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Protection by Tetramethylpyrazine in Acute Absolute Ethanol-Induced Gastric Lesions

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Key Words

Cytoprotection · Absolute ethanol · Tetramethylpyrazine · Hemoglobin · Hematocrit · Malondialdehyde · Acute gastric damage

Abstract

Acute oral administration of absolute ethanol (1.0 ml/kg) to fasting rats produced extensive necrosis of the gastric mucosa within 1 h. Pretreatment 30 min before administration of ethanol with oral tetramethylpyrazine (TMP) prevented this necrosis. Gross examination of the gastric mucosa of rats that received TMP showed fewer gastric lesions than that of rats who did not receive TMP. TMP pretreatment in rats exhibited superoxide scavenging activity in absolute ethanol-induced lipid peroxidation in gastric mucosal homogenates. TMP added in vitro to gastric homogenates made from control rats also showed scavenging activity. We conclude that the gastric protective mechanism of TMP could be attributed, at least in part, to its ability to inhibit lipid peroxidation and hence indirectly protect the gastric mucosa from oxidative stress.

Previous studies have shown that gastric ulcers are often associated with ingestion of aspirin [7, 13] and absolute ethanol [25]. Acute absolute ethanol administration often leads to tissue damage, especially of the gastric mucosa in the rat, and this model is useful for studying drugs with possible antiulcer action [25]. Tetramethylpyrazine (TMP) is a pharmacologically active constituent of *Ligusticum wallichii* French [12]. It not only blocks the entry of extracellular calcium through calcium channels but also inhibits the release of intracellular stored calcium in vascular smooth muscle cells [12]. TMP has been used to treat ischemic heart disease [2]. Previous research has shown that TMP protects the myocardium [1] and has a protective effect on reserpine-induced acute hemorrhagic mucosal lesions in rats [19]. The aims of the present study were to investigate whether TMP administration per os protects the gastric mucosa from absolute ethanol-induced lesions in rats, and if TMP does provide protection, what is the potential mechanism?

Materials and Methods

Animals and Treatments

Male Wistar rats (about 150–200 g) were purchased from the animal center, College of Medicine, National Yang-Ming University. They were kept for at least 1 week on commercial diets (Fu-So Co.,

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Taipei, Taiwan) under controlled conditions (25 ± 1°C, 55 ± 5% humidity) with free access to food and water. The rats were randomly divided into five groups with 10 animals each. Group 1 (control) received saline (1.0 ml/kg, p.o.), group 2 received absolute ethanol (1.0 ml/kg, p.o.) and groups 3–5 received TMP orally at doses of 50, 100 and 150 mg/kg, respectively, 30 min before oral administration of 1.0 ml/kg absolute ethanol. The animals were killed 1 h after absolute ethanol administration. The gastric lesions were evaluated according to an index described by Zhang et al. [25].

Assessment of Stomach Lesions

Blood samples were collected from the carotid artery, allowed to coagulate at room temperature for 1 h and then subjected to hemoglobin and hematocrit assays according to the method described by Mock et al. [11] and Viau et al. [17], respectively.

Assessment of gastric lesions was also carried out by gross pathological examination of the stomach mucosa. Massive gastric hemorrhage was observed in the rats' stomachs 1 h after oral administration of 1.0 ml/kg absolute ethanol. The stomachs were then dissected along the greater curvature, and the mucosa was examined for the number of absolute ethanol-induced mucosal lesions according to the method described by Gutierrez-Cabano [4] and Gutierrez-Cabano and Raynald [5]. Gutierrez-Cabano and Raynald [5] reported that the intragastric administration of clarithromycin, a macrolide antibiotic, macroscopically protected the rat gastric mucosa from 96% of ethanol-induced lesions. This protective effect was dose dependent, the reduction of lesions being 92.3, 81.4, 52.2 and 5.4% at doses of 400, 200, 100 and 50 mg/kg, respectively [5]. Gutierrez-Cabano [4] also suggested that the protective effect afforded by intragastric polyethylene glycol 400 against ethanol-induced gastric mucosal damage is partially mediated by alpha2-adrenoceptors, and a mucus-dependent mechanism may be involved.

