Background: The exposure of gastric mucosa to damaging factors, such as ethanol, water restraint stress, or ischemia followed by reperfusion, produces pathological changes: inflammatory process, hemorrhagic erosions, even acute ulcers. The base of these changes is a disturbance of protective mechanisms and disruption of gastric mucosal barrier. Previous studies pointed out the role of disturbances of gastric blood flow, mucus secretion and involvement of prostaglandins and nitric oxide formation in the pathomechanism of gastric mucosa lesions. The role of reactive oxygen species (ROS) in these processes has been little studied.

Aim: The purpose of our present investigations is to explain the participation of ROS in acute gastric mucosal damage by various irritants.

Material and methods: Experiments were carrying out on 80 male Wistar rats. To assess gastric blood flow (GBF) laser Doppler flowmeter was used. The area of gastric lesions was established by planimetry. The levels of proinflammatory cytokines were measured by ELISA technique. The colorimetric assays were used to determine of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) as well as superoxide dismutase (SOD) activity.

Results: We demonstrated that 3.5 h of water immersion and restraint stress (WRS), 30 min of gastric ischemia followed by 60 min of reperfusion or intragastric administration of 100% ethanol, all resulted in appearance of acute gastric mucosal lesions accompanied by a significant decrease of gastric blood flow. These lesions are also accompanied by the significant increase of proinflammatory cytokines including interleukin–1 beta (IL-1β) and tumor necrosis factor alpha (TNFα) plasma level.

Biological effects of ROS were estimated by measuring tissue level of MDA and 4-HNE, the products of lipid peroxydation by ROS, as well as the activity of SOD, the scavanger of ROS. It was established that 3.5 h of water immersion and restraint stress (WRS), 30 min of gastric ischemia followed by 60 min of reperfusion or intragastric administration of 100% ethanol lead to significant increase of MDA and 4-HNE mucosal level, accompanied by a decrease of SOD activity (significant in WRS and ethanol application).

Conclusions: The pathogenesis of experimental mucosal damage in rat stomach includes the generation of ROS that seem to play an important role, namely due to generation of lipid peroxides, accompanied by impairment of antioxidative enzyme activity of cells.

Key words: ethanol, water immersion restraint stress, ischemia and reperfusion, MDA, SOD, IL-1β, TNFα.

INTRODUCTION

A variety of factors produce damage of gastric mucosa, including: systemic events such as thermal stress, or local mucosal application of various irritants that are commonly named breakers of gastric mucosal barrier (1, 2). This mucosal barrier is composed by epithelial cells with tight junctions and superimposed layer of mucus. The aim of this
barrier is to protect the mucosa against damage of deeper structures by hydrogen ions (H⁺) and other noxious substances originating from the gastric lumen (3). The endogenous prostaglandins (PGs) play an important role in the maintenance of mucosal integrity, which include continuous secretion of bicarbonate anions (HCO₃⁻) and a mucus production in the stomach and duodenum (1, 4). The imbalance between gastrotoxic agents and protective mechanisms results in an acute inflammation. The interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNFα) are major proinflammatory cytokines, playing important role in production of acute inflammation (5). This acute inflammation is accompanied by neutrophils infiltration of gastric mucosa.

Neutrophils produce superoxide radical anion (O₂•⁻), which belongs to group of reactive oxygen species (ROS). Superoxide radical anion reacts with cellular lipids, leading to the formation of lipid peroxides, that are metabolized to malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). Organism has several enzymatic systems, which scavenge ROS and prevent their destructive action. The major antioxidative enzyme is superoxide dismutase (SOD) (6).

Three types of superoxide dismutase (SOD) can be distinguished: cytoplasmatic, mitochondrial and extracellular. SOD catalyzes the dismutation of superoxide radical anion (O₂•⁻) into less noxious hydrogen peroxide (H₂O₂), that is further degraded by catalase or glutathione peroxidase. Catalase is an enzyme which accelerates degradation of H₂O₂ into water and oxygen (7). The second pathway of H₂O₂ metabolism depend on activity of glutathione peroxidase (GPx) and cooperating glutathione reductase. The reduction of H₂O₂ into water by GPx is accompanied by the conversion of glutathione from reduced form (GSH) into oxidized form (GSSG) (8-10) (Fig. 1).

