

Palmul-tang, a Traditional Herbal Formula, Protects against Ethanol-induced Acute Gastric Injury in Rats

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Objectives: *Palmul-tang* (*hachimotsu-to* in Japanese and *bawu-tang* in Chinese) is a mixture of eight herbs. It is traditionally used for the treatment of anemia, anorexia, general weakness, and female infertility in China, Japan, and Korea. In this study, we investigated the protective effects of *Palmul-tang* water extract (PTE) against ethanol-induced acute gastric injury in rats.

Material and Methods: Acute gastric lesions were induced by intragastric administration of 5mL/kg body weight of absolute ethanol to each rat. Control group rats were given PBS orally and the ethanol group (EtOH group) received absolute ethanol (5mL/kg) by oral gavage. The positive control group and the PTE group were given oral doses of omeprazole (50mg/kg) or PTE (400mg/kg), respectively, 2 h prior to the administration of absolute ethanol. The stomach of each animal was excised and examined for gastric mucosal lesions. To confirm the protective effects of PTE, we evaluated the degree of lipid peroxidation, the level of reduced glutathione (GSH), and the activities of the antioxidant enzymes catalase, glutathione-S-transferase, glutathione peroxidase, and glutathione reductase in the stomach.

Results: PTE reduced ethanol-induced hemorrhage and hyperemia in the gastric mucosa. PTE reduced the increase in lipid peroxidation associated with ethanol-induced acute gastric lesions and increased mucosal GSH content and the activities of antioxidant enzymes.

Conclusion: These results indicate that PTE protects gastric mucosa against ethanol-induced acute gastric injury by increasing antioxidant status. We suggest that PTE could be developed as an effective drug for the treatment of acute gastric injury.

Key Words : *Palmul-tang*, herbal formula, ethanol, acute gastric injury, antioxidative effect

Introduction

Oxidative stress is generally defined as excess formation or insufficient removal of reactive oxygen species (ROS) and plays a critical role in the development of various diseases^{1,2}. Oxidative stress induces degradation of cell membrane lipids, resulting in cell damage. This process is known as lipid peroxidation and produces malondialdehyde (MDA) as

an end product. Organisms have antioxidant defense systems to reduce oxidative stress and lipid peroxidation. The human antioxidant defense system includes reduced glutathione (GSH), catalase, glutathione-S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR), and superoxide dismutase (SOD)³. Oxidative stress plays an important role in gastric injury because the gastric mucosa is continuously exposed to factors such as ethanol, stress, and non-

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steroidal anti-inflammatory drugs⁴⁻⁶). These agents induce oxidative stress, which increases lipid peroxidation and decreases the activity of antioxidant enzymes, resulting in acute gastric damage, including hemorrhage, congestion, edema, erosion, and ulcers^{7,8}). Treatment of acute gastric injury is focused on improving antioxidant status and reducing lipid peroxidation. Indeed, many antioxidants exhibit protective effects against ethanol-induced acute gastric injury^{9,10}).

Palmul-tang (*Bawu-tang* in Chinese, *Hachimotsu-to* in Japanese), an oriental traditional herbal formula, is composed of eight herbs (Table 1). The effects of *Palmul-tang* extract (PTE) have been confirmed by several researchers. Previous studies have shown that PTE enhances female reproductive function by preventing apoptosis and DNA damage, promoting cell proliferation¹¹), conferring an antiallergic effect¹²), and protecting against hypoxic damage in H9c2 cardiomyoblast cells¹³). Its constituent crude herbs and compounds also showed pharmacological effects. *Atractylodes rhizome*¹⁴), *Angelicae gigantis radix*¹⁵), *Ginseng radix*¹⁶), *Glycyrrhizae radix*¹⁷), *Hoelen*¹⁸), and *Paeoniae radix*¹⁹) showed anti-inflammatory and antioxidative effects. *Atractylodes rhizome*²⁰), *Glycyrrhizae radix*²¹), and *Ginseng radix*²²) attenuated experimentally induced gastric injury. In addition, glycyrrhizin²³), ferulic acid²⁴), liquiritin²⁵), and paeoniflorin²⁶) have antioxidative effects. Previous studies showed that glycyrrhizin²⁷)

