ANALYSIS OF CYTOPROTECTION USING IN VITRO MODELS: MECHANISMS OF CELLULAR INJURY AND ITS PREVENTION ON FRESHLY ISOLATED AND CULTURED CELLS

Ph.D. DISSERTATION

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UNIVERSITY MEDICAL SCHOOL OF PÉCS

PÉCS, 1998

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1998

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ABBREVIATIONS

BPC - body protection compound

DMEM - Dulbecco`s modified Eagle`s medium

EB - ethidium bromide

EtOH – ethanol

FCS – fetal calf serum

GMCs - gastric mucosal cells

HCl - hydrochloric acid

Hep G2 - human hepatocellular carcinoma cell line

H. pylori - Helicobacter pylori

IND - indomethacin

LDH - lactate dehydrogenase

NO - nitric oxide

NSAIDs - nonsteroidal antiinflammatory drugs

PGE_2 - prostaglandin E_2

PGI_2 - prostacyclin

PGs - prostaglandins

SDH - succinic dehydrogenase

Sp2/0-Ag14 - mouse myeloma cell line

TB - Trypan Blue

1. GENERAL INTRODUCTION

1.1. GASTRIC CYTOPROTECTION

1.1.1. General aspects

Peptic ulcer is a widely spread disease in the world. The main place of its development is the upper gastrointestinal tract, where in natural conditions very aggressive compounds can be found (e.g. pepsin and hydrochloric acid). In healthy relationships these aggressive factors cannot necrotize the surface epithelium, because they are in equipment with the protective agents (Table 1.)

The main curative way to prevent and heal the peptic ulceration was the decrease or inhibition of gastric acid secretion. At the end of 1970's a new field appeared in the experimental ulcer research, namely the protective side. In 1978 Chaudhury and Jacobson described firstly the term of cytoprotection (Chaudhury and Jacobson, 1978).

Table 1

Endogenous defensive and aggressive factors in the pathogenesis of peptic ulcer disease.

DEFENCE	AGGRESSION
Mucus	Hydrochloric acid
Prostaglandins	Pepsin
Bicarbonate	Gastrin
Sulfhydryls	Free radicals
Interleukin-1	Proteases
Superoxide dismutase	Leukotrienes
Catalase	Helicobacter pylori
Nitric oxide	Impaired blood flow
Dopamine	Dysmotility

Cytoprotection was internationally accepted after Robert`s publication in 1979: the gastric mucosa was protected against necrotizing agents (alcohol, HCl, NaOH, hypertonic NaCl and thermal injury) by exogenous prostaglandins without any inhibition of gastric acid secretion (Robert et al., 1979). This publication gave a new impulse and field for the experimental ulcer research, the quickly beginning experiments described more and more new cytoprotective drugs. These results were published in several books (Recent Advances in Gastrointestinal Cytoprotection, 1984; Advances in Gastrointestinal Cytoprotection: Topics 1987, 1989; Cell Injury and Protection in the Gastrointestinal Tract: From Basic Sciences to Clinical Perspectives, 1993; Twenty Five Years of Peptic Ulcer Research in Hungary: From Basic Sciences to Clinical Practice (1971-1995), 1997).

There are a lot of mechanisms involved in the phenomenon of gastric cytoprotection. In the following chapters the wide range of organ factors will be overviewed which also play an important role in the development of gastric cytoprotection as the protective or aggressive side.

1.1.1.1. The role of central nervous system

The brain and the gastrointestinal tract are in a strong connection as the parts of the brain-gut axis. Clinical observations verified that affective or emotional state influences the gastrointestinal function (Schindler and Ramchandari, 1991). In patients with ulcer disease stress and anxiety are associated with the active ulcer phase (Feldman et al., 1986). In addition, acute gastric mucosal lesions are serious complications of different type of stress situations e.g. burning, trauma or infection. Stress ulcer can be used as experimental ulcer model in cytoprotective investigations (Kitajima et al., 1989; Tóth, 1989).

In 1965 Strang reported a high incidence of gastroduodenal ulcer disease in patients with Parkinson`s disease, which is related to a central dopamine deficiency (Strang, 1965). Szabo began the systematic study of a role for dopamine in duodenal ulcer disease, and proved the significant pathogenetic role for dopamine depletion in the genesis of experimental ulcer models (Szabo, 1979; Szabo et al., 1982). In addition, there was experimental evidence for a selective dopamine neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced massive nigrostriatal dopamine loss and duodenal ulcers in rats (Szabo et al., 1985a).

Dopamine agonist and promoters (L-dopa, bromocriptine, apomorphine and D-amphetamine) reduce, while dopamine antagonists (haloperidol, pimozide, domperidone) worsen gastric lesions produced by restraint-cold stress (Glavin and Dugani, 1987). Dopamine also has a significant role as an endogenous gastroprotective agent, especially in adaptive cytoprotection (MacNaughton and Wallace, 1989).

These samples are only a part of the role of central nervous system in the phenomenon of gastric cytoprotection, there are many other mechanisms involving in the regulation of brain-gut axis.

1.1.1.2. The vagal nerve

Almost the whole gastrointestinal tract (from the stomach to the first part of large bowel) is innervated by the vagal nerve. In physiological circumstances it plays an important role among others in the regulation of gastrointestinal motility and secretion of digestive juices.

In addition, the role of vagal nerve in the phenomenon of cytoprotection was independently proved by Mózsik and Miller in the early 80s: after bilateral surgical vagotomy the prostaglandin-induced protection and adaptive cytoprotection were failured (Mózsik et al., 1982; Miller et al., 1983).

The exact mechanisms of the role of vagal nerve in gastric mucosal protection are unknown, but some pathways can be suggested based on experimental results:

- Decreased gastric emptying (Mózsik et al., 1992a);
- Decreased synthesis of prostaglandins (Bódis et al., 1990);
- Increased vascular permeability (Király et al., 1990; Mózsik et al., 1993a; Karádi et al., 1994);
- Changes in the gastric mucosal protective properties of SH-groups (Mózsik et al., 1990);
- Changes in biochemical mechanisms (Mózsik et al., 1992b);
- Changes in the regulatory mechanisms between the membrane-bound ATP-dependent energy systems (Vincze et al., 1993);
- Significantly altered regulatory pathways to protective agents (Mózsik et al., 1995b).

These results emphasize that the vagal nerve has an important role both in the development of gastric mucosal damage (by increasing aggressive factors) as well as in the development of gastric cytoprotection.

1.1.1.3. Blood flow and microcirculatory changes

The intact blood flow is basically necessary for the maintenance of gastric mucosal integrity. Most of the cell and tissue injury is produced by hypoxia, especially ischemia due to impaired blood flow. Hypoxia might cause reversible or irreversible cell injury. In addition, the reestablished blood flow often aggravates the damage because of generation of free radicals (Szabo and Nagy, 1992).

The changes in blood flow are not only alterations of neural and metabolic control but are the result of prior damage to the vascular endothelium. The acute mucosal damage in the stomach is manifested as a hemorrhagic lesion. Vascular injury is a very early event in the pathogenesis of chemically induced gastric erosions (Szabo et al., 1985b). Lacy and Ito microscopically analyzed the changes in gastric mucosa during cytoprotection induced by prostaglandins, and reported that only the hemorrhage and not the surface epithelial lesions was prevented (Lacy and Ito, 1984). Further studies showed that cytoprotective prostaglandins and sulfhydryls preserved the microvascular injury (Szabo, 1984), and they maintained blood flow in subepithelial capillaries after topical administration of necrotizing agents (Pihan et al., 1986).

The changes in gastric mucosal blood flow are also regulated by neural and hormonal influence, and these effects are important in the development of gastric cytoprotection. It is mostly mediated by the local release of vasodilatator peptides (e.g. tachykinin and calcitonin gene related peptide, CGRP) contained in sensory nerve endings (Holzer et al., 1991). Topical administration of capsaicin evokes the release of neuropeptides and causes a long-lasting enhancement in the microcirculation of the rat stomach and jejunum. It is not mediated through vagal or sympathetic pathways, only by stimulation of capsaicin sensitive sensory nerves (Abdel-Salam et al., 1996).

These mechanisms represent only a part but underline the important role of the vascular compound in the development of gastric cytoprotection.

1.1.1.4. Mucosal barrier

The intraluminal surface of the stomach is covered with mucus layer. This is a protective agent against the aggressive factors such as hydrochloric acid and pepsin presented in physiological conditions the lumen of the stomach. Gastrointestinal mucus occurs as a stable, water-insoluble gel adherent to the

mucosal surface. It has a variable thickness, about 50-450 μm (median 180 μm) in man (Kerss et al., 1982).

The most important function of the mucus in the stomach and duodenum is the protection of the mucosa. It 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 (Flemström and Garner, 1982). In addition, the adherent mucus gel protects the mucosa from proteolytic attack of intraluminal pepsin. Pepsin in the gastric lumen progressively digests the mucus layer, but the loss is replaced by secretion of new mucus. This dynamic balance can be damaged following exposure to excess pepsin and acid but not acid alone (Leonard and Allen, 1986).

It must be point out that the adherent mucus gel layer is not a diffusion barrier to necrotizing agents used in cytoprotective investigations such as bile salts, hypertonic saline, ethanol and nonsteroidal antiinflammatory drugs (Matúz, 1992).

1.1.1.5. Nitric oxide and gastric mucosal protection

In 1987, it was shown that vascular endothelial cells could synthesize nitric oxide (NO), and this gas acted as a labile humoral-like messenger that relaxed vascular smooth muscle (Palmer et al., 1987). NO is a colorless gas that is slightly soluble in water. In biologic systems NO has a half-life of <5 seconds. Vascular endothelial cells synthesize NO enzymatically from L-arginine (Palmer et al., 1988). In humans NO is formed in both arteries and veins and contributes to the control of basal and stimulated regional blood flow (Vallance et al., 1989a; Vallance et al, 1989b). Endothelium-derived NO can also diffuse into nearby adhering platelets in the lumen of the blood vessels to inhibit platelet adhesion and aggregation (Radomski et al., 1987).

Regarding to these facts, NO also has to play a role in gastric cytoprotection altering the mucosal blood flow and platelet aggregation. Several experimental results can suppose this hypothesis. Topical mucosal application of a NO solution or of glyceryl trinitrate or nitroprusside reduces the severity of ethanol-induced hemorrhagic mucosal damage (MacNaughton et al., 1989). Intravenous administration of nitroprusside also inhibits mucosal damage (Wallace et al., 1989a). Administration of inhibitors of NO synthesis alone did not lead to acute gastric mucosal damage in the rat. However, inhibition of NO synthesis after depletion of vasodilator neuropeptides with chronic capsaicin pretreatment or after inhibition of prostacyclin synthesis with indomethacin did induce substantial mucosal injury (Whittle et al., 1990).

In pathological circumstances the development of mucosal ulceration could be related to decreased endothelial production of vasoactive mediators with protective actions such as NO. In pharmacological point of view an increased mucosal level of NO could be protective against gastric mucosal injury in high risk patients.

1.1.1.6. Prostaglandins and nonsteroidal antiinflammatory drugs

Nonsteroidal antiinflammatory drugs (NSAIDs) are commonly used for rheumatic disorders, fever, pain and inflammation. Generally these drugs are well tolerated, but in a significant minority gastrointestinal side effects may result in serious complications. The main action of NSAIDs is inhibiting cyclooxigenase enzyme activity and by this way decreasing the synthesis of novel inflammatory mediators, namely prostaglandins (Vane, 1971).

Inflammation is the response of living tissues to injury. It involves a complex array of enzyme activation, mediator release, extravasation of fluid, cell migration, tissue breakdown and repair. Prostaglandin E_2 is the predominant eicosanoid

detected in inflammatory conditions. Prostaglandins belong to a class of polyunsaturated fatty acids derived from arachidonic acid, a phospholipid component present in all cell membrane. Arachidonic acid is released from the cell membrane into the cytoplasm by phospholipase A_2 and is converted to prostaglandins or leukotrienes by the enzymes cyclooxigenase and 5-lipoxigenase, respectively. Prostaglandins are tissue specific, in the gastrointestinal mucosa PGE_2, PGI_2 and PGF_{2alpha} are the most prevalent eicosanoids. Prostaglandins inhibit gastric acid secretion endogenously in the stomach, and in a high dose exogenously. They have also an important role in gastrointestinal mucosal defense like a cytoprotective agent (Robert et al., 1979).

Recently two types of cyclooxigenase have been identified: COX-1 and COX-2. Both enzymes have a similar molecular weight and active sites for the attachment of arachidonic acid or NSAIDs, although the active site on COX-2 is larger and can accept a wider range of structures as substrates (Vane and Botting., 1995).

COX-1 is constitutively expressed in most tissues, and performs a "housekeeping" function to synthesize prostaglandins which regulate normal cell activity. The concentration of the enzyme is stable, but small increases can occur in response to stimulation with hormones or growth factors (DeWitt, 1991). Little or no COX-2 is found in resting cells but its expression can be largely increased after exposure of cells to bacterial lipopolysaccharide, phorbol esters, cytokines or growth factors. COX-2 can be produced in many cells and tissues and its induction inhibited by glucocorticoids such as dexamethasone (Masferrer et al., 1990).

NSAIDs are the potent inhibitors of cyclooxigenase enzyme, and the differences in action and side effects of different drugs can be explain by their different activity on COX-1 and COX-2. Piroxicam 250, aspirin 166 and indomethacin 60 times more active on COX-1 than COX-2, but some compounds e.g. meloxicam, naproxen and diclofenac are good selective inhibitors of COX-2 (Vane and Botting, 1995). The pharmacological profiles of COX-1 and COX-2 are different, so it will be possible to develop new antiinflammatory drugs which are specific inhibitors of COX-2 and will not have gastrointestinal side effects.

1.1.1.7. A new aggressive factor: Helicobacter pylori

Nowadays Helicobacter pylori (H. pylori) is one of the most frequent field in gastroenterology. This bacterium was firstly isolated from a patient with chronic gastritis in 1983 (Warren, 1983), and since then it has been linked to gastrointestinal diseases in human. Epidemiological studies proved the presence of this bacterium in biopsy samples from patients suffering gastritis, gastric and duodenal ulceration (Taylor et al., 1991; Moss et al., 1992). In addition, there is a doubt that H. pylori is in connection with gastric carcinoma and lymphoma (Loffeld et al., 1990).

How does this bacterium cause gastrointestinal diseases? There are many explanations to this question. Some papers emphasized the direct cellular effect of bacterial toxins (Figura et al., 1994), the breakdown of the mucus layer (Sarosiek et al., 1994), the role of H. pylori exotoxins includes urease, which catalyses the production of ammonia (Nagy et al., 1996), proteases, phospholipase A_2 and platelet-activating factor (Wyle et al., 1993), and the interactions between the bacterial unusual fatty acids and gastric parietal cells (Beil et al., 1994).

While H. pylori persists in a living organism, the host's immune system has to play an important role in inflammation, and immune reactions can lead to development of cell injury. In the last century Robert Koch described his four principles for acceptance of the infectious origin of different diseases (Jawetz et al., 1982), namely 1. microorganism must regularly be isolated from the caches of the illness; 2. it must be grown in pure culture in vitro; 3. when such a pure culture is inoculated into susceptible animal species, the typical disease must be resulted; and 4. microorganism must be again isolated from the tissues of experimentally induced disease. Koch's principles also exist in our days for the acceptance of a possible factor as an infectional aetiology for a disease. The first, second and fourth principle can be proved in different observations, however, in the case of H. pylori all criteria never were proved together neither in humans nor in experimental circumstances (Graham, 1995; Ross et al., 1992). Marshall's observations (when he drank a broth of H. pylori) (Marshall et al., 1985) clearly indicated that H. pylori

(at least alone) cannot cause peptic ulcer. The endoscopic studies at the 10th day after the infection proved the development of gastritis (without appearance of gastric and duodenal ulcer), which disappeared before 14 days. One possible conclusion of Marshall's observations is that H. pylori might produce gastritis, however, it cannot produce gastroduodenal ulcer in humans.

Koch's third postulate was not proved until now, namely there is no experimental model in which the cultured H. pylori can produce peptic ulcer (or only gastroduodenal ulcer). It was impossible to induce gastritis in rats by feeding H. pylori suspensions for seven days period, however, the presence of H. pylori was demonstrated in the gastric mucosa (Ross et al., 1992).

The question remains open about a pathogenetic or opportunist role of H. pylori in the aetiology of peptic ulcer and low grade gastric lymphomas. The intensive experimental and clinical observations might answer these problems and clear up the exact role of H. pylori in human diseases.

1.1.1.8. Free radicals and scavenger compounds

Free radicals are well known common pathways in cellular injury due to lipid peroxidation and membrane damage (in details see chapter 2.1.1.). In the living organisms it can be manifested among others as a tissue necrosis or as a genetic malformation e.g. development of malignancies.

During gastrointestinal mucosal damage the necrotizing agents induce an expressive free radical formation. Its possible role was suggested by the fact that some scavenger compound has cytoprotective properties (Jávor et al., 1986). In addition, gastric mucosa contains a high level of glutathione, which is able to protect the cells against free radicals (Boyd et al., 1979).

The scavenger compounds-induced gastric cytoprotection is manifested in about 15-60 min after administration of necrotizing agents (Vincze et al., 1991a). This observation shows that in the gastric mucosa the primary event is the direct cellular and tissue damage, and this effect induces a free radical formation, which can be protected by scavengers.

Carotenoids are widely used cytoprotective agents in gastrointestinal observations. Besides their scavenger properties, a wide range of retinoids have vitamin A activity which can promote the epithelial restitution and ulcer healing (Mózsik et al., 1993b). It could be another explanation for the action in the later phase of tissue injury.

Regarding to these facts cytoprotection induced by scavenger compounds is a very complex and multifactorial phenomenon.

1.2. IN VIVO ORGANOPROTECTION

1.2.1. Experimental results

The aim of the next chapters is to summarize the main approaches of a part of in vivo observations carried out in animal models. These results represent the mechanisms of organoprotection usually in rats. The effects induced by necrotizing agents and organoprotective factors are very complex and multifactorial events, it is impossible to decide whether organo- or cytoprotection is more important in the development of the beneficial results.

1.2.1.1. Prostaglandins and prostacyclin

In 1979 prostaglandins (PGs), namely 16,16-dimethyl PGE_2 was firstly described as a cytoprotective agent (Robert et al., 1979). The effect of PGs is a very complex mechanisms. After bilateral surgical vagotomy the protective effect induced by PGs is failured (Mózsik et al., 1982), and the stimulation of the vagal nerve enhances the tissue formation and release of gastric mucosal PGs (Singh, 1980).

On the other hand, other compounds e.g. sulfhydryls are also necessary for the gastroprotective effect of PGs (Johansson and Bergström, 1982).

The PGs-induced protection manifests in the early phase after administration of necrotizing agents (Sütő et al., 1989), this is the phase of the early vascular and mucosal injury (Szabo et al., 1985b). Microscopic studies showed that initially only the deep hemorrhagic lesions is prevented by PGs (Lacy and Ito, 1984). It seems that one of the main function of these compounds is the maintenance of subepithelial blood flow (Szabo and Pihan, 1987).

According to these facts PGs-induced gastric protection is originated from many mechanisms. Further experiments are required at cellular level to assess the direct cellular events of this phenomenon.

1.2.1.2. Histamine and pentagastrin

Histamine and pentagastrin are novel aggressive factors in the stomach due to stimulation gastric acid secretion. The cellular mechanisms involved in the gastric acid secretion of rats in ethanol-induced mucosal damage and prostacyclin (PGI_2)-induced gastric cytoprotection have been studied: atropine, adrenalin, cimetidine, prostacyclin, actinomycin D, Degranol (mannomustine) inhibited the gastric acid secretion and ethanol-induced mucosal damage (Mózsik et al., 1982), while surprisingly histamine, pentagastrin and 2,4-dinitrophenol (which enhanced the gastric acid secretion in a short time period) stimulated the PGI_2-induced gastric cytoprotection (Mózsik et al., 1995a; Bódis et al., 1996a; Bódis et al., 1997a). So the drugs stimulating the aggressive side (HCl) of the stomach, could enhance the PGI_2-induced gastric cytoprotection. Furthermore, it was proved that the intact vagus is basically necessary for the receiving gastric cyto- and mucosal protection by atropine, cimetidine, PGI_2 and β-carotene (Mózsik et al., 1995a).