![Fig. 1. Overall metabolism of formation of reactive oxygen species (ROS) in an organism.](image-url)
The role of ROS metabolism in the gastrotoxic processes has been little studied. That is why it was important to determine the significance of MDA, SOD and afore mentioned above cytokines in cases of gastric barrier disturbances and following gastric mucosa damage.

The methods, which are predominantly used in experimental induction of gastric lesions in animals include: intragastrical ethanol administration (11, 12), water immersion restraint stress (13, 14), as well as ischemia followed by reperfusion (15, 16). The aim of our present investigations is to explain the participation of ROS in gastric mucosal damage by various irritants.

MATERIAL AND METHODS

Experiments were carried out on 80 male Wistar rats, weighing about 200g. The animals were fasted for 24 h before all studies. Studies were approved by the Ethic Committee for Animal Research of Jagiellonian University.

Production of gastric lesions

The animals were divided into 4 groups. In 1st group gastric lesions were produced by intragastric (ig.) application of ethanol, in 2nd group- by water restraint stress, in 3rd group-by ischemia followed by reperfusion and in 4th group vehicle saline was used and animals did not undergo any procedures.

In 1st group 1,5 ml of 100% ethanol was applied, using a metal orogastric tube, as described previously (17). 2nd group of animals underwent 3.5 h of water immersion restraint stress in temperature 23°C, using the method originally proposed by Takagi et al. (18). In 3rd group a celiac artery was clamped, to evoke 30 min of ischemia. After this time the celiac artery was opened (60 min reperfusion). It was performed by modified method originally proposed by Wada et al.(16, 19).

Determination of gastric blood flow and area of lesions

The evaluation of gastric lesions was performed 1 h following ig. administration of 100% ethanol, 3.5 hours after the start of water immersion restraint stress and after 60 min of reperfusion.

To assess gastric blood flow (GBF) laser Doppler flowmeter (Laserflo, model BPM 403A, Blood Perfusion Monitor, Vasamedics, St. Paul, Minnesota, USA) was used. The animals were anaesthetized with Vetbutal 50mg/kg (Biowet, Pulawy, Poland), then the abdomen was opened and the stomach was exposed to assess the GBF. Blood flow was measured on anterior and posterior wall of stomach. The mean values of these measurements were calculated and expressed as percent change from value recorded in the intact mucosa (11, 12).

To establish the number and area of gastric lesions computerized planimetry (Morphomat, Carl Zeiss, Berlin, Germany) was used, as described previously (13). Results are expressed as mm².

Determination of plasma IL-1β and TNFa levels

A venous blood sample was withdrawn from the vena cava into EDTA-containing vials in order to determine the plasma level of interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNFa) by ELISA technique (BioSource International, Camarillo, CA, USA). Details of this method were described previously (5,16).
**Measurement of lipid peroxidation**

For lipid peroxidation in investigated groups, the determination of the levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) was carried out and their levels were used as indicators of lipid peroxidation. The procedure of MDA and 4-HNE determination was following: 600 mg of gastric mucosa was excised. Then 20 ml 0.5 M BHT (butylated hydroxytoluene) was added in order to prevent sample oxidation. This sample was subsequently homogenized in 20 mM Tris for 15 sec. in pH=7.4. Then homogenate was centrifuged (3000´g at 4°C for 10 min.). Obtained clear supernatant was stored at -80°C prior to testing.

The colorimetric assay for lipid peroxidation (Bioxytech LPO-586, Oxis, Portland, USA) was used to determine of MDA and 4-HNE tissue concentration. This assay is based on the reaction of a chromogenic reagent N-methyl-2-phenylindole with MDA and 4-HNE at 45°C. This reaction yields a stable chromophore with maximal absorbance at 586 nm. This absorbance was measured by spectrophotometer Marcel s300, Warsaw, Poland. Results were expressed as nanomol per gram of tissue (nmol/g).

**Determination of SOD activity**

To determine activity of superoxide dismutase (SOD), a sample of gastric mucosa was taken, as described above. The colorimetric assay for assessment of SOD activity (Bioxytech, SOD-525, Oxis, Portland, USA) was used. This method is based on the SOD-mediated increase in the rate of autooxidation of tetrahydrobenzofluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. This absorbance was measured by spectrophotometer Marcel s300, Warsaw, Poland. Outcomes were expressed as units per gram of tissue (U/g).

