Protective effect of Pu-erh tea extracts against ethanol-induced gastric mucosal damage in rats

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Abstract. Pu-erh tea has become a focus of research due to its reported biological activities, including anti-oxidation, anti-inflammation and anti-immunosenscence. The present study was performed to evaluate the potential gastroprotective function of Pu-erh tea extracts against ethanol-induced gastric mucosal damage in rats. Sprague Dawley rats were randomly divided into seven groups: A normal control, a model control, a cimetidine (0.08 g/kg) group, three Pu-erh tea extracts groups (low, moderate and high-dose; 0.50, 1.00 and 1.50 g/kg, respectively), and a green tea powder (1.00 g/kg) group. The normal and model groups were pre-treated with distilled water while the other groups were respectively administered cimetidine, Pu-erh tea extracts and green tea powder for 14 days. Then, absolute ethanol was orally administered to the rats of all groups excluding the normal controls. The effects of the pretreatments on gastric mucosal injury were evaluated by gross assessment of gastric lesions, examination of histopathology and determination of myeloperoxidase (MPO) activity and asymmetric arginine (ADMA) concentration in the gastric mucosal homogenate. Pre-treatment with cimetidine or Pu-erh tea extracts markedly suppressed the formation of ethanol-induced gastric lesions. Furthermore, clear decreases in MPO activity and ADMA concentration in the gastric mucosal homogenate were observed following pretreatment with cimetidine or Pu-erh tea extracts. The anti-gastric ulcer activity of green tea was less than that of Pu-erh tea. Overall, these effects of Pu-erh tea extracts may be due to potential functions in protecting the gastric mucus layer and suppressing inflammation.

Key words: gastric mucosal damage, Pu-erh tea extracts, rats, myeloperoxidase, asymmetric arginine

Introduction

Pu-erh tea is a type of fermented tea that incorporates microorganism metabolites during the fermentation process (1). It is prepared from processed leaves and buds of the broad-leaf variety of the tea plant [Camellia sinensis var. assamica (L.) O. Kuntze; Theaceae], primarily grown in Yunnan province of China (2). Pu-erh tea is widely consumed worldwide as part of a normal diet. Its chemical components and properties vary due to the time of year that the tealeaves are harvested and the fermentation methods used. The main components of Pu-erh tea extracts include tea polyphenol, tea pigment, tea polysaccharide and alkaloids (3). In previous years, studies on the potential health-beneficial effects of Pu-erh tea have identified a range of biological activities, including anti-oxidation (4), anti-obesity (5), anti-inflammation, anti-immunosenscence (6), anti-hyperlipidemic (7), antitumor (8), antiviral (9) and antibacterial (10) effects. However, its potential action in reducing or limiting gastric mucosal injury and the mechanisms associated with these effects have not been experimentally clarified.

It is established that gastric mucosal injury occurs due to an imbalance between mucosal defensive and aggressive factors (11). Gastric mucosa is frequently exposed to HCl, pepsin, bile acids, ethanol, non-steroidal anti-inflammatory drugs (12), Helicobacter pylori toxins and other noxious substances (13,14). Defensive mechanisms manifest through the activation of various mucosal protection lines, including of mucus and bicarbonate secretion, the mucosal barrier itself, gastric micro-circulation (15) and the renin-angiotensin system (16).

The biological functions of Pu-erh tea in the stomach are broad according to previous literature, and include detoxification, promotion of food digestion, regulation of gastrectasia and removal of fats (17). However, there is a lack of data on the gastro-protective activity of Pu-erh tea. In the present study, experiments were designed to investigate the effect and mechanisms of Pu-erh tea extracts on preventing gastric mucosa injury in rats induced with ethanol, namely by histopathological examination and determination of the levels of myeloperoxidase (MPO) and asymmetric arginine (ADMA) in the stomach tissue. MPO activity is regularly used as an indicator for evaluating the progression of intestinal ulcers: it is known to be increased in...
the ulcerated condition and to be reduced through the healing process (18). Meanwhile, it has been demonstrated that ADMA facilitates in gastric mucosal injury: acute administration of high-dose ethanol significantly increased the gastric ulcer index, which was concomitant with an increase of ADMA, while the increased level of ADMA was suppressed by the resveratrol analog BTM-0512 (19). Therefore, these indicators were used in the present study to determine the protective effect of Pu-erh tea extracts against ethanol-induced gastric mucosal damage in rats.

