

# Theaflavins attenuate ethanol-induced oxidative stress and cell apoptosis in gastric mucosa epithelial cells via downregulation of the mitogen-activated protein kinase pathway

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**Abstract.** Ethanol-induced diseases of the gastric mucosa are the most common and refractory diseases of gastrointestinal system in clinic, and are mediated by oxidative stress and apoptosis pathways. Theaflavins (TFs) are considered to be antioxidants. The present study aimed to determine the molecular mechanism underlying the ability of TFs to attenuate ethanol-induced oxidative stress and apoptosis in GES-1 gastric mucosa epithelial cells. A Cell Counting Kit-8 (CCK-8) assay was performed to investigate the cell viability of GES-1 cells following administration of ethanol (0.5 mol/l) and subsequent treatment with TFs (20, 40 and 80  $\mu$ g/ml) for specific time intervals. A carboxyfluorescein diacetate succinimidyl ester assay was used to measure proliferation and further investigate the results of the CCK-8 assay. Flow cytometry was performed to measure reactive oxygen species (ROS) levels and the apoptosis rates of GES-1 cells. Furthermore, levels of oxidative stress-associated factors, including malondialdehyde, superoxide dismutase and glutathione, were investigated using commercial kits. Reverse transcription-quantitative polymerase chain reaction and western blot assays were performed to determine the expression levels of apoptosis-associated factors, as well as the phosphorylation levels of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 kinase (p38). The results of the present study demonstrated that treatment with ethanol inhibited GES-1 cell proliferation, and enhanced ROS levels and apoptosis rates, potentially via downregulation of B-cell lymphoma-2 (Bcl-2) expression and upregulation of Bcl-2-associated X and caspase-3 expression levels, as well as enhancing the

phosphorylation levels of ERK, JNK and p38. However, treatment with TFs was revealed to attenuate the effects of ethanol administration on GES-1 cells in a dose-dependent manner. In conclusion, TFs may attenuate ethanol-induced oxidative stress and apoptosis in gastric mucosa epithelial cells via downregulation of various mitogen-activated protein kinase pathways.

## Introduction

Ethanol-induced gastric mucosa injury represents one of the most refractory and frequently represented disease group in clinical settings, including gastritis, gastric ulcers and gastric mucosal stress injury (1). Patients with gastric mucosa injury commonly suffer from hyperemia, edema, erosion and hemorrhage (2). Chronic alcohol consumption is one of the most important factors contributing to the high incidence and prevalence of gastric mucosa injury (3). Ethanol-induced gastric mucosal injury (acute or chronic), with the imbalance of protective and injury-inducing factors of the mucosa (4), maybe attenuated by the regulation of anti-inflammatory and antioxidant pathways (5,6). Reactive oxygen species (ROS) have previously been demonstrated have important roles in disease pathogenesis and to be involved in complex physiological processes associated with oxidative stress, such as cell signaling and apoptosis (7-9). In clinical and animal model studies, chronic alcohol consumption was revealed to be associated with suppressed superoxide dismutase (SOD) activity, reduced glutathione (GSH) levels and the oxidation of GSH/glutathione disulfide (GSSG) redox potentials, as well as enhanced levels of the lipid peroxide product, malondialdehyde (MDA) (10-12).

The major bioactive polyphenols of tea (*Camellia sinensis*) are epigallocatechin gallate (EGCG) (13), a member of the catechin group of polyphenols found in tea, and theaflavins (TFs), which are a polymerized type of catechin (14). Tea polyphenols, which are predominantly extracted from green tea, have been extensively investigated (15). However, despite the beneficial properties of black tea, the polyphenolic compound TF derivatives have not been widely studied. Previous studies investigating black tea have revealed that it exhibits chemopreventive and chemotherapeutic effects (16,17). There are

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four major TFs in black tea: TF1, TF-3-gallate, TF-3'-gallate and TF-3, 3'-digallate (TF3), of which TF3 is the most abundant (18,19). Tea polyphenols serve predominantly as antioxidants; and they have also been demonstrated to serve as anticancer agents in numerous preclinical and clinical studies (20,21). TFs have been reported to attenuate inflammatory responses in human gingival fibroblasts as a result of antibacterial properties (22), and to suppress the secretion of inflammation-associated factors and enhance the secretion of antimicrobial peptides in oral epithelial cells *in vitro* (23). An investigation using animal models with oxidative stress revealed that tea polyphenols functioned as antioxidants primarily by scavenging ROS and attenuating the suppression of the activity of antioxidant enzymes, such as SOD and GSH (24). Furthermore, TFs have been demonstrated to suppress hematopoietic stem cell (HSC) senescence and reduce oxidative stress to protect mouse HSCs from radiation injury *in vivo* (25).