and paeoniflorin²⁸) protect against irritant-induced gastric mucosal injury. In oriental medicine, PTE is used for the treatment of symptoms such as dizziness, pale or sallow complexion, discomfort of digestion which are caused by the deficiency of *Qi* and blood. Because *Qi* and blood are produced in the spleen, *Qi* and blood are sufficiently maintained in body when *Qi* of the spleen is intense. The spleen is also closely related with digestion and dietary absorption. If stomach diseases such as gastritis or gastric ulcer occur, the production of *Qi* and blood in the spleen is insufficient. Therefore, PTE supplements the *Qi* and blood in the body via tonifying the *Qi* of the spleen, resulting in attenuation of gastric damage.

Considering this information, we hypothesized that PTE would attenuate absolute ethanol-induced gastric injury via enhancement of antioxidative status. Therefore, we evaluated the protective effects of PTE on ethanol-induced acute gastric injury in rats.

Materials and Methods

1. Preparation of PTE

The PTE was prepared in our laboratory (Table 1) from a mixture of chopped crude herbs. Before performing the study, the identity of each crude herb was confirmed by a pharmacist and by professors at the College of Oriental Medicine. PTE was extracted

Table 1. Composition of PTE

Scientific name	Amount (g)	Company of purchase	Source
<i>Ginseng Radix Alba</i>	4.5	Omniherb	Korea
<i>Cnidii Rhizoma</i>	4.5	Omniherb	Korea
<i>Rehmanniae Radix Preparata</i>	4.5	Omniherb	Korea
<i>Hoelen</i>	4.5	HMAX	China
<i>Glycyrrhizae Radix</i>	4.5	HMAX	China
<i>Angelicae Gigantis Radix</i>	4.5	Omniherb	Korea
<i>Paeoniae Radix</i>	4.5	Omniherb	Korea
<i>Atractylodis Rhizoma</i>	4.5	Omniherb	Korea
Total amount	36.0		

in distilled water at 100°C for 2 h. The solution was evaporated to dryness and freeze-dried (yield: 25.9%). Lyophilized PTE (200mg) was dissolved and mixed in distilled water (20mL). The solution was filtered through a SmartPor GHP syringe filter (0.2µm pore size, Woongki Science, Seoul, Korea).

2. Reagents and materials

Ferulic acid and 5-hydroxymethylfurfural (HMF) were purchased from Sigma-Aldrich (St Louis, MO, USA). Albiflorin, paeoniflorin, and glycyrrhizin were produced by Wako (Osaka, Japan). Liquiritin and nodakenin were purchased from NPC BioTechnology Inc. (Daejeon, Korea). The purity of all reference standards was greater than 98.0%. HPLC-grade methanol, acetonitrile, and water were obtained from J.T. Baker (Phillipsburg, NJ, USA). Glacial acetic acid was of analytical reagent grade (Junsei, Tokyo, Japan). The materials for the PTE were purchased from Omniherb (Yeongcheon, Korea) and HMAX (Chungbuk, Korea). A voucher specimen (2008-KE05-1~KE05-8) has been deposited at the Herbal Medicine EBM Research Center, Korea Institute of Oriental Medicine.

3. Preparation of standard solutions and calibration curves

Standard stock solutions of 5-HMF, albiflorin, paeoniflorin, liquiritin, ferulic acid, nodakenin, and glycyrrhizin (all 1,000µg/ml) were prepared in methanol and stored below 4°C. Working standard solutions were prepared by serial dilution of stock solutions with methanol. Calibration curves were obtained by assessment of peak areas from standard solutions with the following concentration ranges: 5-HMF, 6.25-50.00µg/mL; albiflorin, ferulic acid, and glycyrrhizin, 3.13-25.00µg/mL; paeoniflorin, 9.38-150.00µg/mL; liquiritin, 12.50-100.00µg/mL; and nodakenin, 4.69-75.00µg/mL.