$FeCl_2$-Stimulated Lipid Peroxidation in Rat Stomach Homogenate (in vitro)

Rat stomach homogenate was prepared according to the method described by Hongo et al. [6]. The inhibitory effect of TMP on $FeCl_2$-induced lipid peroxidation in normal rat stomach homogenate was determined by the thiobarbituric acid (TBA)-malondialdehyde (MDA) adduct according to the modified method described by Yuda et al. [23]. A mixture containing 0.5 ml of normal stomach homogenate, 0.1 ml of Tris-HCl buffer (pH 7.2), 0.05 ml of 4 m*M* $FeCl_2$ and 0.05 ml of 4 m*M* of various concentrations of TMP (0.01, 0.1 and 1.0 mg/ml) was incubated for 1 h at 37°C for homeostasis. After incubation, 9 ml of distilled water and 2 ml of 0.6% TBA were added to 0.5 ml of the incubated mixture and shaken vigorously. The mixture was then incubated for 30 min in a 95°C water bath. After cooling, 5 ml of *n*-butanol was added and the mixture was again shaken vigorously. The *n*-butanol layer was separated by centrifugation at 1,000 *g* for 10 min, and the upper *n*-butanol layer was collected to measure MDA production, in units of pmol/mg protein, at 532 nm on a spectrophometer [20].

Assessment of Lipid Peroxidation Activity (in vivo)

In order to evaluate the inhibitory activity of TMP on the lipid peroxidation generated assay system, portions of rat stomach tissue dissected from groups 1–5 were sliced and homogenized (13,000 rpm, 3 min) with 25 m*M* Tris-HCl buffer (pH 7.2; 10% w/v) [17]. In a glass test tube, 0.1 ml of stomach homogenate was incubated in a shaking water bath for 1 h at 37°C in Tris-HCl buffer (pH 7.2), in the presence of various concentrations of TMP (50, 100 and 150 mg/kg). To this mixture, 1.5 ml of 1.0% TBA and 1.5 ml of 20% acetic acid were added and the mixture was further incubated for 1 h in a 95°C water bath. After cooling, 5 ml of *n*-butanol was added and the mixture was again shaken vigorously. The *n*-butanol layer was separated by centrifugation at 1,000 *g* for 10 min, and the upper *n*-butanol layer was collected to measure MDA production, in units of nmol/mg protein, at 532 nm on a spectrophometer [20].

Scavenging Activity of Free Radicals: Cytochrome c Test

The absolute ethanol-induced superoxide anion production in rat stomach was assayed according to the method described by McCord and Fridovich [10]. Xanthine oxidase converts xanthines to uric acid, simultaneously yielding its by-product superoxide anions, followed by direct reduction of ferricytochrome c to ferrocytochrome c, which has a specific UV absorbance at a wavelength of 550 nm. When a compound shows superoxide scavenging activity, there is a decrease in the UV absorbance spectrum in the reduction of ferricytochrome c.

Quantitative Evaluation of the Degree of Gastric Mucosal Injury

According to the method described by Umemoto et al. [16], the photographs in figures 1 and 2 were obtained using a digital camera (Epson Photo 700). One representative digital picture was chosen which showed a moderate degree of gastric mucosal injury and red color. The digital picture was downloaded onto a computer and the gastric lesions were processed by image analysis with PhotoShop software, by means of which the red color was quantified (press 256 level histogram) and expressed.

Drugs and Chemicals

Absolute ethanol, hemoglobin and hematocrit kits, TBA, sodium dodecyl sulfate, ferric chloride, *n*-butanol and cytochrome c were purchased from Sigma Chemical Company (St. Louis, Mo., USA). Acetic acid was obtained from Yon-chi Chemical Company in Taipei, Taiwan. TMP was a gift from the Institute of Chinese Materia Medica, China Academy of Traditional Chinese Medicine, Beijing, People's Republic of China.

