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The protective effect of vitamin C, vitamin E and selenium combination therapy on ethanol-induced duodenal mucosal injury

M Koyuturk*[,1], S Bolkent[2], S Ozdil[3], S Arbak[4] and R Yanardag[5]

[1]*Department of Histology and Embryology, Faculty of Medicine, Kadir Has University, 80810 Gayrettepe, Turkey;*
[2]*Department of Biology, Faculty of Science, Istanbul University, 34459 Vezneciler, Turkey;*
[3]*Department of Internal Medicine, Istanbul Medical Faculty, Istanbul University, 34390 Capa, Turkey;*
[4]*Department of Histology and Embryology, Marmara Medical Faculty, Marmara University, 81326 Haydarpasa, Turkey;*
[5]*Department of Chemistry, Faculty of Engineering, Istanbul University, 34850 Avcılar, Istanbul, Turkey*

In this study, the effect of a combination of vitamin C, vitamin E and selenium on ethanol-induced duodenal mucosal damage in rats was investigated morphologically and biochemically. The duodenal mucosal injury was produced by oral administration of 1 mL of absolute ethanol to each rat. Animals received vitamin C (250 mg/kg), vitamin E (250 mg/kg) and selenium (0.5 mg/kg) for 3 days and absolute ethanol 1 hour after last antioxidant administration and were sacrificed 1 hour after absolute ethanol. Extreme degeneration in intestinal mucosa of rats given ethanol was observed morphologically. In addition, an increase in neuronal nitric oxide synthase immunoreactive areas was observed in the rats of the group given ethanol. On the other hand, a normal morphological appearance and a decrease in neuronal nitric oxide synthase immunoreactive areas were detected in the rats given ethanol+vitamin C+vitamin E+selenium. In the group to which ethanol was administered, an increase in serum cholesterol and a decrease in serum albumin levels were determined. On the other hand, in the group to which ethanol+vitamin C+vitamin E+selenium were administered, serum cholesterol value decreased, and the serum albumin level increased. As a result, we can say that the combination of vitamin C, vitamin E and selenium has a protective effect on ethanol-induced duodenal mucosal injury. *Human & Experimental Toxicology* (2004) **23,** *391–398*

Key words: duodenal injury; ethanol; nitric oxide synthase; selenium; vitamin C; vitamin E

Introduction

Gastrointestinal mucosal lesions are caused by various agents such as stress,[1,2] ischemic-reperfusion,[3] aspirin as nonsteroidal anti-inflammatory drug,[4,5] 70% ethanol,[6] 80% ethanol,[7] 96% ethanol,[8,9] absolute ethanol,[6,9] acetic acid,[10,11] indomethacin and reserpin.[12,13] It is known that free radical production increased significantly in the tissue damage. It is shown that pretreatment with vitamin C or vitamin E prior to the administration of ethanol inhibited generation of free radicals and DNA strand breaks in the liver.[14] It was recently reported that vitamin E might exert a protective effect against nonsteroidal anti-inflammatory drug-induced gastric mucosal injury.[5] The protective effects of selenium seem to be primarily associated with its presence in the glutathione peroxidases, which are known to protect DNA and other cellular components from damage by oxygen radicals.[15] A number of investigations have revealed that vitamin C, vitamin E and selenium levels were decreased by exposure to ethanol.[2,14,16] Selenium alone has been shown to produce significant anti-ulcer activity,[13] and cells adequately supplied with selenium are less susceptible to the damaging effects of endogenously or exogenously generated oxygen radicals.[15] Vitamin C and vitamin E or vitamin E and selenium exert a synergistic effect in the prevention of biological membranes from oxidants.[14,17]

Nitric oxide synthase (NOS) is constitutively present in rat small intestine; the predominant form (90%) is neuronal nitric oxide synthase

*Correspondence: Meral Koyuturk, Kadir Has University Medical Faculty, Histology and Embryology Department, Vefa Bey S. N. 5 80810 Gayrettepe, Istanbul, Turkey
E-mail: mkoyuturk1@yahoo.com

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(nNOS).[18] The relationship between the degree of gut injury and nNOS activity is reported.[19] Nitric oxide (NO) is a multifunctional messenger that is involved in a wide range of physiological processes in many systems.[20] Intracellular NO can regulate oxidants through its ability to react rapidly with radical oxygen species. There is clear evidence for protective effects of NO on excess production of reactive oxygen species.[21] A moderate concentration of NO protects the cells against oxidative stress and plays an important role as regulatory mediator in various signalling processes.[22]

The present morphological and biochemical study was undertaken to investigate the protective effect of a combination of vitamin C, vitamin E and selenium on duodenal mucosal injury produced by ethanol. In addition, we aimed to investigate the role of nNOS expression in ethanol-induced duodenal damage and the relationship between nNOS and antioxidants such as vitamin C, vitamin E and selenium.

