

## Dose-Dependent Effects of Verapamil on Ethanol-Induced Gastric Lesions in Rats

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The effects of graded doses of verapamil on ethanol-induced stomach mucosal damage were studied in rats. Gastric lesions were induced *in vivo* by oral administration of 80% ethanol and evaluated with regard to ulcer index, gastric mucus content, free and total acidity, lipid peroxidation and nonprotein sulfhydryl groups. Orally administered ethanol markedly increased the ulcer index and lipid peroxidation. Pretreatment of rats with verapamil (1, 5, and 25 mg/kg *i.p.*) was carried out 1 h before the administration of ethanol. Verapamil showed a protective effect against ethanol-induced mucosal damage only at high dose (25 mg/kg). Verapamil dose dependently decreased the total acidity, lipid peroxidation, and nonprotein sulfhydryl content. Verapamil 25 mg/kg also increased significantly the gastric mucus secretion. L-arginine (100 mg/kg) or L-nitroarginine (100 mg/kg) with verapamil were also administered to the animals to determine the role of nitric oxide in the mechanism of the gastroprotective activity of verapamil (25 mg/kg). The results indicate that reduced acidity and lipid peroxidation and increased mucus secretion participate in the protective effects of verapamil against ethanol damage. On the other hand, a decrease in the nonprotein sulfhydryl content was observed with decreased gastroprotective effects of verapamil.

**Key words** — verapamil, ethanol, gastric lesions

### INTRODUCTION

Ethanol-induced gastric mucosal injury is suggested to involve the stimulation of oxygen-derived free radicals,<sup>1)</sup> stimulation of histamine and seroto-

nin,<sup>2)</sup> release from gastric mast cells, a reduction in the mucosal level of sulfhydryl compounds and prostaglandin E<sub>2</sub>,<sup>2)</sup> intracellular calcium depletion in the gastric glandular mucosa,<sup>3,4)</sup> and an increase lipid peroxidation.<sup>5)</sup> It is known that the calcium channel blockers verapamil,<sup>6)</sup> nitrendipine and diltiazem<sup>7,8)</sup> have gastroprotective effects.

There are controversial reports on the effects of verapamil on ethanol-induced gastric lesions. Some of them suggested that verapamil increased injury,<sup>4,7,9)</sup> whereas others indicated that verapamil decreased ethanol-induced gastric damage<sup>10–12)</sup> and Verapamil at low doses (2.5–5 mg/kg) worsened,<sup>4,7,9)</sup> and at high doses (10–20 mg/kg) lessened<sup>10–12)</sup> ethanol-induced gastric lesions.

The present study was designed to assess the involvement of mucus secretion, acidity, lipid peroxidation, and nonprotein sulfhydryl (NP-SH) compounds that are important factors in the gastric defense mechanisms in the possible gastroprotective effects of verapamil against ethanol induced gastric injury and to determine the optimal gastroprotective dose of verapamil. Furthermore, we aimed to investigate the role of nitric oxide (NO) in the ethanol-induced gastric lesions and in the gastroprotective effects of verapamil.

### MATERIALS AND METHODS

All experiments for animal testing were approved by the Eskisehir Osmangazi University School of Medicine Animal Use and Care Committee. Albino male rats weighing 250–300 g were used in the present study. The albino rats were purchased from the Laboratory Animal Production Center, Department of Pharmacology, Eskisehir Osmangazi University School of Medicine. The animals were maintained in single cages and were deprived of food for 16 hr before the experiments. Free access to water was allowed until 1 hr before the beginning of experiments. Verapamil (1, 5, and 25 mg/kg), L-arginine (100 mg/kg), L-nitroarginine (L-NNA, 100 mg/kg), or saline was injected intraperitoneally 1 hr before the induction of gastric mucosal damage.

The rats were divided into nine groups: group 1, control (saline); group 2, ethanol; group 3, ethanol + verapamil (1 mg/kg); group 4, ethanol + verapamil (5 mg/kg); group 5, ethanol + verapamil (25 mg/kg); group 6, ethanol + L-arginine (100 mg/kg); group 7, ethanol + L-arginine (100 mg/kg)+ Verapamil (25 mg/kg); group 8, ethanol +L-NNA

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(100 mg/kg); and group 9, ethanol + L-NNA (100 mg/kg) + Verapamil (25 mg/kg).