Data Analysis

Data were shown as mean ± SE (n = 10). Statistical significance was assessed by one-way analysis of variance coupled with Dunnett's test or Newman-Keuls test. The level of significance was $p < 0.05$.

Results

Effect of TMP on Absolute Ethanol-Induced Gastric Lesions

Normal rat stomach and the gastric lesions induced by oral administration of 1.0 ml/kg absolute ethanol are shown in figures 1A and B, respectively. TMP was found to dose-dependently prevent the formation of gastric lesions induced by acute absolute ethanol administration (fig. 2A–C).

Severe gastric damage was observed from the outside of the damaged stomach as indicated by the appearance of

thick black or red lines. After opening the stomach, lesions were found in the gastric mucosa. Gastric lesions were located mostly in the corpus (the portion which secretes acid and pepsin); no gross lesions developed in the forestomach (the nonsecreting portion which is covered with squamous epithelium). Histologically, the absolute ethanol-induced gastric lesions consisted of necrosis which extended down through about two thirds of the gastric mucosa (involving the surface epithelium and the region of mucosal neck cells and parietal cells; not shown). Oral administration of 1.0 ml/kg absolute ethanol produced gross mucosa lesions in the stomach within 1 h. These lesions had hemorrhages which were linear or dotted in shape (fig. 1B). Table 1 shows that oral administration of 1.0 ml/kg absolute ethanol did not affect the hemoglobin and hematocrit levels. Oral administration of various doses of TMP (1.0 ml/kg) also did not affect hemoglobin and hematocrit levels.

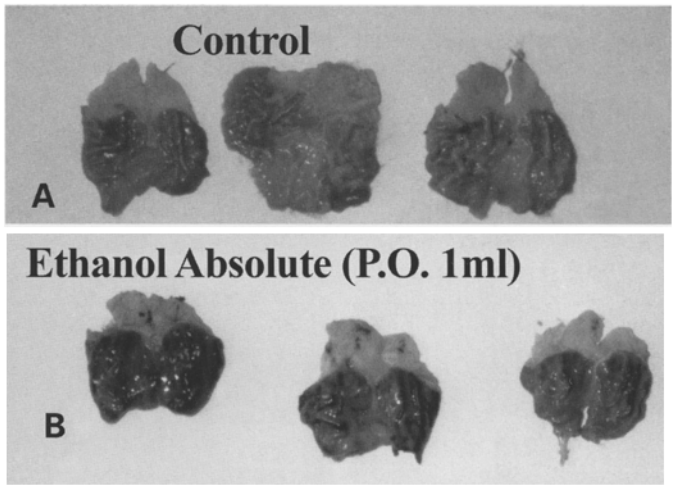

Fig. 1. Gastric lesions produced by absolute ethanol. One milliliter of absolute ethanol was administrated orally and the animals were killed 1 h later. The stomachs of the animals were dissected along the greater curvature. **A** Control animal. **B** Rat which received absolute ethanol. In **B**, extensive necrotic lesions can be seen in the corpus, of which the antrum was almost intact grossly. The forestomach (upper white portion) remained intact.