What can be suggested as the biochemical background of the in vivo cytoprotective effects of these aggressive drugs? The inhibitory drugs such as cimetidine and atropine decrease the cell metabolism, while pentagastrin, histamine and 2,4-dinitrophenol (for a short time) increase it. The PGI_2-induced gastric cytoprotection is decreased by gastric acid inhibitory drugs, however, histamine, pentagastrin and 2,4-dinitrophenol can stimulate it. This effect partly can be explained by acting at the level of receptors due to reduction of ATP-ADP transformation and increasing the cellular cAMP content (in the case of histamine and pentagastrin) (Mózsik et al., 1988), but the mechanism of 2,4-dinitrophenol is quite different (inhibition the respiratory chain). With this knowledge of the facts metabolic components have to play a role in the protection of gastric mucosal damage.

However, further observations are required at the level of the cells to decide whether organo- or cytoprotection takes a part in the histamine- and pentagastrin-induced gastric mucosal protection.

1.2.1.3. Scavenger compounds

Some compounds, namely retinoids (vitamin A, beta-carotene, beta-cryptoxanthin, zeaxanthin) have scavengers property besides their cytoprotective effect (Jávor et al., 1986). The possible role of oxygen free radicals in gastric mucosal lesions was also based on this observation. The gastric cytoprotection is not depended on presence of vitamin A activity, beta-ionone ring, number of unsaturated links and chemical structure of terminal part of molecules (Mózsik et Jávor, 1991).

Beta-carotene is one of the widely used scavengers in cytoprotective investigations. It did not decrease the gastric secretory response in pylorus-ligated rats, but it was able to prevent the gastric mucosal damage induced by ethanol, hydrochloric acid or indomethacin (Mózsik et al., 1991a). One of the possible explanation of the beneficial effect of beta-carotene is based on its scavenger property. It was proved that free radicals play a role in ethanol-induced mucosal damage (Pihan et al., 1987), but it is only a part of the action of beta-carotene. However, the intact vagal nerve and adrenals are basically necessary for the development of beta-carotene-induced gastric mucosal protection. In addition, this process is not mediated by prostaglandins, and might be partly mediated by SH-groups (Vincze et al., 1997). Time course studies proved that the action of scavengers manifested only at later phase of mucosal damage despite with prostaglandins (Vincze et al., 1991).

It seems that there are many factors presenting the scavengers-induced organoprotection, among others there must be direct cytoprotective effect due to elimination of free radicals.

1.2.1.4. Body protection compound, BPC

A new human gastric juice peptide (code-named body protection compound - BPC, M.W. 40.000) was isolated by Sikiric et al., which is generally described as an organoprotective agent (Sikiric et al., 1991a,b; Mózsik et al., 1991b). In experimental models it has beneficial effect among others on the gastrointestinal tract (stomach, pancreas, small and large intestine), kidney, brain, liver. After several biochemical procedures a 15 amino acid fragment (BPC 157), though to be essential for the activity of entire peptide, was characterized (Sikiric et al., 1993a). Although the exact mechanisms of this prevention are yet unknown, they are probably mediated in adrenal, parathyroid, thyroid and ovarian pathways (Sikiric et al., 1992). Recently this natural compound BPC 157 was synthesized and named PL-10 substances, which also show the in vivo general protective effect (Erceg et al., 1997).

A significant pool of BPC has been immunohistochemically demonstrated in the stomach as well as in the brain (Sikiric et al., 1993a). Based on the protective effects of different peptides given centrally and/or peripherally, on a relatively short half time, on a small molecule size (15 amino acids) it seems that BPC primary has special gut peptide properties. In addition, its role in the induction of the healing processes appears to be very likely (Sikiric et al., 1994). Acute toxicology shows a very high therapeutic index, since no death or pathologic changes were observed despite the administration of a very high dosage (e.g. 50 mg/kg) (Sikiric et al., 1993a).

The wide range of benefit actions on different organs can be explained only by direct cellular effect - by direct cytoprotection. Regarding to these facts, after further investigations BPC also might have a part in human therapeutics as an antiulcer drugs.

2. CYTOPROTECTION OR ORGANOPROTECTION?

In the early 80s some research teams suggested that the terminology of "organoprotection" is more suitable for the phenomenon of cytoprotection. The word "gastric cytoprotection" originally means the protection of gastric mucosal cells, but in vivo experiments many other factors e.g. central nervous system (Grijalva et al., 1990), vagal nerve (Mózsik et al., 1993a), blood flow (Szabo et al., 1985b; Guth et al., 1984), vascular permeability (Szabo et al., 1985b) and the rapid epithelial restitution from surviving neck cells (Lacy and Ito, 1984) play an important role in the mechanisms of defence. On the other hand, cytoprotection is not a general phenomenon: the protective effects are not the same in different models, and it is not general in different parts of the gastrointestinal tract.

Regarding to these facts the terms of organoprotection and cytoprotection differ from each other. Organoprotection is a complex phenomenon, it consists many other factors from other organ systems e.g. vascular, hormonal and neural effects, while cytoprotection means the direct cellular events induced by toxic agents or drugs.

New methods are required for the complex analysis of cytoprotection to study the direct cellular effect of the in vivo organoprotective agents. There are two possibilities for in vitro observations: freshly isolated cells (short term culture) and cell lines (long term culture). In the following chapters the mechanisms of cell injury, the advantages and disadvantages of these two kind of cell cultures will be overviewed.

2.1. CYTOPROTECTION AT CELLULAR LEVEL

2.1.1. The mechanisms of cell injury

Cell injury (reversible or irreversible) occurs as a result of single or multiple causes, and it develops through multiple steps and pathways. These pathways are mostly nonspecific: ischemic, chemical or physical factors induce same or similar manifestations.

The five common causes of cell injury are:

1. *Hypoxia and ischemia*
    - a. Decreased blood supply
    - b. Decreased O2-carrying capacity
    - c. Diminished tissue oxidative enzymes
2. *Chemicals*
    - a. Natural (e.g. aflatoxin) vs. synthetic
    - b. Water vs. lipid solubility
3. *Biologic agents*
    - a. Viruses, rickettsiae, bacteria, fungi, parasites
    - b. Immunological reactions
4. *Physical factors* - usually irreversible cell injury
    - a. Mechanical force e.g. trauma, pressure
    - b. Temperature extremes: burn, frost-bite
    - c. Electric shock
    - d. Ionizing radiations
5. *Genetic defects* - slowly developing
    - a. Chromosomes (autosomes or sex chromosomes)
    - b. Plasma membrane regulating receptors or transport processes
    - c. Enzymes regulating catabolism or anabolism

Although there is no common final pathway of cell injury and death, a few common chemical entities should be considered. One of them is the free radical activity. These activated oxygen species and free radicals have very short half lives but are extremely reactive damaging to the environment where they are generated. The main mechanism of injury caused by free radicals is lipid peroxidation which destruates the polyunsaturated fatty acids-containing biological membranes. The other common pathway of cell injury is the alteration in calcium homeostasis. Ischaemia, chemicals or microbiologic agents may influence the huge gradient between extracellular and intracellular free calcium concentrations. These changes result the poisoning of mitochondrial oxidative processes, interference with normal receptor-membrane functions, and possible activation of phospholipase A_2 which leads to degradation of membrane phospholipids.

The early morphologic changes in cell injury may only be observed by electron microscopy. Plasma membrane alterations reflect the disturbances in ion and volume regulations induced by loss of ATP. These include cell swelling, formation of cytoplasmic blebs, blunting and distortion of microvilli, creation of myelin figures, and loosening of intracellular attachments. These changes can occur rapidly and are readily reversible. In late stages, breaks are seen in both the membranes enclosing the cells and organelles. The irreversible phase of cell injury is characterized by the loss of mitochondrial and membrane functions.

Cell and tissue injury in the gastrointestinal mucosa is also a very complex phenomenon. In addition to the direct toxic effect of a drug, one has to take into consideration an ischemic component and a role of vascular injury as well as the damaging action of acid and pepsin that are normally present in the lumen of the upper gastrointestinal tract. In the other hand it is possible to preserve most of the structure and function of a tissue and an organ despite the fact that thousands of cells might be killed. However, it is important to underline the differences between cell and tissue injury: cells might be sacrificed while the tissue structure and function are not markedly altered - they might actually be "protected". The differences between cytoprotection and organoprotection are also based on this fact.

2.1.2. Short term and long term cell culture systems

The new exact way in the complex analysis of cytoprotection is the studies on freshly isolated cells (short term culture) or on primary/continuous (long term) cultures. By this way all of the surrounding effects originated from other organs can be eliminated, and the behavior of the single cells can be examined. These methods are suitable for direct cytoprotective investigations.

In the analysis of gastrointestinal cytoprotection cell isolation can be performed from the stomach (usually from the rats, because *in vivo investigations* were carried out also on these animals). The other advantage of the cell isolation is that one study requires only one or two rats. The animals are anesthetized and killed without any painful procedures.

Gastric mucosa is consists of several cell types. The luminal surface is lined with surface mucous cells, in the glandular regions mucous neck cells, chief cells, parietal cells, endocrine-like cells and undifferentiated cells can be found (Fig. 1).

By the help of digestion with enzyme preparations e.g. pronase, collagenase mixed population of gastric mucosal cells can be isolated (Nagy et al., 1994). In a short term culture the life time of the cells is about 6-8 hours, they are suitable only for short-term observations. In addition, the surface receptors can be damaged during the isolation processes and there is not enough time for regeneration. These freshly isolated cells are a good targets for toxicological studies, however, their receptors can be stimulated only with pharmacological (not physiological) doses of drugs (Nagy et al., 1994).

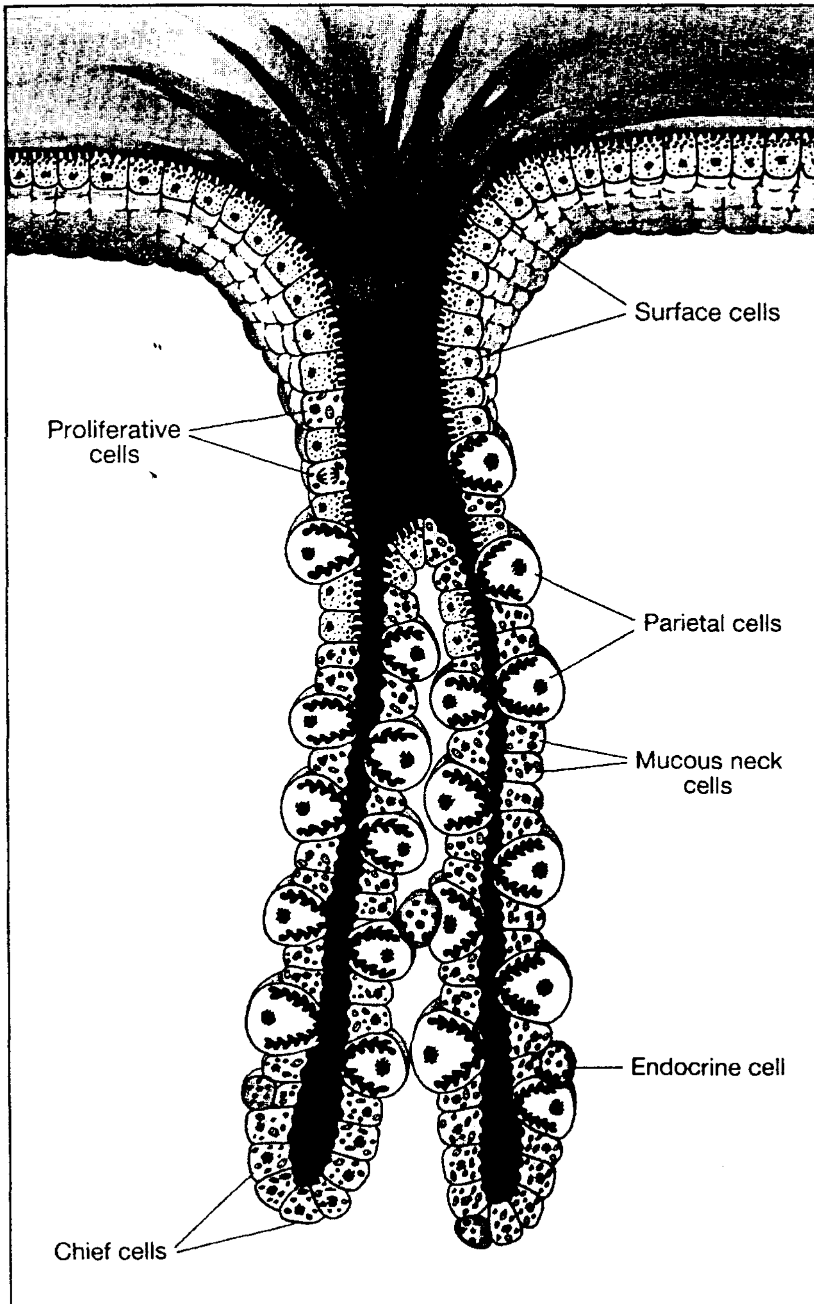

Fig. 1

Schematic diagram of a gland in the glandular stomach representing the different cell types which can be isolated by enzymatic digestion.

The usage of long term cell cultures in cytoprotective investigations have several advantages. Long term cell cultures can be continuous cell lines (usually they are malignant cells) or primary cultures from acute isolation and enrichment.

Cell lines are always in the same circumstances, the observations are well reproducible, these cells are not influenced by other effects. The experiments can be long term studies, the surface receptors are unharmed.

The primary cultures of the cells especially from the stomach are good targets for pharmacological observations, for remodelling the different physiological regulating pathways and biochemical processes in the cells.

Parietal cells are highly differentiated cells with a finite life span, they can be cultured for 1-2 weeks, the cell number in the culture is decreased by 5-10% per day (Chew et al., 1989). After isolation parietal cells must be enriched from the mixed population. The partially enriched parietal cells are rapidly overgrown with mucous-containing and fibroblast-like cells. By the methods of Chew et al. 100% purity can be approached. Stimulation by histamine and carbachol induces morphological changes in the parietal cells: there is a significant increase in cell size and canalicular microvilli are appeared. These events can be blocked by pre-addition of receptor antagonists (Soroka et al., 1993; Chew, 1994).

The advantages and disadvantages of the different cell culture models are summarized in Table 2.

Table 2

Advantages and disadvantages of different types of cell culture models.

	Advantages	**Disadvantages**
Short term culture	Easy methodology	Short life time Possible receptor damage
Continuous culture	Well reproducible Long-term studies	Malignant cells
Primary culture	Long-term studies	Difficult to culture

2.2. THE AIMS OF THIS STUDY

The aim of this study was to analyze the in vitro effect of some in vivo aggressive and protective agents. The in vitro observations were carried out

- on a **short term** (freshly isolated gastric mucosal cells from the rats) and

- on a **long term** (Sp2/0-Ag14 - a non-secreting mouse myeloma cell line as an internationally accepted general cell model) **cell culture `models.**

By the help of these cultures the direct cellular effect of the aggressive factors and agents having in vivo organoprotection were analyzed, namely:

1) Direct short term and long term cellular effect of ethanol;

2) Direct cellular effect of indomethacin without or with application of other aggressive factors;

3) Direct cellular effect of the homogenate of human pathogenic Helicobacter pylori;

4) The prostacyclin-induced direct cytoprotection against ethanol- and indomethacin injury;

5) Direct cellular effect of histamine and pentagastrin;

6) Cytoprotection induced by PL-10 substances (synthesized parts of BPC);

7) The effect of scavengers in long term cell injury.

3. SHORT TERM CULTURES

3.1. MATERIALS AND METHODS

3.1.1. Preparation of mixed gastric mucosal cells from rats

Gastric mucosal cells (GMCs) from 1-2 unfasted Sprague-Dawley rats were isolated by the method of Nagy et al. (Nagy et al, 1994). Briefly, the segments of glandular stomach without blood vessels and surrounding connective tissue were sequentially incubated in a physiologic solution containing 0.5 mg/ml pronase E (type XXV, Sigma Chemical Co., St. Louis, USA) and 10^{-3} M EGTA (Sigma Chemical Co., St. Louis, USA). After several washing cells were resuspended and kept in shaking water bath at 37°C in a solution (0.157 M, pH 7.4) produced freshly with the following ingredients (mM): 98.0 NaCl (Reanal, Budapest, Hungary), 5.8 KCl (Reanal, Budapest, Hungary), 2.5 NaH_2PO_4 (Reanal, Budapest, Hungary), 5.1 Na pyruvate (Reanal, Budapest, Hungary), 6.9 Na fumarate (Sigma Chemical Co., St. Louis, USA), 2.0 glutamine (Sigma Chemical Co., St. Louis, USA), 24.5 HEPES Na (Sigma Chemical Co., St. Louis, USA), 1.0 Trizma base (Reanal, Budapest, Hungary), 11.1 D-glucose (Reanal, Budapest, Hungary), 1.0 $CaCl_2$ (Reanal, Budapest, Hungary), 1.0 $MgCl_2$ (Reanal, Budapest, Hungary) and 2.0 mg/ml (w/v) bovine serum albumin (Reanal, Budapest, Hungary). Examinations were carried out also in this solution. 85-95 % initial viability of the isolated cells was maintained for 6-7 h.

Mixed population of freshly isolated gastric mucosal cells contains about 20-25 % parietal cells and approximately 40-40 % chief and mucous producing cells (Fig. 2).

3.1.2. Determination of cell viability

Cell viability can be determined at different levels of the cells, namely at the level of the membrane, at the level of mitochondria and at the level of the nucleus (Fig. 3).

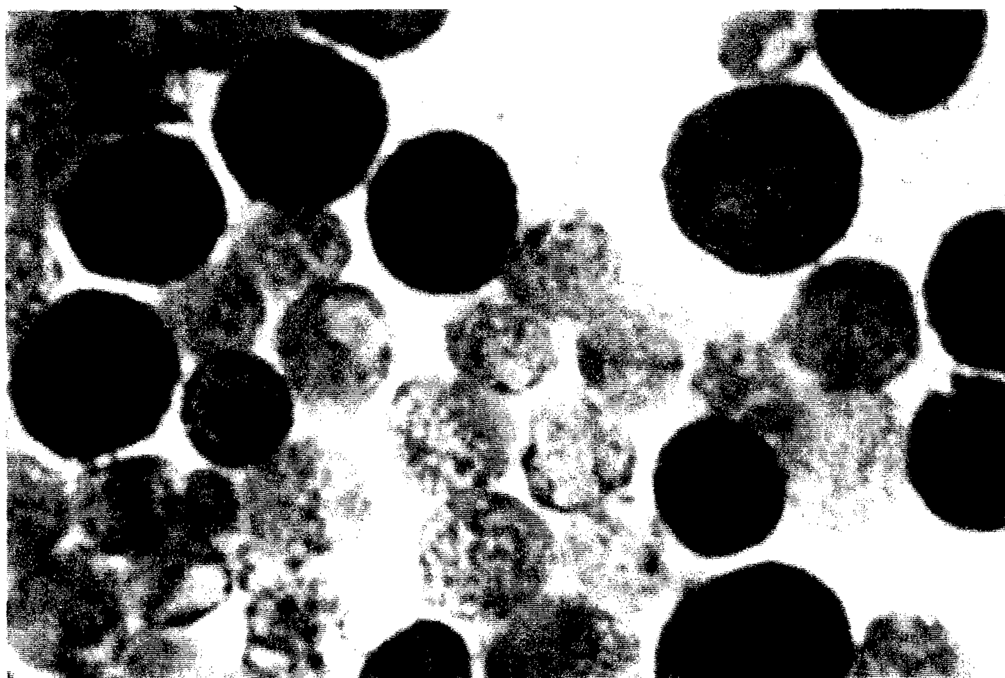

Fig. 2

Freshly isolated rat gastric mucosal cells by Congo red staining. The big bright red cells are parietal cells, the smaller ones are chief cells, while the smaller cells with gray cytoplasm are mucous producing cells.

