ml and 15 mg/ml, respectively, all within the range of clinical application. The parameters studied are listed in Table I.

Table I

Investigated parameters

Malonyl dialdehyde (MDA)	nmol/ml RBC
Reduced glutathione (GSH)	μmol/ml RBC
Glutathione peroxidase (GPX) activity	μmol GSH/min/ml RBC
Superoxide dismutase (SOD) activity	U/g Hb
Time of full acid haemolysis	sec

Malonyl dialdehyde (MDA) was determined from heparinized blood, according to Placer et al. [10] using thiobarbituric acid. Superoxide dismutase (SOD) activity was measured by the method of Misra and Fridovich [8], based on the inhibition of formation of adrenochrom by SOD in haemolysatum. The level of reduced glutathione was determined in haemolysatum according to Reichard and Galvin [12] using dinitrotetrazolium blue. The activity of glutathione peroxidase (GPX) was measured by the method of Sedlak and Lindsay [13].

Previously the method of acid haemolysis was mainly used for the diagnosis of paroxysmal nocturnal haemoglobinuria. Recently we have elaborated a modified method according to which the haemolytic process is performed in an aggregometer. The gradual increase of the transmission of the erythrocyte suspension can be registered by a potentiometric recorder in the form of a dynamic haemolysis curve. By detecting changes of the time of full haemolysis, this procedure seems to be applicable for the investigation of the membrane damaging and cytoprotective effect of different agents [1]. The principle of the method is shown in Figure 1. The shape of a dynamic haemolysis curve and the change of pH during the haemolytic process are presented in Figure 2.

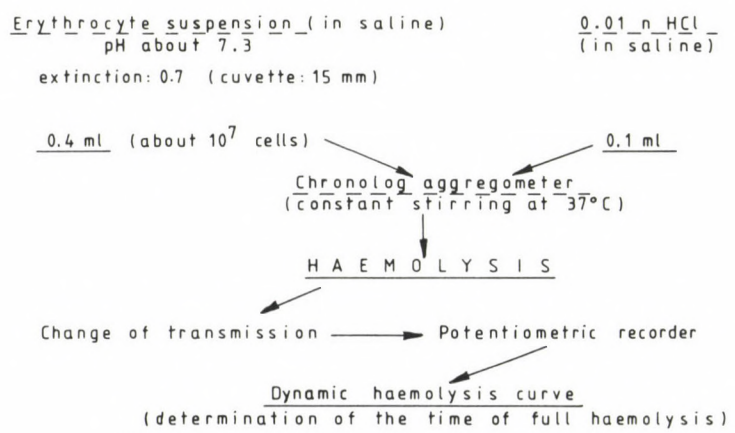

Fig. 1. Investigation of acid haemolysis in aggregometer

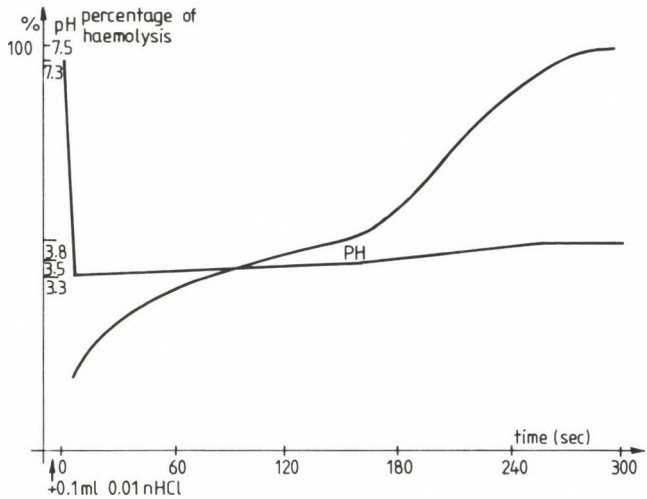

Fig. 2. Dynamic haemolysis curve and the change of pH

Results

The level of MDA represents the lipid peroxidation of the membrane, the amount of reduced glutathione is in correlation with the free radical scavenger capacity of the cell, while SOD and GPX play essential role in the elimination of different free radicals. The changes of the time of full haemolysis represent the degree of membrane damage caused by the different drugs. Results are summarized in Table II.

Table II

Changes of different parameters after the incubation of human RBCs for 2 hours (n = 12)

Name of drug	MDA nmol/ml RBC	GSH μmol/ml RBC	SOD U/g Hb	GPX μmol GSH/min/ml RBC	time (sec)
Legalon^{R}	154 ± 20	0.52 ± 0.08	1582 ± 76^{+}	21.4 ± 2.2	294 ± 36
Adriblastina^{R}	188 ± 30^{+}	0.49 ± 0.06	1198 ± 152	17.7 ± 0.8	198 ± 24^{++}
Paracetamol	130 ± 16	0.27 ± 0.06^{++}	1376 ± 87	18.4 ± 1.4	204 ± 20^{++}
Control (salina)	140 ± 18	0.50 ± 0.08	1311 ± 112	19.0 ± 1.3	264 ± 20

All data are presented with ± SD.
+: $p < 0.05$; ++: $p < 0.01$

Discussion

The level of MDA was significantly enhanced after incubation of red blood cells with Adriblastina^R ($p < 0.05$), which is in good correlation with the observation of Mimnaugh et al. [7], who found that some of the effects of adriamycin on mitochondrial morphology and biochemical function may be mediated by adriamycin-enhanced reactive oxygen-dependent mitochondrial lipid peroxidation. The other drugs did not influence the level of MDA significantly.

The hepatocellular injury caused by an overdose of paracetamol is a good example of direct, metabolite-related hepatotoxicity. The toxic metabolite preferentially conjugates with intracellular glutathione and when this is exhausted, it arylates essential nucleophilic macromolecules, thus producing hepatic necrosis [15]. Others found that the administration of paracetamol to rabbits and to human volunteers did not affect plasma glutathione levels [2]. However, the amount of glutathione in plasma is low as compared to its concentration in red blood cells (1:0.001 of the concentration there), therefore it cannot represent the latter. We found that the level of reduced glutathione in erythrocytes was markedly depleted by paracetamol ($p < 0.01$), while Legalon^R caused a slight increase. On the other hand, the activity of both enzymes studied were enhanced under the effect of Legalon^R ($p < 0.05$ for SOD), which leads – together with the above-mentioned increase of glutathione level – to an increased free radical eliminating capacity of the GPX system and SOD as well. Adriblastina^R decreased the activity of both enzymes, while paracetamol did not exert a specific effect. The time of full haemolysis showed an excellent correlation with the above results: Adriblastina^R and paracetamol decreased significantly the time of full haemolysis ($p < 0.01$ for both), while the incubation with Legalon^R resulted in certain increase.

These observations suggest that Adriblastina^R (doxorubicin), first of all, enhances the lipid peroxidation of the red blood cell membrane, but it has also certain negative effect on the free radical scavenger mechanisms. Paracetamol causes a depletion of intracellular glutathione level, due to which the free radical eliminating capacity of the GPX system is reduced, thus free radicals may provoke lipid peroxidation in the membrane of erythrocytes earlier and easier. Legalon^R seems to be able to enhance the activity of both superoxide dismutase and glutathione peroxidase, which may explain the protective effect of the drug against free radicals of different type and on the other hand gives an explanation for the increase of the time of full haemolysis, due to the enhanced resistance of the red blood cell membrane against hydrochloric acid under the effect of Legalon^R.

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ENDOGENOUS SCAVANGER LEVELS (VITAMIN E AND A) OF PATIENTS WITH CHRONIC ALCOHOLIC LIVER DISEASE UNDER DIFFERENT ENVIRONMENTAL CONDITIONS

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Serum level of endogenous scavangers (E and A vitamin) was studied in groups of patients with various chronic alcoholic liver diseases and in a healthy control group on polluted and non-polluted areas.

Vitamin levels in patients with chronic liver disease are diminished in comparison to the healthy in general, but mainly in the cirrhotic group. Diminution of vitamin E levels was observed in earlier phase of liver disease than that of vitamin A levels. Patients and healthy control on polluted area showed more expressed diminution of vitamin levels than the same groups on non-polluted area. Free radical parameter (RBC diene conjugate content) and characteristic alcoholic parameters (serum GOT, gamma-GT, cholesterol level and liver GOT, gamma-GT content in biopsy specimen) were used to explain the differences between the same investigated groups on polluted and non-polluted areas.

As conclusion can be supposed that industrial pollution of environment has a worsening effect in diseases with free radical mechanism.

Keywords: endogenous scavengers, environment and alcoholic liver disease, free radical diseases and environment.

Development of chronic alcoholic liver disease is considered as a consequence of long-term free radical attacks, which take place owing to the metabolism of ethanol [8]. Duration and so the celerity of progressive phase of liver damage are related to the quantity and the time of exposure of free radical loading mainly besides genetic and immunologic factors.

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Theoretical possibility of additional, that is of environmental free radical effects during the long-term alcohol consumption raises the question of their influencing role besides alcohol in progressive processes of liver damage [4, 6, 14].

Substances evoking free radicals with untoward biologic effects occur owing to industrial pollution in some areas of human environment [13, 14, 16]. Although chemical characteristics and mechanism of the free radical evoking potency of polluting substances have basic differences, their effects can be measured by the common biochemical consequences, e.g. by free-radical parameters, methods of which are elaborated hypothetically as well as in practice [1, 2, 3, 15].

Supposing, that characteristics of ethanol consumption do not differ within the various areas of our country, environmental influences can be established by comparison of radical effects with patients in polluted and non-polluted areas.

The aim of our study was to establish the effect of environmental pollutions on the chronic alcoholic liver diseases. The basis of comparison was the estimation of scavanging and cytoprotective (E and A) vitamin levels of patients in polluted and non-polluted areas.

Polluting materials with free radical evoking effects were not analysed in this study, on one hand, because of their common consequences on the corresponding parameters and because of their simultaneous effects, and, on the other hand, because the polluted and the non-polluted state is well known about the two areas which the investigated persons are coming from.

Material and methods

Chronic alcoholic liver disease of patients was characterized besides conventional clinical data by histologic examination of liver biopsy specimens. Patients without cirrhosis were not classified in subgroups because of calculation technic. Healthy blood donors served as controls.

Serum vitamin levels were used as the main basis of comparison; they were compared in various groups of patients with various diagnoses and in the control independent of the two areas, as well as in the similar groups of the two areas.

Some other free radical (diene conjugate), biochemical tests from the serum and biopsy specimens of the patients were estimated and analysed statistically with the aim of explanation and true interpretation of the results as follows:

1. Comparison of the vitamin level of patients with and without biochemical activity of the disease was carried out, 2. connection between some characteristic biochemical parameter of alcoholic liver disease (gamma-GT, GOT in the serum and in the biopsy material, serum cholesterol and vitamin levels) and RBC (red blood corpuscle) diene conjugate content was studied, and 3. as a very simplified information about the nutritive factors in alcoholics the correlation of both measured vitamin level with each other in patients and in a healthy group was also studied.

All the methods used are demonstrated in Table I.

Table I

Methods of investigations

Serum vitamin (A and E) levels:	HPLC (Varian 2000) separating column: BST SI 100S 10 C18 detectors: vitamin E: UV 292 nm vitamin A: spectrofluorimeter 325/500 nm
Diene conjugate extracts from RBC membrane:	AOAC Official Methods of Analysis, 1984
Enzyme activities:	GOT, GPT: optimalized kinetic UV test gamma-GT gGT – 4 – NA (Protein: Biuret reaction)
cholesterol level:	Allain, C.C. et al.: Clin. Chem. 20: 470-475. 1974.

Statistical analysis were done as follows: unpaired Student's *t*-test, analysis of variance and calculation of correlations.

Results

Figure 1 shows the serum vitamin E level of all patients and of controls. The serum vitamin E level was diminished in all groups of patients in comparison to healthy controls significantly.

Figure 2 shows serum vitamin A levels in various groups. Vitamin A level was diminished in the cirrhotic group and as a consequence, in the group with heterogenous diagnoses in comparison to the healthy controls significantly.

The mean vitamin E level of the healthy group and the mean levels of the investigated two types of vitamins (E and A) in the cirrhotic group from the polluted area were lower than the same values of the analogous groups from the non-polluted area. Vitamin levels in all of similar comparisons to the earlier showed the same tendency (with the exception of one connection) however, the differences have not reached significance (Fig. 3).