**Statistical analysis**

Results are expressed as means ±SEM. Statistical analysis was done using nonparametric Mann-Whitney test. Differences with p<0.05 were considered as significant.

**RESULTS**

**Assessment of gastric lesions and gastric blood flow**

*Figs 2 and 3* exhibit the mean lesion area in the stomach of tests with ethanol or ischemia-reperfusion (I/R) model and mean ulcer number in WRS model, as well as gastric blood flow. Intact mucosa did not show any macroscopic lesions (0 mm²) and the gastric blood flow (GBF) in this intact mucosa (48 ± 6 ml/min/100g of tissue) was accepted as the control value (100%). Application of ig. 100% ethanol produced numerous gastric mucosal lesions with an area averaging about 98.4 ± 10 mm². The GBF was reduced to the value 59 ± 6% as compared to intact mucosa. Ischemia of 30 min, followed by 60 min reperfusion, induced erosions in gastric mucosa with lesion area averaging 25.3 ± 9 mm² and GBF reduced to the value 64 ± 5% (*Fig. 2*). 3.5 hours of WRS resulted in induction of damages (mean lesion number about 24 ± 4) and GBF reduced to 46 ± 7% (*Fig. 3*).
Plasma levels of IL-1β and TNFα

Plasma concentrations of IL-1β in intact (control) animals averaged 3 ± 0.5 pg/ml. After 100% ethanol administration the IL-1β level increased to 9.2 ± 4.8 pg/ml. The observed increase of IL-1β level after 30 min ischemia followed by 60 min reperfusion, was negligible. After 3.5 h WRS marked and significant increase in IL-1β level was noticed, averaging 48.8 ± 13.59 pg/ml (Fig. 4).
In case of rats without any experimental procedures (intact mucosa), concentration of TNFα remained at low level (1.6 ± 0.4 pg/ml). Application of 100% ethanol resulted in a significant increase of TNFα level (7.95 ± 1.64 pg/ml), with small, but also significant increase was observed in the group with 3.5 h of WRS (4.23 ± 1.2 pg/ml). An increment of TNFα after ischemia-reperfusion, appeared to be negligible and did not reach statistical significance (Fig. 5).

![Fig. 4. The plasma level of interleukin -1β (IL-1β) in rats exposed to application of 100% ethanol intragastrically (i.g.), or 3.5 h of WRS (temp. 23°C), or 30 min of ischemia followed by 60 min of reperfusion. Results are mean ± SEM. Asterisk (*) indicates significant changes as compared with the control group.](image)

![Fig. 5. The plasma level of tumor necrosis factor alpha (TNFα) in rats with i.g. application of 100% ethanol, or 3.5 hours of WRS (temp. 23°C), or 30 min ischemia followed by 60 min reperfusion. Results are mean ± SEM. Asterisk (*) indicates significant changes as compared with the control group.](image)
Measurement of lipid peroxidation

Malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) in tissue are accepted as major products of lipid peroxidation. They are considered indicators of mucosa injuring by ROS. Concentration of MDA and 4-HNE in intact mucosa was at very low level, near to the analytical limit of detection, averaging $5.96 \pm 0.04 \text{ nmol/g}$ of tissue. After administration of 100% ethanol, the levels of MDA and 4-HNE almost doubled value ($10.01 \pm 1.71 \text{ nmol/g}$). In case of 3.5 h WRS the level of lipid peroxides metabolites increased to $15.76 \pm 0.95 \text{ nmol/g}$. The value of MDA and 4-HNE mucosal concentration, for I/R model, averaged $10.75 \pm 1.6 \text{ nmol/g}$. These outcomes, in all investigated groups, were significantly higher, as compared with the values obtained in the intact mucosa (Fig. 6).

**Fig. 6.** Concentration of MDA and 4-HNE (nmol/g) in the gastric mucosa in rats exposed to application of 100% ethanol intragastrically (ig.), or 3.5 h of WRS (temp. 23°C), or 30 min ischemia followed by 60 min reperfusion. Results are mean ± SEM. Asterisk (*) indicates significant changes as compared with the control group.