3.1.2.1. Trypan Blue exclusion test

Trypan Blue (TB) is excluded by viable cells, while taken up by damaged cells staining the cytoplasm blue (Baur et al, 1975). 0.4 % TB (Sigma Chemical Co., St. Louis, USA) was mixed with the same volume of cell suspension and 5 min later the rate of stained (dead) and unstained (viable) cells were calculated as % after counting 100 cells in hemocytometer.

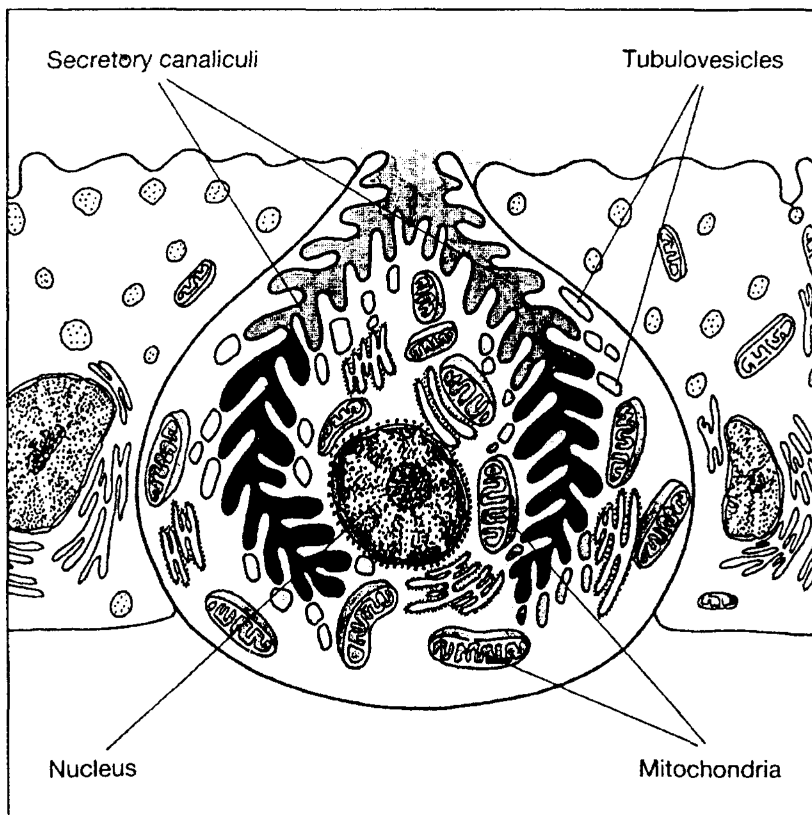

Fig. 3

The levels of cell injury. Membrane damage can be determined by Trypan Blue exclusion test and lactate dehydrogenase assay, mitochondrial function can be measured by succinic dehydrogenase assay, while nuclear damage can be assessed by ethidium bromide-DNA fluorescence method.

3.1.2.2. Biochemical assays

3.1.2.2.1. Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) enzyme can be found in the cytoplasm of the cells. If it presents in the supernatant, it can indicate the membrane damage of the cells. Its activity was determined in samples of both toxic agent-free supernatant and cell pellet destroyed by freeze. The colorimetric assay was based on reduction of NAD^+ to NADH catalyzed by LDH in presence of lactate as substrate (Bergmeyer and Bern, 1974). The color product of the reduction of phenazine metosulphate (Sigma Chemical Co., St. Louis, USA) and tetrazolium salt isonicotinic acid hydrazid (Sigma Chemical Co., St. Louis, USA) was measured at 520 nm on Hitachi 124 spectrophotometer. The results were expressed as mU/min/10^6 cells.

3.1.2.2.2. Succinic dehydrogenase assay

Succinic dehydrogenase (SDH) enzyme can be found in the mitochondria. The mitochondrial integrity was tested in $2x10^6$ previously treated and redispersed cells (Mosmann, 1983). The color formazan product was quantified by Hitachi 124 spectro-photometer at 500 nm and calculated as nmol/min/$2x10^6$ cells.

3.1.2.2.3. Ethidium bromide-DNA fluorescence assay

The nuclear damage of cells was assessed by nuclear fluorescence due to the ethidium bromide-DNA binding (Dey and Majumder, 1988). 10^7 cells were mixed with EB solution (Sigma Chemical Co., St. Louis, USA), and the fluorescence intensity was measured in Hitachi F-3000 fluorescence spectrophotometer at 325-585 nm (excitation-emission). The results were expressed as arbitrary fluorescence units/10^7 cells.

3.1.3. Toxicological studies

3.1.3.1. Ethanol

Cells were incubated with different concentration of ethanol (EtOH) (Reanal, Budapest, Hungary) such as 5, 10, 15, 20 and 50% (v/v) for 5 min in shaking water bath at 37°C. Equivalent volume of both diluted toxic agent and cell suspension were used. At the end of 5 min incubation cells were separated from the supernatant by centrifugation (1000 G, 5 min). Cell pellet was carefully resuspended and incubated for 10 min in water bath, then it was centrifuged again to get the toxic agent-free supernatant and resuspended cells for further biochemical assays.

3.1.3.2. Indomethacin

Cells were incubated with 10^{-8}-10^{-3} M indomethacin (IND) (Chinoin, Hungary) alone or combined with 15% EtOH for 5 min in shaking water bath at 37°C. The further processes were the same as described above.

1.3.3. Helicobacter pylori

Helicobacter pylori (H. pylori) was obtained from biopsy samples of patients with gastritis, and they were cultured in blood agar base. The existence of infection with H. pylori was proven by histology and urease positivity. The bacterial suspensions (10^8 bacteria/ml in 20 mM Tris/HCl buffer, pH 7.0) were sonicated on ice (power: 30 W) in six consecutive treatments lasting 30 seconds. Isolated GMCs were incubated with sonicated H. pylori (10^6-10^8 bacteria/ml) for 30 min in shaking water bath at 37 °C. At the end of 30 min incubation cells were separated from the supernatant by centrifugation, the following examinations were carried out as described above.

3.1.4. Pharmacological and cytoprotective investigations

3.1.4.1. Prostacyclin

Cells were preincubated with 10^{-4}-10^{-6} M prostacyclin (PGI_2Na) (Chinoin, Hungary) for 60 min in shaking water bath at 37 °C. After this treatment cells were incubated with 15% EtOH and 10^{-3} M IND for 5 min as described above.

1.4.2. Histamine

The isolated rat gastric mucosal cells were incubated with 10^{-8}-10^{-6} M histamine (Peremin, Chinoin, Hungary) for 60 min with or without 10^{-4} M prostacyclin. After this treatment cells were incubated by 15% EtOH alone or combined with 10^{-3} M IND for 5 min. The possible acid secretion was observed by measurements of pH in the incubating medium (Radelkis, Hungary).

3.1.4.3. Pentagastrin

Cells were preincubated with 10^{-8}-10^{-6}-10^{-4} M pentagastrin (Peptavlon, ICI, UK) alone or combined with 10^{-4} M PGI_2 for 60 min in shaking water bath at 37 °C. After this treatment cells were incubated with 15% EtOH for 5 min as described above.

3.1.4.4. PL-10 substances

PL-10 substances are the synthesized variants of the originally described pentadecapeptide BPC 157 (Gly Glu Pro Pro Pro Gly Lys Pro Ala Asp Asp Ala Gly Leu Val, M.W. 1419) (Sikiric et al, 1993a). Four compounds were tested dissolved in saline:

PL-10.1.AK15-2 - a 15 amino acid compound produced by chemical synthesis from the second batch;

PL-10.1.AK15-3 - a 15 amino acid compound produced by chemical synthesis from the third batch;

PL-10.1.AK15-4 - a 15 amino acid compound produced by chemical synthesis from the fourth batch;

PL-10.1.AK14-2 - a 14 amino acid compound produced by chemical synthesis from the second batch.

Cells were incubated with 5-50-500 ng/ml-5 μg/ml doses of four PL-10 substances (PLIVA, Croatia) for 60 min in shaking water bath at 37°C. After this treatment 15% ethanol (EtOH) were used for 5 min as toxic agent. The following procedures were the same as described above.

3.1.5. Statistics

Values in figures and text were expressed as means ± SEM. Comparisons were performed by unpaired Student's t test or by analysis of variance (ANOVA) followed by unpaired Student's t test. P values were considered significant at $p<0.05$.

3.2. RESULTS

3.2.1. Toxicological studies

3.2.1.1. Effect of ethanol

5, 10, 15, 20 and 50% of EtOH concentration-dependently decreased the cell viability detected by TB exclusion test (Fig. 4). EC_{50} = 13.5 %

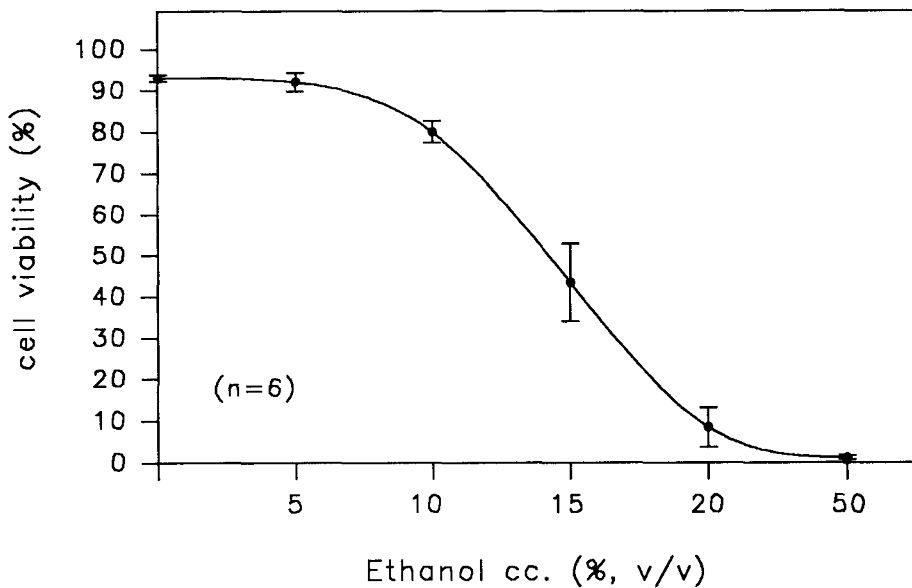

Fig. 4

Changes in the viability of acutely isolated gastric mucosal cells after 5 min incubation of 5-50% (v/v) ethanol detected by Trypan Blue exclusion test.

LDH activity concentration-dependently increased in the supernatant and at the same time it decreased in the cell pellet after ethanol treatment (Fig. 5). At high concentration of ethanol (50%) enzyme activity could not been detected because of inactivation.

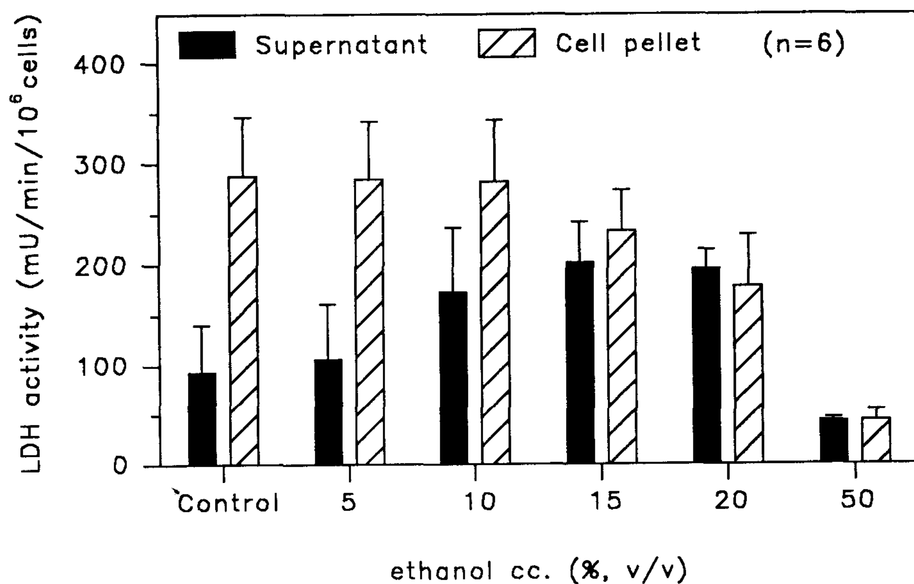

Fig. 5

LDH activity in the cell pellet and supernatant in freshly isolated rat gastric mucosal cells after 5 min incubation of 5-50% (v/v) ethanol.

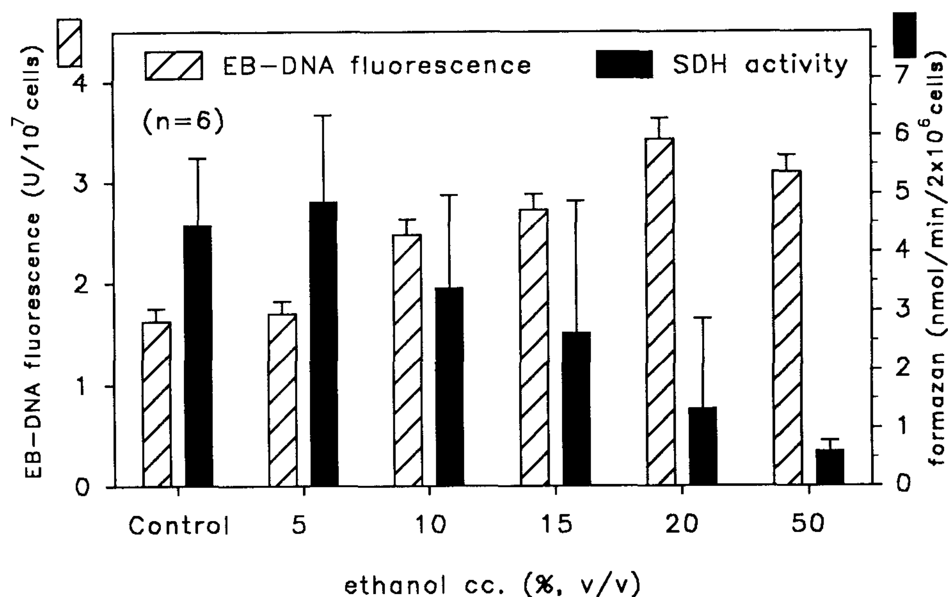

Fig. 6

EB-DNA fluorescence and SDH activity in freshly isolated rat gastric mucosal cells after 5 min 5-50% (v/v) ethanol treatment.

SDH activity was concentration-dependently decreased parallel with the destruction of the cells. EB-DNA fluorescence was increased after ethanol treatment, it showed the nuclear damage of the cells (Fig. 6).

3.2.1.2. Effect of indomethacin

After 5 min incubation of 10^{-8}-10^{-3} M IND only the high doses (10^{-4}-10^{-3} M) of IND decreased significantly ($p<0.01$) the number of viable cells (Fig. 7).

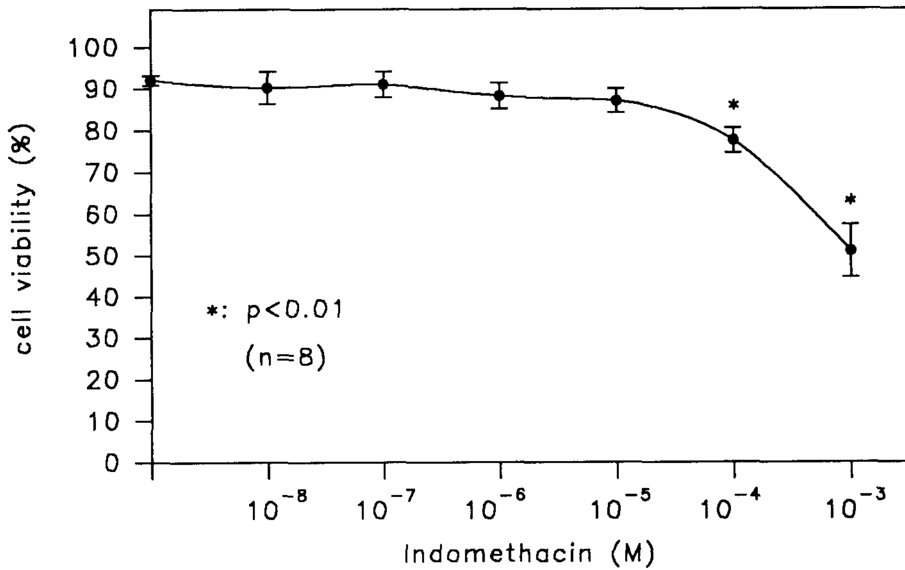

Fig. 7

Changes in the viability of acutely isolated gastric mucosal cells after 5 min incubation of 10^{-8}-10^{-3} M indomethacin (IND) detected by Trypan Blue exclusion test. * : $p<0.01$ compared with untreated cells.

3.2.1.3. Combined effect of ethanol and indomethacin

Using combined these toxic agents IND in all doses significantly ($p<0.05$, $p<0.02$, $p<0.01$) aggravated the 15% EtOH-induced cell injury determined by TB exclusion (Fig. 8). LDH activity in the cell pellet (Fig. 9) and SDH activity (Fig. 10) were decreased by combined treatment. LDH activity in the supernatant (Fig. 9) and EB-DNA fluorescence (Fig. 10) increased after 10^{-3} M IND plus 15 % EtOH treatment.

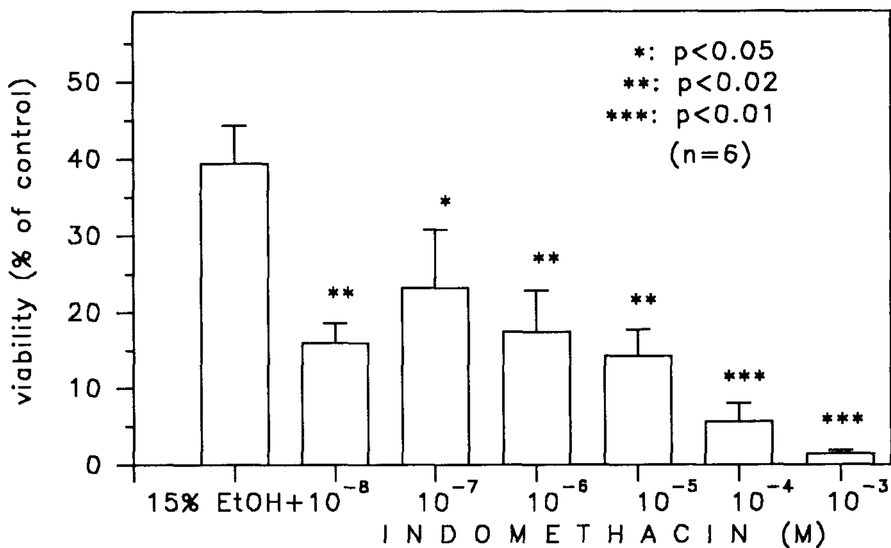

Fig. 8

Changes in the viability of acutely isolated gastric mucosal cells after 5 min incubation of 15% (v/v) ethanol (EtOH) and 10^{-8}-10^{-3} M indomethacin (IND) detected by Trypan Blue exclusion test (% of control). * : $p<0.05$, ** : $p<0.02$, *** : $p<0.01$ compared with 15% EtOH treatment.