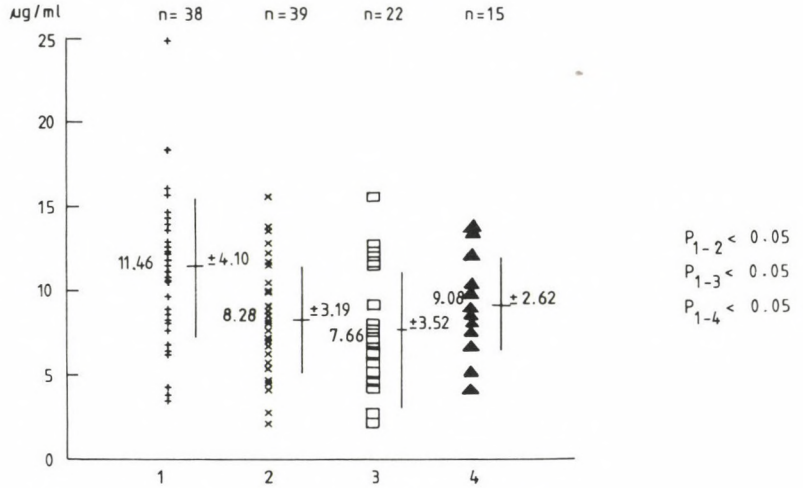

Fig. 1. Serum E vitamin level of all patients and of the controls:
1. Group of the healthy blood donors,
2. all patients with chronic liver diseases,
3. group of cirrhotic patients,
4. patients with various forms of chronic alcoholic liver diseases without cirrhosis

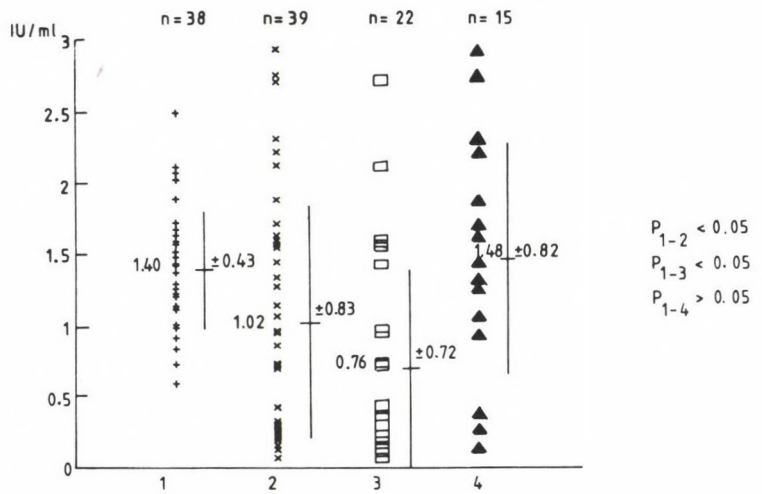

Fig. 2. Serum A vitamin level of all patients and of the controls:
1. Group of the healthy blood donors,
2. all patients with chronic liver diseases,
3. group of cirrhotic patients,
4. patients with various forms of chronic alcoholic liver deseases without cirrhosis

Comparisons were carried out in groups of patients with an expressed and without any biochemical activity independent of areas and diagnoses (Fig. 4). The results showed no statistical differences.

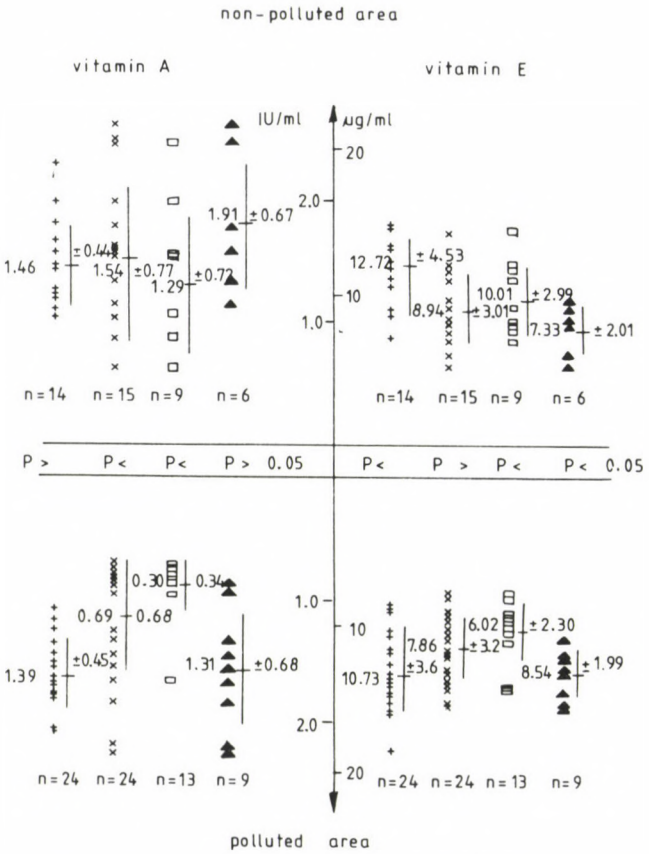

Fig. 3. Comparison of vitamin levels (A and E vitamins) of patients with chronic alcoholic liver diseases and of the healthy controls in non-polluted (above) and in polluted (below) areas.
1. Groups of healthy blood donors,
2. groups of all patients with chronic alcoholic liver diseases,
3. groups of patients with alcoholic cirrhosis,
4. groups of patients with various forms of chronic alcoholic liver diseases

The connection between a very sensitive free-radical parameter in the early phase of free-radical damage, diene conjugate extract from RBC and characteristic parameters of alcoholic liver damage as well as vitamin levels are demonstrated in Table II.

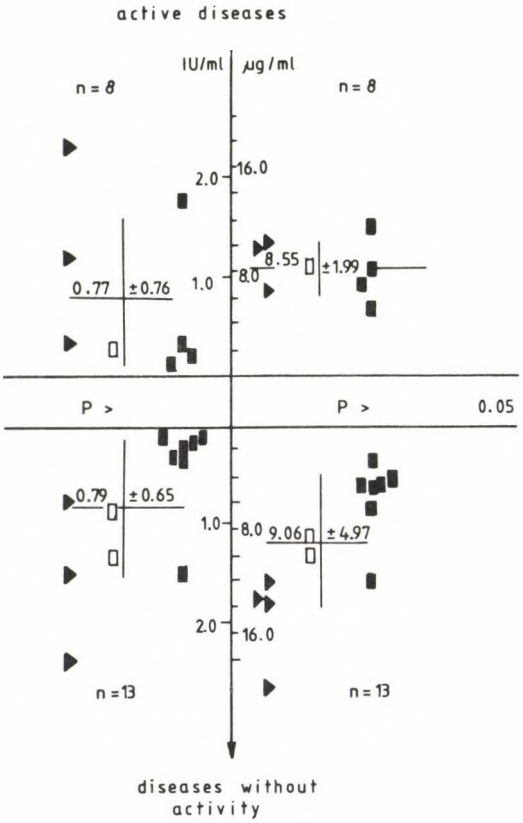

Fig. 4. Comparison of vitamin levels (A and E) of patients with (above) and without (below) biochemical activity of the disease. Signs: ■ cirrhosis hepatis, ▶ chronicus alcoholic hepatis, □ alcoholic fatty liver

Diene conjugate level showed a positive correlation with gamma-GT content of biopsy material on high significance level, a nearly significant negative correlation with vitamin levels (more expressed in the case of vitamin E), and no tendency of correlation with the other parameters of alcoholic liver disease. Levels of the two vitamins investigated showed in the healthy controls a positive correlation with each other, but their connection in the patient groups showed a dissociation (Fig. 5).

Table II

Correlation between RBC diene conjugate extract values and characteristic parameters of chronic alcoholic liver disease in serum and in the bioptic material of the liver

In biopsy material					In serum	
borderland values	observed	n	test	n	observed	borderland values
0.8114	0.0418	5	SGOT	17	0.0884	0.4683
0.8114	0.9435*	5	gamma-GT	17	0.2141	0.4683
			vitamin E	9	-0.6053*	0.6319
			vitamin A	9	-0.5105*	0.6319
			cholesterol	17	0.0488	0.4683

*$p \leq 0.05$

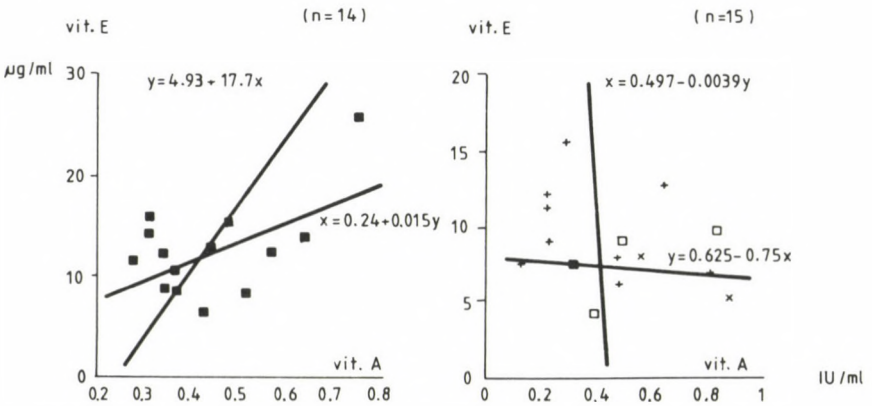

Fig. 5. Correlation of studied vitamin levels (A and E) in a healthy and in a patient group. Signs: ▪ healthy, □ alcoholic fatty liver, X chronic alcoholic hepatitis, + cirrhosis hepatis

Discussion

Supposing as "0"-hypothesis of study the similar drinking habits within the two populations investigated, differences between levels of scavanger (E) and cytoprotective (A) types of vitamins can be explained with the causative role of additive factors only. Biochemical activity of the disease did not give explanation of these differences. Changes in vitamin levels showed a tendency of correlation with the other free-radical parameter of the early phase of free-radical damages, the diene conjugate value, which is in strong correlation with the most dynamic biochemic parameters (gamma-GT value) of alcoholic liver disease [5, 11, 17].

The known scavanging function of vitamin E can explain its changes in alcoholic liver diseases, but not the differences between patients in the two areas [7, 15]. Changes of vitamin A level can be explained by its changed metabolism in alcoholics, which depends partly on alcoholic enzyme induction and partly on its role in cytoprotection, but their differences between the two areas can be explained by different influencing factors only [9, 10]. Diminution of A vitamin level is not proportional with the changes of vitamin E level in patients; this observation is controverse for a nutritional factor in the mechanism of changes. Our results suggest an important role of additive free-radical loading, e.g. of environmental factors in addition to alcoholic effects on the diminution of scavanging and cytoprotective vitamin levels in patients with chronic alcoholic liver disease; the observed differences in the two areas between the scavanging and cytoprotective vitamin levels suggest a higher demand of these vitamins in polluted areas. Further investigations are needed to establish the clinical significance of environmental factors in the progression of free-radical mediated diseases.

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POST-CHOLECYSTECTOMY SYNDROME AND MAGNESIUM DEFICIT

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The serum level of magnesium and calcium was systematically measured in patients with gallstones before and after cholecystectomy. It was found that 60 percent of the operated patients suffered of different digestive syndromes in association with magnesium deficiency, while 40 per cent of patients had the same complaints in association of magnesium and calcium deficiency. When magnesium and/or magnesium plus calcium was supplemented these syndromes could be decreased significantly. In the latter case, an optimal ratio of magnesium/calcium is needed in the supplementary therapy.

Keywords: cholecystectomy, deficiency of magnesium and calcium after cholecystectomy, supplementation of magnesium and calcium after cholecystectomy

According to data in literature, 20–30% of the cholecystectomized patients develop a post-cholecystectomy syndrome (PCES) after months or years. The etiopathogenesis of this syndrome is clear in certain cases (choledochal lithiasis, choledochal stricture, cholesterolosis, obstructive papillitis, pancreatic duct stenosis), but there are other instances when anatomical lesions cannot be evidenced [12, 13, 16]. This fact suggests functional disturbances. According to Durlach, the functional disturbances of the digestive tract and biliary tree are magnesium-dependent [9]. Magnesium participates in the biocatalyzation of over 360 biochemical reactions. Thus, it takes part in the synthesis of gastric-juice enzymes and mucins and of digestive polypeptide hormones such as pancreozymin-cholecystokinin the role of which in the bile-ducts physiology is well known [9, 10]. Magnesium is indispensable for the synthesis of macroergic compounds and release of the energy stored in these compounds, as well as for the synthesis of hydrogen and electron transporters [1, 2, 3, 4, 5, 6, 7, 8, 11, 14, 15, 17, 20, 21]. All these evidence the cytoprotective role of this

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quasi-totalitary intracellular ion. Disturbance of its homeostasis entails a lot of functional imbalance.

Starting from these considerations, in this study we investigated the extent to which magnesium deficit is involved in PCES etiopathogenesis.

Material and methods

The study included 40 cholecystectomized patients with PCES and magnesium deficit (MD), in which organic lesions of the remaining bile ducts were excluded by imaging and endoscopic methods. Twelve of these patients already had records before surgery. The patients were followed up for a mean period of 12.7 months, using test-sheets with the following data: age, sex, levels of serum and erythrocytic magnesium, serum calcium, disturbances of the central and peripheral nervous system, digestive, trophic and functional disturbances. All the patients received substitution therapy with TIOMAG (Mg gluconate + methionine), according to a regimen devised in the 3rd Medical Clinic of Cluj-Napoca. Magnesium and calcium dosage was performed by a photocomplexometric method.

Results

Mean age of the study group was 43.6 years (Fig. 1). 83.3% of the patients were women (Fig. 2). Cholecystectomy was performed for the affections presented in Table I.