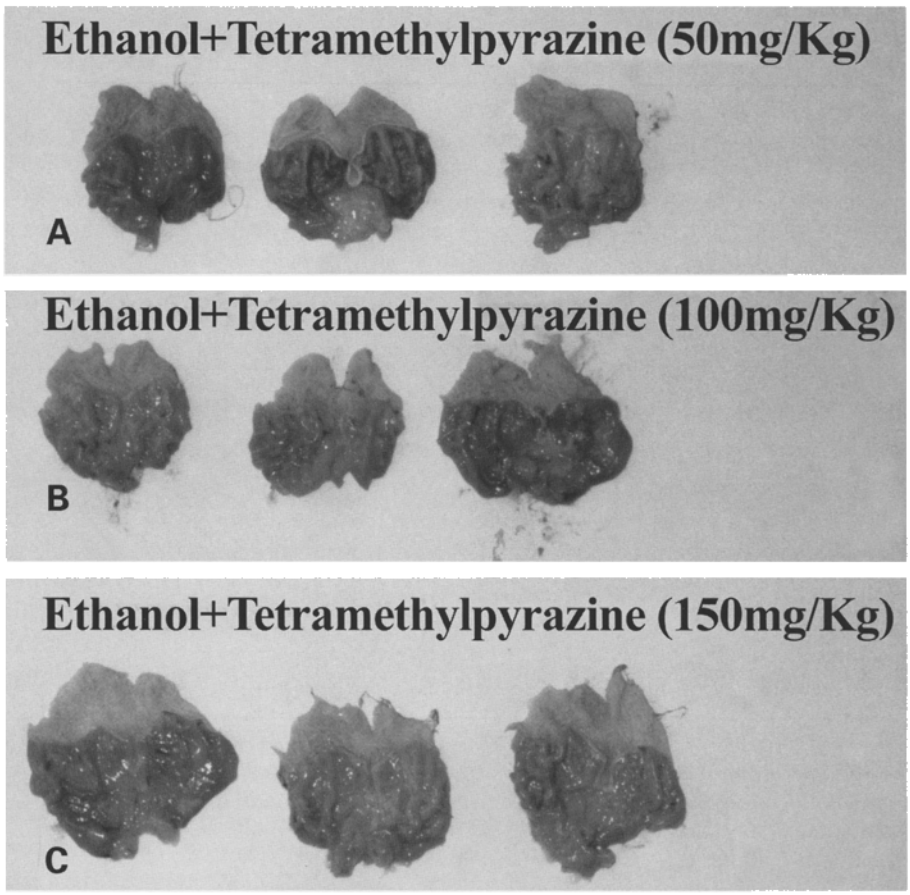

Fig. 2. Gastric cytoprotection by various doses of TMP against absolute ethanol-induced gastric lesions. One milliliter of absolute ethanol was given orally and the animals were killed 1 h later. **A**, **B** and **C** represent animals who received TMP at a concentration of 50, 100 and 150 mg/kg, respectively. All three TMP doses were shown to prevent the formation of gastric lesions induced by acute absolute ethanol administration.

Table 1. Effect of various oral doses of TMP on absolute ethanol (AE)-induced hemoglobin (Hb) and hematocrit (Hct) levels

Groups	Hb, g/dl	Hct, %
Saline control (1.0 ml/kg, p.o.)	12.8 ± 0.4	36.7 ± 1.8
AE (1.0 ml/kg, p.o.)	12.5 ± 0.6	32.5 ± 2.1
AE + TMP (50 mg/1.0 ml/kg)	12.4 ± 0.6	33.9 ± 1.7
AE + TMP (100 mg/1.0 ml/kg)	12.0 ± 0.4	34.8 ± 1.4
AE + TMP (150 mg/1.0 ml/kg)	12.4 ± 0.6	37.2 ± 1.8

Table 2. Inhibitory effect of various doses of TMP on $FeCl_2$-induced or $FeCl_2$ plus absolute ethanol (AE)-induced lipid peroxidation in the rat stomach homogenate in vitro

Groups	Concentration of TMP, mg/ml	MDA pmol/mg protein	Inhibition rate, %
Control		0.8 ± 0.089	
$FeCl_2$		61 ± 0.26***	
$FeCl_2$ + AE (0.1 ml)		66 ± 0.77###	
$FeCl_2$ + AE (0.5 ml)		96 ± 15##	
$FeCl_2$ + TMP	0.01	1.6 ± 0.25##	97.4
$FeCl_2$ + TMP	0.1	0.4 ± 0.081##	99.3
$FeCl_2$ + TMP	1.0	0.5 ± 0.096##	99.3

Each value represents the mean ± SE (n = 10). *** $p < 0.001$ compared to the control group; ## $p < 0.05$, ### $p < 0.001$ compared to the $FeCl_2$ group (Newman-Keuls test). A p value less than 0.05 was considered significant.