**Induction of Gastric Mucosal Damage by Ethanol** — One milliliter of 80% ethanol in water was given intragastrically to each animal using a gavage needle. The animals were killed with an injection of thiopental (100 mg/kg) i.p. 1 hr after the administration of ethanol.<sup>5)</sup>

**Acid Determination** — The gastric contents were collected by washing with 1 ml of saline and subsequently centrifuged. The total and free gastric acid was determined using NaOH 0.1 M. Methyl orange and phenolphthalein were used as indicators.<sup>13,14)</sup>

**Measurement of Gastric Lesions** — The stomachs were excised, opened along the greater curvature, and examined for ulcers under a light with a magnifying glass. Each was measured along the greatest length of lesions (mm); five petechiae were considered equivalent to a 1-mm ulcer.<sup>15)</sup>

**Determination of Gastric Mucus** — The corpus of the stomach was separated into two parts. One part was weighed and used for the determination of gastric mucus according to the modified procedure of Corne *et al.*<sup>16)</sup> using Alcian blue and evaluated spectrophotometrically.<sup>17)</sup> Briefly 0.5 g of the glandular segment of the stomach (corpus + antrum) was placed in 10 ml of 0.1% Alcian blue solution in sucrose 0.16 M, buffered with sodium acetate 0.05 M adjusted to pH 5.5 with HCl and left to stain for 2 hr. Uncomplexed dye was removed by two washes of 15 and 45 min in sucrose 0.25 M. Dye complexed with mucus was eluted by immersion in 5 ml of a MgCl<sub>2</sub> solution 0.5 M for 2 hr. The dye extract was shaken briefly with equal volumes of diethyl ether and then centrifuged at 3600 rpm for 10 min. The concentration of the dye in the aqueous layer was then determined spectrophotometrically at 598 nm (Metrolab 330 spectrophotometer) using the standard curve of Alcian blue. The mucus content ( $\mu\text{g/g}$  wet tissue) was then calculated.

**Determination of NP-SH Compounds** — The other part of the corpus was used for the determination of NP-SH. The content of NP-SH compounds ( $\mu\text{mol/g}$  wet tissue) was measured spectrophotometrically at 412 nm with the method of Sedlak and Lindsay.<sup>5,18)</sup> From each stomach 0.4 g of the glandular part was removed and homogenized in 8 ml of ice-cold EDTA 0.02 M. Aliquots of the homogenate (5 ml) were mixed with 4 ml of water and 1 ml of 50% trichloroacetic acid. The mixture was centrifuged at 3000 rpm for 15 min. Two milliliters of each supernatant were mixed with 4 ml of Tris buffer

0.4 M (pH 8.9) and 0.1 ml of 5,5-dithio-bis-2-nitro benzoic acid. Five minutes later, the absorbance of the mixture was determined at 412 nm. The content of the NP-SH compounds ( $\mu\text{mol/g}$  wet tissue) was then calculated with reference to a standard curve constructed using a standard glutathione solution.

**Determination of Lipid Peroxidase** — The gastric fundus was used for the determination of lipid peroxidase. The method reported by Utley *et al.*<sup>19)</sup> was followed and the spectrophotometric measurements were performed at 535 nm. The malonaldehyde content (nmol/g wet tissue) (index of the magnitude of lipid peroxidation) was then calculated.<sup>5,19)</sup> The fundus was removed, weighed and homogenized in KCl 0.15 M (at 4°C) (Kika-VerkeT 25 B homogenizer) to give a 10% w/v homogenate. Aliquots of the homogenate (1 ml) were incubated at 37°C for 3 hr in a metabolic shaker. Then 1 ml of a 10% aqueous trichloroacetic acid solution was added and mixed. The mixture was centrifuged at 2400 rpm for 10 min. One milliliter of the supernatant was removed and mixed with 1 ml of 0.67% 2-thiobarbituric acid in water and placed in a boiling water bath for 10 min. The mixture was cooled and diluted with 1 ml of distilled water. The absorbance of the solution was read at 535 nm. The content of malonaldehyde (nmol/g wet tissue) was then calculated by reference to a standard curve of malonaldehyde solution.

Results are expressed as mean + S.E. with n-8 animals/group. The significance of differences was analyzed using analysis of variance (ANOVA). Values of  $p < 0.05$  were taken to indicate statistical significance.