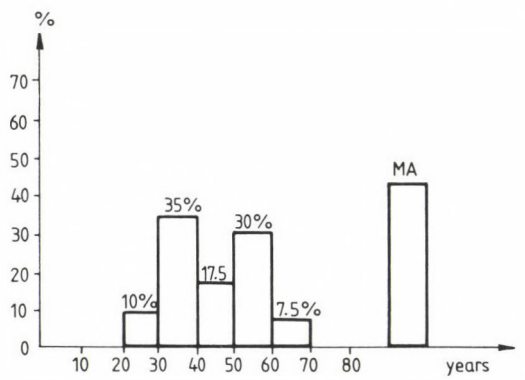

Fig. 1. Distribution by age decades of patients with solitary magnesium and magnesium plus calcium deficiency. Mean age = 43.6 years

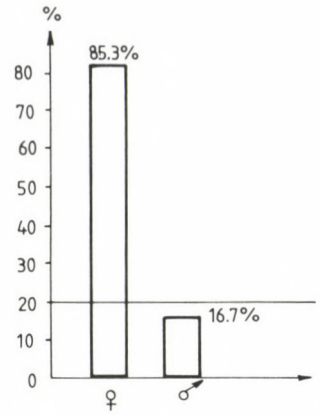

Fig. 2. Sex distribution of patients with magnesium deficiency

Table I

Diseases which indicated cholecystectomy

Disease	No. of patients	Percentage
Biliary lithiasis	35	87.5
Choledochal lithiasis	2	5
Gallbladder cholesterolosis	1	2.5
Gallbladder malformation	1	2.5
Non-lithiasic cholecystitis	1	2.5

Solitary MD was found in 60% of the patients; the other 40% of the patients had simultaneous magnesium and calcium deficit (Fig. 3).

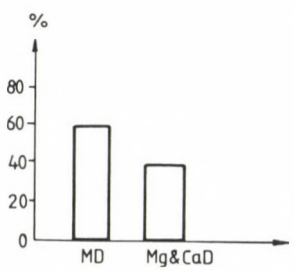

Fig. 3. Relation between solitary magnesium deficiency (MD) and simultaneous Mg and Ca deficiencies

Normal Ca/Mg balance was restored by standard TIOMAG therapy (Fig. 4).

Substitution therapy

regimen	
vit. B_1	300–600 mg Mg
vit. B_6	60–100 mg
Ca^{++}	250–750 mg
	185 mg (2×1 tabl. Ca lact.)

Fig. 4. Substitution therapy regimen

The mean time necessary to rebalancing is presented comparatively with two groups (Fig. 5): one with spasmophilia with digestive manifestations (but not biliary) and another with spasmophilia without digestive manifestations.

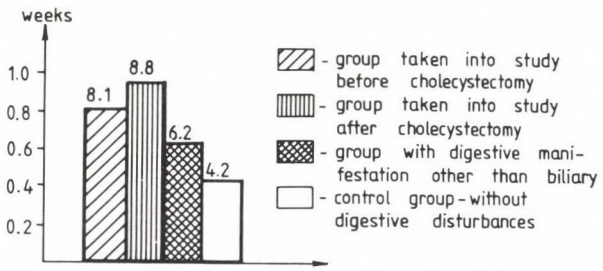

Fig. 5. Mean time required by Mg/Ca rebalancing

The mean values of serum and erythrocytic magnesium are given in Fig. 6.

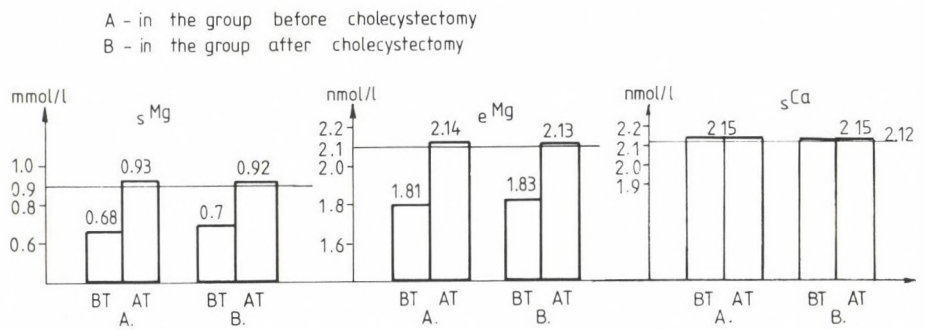

Fig. 6. Magnesium deficiency (MD) correction by TIOMAG treatment in patients with cholecystectomy (before and after)

Frequency of digestive manifestations is presented in Fig. 7. After substitution therapy these manifestations disappeared.

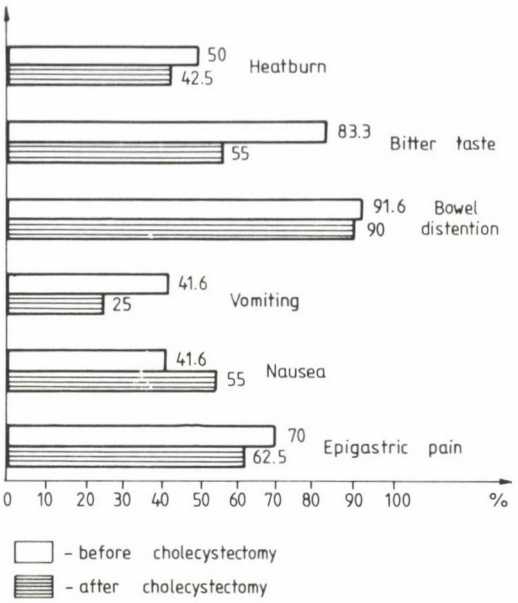

Fig. 7. Frequency of digestive syndrome manifestations in patients

In 25% of the patients, who discontinued TIOMAG treatment for more than 2 months, digestive disturbances and MD recurred. After resumption of treatment and MD correction, these disturbances disappeared again, which proves their magnesium-dependence and reversibility.

Discussion

The TIOMAG preparation is composed of Mg gluconate and methionine [18]. This association is based on the metabolic interaction of magnesium with methionine and its metabolites, which supply 80% of sulphur requirements in the body [19] (Fig. 8).

In order to establish an adequate therapeutic regimen, it is necessary to know the magnesium levels and Mg/Ca ratio, because in the case of an associated hypocalcemia Ca deficit must also be corrected. This can be done simultaneously with Mg substitution therapy, by administering a mean dose of 186 mg Ca^{2+}/day. Observance of this dose is very important, for the range in which Ca–Mg antagonism does not manifest is very narrow.

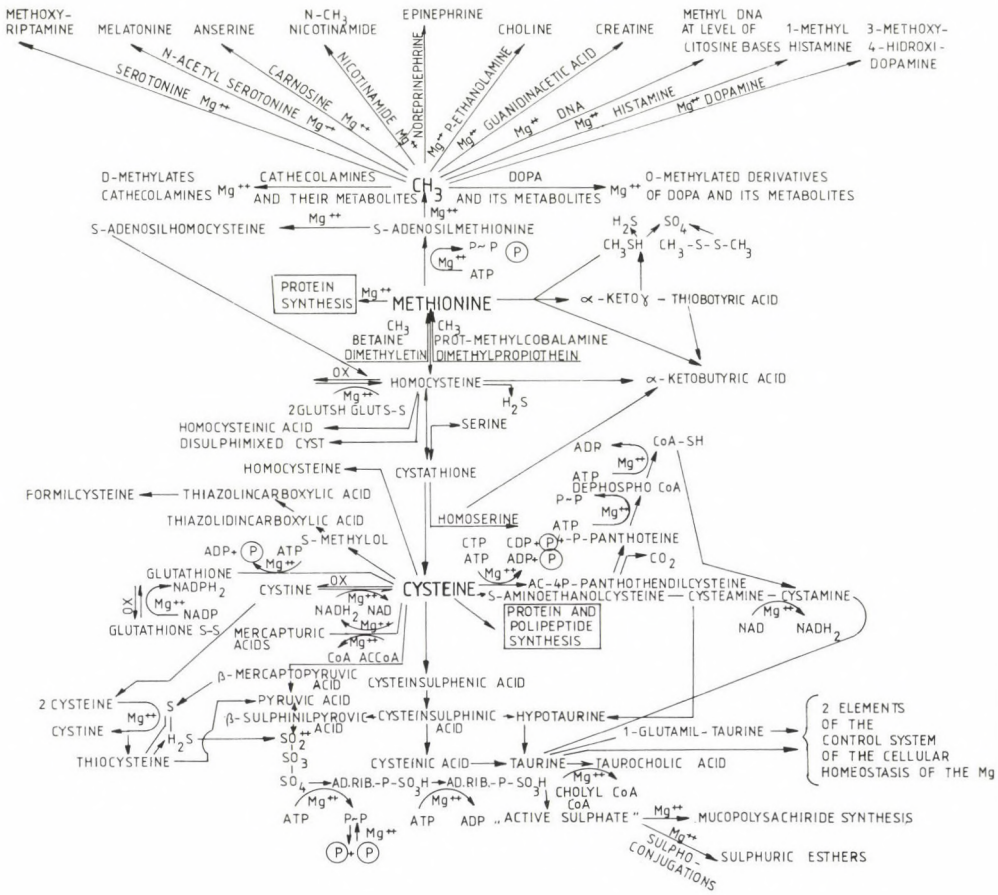

Fig. 8. Metabolic interactions of Mg with methionine and its sulfurated metabolites

The time required for Mg/Ca rebalancing was prolonged in the group studied. The cause of this prolonged time is probably the absence of gallbladder and intermittent bile evacuation into the duodenum, with consequences on magnesium intestinal absorption.

The results obtained show that functional hepato-biliary and gastrointestinal disturbances in the group studied are magnesium-dependent. TIOMAG treatment eliminates these disturbances, reestablishing normal function of hepatocytes and bile ducts. Discontinuation of treatment for more than 2 months leads to recurrence to the above-mentioned symptoms. Resumption of TIOMAG therapy makes the symptoms subside again. Systematic magnesium administration, according to requirements, under clinical and biochemical follow-up, prevents recurrence of PCES manifestations.

These findings demonstrate the cytoprotective role of our preparation on the hepatocyte and bile ducts.

Conclusions

1. Functional manifestations of PCES subsided after TIOMAG substitution therapy, together with MD correction.
2. Discontinuation of substitution therapy determined recurrence of clinical symptoms, which subsided again after the treatment was resumed.
3. The findings demonstrate the dependence of PCES functional manifestations on MD.
4. Continuation of substitution therapy with a maintenance dose prevents recurrence of PCES symptoms.

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PANCREATIC CYTOPROTECTION: NEW APPROACHES

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A brief report is given on the possible role of oxygen-derived free radicals and cholecystokinin in the pathogenesis of experimentally induced acute pancreatitis. Furthermore, use of scavangers (superoxide dismutase, catalase), CCK-receptor antagonists and somatostatin are discussed in the therapy of acute pancreatitis induced in animal models. It is suggested that both the term of direct pancreatic cytoprotection of the above-mentioned agents and the validity of the animal models used for induction of acute pancreatitis have to be reconsidered.

Keywords: pancreas, cytoprotection, pancreatitis, free radicals, scavangers, CCK-receptor antagonists, somatostatin

Aim of researchers studying the pathogenesis of the acute pancreatitis is to find out a common denominator (trigger) which initiates the pathological process in the gland resulting in pancreatic autodigestion. In the following I am making an attempt to outline the new tendencies to clear up such a denominator (trigger) and by this way to establish a causal understanding and therapy of the disease.

The following factors are discussed:

1. the pathological role of the free radicals and the therapeutic use of the free radical scavangers,
2. use of cholecystokinin receptor antagonists,
3. use of trypsin inhibitors and
4. use of somatostatin in experimental acute pancreatitis.

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1. Oxygen-derived free radicals

In the past few years more and more publications appeared in which, an attempt was made to demonstrate oxygen metabolites as causative factors in initiating autodigestion of the exocrine pancreas. Then, the investigators attempted to combat oxygen-derived free radicals and by this way to influence favourably the outcome of acute pancreatitis provoked experimentally by different agents, like local ischaemia, bile acids, ethanol of even by hormonal hyperstimulation of the gland.

The theoretical base of such studies is that both vascular endothelial injury, resulting in increased local capillary permeability, furthermore neutrophilic inflammation are demonstrable in the onset the disease. In the capillary wall, activation of xanthine dehydrogenase-oxidase system, in neutrophils a reduction of a plasma-membrane associated NADPH oxidase can generate oxygen-derived toxic products including the superoxide free radical, hydrogen hyperoxide and the hydroxy radical [11, 27–29, 35].

The mechanism of this biochemical process is, that the xanthine dehydrogenase-oxidase system of the capillary endothelial cells is activated to xanthine oxidase for example by ischaemic injury, pancreatic ductal obstruction or by chymotrypsin [28]. Hypoxanthine is produced from ATP during the ischaemia. In the presence of oxygen, xanthine oxidase reduces hypoxanthine to xanthine and generates toxic superoxide radicals (superoxide free radical hydroxy radical and hydrogen hyperoxide) [28, 35]. All these toxic oxygen metabolites are normally detoxified by the endogenous free radical scavangers, like intracellular superoxide dismutase (SOD) and catalase. Without their detoxification these oxygen metabolites damage the capillary wall or attack the lipid membranes and proteins. Interaction of fatty acids of the lipid membranes and the oxygen-derived free radicals lead to lipid peroxidation. The process produces aldehydes. Aldehydes generated from lipid peroxidation can disrupt the membrane architecture by introducing hydrophilic moieties into the hydrophobic lipids [35]. Aldehydes can be transported into other organs by attacking them [32, 33]. Malonaldehyde is produced during the peroxidation of fatty acids containing three of more double bounds.

