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Protective Effects of Ascorbic Acid, DL-α-Tocopherol Acetate, and Sodium Selenate on Ethanol-Induced Gastric Mucosal Injury of Rats

SADAKAT OZDIL,[1] REFİYE YANARDAG,[2] MERAL KOYUTURK,*,[3] SEHNAZ BOLKENT,[4] AND SERAP ARBAK[5]

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

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ABSTRACT

In this study, the effect of ascorbic acid (vitamin C), DL-α-tocopherol acetate (vitamin E), and sodium selenate (selenium) on ethanol-induced gastric mucosal injury in rats was investigated morphologically and biochemically. The gastric mucosal injury was produced by 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 d 1 h prior to the administration of absolute ethanol. In gastric mucosa of rats given ethanol according to control groups, neuronal nitric oxide expression decreased. This immunoreactivity was much lower in the group given ethanol+vitamin C+vitamin E+selenium than the control group and the ethanol-induced group. Scanning electron microscopic evaluation of the ethanol-induced group, when compared to control groups, revealed degenerative changes in gastric mucosa, whereas a good arrangement in surface topography of gastric mucosa in the group given ethanol + vitamin C+vitamin E + selenium was observed. In the group administered ethanol,

* Author to whom all correspondence and reprint requests should be addressed.

a reduction of the stomach glutathione (GSH) and serum total protein levels and increases in serum sialic acid, triglycerides, and stomach lipid peroxidation (LPO) levels were observed. Vitamin C+vitamin E+Se administration to alcohol-treated rats significantly increased the serum total protein, triglyceride levels, and stomach GSH levels and significantly lowered the levels of serum sialic acid and stomach LPO compared to untreated alcohol-supplemented rats. As a result of these findings, we can say that the combination of vitamin C, vitamin E, and selenium has a protective effect on ethanol-induced gastric mucosal injury of rats.

Index Entries: Ethanol; gastric mucosa; ascorbic acid; α-tocopherol acetate; sodium selenate; rat.

INTRODUCTION

Acute gastric mucosal injury is caused by various agents such as ethanol *(1,2),* stress *(3),* ischemic reperfusion *(4),* nonsteroidal anti-inflammatory drug *(5),* acetic acid *(6),* indomethacin, and reserpine *(7).* Many studies have demonstrated that inhibition of the enzyme involved in oxygen radical generation markedly reduced gastric mucosal injury induced by various agents. Oxygen radicals have been proposed to be pathogenic factors in acute gastric mucosal injury in animals and human, and extracellularly generated reactive oxygen metabolites can be directly toxic to gastric mucosal cells in vitro and in vivo *(8).* The mechanism of ethanol-induced gastric mucosal injury is unclear and several factors have been implicated. It has been reported that oxygen-derived radicals could play an important role in the pathogenesis of ethanol-induced gastric mucosal damage and that free-radical production increased during exposure to ethanol *(9–11).*

Selenium (Se) is an essential component of the antioxidant defense system that is known to protect DNA and other cellular components from damage by oxygen radicals *(12,13).* It is located at the catalytic site of the enzyme glutathione peroxidase (GPx), and gastrointestinal glutathione peroxidase (GI-GPx) is a member of the glutathione peroxidase family *(14).* One of the other antioxidant agents in our study is vitamin E, known as dietary antioxidant and it inhibits peroxidation of membrane lipids *(15)* in gastric mucosal injury *(16).* Vitamin C scavenges reactive oxygen and nitrogen species and can thereby prevent oxidative damage to important biological macromolecules such as DNA, lipids, and proteins *(17).* High dietary ascorbic acid intake appears to protect against gastric cancer. This could be because of its action as a scavenger of reactive radical species formed in the gastric mucosa *(18).* Neuronal nitric oxide expression has been reported in rat gastric mucosa and NO could influence muscle tone as well as exocrine and endocrine functions *(19).* Nitric oxide plays a cytoprotective or cytotoxic role depending on its concentration *(20).*

The aim of the present study was to determine whether a combination of vitamin C, vitamin E, and selenium had a protective effect on gastric

mucosal injury induced by ethanol and the effects of antioxidant combination to regulation of neuronal nitric oxide synthase activity.