4. HPLC analysis

We performed simultaneous analysis using a Shimadzu LC-20A HPLC system (Shimadzu Co., Kyoto, Japan), consisting of a solvent delivery unit, an inline degasser, a column oven, an autosampler, and a photodiode array (PDA) detector. The data processor used LC solution software (Version 1.24). The analytical column was a Gemini C18 (250mm×4.6mm; particle size, 5µm; Phenomenex, Torrance, CA, USA) and was maintained at 40°C. The mobile phases consisted of 1.0% (v/v) aqueous acetic acid (A) and 1.0% (v/v) acetic acid in acetonitrile (B). The gradient flow was as follows: (A)/(B)=95/5 (0min)→(A)/(B)=40/60 (40min)→(A)/(B)=0/100 (45min; hold for 5min)→(A)/(B)=95/5 (55min). The analysis was carried out at a flow-rate of 1.0mL/min with PDA detection at 230nm (for albiflorin and paeoniflorin), 254nm (for glycyrrhizin), 280nm (for 5-HMF and liquiritin), 320 nm (for ferulic acid), and 330 nm (for nodakenin). The injection volume was 10µL.

5. Acute gastric injury

Specific pathogen-free male Sprague-Dawley rats, weighing 200-250g (aged 6 weeks) were purchased from the Orient Co. (Seoul, Korea) and used after a week of quarantine and acclimatization. The animals were kept in a room at 23±3°C with a relative humidity of 50% under a controlled 12h/12h light/dark cycle. The rats were given a standard rodent chow and sterilized tap water *ad libitum*. All experimental procedures were performed in compliance with the NIH Guide for the Care and Use of Laboratory Animals and the National Animal Welfare Law of Korea

Acute gastric lesions were induced by intragastric administration of absolute ethanol in according to a previously described method²⁹⁾. Twenty-eight rats were allocated to one of four groups and fasted for 18 h before the experiment. Rats in the control group were given PBS orally (5ml/kg body weight), and

the absolute-ethanol group (EtOH group) received absolute ethanol (5mL/kg body weight) by oral gavage. Rats in the positive control group were given omeprazole (50 mg/kg body weight) by oral administration 2 h prior to the administration of absolute ethanol. Omeprazole was used as a positive control drug because it possesses anti-inflammatory and antioxidant activities and has been widely used for the treatment of gastritis³). The dose of PTE was based on the preliminary study. In the preliminary study, PTE was administered to SD rats at dose levels of 200 and 400mg/kg. The reduction of gastric mucosal lesions was detected in 400 mg/kg treated animals more than 200mg/kg treated animals in macroscopic examination. The fourth group received PTE (400mg/kg body weight) 2 h prior to absolute ethanol administration.

6. Macroscopic examination of gastric mucosa

Animals were sacrificed using an overdose of 50mg/kg pentobarbital administered 1 h after they received the absolute ethanol treatment. The stomach was removed from each animal, and opened along the greater curvature. The tissue was gently rinsed in PBS. The stomach was stretched out a piece of cork with the mucosal surface facing upward, and was examined in a standard position for gastric mucosal lesions. Photographs of hemorrhagic erosions in the stomach were taken with a Photometric Quantix digital camera. After the gastric lesions were photographed, the stomach tissue was stored at -70°C for biochemical analysis.

7. Biochemical analysis

The stomach tissue was cut into small pieces and homogenized (1/10w/v) with tissue lysis/extraction reagent and a protease inhibitor (Sigma, MI, USA). The homogenates were centrifuged at 12,000 rpm for 10min at 4°C to precipitate cell debris, and the supernatant was used to measure levels of malondial-

dehyde (MDA), GSH, catalase, GST, GPx, and GR. Total protein was determined using a protein assay reagent (Bio-Rad Laboratories).