Materials and methods

Animals. Sprague-Dawley male rats (n=126) were obtained from the Vital River Laboratories Co., Ltd. (Beijing, China), the weight of which ranged from 180 to 220 g. They were group-housed (5 rats per cage) in standard and pathogen-free environmental conditions (22±1˚C, 60±5% humidity, 12-h light/dark cycle) with free access to a standard commercial diet and water ad libitum. Animals were acclimatized to the environment for at least one week. The study protocols were approved by the Tasly Laboratory Animal Welfare and Ethics Committee of Tasly Pharmaceuticals, Inc. (Tianjin, China), and conducted according to the rules of animal experimentation and the guide for the Care and Use of Laboratory Animals of Tasly Pharmaceuticals, Inc. The rats were randomly divided into seven groups (n=18 per group).

Chemicals and drugs. An aqueous extract of Pu-erh tea was provided by Tasly Pharmaceuticals, Inc., which was dissolved in distilled water and prepared as described previously (1).

The ingredients in fermented Pu-erh tea include caffeine, polyphenols, γ-aminobutyric acid, theanine, statin, polysaccharides (3,20-22), theaflavins, thearubigins and theabrowins (23-26).

Cimetidine, used as a reference drug in this study, was obtained from GlaxoSmithKline (Shanghai, China) and dissolved in distilled water. A standardized powder of green tea was purchased from Damin Foodstuff (Zhangzhou Co., Ltd., (Zhangzhou, China). Hematoxylin and eosin were provided by Muto Pure Chemicals Co., Ltd. (Tokyo, Japan). Absolute ethanol, formalin, paraffin and dimethylbenzene were supplied by Rionlon (Tianjin) Industry Co., Ltd. (Tianjin, China). ELISA kits for rat MPO (catalogue no. CK-E30635) and rat ADMA (catalogue no. CK-E30769) were provided by Shanghai Bogoo Biotechnology Co., Ltd. (Shanghai, China). Other reagents used in the study were of analytical grade or higher without further purification.

Ethanol-induced gastric lesion and pharmacological intervention. The rats in normal and model groups were intragastrically (i.g.) administered 10 ml/kg distilled water, while those in other groups were administered 0.08 g/kg cimetidine (i.g.), Pu-erh tea extracts at 0.50, 1.00 and 1.50 g/kg, and 1.00 g/kg green tea powder, respectively. The animals were administered with the test drugs or distilled water between 8:00-9:00 am once a day for 14 consecutive days. Acute gastric lesions were created by intragastric application of absolute ethanol according to a common method (27). The rats, excluding those in the normal control group, were orally administrated absolute ethanol (5 ml/kg) on day 15 after being fasted for 24 h but with free access to water. At 60 min after ethanol administration, the rats were euthanized by cervical dislocation following an overdose of diethyl ether anesthesia (28-30) and the stomachs were immediately excised. Each stomach was opened along the greater curvature as described by Balan et al (31) and rinsed in cold saline solution. Half of the stomachs in each group were fixed in 10% buffered formalin at room temperature for 15 min for anatomical examination, while the others were immediately preserved in liquid nitrogen (-196˚C) for determination of MPO and ADMA.

Gross assessment of gastric lesions. After 15 min, the stomachs in 10% buffered formalin were removed. Photographs of the gastric mucosa were taken, and the mucosal lesions were scored by a laboratory animal technician blinded to the experimental protocol. The length and width of each injured area of the gastric mucosa were measured with a vernier caliper. Gastric mucosal ulcer index (UI) was determined according to Table I, following the guidelines of the Technical Standards for Testing and Assessment of Health Food (32), and calculated according to the following formula: UI = spot erosion point + erosion length points + (erosion width points x2). The inhibitory rate (%) was calculated by the formula: I (%) = [(Ulcontrol - UItreated)/Ulcontrol] x 100% (33).

Histopathological examination. The formalin-fixed stomach tissues were embedded in paraffin wax and gradient dehydrated in increasing concentrations of ethanol (70-100% v/v). The specimens were sectioned (4-μm thick) and stained with hematoxylin (~1.5 min) and eosin (30 sec) at room temperature for histopathological examination. An epithelial damage scoring system (34) was applied to rate histopathological changes, including congestion, edema, hemorrhage, degeneration and necrosis in the gastric mucosa, by light microscopy (x400), as described in Table II. The total score of pathological changes was calculated by the formula: Total score = hyperemia points + (bleeding points x2) + (degeneration and necrosis points x3) (34).