Materials and methods

Animals
Forty, 4–5-month old, adult female Sprague–Dawley rats, weighing 200–250 g, obtained from DETAM (Istanbul University Centre for Experimental Medical Research and Application) were used in this study. The experiments were reviewed and approved by the local institute's Animal Care and Use Committees. The animals were fed with pellet chow and tap water *ad libitum* before the experiments and fasted for 24 hours prior to the experiments. All rats were clinically healthy.

Experimental design and treatment of animals
The animals were randomly divided into four groups. Group I: intact animals (control). Group II: control animals receiving vitamin C (250 mg/kg/day), vitamin E (250 mg/kg/day) and sodium selenate (0.5 mg/kg/day) for 3 days. Group III: animals receiving 1 mL absolute ethanol. Group IV: animals received vitamin C, vitamin E and selenium (in the same doses) for 3 days and absolute ethanol 1 hour after last antioxidant administration, and sacrificed 1 hour after absolute ethanol. The antioxidants and absolute ethanol were given to rats by gavage.

Animal model for duodenal mucosal lesions
Duodenal damage was induced by oral administration at a constant volume by 1 mL absolute ethanol per rat. The animals were sacrificed by ether 1 hour after treatment with absolute ethanol.

Light microscopical study
First part of duodenum was taken from animals which were fasted overnight, under ether anaesthesia. The tissues which were fixed in Bouin's solution and subsequently processed using traditional paraffin embedding techniques for preparation of paraffin sections were stained with Masson's triple dyes and Periodic-Acid-Schiff for histological evaluation.

Immunohistochemical study
Same paraffin blocks of duodenal specimens that were prepared for light microscopic assay were used for immunohistochemical evaluation. Slides were deparaffinized in toluol and hydrated in ethanol series. Slides were treated with 0.3% Triton-X 100 for 10 min and then rinsed in phosphate-buffer saline (10 mM, pH 7.5). Antigen retrieval was performed in 0.01 M citrate buffer (pH 6). A Histostatin Plus (Zymed Laboratories, San Francisco, USA) broad-spectrum kit of the streptavidin–biotin system was then applied. Sections were covered with blocking serum for 20 min to prevent non-specific binding. They were then incubated with nNOS antibody at 1:100 dilution (Transduction Laboratories, Lexington, USA) overnight at 4°C. Slides were incubated for 20 min with biotinylated secondary antibody then incubated with the streptavidin–peroxidase conjugate for 20 min. The enzyme activity was developed using aminoethylcarbazole (AEC). The sections were counterstained with haematoxylin. Negative control sections were prepared by substituting the nNOS antibody with phosphate-buffer saline. Staining intensity was rated as weak (+), moderate (++) or strong (+++) by two different, blinded observers.

Electron microscopical study
For scanning electron microscopy, duodenal tissue samples are prefixed for 2 hours in a 2% phosphate-buffered glutaraldehyde solution (0.1 M, pH 7.2), postfixed for 1 hour in a 1% phosphate-buffered osmium tetroxide solution and passed from increasing alcohol and amyl acetate series. After drying the tissue samples with a BIORAD 'Critical Point Dryer' and gold coating with a BIORAD SC 502, tissue samples were examined under a JEOL 5200 JSM scanning electron microscope.

Biochemical study
Biochemical investigations of cholesterol and albumin in serum were measured by means of an autoanalyser (Targa 3000 Biotechnica).