Lipid peroxidation was estimated by determination of MDA using a thiobarbituric acid-reactive substances (TBARS) assay kit (BioAssay Systems, CA, USA). In brief, a 100µL aliquot of homogenate was mixed with 100µL of 10% trichloroacetic acid and incubated for 15min on ice. The mixture was centrifuged at 12,000rpm for 5min at 4°C and then 200µL of the supernatant was mixed with 200µL of thiobarbituric acid and incubated at 100°C for 60min. After the mixture had cooled, absorbance at 535nm was measured. The results are expressed as nmol of MDA/mg protein. The levels of reduced glutathione (GSH) were measured using a GSH assay kit (Cayman, Ann Arbor, MI), which involves an optimized enzymatic recycling method and glutathione reductase. The sulfhydryl group of GSH reacts with 5, 5-dithio-bis-2-nitrobenzoic acid (DTNB) and produces a yellow-colored 5-thio-2-nitrobenzoic acid (TNB). A mixed disulfide, GSTNB (between GSH and TNB), is produced concomitantly and is reduced by glutathione reductase to recycle the GSH, producing more TNB. The rate of TNB production is directly proportional to this recycling reaction which is in turn directly proportional to the concentration of GSH in the sample. The absorbance of TNB at 410nm was used to estimate the amount of GSH in the sample. The GSH level was expressed as µmol/mg protein.

Glutathione-S-transferase (GST) activity was determined by measuring the conjugation of 1-chloro-2, 4-dinitrobenzene (CDNB) with reduced glutathione using GST assay kit (Cayman). Conjugation is accompanied by an increase in absorbance at 340nm and rate of increase is directly proportional to the GST activity in the sample. Glutathione peroxidase (GPx) activity was measured indirectly via a coupled reaction with glutathione reductase using a GPx assay kit (Cayman). Oxidized glutathione, produced upon reduction of hydroperoxide by GPx, is recycled to its reduced

state by GR and nicotinamide adenine dinucleotide phosphate (NADPH). The oxidation of NADPH to NADP⁺ is accompanied by a decrease in absorbance at 340nm. Glutathione reductase (GR) activity was determined by measuring the rate of NADPH oxidation using a GR assay kit (Cayman). Oxidation of NADPH to NADP⁺ is accompanied by a decrease in absorbance at 340 nm. Catalase activity was measured according to the peroxidatic function of catalase using a catalase assay kit (Cayman). This method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of hydrogen peroxide. The formaldehyde produced is measured colorimetrically at 540nm with 4-amino-3-hydrazino-5-mercapto-1, 24-triazole (Purpald) as the chromagen. The activities of catalase, GST, GPx, and GR, were expressed as U/mg protein.

8. Statistical analysis

Data are expressed as means ± standard deviations (SDs). Statistical significance was determined using analysis of variance (ANOVA). If a test showed a significant difference between groups, the data were analyzed by a multiple comparison procedure using Dunnett's test³⁰. Statistical analysis was performed

using SYSTAT version 10. The levels of significance were set as $p < 0.05$ and $p < 0.01$.

Results

1. HPLC analysis of PTE

A three-dimensional chromatogram was obtained using an HPLC-PDA detector. Under optimized chromatography conditions, seven components were eluted within 45min using mobile phases consisting of solvent A (1.0% v/v aqueous acetic acid) and solvent B (1.0% v/v acetic acid in acetonitrile). The three-dimensional HPLC chromatogram for PTE is shown in Fig. 1. The retention times of the components were 8.166min (5-HMF), 16.21min (albiflorin), 17.16min (paeoniflorin), 19.23min (liquiritin), 19.87min (ferulic acid), 20.36min (nodakenin), and 39.96min (glycyrrhizin). The linearity of the peak area (y) versus concentration (x, µg/mL) curve for each component was used to calculate the contents of the main components in the PTE. The correlation coefficients (r²) of calibration curves for the seven constituents was greater than 0.9998. The concentrations of the seven components in PTE ranged from 0.63 to 6.22 mg/g (Table 2).

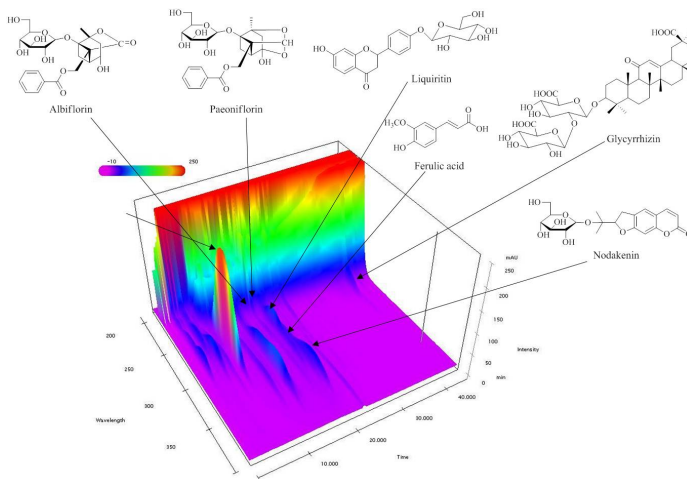


Fig. 1.