Table 3. Inhibitory effect of various doses of TMP on absolute ethanol (AE; 1.0 ml/kg, p.o.)-induced lipid peroxidation in the rat stomach homogenate in vivo

Groups	Dose of TMP mg/kg, p.o.	MDA nmol/mg protein	Inhibition rate, %
Normal control		0.064 ± 0.001	–
AE		0.113 ± 0.01***	–
AE + TMP	50	0.110 ± 0.001	2.7
AE + TMP	100	0.075 ± 0.002##	33.6
AE + TMP	150	0.063 ± 0.002###	44.2

Each value represents the mean ± SE (n = 10). *** $p < 0.001$ compared to the normal control group; ## $p < 0.05$, ### $p < 0.001$ compared to the AE group (Newman-Keuls test). The inhibition rates were compared between each of the AE + TMP groups and the AE group. A p value less than 0.05 was considered significant.

Table 4. IC_{50} of three different concentrations of TMP in the in vitro assay of the inhibition of lipid peroxidation

Groups	IC_{50}, μM
TMP (0.001 mg/ml)	0.122 ± 0.0001
TMP (0.1 mg/ml)	0.121 ± 0.0002
TMP (1.0 mg/ml)	0.115 ± 0.0001

The data shown are those derived from a concentration response tested with three different concentrations of TMP. Each value represents the mean ± SE of three independent assays in concentration determination studies; each assay was done in triplicate.

Table 5. Image analysis of absolute ethanol (AE)-induced gastric lesions and the protective effects of various concentrations of TMP

Groups	Red histogram, mm
Normal control	220.3 ± 22.4
AE (1.0 ml/kg, p.o.)	154.3 ± 52.5*
TMP (50 mg/kg)	154.0 ± 37.6
TMP (100 mg/kg)	186.7 ± 20.8#
TMP (150 mg/kg)	196.3 ± 20.9#

* $p < 0.0001$ compared to the normal control group; # $p < 0.0001$ compared to the absolute ethanol (1.0 ml/kg) group (one-way analysis of variance coupled with Dunnett's test). A p value less than 0.05 was considered significant.

Inhibitory Effect of TMP on Tissue Lipid Peroxidation in Absolute Ethanol-Induced Acute Gastric Lesions (in vitro)

Fe^{2+} is well known to stimulate lipid peroxidation in liver microsomes and mitochondria [23]. As expected, Fe^{2+} also stimulates lipid peroxidation in stomach mucosa. Various concentrations of TMP (0.01, 0.1 and 1.0 mg/ml) added in vitro could dose-dependently inhibit $FeCl_2$-stimulated lipid peroxidation in rat stomach homogenate (table 2).

As shown in table 2, the two concentrations of absolute ethanol dose-dependently increased $FeCl_2$-stimulated lipid peroxidation in the rat stomach homogenate.

Inhibitory Effect of TMP on Tissue Lipid Peroxidation in Absolute Ethanol-Induced Acute Gastric Lesions (in vivo)

Table 3 shows the inhibitory effect of pretreatment with various concentrations of TMP (50, 100 and 150 mg/kg) on lipid peroxidation in homogenate made from the stomachs of pretreated rats (table 3). TMP dose-dependently inhibited the lipid peroxidation-induced MDA formation in vivo.

Cytochrome c Test in vitro

In the cytochrome c test [15], the IC_{50} of three different concentrations of TMP (0.01, 0.1 and 1.0 mg/ml) in the in vitro assay of inhibition of lipid peroxidation ranged from 0.122 ± 0.0001 to 0.115 ± 0.0001 μM. TMP at a concentration of 1.0 mg/ml exhibited the strongest superoxide scavenging activity (table 4).