Activity of SOD in tissues

Enzymatic activity of superoxide dismutase (SOD) is a measure of antioxidative properties of cells. In intact gastric mucosa activity of SOD reached high level, i.e. $347.3 \pm 58.77 \text{ U/g}$ of tissue. 100% ethanol, applied ig, resulted in negligible and insignificant decrease of SOD activity ($309.73 \pm 28.77 \text{ U/g}$). The mucosa of animals, which underwent 3.5 h WRS, showed significant decrease of SOD activity, averaging $245.18 \pm 22.94 \text{ U/g}$. The lowest activity of SOD was observed after ischemia-reperfusion, averaging $185.936 \pm 41.93 \text{ U/g}$ (Fig. 7).
DISCUSSION

Previous studies focused on the participation of ROS in pathogenesis of diseases resulting from ischemia and reperfusion of organs, supplied by terminal arteries with weekly developed collateral circulation, for example the heart (20), the brain (21, 22), or the kidney (23, 24). In digestive system investigations on ROS predominantly concerned the pancreas (25) and small intestine (26). The investigations were also related to the biological effects of oxidative stress on the liver (27). Little information is available regarding the formation of ROS in gastric mucosa, exposed to various damaging factors. Erin et al. (28) attempted to explain the mechanism of radical production. He examined pathomechanisms of gastric mucosa damage, resulting from thermal stress. Especially, the role of C fibers in this model was considered. Animals in Erin’s model, underwent thermal stress, in temperature 6°C, during 4 h. Erin et al. failed to observe any significant changes in MDA level in stressed stomach. In our investigations we applied different approach, namely water immersion restraint stress (WRS) in temperature 23°C, during 3.5 h. Under these stress conditions a significant increase of MDA level after WRS, accompanied by decrease of enzymatic activity of antioxidative enzyme-superoxide dismutase (SOD) were observed.

Fig. 7. SOD activity (U/g) in the gastric mucosa in rats exposed to application of 100% ethanol intragastrically (i.g.), or 3.5 h of WRS (temp. 23°C), or 30 min ischemia followed by 60 min reperfusion. Results are mean ± SEM. Asterisk (*) indicates significant changes as compared with the control group.
Previous research on metabolism of ROS in gastric mucosa focused on the effects of *Helicobacter pylori* infection. Davies *et al* (29) showed that such infection of human gastric mucosa resulted in an increase of ROS production, measured by chemiluminometry, as compared with healthy mucosa. Experiments, carried out in our unit focused on preparing mice or rat model with *Helicobacter pylori* infection, preceded by a decrease of gastric mucosae integrity, using ischemia followed by reperfusion (16). In this investigations we confirmed that exposed gastric mucosa to oxidative stress, induced by occlusion of celiac artery (19), leads to the generation of lipid peroxides, as expressed by an increase of tissue level of MDA accompanied by impairment of antioxidative defense mechanisms, such as decrement in SOD activity. It is of interest that gastric mucosal lesions in rats induced by I/R can be attenuated by SOD, catalase and NO-synthase inhibitors, suggesting that ROS cooperate with NO in I/R-induced gastric damage and that reduction in ROS and NO toxicity is required to prevent the formation of acute gastric lesions by I/R (30).

Ethanol in high concentration (100%) used in this study is a classic model of mucosal barrier injury (11, 12). However, the participation of ROS in this model of acute gastric lesions has not been studied. Experiments carried out till now focused on measurement of MDA level, or its derivatives, in rat’s liver after ethanol application (31). Alterations in SOD activity in rat cerebellum under an influence of ethanol were also investigated (32). No study was undertaken to determine the role of SOD activity and MDA in animal model with ethanol-induced gastric damage, but only the effect of nitric oxide on prostaglandins synthesis (33) has been examined in pathogenesis of acute ethanol-induced mucosal injury.

Results of our experiments indicate that the production of gastric mucosal lesions by 100% ethanol, 3.5 h of WRS, or I/R, lead to decrease of gastric blood flow, increment of inflammatory changes, expressed by increase IL-1β and TNFα levels, as well as generation of ROS. Intensification of ROS production results in lipid peroxidation, expressed by tissue increment of MDA and 4-HNE levels. These phenomena are accompanied by impairment of antioxidative properties of cells, what is supported by our finding of the decrease of SOD activity in gastric mucosa.

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