Table 2. The contents of seven marker compounds in PTE (n=3).

Batch (#)	Content (mg/g)																				
	5-HMF			Albiflorin			Paeoniflorin			Liquiritin			Ferulic acid			Nodakenin			Glycyrrhizin		
	Mean	SD	RSD (%)	Mean	SD	RSD (%)	Mean	SD	RSD (%)	Mean	SD	RSD (%)	Mean	SD	RSD (%)	Mean	SD	RSD (%)	Mean	SD	RSD (%)
1	1.82	0.002	0.135	1.23	0.007	0.580	6.16	0.079	1.287	3.80	0.064	1.681	0.63	0.006	0.928	3.03	0.013	0.426	2.91	0.002	0.052
2	1.82	0.003	0.155	1.29	0.010	0.746	6.10	0.051	0.835	3.77	0.020	0.533	0.64	0.004	0.622	3.07	0.024	0.778	2.92	0.003	0.105
3	1.82	0.002	0.133	1.30	0.006	0.488	6.22	0.071	1.146	3.79	0.012	0.322	0.64	0.005	0.783	3.00	0.007	0.219	2.91	0.002	0.072

2. PTE reduced ethanol-induced acute gastric injury

Gross examination of gastric mucosa showed that the EtOH group had gastric mucosal injuries (hemorrhage and hyperemia) (Fig. 2), whereas no abnormalities or lesions were found in the normal control group. In contrast, PTE pretreatment attenuated gastric mucosal injury compared with the EtOH group to a degree similar to that in the omeprazole-treated group.

3. PTE reduced the increase in lipid peroxidation and GSH content in ethanol-induced acute gastric lesions

The concentration of MDA, an end product of lipid peroxidation, was greater in the EtOH group (13.32±1.32nmol/mg protein, p<0.05) than in control rats (10.09±0.66 nmol/mg protein) (Fig. 3A). The MDA level in PTE-treated rats differed significantly (9.84±1.25nmol/mg protein, p<0.05) from those in

the EtOH group. Also, in the omeprazole-treated group (8.73±1.08nmol/mg protein, p<0.01) was observed a significant reduction compared with the EtOH group. Conversely, the content of GSH in the stomachs of the EtOH group (32.30±1.09µmol/mg protein, p<0.01) was less than that in the control group (45.99±2.05 µmol/mg protein), and that in the PTE-treated groups was significantly greater (44.19±2.05µmol/mg protein, p<0.01) than that in the EtOH group (Fig. 3B).

4. PTE attenuated the ethanol-induced decrease in the activities of antioxidant enzymes

As shown in Fig. 4A, catalase activity in the EtOH group (238.13±28.07U/mg protein, p<0.01) was less than that in the control (326.23 ± 29.08 U/mg protein). However, PTE treatment resulted in a significant increase (301.41±24.88U/mg protein, p<0.01) in catalase activity compared with that of the EtOH group. Also, the omeprazole-treated group (283.55±

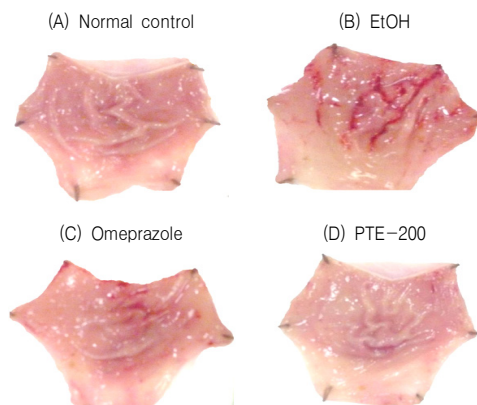


Fig. 2.