Determination of MPO and ADMA. The segments of stomach tissue with ulcers were processed into 20% tissue homogenate in cold saline with a UP400S ultrasonic processor (Ningbo Xinzhi Bio-tech Co., Ltd., Ningbo, China). The rat MPO and ADMA ELISA kits were then used to determine MPO activity and ADMA concentration in the homogenate, according to the manufacturer’s protocols. Optical absorbance (O.D.) at 450 nm was recorded with a Tecan Infinite 200 Microplate Reader (Tecan Group, Ltd., Mannedorf, Switzerland). MPO

### Table I. Gross scoring system for gastric mucosal lesions.

| Gastric mucosal lesion | Points |
|-----------------------|--------|
| Spot erosion (no.)    | 1      |
| Erosion length (mm)   | 1-5    |
| Erosion width (mm)    | 1-2    |

The ingredients in fermented Pu-erh tea include caffeoylethylbyric acid, theanine, statin, polysaccharides (3,20-22), theaflavins, thearubigins and theabrowins (23-26).
activity or ADMA concentration in the samples was then determined by comparing the O.D. value of the samples to the standard curve.

**Statistical analysis.** The results from each group were expressed as the mean ± standard error of mean. The data were analyzed by one-way analysis of variance with Fisher’s least significant difference post hoc analysis. Statistical analysis was performed with SPSS 16.0 (SPSS, Inc., Chicago, IL, USA), and P<0.05 was considered to indicate statistical significance.

**Results**

Protective effect of the extracts in gastric lesions based on gross evaluation. Cimetidine and moderate-to-high-dose Pu-erh tea extracts (1.00 and 1.50 g/kg) administered prior to alcohol-induced gastric injury significantly decreased the ulcer index in rat gastric mucosa, compared with the model control (P<0.01; Figs. 1 and 2). From initial observations, it was noted that the middle-to-high doses of Pu-erh tea extracts markedly suppressed the formation of damage; a notable phenomenon was that mucosal folds in the rat stomach became flatter following treatment with the extracts (0.50-1.50 g/kg;
Additionally, the severity of ethanol-induced gastric mucosal damage appeared to be dose-dependently reduced by the pretreatment with Pu-erh tea extracts (Figs. 1 and 2). The inhibitory rate of the high-dose Pu-erh tea extracts (1.50 g/kg) was 71.52%, which was higher than that of cimetidine (46.73%). Meanwhile, gastric mucosal injury in rats pre-treated with green tea powder (1.00 g/kg) was not significantly improved, though its inhibitory rate was 24.81%.

Protective effect of the extracts against in gastric lesions based on histopathological evaluation. Histopathological assessment of the gastric tissues was subsequently performed (Fig. 3). Under high-power light microscopy, the gastric mucosa of rats in the normal control was smooth and flat. The layers of the gastric mucosa exhibited clear boundaries, and there were no signs of pathological changes such as hyperemia, bleeding or epithelial cell degeneration or necrosis (Fig. 3A). In the model control, however, relatively increased damage to the gastric mucosa was identified (Fig. 3B): The surface of the gastric mucosa was uneven and exhibited erosion, ulcers and bleeding; furthermore, marked pathological changes including hyperemia, bleeding and epithelial cell degeneration and necrosis were observed. Gastric mucosal damage in rats pre-treated with cimetidine or high-dose Pu-erh tea extracts (1.50 g/kg) was clearly alleviated (Fig. 3C and G), compared with that of the model control. Notably, the mucosal superficial layer of rats pre-treated with high-dose Pu-erh tea extracts was intact and the submucosal layers were only slightly congested (Fig. 3G). On scoring of the damage, the model group was determined to have a score for hyperemia of 1.22±0.16, for bleeding of 2.11±0.37, and for epithelial cell degeneration or necrosis of 3.33±0.25 (Table III). Thus, the score for total pathological change in the model control was 15.44±0.56, which was significantly higher than that of the normal control (0.38±0.20; P<0.05); whereas, the total scores for rats in the Pu-erh tea extract groups were significantly decreased compared with the model group (P<0.05), to 9.75±1.16 in the middle-dose group (1.00 g/kg; Table III). Green tea powder at the dose of 1.00 g/kg did not exert significant protection in the gastric mucosa (Fig. 3D and Table III).