Besides the capillary endothelial and leukocytic sources of oxygen metabolites other sources of oxidants do exist: they are among others the prostaglandin synthetase and lipoxygenase systems, etc. [35].

Sixteen years ago we were the first who called attention to the process of the *in vivo lipid peroxidation* in the process of the experimentally induced acute pancreatitis. Intraductal injection of linoleic acid produced a very extensive necrosis of the rat pancreas and resulted in the increase of the malonaldehyde content of the gland [12]. Joan Braganza [3, 4], who attaches a great importance to the free radicals in the inflammatory pancreatic diseases and in the development of liver

complications of the chronic pancreatitis evaluated our study – in a private letter written to us – as a pioneering work. Recent work of Vollmar et al. [33] demonstrated that intraarterially injected oleic acid in the pancreas induced acute pancreatitis, while arachidonic cascade was activated and both prostacycline, and vasoactive thromboxane A_2 was related into the pancreatic venous blood and lymph. The significance of *degradation products from triglycerides by lipase* was stressed by Nagai et al. [20] in pancreatic cellular disruption. This article proved our results obtained *in vivo* and published 20 years earlier [5, 24]. These studies [5, 24] called attention to the role of pancreatic digestive enzymes like to that of lipase in the initiation of pancreatic self-digestion.

It is obvious that both the products of the damaged capillary endothelium [21, 25], its enzyme (xanthine oxidase) system and the active or activated digestive enzymes like lipase, trypsin, etc. may attack the exocrine pancreatic parenchyma.

Results obtained with superoxide dismutase (SOD) and catalase: From theoretical point of view one has to prevent or treat pancreatic tissue damages by *scavangers*, enzymes like superoxide dismutase which neutralizes O_2 superoxide, or with catalase which neutrolizes H_2O_2, or by antioxidants as vitamins C, E, or by inhibiting the generation of the free radicals by means of allopurinol, a *specific xanthine oxidase inhibitor.*

In caerulein-induced acute pancreatitis in the rat pancreatic malonaldehyde concentration is elevated [8, 29] and SOD activity of the gland disappeared [8]. Similarly, the chemoluminiscence of the pancreas, which is dependent on oxygen free radical activity increased in caerulein- and taurocholate-induced pancreatitis [14]. Protective effect of SOD and catalase was demonstrated in caerulein-induced pancreatitis *in vivo* [8, 29, 36] in pancreatitis induced by common bile duct obstruction [15], and in pancreatitis induced by intraarterial FFA, ethanol, or ductal obstruction + secretin administration in the perfused pancreas [27]. In another study oedema and inflammatory response was not affected by SOD and catalase treatment in caerulein-induced acute pancreatitis [29].

Active oxygen (lipoxygenase) metabolites were transported from the pancreas via lymphatic pathway in haemorrhagic porcine pancreatitis [32]. Pulmonary complications induced by experimental haemorrhagic pancreatitis were prevented by pretreatment with SOD and catalase [6]. Allopurinol, a specific xanthine oxidase inhibitor, improved the histological damage in the pancreas induced by ischemia [28].

2. Cholecystokinin receptor antagonists in the cytoprotection of the pancreas

It is well known that supramaximal doses of caerulein induce acute oedematous pancreatitis in the adult rat [16]. Since the decapeptide caerulein has the same amino-acid sequence of its C-terminal as the CCK, the question arises if

application of cholecystokinin receptor-antagonists do have a favourable effect on such pancreatic tissue damage.

Application of L364,718 a specific cholecystokinin receptor antagonist decreased tissue damage of the pancreas induced by caerulein but it had no effect on the pancreatitis, induced by oleic acid or by ischemia, or on damage of the canine pancreas initiated by common bile duct obstruction combined with coadministration of secretin [22a]. Proglumide and benzotript exert a protective effect on caerulein-induced pancreatitis of the rat [22]. The course of pancreatitis induced by choline-deficient ethionine supplemented diet was aggravated by hydrocortisone. Treatment with lorglumide, a specific CCK-receptor antagonist, however, completely abolished the adverse effect of hydrocortisone on the diet-induced pancreatitis [13]. Glucocorticoids increased the sensitivity of the acinar cells to secretagogues' stimulation [10].

3. Effect of synthetic proteinase inhibitors (Foy 305: Camostate, Foy: Gabexate mesilate) on experimentally induced acute pancreatitis

Synthetic proteinase inhibitors are effective in inhibiting activity of trypsin, production of vasoactive substances, etc. It is suggested that small amount of activated trypsin was released from trypsinogen and it activated the zymogen forms of the stored and secreted digestive enzymes. Theoretically, use of proteinase inhibitor seems to be justified in acute pancreatitis.

Prophylactic administration of camostate had a favourable effect on the course of the bile-induced-pancreatitis and its use was beneficial on the survival rate and tissue damage in the diet-, and bile-salt-induced experimental pancreatitis [18]. Camostate dissolved in peritoneal lavage fluid in taurocholate pancreatitis improved its therapeutical efficiency [19]. Similarly, intraductally administered gabexate mesilate inhibited the intrapancreatic trypsin activity and increased the survival rate in taurocholate pancreatitis of dogs [34].

4. Use of somatostatin in acute pancreatitis

The beneficial effect of somatostatin in acute pancreatitis is debated [1, 7, 9]. One group of authors published that use of somatostatin in acute human pancreatitis is not proved to be better than the traditional medical treatment in the disease [9]. Other group of authors found that somatostatin seemed to reduce the local complications of the human acute pancreatic disease [7]. It is obvious that somatostatin can reduce the serum level of pancreatic digestive enzymes in experimental acute pancreatitis [2, 17]. We had the same results [30, 30a]. However, we found that somatostatin applied for more than 4 days after induction of caerulein-

pancreatitis inhibited the replication process (restauration of DNA content) in the pancreas during the regenerative phase of the pancreatitic process [30, 30a] (Fig. 1).

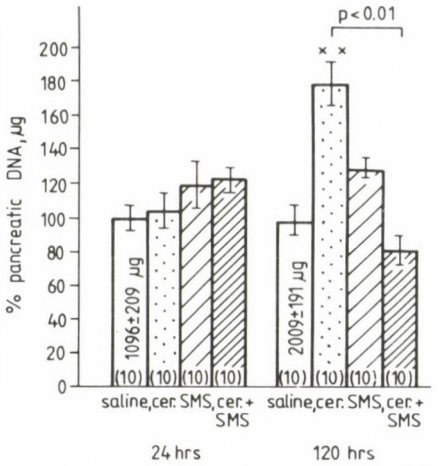

Fig. 1. The effect of caerulein-induced pancreatitis and its treatment with Sandostatin (4 µg/kg, 2x/day for 5 days) of the % change of pancreatic DNA at the 2nd and 5th day after the induction of pancreatitis. Saline and Sandostatin (SMS), did not change the DNA content of the intact pancreas given for 5 days. Induction of caerulein pancreatitis significantly increased the DNA content (regenerative phase) of the injured gland compared to saline control ($P < 0.01$). SMS given for 5 days counteracted the increase in DNA content evoked by caerulein pancreatitis. *Groups: saline* = intact control treated with saline, *SMS* = intact rats treated with SMS as above, *cer.* = caerulein-induced acute pancreatitis treated with saline, *cer. + SMS* = caerulein-induced pancreatitis treated with SMS as above. 10–10 Male Wistar rats weighing 90 g in average are in each group. Mean ± S.E.M. are shown

The term of pancreatic direct *cytoprotection* includes the property of some agents by which they prevent damage of the pancreas without they would have or exert an explicit pancreatic antisecretory activity [23]. In this respect, however, the CCK-receptor antagonists or somatostatin have an inhibitory action on pancreatic secretion and growth [26, 31]. Thus, their therapeutic effect on the pancreas cannot be yet determined as direct pancreatic cytoprotection. Indeed, it is to be cleared, how scavangers do influence pancreatic secretion and growth.

In conclusion: Although there are new tendencies in approaching and understanding better the pathogenesis of acute pancreatitis and by this way the pancreatic cytoprotection we cannot conclude

(i) whether the initiation of the pathological process is due to an extracellular or to an intracellular trigger or rather to a mechanism induced by both denominators,

(ii) whether the common trigger of the autodigestive process has vascular endothelial or even digestive enzymatic character or both of them.

The failure of such conclusions may be attributed to the fact that the animal models which we use in our studies may be irrelevant or do not imitate the real, causal pathological process initiating and pursuing the autodigestive process in the human gland.

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THE EFFECT OF SOMATOSTATIN, GABEXATE MESILATE AND DEXTRAN 40 ON THE MICROCIRCULATION IN SODIUM TAUROCHOLATE-INDUCED PANCREATITIS

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In vivo microscopy was performed to assess the effect of dextran 40, gabexate mesilate and somatostatin on the microcirculation in sodium taurocholate-induced pancreatitis in rats. Intraductal infusion of 0.4 ml of a 4% solution of sodium taurocholate decreased capillary blood flow, induced capillary stasis and increased vascular permeability in the head of the pancreas. Dextran 40, gabexate mesilate and somatostatin improved capillary blood flow in the initial phase of acute pancreatitis significantly and prevented stasis in 5 of 9, 3 of 8 and 7 of 10 ($p < 0.05$) cases. Only dextran 40 reduced the increase of vascular permeability. Decrease of capillary blood flow, capillary stasis and vascular permeability changes are important factors contributing to the pathogenesis of sodium taurocholate-induced pancreatitis. Dextran 40, gabexate mesilate and somatostatin exert a beneficial effect on the microcirculatory changes in this model of acute pancreatitis.

Keywords: acute pancreatitis, sodium taurocholate, microcirculation, *in vivo* microscopy, blood flow, microvascular permeability

Several attempts have been made to influence the fatal course of acute pancreatitis. These include the inhibition of exocrine pancreatic secretion, the prevention of autodigestion by application of proteinase inhibitors and hemodilution by infusion of plasma expanders to support circulation. In animal models pretreatment with somatostatin [23], gabexate mesilate [24] or hemodilution with dextran [11] have revealed promising results.

The intraductal infusion of sodium taurocholate into the pancreatic duct leads to a well reproducible type of pancreatitis with hemorrhagic necrosis [1–3]. We

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recently demonstrated that stasis, ischemia and permeability changes of the microcirculation are early microcirculatory events in this model [13]. The microcirculatory changes were observed predominantly in the head of the pancreas, where hemorrhagic necrosis occurs, while in the corpus and the tail of the pancreas the microcirculation remains intact. Studies with *in vivo* microscopy in bile-induced pancreatitis in the cat revealed that hemodilution with dextran improved the microcirculation within the pancreas [11]. Pretreatment with somatostatin in bile-induced pancreatitis in the dog prevented hemorrhagic necrosis [23].

Gabexate mesilate or camostate, two proteinase inhibitors, showed some beneficial effect in pancreatitis induced with taurocholate [15], choline-deficient ethionine-supplemented diet (CDE) [15, 19] or cerulein [24].

Our previous investigations [13] revealed that microcirculatory changes contribute to the pathogenesis of taurocholate-induced pancreatitis. In this study we demonstrate that somatostatin, gabexate mesilate and dextran influence the taurocholate-induced microcirculatory changes and prevent stasis.

Materials and methods

Female Wistar rats (180–250 g body wt) were anaesthetized with sodium pentobarbital (6 mg/100 g ip). The operations were performed on an operation blanket (Harvard) and the body temperature was maintained at 37 °C. For the intravenous application of drugs and tracers the vena cava was cannulated. The duodenal loop was carefully mobilized and fixed to a metal plate that was kept at 37 °C. The exposed pancreas was superfused with saline (37 °C). The pancreatic duct was cannulated via the papilla of Vater with a 3 cm plastic tube. The volume of the tube was < 0.01 ml. The start of the experiment (t = −20 min) was defined as the ligation of the common bile duct.

In vivo microscopy was performed using an epi-illumination microscope (Leitz) with ×10 and ×25 objective lenses (Leitz, Fluotar 10/0.45 and 25/0.6). The light of a mercury vapor lamp passed through a filter set with an excitation filter of 450–490 nm, a dichroic mirror (510 nm) and a barrier filter (515 nm). A cadmium selenide video camera (Siemens) was connected to the microscope and the images were recorded using a video cassette recorder (Panasonic). A video analyzer system (Datacube) digitized every image into 512 × 512 pixels. A pixel with 8 bits resolves 256 grey values.

Microcirculatory stasis, capillary blood flow and permeability changes were measured off line. The time from the start of the intraductal infusion to the complete stop of the microcirculatory blood flow within the area under observation was defined as the time of total stasis. The time when the first stop in a single capillary occurred was defined as the time of partial stasis. Capillary blood flow was assessed with a semiquantitative scale from 0 to 4 with the following definition: 0: stasis; 1: flow with clear particle identification and intermittant stops; 2: flow with clear particle identification and no stops; 3: particle identification just possible; and 4: no particle identification possible. Permeability changes were measured with the image analyzing system. The changes of average grey values of a rectangular area within a pancreatic lobule were measured. Extravasation of the fluorescent tracer increased the light intensities within these rectangles.