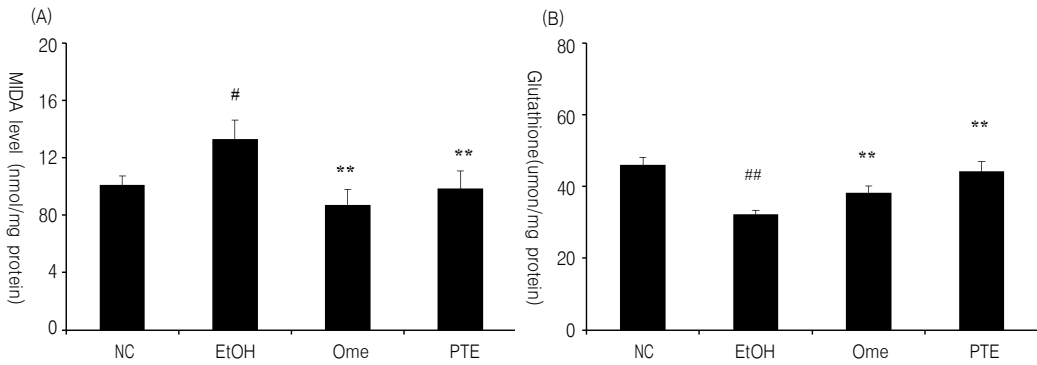


Fig. 3.

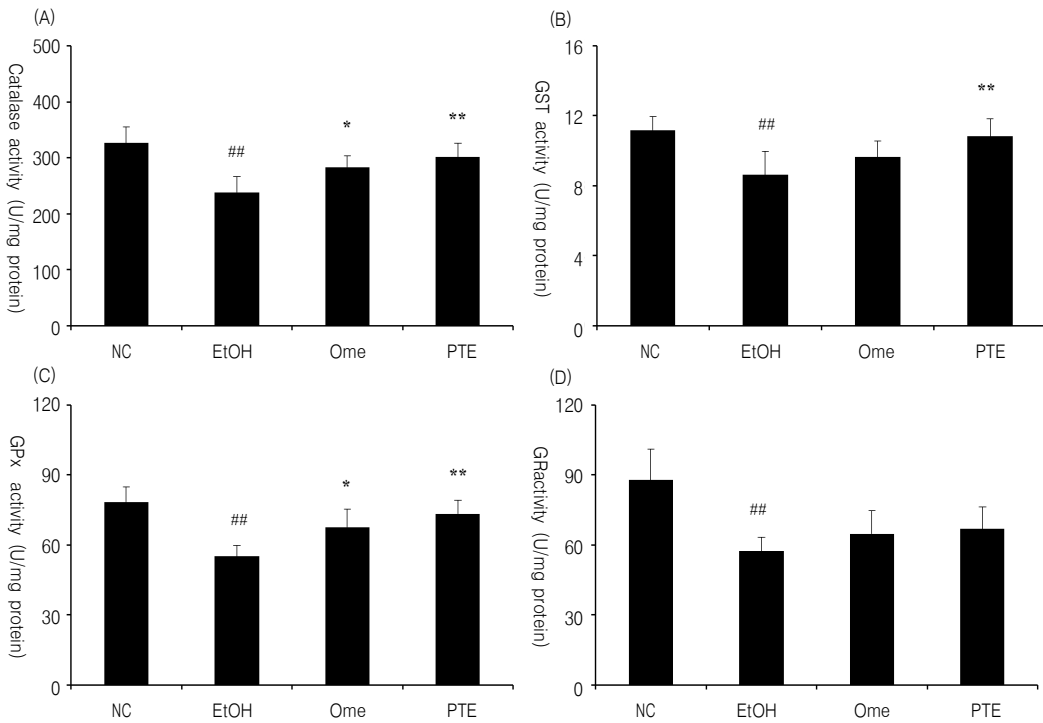


Fig. 4.

20.47U/mg protein, $p < 0.05$) showed a significant increase compared with the EtOH group. GST (10.83 ± 1.00 U/mg protein, $p < 0.01$) and GPx activities (73.33 ± 5.81 U/mg protein, $p < 0.01$) in the PTE-treated group increased markedly more than those in the EtOH group (8.63 ± 1.34 U/mg protein and 55.12 ± 4.83 U/mg protein in GST and GPx, respectively), as well as the results

of catalase activity (Fig. 4B and 4C). Although GR activity increased to a greater extent in the PTE-treated group (66.91 ± 9.56 U/mg protein) than EtOH group (57.34 ± 6.06 U/mg protein), the difference was not significant (Fig. 4D).