Effect on MPO activity and ADMA concentration in mucosal tissue. MPO activity in gastric tissue homogenate of the model control group was significantly increased compared with that of the normal control group (2,032.59±69.63 vs. 1,778.13±20.58 U/l, P<0.05; Fig. 4). The concentration of ADMA was also significantly higher in the model group compared with that in the normal control group (1,411.25±20.85 vs. 1,233.39±27.30 pmol/ml, P<0.05; Fig. 5). The activity of MPO and the concentration of ADMA in rats pre-treated with cimetidine was 1,811.07±32.50 U/l (Fig. 4) and 1,242.37±24.76 pmol/ml (Fig. 5), respectively, which were reduced significantly compared with that of the model.
Similarly, MPO activity in the low and high-dose Pu-erh tea extract groups (P<0.01), and ADMA concentration in all three extract treatment groups (0.50 and 1.50 g/kg: P<0.05; 1.00 g/kg: P<0.01) were also significantly attenuated compared with their levels in the model control group (Figs. 4 and 5).

**Discussion**

Gastric mucosal damage is caused by an imbalance between the protective and aggressive mechanisms in the mucosa, and is considered the net result of the actions of several endogenous factors and aggressive exogenous factors (11). The integrity of the gastric mucosa primarily depends on efficient protection of the gastric mucosal barrier, through maintaining defenses such as the gastric mucus layer, the mucus-bicarbonate barrier and mucosal microcirculation (14), which may be damaged by internal factors and external stimuli factors (11). Damage to the mucosa caused by internal factors and external stimuli is accompanied with the production of a number of inflammatory mediators and cytokines (35). Gastric mucosal injury may occur when noxious factors attack the intact mucosal defense, or when the mucosal defensive mechanisms are impaired (14). The gastric mucus layer is the first line of defense that serves to protect stomach tissue from external stimuli (14). Mucosal defensive factors enable the mucosa to remain intact despite its frequent exposure to external substances and ranging temperature, pH and osmolarity, and notably, to substances with detergent or cytotoxic action and bacterial products that induce local and systemic inflammatory reactions (36,37).

Ethanol is commonly used to induce ulcers in experimental animals, and causes acute gastric mucosal damage (38). Oral administration of absolute ethanol to rats produces typical characteristics of alcohol injury including linear hemorrhagic lesions, extensive submucosal edema, inflammatory cell infiltration and epithelial cell loss in stomach tissue (39). Ethanol produces necrotic lesions in the gastric mucosa through its direct toxic effect, as well as by reducing the secretion of bicarbonates and the production of mucus and defensive factors (40,41).

| Group                        | Hyperemia  | Bleeding  | Epithelial cell degeneration/necrosis | Pathological changes, total score |
|------------------------------|------------|-----------|--------------------------------------|----------------------------------|
| Normal                       | 0.38±0.20  | 0.00±0.00a| 0.00±0.00d                          | 0.38±0.20d                       |
| Model                        | 1.22±0.16a | 2.11±0.37b| 3.33±0.25b                          | 15.44±0.56b                      |
| Cimetidine (g/kg)            | 1.11±0.12  | 2.00±0.31  | 1.56±0.31d                          | 9.78±0.79c                       |
| Green tea powder (g/kg)      | 1.11±0.12  | 1.67±0.35  | 3.11±0.41                           | 13.78±1.50                       |
| Pu-erh tea extracts (g/kg)   |            |           |                                      |                                  |
| 0.5                          | 1.11±0.12  | 1.78±0.42  | 3.44±0.26                           | 13.89±1.26                       |
| 1.0                          | 1.25±0.25  | 1.25±0.37  | 2.00±0.76c                          | 9.75±1.16c                       |
| 1.5                          | 1.11±0.12  | 0.56±0.19c | 1.33±0.18d                          | 6.22±0.77d                       |

Data were expressed as the mean ± standard deviation. (n=9). *P<0.05, **P<0.01 vs. normal control group; *P<0.05, **P<0.01 vs. model control group.
Gastric mucus when secreted in sufficient quantity is an important factor for the functioning of the gastric mucosa. It consists of a viscous, elastic, adherent and transparent gel, formed by water and glycoproteins, that covers the surface of the gastrointestinal mucosa (42). The protective properties of the mucus barrier depend not only on the gel structure but also on the amount or thickness of the layer covering the mucosal surface (28). In the present study, it was noted that folds of gastric mucosa were flattened and gastric damage was markedly reduced in rats pretreated with Pu-erh tea extracts, compared with the model control. Furthermore, the pretreatment with Pu-erh tea extracts at moderate and high-dose caused significant reduction in the UI and inhibition of gastric mucosal injury. A study by Scoparo et al (43) demonstrated that the fraction of green and black tea containing heteropolysaccharides reduced gastric lesions induced with ethanol and protected gastric mucosa tissue. In the current study, it was identified that Pu-erh tea extracts clearly suppressed the formation of the gastric ulcer, and exhibited a higher inhibitory rate on gastric ulcer formation than green tea. This observed effect is probably associated with the high content of polysaccharides present in Pu-erh tea extracts (43).