Protocols

At t = −15 min a 5% solution of fluorescein isothiocynate (FITC)-dextran (Sigma, molecular weight 150,000 u) was injected intravenously (dose 0.04 ml/100 g) followed by an infusion of one of the therapeutic substances. Group 1 received saline i.v., group 2 dextran 40 (160 mg/kg bolus and 450 mg/kg × h i.v.), group 3 gabexate mesilate (3.2 mg/kg bolus and 9 mg/kg × h i.v.) and group 4 somatostatin (0.22 mg/kg bolus and 0.65 mg/kg × h i.v.). 15 minutes later (t = 0 min) pancreatitis was induced in all groups by intraductal infusion of 0.4 ml of 4% sodium taurocholate for 5 min 20 s. The images of the microcirculation were recorded from t = −5 min to t = 30 min.

Statistics

All values are reported as means ± SE. The unpaired Student's *t*-test was used to determine statistical differences between groups. The statistical differences between proportions were tested with the Fisher–Yates exact test.

Results

Gross observations

Intraductal infusion of 0.4 ml of a 4% solution of sodium taurocholate leads to haemorrhagic necrosis in the head of the pancreas in all rats of group 1. In the corpus and tail the pancreas developed edema. Dextran 40, gabexate mesilate or somatostatin prevented hemorrhagic necrosis in 4 of 9, 2 of 8 and 7 of 10 cases, respectively.

Table I

The effect of pretreatment (t = −15 min) with dextran 40 (160 mg/kg bolus i.v. + 450 mg/kg × h), gabexate mesilate (3.2 mg/kg bolus i.v. + 9 mg/kg × h) or somatostatin (0.22 mg/kg bolus i.v. + 0.65 mg/kg × h) on the incidence of stasis and on total and partial stasis times in the head of the pancreas after intraductal infusion (t = 0 min until t = 5 min 20 s) of 0.4 ml sodium taurocholate (4% solution)

Pretreatment	Partial stasis (s)	n_1	Total stasis (s)	n_2
saline	93 ± 27	8/8	232 ± 47	8/8
dextran 40	242 ± 142	8/9	121 ± 37	4/9
gabexate mesilate	120 ± 24	8/8	175 ± 39	5/8
somatostatin	173 ± 60	5/10	223 ± 45	3/10*

Stasis values are means ± SE; n_1: incidence of partial stasis vs. no. of experiments; n_2: incidence of total stasis vs. no. of experiments. *$p < 0.05$ (Fisher–Yates exact test).

Microcirculatory changes

Sodium taurocholate induced total stasis of the microcirculation within 3 to 4 min (Table I). The time of stasis was not influenced by the therapeutic agents. Somatostatin significantly prevented the incidence of stasis.

Figure 1 illustrates the effect of intraductal infusion of sodium taurocholate on capillary blood flow. Sodium taurocholate lead to a sudden decrease of capillary blood flow. Dextran 40, gabexate mesilate and somatostatin prevented this decrease of blood flow significantly.

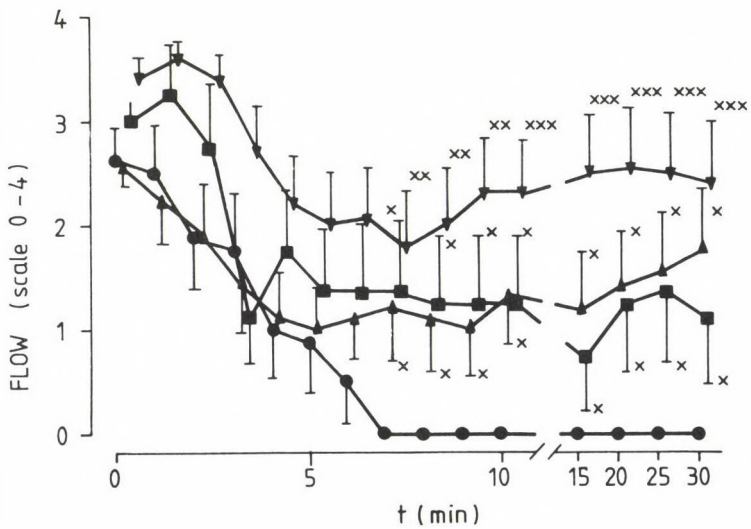

Fig. 1. The effect of pretreatment (t = −15 min) with saline •, dextran 40 ▲ (160 mg/kg bolus i.v. + 450 mg/kg × h), gabexate mesilate ■ (3.2 mg/kg bolus i.v. + 9 mg/kg × h) or somatostatin ▼ (0.22 mg/kg bolus i.v. + 0.65 mg/kg × h) on capillary blood flow in the head of the pancreas after intraductal infusion (t = 0 min until t = 5 min 20 s) of 0.4 ml sodium taurocholate (4% solution). *p < 0.05, **p < 0.01, ***p < 0.001 compared to saline group

Figure 2 demonstrates the increase of vascular permeability induced by sodium taurocholate. In group 1 sodium taurocholate increased the permeability during the first minutes. Then stasis of the microcirculation occurred and no fluorescent tracer could extravasate any more so that no further increase of the measured light intensities was noted. The prevention of stasis by the protective agents preserved blood flow and the transport of FITC-dextran to the damaged capillaries was maintained. This resulted in an increase of the measured light intensities. Somatostatin prevented stasis of the microcirculation most efficiently, but had no effect on the permeability changes induced by taurocholate. This resulted in the

strongest extravasation of FITC-dextran in this group. Gabexate mesilate was less effective in preventing stasis in a lower degree of extravasation of FITC-dextran compared to the somatostatin group, because it had no beneficial effect on the increased vascular permeability. Dextran 40 preserved blood flow more effectively than gabexate mesilate and prevented stasis in 4 of 9 cases, but marked extravasation of FITC-dextran did not take place in those experiments.

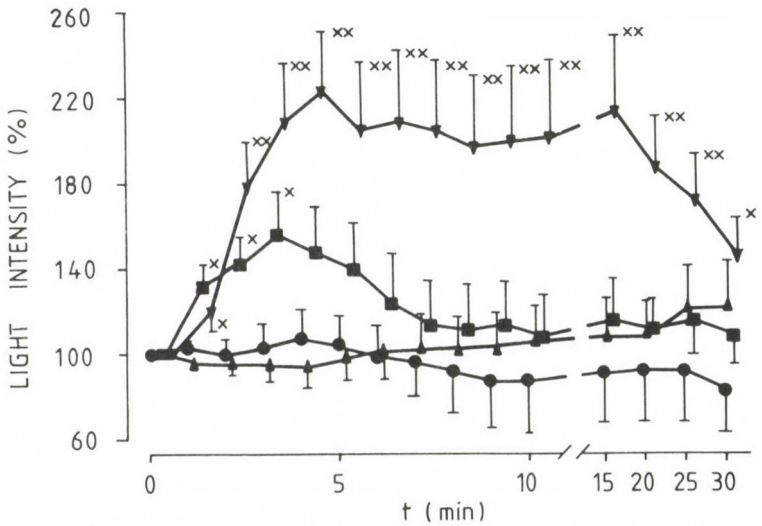

Fig. 2. The effect of pretreatment (t = −15 min) with saline •, dextran 40 (160 mg/kg bolus i.v. + 450 mg/kg × h), gabexate mesilate ▪ (3.2 mg/kg bolus i.v. + 9 mg/kg × h) or somatostatin (0.22 mg/kg bolus i.v. + 0.65 mg/kg × h) on vascular permeability changes in the head of the pancreas after intraductal infusion (t = 0 min until t = 5 min 20 s) of 0.4 ml sodium taurocholate (4% solution). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to saline group

Discussion

Infusion of sodium taurocholate into the pancreatic duct of rats leads to hemorrhagic necrosis, decrease of blood flow, stasis of the microcirculation and to an increase of vascular permeability [13]. Sodium taurocholate administration induces a severe type of pancreatitis [1, 2] that is usually therapy-resistant. However there have been few reports about successful pretreatment, for example with the proteinase inhibitor camostate [15]. In this study we demonstrate the protective effect of dextran 40, gabexate mesilate and somatostatin on the pancreatic microcirculation in the experimental model of taurocholate-induced pancreatitis. The three therapeutic agents used in this study prevented microcirculatory stasis and improved capillary blood flow. Only dextran could prevent the permeability changes induced by taurocholate.

There is a considerable body of evidence that dextrans exert beneficial effects in the treatment of experimental pancreatitis [4–8, 11–12, 16, 21]. Dextrans reduce blood viscosity and thereby improve pancreatic microcirculation. Despite the reduction of the concentration of circulating erythrocytes by hemodilution with dextrans the oxygen supply of the tissue is improved [18]. Klar et al. [11] found a significant protection of pancreatic capillary perfusion correlating with a limitation of parenchymal destruction in a model of bile-induced pancreatitis in rabbits. This is in good agreement with our results. We additionally measured a reduction of the extravasation of the fluorescent tracer FITC-dextran, indicating a protective effect of dextran 40 on taurocholate-induced microvascular permeability changes. In the hamster cheek pouch LTB_4-induced vascular permeability could only be prevented by dextran sulfate and not by dextran [20]. LTB_4-induced permeability is neurophil-dependent. The taurocholate-induced vascular permeability changes preceed the induction of leukocyte rolling and leukocyte adherence in the microcirculation (K. Kusterer unpublished observations), indicating that taurocholate-induced permeability is leukocyte-independent. Dextran 40 might interfere with the detergent action of taurocholate, thus attenuating the direct toxic effect of taurocholate on microvessels.

The pathomechanism of acute pancreatitis involves the intraparenchymal activation of pancreatic enzymes resulting in autodigestion of the gland. Gabexate mesilate is a proteinase inhibitor with a broad spectrum inhibiting not only trypsin and kallikrein, but also plasmin, thrombin, factor Xa, elastase, C_1 esterase and phospholipase A_2 [9]. Prophylactic application of gabexate mesilate or camostate showed some beneficial effect in CDE-induced pancreatitis [15, 19], cerulein-induced pancreatitis [24] and sodium taurocholate-induced pancreatitis [15]. The therapeutic effect of gabexate mesilate in acute pancreatitis has been ascribed to the inhibition of pancreatic enzyme activity, like trypsin, thus preventing autodigestion [24].

Marks et al. [17] found measurable activation of intrapancreatic proteinases not until 15 min after the induction of pancreatitis. The dramatic microcirculatory changes following taurocholate administration occur within the first minutes and probably preceed the activation of intrapancreatic proteinases in this model. Gabexate mesilate inhibits blood coagulation by inactivating thrombin and faxtor Xa [9].

In this study we demonstrate that gabexate mesilate is capable of preserving microcirculatory blood flow in acute pancreatitis. Therefore it might well be that the beneficial effect of gabexate mesilate in acute pancreatitis is not only related to the inhibition of autodigestion, but also to the protection of pancreatic blood flow.

The protective effect of somatostatin is attributed to the inhibition of exocrine pancreatic secretion. Pretreatment with somatostatin in bile-induced acute pancreatitis prevented hemorrhagic necrosis [23]. In sodium taurocholate-induced

pancreatitis no beneficial effect could be observed [14, 22]. In our study somatostatin was more effective in preventing stasis and drop of blood flow than dextran or gabexate mesilate.

In a recent study [13] we demonstrated regional differences in blood flow distribution within the pancreas. In sodium taurocholate-induced pancreatitis necrosis occurred predominantly in the head of the pancreas and ischemia and stasis were important pathogenetic factors there. In the corpus and tail the blood flow was preserved. Somatostatin might reduce total pancreatic blood flow [22], nevertheless it is more important that it prevents stasis and decrease of blood flow during acute pancreatitis in the most severely affected areas where hemorrhagic lesions develop. On the other hand, microcirculatory changes occur within minutes after the induction of acute sodium taurocholate-induced pancreatitis [13]. Schröder et al. [22] performed the measurements one and five hours after the induction of pancreatitis, which might be too late to demonstrate a beneficial effect of somatostatin on pancreatic microcirculatory blood flow. Taurocholate-induced vascular permeability changes were not prevented by somatostatin. In fact we measured more extensive extravasation of FITC-dextran in the somatostatin group than in all other groups. Nevertheless somatostatin prevented the occurrence of haemorrhage macroscopically. Intact blood flow might be more important for the protection of tissue integrity than prevention of vascular permeability changes in the treatment of acute pancreatitis.

The beneficial effect of gabexate mesilate in various models of acute pancreatitis has been attributed to the inhibition of proteinases preventing the autodigestion of the gland [9, 24]. Somatostatin inhibits exocrine pancreatic secretion which was assumed to influence the fatal course of acute pancreatitis [23]. Kaplan [10] hypothesized that disturbances of blood flow are essential factors in the pathogenesis of acute pancreatitis and suggested a unified concept. Our study revealed that somatostatin, gabexate mesilate and dextran are capable to prevent the previously characterized microcirculatory disturbances in the model of sodium taurocholate-induced pancreatitis in the rat. This supports the concept that the microcirculation is of essential importance in the pathogenesis of acute pancreatitis. It might well be that microcirculatory disturbances are the initial factors triggering the following pathophysiological events.