## RESULTS

The doses of verapamil (even 25 mg/kg) were well tolerated by the animals. We did not notice a significant adverse signs. Ethanol significantly increased the ulcer index and lipid peroxidation and significantly decreased gastric wall mucus and NP-SH compounds. There was no significant change in free acidity in any group. Verapamil at only the 25 mg/kg dose showed gastroprotective activity against ethanol-induced lesions but low doses of verapamil had no gastroprotection (Table 1). They 25 mg/kg dose of verapamil reduced the ulcer index, NP-SH compounds, and total acidity and lipid peroxidation, and increased gastric wall mucus (Table 1). Ethanol decreased NP-SH compounds and

**Table 1.** Effects of Verapamil on Ethanol-Induced Ulcer Index, Wall Mucus, Total Acidity, Lipid Peroxidation and NP-SH Compounds in Rat Stomach

Group (n:8 in each)	Ulcer index	Mucus secretion ( $\mu\text{g/g}$ tissue)	Total acidity (meq/l)	Lipid peroxidation (nmol/g)	NP-SH ( $\mu\text{mol/g}$ tissue)
Control	0	9.30 $\pm$ 0.93	6.14 $\pm$ 1.44	24.50 $\pm$ 1.49	12.10 $\pm$ 0.78
Ethanol	69.00 $\pm$ 3.70*	7.74 $\pm$ 0.49*	8.63 $\pm$ 1.91	44.40 $\pm$ 9.10*	6.18 $\pm$ 0.76*
Ethanol + verapamil (1 mg/kg)	66.00 $\pm$ 4.27	9.20 $\pm$ 0.49	6.50 $\pm$ 0.98	31.60 $\pm$ 2.53	2.43 $\pm$ 0.57*,**
Ethanol + verapamil (5 mg/kg)	69.13 $\pm$ 5.46	9.30 $\pm$ 0.57	4.20 $\pm$ 0.51**	25.60 $\pm$ 1.92	2.47 $\pm$ 0.22*,**
Ethanol + verapamil (25 mg/kg)	39.50 $\pm$ 7.37**	10.25 $\pm$ 1.04**	5.20 $\pm$ 0.71**	16.10 $\pm$ 1.24**	6.87 $\pm$ 0.57*
Ethanol + L-arg (100 mg/kg)	128.30 $\pm$ 16.86*,**	11.40 $\pm$ 0.90**	16.57 $\pm$ 2.53*,**	25.20 $\pm$ 1.98**	4.34 $\pm$ 1.18*
Ethanol + L-arg (100 mg/kg) + verapamil (25mg/kg)	81.66 $\pm$ 17.67	10.80 $\pm$ 0.90**	7.83 $\pm$ 1.33	20.20 $\pm$ 0.95**	5.15 $\pm$ 1.19*
Ethanol + L-NNA (100 mg/kg)	59.00 $\pm$ 12.38	7.80 $\pm$ 0.27	9.00 $\pm$ 0.29*,**	24.95 $\pm$ 1.18**	5.25 $\pm$ 1.22*
Ethanol + L-NNA (100 mg/kg) + verapamil (25 mg/kg)	79.00 $\pm$ 11.12	9.46 $\pm$ 1.65	11.88 $\pm$ 2.06	32.95 $\pm$ 4.79	4.01 $\pm$ 0.77*

\* $p < 0.05$  (compared with control group); \*\* $p < 0.05$  (compared with ethanol group). All values are given as mean  $\pm$  SEM.

increased lipid peroxidation (Table 1) in gastric tissue. The ulcer index was increased due to these effects of ethanol. When L-arginine (100 mg/kg) was administered with ethanol, the ulcer index and total acidity increased significantly, although gastric mucus production increased and lipid peroxidation decreased significantly. The addition of verapamil (25 mg/kg) to the combination of ethanol and L-arginine suppressed only the total acidity, but did not affect the other parameters. The administration of L-NNA with verapamil did not change any of the parameters except for total acidity (Table 1). The combination of ethanol + verapamil with L-arginine or L-NNA did not reduce ulcer index (Table 1).

## DISCUSSION

Our experiments have shown that verapamil has gastroprotective activity at the 25 mg/kg dose given 1 hr before ethanol administration. It significantly reduced gastric mucosal injury produced by intragastric instillation of ethanol (v/v 80%). In the present study, the ulcer index and lipid peroxidation were higher and the level of NP-SH compounds and gastric mucus were lower in the group given ethanol than in the intact animals, as shown in previous reports.<sup>2,4,5</sup> Low doses of verapamil were ineffec-

tive in reducing ethanol-induced gastric lesions. It appears that low doses of verapamil could not sufficiently stimulate the production of NP-SH compounds and inhibit lipid peroxidation.