MPO, a biomarker for neutrophil-dependent inflammation, is mainly released from neutrophils, and therefore is also an essential marker for normal neutrophil function. MPO and other tissue-damaging substances including reactive oxygen metabolites and cytotoxic proteins are released into the extracellular space when neutrophils are stimulated (44,45). Ethanol administration causes an increase of mucosal MPO activity, which thus indicates that the level of activated neutrophils secreting oxygen radicals is increased (46,47). In the present study, absolute ethanol induced an increase of MPO activity in the model control compared with the normal control. The pretreatment of Pu-erh tea extracts prior to this alcohol induction suppressed the release of MPO, compared with the model control, which may indicate that the degree of inflammation induced by neutrophils was also inhibited.

ADMA is the endogenous inhibitor of nitric oxide synthase (NOS), and has been implicated in pathophysiology of the upper gastrointestinal tract (48). The generation of high levels of ADMA suppresses nitric oxide (NO) production through inhibition of NOS activity (49). NO, when serving as a potent vasodilator, increases blood flow in the gastric mucosa, inhibits the secretion of gastric acid and potentiates the secretion of mucus and bicarbonate, which thus protect the gastric mucosa against damage induced by a variety of corrosive substances and noxious agents (48). However, the biological life of ADMA is limited, as it may be hydrolyzed into L-citrulline and dimethylamine by dimethylarginine dimethylaminohydrolase (DDAH) excreted from the kidneys (50). A previous study focused on the effect of ADMA in gastric mucosal injury induced by ethanol, cold stress and indomethacin, and identified that ADMA levels were increased in gastric juice in animal models of gastric mucosal lesions (51). Furthermore, NO biosynthesis and DDAH activity in the stomach may be significantly inhibited in animals exposed to ethanol, stress and indomethacin (50-52). A study by Yi et al (53) study demonstrated that there were crude polyphenols in Dragon-pearl green tea, which increased the level of NO, improved microcirculation in the gastric mucosa, cleared oxygen free radicals and strengthened the protective function of the mucosal barrier. Adhikary et al (54) observed that both black tea and theaflavins suppressed various inflammatory modulators and inducible NOS-mediated nitric oxide synthesis during gastric ulcer healing. In the current study, the concentration of ADMA in the model control was significantly increased, compared with that in the normal control. The administration of Pu-erh tea extracts prior to ethanol-induced injury exerted clear protective effects against the gastric mucosal injury, and the concentration of ADMA was significantly decreased, compared with the model control. Furthermore Pu-erh tea extracts were identified to decrease the concentration ADMA to a greater extent than green tea, which is probably due to the higher content of theaflavins in Pu-erh tea extracts (54).

In conclusion, the study present focused on the protective effect of Pu-erh tea extracts in the gastric mucosa. Absolute ethanol was used to induce gastric mucosal injury and the action of Pu-erh tea extracts was investigated. The extracts exerted significant protective effects against the gastric mucosal damage. The human equivalent of the rat dose 1.00 g/kg is 0.16 g/kg, according to the dose conversion relationship of the Food and Drug Administration (55), which is equivalent to physiological tea consumption in humans. The effect of Pu-erh tea extracts was greater than that of green tea powder. Pretreatment with Pu-erh tea extracts prior to the intragastrical administration of absolute ethanol inhibited the activity of MPO and decreased the concentration of ADMA compared with the pre-administration of distilled water. These protective effects of Pu-erh tea extracts against gastric mucosal damage may be due to its preservation of the gastric mucus layer as well as roles in decreasing inflammation and increasing NO production.

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Availability of data and materials
The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors’ contributions
JY and WZ were the principal investigators responsible for the study and contributed to the design of the experiments, and JY wrote the original manuscript. YG and JD were responsible for analyzing the experimental data. XL, PT and YL were responsible for collecting experimental data. XM and YZ was the leader of the research group, and was responsible for the study design and guidance, and for checking the accuracy and authenticity of the manuscript.

Authors' contributions
Ethics approval and consent to participate

The study protocols were approved by the Tasly Laboratory Animal Welfare and Ethics Committee of Tasly Pharmaceuticals, Inc. (Tianjin, China), and conducted according to the rules of animal experimentation and the guide for the Care and Use of Laboratory Animals of Tasly Pharmaceuticals, Inc.

Consent for publication

Not applicable.

Competing interests

WZ, XL, PT and YL are research fellows and XM is vice director of Tasly Pharmaceuticals, Inc., Tianjin, China. All other authors declare no competing interests.

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