MATERIALS AND METHODS

Animals

In this study, 40, 4- to 5-mo-old female Sprague–Dawley rats (Istanbul University Centre for Experimental Medicine Research and Application [DETAM]) were used in metal cages maintained at normal room temperature. The animals were given a standard chow diet and tap water *ad libitum* before the experiments and fasted for 24 h prior to the experiments. All rats were clinically healthy.

Experimental Design and Treatment of Animals

A total of 40 rats were divided into 4 groups: group I: intact animals (control) (n=10); group II: control animals receiving vitamin C (250 mg/kg, orally, daily, for 3 d), vitamin E (250 mg/kg, orally, daily, for 3 d), and Se (0.5 mg/kg, orally, daily, for 3 d) (n=10); group III: animals receiving 1 mL absolute ethanol (n=10); group IV: animals receiving vitamin C, vitamin E, and Se for 3 d 1 h prior to the administration of absolute ethanol (in the same dose and time) (n=10). Animals were fasted overnight (18 h) prior to the experiment, but they were allowed free access to water. The rats were sacrificed at 1 h after ethanol exposure to ether.

Animal Model for Gastric Mucosal Lesions

The gastric mucosal lesions were produced intragastrically at a constant volume by 1 mL absolute ethanol per rat. The animals were killed 1 h after treatment with absolute ethanol and the stomachs were removed and then opened along the lesser curvature.

Immunohistochemical Study

The same paraffin block of morphological study of 3 μm was cut and then placed on poly-L-lysine-coated glass slides. Slides were deparaffinized in toluol and hydrated in an ethanol series. The tissue was permeabilized with 0.3% Triton X-100 for 10 min and then rinsed in phosphate-buffered saline (PBS) (10 m*M*, pH 7.5). For antigen retrieval, the slides were pressure cooked in 0.01 *M* citrate buffer using a standard household pressure cooker. When the pressure indicator valve has risen after about 5 min, sections were incubated for 5 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide. A Histostatin Plus (Zymed Laboratories, USA) broad-spectrum kit of the streptavidin–biotin system was then employed. Sections were covered with blocking serum for 20 min to block nonspecific binding sites. They were then incubated with neu-

ronal nitric oxide synthase (nNOS) antibody overnight (Transduction Laboratories, Lexington, KY) at 4°C at 1 : 100 dilution. Slides were incubated for 20 min with biotinylated secondary antibody and then incubated with the streptavidin–peroxidase conjugate for 20 min. The enzyme activity was developed using aminethylcarbazole (AEC) and then the sections were counterstained with hematoxylin. Negative control sections were prepared by substituting the nNOS antibody with PBS.

Electron Microscopical Study

For scanning electron microscopy, tissue samples were prefixed for 2 h in a 2% phosphate-buffered glutaraldehyde solution (0.1 *M,* pH 7.2) and postfixed for 1 h in a 1% phosphate-buffered osmium tetroxide solution and passed from increasing alcohol and amyl acetate series. After drying the tissue samples with a Bio-Rad "Critical Point Dryer" and gold coating with a Bio-Rad Sputter Coater (SC 502), tissue samples were examined under a JEOL 5200 JSM scanning electron microscope.

Biochemical Assays

Biochemical investigations were made in serum and tissue. Biochemical investigations of triglyceride and total protein in serum were measured by means of an autoanalyzer (Targa 3000; Biotechnica). Serum sialic acid level was determined by the Lorentz methods *(21).*

For biochemical analyses, tissue samples of the stomach were washed with physiological saline and kept frozen until the day of the experiments. Stomach was then homogenized in cold 0.9% NaCl with a glass homogenizer to make up 10% homogenate (w/v). These homogenates were centrifuged. The clear supernatants were used for protein, lipid peroxidation (LPO), and glutathione (GSH) analyses.

The LPO levels were determined according to the method of Ledwozwy *(22).* In brief, the adducts formed following boiling tissue homogenate with thiobarbituric acid and extracted with *n*-butanol. The difference in optical density at 532 nm is a measure of the stomach malondialdehyde (MDA) content as a measure of thiobarbituric acid reactive species (TBARS), which is undertaken as an index of lipid peroxidation.