Discussion

In the present study, we investigated the protective effects of PTE on absolute ethanol-induced acute gastric injury in rats. Animals treated with absolute ethanol developed severe gastric injury (hemorrhage and hyperemia), elevated lipid peroxidation, and reductions in activities of antioxidative defense components (GSH, catalase, GST, GPx and GR). In contrast, in PTE-treated animals, ethanol-induced gastric injury and lipid peroxidation were attenuated and the activities of antioxidant enzymes were elevated.

In normal metabolism, there is a balance between the generation of ROS and the antioxidant defense system. However, pathological conditions induced by various chemicals and stress increase the production of ROS, causing oxidative stress, which results in an increase in lipid peroxidation and decreases the activities of antioxidant enzymes in various organs¹. Lipid peroxidation is usually assessed using MDA level. MDA, an end product of lipid peroxidation, is a major reactive aldehyde formed during peroxidation of polyunsaturated fatty acids in biological membranes³¹. GSH acts as a nonenzymatic antioxidant both intra- and extracellularly in conjunction with various enzymatic processes that reduce levels of hydrogen peroxide and hydroperoxides³². In our study, absolute ethanol induced gastric lesions increased the level of lipid peroxidation, and reduced the level of GSH, as was previously described³³. Conversely, PTE reduced lipid peroxidation and increased gastric tissue GSH content. These results suggest that PTE reduces ethanol-induced oxidative stress in gastric tissue.

Antioxidant enzymes such as catalase, GST, GPx, and GR induce direct or indirect enzymatic processes that decrease oxidative stress caused by ROS. Of the antioxidant enzymes, catalase is ubiquitously present in all aerobic cells, where it catalyzes the decomposition of hydrogen peroxide to water and oxygen³⁴. GST is a soluble protein located in the cytosol and the

endoplasmic reticulum that catalyzes conjugation of GSH with many xenobiotics and their reactive metabolites^{35,36}. This activity detoxifies endogenous compounds such as peroxidized lipid and promotes the breakdown of xenobiotics. GR reduces glutathione disulfide (GSSG) to the sulfhydryl form, GSH, which is an important cellular antioxidant³⁷. Several studies have shown that absolute ethanol increases lipid peroxidation and reduces the activities of antioxidant enzymes in gastric tissue^{6,33}. In our study, absolute ethanol induced gastric lesions and decreased the activities of antioxidant enzymes (GST, catalase, GPx, and GR) compared with those in the normal control group. In contrast, PTE treatment significantly increased the activities of antioxidant enzymes in gastric tissue compared with those of animals that received ethanol alone. These results are similar to those of previous studies that evaluated the protective effects of antioxidants against ethanol-induced gastric injury^{9,10,38}. Thus, we consider that PTE attenuated ethanol-induced gastric injury via its antioxidant activity.

Rat models of ethanol-induced gastric injury are considered reliable tools for studying the pathogenesis of acute gastric injury²⁹. Previous studies showed that absolute ethanol-induced gastric lesions are characterized by hemorrhage, ulceration, and hyperemia^{7,8}. In our study, we observed gastric mucosal changes in animals treated with absolute ethanol, but the degree of change was less in animals treated with PTE and ethanol than in animals treated with ethanol alone. Considering these observations, oral administration of PTE appears to attenuate ethanol-induced acute gastric injury.

PTE induces to activate the defense mechanism for various toxic materials in various organs via improvement of spleen function in oriental medicine. The present study showed that PTE reduced ethanol-induced gastric injury via antioxidant status in stomach. Therefore, we considered that protective effects of PTE via increase in the antioxidant status are closely related the enhanced physiological conditions induced

by PTE administration, including increased *Qi* and blood in the body. This is the first study to demonstrate the protective effects of PTE in ethanol-induced acute gastric injury of rats. This protective effect was caused in part by the antioxidative activity of PTE. We suggest that PTE may be a useful drug for the treatment of acute gastric injury.

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