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ADEQUATE ENZYMATIC SUBSTITUTION IN TREATING EXOCRINE PANCREATIC INSUFFICIENCY

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The chymotrypsin in the stool test was used to monitor adequate enzymatic substitution in treating exocrine pancreatic insufficiency with 18 patients (16 suffering from chronic pancreatitis and 2 having passed duodenopancreatectomy due to pancreatic cancer). This test helps to identify pancreatic insufficiency and can be successfully used in monitoring the adequate amount of pancreatic substitute, which, we have found, differs from patient to patient. The dosage can be higher in cases of chronic pancreatitis than in those required after duodenopancreatectomy.

Keywords: enzyme replacement therapy, chymotrypsin

Medical treatment of pancreatic exocrine insufficiency is based on the oral administration of pancreatic enzymes. The effectiveness of pancreatic replacement is commonly assessed by measuring the reduction in degrees of steatorrhoea [7, 9], the duodenal recovery of enzyme activity after oral administration [5, 13] or by cholesteryl octanoate breath test [11]. Attention was focussed on lipase and other pancreatic esterases, which are irreversibly inactivated below pH 4 [3, 12]. Trypsin and other proteases are much sturdier enzymes and it was found that the treatment of azotorrhoea (protein malabsorption) offers no problems despite the fact that edema, hypoproteinemia, and weight loss are common, especially in cases of severe exocrine insufficiency. The degree of pancreatic insufficiency is tested by various, often laborious and expensive procedures [2]. Relatively simple and reliable is the determination of chymotrypsin activity in stool. The principle of this method is based on the determination of p-nitroaniline after the cleavage from the substrate – Boehring [1].

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Methods

We studied 18 patients (16 suffering from chronic pancreatic cancer) with severe exocrine pancreatic insufficiency. All patients showed clinical symptoms of chronic pancreatic insufficiency, biochemical test resulted abnormal (all had abnormal parabenzoic acid test results (PABA – Spofagnost) showing less than 25% PABA recovery in urine after six hours). Pancreozymin-Secretin test was performed in 5 patients with pathological results, all patients having decreased chymotrypsin activity in stool (less than 3 U/g). Four patients had pancreatic calcifications in abdominal X-rays, ultrasonography also was abnormal with all of them. Five patients required insulin (20–40 units per day) because of pancreatic diabetes. Enzyme substitution was carried out with pancreatin (Kreon Kali Chemie, FRG). This preparation contains 300 mg of pancreatin with an activity of 8 000 U lipase, 9 000 U amylase, and 450 U proteases per capsule (manufacturer's data). Each patient took capsules with meals several times a day. Chymotrypsin activity was tested with Boehringer's Chymotrypsin in the stool test. For the stool chymotrypsin determination we use the following reference ranges: the normal range is above 6 U chymotrypsin/g stool, the borderline being between 3–6 U/g, and the pathological range being below 3 U chymotrypsin/g stool. We used this test repeatedly every 10 days during the substitution therapy, which we carried out with the increased dosage of pancreatin to reach more than 6 U chymotrypsin/g stool.

Results

The amount of enzymatic substitute required to reach more than 6 U chymotrypsin activity/g stool differed from patient to patient, i.e. 6–24 capsules/day (Fig. 1). During the therapy with such patients we observed that there is an almost linear relation between the increase of the chymotrypsin activity in stool and the rising amount of the substitution, and thus it was possible to extrapolate the adequate amount of enzymatic substitution from two adjoining values (Fig. 2). Most patients on this regime became relatively asymptomatic with diarrhoea alleviation and pain relief. Two patients with severe signs of malnutrition (edema, hypoproteinemia, low serum cholinesterase) achieved a satisfactory nutritional status and their weight loss stopped. Two patients after duodenopancreatectomy required 5 400 and 6 300 mg of pancreatin/day, respectively, while patients suffering from severe pancreatitis required more (up to 7 200 mg pancreatin/day) to reach our limit of chymotrypsin activity in stool per day. Required doses of insulin in patients with pancreatic diabetes dropped (4–12 units per day). We did not meet any adverse effects of substitution despite high doses of pancreatin.

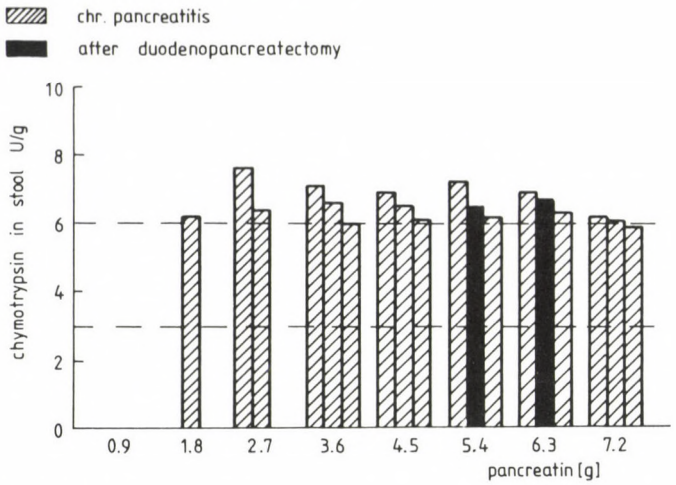

Fig. 1. Results of various treatment of chymotrypsin activity in stool in patients with chronic pancreatitis [16] and after duodenopancreatectomy [2]. Chymotrypsin in stool – decision limits: under 3 U/g – pathological range, 3 to 6 U/g – tentative area, above 6 U/g – normal range

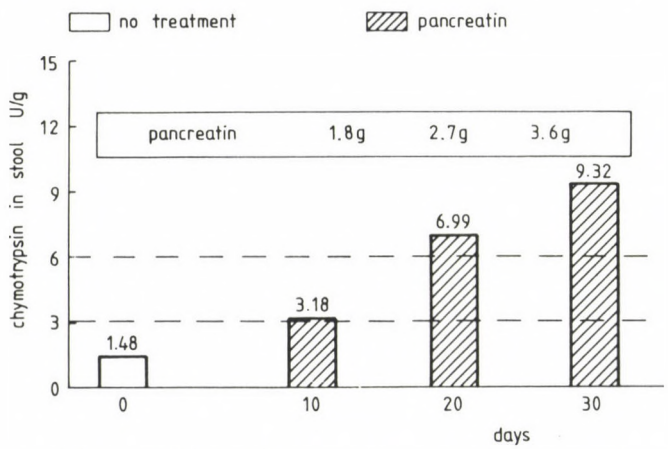

Fig. 2. Effect of treatment with increased dosage of pancreatin on chymotrypsin in stool in patient with chronic pancreatitis (male 42 years old)

Discussion

Pancreatic replacement therapy, an important part of the complex therapy, generally improves the nature and frequency of the stool but seldom restores entirely fecal fat to normal levels. This treatment improves absorption and nutrition and it is indicated not only in obviously underweight patients with gross steatorrhoea, but in all with small amount of active proteases in stool. Chymotrypsin is here an indicator

of other enzymes. Pancreatic substitution should be used where deficiency or malabsorption of protein has depressed pancreatic enzyme secretion [4, 16]. Malabsorption may result in an inadequate supply of aminoacids to the rest of pancreas, and with intestinal disease, it may result in an inadequate release of secretin and CCK – pancreozymin to stimulate the pancreas [8]. Pancreatic extracts are usually prepared from cow or hog pancreas. Enteric preparations are better because lipase is inactivated in stomach when the pH is below 4.5, and trypsin is inactivated when the pH reaches 3.5. The choice of preparation and the dose should be titrated according to the patient's requirement. Pancreatic replacement therapy has been observed to be more effective when given at an hour's intervals during the day [6, 10], but we used conventional regime with 6–7 meals/day (each meal with 1–4 caps of pancreatin). This therapy is rather expensive and should be as adequate as possible. Chymotrypsin in stool test helps to identify pancreatic insufficiency and can be successfully used to monitor the amount of pancreatic substitute, which differs from patient to patient. The dosage can be higher in cases of chronic pancreatitis than in those found after duodenopancreatectomy. The presence of enzymatic inhibitors may offer some explanation but beneficial effect of systemic enzyme therapy should also be taken in consideration [14, 15]. Pancreatic enzyme replacement therapy is important in all cases of pancreatic exocrine insufficiency and may be tested in the same way.

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ANTIINFLAMMATORY EFFECT OF A PROTEIN KINASE C INHIBITOR (K-252a) ON THE DEVELOPMENT OF THE DEXTRAN-INDUCED PAW EDEMA IN THE RAT (PRELIMINARY RESULTS)

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The effect of a metabolite of Nocardiopsis sp. as a protein kinase C inhibitor from microbial origin was investigated on the onset and development of dextran-induced paw edema in the rat. It was published that this compound (K-252a) interferes with histamine release from mast cells, while dextran-induced paw and nose edema are induced by vasoactive agents, like histamine etc., released from the disrupted mast cells. The antiinflammatory effect of the K-252a is effectuated by the inhibition of protein kinase C. Groups of male Wistar rats with 180–200 g b.w. were used; each group consisted of 10–10 rats. The following groups were consisted: rats given orally DMSO (control), rats given 1 mg/kg, or 3 mg/kg b.w. of K-252a dissolved in DMSO and given p.o. one hour before dextran injection. Dextran (BDH Chem. LTD, molW: 200.000, England) was injected intraperitoneally in 10% solution, in a dose of one ml/100 g b.w. Volume of the hind leg was measured by a mercury plethysmometer. Time-sequence of the edema was followed.

Increase in volume of hind leg paw was related to its 0-min volume in %. Results were analyzed by the Kruskal–Wallis-test. Edema of the legs and noses appeared in each of the control rats in one hour. The 1 mg/kg dose of K-252a retarded the appearance of the edema by 1 hour, the 3 mg/kg dose, however, prevented its onset for 4 hours. Conclusion: K-252a is a potent antiinflammatory agent against dextran in the rat through its inhibitory effect on the release of vasoactive agents from mast cells and that of protein kinase C.

Keywords: dextran-induced edema, K-252a protein kinase C inhibitor

Ohmori et al. [2] have published that K-252a a novel metabolite of Nocardiosis sp. inhibits both protein kinase C and calmodulin. The effect of this compound was more effective on protein kinase C than on calmodulin. K-252a has inhibitory effect

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on cyclooxygenase, lypoxygenase cascade and phospholipase A_2 activity. It counteracts the release of histamine from peritoneal mast cells of the rat [3].

As it is known dextran given intraperitoneally induces paw and nose edema in the rat, which is elicited by vasoactive substances released from disrupted mast cells [1]. The dextran-induced paw edema has an anaphylactoid origin. The antiinflammatory effect of the K-252a compound is effectuated by the inhibition of protein kinase C. Starting from this effect of the agent we investigated how K-252a does influence the formation of paw edema of rats induced by intraperitoneal administration of dextran.

Material and methods

Groups of male Wistar rats weighing 180–200 g b.w. were used. The following groups were constructed: rats given orally 1 ml of DMSO (control): rats given orally one or 3 mg/kg b.w. of K-252a dissolved in DMSO and given orally one hour before dextran injection. Each group consisted of 10–10 rats. Dextran (BDH, molW: 200 000) was injected intraperitoneally in 10% solution in a dose of 1 ml/100 g b.w. Volume of the hind limb was measured using a mercury displacement plethysmometer. The per cent increase in paw volume with respect to its 0-min volume was calculate in each case. The volume of the paw was measured at 30 min intervals. Statistical analysis was performed by the Kruskal–Wallis test.

Results and discussion

Dextran-induced edema of the legs and noses appeared in each of the control rats in one hour after induction. The 1 mg/kg dose of K-252a retarded the appearance of the edema by one hour, the 3 mg/kg dose, however, prevented its onset for 4 hours (Fig. 1).

K-252a at doses 1 and 3 mg/kg given orally elicited a dose-dependent inhibition of dextran-induced paw and nose edema in the rat. Similarly, such and higher doses of this agent counteracted the carragenin-induced local paw edema and the crotonoil-induced ear edema in the rat.

K-252a seems to exert its antiinflammatory effect by modifying mast cells' function participating in the inflammatory process by inhibiting protein kinase C activity and by this way it inhibits release of vasoactive agents, and probably that of lipopolysaccharides and arachidonic acid.