Reduced glutathione (GSH) was determined according to the method by Beutler using Ellman's reagent. The procedure is based on the reduction of Ellman's reagent by SH groups to form 5,5'-dithio-bis(2-nitrobenzoic acid), which has an intense yellow color that is measured spectrophotometrically at 412 nm using a Shimadzu spectrophotometer *(23).* The protein content in the supernatants was determined by the Lowry method *(24).*

Statistical Study

The results were evaluated using an unpaired *t*-test and analysis of variance (ANOVA) using the NCSS statistical computer package *(25).*

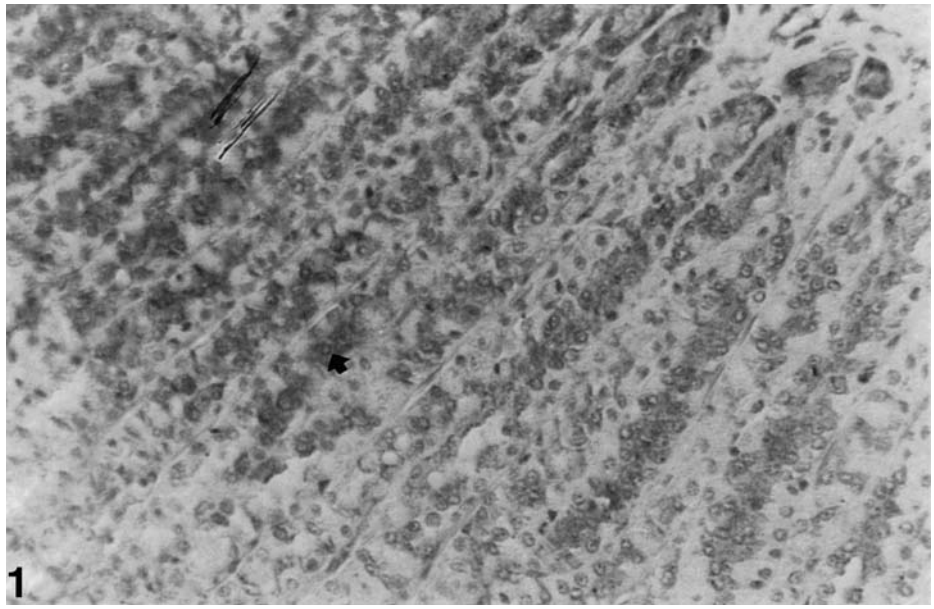

Fig. 1. nNOS immunreactivity (◗) in the oxyntic gland in gastric mucosa of control rats (magnification: ×520).

RESULTS

Immunohistochemical Results

The nNOS immunoreactivity was determined in the lower half of the oxyntic gland in the gastric mucosa. Strong immunoreactions were located in parietal and chief cells of control gastric mucosa and also the intensity of enteroendocrine cells immunoreactions was quite lower than the other cells (*see* Fig. 1). Similar reactions were found in the control group given an antioxidant (*see* Fig. 2). The intensity and number of immunoreactive cells decreased in the ethanol-induced gastric mucosa (*see* Fig. 3). The reaction of the group given ethanol+antioxidant was lower than control group and much lower than the ethanol-induced group (*see* Fig. 4).

Electron Microscopic Results

Scanning electron microscopic evaluation of the control groups revealed a good surface topography of gastric mucosa (*see* Fig. 5). In the group given absolute ethanol, extreme mucosal degeneration, exfoliation in epithelial cells, epithelial cell separation, deep erosions and gastric pits, exposure of basal lamina, fibrin deposits, and deformation in erythrocytes were revealed (*see* Fig. 6). In the group given ethanol +vitamin C+vitamin E+ Se, a good epithelial arrangement in surface topography of gastric mucosa except for minimal epitelial cell separation was observed (*see* Fig. 7).