Statistical analysis
The results were evaluated using an unpaired *t*-test and ANOVA variance analysis using the NCSS statistical computer package.[23]

Results

Light microscopical results
As light microscopic results, expansion and compression of the villi, ruptures and discontinuity in the epithelium of the end of the villi, oedema in the inside of villi and submucosa, hyperaemia in the capillaries, an increase in the mononuclear cell infiltration, a decrease in PAS positive reaction were observed in duodenum of all rats of the group given ethanol, according to controls (Fig. 1A, B). On the other hand, the same structures as the controls were determined in duodenum of all animals of the group given ethanol+vitamin C+vitamin E+selenium. In addition, an increase in PAS positive reaction and mucus was noticed (Fig. 1C).

nNOS immunoreactivity in duodenum was determined in all groups of rats and in the same density (+++) (Fig. 2A–C). nNOS was localized in nerve fibres of circular muscle layer and ganglia cells of myenteric plexus. It was observed that immunoreactive areas increased in circular muscle layer and ganglia cells in duodenum of all rats of the group given ethanol (Fig. 2B). These immunoreactive areas were decreased in duodenum of all animals of the group given ethanol+vitamin C+vitamin E+selenium according to the experimental group and were almost the same as in control groups (Fig. 2C). In the control group given antioxidants, immunoreactive areas were found the same as with intact control group.

Electron microscopical results
Scanning electron microscopical evaluation of Group I and Group II revealed good duodenal mucosa integrity with intact enterocytes (Fig. 3A, B). Scanning electron microscopic results of the etha-