It was reported that ethanol-induced gastric mucosal injury resembles an inflammatory response.<sup>20</sup> Additionally, it was shown that there was a significant inhibition of mucosal synthesis of PGE<sub>2</sub> following the administration of ethanol.<sup>2</sup> Gastric mucosal levels of SH compounds also decreased following exposure to stress or ethanol.<sup>21</sup> It was suggested that endogenous gastric acid production plays a small or negligible role in the pathophysiology of acute injury induced by ethanol<sup>1</sup> because drugs have acid antisecretory properties such as cimetidine and atropine failed to prevent ethanol-induced gastric injury.<sup>1,22</sup> Gastric mucus plays an important role in the pathophysiology of acute mucosal damage following the application of necrotizing agents.<sup>23</sup> The factors implicated in the mechanism of ethanol-induced gastric mucosal injury are oxygen-derived radicals, products of arachidonate metabolism, and mast cell secretory products.<sup>19</sup> Endogenous gastroprotective factors, such as prostaglandins, sensory neuropeptides, and gastrointestinal hormones participate in the functional and mechanical preservation of the gastric mucosa.<sup>24</sup> The protective effect of verapamil on ethanol-induced ulcers may

reside on its ability to inhibit gastric lipid peroxidation (Table 1). It was suggested that an increase in gastric NP-SH content limits the production of oxygen-derived free radicals.<sup>25)</sup> However, the results of this study showed that verapamil decreased the NP-SH content at low doses.

Endogenous NO produced by constitutive NO synthase regulates mucosal perfusion and has been suggested to protect the gastrointestinal mucosa against various stimuli.<sup>26)</sup> But the overproduction of NO may be involved in gastrointestinal injury<sup>27)</sup> and dysregulation of cytosolic calcium.<sup>28)</sup> The intensifying effect of L-arginine on ethanol-induced gastric damage can be explained by NO overproduction. Some reports have shown that L-NAME is protective against ethanol-induced gastric damage.<sup>29,30)</sup> Gastroprotective activity of NO against ethanol-induced ulcers has been reported in rats.<sup>31,32)</sup> However, an ulcerogenic as well as a protective effect of NO was demonstrated in the injury of rat gastric mucosa.<sup>33)</sup> NO donors at higher concentration or induction of NOS have been demonstrated to damage the gastric mucosal cells.<sup>34,35)</sup> It has been reported that certain calcium channel antagonists modulate NO synthesis by altering the induction of NO synthase.<sup>36)</sup> It was shown that verapamil, diltiazem, nifedipine and amlodipine strongly influence the coronary endothelial L-arginine NO system in isolated rat heart.<sup>37)</sup> Dihydropyridine and benzodiazepine calcium channel blockers were reported to enhance the production of endothelium-derived NO whereas verapamil did not to have a discernible effect on the NO system in human coronary artery endothelial cells *in vitro*.<sup>38)</sup> Another study reported that verapamil markedly increased the formation of nitrite in cardiac myosites in response to lipopolysaccharide and interferon-gamma, but not in vascular smooth muscle or mesangial cells.<sup>36)</sup> Mucosal NO, was demonstrated to markedly reduce the gastric mucosal damage in rats treated with acidified ethanol.<sup>39)</sup> It appears that NO has both protective and pathologic effects on the gastric tissue.<sup>40)</sup> The results obtained in rats treated with ethanol and L-arginine revealed an increase in gastric ulcers and this may be because the high concentration of NO. When verapamil (25 mg/kg) was administered with ethanol and L-arginine, the ulcer index was reduced insignificantly. NP-SH compounds were markedly reduced in gastric tissue after ethanol and verapamil challenge.

It appears that lipid peroxidation, NP-SH compounds and mucus production are crucial parameters in evaluating gastric mucosal injury after adminis-

tration of ethanol. Non enzymatic oxidation could occur by estimating lipid peroxidation. Because non-enzymatic lipid peroxidation might be involved in above changes, it is conceivable that the protective effects of verapamil and NO-producing precursors on ethanol-induced damage are due, at least partially, to the suppression of non-enzymatic lipid peroxidation. Furthermore, mucosal NO in the gastric defense system is released after activation by mild irritants to induce cytoprotection.<sup>39,40)</sup>

The present study showed that pretreatment of rats with verapamil 25 mg/kg reduced the ulcer index, but the lower doses were insufficient to protect against ethanol-induced gastric damage. These results suggest that both the dose of verapamil and the concentration of ethanol affect its gastroprotective activity. Additionally, the common factors in the protective effect of verapamil seem to be its ability to decrease lipid peroxidation and total acidity and to increase gastric wall mucus; stimulating NO production and decreasing the NP-SH content may adversely affect the gastroprotective activity.<sup>41)</sup> However, it appears that the role of NO may be unclear in the pathophysiology of ethanol-induced ulcers and the gastroprotective activity of verapamil.

Calcium antagonists are commonly used to treat several cardiovascular diseases including hypertension, angina pectoris, and arrhythmia. Therefore the gastric effects of verapamil may play a beneficial role in patients who have cardiovascular symptoms and also consume alcohol.

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