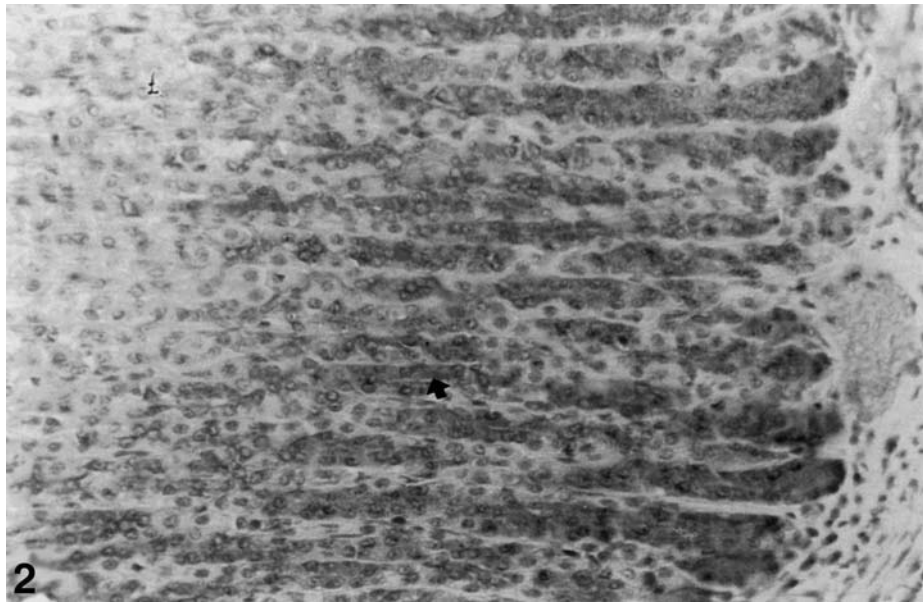

Fig. 2. nNOS immunre activity (◗) in the oxyntic gland in gastric mucosa of control rats given vitamin C, vitamin E, and selenium (magnification: ×520).

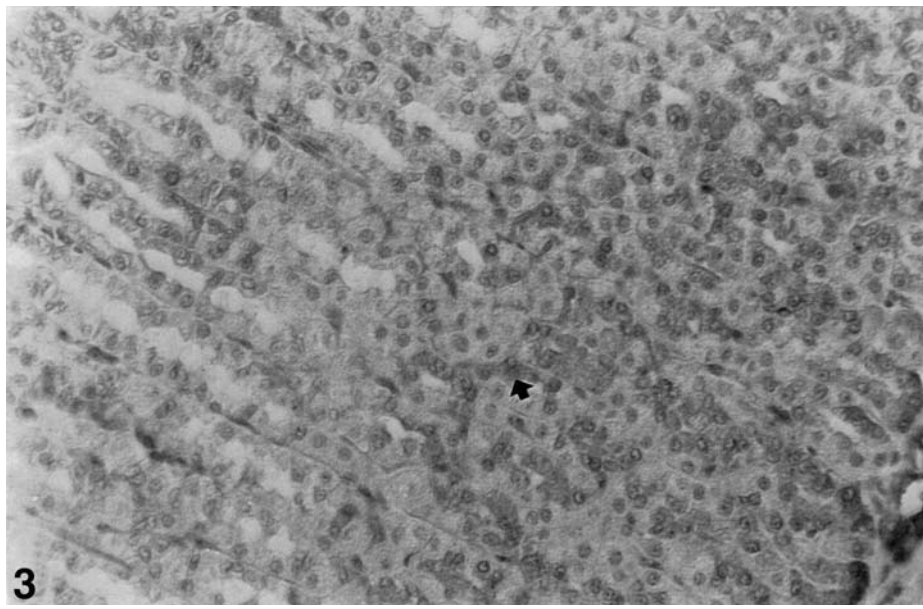

Fig. 3. A decrease in nNOS immunreactivity (◗) in the oxyntic gland in gastric mucosa of the rats given ethanol (magnification: ×520).