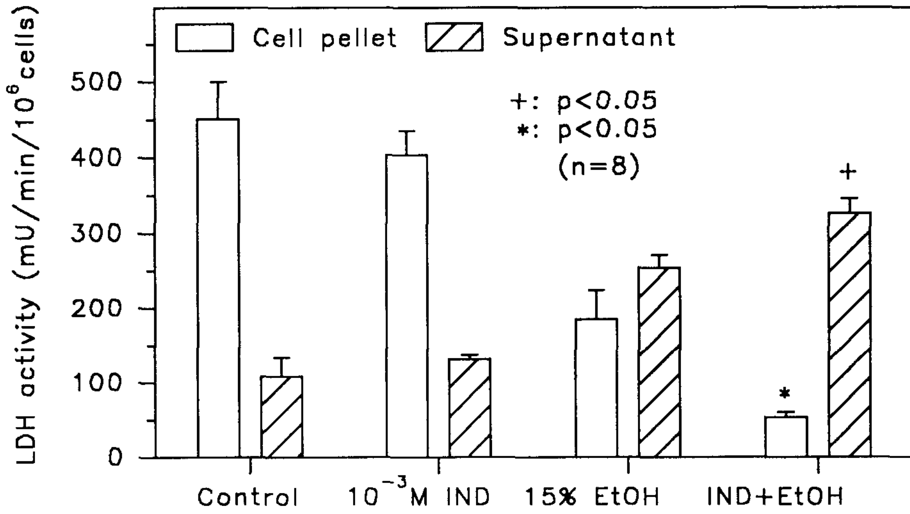

Fig. 9

Lactate dehydrogenase activity in the cell pellet and supernatant of 10^6 acutely isolated gastric mucosal cells after 5 min incubation of 15% (v/v) ethanol (EtOH) and 10^{-3} M indomethacin (IND). * : $p < 0.05$ compared with 15% EtOH treatment.

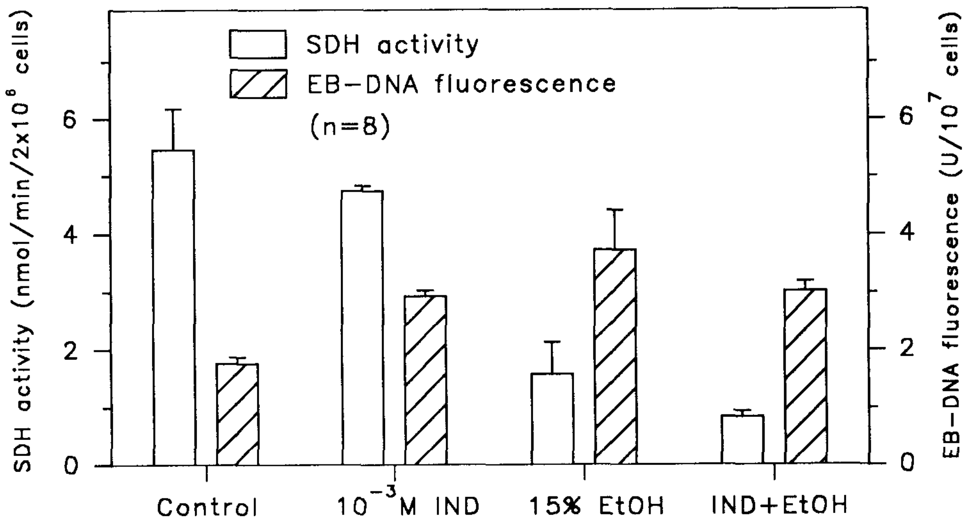

Fig. 10

Quantity of formazan synthesized by succinic dehydrogenase in $2x10^6$ acutely isolated gastric mucosal cells and intensity of ethidium bromide-DNA fluorescence (arbitrary units) in 10^7 acutely isolated gastric mucosal cells after 5 min incubation of 15% (v/v) ethanol (EtOH) and 10^{-3} M indomethacin (IND).

3.2.1.4. Direct cellular effect of Helicobacter pylori combined with 15% ethanol

10^6-10^8 sonicated bacteria/ml had no direct cellular toxicity on freshly isolated rat GMCs. In addition it did not aggravate the 15% EtOH-induced cellular damage detected by Trypan Blue exclusion test (Fig. 11), LDH activity (Fig. 12) or ethidium bromide-DNA fluorescence (Fig. 13).

2.1.5. Combined effect of Helicobacter pylori and indomethacin

30 min preincubation by sonicated H. pylori (10^6-10^8 bacteria) did not aggravate the 10^{-8}-10^{-3} M IND-induced cell injury on freshly isolated rat GMCs detected as well as Trypan Blue exclusion test (Fig. 14) or SDH activity (Fig. 15).

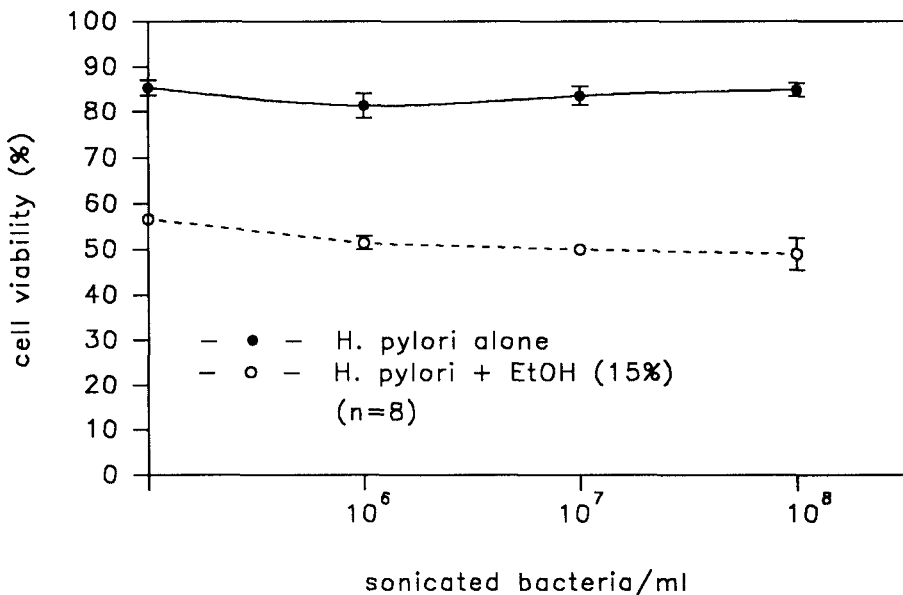

Fig. 11

Effect of 30 min incubation of sonicated 10^6-10^8 bacteria/ml H. pylori on the viability of freshly isolated rat gastric mucosal cells detected by Trypan Blue exclusion test without or with 5 min 15 % ethanol treatment.

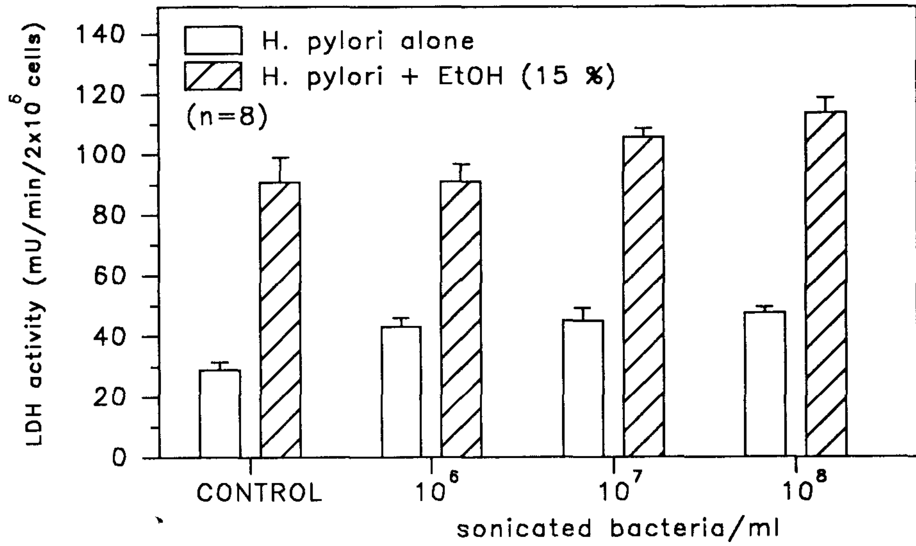

Fig. 12

Changes in lactate dehydrogenase activity of freshly isolated rat gastric mucosal cells after 30 min incubation by sonicated H. pylori (10^6-10^8 bacteria/ml) without or with 5 min 15 % ethanol (EtOH) treatment.

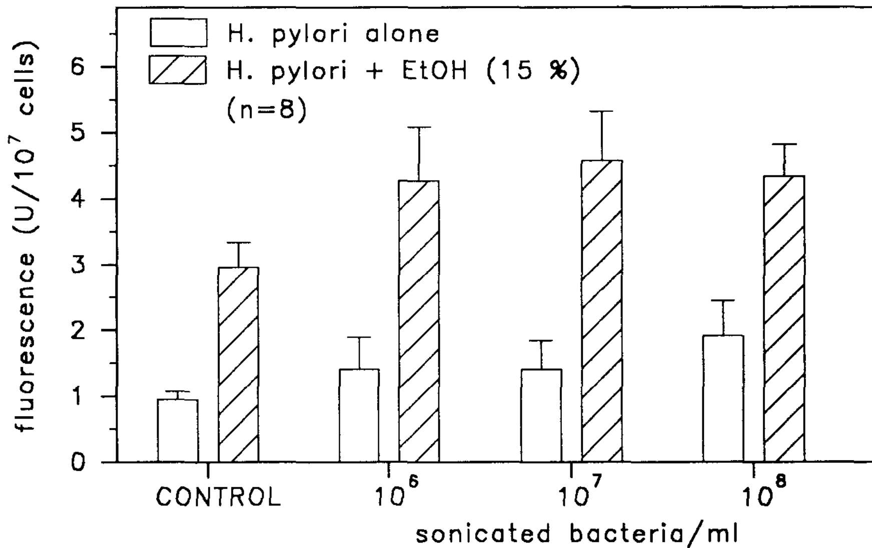

Fig. 13

Changes in ethidium bromide-DNA fluorescence of freshly isolated rat gastric mucosal cells after 30 min incubation by sonicated H. pylori (10^6-10^8 bacteria/ml) without or with 5 min 15 % ethanol (EtOH) treatment.

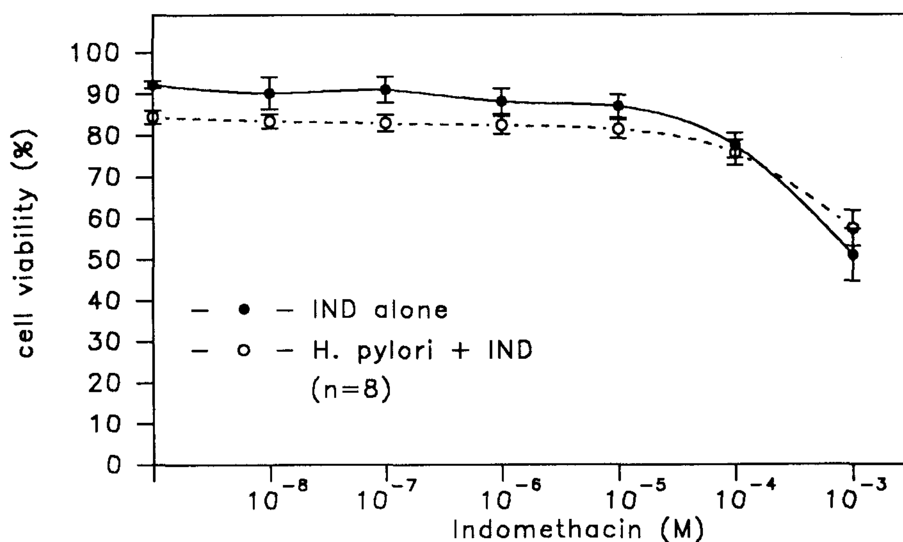

Fig. 14

Effect of 10^{-8}-10^{-3} M indomethacin (IND) on the viability of freshly isolated rat gastric mucosal cells detected by Trypan Blue exclusion test without or with 30 min incubation by sonicated H. pylori (10^7 bacteria/ml).

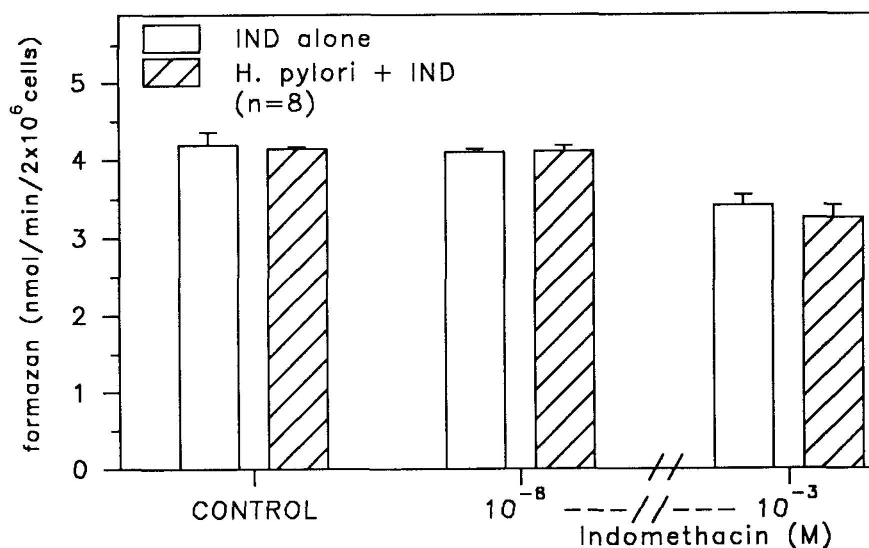

Fig. 15

Changes in succinic dehydrogenase activity of freshly isolated rat gastric mucosal cells after combined treatment of 10^{-8}-10^{-3} M indomethacin (IND) and sonicated H. pylori (10^7 bacteria/ml).

3.2.2. Pharmacological and cytoprotective investigations

3.2.2.1. Effect of prostacyclin preincubation

60 min preincubation of 10^{-6}-10^{-4} M PGI_2 did not prevent the 15% EtOH-induced cellular damage, but it significantly ($p<0.05$) protected the acutely isolated rat gastric mucosal cells against the combined effect of 10^{-3} M IND and 15 % EtOH detected by Trypan Blue exclusion test (Fig. 16). In the case of biochemical studies (SDH and LDH activity, EB-DNA fluorescence) these changes were not significant.

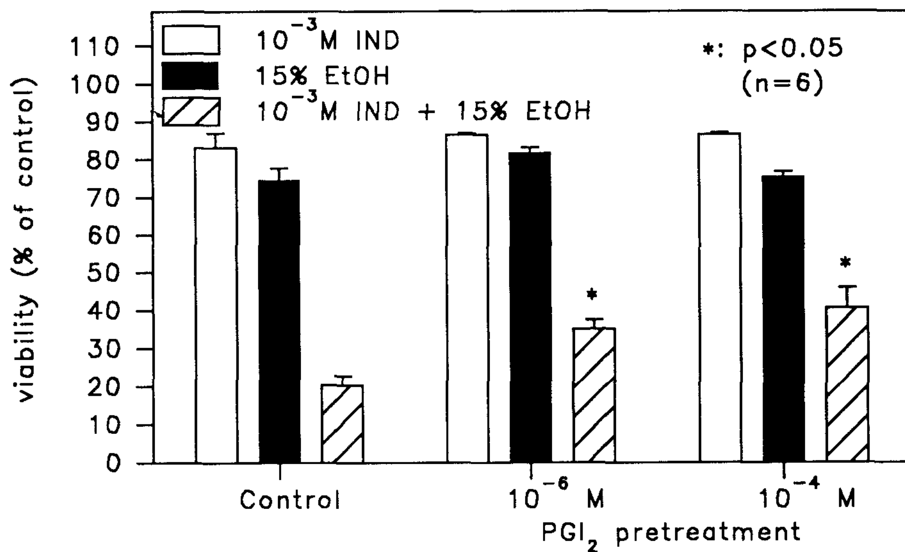

Fig. 16

Effect of 60 min incubation of 10^{-6}-10^{-4} M PGI_2 on freshly isolated rat GMCs detected by Trypan Blue exclusion test after 5 min 15% EtOH and 10^{-3} M IND treatment. *: $p<0.05$ (compared with 15% EtOH+10^{-3} M IND treatment).

3.2.2.2. Effect of histamine

60 min preincubation by 10^{-8}-10^{-6} M histamine did not cause any changes in the viability of rat GMCs. There was no measurable acid secretion in our incubating medium detected by phenol red indicator system. Combined with 5 min 15% EtOH treatment 10^{-6} M histamine significantly ($p<0.05$) aggravated the EtOH-induced cellular damage detected by Trypan Blue exclusion test and SDH activity (Figs. 17, 18).

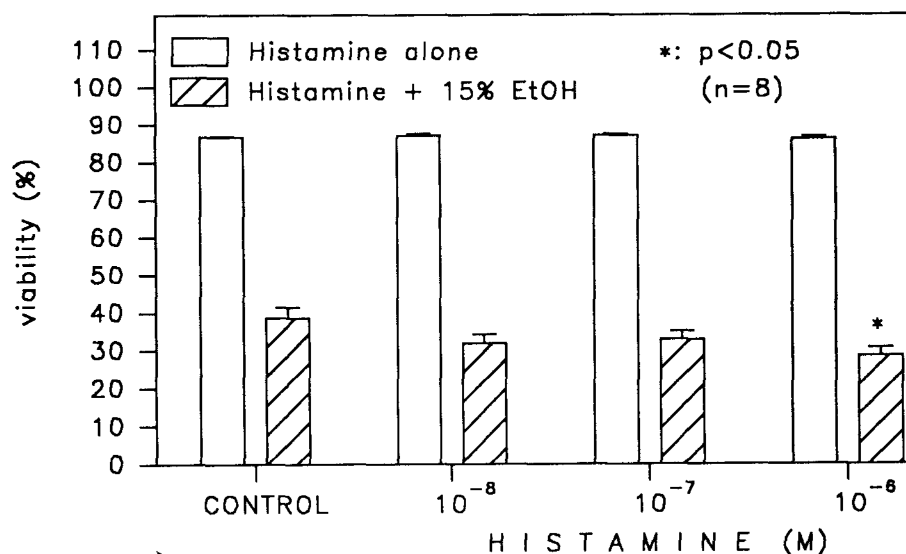

Fig. 17

Effect of 60 min incubation of 10^{-8}-10^{-6} M histamine on the viability of freshly isolated rat gastric mucosal cells detected by Trypan Blue exclusion test without or with 5 min 15 % ethanol (EtOH) treatment. * : $p<0.05$ compared with 15% EtOH-treated control.

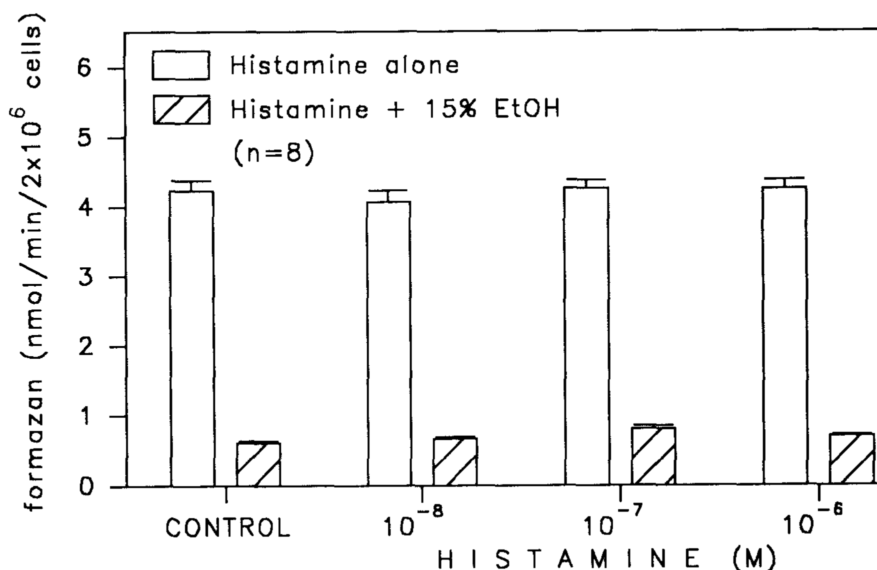

Fig. 18

Changes in succinic dehydrogenase activity of freshly isolated rat gastric mucosal cells after 60 min incubation by 10^{-6}-10^{-8} M histamine without or with 5 min 15 % ethanol (EtOH) treatment.