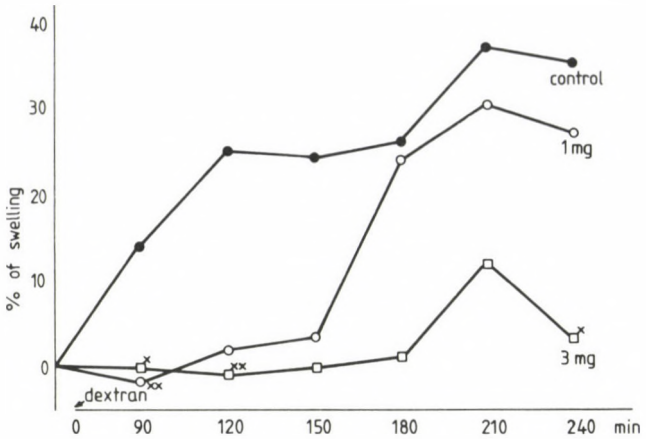

Fig. 1. The appearance of dextran-induced edema of the rat hind limb is retarded by 1 mg/kg b.w. of K-252a and inhibited by 3 mg/kg b.w. dose of K-252a given orally one hour before dextran injection

'--' DMSO was given orally 1 h before dextran (10%., i.p.) injection

o--o 1 mg/kg b.w., K-252a was given orally 1 h before dextran (10%., i.p.) injection

□-□ 3 mg/kg b.w. K-252a was given orally 1 h before dextran (10%., i.p.) injection

x significantly different from control at $P < 0.05$ level

xx significantly different from control at $P < 0.01$ level

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THIRD INTERNATIONAL SYMPOSIUM ON GASTROINTESTINAL CYTOPROTECTION

The Take Home Message

Chairmen: Gy. Mózsik (Pécs, Hungary) and A. Pár (Pécs, Hungary)

Gy. Mózsik: Ladies and Gentlemen! Now we arrived to the last but important part of this Symposium. We would like to draw some general conclusions from the presentations of this Symposium as well as from the literature.

The form of *The Take Home Message* comes from the Harvard Medical School of Boston (MA, U.S.A.). The main point of this was that the students, who had finished their studies at the University, received some important (unimportant) message from their teachers for their life. This university custom was adapted for different international meetings or symposia. We would like to follow this tradition in this symposium.

The methodology used now differs from the general practice, namely professor Pár and myself will give a short summary (message) on this Symposium. Naturally we will give possibility for participants of the Symposium to express their opinion(s).

First of all, we have to commemorate professor *André Robert*, who departed this life on 20th May, 1991, after a long fight with a chronic disease. He was a very kind friend of the Hungarian ulcer researchers, and participated in many symposia organized by Hungarian physicians. His first visit took place in 1976 at Parádfürdő, Hungary, when the Third International Conference on Experimental Ulcer was organized by me. Thereafter the Standing Committee of International Conference on Experimental Ulcer was formally established. This series on Experimental Ulcer was as follows. The First Conference was organized by prof. C. J. Pfeiffer (at Copenhagen, Denmark, 1970). The Second meeting was organized by prof. Th. Georghui (Cologne, F.R.G., in 1973). The Third one in Parádfürdő, Hungary by me. The Fourth Conference was organized by professor S. Umehara (Tokyo, Japan, 1980), the Fifth one by S. Szabo (Boston, U.S.A., 1985), the Sixth meeting by professor Racmilewitz (Jerusalem, Israel, 1988) and the Eighth Conference by professor Sewing (Berlin, F.R.G., 1991). The Forthcoming Conference on Experimental Ulcer will be held in Kyoto, Japan and it will be organized by professor S. Okabe.

Akadémiai Kiadó, Budapest

Professor *André Robert* was the most creative participant of these meetings and he was the father of the International Standing Committee. He participated in several other meetings in Hungary. His last participation was registered in 1990 by the 2nd International Symposium on Experimental and New Therapeutic Approaches to Ulcer Disease in Pécs, Hungary (this symposium was a satellite symposium of the Congress of International Union of Pharmacological Sciences (IUPHAR) to be held in Amsterdam, The Netherlands). His memorial lecture on the "Discovery of Gastric Cytoprotection" was recorded by the TV. He could give only one more review paper in his field in Praha (Czechoslovakia) at 1990.

A review paper on his personal and scientific life will be published in the journal of *Experimental and Clinical Gastroenterology*.

I would like to point up some remarks:

1. One of the most problematic questions is the terminology of *cytoprotection*. The definition given by Prof. André Robert (1979) was clear concerning to gastric mucosal protection, however, this criterion was not covered by the terminology of "cytoprotection" of small intestinal, large bowel, liver and pancreas. In the case of the mentioned organs, no correct secretory measurements were done. As Dr. Gyires emphasized in her lecture, we cannot give a general meaning of the "gastric cytoprotection", because a compound might have cytoprotective effect in one model, but none in an other one, furthermore this compound may produce mucosal damage in different other models. Similar observations were presented by others in the literature. These arguments indicated that we need to modify the terminology of *cell protection* instead of *cytoprotection*. As cell protection cannot be separated from *cell injury*, thereafter we will use the terminology of *cell injury and protection in the gastrointestinal tract*;

2. Professor Szabó comprehensively overviewed the current status of the mechanism of mucosal injury and protection. Multiple mechanisms are involved in both processes. Different pathways or cellular mechanisms participate in the development of mucosal injury and protection. The importance of tissue hypoxia or ischemia, biogenic agents (Heliobacter pylori, viruses) endothelins, eicosanoids (leucotrienes, thromboxanes, prostaglandins), platelet activating factor, oxygen free radicals, ammonia, HCl, bile acids, was critically evaluated. It was interesting to note that some components (prostaglandins, histamine, etc.) have biphasic effects.

3. It was interesting to hear that the researchers have tried to summarize the central, peripheral and cellular mechanisms of cell injury and protection, however, in case of liver cell injury and protection we could notice the peripheral (dominantly the immunological) mechanisms only. It is true that the immunological responses of different organs cannot be separated from their innervation, however, in case of liver cell injury and protection this question remained unclear.

4. We can speak on different axes in the gastrointestinal tract, namely brain-stomach axis, brain-gut axis and brain-pancreas axis. These differ from each other, however, some similarities do exist. We never heard about brain-liver axis. At this moment on the basis of lectures presented in this Symposium we do not know whether this axis exists or not.

5. The physicochemical, namely hydrophylic or lypophylic, properties of chemicals are important in the development of the gastrointestinal and liver cell injury and protection. These facts were emphasized clearly by prof. Rainsford, Bommelaer, Szalay and Lirussi. This is one of the reasons why we need to organize similar type symposium for the researchers working in this field;

6. We heard interesting results on the different growth factors. Dr. Vattay presented a paper on the fibroblast growth factor and on its importance in the healing of chronic duodenal ulcer in animals. Prof. Sikiric presented an overview on the body protecting compound (BPC). The physiological and biochemical mechanisms of this compound are unknown, however, it possibly seems to have the characteristics of growth factor;

7. The physicochemical properties of NOSAC play an important role in the development of gastrointestinal mucosal damage. It was emphasized in the excellent lecture given by prof. Rainsford. Similar observations were presented by Hermecz on the chemical structures of protecting compounds againts the gastric mucosal damage;

8. An absolute new finding was presented by Dr. Beinborn. His results clearly indicated the existence of PGE_2 receptors in the cell membrane. We have more correct information on the cellular pathways of defense mechanisms of GI mucosa;

9. Dr. Bálint and others have emphasized that the intracellular signal molecules are involved in the development of cell injury and protection. These mechanisms are connected to cellular energy systems, which can be concretized in the drug actions on pump systems for electrolytes and general regulation of cells;

10. The excellent overview presentation of prof. Pfeiffer has demonstrated that we can answer our scientific question(s) simply when we use different domestic animal models. This trend of research gives a new possibility to evaluate the development of gastrointestinal mucosal damage and prevention;

11. The possible role of oxygen free radicals was emphasized in the development of cell injury and protection in the small intestine and large bowel, however, this field became wider than only the close mechanisms due to oxygen free radicals (see the observation on the field of small intestine);

12. Many new observations were presented by the Pécs team on the possible role of intact vagal nerve in the development of gastrointestinal mucosal protection. It is true that the details of these questions are unknown, but it is clear that the intact vagal nerve is basically necessary for the development of gastric mucosal protection produced by different agents (prostacyclin, β-carotene, small doses of antisecretory

agents). The fact that β-carotene is a typical scavenger molecule and its beneficial effect is abolished by acute surgical vagotomy, indicates that the scavenger effect is only a part of the total mucosal protecting effect (no intact vagal nerve is necessary for receiving the scavenger effect). On the other hand, a lot of observations (Drs. Glavin, Henke, Murison) pointed to a higher organization of the development of GI cell injury and protection;

13. This Symposium was the first to demonstrate the whole series of GI mucosal injuries and protection, including the esophageal, gastric, small intestinal, large bowel, liver and pancreatic cell injury and protection.

A. Pár: During our morning session I think we could learn a lot about the role of free radicals and lipid peroxidation as well as the exogenous and endogenous scavengers in toxic liver injuries. It seemed that the tissue damaging and even the healing mechanisms, that is cell necrosis and regeneration may have similar molecular backgrounds both in the gastrointestinal ulcers and hepatic cell damages. Well documented clinical trials and animal experiments, furthermore *in vitro* model studies presented at this session, suggested that we possess some true hepatoprotective agents, either in form of natural antioxidants such as flavonoids, or the endogenous metabolite of methionine, or the ursodeoxycholic acid, finally essentially phospholipids. All these substances may serve the treatment of different forms of liver disease in the future.

The presentations reflected the development in the knowledge on hepatoprotection having taken place from the last cytoprotection symposium. The results are encouraging for further studies, for a deeper insight, and to obtain newer effective therapeutic and preventive measures.

May I give my take home message with the words of *Hans Popper*, who was one of the greatest hepatologist in this century.

"... The future of hepatology will depend not only on the asking right questions but on discarding wrong questions in biological studies. Code of Maimonides: May I always be able to discover today the errors of yesterday and to obtain a new light tomorrow on what I am sure today!

Secondly, in clinical investigations for new therapeutic modalities, long term follow-ups with large groups of patients, controlled prospective trials must be performed, where impressions must be replaced by facts."

Thank you!

Gy. Mózsik: We learned an important thing concerning the organization of this type of Symposium. Independently we went over the cell injury and protection of the whole GI tract (and that is a very good thing), this is what we have to reconsider in the future on the method of Symposium organization. As to my opinion a better

organization can be achieved if we identify some common etiological events (which probably are common in the development of GI cell injury and protection) which should be taken as the main questions and these points should be evaluated concerning different organs (tissue hypoxia, vascular events, cellular metabolism, oxygen free-radicals, time sequence-analysis of changes obtained in the different examined parameters, hydrophylic and lipophylic properties of components producing mucosal damage and protection, etc.).

We also would like to express our best thanks for the participants and organizers.

In lack of anymore comments the Symposium is closed. We will meet in 1995 at Pécs, Hungary.

The Proceedings of this Symposium will be published as regular papers in *Acta Physiologica Hungarica* (1992). The following title will be used for the proceedings: *Cell injury and protection in gastrointestinal tract: from basic sciences to clinical perspectives.*

A critical evaluation was published by professor K.D. Rainsford (Hamilton, Canada) on the *Third International Symposium on Gastrointestinal Cytoprotection*, entitled

Report on the Third International Symposium on Gastrointestinal Cytoprotection, Pécs, Hungary, October 7–8th, 1991.

This paper was published in journal of *Inflammopharmacology*, 1991, 1:1019–1028.

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CONTENTS

Introductory remarks
Gy. Mózsik, A. Pár, G. Csomós, M. Kitajima, M. Kondo, C.J. Pfeiffer, K.D. Rainsdorf, P. Sikirić, S. Szabo 3

Basic, central, peripheral and cellular mechanisms of gastrointestinal cytoprotection

Pathways, mediators and mechanisms of gastroduodenal mucosal injury
S. Szabo, L. Nagy 9
Mechanisms of NSAID-induced ulcerogenesis: structural properties of drugs, focus on the microvascular factors, and novel approaches for gastro-intestinal protection
K.D. Rainsford 23
ATP breakdown and resynthesis in the development of gastrointestinal mucosal damage and its prevention in animals and human (An overview of 25 years ulcer research studies)
Gy. Mózsik, Ágnes Király, G. Sütő, Á. Vincze 39
Gastric anti-ulcerogenic drug effect. A possible mechanism of its molecular basis
G.A. Bálint 81
The significance of the gastroprotective effect of body protection compound (BPC): modulation by differenct procedures
P. Sikirić, M. Petek, R. Ručman, S. Seiwerth, Z. Grabarević, I. Rotkvić, V. Jagić, B. Turković, B. Mildner, M. Duvnjak, A. Cviko, M. Kolega, M. Dodig, A. Sallmani, S. Djačić, D. Lucinger, S. Mikulandra, D. Erceg 89
Ethanol and the antioxidant defense in the gastrointestinal tract
C.H. Cho, C.J. Pfeiffer, H.P. Misra 99
Brain-gut relationships: gastric mucosal defense is also important
G.B. Glavin, A.M. Hall 107
Stress ulcer modulation by limbic system structures
P.G. Henke, A.Ray 117
Individual behavioral characteristics and extent of stress-induced gastric ulceration in rats
R. Murison, A. Skjerve 127