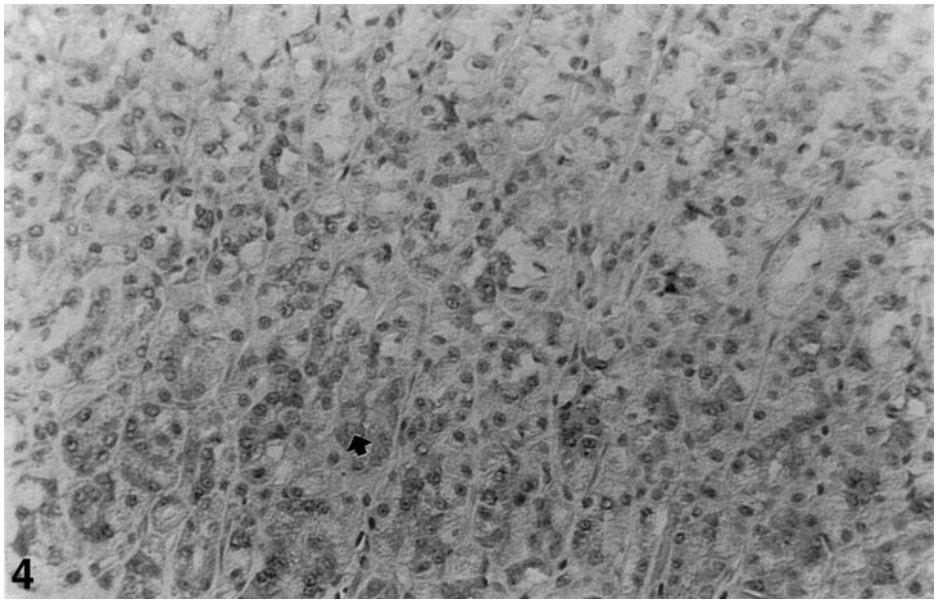

Fig. 4. The much lower immunreactivity (⧫) in the oxyntic gland in gastric mucosa of the rats given ethanol+vitamin C+vitamin E+ Se according to control groups and ethanol-induced group (magnification: ×520).

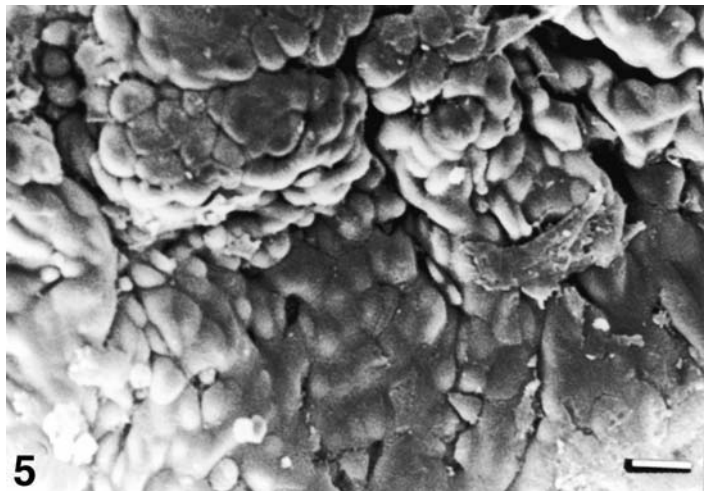

Fig. 5. Normal surface topography in the control group (bar= 10 μm).

Biochemical Results

The results of the present experiment are shown in Tables 1 and 2. The serum total protein levels in the ethanol group (7.69 ± 0.22 g%) were found to be significantly lower than in the control group (8.12 +0.47 g%)

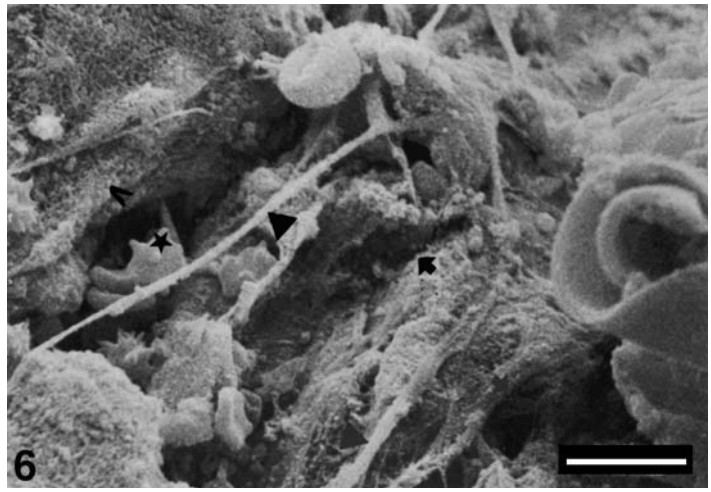

Fig. 6. Separation in epitheliall cells (◗), exposure of basal lamina (V), fibrin deposits (►), and deformation in erythocytes (★) (bar= 10 μm).