3.2.2.3. Combined effect of histamine and prostacyclin

60 min incubation of histamine in combination with 10^{-4} M PGI_2 did not change the viability of freshly isolated rat GMCs detected by Trypan Blue exclusion (Fig. 19), but significantly ($p<0.05$) increased the 1 hr-survival of the cells detected by SDH activity (Fig. 20).

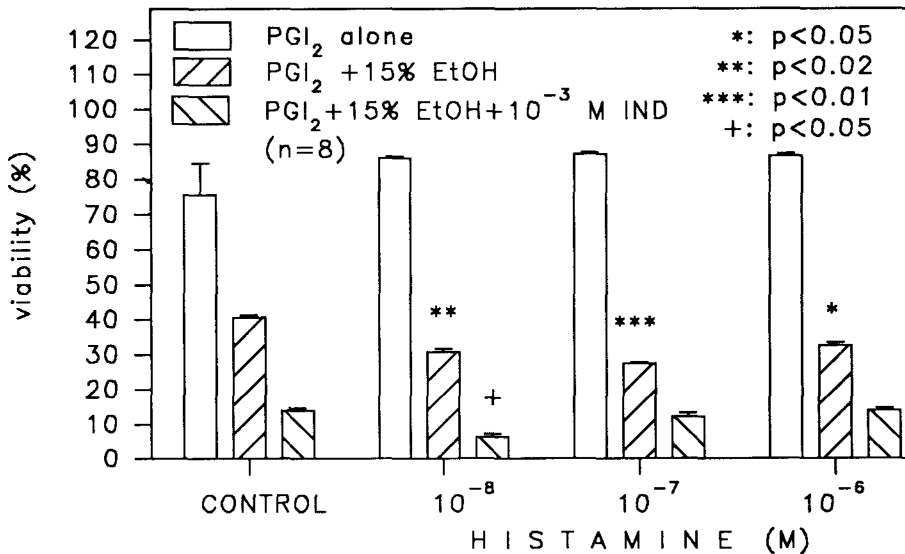

Fig. 19

Effect of 60 min incubation of 10^{-6}-10^{-8} M histamine and 10^{-4} M PGI_2 on the viability of freshly isolated rat gastric mucosal cells detected by Trypan Blue exclusion test without or with 5 min 15 % ethanol (EtOH) and 10^{-3} M indomethacin (IND) treatment. * : $p<0.05$, ** : $p<0.02$, *** : $p<0.01$ compared with PGI_2 and EtOH treated control. + : $p<0.05$ compared with PGI_2, EtOH and IND treated control.

3.2.2.4. Combined effect of histamine, prostacyclin, ethanol and indomethacin

60 min incubation of 10^{-8}-10^{-6} M histamine combined with 10^{-4} M PGI_2 significantly aggravated the 15% EtOH-induced cell injury detected by Trypan Blue exclusion test (Fig. 19). Surprisingly the lower doses of histamine caused higher

cellular damage. SDH activity (showing the 1 hr-survival) did not suppose these negative changes (Fig. 20). The same tendency could be seen after combined 5 min incubation of EtOH and IND: 10^{-8} M histamine significantly ($p<0.05$) aggravated the cell injury (Fig. 19), but this effect disappeared by SDH activity (Fig. 20).

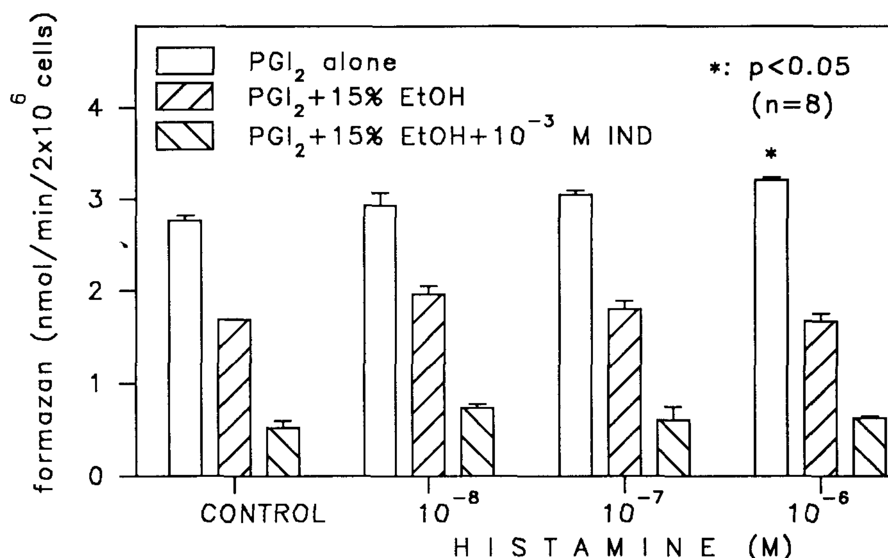

Fig. 20

Changes in succinic dehydrogenase activity of freshly isolated rat gastric mucosal cells after 60 min incubation of 10^{-6}-10^{-8} M histamine and 10^{-4} M PGI_2 without or with 5 min 15 % ethanol (EtOH) and 10^{-3} M indomethacin (IND) treatment. * : $p<0.05$ compared with PGI_2 treated control.

3.2.2.5. Pentagastrin

60 min preincubation of 10^{-8}-10^{-6} M pentagastrin did not cause any changes in the viability of GMCs. After 5 min incubation of 15% EtOH 10^{-4} M pentagastrin significantly ($p<0.05$) aggravated the EtOH-induced cellular damage (Fig. 21).

After 60 min incubation of 10^{-8}-10^{-6}-10^{-4} M pentagastrin combined with 10^{-4} M PGI_2 we also could not observe any protective effect against EtOH-induced cell injury (Fig. 21).

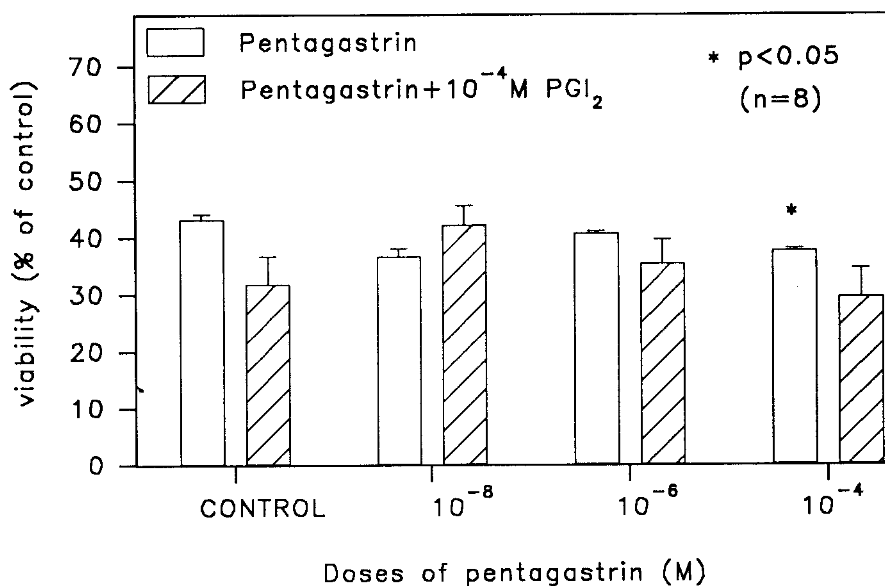

Fig. 21

Changes in the viability of freshly isolated rat GMCs detected by Trypan Blue exclusion test after 60 min incubation of pentagastrin alone or combined with 10-4 M PGI_2 and 5 min 15% EtOH treatment. *: $p<0.05$ compared with 15% EtOH treated control.

3.2.2.6. PL-10 substances

3.2.2.6.1. PL 10.1.AK15-4 and PL 10.1.AK15-2

60 min incubation of these substances with rat gastric mucosal cells did not aggravate the EtOH-induced cell injury, however, we could not observed any protective effect against 15% EtOH tested by Trypan Blue exclusion test and SDH activity (Figs. 22, 23).

2.2.6.2. PL 10.1.15AK-3

There was a **significant cellular protection** detected by Trypan Blue exclusion test after 60 min incubation of 5 µg/ml dose of PL 10.1.15AK-3 against 15% EtOH in rat GMCs ($p<0.05$, p=1.6417E-6 analyzed by ANOVA), but we could not observe it by SDH activity (Fig. 24).

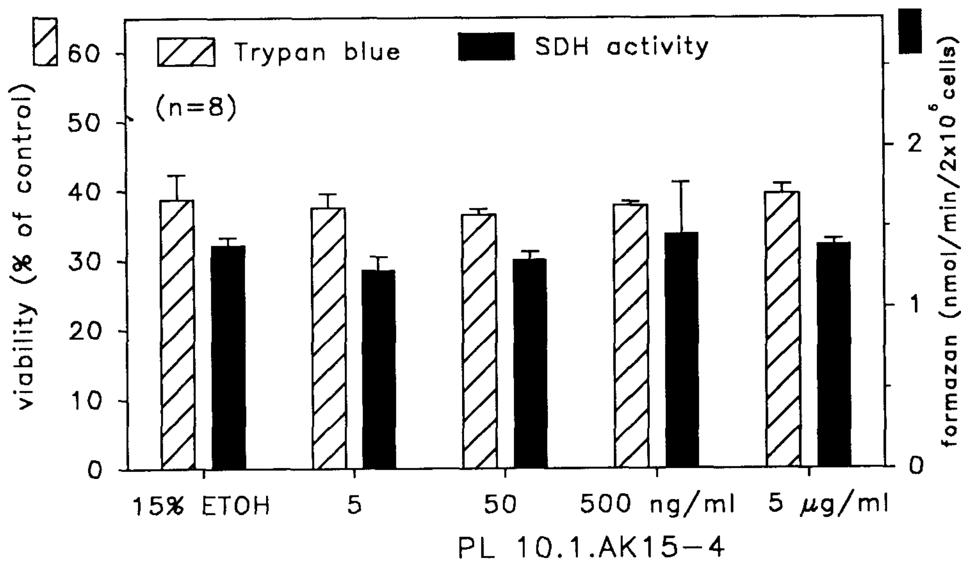

Fig. 22

Changes in the viability detected by Trypan Blue exclusion test and SDH activity of isolated rat gastric mucosal cells after 60 min incubation of PL 10.1.AK15-4 and 5 min 15% ethanol (EtOH) treatment.

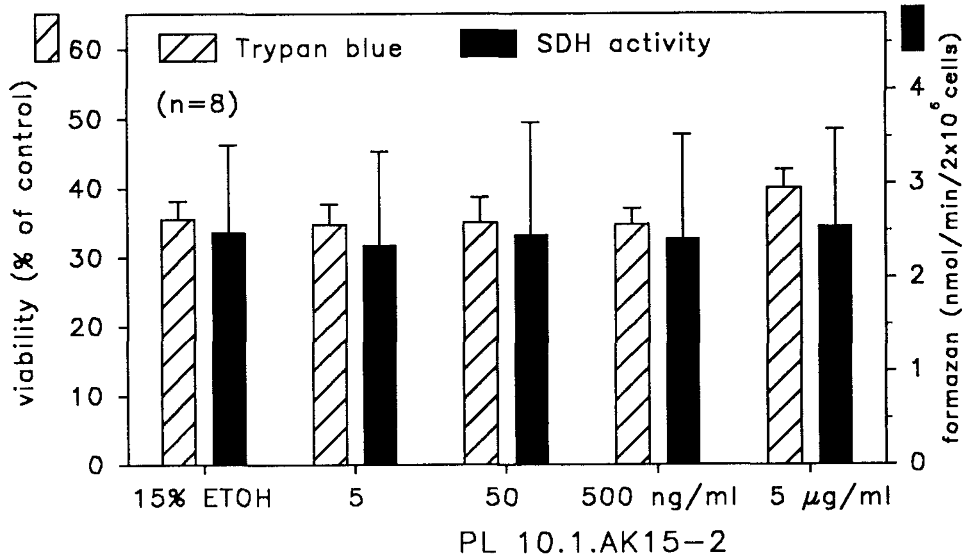

Fig. 23.

Changes in the viability detected by Trypan Blue exclusion test and SDH activity of isolated rat gastric mucosal cells after 60 min incubation of PL 10.1.15AK-2 and 5 min 15% ethanol (EtOH) treatment.

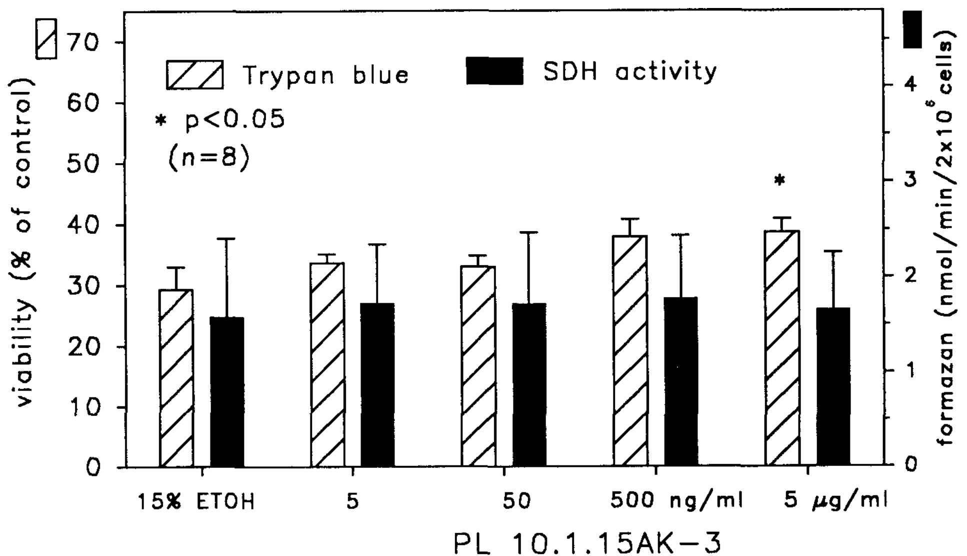

Fig. 24

Changes in the viability and SDH activity of isolated rat gastric mucosal cells after 60 min incubation of PL 10.1.15AK-3 and 5 min 15% ethanol (EtOH) treatment. *: $p<0.05$ (15% EtOH vs. PL-10 preincubation+15% EtOH).

3.2.2.6.3. PL 10.1.AK14-2

We detected a **significant and dose-dependent protection** by Trypan Blue exclusion test in freshly isolated rat GMCs after 60 min incubation of PL 10.1.AK14-2 against 15% EtOH ($p<0.001$, $p=1.4576E-6$ analyzed by ANOVA). The biochemical examinations did not support these significant changes: we could detect only a protective tendency by SDH activity (Fig. 25).

This significant cytoprotection can be represent by the help of a dose-response curve: ED_{50}=50 ng/ml, r=0.9982 (Fig. 26).

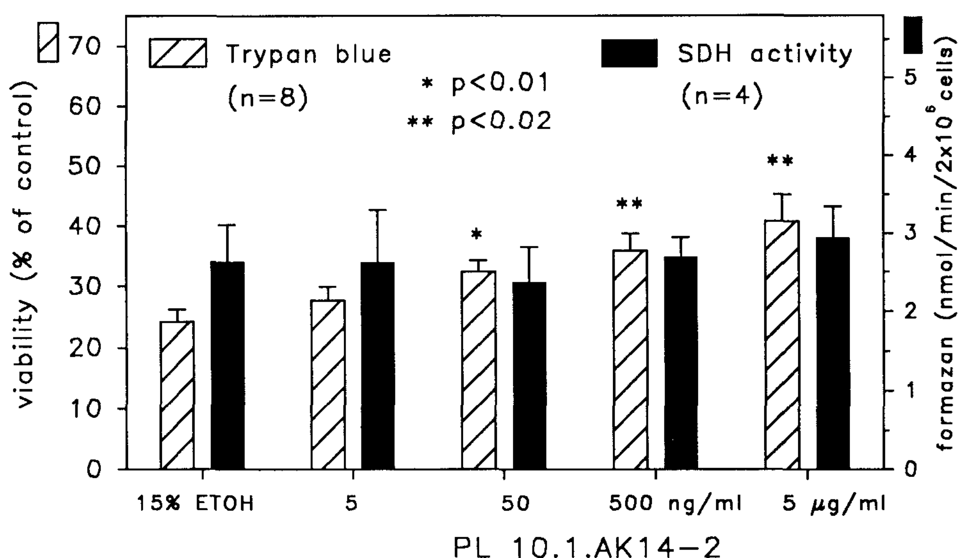

Fig. 25

Changes in the viability detected by Trypan Blue exclusion test and SDH activity of rat gastric mucosal cells after 60 min incubation of PL 10.1.AK14-2 against 5 min 15% ethanol (EtOH) treatment. *: $p<0.02$ **: $p<0.01$ (15% EtOH vs. PL-10 preincubation+15% EtOH)

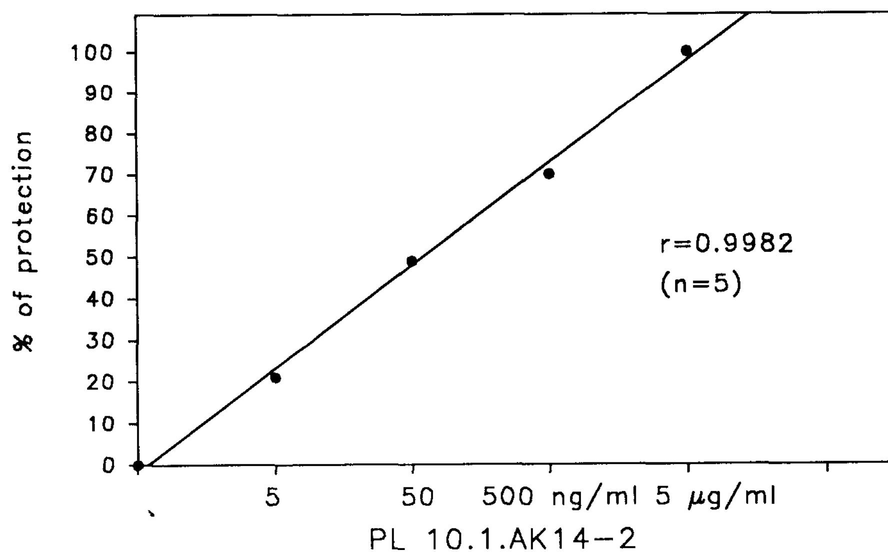

Fig. 26

Dose-response curve for PL 10.1.AK14-2 substance. The protection is expressed in percent values, the effect of 5 μg/ml dose was regarded as 100% (Y=24.9X-1.8).

4. LONG TERM CULTURES

4.1. MATERIALS AND METHODS

4.1.1. Standard continuous cell lines

4.1.1.1. Sp2/0-Ag14 cell line

Sp2/0-Ag14 (CRL 1581) is a non-secreting mouse myeloma cell line obtained from American Type Culture Collection (ATCC). These cells do not secrete any immunglobuline, in biological point of view they are internationally accepted as a general cell model (Schulman et al, 1978). Cells were cultured in Dulbecco`s modified Eagle`s medium (DMEM) containing 10% fetal calf serum (FCS) and maintained in humidified incubator in 95% air and 5% CO_2 at 37 °C.

4.1.1.2. Hep G2 cell line

Hep G2 (HB 8065) is a human hepatocellular carcinoma cell line obtained from American Type Culture Collection (ATCC). Cells are cultured in Dulbecco`s modified Eagle`s medium (DMEM) containing 10% fetal calf serum (FCS) and maintained in humidified incubator in 95% air and 5% CO_2 at 37 °C.