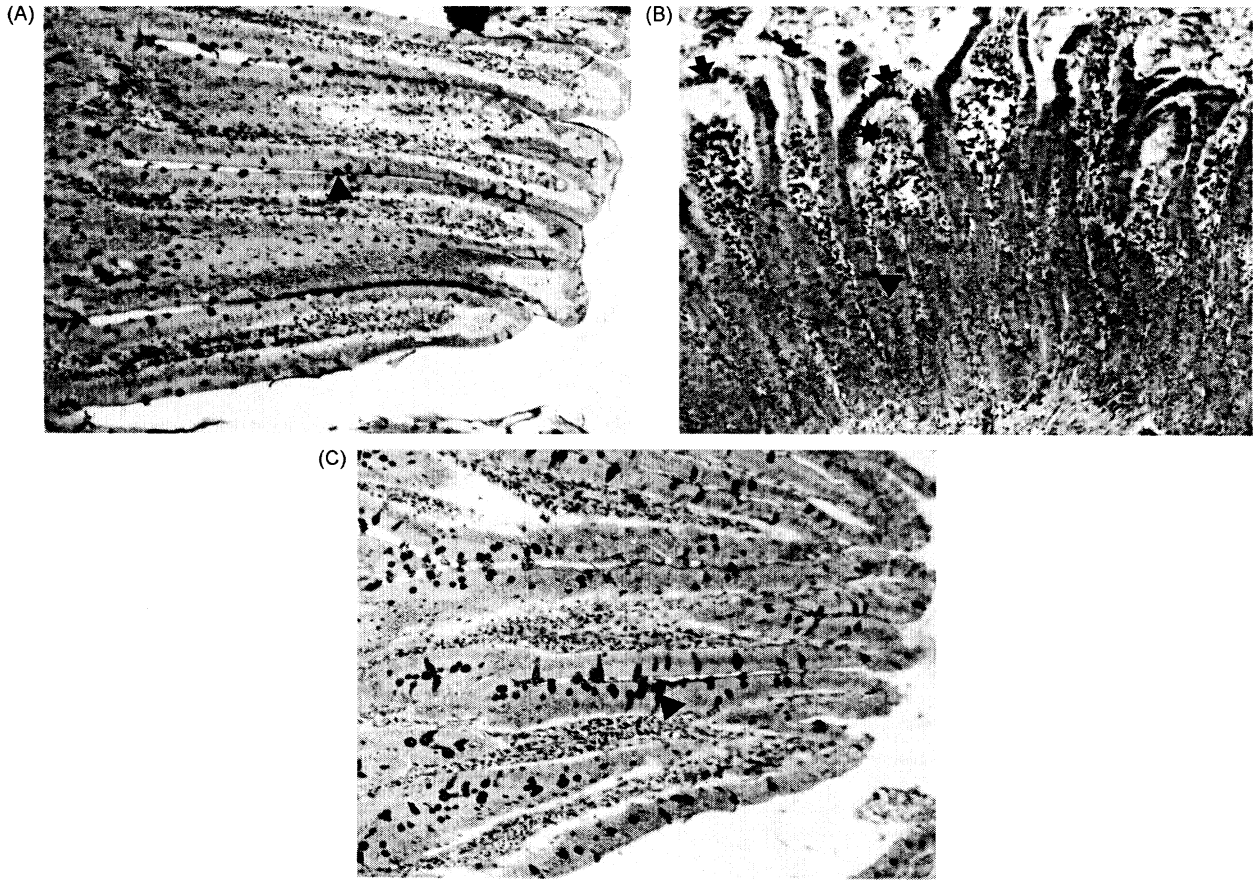

Figure 1A. A normal histological appearance of intestinal tissue of control rats. PAS positive reaction (▲). B. The histological appearance of intestinal tissue of rats given ethanol. Expansion and compression of the villi (→), oedema in the inside of villi and submucosa (*) and a decrease in PAS positive reaction (▲). C. The histological appearance of intestinal tissue of rats given ethanol+vitamin C+vitamin E+selenium. The morphology of intestinal tissue was noticed to be nearly the same as those of the controls. An increase in PAS positive reaction (▲). PAS. ×240.

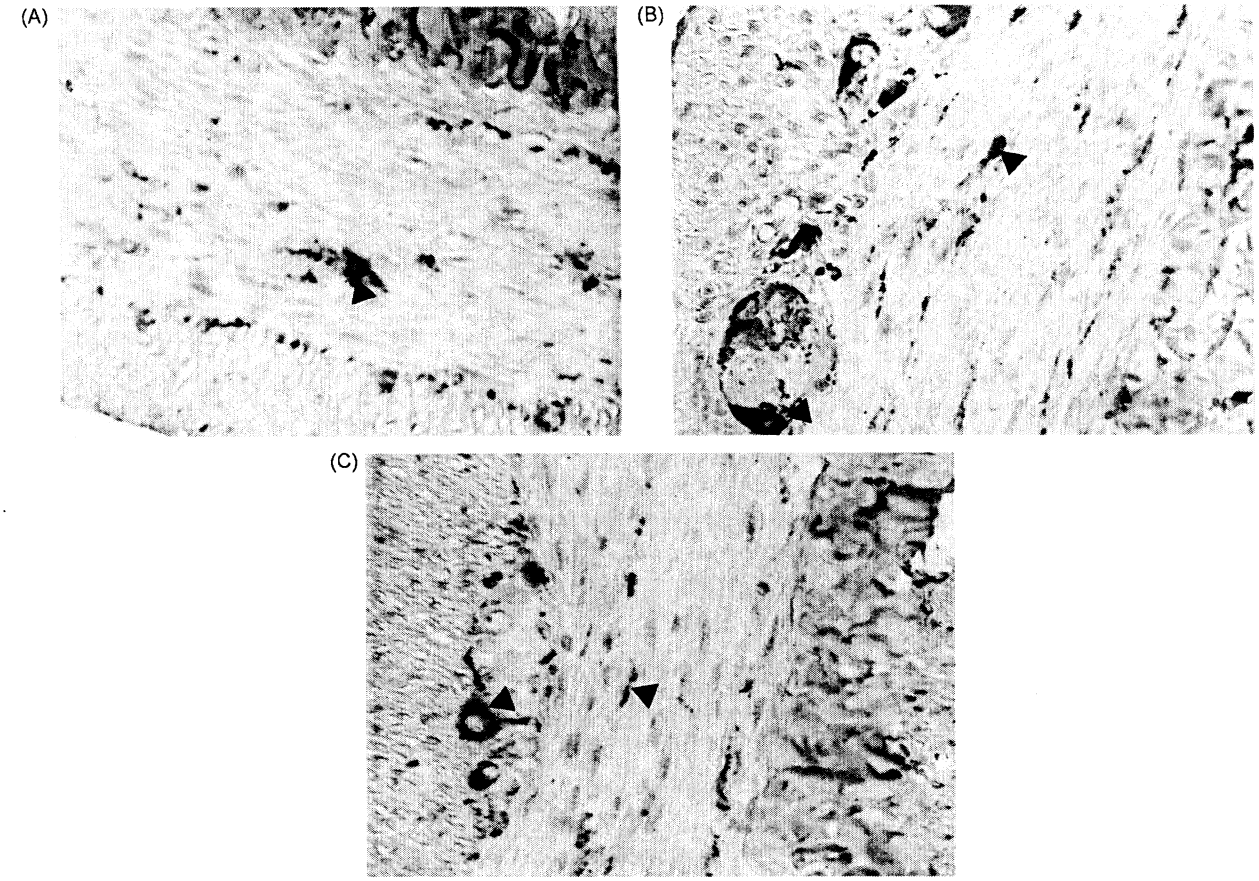

Figure 2A. nNOS immunoreactivity (▲) in circular muscle layer and myenteric plexus of control rats. B. An increase in nNOS immunoreactivity (▲) in circular muscle layer and myenteric plexus of rats given ethanol. C. A decrease in nNOS immunoreactivity (▲) in circular muscle layer and myenteric plexus of rats given ethanol + vitamin C + vitamin E + selenium. × 520.