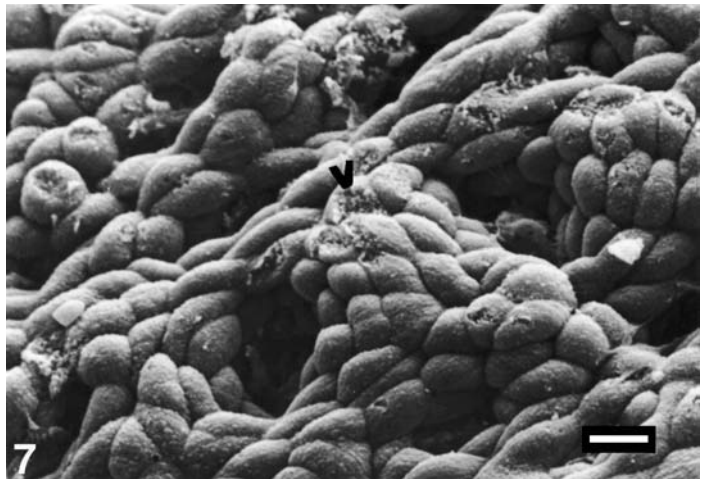

Fig. 7. Same topography as those of the controls in the group given ethanol +vitamin C+vitamin E+ Se (bar= 10 μm).

(p=0.005). In the ethanol + vitamin C+vitamin E+Se group (9.02 ± 0.30 g%), they were high compared to the ethanol group (p= 0.0001) (*see* Table 1).

Serum triglyceride levels in ethanol group (76.34 ±2.83 mg%) were significantly increased compared to control group (64.45 ±1.35 mg%). In ethanol +vitamin C+vitamin E+Se group, serum triglyceride levels were high compared to the ethanol group (p=0.027) (*see* Table 1). Serum sialic acid levels in the ethanol group (2.62 ± 0.22 mmol/L) were significantly higher than in the control group (1.95 ± 0.30 mmol/L). Serum sialic acid

Table 1
Mean Levels of Serum Parameters*

Groups	n	Total Protein g %	Pt-test	Triglyceride mg %	Pt-test	Sialic Acid mmol/l	Pt-test
Control	10	8.22 ± 0.47	0.964	64.45 ± 1.35	0.0001	1.95 ± 0.30	0.224
Control+VitC+E+Se,	10	8.20 ± 0.33		88.75±1.49		2.36 ± 0.55	
Ethanol	10	7.69 ± 0.22[a]	0.0001	76.34 ± 2.83[b]	0.027	2.62 ± 0.22[c]	0.002
Ethanol+Vit+C+E+Se	10	9.02 ± 0.30		81.36 ± 5.46		1.92 ± 0.46	
P_{ANOVA}		0.0001		0.0001		0.012	

* Mean±SD; *n*=number of animals.
[a] p=0.0001 versus control groups.
[b] p=0.0001 versus control groups.
[c] p=0.001 versus control groups.

Table 2
Mean Levels of Stomach Glutathione and Lipid Peroxidation for All Groups*

Groups	n	GSH nmol GSH/ mg protein	$P_{t\text{-test}}$	LPO nmol MDA/mg protein	$P_{t\text{-test}}$
Control	10	17.66 ± 0.51	0.0001	0.69 ± 0.08	0.0001
Control+Vit C+E+Se	10	14.84 ± 0.43		1.63 ± 0.07	
Ethanol	10	9.84 ± 0.93[a]	0.0001	2.99 ± 0.18[b]	0.0001
Ethanol + Vit.C+E+Se	10	13.54 ± 0.75		1.36 ± 0.11	
P_{ANOVA}		0.0001		0.0001	

* Mean ± SD; *n*=number of animals.
[a] p=0.0001 versus control groups.
[b] p=0.0001 versus control groups.

levels were decreased in the ethanol +vitamin C+vitamin E+Se group (1.92 ± 0.46 mmol/L) compared to the ethanol group (*see* Table 1).

The mean stomach LPO and GSH levels of four groups are given in Table 2. According to Table 2, a significant difference in the stomach GSH levels of the four groups was observed (p=0.0001). Alcohol administration significantly reduced the GSH levels in rat stomach tissues compared to control animals. Administration of vitamin C+vitamin E+Se to rats increased the stomach GSH content in ethanol groups.

Stomach LPO levels in ethanol group were significantly increased compared to the control group (p= 0.0001). Administration of vitamin C+vitamin E+Se caused a significant decrease in the LPO levels in the ethanol group (p=0.0001), but vitamin C+vitamin E+Se caused a significant increase in LPO levels in the control groups (p= 0.0001).