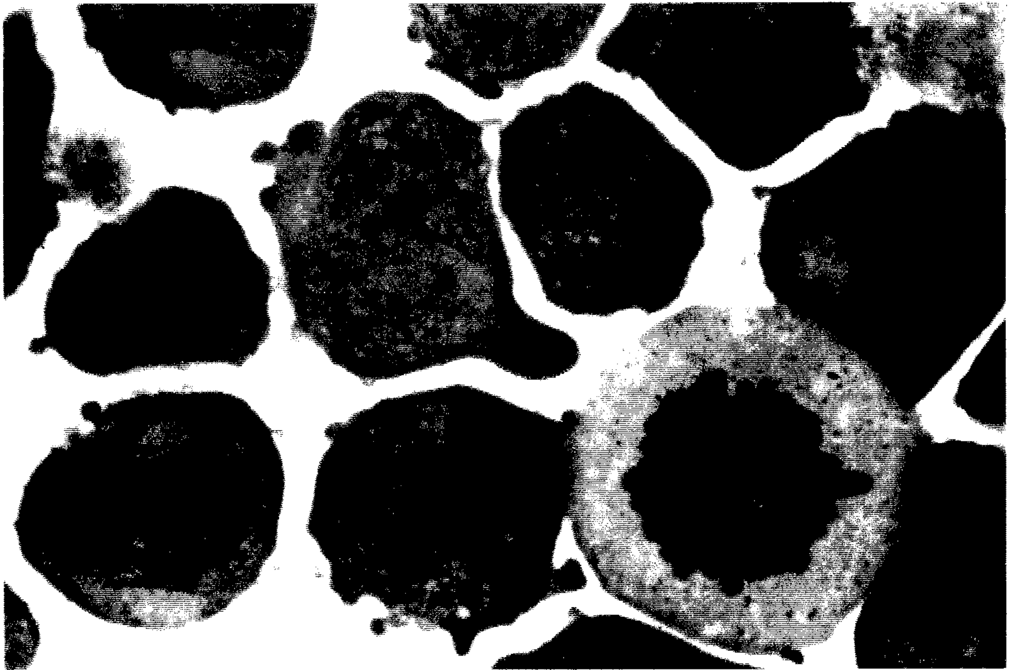

Fig. 27

Light microscopic visualization Sp2/0-Ag14 cells stained by the May Grünwald/Giemsa technique. The large cell is in the mitotic stage showing the intensive reproduction.

4.1.2. Toxicological studies

4.1.2.1. Ethanol

10^5 Sp2/0-Ag14 and Hep G2 cells were incubated with 1, 5, 10, 15 and 20% of ethanol (EtOH) (Reanal, Budapest, Hungary) diluted in DMEM + 10% FCS for 5 min. After this treatment cells were washed out and resuspended in the culture medium. Viability was assessed by Trypan Blue exclusion test at 5 min, 60 min, 4 hr and 24 hr after EtOH treatment.

4.1.2.2. Pyrazole

Pyrazole is an alcohol dehydrogenase enzyme inhibitor compound (Goldberg et Rydberg, 1969). During 1, 5, 10, 15 and 20 % EtOH treatment and 24 hr incubation 10^5 Sp2/0-Ag14 and Hep G2 cells were coincubated with 10^{-5} M pyrazole (Sigma Chemical Co., St. Louis, USA). Viability was determined by Trypan Blue exclusion test at 5 min, 60 min, 4 hr and 24 hr after EtOH treatment.

4.1.2.3. Indomethacin

Sp2/0-Ag14 cells were incubated with 10^{-8}-10^{-3} M doses of indomethacin (IND) (Chinoin, Hungary) for 5 min alone or in combination with 15% EtOH. Cell viability was determined by TB exclusion test immediately after the treatment.

4.1.3. Determination of free radicals by electron spin resonance (ESR)-spin trapping method

ESR measurements were carried out on a Bruker ESP300E spectrometer operating at X-band and equipped with 100 kHz field modulation. Free radicals were generated by a Fenton-type system consisting of ferrous complexes and hydrogen peroxide, using alpha-phenyl-N-tert-butylnitrone (PBN) as the spin trap. Spin trapping experiments were performed as follows: 150 mM PBN in water and untreated or EtOH-treated Sp2/0-Ag14 cell suspension (10^7/ml in DMEM without FCS) were mixed thoroughly. After addition of iron complexes (1 mM $FeCl_2$ in 0.0012 n HCl) and H_2O_2 (0.3 % stock solution, 8.8 mM), the sample was immediately injected into an ESR flat quartz cell. Typical time between mixing the reagents and the beginning of ESR signal acquisition was 1 min. Spectra were

recorded at room temperature using 10 mW microwave power and 0.143 mT modulation amplitude. Measurements were carried out at 5, 60, 120, 180 and 240 min after the 5 min EtOH-treatment. The presence of the spin adduct was detected by integrated spectra.

4.1.4. Cytoprotective investigations

4.1.4.1. Scavenger compounds

10^5 Sp2/0-Ag14 cells were incubated with 15 % EtOH for 5 min, then cells were washed out. During and after EtOH incubation cells were coincubated with the following agents for 24 hr:

10^{-4}-10^{-3} M glutathione (GSH) (Sigma Chemical Co., St. Louis, USA)
10^{-4}-10^{-3} M D-mannitol (Sigma Chemical Co., St. Louis, USA)
10^{-3} M dimethyl sulfoxide (DMSO) (Reanal, Budapest, Hungary)
10^{-4}-10^{-3} M 5,5-dimethyl-1-pyrroline N-oxide (DMPO) (Sigma Chemical Co., St. Louis, USA)

4.1.4.2. PL-10 substances

Sp2/0-Ag14 cells were incubated by 5-50-500 ng/ml-5 µg/ml doses of four PL-10 substances (PL-10.1.AK15-2, PL-10.1.AK15-3, PL-10.1.AK15-4, PL-10.1.AK14-2, for further information see chapter 3.1.4.4.) for 60 min, after it cells were treated by 15% EtOH for 5 min. Cell viability was determined by TB exclusion test and SDH activity immediately after EtOH treatment.

4.1.5. Determination of cell viability

4.1.5.1. Trypan Blue exclusion test

See Chapter 3.1.2.1.

4.1.5.2. Succinic dehydrogenase assay

See Chapter 3.1.2.2.2.

4.1.5.3. Lactate dehydrogenase assay

See Chapter 3.1.2.2.1.

4.1.6. Statistics

Values in figures and text are expressed as means + SEM. Comparisons were performed by unpaired Student`s t test. P values were considered significant at $p<0.05$.

4.2. RESULTS

4.2.1. Toxicological studies

4.2.1.1. Direct cellular effect of ethanol on Sp2/0-Ag14 cells

1, 5, 10, 15 and 20% EtOH concentration-dependently decreased the viability of Sp2/0-Ag14 cells (Fig. 28). There was no essential difference in cell viability between 5 and 60 min, while in 4 hr after EtOH treatment the cell destruction was increased at 15 % EtOH concentration. At 24 hr an expressive decrease began in the cell viability, which was significant at 15 % EtOH concentration ($p<0.05$ vs. control).

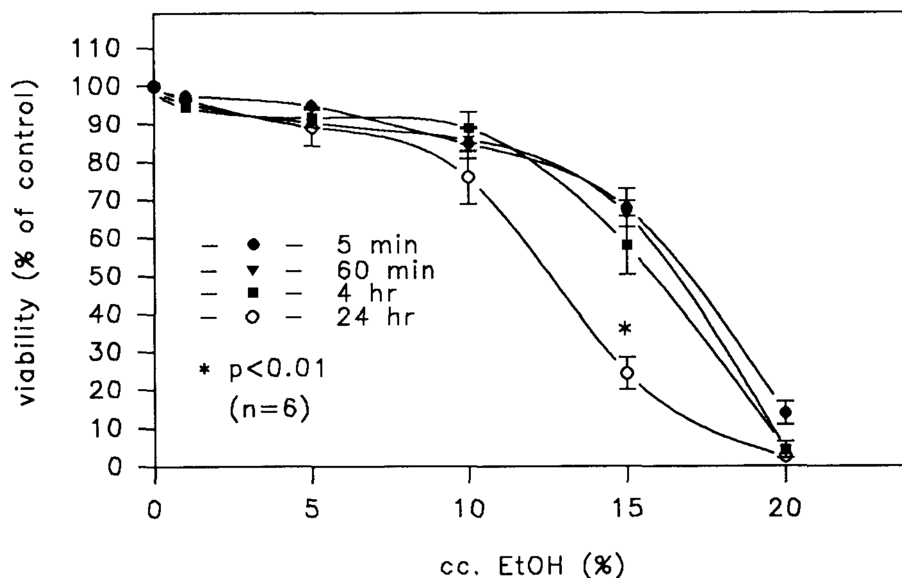

Fig. 28

Effect of 5-min ethanol (EtOH) incubation on Sp2/0-Ag14 cells. Viability was detected by Trypan Blue exclusion test at 5, 60 min, 4 and 24 hr after EtOH exposure. *: $p<0.05$ vs. *cell viability at the time of 5 min.*

4.2.1.2. Direct cellular effect of ethanol on Hep G2 cells

1, 5, 10 and 15% of EtOH concentration-dependently, but very slightly decreased the cell viability at the time of 5 min, 60 min, 4 hr and 24 hr. There was a great fall in the viability between 15 and 20% EtOH at all counting time (Fig. 29). Secondary cell destruction could not be seen in Hep G2 cells.

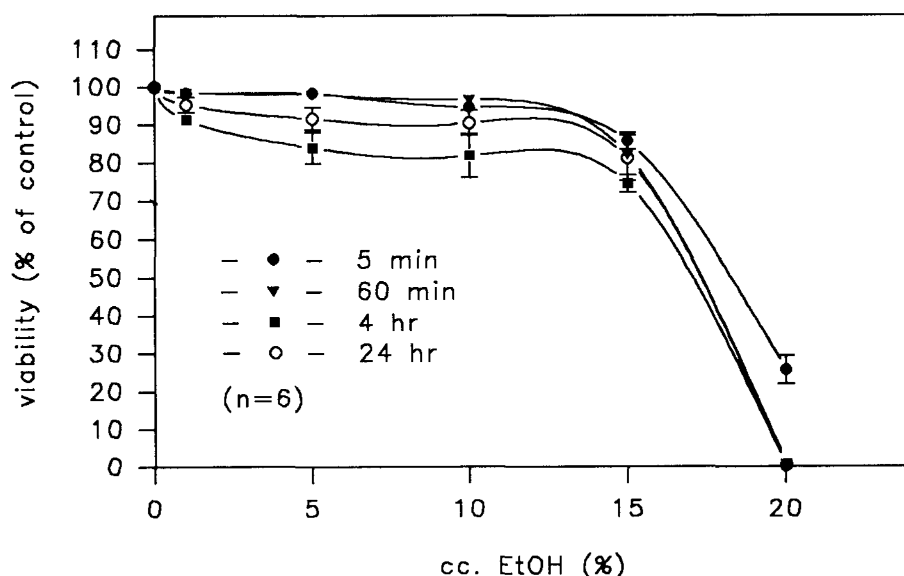

Fig. 29

Effect of 5-min ethanol (EtOH) incubation on Hep G2 cells. Viability was detected by Trypan Blue exclusion test at 5, 60 min, 4 and 24 hr after EtOH exposure.

4.2.1.3. Effect of pyrazole on EtOH-induced cellular damage in Sp2/0-Ag14 and Hep G2 cells

10^{-5} M pyrazole was not toxic for both types of cells. In Sp2/0-Ag14 cells there was no difference in the viability between 1, 5, 10, 15 and 20 % EtOH coincubated with 10^{-5} M pyrazole and only EtOH-treated cells (Fig. 30). The significant secondary cell destruction also could be seen in this model ($p<0.05$).

In Hep G2 cells 10^{-5} M pyrazole aggravated the EtOH-induced cellular damage, the resistance against EtOH disappeared. At 10% EtOH a significant decrease was detectable in 24 hr after EtOH exposure, which show the same tendency like Sp2/0-Ag14 cells (Fig. 31).

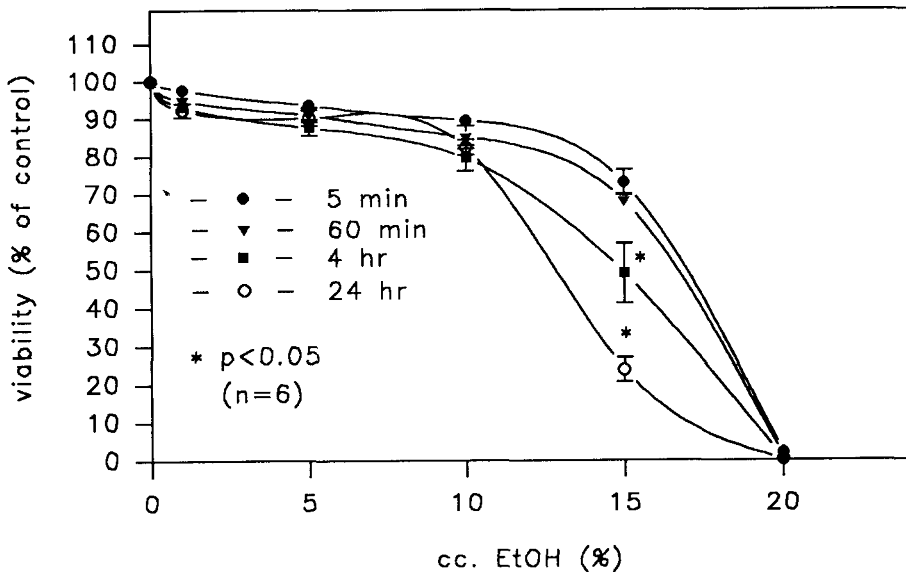

Fig. 30

Effect of pyrazole on Sp2/0-Ag14 cells. After 5 min ethanol (EtOH) incubation cell viability was detected by Trypan Blue exclusion test at 5, 60 min, 4 and 24 hr. During and after EtOH exposure cells were coincubated with 10^{-5} M pyrazole. *: $p<0.05$ vs. cell viability at the time of 5 min.

4.2.1.4. Effect of indomethacin on Sp2/0-Ag14 cells

10^{-8}-10^{-3}M IND alone had no effect on the viability of Sp2/0-Ag14 cells. (Fig. 32).

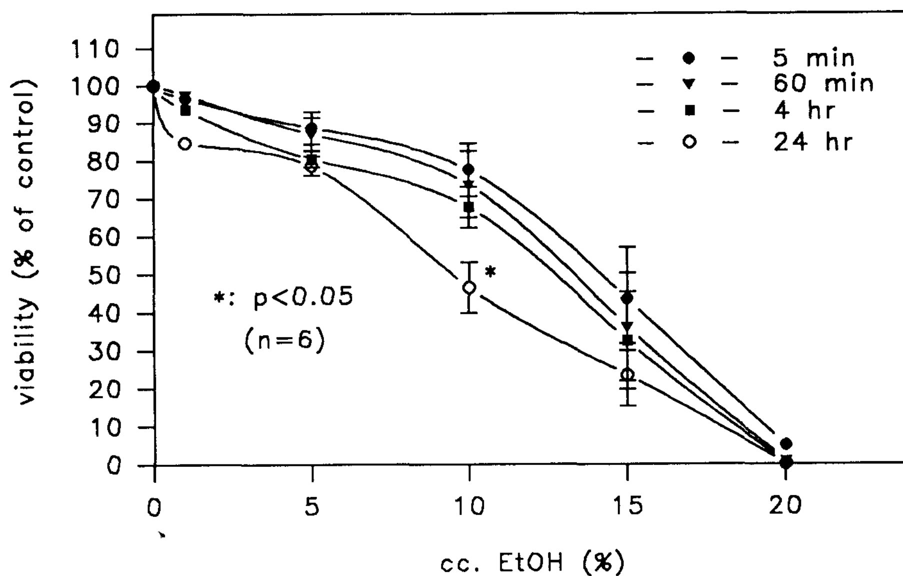

Fig. 31

Effect of pyrazole on Hep G2 cells. After 5 min ethanol (EtOH) incubation cell viability was detected by Trypan Blue exclusion test at 5, 60 min, 4 and 24 hr. During and after EtOH exposure cells were coincubated with 10^{-5} M pyrazole. *: $p<0.05$ vs. cell viability at the time of 5 min.

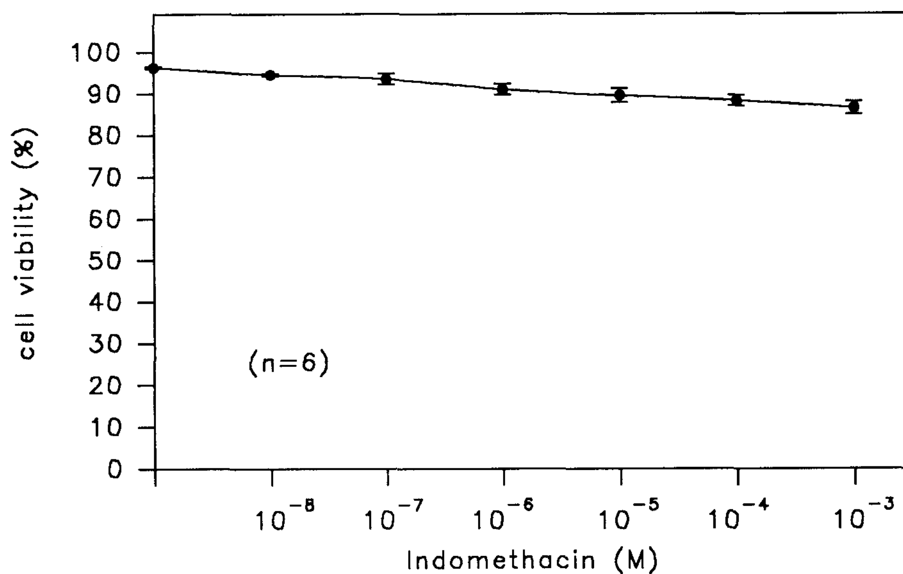

Fig. 32

Changes in the viability of Sp2/0-Ag14 cells after 5 min incubation of 10^{-8} - 10^{-3} M indomethacin (IND) detected by Trypan Blue exclusion test.

4.2.1.5. Combined effect of ethanol and indomethacin on Sp2/0-Ag14 cells

Using combined these toxic agents 10^{-3} M IND significantly aggravated the 15% EtOH-induced cell injury detected by Trypan Blue exclusion test, SDH and LDH activity (Figs. 33, 34).

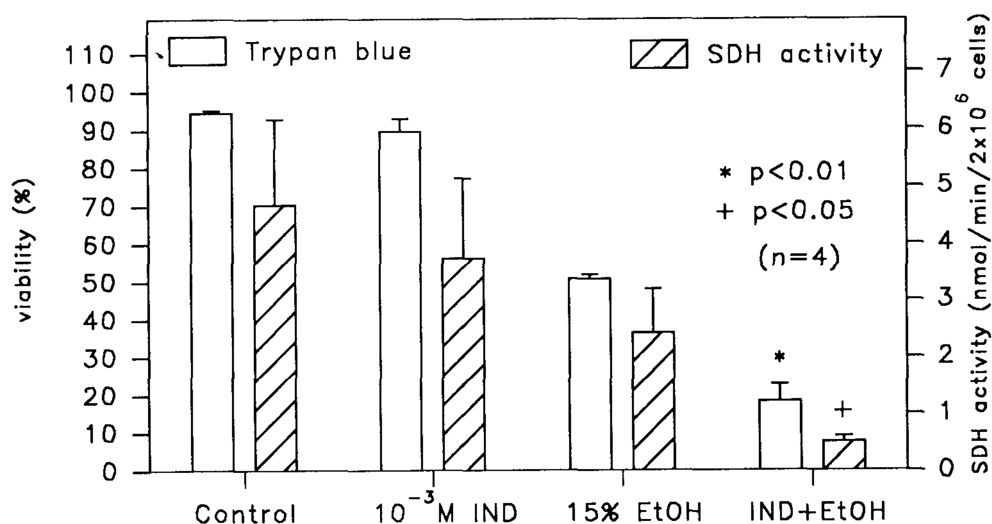

Fig. 33

Changes in the viability of Sp2/0-Ag14 cells after 5 min incubation of 15% (v/v) ethanol (EtOH) and 10^{-3} M indomethacin (IND) detected by Trypan Blue exclusion test and SDH activity. *: $p<0.01$, +: $p<0.05$ compared with 15% EtOH treatment.