nol-administered group, when compared to control groups, presented a loss of epithelium from the villi with exposure of underlying lamina propria. Haemorrhagic regions with deep erosions and fibrin deposits indicated an extreme degeneration of villar surface topography (Figure 3C). Scanning electron microscopical investigation of the ethanol + vitamin C + vitamin E + selenium-administered group showed a good villar epithelial arrangement in surface topography of villi. Closely packed enterocytes with mucous secretion demonstrated a significant reduction in the severity of mucosal injury by antioxidant treatment (Fig. 3D).

Biochemical results

Serum cholesterol and albumin values are presented in Table 1. From the obtained results, values of cholesterol in serum in the group administered ethanol have shown no significant difference when compared with the control group ($P_{t\text{-test}} = 0.161$). Also, a significant decrease was noted in the group administered ethanol + vitamin C + vitamin E + selenium compared to the groups administered ethanol ($P_{t\text{-test}} = 0.0001$). According to Table 1, a significant difference in the serum cholesterol levels of four groups was observed ($P_{\text{ANOVA}} = 0.0001$). In this study, a statistically significant decrease was observed in the serum albumin values of the group administered ethanol, in comparison with the control group ($P_{\text{value}} = 0.020$). Also, an insignificant increase was detected in the group administered ethanol + vitamin C + vitamin E + selenium, when compared to the group administered ethanol ($P_{\text{value}} = 0.138$). According to Table 1, a significant difference in the serum albumin levels of the four groups was observed ($P_{\text{ANOVA}} = 0.080$).

Discussion

Duodenal ulceration may heal despite hyperchlorhydria, suggesting that the integrity of the duodenal mucosa is more crucial than the state of acid secretion in the mechanism of development of this ulceration. It is suggested that oxygen-derived free radicals are detrimental to the integrity of the duodenal mucosa and that in the rat oxygen-derived