DISCUSSION

Ethanol is a direct systemic toxin that produces injury to all tissue, depending on dose and duration of exposure *(26,27).* Acute gastric mucosal damage is produced by various mucosal damaging agents such as ethanol, aspirin, strong acids and bases, and stress *(28).* It has been reported that free radicals could play an important role in pathogenesis of acute gastric lesions induced by ethanol and that free-radical production increased during exposure to ethanol *(29,30).*

The low amounts of GI-GPx protein has been reported in selenium deficiency. The function of GI-GPx has been discussed as a primary barrier against the absorbtion of ingested hydroperoxides *(31).* The protective effects of selenium reported via inhibition of gastric lesions by the mucosal regeneration of prostaglandins *(32).* Also, an increasing GPx level was reported in combined protective effect of selenium and ascorbic acid against alcohol-induced oxidative stress *(33).*

α-Tocopherol interacted with the biological reductants NADH and NADPH in microsomal membranes. Protection of vitamin E was reported by inhibiting lipid peroxidation and accumulation of activated neutrophils *(5).* Another study on ethanol-induced gastric lesions reported reduced basal gastric acid secretion with treatment of Se and vitamin E when given individually. The better inhibition of gastric acid secretion was reported with the combination effects of these agents *(7).* However, some authors reported that neither gastric acidity nor mucus protection are involved in the gastroprotective effects of vitamin E *(34,35).* The antioxidant synergism was reported also between α-tocopherol and ascorbic acid *(36).* Vitamin E shares with vitamin C the ability to inhibit nitrosamine formation in the stomach *(37).* Vitamin C acts as a reducing compound in the aqueous part of the cell and vitamin E acts as scavenging free radicals and stabilizes the membrane in the cell *(38).* Vitamin E administration was reported to reduce protein glycosylation in diabetic patients and a similar inhibiting

effect of vitamin C *(39).* Vitamin C also prevents oxidative lesions in DNA that include base modifications, sugar damage, and strand breaks *(40).*

Several findings reported that NO is important for normal gastric function. NO is necessary for the intracellular barrier of the gastrointestinal epithelium *(41).* The protective effects of NO was also reported via increasing mucus secretion and regulation of gastric acid secretion *(42,43).* Nitric oxide is a molecule capable of reacting via multiple pathways to modulate lipid oxidation reactions and effects on the inflamatory process *(44).* Nitric oxide synthase decreased the ischemia-reperfusion-induced gastric mucosal injury and the effects of NO was reported as an antioxidant defensive role by maintaining mucus, glutathione, and glutathione peroxidase *(45).* Neuronal nitric oxide expression was determined in parietal cells, chief cells, mucosecretory cells of gastric epithelium, and some enteroendocrine cells. The findings of studies reported that NO might influence parietal cell secretion directly as an intracellular signaling molecule and/or indirectly by acting on adjacents cells *(19,46).* In our study, immunoreactions of nNOS decreased ethanol-induced gastric mucosa lower than the normal group. The antioxidant and ethanol group immunreactions were decreased compared to the ethanol-induced group. According to these findings, the visible healing in the cases of antioxidant and ethanol group is independent of nNOS expressions.

Our study showed that antioxidants improved the integrity of gastric mucosal epithelium and reduced the degree of damage in the mucosal architecture. The mucosal injury is the result of the hazardous effect of ethanol, which rapidly penetrates gastric mucosa, causing membrane damage. The prominent epithelial damage of the ethanol-induced group could be the result of increased lipid peroxidation of cell membranes, leading to cell death. 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 *(47).* Microscopic evaluation of gastric mucosa of the antioxidant treated group revealed a significant reduction in injury formation. We could correlate these findings with the free-radical-trapping activity of antioxidants.

Antioxidants are essential in preventing the cellular damage caused by free radicals and free-radical-modified LPO. In normal metabolism, there is a balance between the generation of free radicals and the antioxidant defense mechanism. Chronic ingestion of alcohol upsets this balance, and there is ample evidence to demonstrate the oxidative stress induced by ethanol *(3).* Antioxidants prevent new radical species formation by converting existing free-radical species into less harmful molecules or by preventing the transformation of free radicals from other molecules *(48).* The protective effect of some antioxidants has been demonstrated light microscopically in the treatment of gastric mucosal injury *(28).* Low-molecular-weight antioxidants also play important roles in preventing free-radical damage. Vitamin E disrupts the chain reaction of lipid peroxidation by scavenging intermediate peroxyl radicals and also traps mutagenic elec-

trophilics such as NO *(49).* Some studies have shown that vitamin E has a protective effect against gastric mucosal injury induced by ischemia reperfusion and other insults *(50).* In addition, it has been demonstrated that macroscopically vitamin E has a protective effect against gastric mucosal injury induced by NSAIDs (nonsteroidal anti-inflammatory drugs) *(50).* Vitamin C or ascorbic acid is a naturally occurring free-radical scavenger, and as such, its presence assists various other mechanisms in decreasing the numerous distruptive free-radical processes *(3)* from taking place, including LPO. Recent studies have shown that both vitamins E and C are reduced in alcoholics. Chakrabarthy et al. *(51)* have shown that ascorbate protects guinea pig tissues from LPO both in vivo and in vitro. Selenium is an essential part of the enzyme glutathione peroxidase, which functions as part of an antioxidant.