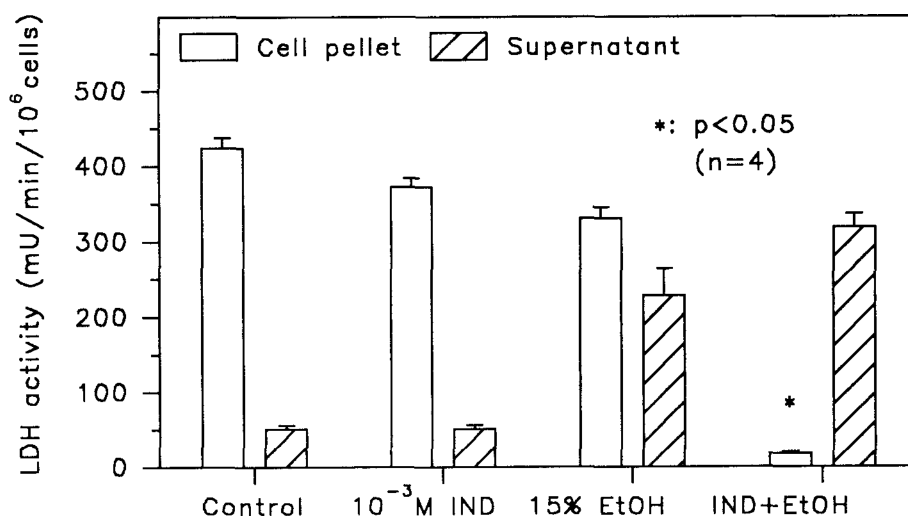

Fig. 34.

Lactate dehydrogenase activity in the cell pellet and supernatant of 10^6 Sp2/0-Ag14 cells after 5 min incubation of 15% (v/v) ethanol (EtOH) and 10^{-3} M indomethacin (IND). *: $p<0.05$ compared with 15% EtOH treatment.

4.2.2. Free radical activity in ethanol-induced cellular damage in Sp2/0-Ag14 cells

After EtOH-treatment the free radical activity was increased compared with untreated cells detected by ESR-spin trapping method (Fig. 35).

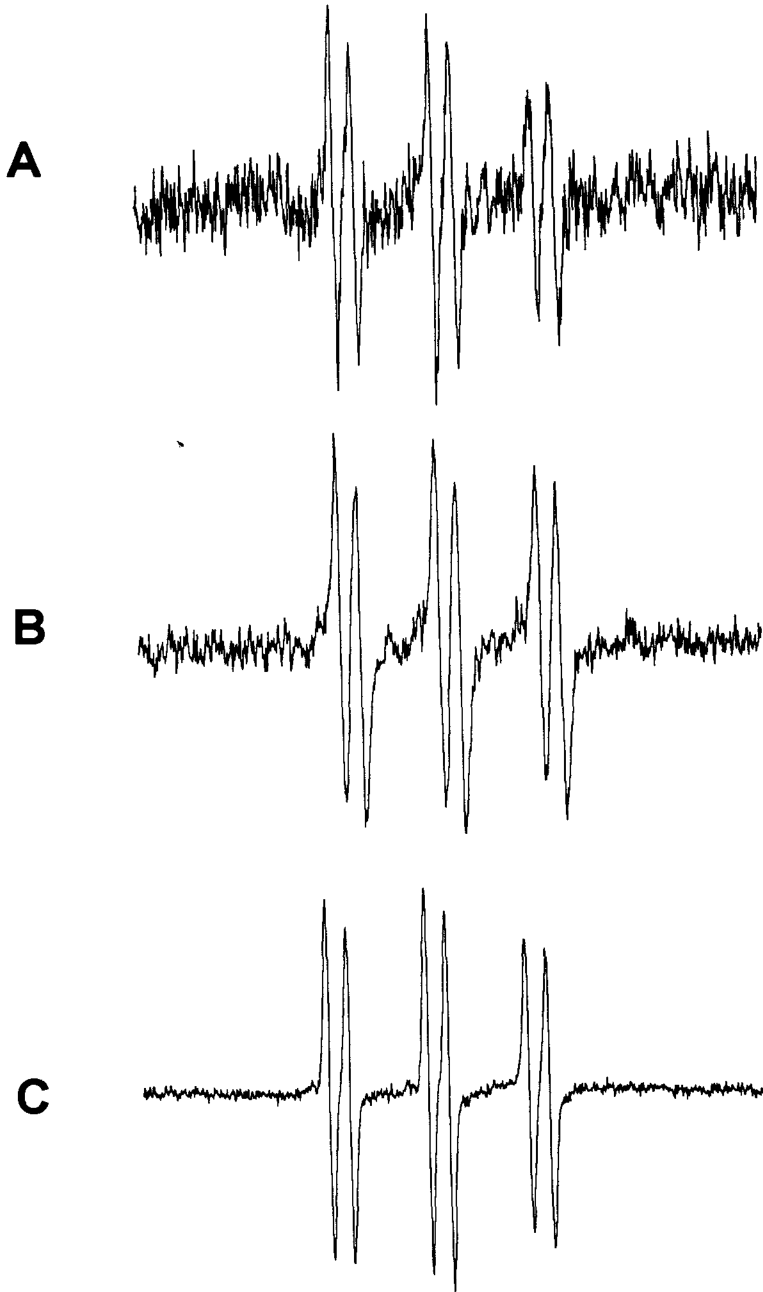

Fig. 35

ESR spectra recorded 5 min after 15% ethanol (EtOH) exposure. (A) EtOH treated cells plus PBN in buffer solution. (B) Untreated cells plus PBN in buffer solution. (C) Buffer solution plus PBN.

The values of corrected double integral represent the following changes (Fig. 36):

a) The free radical activity showed a saturation curve both in untreated and EtOH-treated cells;

b) The EtOH treatment induced higher free radical activity during the measurement;

c) The free radical activity was increased immediately after the EtOH treatment.

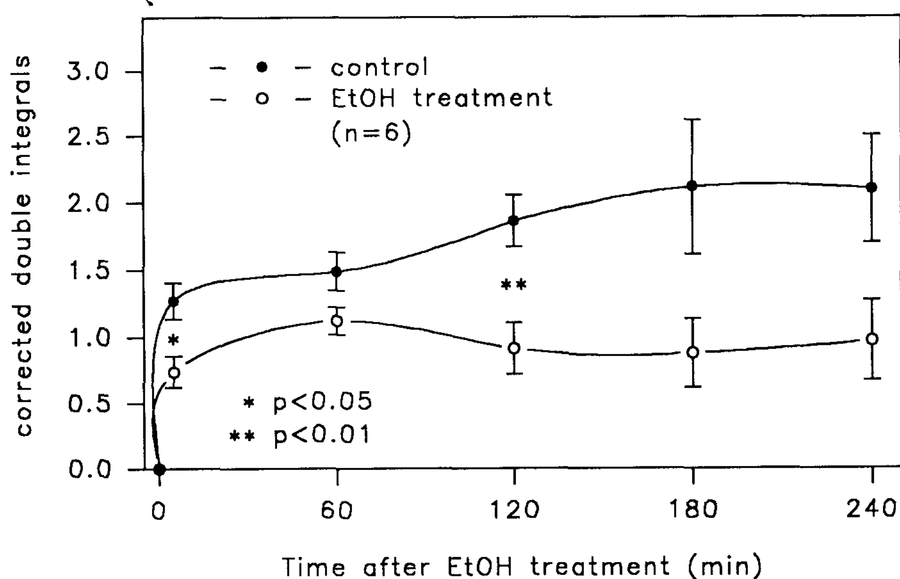

Fig. 36

Curves representing the values of corrected double integral in control and ethanol (EtOH)-treated cells. After 5 min exposure of EtOH free radical activity was detected by ESR-spin trapping method at 5, 60, 120, 180 and 240 min.

4.2.3. Cytoprotective investigations

4.2.3.1. Cytoprotection induced by scavenger compounds

10^{-3} M DMPO and GSH significantly prevented the cells against 15% EtOH injury at the time of 4 hr ($p<0.05$), but this protection disappeared at 24 hr (Figs. 37, 38). Coincubation of Sp2/0-Ag14 cells with 10^{-3} M DMSO significantly prevented the 15% EtOH-induced secondary cell destruction only at the time of 24 hr ($p<0.01$). (Fig. 39). 10^{-3} M D-mannitol was not protective in this model. None of the examined compounds was toxic for the cells.

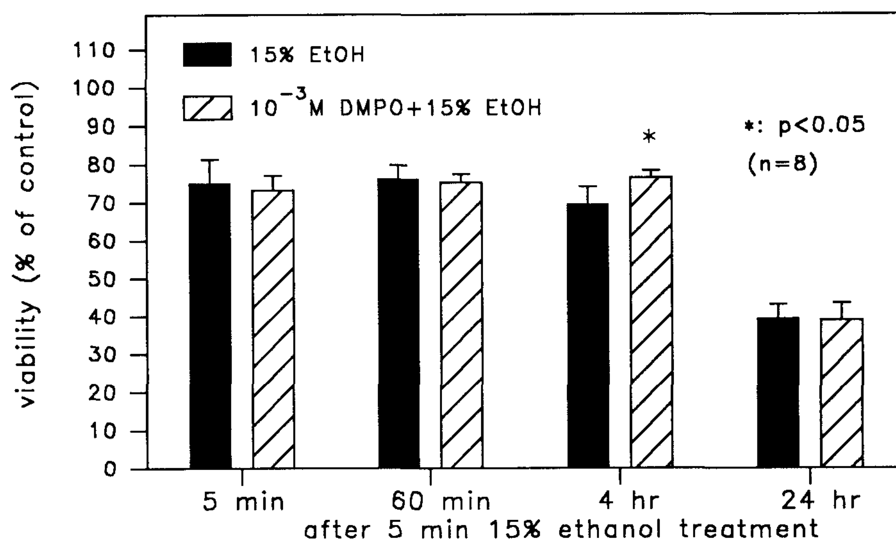

Fig. 37

Effect of 5,5-dimethyl-1-pyrroline N-oxide (DMPO) on Sp2/0-Ag14 cells. During and after 5 min exposure of 15 % ethanol (EtOH) cells were coincubated with 10^{-3} M DMPO, cell viability was detected by Trypan Blue exclusion test at 5, 60 min, 4 and 24 hr. * : $p<0.05$ compared with EtOH-treated cells.

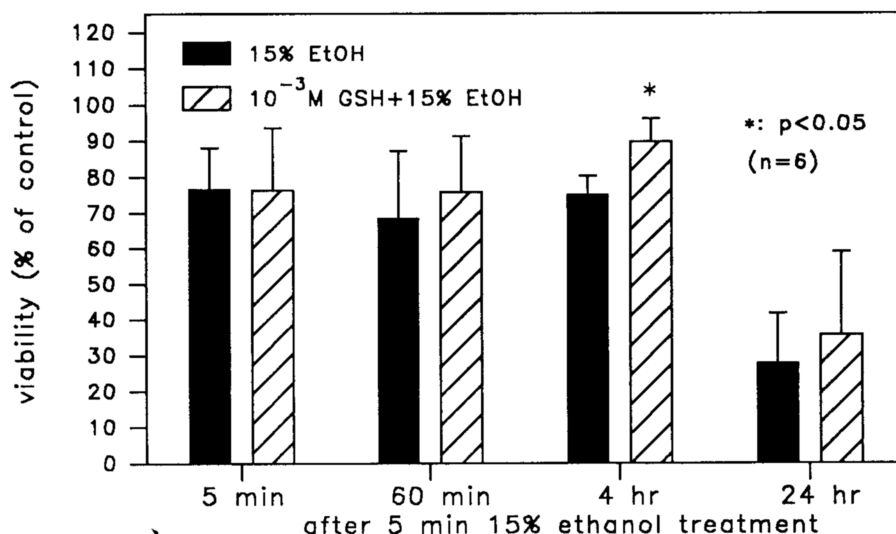

Fig. 38

Effect of glutathione (GSH) on Sp2/0-Ag14 cells. During and after 5 min exposure of 15 % ethanol (EtOH) cells were coincubated with 10^{-3} M GSH, cell viability was detected by Trypan Blue exclusion test at 5, 60 min, 4 and 24 hr. * : $p<0.05$ compared with EtOH-treated cells.

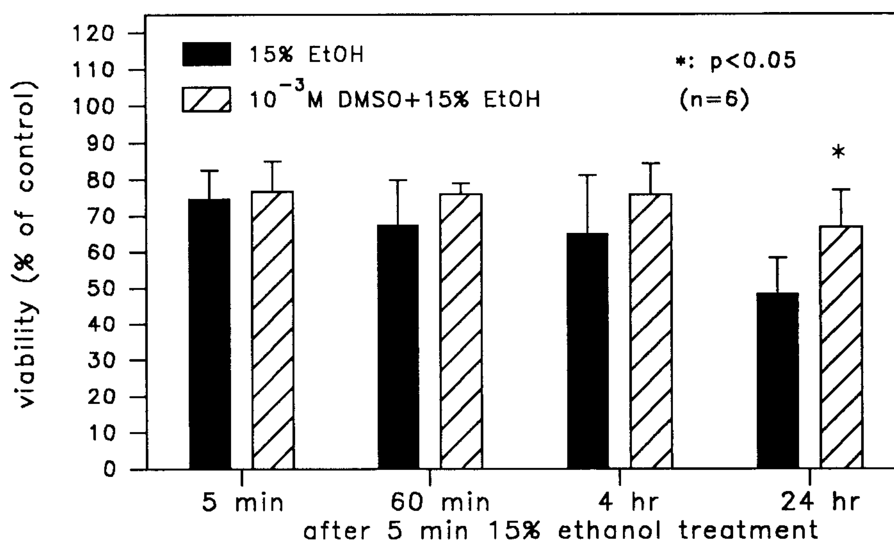

Fig. 39

Effect of dimethyl sulfoxide (DMSO) on Sp2/0-Ag14 cells. During and after 5 min exposure of 15 % ethanol (EtOH) cells were coincubated with 10^{-3} M DMSO, cell viability was detected by Trypan Blue exclusion test at 5, 60 min, 4 and 24 hr. * : $p<0.01$ compared with EtOH-treated cells.

4.2.3.2. Direct cytoprotective investigations by PL-10 substances

4.2.3.2.1. PL 10.1.AK15-4

60 min incubation of this substance with cultured mouse myeloma cells (Sp2/0-Ag14) did not aggravated the EtOH-induced cell injury, however, we could not observed any protective effect against 15% and 20% EtOH tested by Trypan Blue exclusion test (Fig. 40).

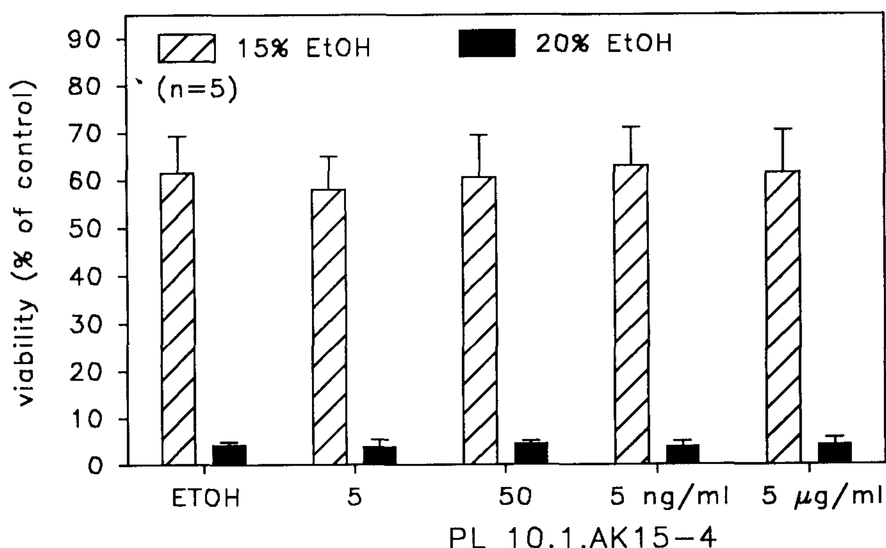

Fig. 40

Changes in the viability of Sp2/0-Ag14 cells after 60 min incubation of PL 10.1.AK15-4 and 5 min 15% and 20% ethanol (EtOH) treatment.

4.2.3.2.2. PL 10.1.15AK-3 and PL 10.1.AK15-2

After 60 min incubation of these substances we could not observe any protective tendency against 5 min 15% EtOH treatment, and there were no changes in SDH activity (Fig. 41).

4.2.3.2.3. PL 10.1.AK14-2

We could not detect any cytoprotection after 60 min incubation of PL 10.1.AK14-2 against 15% EtOH in Sp2/0-Ag14 cells by Trypan Blue exclusion test and SDH activity (Fig. 42).

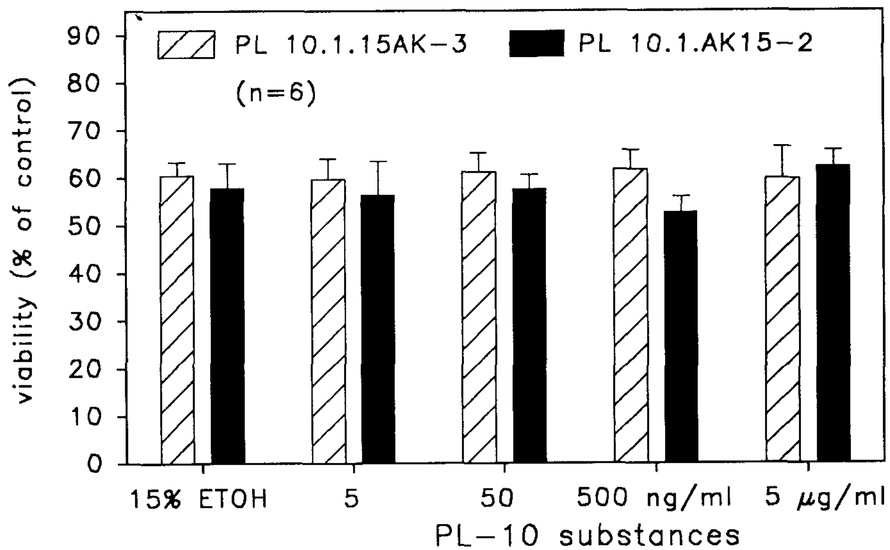

Fig. 41

Effect of 60 min incubation of PL 10.1.15AK-3 and PL 10.1.AK15-2 against 15% ethanol (EtOH) on the viability of Sp2/0-Ag14 cells detected by Trypan Blue exclusion test.

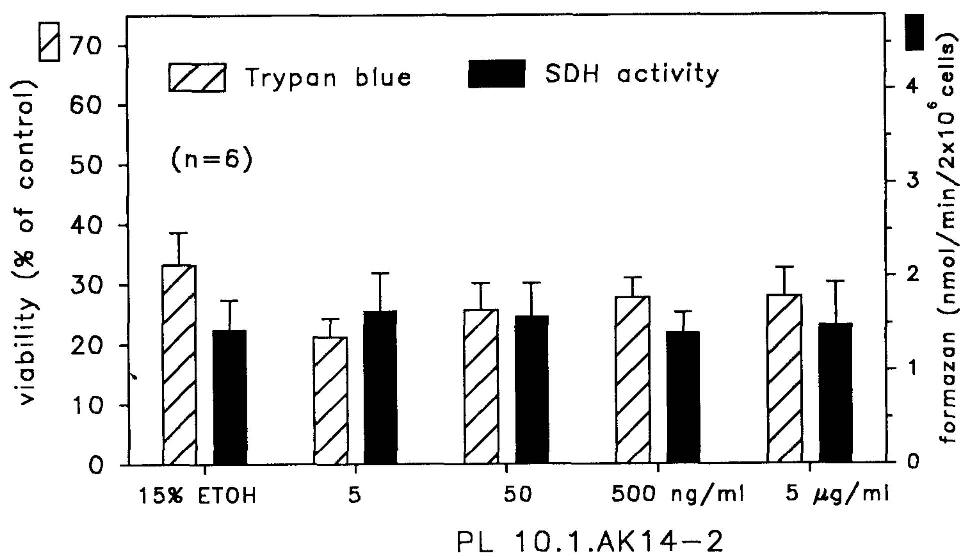

Fig. 42

Changes in the viability detected by Trypan Blue exclusion test and SDH activity of mouse myeloma cells (Sp2/0-Ag14) after 60 min incubation of PL 10.1.AK14-2 against 5 min 15% ethanol (EtOH) treatment.