Quantitative Evaluation of the Degree of Gastric Mucosal Injury

From the quantitative image analysis of the degree of gastric mucosal injury, the gastric mucosal injury of the absolute ethanol-treated group showed a shift in the red histogram to the left side, i.e. loss of the red color (red histogram of the absolute ethanol model stomach: 154.27 ± 52.46 mm) (table 5). The therapeutic effect of various doses of TMP on the injury to rat gastric mucosa can be expressed as red color retained in the histogram. Although pretreatment with TMP at 50 mg/kg did not result in retention of the red color, the red color was retained in the groups pretreated with 100 and 150 mg/kg TMP ($p < 0.05$) (100 mg/kg: 186.72 ± 20.76 mm; 150 mg/kg: 196.28 ± 20.99 mm); these values were quite similar to that of normal control rat stomach (220.34 ± 22.40 mm).

Discussion

TMP is an important constituent of the medicinal plant *Ligusticum wallichii* French. It has been considered a true calcium antagonist [1, 12] and could be used clinically as a useful therapeutic agent in ischemic heart disease. In other tissues, it acts to suppress coronary vasoconstriction and ischemic changes produced by endothelin-1 [24]. TMP has also been reported as a drug to treat cardiovascular disease [8, 9]. It has been shown to possess pharmacological activities such as antiplatelet aggregation [14] and to improve microcirculation [21, 22]. TMP was also found to protect against gastric lesions induced by reserpine by its promotion of the secretion of gastric barrier mucus [19]. However, the mechanism by which TMP protects the gastric mucosa from absolute ethanol-induced damage is still unclear.

It has been reported that a decrease in the quantity of gastric mucosa was associated with gastric mucosa injury induced by aspirin [3]. The relationship between free radical formation and absolute ethanol-induced gastric lesions is still unclear. Our present study showed that absolute ethanol significantly damaged the gastric mucosa (fig. 1), and pretreatment with TMP (fig. 2) could significantly prevent absolute ethanol-induced gastric lesions. Image analysis of gastric lesions further confirmed these protective effects of TMP (table 5). The hematocrit and hemoglobin values of the absolute ethanol-induced gastric ulcer group (table 1) were different from that of aspirin-induced gastric ulcers [18], indicating that the oral administration of aspirin can induce a severe decrease in hemoglobin and hematocrit levels.

Table 1 shows that the oral administration of 1.0 ml/kg absolute ethanol did not significantly interfere with hemoglobin and hematocrit levels. After oral administration of various doses of TMP (1.0 ml/kg), no further interference could be noted.

TMP inhibited absolute ethanol-induced MDA formation in vitro (table 2). In the free radical scavenging assay, TMP was further confirmed to have dose-dependent antioxidant activity in the cytochrome c test (table 2). These findings indicated that a decrease in MDA formation is likely to play an important role in the prevention of gastric lesions induced by absolute ethanol. Local gastric mucosal ischemia has been found to be an important factor in ulcerogenesis [14, 21]. TMP has been shown to be a direct vasodilator and can effectively improve the blood supply to the gastric mucosa. Thus, part of the beneficial effect observed in the present study may be mediated through this hemodynamic mechanism.

Our present study suggested that the production of free radicals may be involved in the pathogenesis of gastric lesions induced by absolute ethanol, and that TMP significantly inhibited the formation of ethanol-induced gastric lesions probably by its ability to inhibit membrane lipid peroxidation and free radical formation or due to its free radical scavenging ability. Further studies will determine the time course of protective action of TMP in relation to the stomach damage induced by absolute ethanol. In conclusion, TMP could be clinically beneficial in the treatment of absolute ethanol-induced acute gastric ulcers. However, detailed studies of its pharmacological properties and mechanisms of action must be performed.

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