Lipid peroxidation mediated by free radicals is considered to be a primary mechanism of cell membrane destruction *(52).* Ethanol has been shown to increase LPO in gastric mucosa *(53).* Some publications have indicated high levels of malondialdehyde (MDA) and conjugated dienes in cell membranes of animals subjected to acute or chronic ethanol intoxication *(54).* Oxidative injury can cause inflammation in the gastric mucosa as a result of infiltration of polymorphonuclear leukocytes into the lesion *(53).* MDA and hydroperoxides levels increased in the alcohol-fed animals and were substantially lower in the alcohol + ascorbic acid-fed animals *(55).* MDA levels in gastric mucosa, which is an index of LPO, increased significantly with ethanol administration (*see* Table 2). The ethanol-induced LPO in gastric mucosa was inhibited by vitamins C and E and Se. The inhibitor activity of antioxidant (vitamins C and E and Se) could result from scvanging reactive oxygen species, which initate LPO. Alternatively, the inhibition could occur at the membrane level by inhibiting oxidoreductase activity *(53).* Glutathione is an important constituent of an intracellular protective mechanism against various toxic stimuli, including oxidative stress. Reduced glutathione is known to be a major low-molecular-weight scavenger of free radicals in the cytoplasm *(56).* The stomach is rich in GSH, where it might serve a protective role similar to that observed in the liver, namely removal of free radicals and maintaining mucosal integrity *(56).* Ethanol rapidly penetrates gastroduodenal mucosa, causing membrane damage, exfoliation of cells, and erosion. Ethanol-induced gastric mucosal damage is associated with a significant reduction in nonprotein sulfhydryl concentration in the rat, dog, and humans *(56).* In our study, ethanol significantly decreased gastric GSH concentration. This reduction could be the result of the oxidation of GSH because of the ethanol-induced generation of toxic oxygen metabolities or the binding of GSH to acetaldehyde generation through the oxidation of ethanol by the gastric alcohol dehydrogenase *(57).* Alternatively, a block in the synthesis of the tripeptide induced by ethanol-generated free radicals might occur. In our study, administration of vitamins C and E and Se significantly increased stomach GSH levels in the ethanol group. This effect might be

the result of the activator role of Se on GSH-Px activity and indicate that these three substances effectively protect membrane integrity.

Many reports indicated that alcohol intake significantly increases serum triglyceride levels resulting in hypertriglyceridemia and fatty liver *(58).* Ethanol oxidation by the alcohol dehydrogenase pathway results in the production of NADH, which might contribute to enhanced lipid synthesis. Ethanol itself is converted into acetate, which is the building block of fatty acid synthesis. Ethanol oxidation has been shown to increase α-glycerophosphate levels *(59),* which can lead to enhance triglyceride synthesis. In our study, serum triglyceride levels were increased by the application of ethanol, but vitamins C and E and Se show that antioxidants prevent the damage caused by ethanol.

Sialic acid is an acetylated derivative of neuraminic acid *(60).* It attaches to nonreducing residues of the carbohydrate chains of glycoproteins and glycolipids *(61).* An increase in serum total sialic acid levels has been previously reported in diabetes, cardiovascular disease, inflammatory disorders, and malignant disease, including breast cancer and different solid tumors and leukemias. Recent studies have shown that sialic acid concentration in serum might be increased in alcoholics *(61).* In our study, the sialic acid levels were significantly higher in the ethanol groups that in control groups. Administration of vitamin C+vitamin E+Se significantly decreases sialic acid in the serum of the ethanol group.

As a result, the microscopical and biochemical evaluations reveal that the combination of vitamin C, vitamin E, and Se has a protective effect on ethanol-induced gastric mucosal injury of rats. However, this protective effect is independent of nNOS expressions.

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