Esophageal protection

Esophageal mucosal protection – Why do we need a special approach?
L. Simon 135

Gastric protection

A review of spontaneous ulcer disease in domestic animals: chickens, cattle, horses, and swine
C. J. Pfeiffer 149
Subcellular localization of prostaglandin E_2 receptors in the gastric mucosa
M. Beinborn, Susanne Netz, U. Staar, K.Fr. Sewing 159
Adenosine: A novel ulcer modulator in stomachs
C.H. Cho 175
5-hydroxytryptamine$_3$-receptor blockade protects against gastric mucosal damage in rats
C.W. Ogle, S-C.G. Hui, B.S. Qiu, K.M. Li 181
Role of mucus in mucosal protection through ethanol and pepsin damage models
Judit Matúz 189
Is acute surgical vagotomy an aggressor to gastric mucosa in pylorus ligated rats with and without indomethacin treatment?
Á. Vincze, Ágnes Király, G. Sütő, Gy. Mózsik 195
Effect of acute surgical vagotomy on the mucosal content of 6-keto-$PGF_{1\ alpha}$, PGE_2 and glutathione after intragastric 96% ethanol treatment in rats
G. Sütő, Ágnes Király, Á. Vincze, Gy. Mózsik 205
Correlation between the cytoprotective effect of beta-carotene and its gastric mucosal level in indomethacin (IND) treated rats with or without acute surgical vagotomy
Ágnes Király, G. Sütő, Á. Vincze, Gy. Tóth, Z. Matus, Gy. Mózsik 213
Acute and chronic surgical vagotomy (SV) and gastric mucosal vascular permeability in ethanol treated rats
Ágnes Király, G. Sütő, Á. Vincze, Gy. Mózsik 219
Synthesis and gastroprotective activity of 4*H*-pyrido[1,2-*a*]pyrimidin-4-ones
I. Hermecz, Judit Sipos, Lelle Vasvári-Debreczy, Klára Gyires, Z. Kapui 225
The mechanism of cytoprotective effect of CHINOIN-127
Z. Kapui, K. Boér, I. Hermecz, Klára Gyires 237
Are all "cytoprotective" drugs gastroprotective?
Klára Gyires 247
The antineoplastic drugs dose dependently inhibit the healing of subacute gastric ulcer produced by punching in rats
E. Ezer 257
Resistance to medical ulcer therapy – possible reasons and management
M. Jablonská 265
Selenium in the blood of patients with colorectal cancer and neoplastic polyp
Gy. Rumi, I. Imre, Cs. Sülle, I. Sarudi, J. Kelemen, Zs. Lassú 275
Measurement of non-steroid antiinflammatory drugs induced gastric microbleeding
T. Beró, L. Nagy, T. Jávor, Á. Vincze 281
Influence of sex hormones on ethanol-induced gastric haemorrhagic erosions in rats
F. László, E. Amani, Cs. Varga, F.A. László 289

Small intestinal injury and protection

The effects of local hyperthermia on the morphology of the small bowel
T. Tóth, A. Fenyvesi, I. Klemm 293
Histamine release and SOD, allopurinol and ranitidine pretreatment in haemorrhagic shock in the rat
I. Zöllei, H. Asakawa, S. Karácsonyi 303
Cholinergic neurons are involved in the effect of substance P on the circular muscle of the guinea pig small intestine
L. Barthó, G. Pethő 311

Large bowel injury and protection

The oxygen-centered radicals scavenging activity of sulfasalazine and its metabolites. A direct protection of the bowel
L. Prónai, Ichikawa Yukinobu, I. Láng, J. Fehér 317

Liver injury and protection

Pathogenesis and management of alcoholic liver injury
A. Pár 325
Role of free-radical fractions in liver diseases
J. Fehér, A. Vereckei, G. Lengyel 351
Cytoprotection in the nineties: experience with ursodeoxycholic acid and silymarin in chronic liver disease
F. Lirussi, L. Okolicsanyi 363
Clinical evidence of hepatoprotection induced by ursodeoxycholic acid
Á. Vincze, I. Patty, T. Jávor, A. Pár 369
The effect of silibinin (Legalon^{R}) on the free radical scavenger mechanisms of human erythrocytes *in vitro*
I. Altorjay, L. Dalmi, S. Sári, S. Imre, Gy. Balla 375
Endogenous scavanger levels (vitamin E and A) of patients with chronic alcoholic liver disease under different environmental conditions
Tünde Horváth, L. Szabó, Z. Nagy, Anna Blázovics, Klára Gergely, T. Schwarcz 381
Post-cholecystectomy syndrome and magnesium deficit
J. Szántay, Doina Varga, P.J. Porr 391

Pancreatic injury and protection

Pancreatic cytoprotection: new approaches
M. Papp 399
The effect of somatostatin, gabexate mesilate and dextran 40 on the microcirculation in sodium taurocholate-induced pancreatitis
K. Kusterer, M. Enghofer, T. Poschmann, K.H. Usadel 407
Adequate enzymatic substitution in treating exocrine pancreatic insufficiency
I. Šimek, K. Kubias, M. Duda 417
Antiinflammatory effect of a protein kinase C inhibitor (K-252a) on the development of the dextran-induced paw edema in the rat (Preliminary results)
M. Papp, H. Kase, G. Gigler 423

The Take Home Message
Gy. Mózsik, A. Pár 427

AUTHOR INDEX

Altorjay, I. 375
Amani, E. 289
Asakawa, H. 303

Bálint, G. A. 81
Balla, Gy. 375
Barthó, L. 311
Barski, J. 219
Beinborn, M. 159
Beró, T. 281
Blázovics, Anna 381
Boér, K. 237

Cho, C.H. 99, 175
Cviko, A. 89

Dalmi, L. 375
Djačić, S. 89
Dodig, M. 89
Duda, M. 417
Duvnjak, M. 89

Enghofer, M. 407
Erceg, D. 89
Ezer, E. 257

Fehér, J. 317, 351
Fenyvesi, A. 293

Gergely, Klára 381
Gigler, G. 423
Glavin, G. B. 107
Grabarević, Z. 89
Gyires, Klára 225, 237, 247

Hall, A. M. 107
Henke, P. G. 117
Hermecz, I. 225, 237
Horváth, Tünde 381
Hui, S-C. G. 181

Imre, I. 275
Imre, S. 375

Jablonská M. 265
Jagić, V. 89
Jávor, T. 281, 369

Kapui, Z. 225, 237
Karácsonyi, S. 303
Kase, H. 423
Kelemen, J. 275
Király, Ágnes 39, 195, 205, 219, 213,
Klemm, I. 293
Kolega, M. 89
Kubias, K. 417
Kusterer, M. 404

Láng, I. 317
Lassú Zs. 275
László, F. 289
László, F. A. 289
Lengyel, G. 351
Li, K.M. 181
Lirussi, F. 363
Lucinger, D. 89

Matus, Z. 213
Mazúz, J. 189
Mikulandra, S. 89
Mildner, B. 89
Misra, H. P. 99
Mózsik, Gy. 39, 195, 205, 213, 219, 427
Murison, R. 127

Nagy, L. 281
Nagy, Z. 381
Netz, Susanne 159

Ogle, C. W. 181
Okolicsanyi, L. 363

Papp, M. 399, 423
Pár, A. 325, 369, 427
Patty, I. 369
Petek, M. 89

Pethő, G. 311
Pfeiffer, C. J. 99, 149
Poschmann, T. 407
Prónai, L. 317

Qiu, B. S. 181

Rainsford, K. D. 23
Ray, A. 117
Rotkvic, I. 89
Rucman, R. 89
Rumi, Gy. 275

Sallmani, A. 275
Sári, S. 375
Sarudi, I. 275
Schwarz, T. 381
Seiwerth, S. 89
Sewing, K. F. 159
Sikirič, P. 89
Simek, I. 417
Simon, L. 135
Sipos, Judit 225
Skjerve, A. 127
Staar, U. 159
Sülle, Cs. 275
Sütő, G. 39, 195, 205, 213, 219

Szabó, L. 381
Szabó, S. 9
Szántay, J. 391
Tóth, Gy. 213
Tóth, T. 293
Turković, B. 899

Usadel, K. H. 407

Varga, Cs. 289
Varga, Diona 391
Varga, P. J. 391
Vasvári-Debreczy, Lelle 225
Vereckei, A. 351
Vincze, Á. 39, 195, 205, 213, 219, 281, 369

Yukinobu, Ichikawa 317

Zöllei, I. 303

acetylcholine, 311
acute surgical bilateral truncal vagotomy, 205
 –, and chronic surgical vagotomy, 219
 –, bilateral surgical vagotomy, 213
 –, pancreatitis, 407
 –, surgical vagotomy, 195
adenosine, 175
adherent mucus, 189
alcohol, 325
allopurinol, 303
amygdala, 117
anti-inflammatory drugs, 23
antineoplastic drugs, 257
antioxidant protection of cells, 351
anti-ulcerogenic effect, 81
ATP synthesis, 39

Barett-esophagus, 135
behavioral characteristics, 127
beta-carotene absorption, 213
blood flow, 407
body protection compound (BPC), 89
bovine, 149
brain-gut axis, 107

calcium, 9
cAMP, 81
catalase, 99
cattle, 149
CCK-receptor antagonists, 399
cellular energy systems, 39
 –, (metabolic) adaptation of gastric mucosa after surgical vagotomy, 39
cGMP, 81
chicken, 149
Chinoin, 127, 237
cholestasis, 369
chronic liver disease, 363
chymotrypsin, 417
cimetidine, 81
cirrhosis, 325
colorectal cancer, 275
cyproheptadine, 181
cytoprotection, 23, 81, 237, 399
cytoprotective agents, 225
 –, prostaglandins, 281

deficiency of magnesium and calcium after cholecystectomy, 391
dehydrogenase, 99
detoxication of drugs, 351
dextran-induced edema, 423
dopamine, 107
drug actions on cellular energy systems, 39
duodenal ulcer, 265
duodenum, 39, 107
dynamic haemolysis curve, 375

endogenous mediators, 9
 –, scavengers, 381
environment and alcoholic liver disease, 381
enzyme replacement therapy, 417
erosive gastropathy, 281
erythrocytes, 375
ethanol, 99, 175, 189, 219, 289
exogenous and endogenous chemicals, 39
experimental gastric ulcer models, 39

free radical, 9, 399
 –, diseases and environment, 381
 –, reactions, 351
 –, scavenger mechanisms, 375
gastric cytoprotection, 213
 –, protection, 205
 –, blood flow, 175
 –, damage, 175, 195, 213, 219
 –, lesion, 181
 –, microbleeding, 281
 –, mucosa, 159, 289
 –, secretion, 265
 –, ulcer, 289
 –, ulceration, 81
gastroduodenal mucosal injury, 9
gastroesophageal reflux desease, 135

gastrointestinal mucosa, 23
gastroprotection, 89, 247
glucose-6-phosphate dehydrogenase, 99
glutathione, 99
GSH, 205
guinea pig ileum, 311
gut, 9

haemorrhagic shock, 303
H_2-antagonists, 265
healing, 257
hepatoprotection, 325, 369
hippocampal formation, 117
histamine release, 303
human gastrin, 39
hydroxyl radicals, 317
hyperalimentation with glucose, 39
hyperthermia, 293

individual differences, 117
indomethacin, 195, 213
inflammatory bowel diseases, 317
irritants to gastric mucosa, 39
in vivo microscopy, 407

jejunal ulcers, 39

6-keto-PGF_1 alpha, 205
K-252a protein kinase C inhibitor, 423

Legalon®, 375
leukotrienes, 23
limbic system, 117
liver disease, 351
L-phenylisopropuladenosine, 175
lysosomes, 159

marker enzymes, 159
metabolism, 325
microcirculation, 407
microvascular permeability, 407
modulations, 89
morphine, 247
–, defense, 99
–, protection, 135
mucus barrier, 189
–, thickness, 189
myentric neurons, 311

necrosis, 293
neoplastic polyps, 275
N-ethylcarboxamidoadenosine, 175
N-ethylmaleimide, 247
non-steroid antiinflammatory drugs, 281

ondansteron, 181
organ ablations, 89
–, centered radicals, 317
–, free radicals, 303

pancreas, 399
pancreatitis, 399
patients, 391
peroxidized lipids, 351
pepsin, 189
peptic ulcer, 237
PGE_2, 205
–, receptors, 159
plasma membranes, 159
porcine, 149
prostacyclin, 28, 237
prostaglandins, 9, 23, 195
proteases, 9
protective effect, 9, 275
pylorus ligation, 195
pyrido [1,2-a] pyrimidine, 225

ranitidine, 303
rat, 89, 175, 181, 293

scavengers, 399
selenium, 275
sex hormones, 289
silymarin, 363
small intestine, 293
SOD, 303
sodium taurocholate, 407
somatostatin, 399
Spargue-Dawley rat, 127
spontaneous ulcer, 149
starvation, 39
stomach stress coping response, 89

stress induced gastric ulceration, 127
–, ulcer, 117
subacute ulcer, 257
substance P, 311
sucralfate, 135
sulfalsalazine, 317
sulphydryls, 9, 247
superoxide dismutase, 99
supplementatics of magnesium and calcium after cholecystectomy, 391
surgical vagotomy, 39
swine, 149
thromboxane, 237
toxicity, 325
toxin exposure, 351
types of mucosal adaptations to chemicals, 39

ulcer, 107
–, therapy, 265
ursodeoxycholic acid, 363, 369

vascular permeability, 219
ways of ATP breakdown, 39

zonal necrosis, 351

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