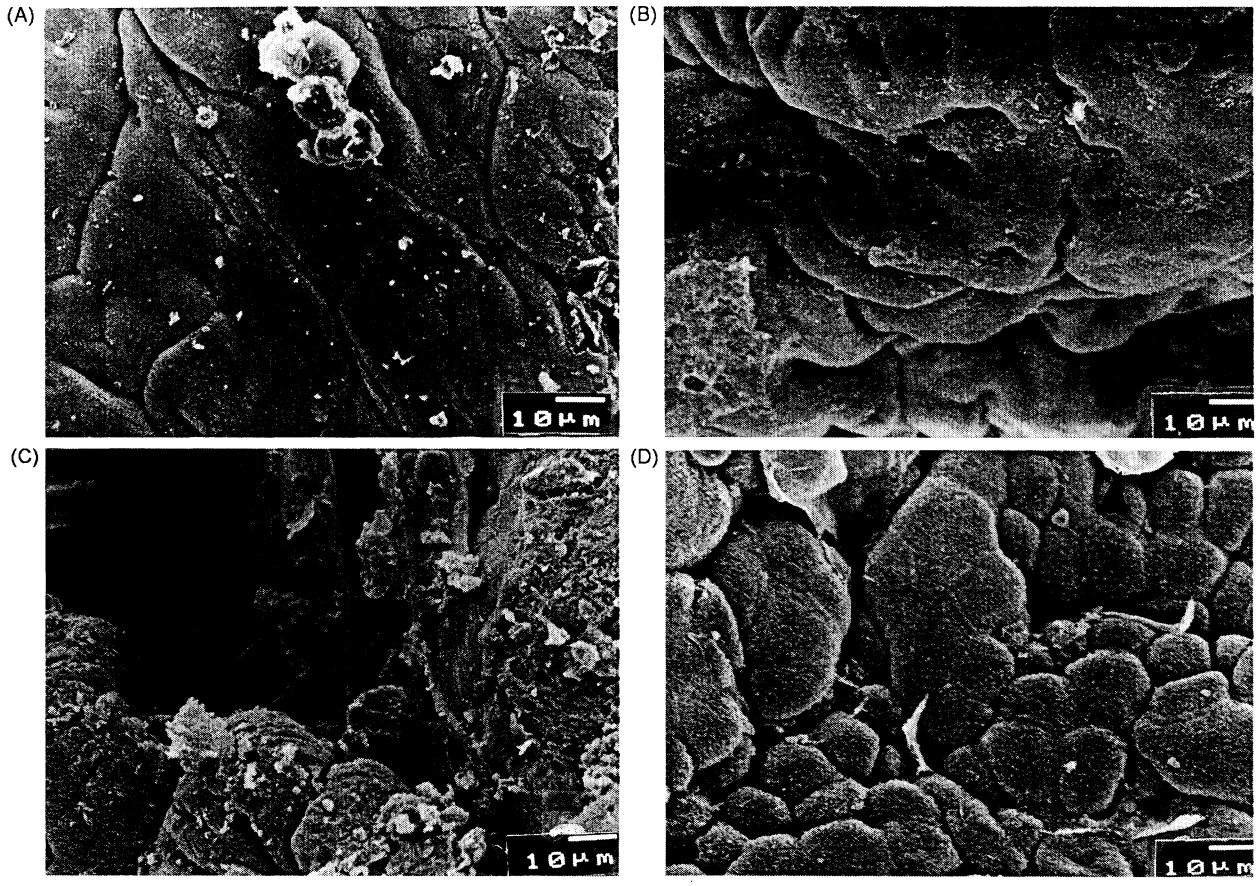

Figure 3A. Control group scanning electron micrograph demonstrates normal surface topography of villar epithelium. B. Scanning electron micrograph of rats receiving vitamin C+vitamin E+selenium. Regular arrangement of enterocytes suggests a normal surface villar topography. C. Scanning electron micrograph of intestinal mucosa of ethanol-administered rat group. Extreme degeneration of surface topography with desquamated villar epithelial cells (▲), deep erosions and haemorrhagic regions with erythrocytes (*) and fibrin deposits. D. Scanning electron micrograph of intestinal mucosa of ethanol+vitamin C+vitamin E+selenium-administered rat group. Surface epithelial topography indicates a good villar epithelial arrangement, tightly packed enterocytes (►) with mucous discharge. Bar: 10 µm.

free radicals are directly implicated in the mechanism of secretagogue-induced acute and chronic duodenal ulceration and that removing these radicals protects the duodenum against ulceration.[24] In addition, it is reported that oral administration of ethanol interrupted the mucosal defence and produced mucosal damage by necrosis or apoptosis, of gastric mucosal cells.[7,25] Our study demonstrated that antioxidants improved the integrity of duodenal epithelium and reduced the degree of damage in the glandular architecture. The mucosal injury due to ethanol administration consisted mainly of separation of the surface epithelium from the underlying lamina propria with complete loss of epithelium.

Table 1 Mean levels of serum cholesterol and albumin for all groups*

Groups	*Cholesterol mg%*	$P_{t\text{-test}}$	*Albumin g%*	$P_{t\text{-test}}$
Control ($n=10$)	56.62±1.04		4.32±0.18	
		0.0001		0.649
Cont. +vit. C+vit. E+Se ($n=10$)	48.68±1.18		4.41±0.58	
Ethanol ($n=10$)	58.84±3.54[a]		3.99±0.36[b]	
		0.0001		0.138
Eth. +vit. C+vit. E+Se ($n=10$)	51.83±2.02		4.19±0.19	
P_{ANOVA}	0.0001		0.080	

*Mean±SD.
n=Number of animals.
[a]$P_{t\text{-test}}=0.161$ versus control groups.
[b]$P_{t\text{-test}}=0.020$ versus control groups.

This fact is due to the hazardous effect of ethanol which rapidly penetrates gastroduodenal mucosa causing membrane damage.