5. DISCUSSION

The aim of this study was to evaluate the in vitro cytoprotective effect of some aggressive factors and in vivo organoprotective agents. These compounds were tested on short term (freshly isolated rat gastric mucosal cells) and long term (Sp2-0/Ag14 and Hep G2 cell line) cultures.

IN ORDER OF THE AIMS IT WAS FOUND THAT:

1) Direct short term and long term cellular effect of ethanol

Ethanol (EtOH) was applied as 5 min incubation on both cell culture models. EtOH concentration-dependently decreased the cell viability, but these results indicate that acutely isolated cells are more vulnerable than cultured cells. After EtOH treatment in the case of Sp2/0-Ag14 cells EC_{50} was higher (16%) than in the case of GMC (13.5%). However, there are differences between the cell lines on dependent of their types: human hepatoma cell line (Hep G2) is more resistance against ethanol probably owing to its metabolic activity.

Immediately after 5 min incubation only the acute direct effect can be detected which is probably due to the lipide solvent property of EtOH. By the help of a long term cell culture model it is possible to analyze the later consequences of a relatively short-term EtOH treatment.

It was found that in 4 and 24 hr after EtOH treatment an unexpected secondary cell destruction could be detected at higher EtOH concentrations. Considering that this phenomenon manifested relatively later after EtOH treatment, it can be regarded as an oxygen free radicals mediated process. ESR-spin trapping detected an increased free radical activity in the cells after EtOH treatment.

Regarding to these results oxygen free radicals play an important role in the long-term cell injury. This result is in agree with Cho et al. who observed an increased catalase activity 1 hr after EtOH treatment in vivo (Cho et al., 1992). They concluded that this high tissue level of catalase can prevent the oxygen free radicals mediated damage. We also could not detect any macroscopic (detected by trypan blue exclusion test) cell injury besides the direct effect of EtOH in the early phases, but our ESR study showed that the free radical activity in EtOH-treated cells increased immediately after EtOH exposure. The secondary cell destruction began relatively later (in 4 and mostly 24 hr), it seems that in the cells this time is necessary for the development of macroscopic damage.

2) Direct cellular effect of indomethacin without or with application of other aggressive factors

Indomethacin (IND) had no damaging effect on stable cultured cells, but its higher doses decreased the viability of isolated cells. The combined treatment with 15% EtOH reduced the viability in both types of cells, but this effect was much milder in Sp2/0-Ag14 studies, while all the GMC were almost destroyed. These results also indicate that cultured cells are more resistant against toxic agents than acutely isolated cells. Though different types of cells were used in this study, nevertheless the differences in their behavior derive rather from the isolation procedure than their types.

In vivo experiments gastrointestinal ulceration can be produced by IND administration (Djahanguiri, 1969; Karádi et al., 1994). IND inhibits the activity of cyclo-oxygenase producing less amount of prostaglandins (PGs) and excessive vasoconstrictor leukotrienes (Rainsford, 1992). On the other hand, IND decreases the mucosal level of adenosine triphosphate (ATP) (Rainsford, 1987), so these factors reduce the gastric mucosal resistance against gastric acid. In our in vitro

study when IND was applied without other aggressive factor such as EtOH, it was not toxic for the cells. But after combined treatment (IND plus EtOH) cell viability was considerably decreased compared to the effect of EtOH. Probably the decrease of the level of endogenous PGs might have a role in the enhanced toxic effect of EtOH. This result is in agreement with Tarnawski et al. (Tarnawski et al., 1988) who observed a protective effect of exogenous PGs on human isolated gastric glands against IND and EtOH injury.

3) Direct cellular effect of the homogenate of human pathogen Helicobacter pylori

In this study the direct cellular effect of sonicated H. pylori was examined on mixed population of freshly isolated rat gastric mucosal cells without and with other aggressive factors (EtOH and IND). This model is suitable to eliminate the additional effect of immune system, which is naturally involved in a bacterial infection. Due to sonication the possible toxic agents (e.g. toxins, unusual fatty acids, proteases) originated from destruction of bacteria can reach the cells in experimental circumstances.

It was found that sonicated H. pylori alone, or in combination with EtOH or IND did not have any direct cellular injury in freshly isolated rat gastric mucosal cells (Bódis et al., 1996b).

What can be the background of this result? There are a lot of data showing the very important role of tissue reactions (e.g. immune system) in the pathogenesis of H. pylori induced diseases. The bacterial supernatant contains novel chemotactic factors, mast cells are involved in immune mechanisms because of IgE response (Aceti et al., 1991). It is possible that IgE-mediated type I hypersensitivity reactions play a role in the production of gastric mucosal damage due to release of mast cell mediators (e.g. TNF, leukotrienes, PAF). Neutrophil activation is also involved in H. pylori infection: isolated surface proteins and

chemotactic factors from bacterial supernatant can induce it. The produced oxygen radicals can be toxic for gastrointestinal mucosa (Chadwick, 1994). Whether these immune responses are primary or consequent to the development of gastritis remains to be established.

One of the other possible explanations is that H. pylori can be a facultative microorganism. Regarding to the very high infectious rate in the population, this bacterium might be only simply an opportunist. However, in the presence of a mucosal lesion it might be a pathogenic factor for gastrointestinal diseases.

4) The prostacyclin-induced direct cytoprotection against ethanol- and indomethacin injury

In this study the in vitro cytoprotective effect of prostacyclin (PGI_2) was analyzed on mixed population of freshly isolated rat GMCs. After preincubation of 10^{-6}-10^{-4} M PGI_2 it could not be found any cytoprotective effect against EtOH injury, but there was a significant cell protection after IND plus EtOH treatment detected by Trypan Blue exclusion test (Bódis et al., 1995).

It is known that PGI_2 has a good cytoprotective effect in vivo against EtOH-induced gastric mucosal damage, and this effect disappears after acute bilateral surgical vagotomy (Mózsik et al., 1991a). The cytoprotective effect of PGI_2 manifests in the early phase after administration of the necrotizing agent (Sütő et al., 1989), according to Szabo et al. this is the phase of the early vascular and mucosal injury (Szabo et al., 1985). Regarding to these facts it is not surprise that without these protective factors (vagal nerve and blood flow) PGI_2 has no direct cytoprotective effect against EtOH-induced cellular damage.

However, exogenous PGI_2 was able to significantly reduce in vitro the aggravating effect of IND. In animal experimental studies PGI_2 was also found to be cytoprotective against IND-induced mucosal injury (Garamszegi et al., 1984).

The main action of IND is inhibiting cyclooxigenase enzyme activity and by this way decreasing the synthesis of prostaglandins (Vane, 1971). It seems that exogenous PGI_2 also has cytoprotective effect against IND at cellular level.

5) Direct cellular effect of histamine and pentagastrin

In this study the cytoprotective effects of histamine and pentagastrin were analyzed on isolated rat gastric mucosal cells.

These results indicated that:

1) Histamine alone did not influence the viability of the cells, but after coincubation with PGI_2 it significantly increased the 1 hr-survival time detected by SDH activity without any aggressive factor;
2) Histamine pretreatment significantly aggravated the 15% EtOH-induced cell injury;
3) Histamine combined with PGI_2 also significantly aggravated the EtOH-induced and EtOH+IND-induced cellular damage detected by Trypan Blue exclusion test, but this significant changes disappeared after 1 hr detected by SDH activity (Bódis et al., 1996a).

It is known that surprisingly both gastric acid secretion inhibiting and stimulating drugs can prevent the EtOH-induced mucosal damage in vivo (Mózsik et al., 1988). Furthermore, it was proved that the intact vagus is basically necessary for the receiving gastric cyto- and mucosal protection by atropine, cimetidine, PGI_2 and β-carotene (Mózsik et al., 1995). The inhibitory drugs such as cimetidine and atropine decrease the cell metabolism, while pentagastrin, histamine and 2,4-dinitrophenol (for a short time) increase it. The PGI_2-induced gastric cytoprotection is decreased by gastric acid inhibitory drugs, however, histamine, pentagastrin and 2,4-dinitrophenol can stimulate it. This effect partly can be explained by acting at the level of receptors due to reduction of ATP-ADP transformation and increasing the cellular cAMP content (in the case of histamine

and pentagastrin) (Mózsik et al., 1988), but the mechanism of 2,4-dinitrophenol is quite different (inhibition the respiratory chain). With this knowledge of the facts metabolic components have to play a role in the protection of gastric mucosal damage.

In vitro circumstances we could not detect any cytoprotective effect of histamine alone or in combination with PGI_2. However, the preincubation of these drugs increased the survival of the cells and could reduce the long-term cell injury. These results show that histamine alone or combined with PGI_2 might stimulate the metabolism of isolated cells which can be detected by SDH activity, but this effect is not enough for the development of cytoprotection (other in vivo factors e.g. vagal nerve are also necessary).

Regarding to these in vivo and in vitro studies the organo- and cytoprotective effect of histamine seems to be different in rats.

The possible cytoprotective effect of pentagastrin was tested on freshly isolated rat gastric mucosal cells.

It was found that:

1) pentagastrin preincubation significantly aggravated the 15% EtOH-induced cell injury;
2) After PGI_2 and pentagastrin treatment also could not been observed any cytoprotective effect against EtOH-induced cellular damage, but the significant aggravating effect of pentagastrin is disappeared (Bódis et al., 1997c).

In vivo pentagastrin has the same cytoprotective and biochemical effect like histamine. These in vitro results also showed similar tendency like histamine, it seems that the background of the effect of these two aggressive factors is based on the same physiological and biochemical processes.

6) Cytoprotection induced by PL-10 substances (synthesized parts of BPC)

PL-10 substances are the synthesized variants of the originally described pentadecapeptide BPC 157 (Sikiric et al., 1993a). In this study the in vitro effect of PL-10 substances was evaluated on freshly isolated rat gastric mucosal cells and on cultured mouse myeloma cell line (Sp2/0-Ag14). After 60 min incubation of these substances (5-50-500 ng/ml-5 μg/ml) we treated the cells by ethanol (15%) to detect the direct cytoprotective effect.

It was found that:

1. PL-10 substances were not toxic for freshly isolated rat GMCs and mouse myeloma cells;
2. PL 10.1.AK14-2 showed a dose-dependent and very significant ($p<0.01$ and $p<0.02$) direct cytoprotective effect in doses of 50 - 500 ng/ml - 5 μg/ml on freshly isolated rat gastric mucosal cells. ED_{50}=50 ng/ml, r=0.9982;
3. PL 10.1.15AK-3 was significantly ($p<0.05$) cytoprotective in dose of 5 μg/ml on freshly isolated rat gastric mucosal cells;
4. These substances were not effective on cultured mouse myeloma cells.

These results show that PL-10.1.15AK-3 and PL 10.1.AK14-2 substances can protect the freshly isolated rat gastric mucosal cells against ethanol injury. This direct cytoprotective effect is stronger and dose-dependent in the case of PL 10.1.AK14-2 substance (ED_{50}=50 ng/ml). This effect is very quick after exposure of the necrotizing agent, because it could not been observed by SDH activity - the duration of this measurement is 1 hr, indirectly it shows the survival of the cells, while Trypan Blue exclusion test reflects the viability immediately after the treatment (Bódis et al., 1997d).

Because it could not been observed any cytoprotection on cultured mouse myeloma cells, it seems that this cellular protection is a specific action for healthy gastrointestinal cells.

Although the exact mechanism of the BPC-induced huge range organoprotection is yet unknown, it seems that systematic processes (e.g. hormonal system (Sikiric et al., 1992) and Selye's general-adaptive-syndrome (Sikiric et al., 1993a) play an important role in the development of this phenomenon. The fact that in vivo BPC 157 is also effective in the rat stomach when given simultaneously with ethanol (Mózsik et al., 1991b) shows a very strong organoprotective and a direct cellular protection. The newly synthesized PL-10 substances (originated from BPC) also show this organoprotection in animal experiments (Erceg et al., 1997), and two of them proved to be cytoprotective in this in vitro study. By this way this study supported the hypothesis of the existence of direct cellular protection and it gave further evidence for the exact mechanism of the BPC-induced beneficial effects.

7) The effect of scavengers in long term cell injury

The effect of scavengers (DMPO, GSH, DMSO and D-mannitol) was analyzed on the free radical-induced secondary cell destruction (detected by ESR-spin trapping) on Sp2/0-Ag14 cell line. The most important in vivo agent beta-carotene cannot be applied in vitro circumstances because of its lipid solubility property.

It was found that GSH and DMPO could prevent the free radicals-induced cell destruction at 4 hr, DMSO was found to be cytoprotective in 24 hr after EtOH exposure, while D-mannitol was ineffective in this model (Bódis et al., 1997a).

Based on these ESR studies, the free radical activity increased immediately after EtOH administration, but it manifested in the decrease of cell viability relatively later, in 4 and 24 hr. GSH and DMPO are not stable molecules in biological systems, their scavenger effects manifested significantly only in the first period. DMSO is a generally used cellular protective agent (e.g. preservation of

the cells by freeze), in addition it is a good hydroxyl radical scavenger (Del Maestro et al., 1980). The major oxygen radical responsible for the EtOH-induced cellular damage is the hydroxyl radical (Mutoh et al., 1990). Previously GSH and DMSO were found to be protective in EtOH-induced cellular damage but immediately after the necrotizing agent (Mutoh et al., 1989). This study gave further information about the dynamics of free radical activity after EtOH injury.

6. CONCLUSIONS

THE NEW RESULTS OF THIS DISSERTATION ARE:

- It was proved by the help of long term cell cultures that there are two main factors in the direct cellular effect of ethanol: a chemical action and an ethanol-induced free radical activity causing secondary cell destruction. In addition, an ESR-spin trapping study was performed in the cell suspension;

- Indomethacin without other aggressive factor do not induce cellular damage, but in the presence of ethanol it aggravates the cell destruction;

- Human pathogen Helicobacter pylori do not have strictly direct cytotoxic effect, in addition, it do not aggravates the other aggressive factor-induced cellular damage;

- The organoprotection induced by prostacyclin have no direct cytoprotective component;

- The histamine- and pentagastrin-induced organoprotection is not presented at cellular level;

- It was proved that in the general beneficial effect of BPC a direct cytoprotection plays an important role;

- A part of the effect of scavengers is a direct cytoprotection, which can be manifested in different time after ethanol exposure on dependence of their chemical structure.

According to these results only a few in vivo "cytoprotective" compounds have direct cellular protection. Most of these agents have other systematic (e.g. vascular, neuronal and hormonal) effect, which influence the development of the protection. The mechanisms of organo- and cytoprotection differ from each other - direct cytoprotection takes only a part in the phenomenon of organoprotection.

Advantages of these methods for possible clinical perspectives

- By the help of these experimental methods the **complex physiological mechanisms** can be examined at cellular level.
- **The toxic effect** of different drugs can also be evaluated at the level of the cells.
- **The scavenger property** of further agents can be estimated in the long term cell culture models.
- These kinds of experiments (cell isolation and cell cultures) are **acceptable for animal experimental ethical requirements**.

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8. ACKNOWLEDGEMENTS

First of all I have to appreciate my tutor, Professor Gyula Mózsik, who has taken care of my scientific work since my university years. Without his help and inspiration this dissertation would never be written.

I also wish to thank to Professor Lajos Nagy for his method of cell isolation, which was the base of my experiments.

Professor Péter Németh from the Immunological and Biotechnological Department and Professor József Belágyi from the Central Research Laboratory helped me with their advises and suggestions, and supported the technical background for my studies in the fields of cell cultures and free radicals.

I would like to thank to my colleagues and Ph.D. fellows for the technical assistance, especially given by Margit Jermás and Rozália Nagy (cytoprotective investigations), Judit Melczer (cell cultures) and Éva Greiner (ESR studies).

My greatest appreciate to my family: my husband, my daughter and my parents for their patient, encouragement and supporting during the years of my experimental work.

9. LECTURES AND PUBLICATION

LECTURES AND POSTERS

1. **Bódis B**, Balaskó M, Csontos Zs, Garamszegi M, Karádi O, Király Á, Sütő G, Vincze Á, Jávor T, Mózsik Gy: A prosztaglandin E_2 és a prosztaciklin szintjének változása akut sebészi vagotómia hatására gyomorban. Magyar Gasztroenterológiai Társaság 32. Nagygyűlése, Balatonaliga, 1990. május 8-12.

2. **Bódis B**, Balaskó M, Csontos Zs, Karádi O, Király Á, Jávor T, Mózsik Gy: Changes of gastric mucosal prostaglandin contents in intact and vagotomized rats, without and with β-carotene treatment. Second International Symposium on Neurobiology of the Brain-Gut Axis: From Basic Science to Clinical Perspectives, Pécs, Hungary, July 13-14, 1990.

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5. **Bódis B**, Csere P, Karádi O, Nagy L, Mózsik Gy: Effect of ethanol, indomethacin and their combination on isolated gastric mucosal cells from the rats. 36th Annual Meeting of the Hungarian Society of the Gastroenterology, Balatonaliga, Hungary, May 24-28, 1994.

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7. **Bódis B**, Karádi O, Németh P, Mózsik Gy: Differences in the effect of indomethacin between HEP G2 and SP2 cell line. Second International Congress of the Worldwide Hungarian Medical Academy, Budapest, Hungary, August 25-27, 1994.

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44. Szabó I, **Bódis B**, Mózsik Gy, Németh P: Gyökfogók által indukált (preventív) hatások stabil (Sp2/0-Ag14 és Hep G2) sejtvonalakon. A fekélybetegség kutatásának 25 éve Magyarországon (1971-1995), Pécs, 1996. március 7-8.

45. Karádi O, **Bódis B**, Mózsik Gy: Akut és krónikus vagotómia és az indometacin-okozta gasztrointesztinális nyálkahártyakárosodás. A fekélybetegség kutatásának 25 éve Magyarországon (1971-1995), Pécs, 1996. március 7-8.

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51. Szabó I, **Bódis B**, Rumi Gy, Németh P, Mózsik Gy: Are the isolated rat gastric mucosal mixed cells proper to use in cytoprotective investigations? 39th Annual Meeting of the Hungarian Society of the Gastroenterology, Balatonaliga, Hungary, June 03-07, 1997.

52. Karádi O, **Bódis B**, Mózsik Gy: Atropine decreases the indomethacin-induced gastrointestinal mucosal lesions after chronic surgical vagotomy. 39th Annual Meeting of the Hungarian Society of the Gastroenterology, Balatonaliga, Hungary, June 03-07, 1997.

53. Mózsik Gy, **Bódis B**, Debreceni A, Karádi O, Király Á, Rumi Gy, Sütő G, Szabó I, Vincze Á: Similarities and differences of gastric mucosal protection produced by PGI2 and β-carotene in rats treated with ethanol, HCl and indomethacin. Alimentary Disease Week, Hong Kong, China, December 12-17, 1997.

54. Szabó I, Bódis B, Rumi Gy, Nagy L, Mózsik Gy: Human and rat isolated gastric mucosal mixed cells as in vitro models for cytotoxic investigations. Alimentary Disease Week, Hong Kong, China, December 12-17, 1997.

PUBLICATION

ABSTRACTS OF UNPUBLISHED LECTURES

1. **Bódis B**, Baláskó M, Csontos Zs, Karádi O, Király Á, Jávor T, Mózsik Gy: Changes of gastric mucosal prostaglandin contents in intact and vagotomized rats, without and with beta-carotene treatment. Dig Dis Sci 1990;35:1015.

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