In addition to the role of oxidative stress, studies have indicated that the underlying molecular mechanisms of ethanol-induced gastric diseases may involve multiple signaling pathways, including apoptosis and mitogen-activated protein kinase (MAPK) pathways, such as extracellular signal-regulated kinase (ERK)1, ERK2, c-Jun N-terminal kinase (JNK) and p38 kinase (p38) MAPK pathways (26,27). Apoptosis is induced by oxidative stress and the subsequent increases in superoxide and hydroxyl radicals, and MAPK pathways have important roles in cell proliferation, differentiation and apoptosis. TFs have previously been revealed to inhibit H<sub>2</sub>O<sub>2</sub> and inflammation-induced apoptosis in neural cells (28,29). Furthermore, the phosphorylation levels of ERK1/2 and JNK have been previously demonstrated to be suppressed by EGCG in epidermal cells (30) and by both EGCG and green tea polyphenols in lung carcinogenesis models (31).

The aim of the present study was to investigate whether TFs may attenuate ethanol-induced oxidative stress in gastric mucosa epithelial cells and to investigate the potential associated underlying molecular mechanisms, including apoptosis and MAPK pathways. The results of the present study indicates that TFs may represent a novel therapeutic agent for the treatment of ethanol-induced injury in gastric mucosa epithelial cells, which may provide insight for future studies investigating ethanol-induced gastric diseases.

## Materials and methods

**Cell culture.** TF3 (>90.0%) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and is approved by the Food and Drug Administration (32,33). GES-1 human gastric mucosa epithelial cells were obtained from the American Type Culture Collection (Manassas, VA, USA) in order to investigate the effects of TF3 on ethanol-induced injury *in vitro*. For the similar reactions to ethanol with primary gastric mucosa epithelial cells, the GES-1 cell line has been widely used in the study of the effects of ethanol on the gastric mucosa, and so the GES-1 cell line was determined to be appropriate for use in the present study (34,35). Cells were cultured in RPMI 1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA)

and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) in a 5% CO<sub>2</sub>-containing humidified incubator at 37°C. When cells reached 70% confluence, cell morphologies were observed under a DMi8 optical microscope (Leica Microsystems GmbH, Wetzlar, Germany).

When cells reached 70% confluence, they were divided into five groups: EtOH group (treated with 0.5 mol/l ethanol for 24 h at room temperature); TF-1, TF-2 and TF-3 groups (respectively treated with 20, 40 and 80 µg/ml TF3 for 6 h, and then 0.5 mol/l ethanol for 24 h, at room temperature) and the Control group (without any treatment). Cell viability and proliferation abilities were subsequently investigated to determine the degree of injury exhibited by GES-1 cells following treatment with ethanol, and whether administration of TF3 attenuates these effects.

**Cell Counting Kit-8 (CCK-8) cell viability assay.** The cell viability of GES-1 cells treated with varying concentrations of TF3 (20, 40 and 80 µg/ml) for 6 h prior to ethanol (0.5 mol/l) treatment were investigated using the CCK-8 assay (Beyotime Institute of Biotechnology, Haimen, China) at specific time intervals (0, 6, 12, 24 and 48 h), according to the manufacturer's protocol. Briefly, cells (5x10<sup>3</sup> cells/well) were seeded with CCK-8 reagent (20 µl) in 96-well plates and incubated for 1 h at 37°C in an atmosphere of 5% CO<sub>2</sub>. The optical density values were analyzed under 450 nm using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA).

**Carboxyfluorescein diacetate succinimidyl ester (CFSE) cell proliferation assay.** The CFSE assay was used to investigate the proliferation ability of cells in all groups following treatment with ethanol for 24 h, with or without pretreatment with 20, 40 and 80 µg/ml TF3 for 6 h. Proliferation was assessed based on the even distribution of CFSE fluorescence when cell division occurs. The CellTrace CFSE Cell Proliferation kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to investigate GES-1 cell proliferation according to the manufacturer's protocol. Cells were mixed with preheated PBS (1 ml) in sterile centrifuge tubes to reach a final concentration of 1x10<sup>6</sup> cells/ml. A total of 2 µl CFSE (5 mmol/l) reagent was added to the mixture, which was subsequently incubated for 10 min at 37°C. Following culture in 10 ml RPMI 1640 containing 10% FBS for 5 min on ice in the dark, cells were seeded into 24-well plates (1x10<sup>5</sup> cells/well) and cultured in an incubator containing 5% CO<sub>2</sub> at 37°C for 4 h. Finally, cells were investigated using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and CellQuest™ Pro Software version 5.1 (BD Biosciences).