The subsequent increase in mucosal permeability together with the release of vasoactive products from mast cells, macrophages and other blood cells may lead to vascular injury, necrosis and ulcer formation.[9] It is suggested that chronic ethanol administration induces oxidative stress, mainly increasing lipid peroxidation of the cell membrane and this leads to increased membrane fluidity, disturbances of calcium homeostasis and finally cell death.[16] It is reported that pharmacological antioxidants could have beneficial effects in reducing the incidence of ethanol-induced changes in cellular lipids, proteins and nucleic acids. The antioxidants considered could act by reducing free radical production, trapping free radicals themselves, interrupting the peroxidation process or reinforcing the natural antioxidant defence.[16] In our other study, it is shown that the administration of vitamin C, vitamin E and selenium to ethanol-induced rats eliminated accumulation of lipid peroxides in the stomach, suggesting that vitamin C, vitamin E and selenium protect against ethanol-induced oxidative damage.[26] The prominent epithelial damage in the ethanol-administered group could be due to increased lipid peroxidation of cell membranes leading to cell death. Microscopical evaluation of gastroduodenal mucosa of the antioxidant-administered group revealed a significant reduction in injury formation. We could correlate these findings with the free radical trapping activity of antioxidants. As is known, ethanol increases membrane lipid peroxidation and production of free radicals and may cause a number of tissue lesions.[27] Free radicals are highly reactive species characterized by one or more unpaired electrons in their outer orbital. These reactive oxygen species are highly reactive and capable of damaging many biological macromolecules such as RNA, DNA, proteins and lipids.[28] The cell membrane consists of phospholipid bilayer, cholesterol and proteins. Phospholipids and cholesterol can be modified by oxidative stress and free radicals.[29] In the present study, the cause of the increase observed in the values of cholesterol and decrease in the albumin levels in the group administered ethanol can be explained by damaged tissue due to the free radicals.

Vitamin E (tocopherol) is an important antioxidant in biological systems and is readily absorbed from intestine. α-Tocopherol is present in the lipid bilayers of biological membranes where it may play a structural role.[30] α-Tocopherol very efficiently scavenges lipid peroxyl radicals and thereby prevents the lipid peroxidation process in an uninhibited chain reaction. Tocopherol deficiency is characterized by a number of chronic health problems; there is damage to cell membranes as a result of increased lipid peroxidation.[31] Ascorbate, the major water-soluble antioxidant, has been shown to efficiently scavenge hypochloride, hydroxyl radicals and peroxyl radicals, and to restore the antioxidant properties of fat-soluble α-tocopherol.[32] Selenium is an essential part of the enzyme glutathione peroxidase, which functions as a part of an antioxidant system to protect membranes and essential proteins from the potentially damaging effects of reactive oxygen and lipid peroxides.[33] The increase in the serum albumin levels and the decrease in the cholesterol values by the application of ethanol and vitamin C, vitamin E and selenium show that antioxidants prevent the damage caused by ethanol.

NO regulates acid and gastric mucus secretion and alkaline production, and is involved in the maintenance of mucosal blood flow.[34] Immunohistochemical studies have shown that the enzyme necessary for NO synthesis is expressed in enteric neurons.[19] It is reported that nNOS immunoreactivity by immunogold staining in small intestine was localized in nerve profiles in myenteric plexus and circular muscle layer.[35] Ethanol intake injures the functional and structural integrity of the intestinal mucosa and causes loss of intestinal barrier function. Abnormal intestinal barrier can allow the penetration of normally excluded luminal substance across the mucosa and can lead to initiation of an inflammatory process and mucosal damage.[21] NO acts as an endogenous mediator modulating both repair and integrity of the tissues, and exhibits gastroprotective properties against different types of aggressive agents.[34] Ethanol absorption is controlled mainly by gastric emptying, because the primary region of ethanol absorption is the small intestine.[36] NO was reported as a neurotransmitter that acts at receptor protein on adjacent neuronal membranes.[37] It was suggested that inhibition of NO synthesis may lead to an increase of intestinal motility.[18] Depending on its concentration, dual roles of NO can expose protective and toxic effects.[38] In this study, the increase of nNOS immunoreactivity in the rats of the group given ethanol may exhibit a role related to regulation of intestinal motility and/or membrane integrity. Immunoreaction levels in the animals of the group given ethanol + antioxidants decreased compared to the group given ethanol as like to control group. The decrease of endogenous nNOS expression in the group given ethanol + antioxidants may be linked to

a regulatory role of antioxidants against mucosal injury.

As a result, the morphological and biochemical evaluations reveal that the combination of vitamin C, vitamin E and selenium has a protective effect on ethanol-induced duodenal injury. The antioxidants supplementation may be useful in alcohol-induced oxidative stress by enhancing the antioxidant capacity, and could play a significant protective role in the acute stage of duodenal injury. In conclusion, food supplementation with vitamin C and E, and selenium can be used in the therapy of ethanol-induced duodenal injury.

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