**ROS detection.** ROS levels were measured using dichlorofluorescein-diacetate (DCFH-DA), an oxygen-sensitive fluorescence probe. Briefly, DCFH-DA (10 µmol/l) was added to EtOH, TF-1, TF-2, TF-3 and Control cell groups (1x10<sup>5</sup> cells/well) in a 6-well plate. Following incubation for 30 min at 37°C in the dark, cells were washed with PBS three times to remove excess dye, and were immediately collected and analyzed using a FACSCalibur flow cytometer (BD Biosciences) and CellQuest™ Pro Software version 5.1 (BD Biosciences).

**Detection of oxidative stress-associated factors.** MDA levels, SOD activity and the GSH/GSSG ratio in the EtOH, TF-1, TF-2, TF-3 and Control cell groups were determined. MDA levels were detected by thiobarbituric acid in a Lipid Peroxidation MDA assay kit (Beyotime Institute of Biotechnology). SOD activities were investigated using Total Superoxide Dismutase assay kit with WST-8 (Beyotime Institute of Biotechnology). Additionally, GSH/GSSG redox potentials were determined using 2,2-dithio-bis-nitrobenzoic acid in the GSH and GSSG assay kit (Beyotime Institute of Biotechnology). All assays were performed according to the manufacturer's protocols.

**Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection.** The apoptosis rates of GES-1 cells were determined using an Annexin V-FITC/PI double-staining assay, according to the manufacturer's protocol (BioVision, Inc., Milpitas, CA, USA). In the present study, cells in the EtOH, TF-1, TF-2, TF-3 and Control cell groups, with the initial concentration of  $1 \times 10^6$  cells/ml, were seeded in a 96-well plate. Following this, Annexin V/FITC (40  $\mu\text{g/ml}$ , 5  $\mu\text{l}$ ) and PI (20  $\mu\text{g/ml}$ , 10  $\mu\text{l}$ ) were added to the cell suspension. Following incubation for 15 min at room temperature, cells were analyzed using a FACSCalibur flow cytometer. A wavelength of 488 nm was used as the exciting light wavelength, and wavelengths of 515 nm (FITC) and 560 nm (PI) were used as detecting light wavelengths.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) from the EtOH, TF-1, TF-2, TF-3 and Control cell groups, and cDNA was obtained using a EN-QuantiTect Reverse Transcription (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocols of 37°C for 10 min, and 85°C for 15 sec. The thermocycling conditions for qPCR were as follows: Initial denaturation at 95°C for 30 sec, followed by 40 cycles of denaturation at 95°C for 5 sec and annealing/extension at 60°C for 30 sec. qPCR was performed in an ABI 7300 Thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a SYBR Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The  $2^{-\Delta\Delta C_t}$  method used to normalize gene expression to the housekeeping gene (GAPDH) (36). The primer sequences used for PCR are presented in Table I.

**Western blot analysis.** Total proteins were extracted from cells in EtOH, TF-1, TF-2, TF-3 and Control cell groups. Cells were lysed using lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 0.02%  $\text{NaN}_2$ , 100  $\mu\text{g/ml}$  phenylmethanesulfonyl fluoride, 1  $\mu\text{g/ml}$  aprotinin, and 1% Triton X-100), and centrifuged at 12,000  $\times g$  for 30 min at 4°C. Total protein concentration was determined via a BCA assay (Beyotime Institute of Biotechnology). Following this, proteins (20  $\mu\text{g/well}$ ) were separated via 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Following blocking using 5% non-fat dry milk for 1 h at room temperature, the membranes were probed using the following rabbit primary antibodies overnight at 4°C: Anti-caspase-3 (cat no. ab13847; 1:500; Abcam, Cambridge, UK), anti-B-cell lymphoma-2 (Bcl-2; cat. no. ab59348; 1:1,000; Abcam), anti-Bcl-2-associated X (Bax; cat. no. ab32503; 1:2,000; Abcam), anti-GAPDH

(cat. no. ab9485; 1:2,500; Abcam), anti-ERK1/2 (cat. no. 4695; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-phosphorylated (p)-ERK1/2 (cat. no. 4370; 1:2,000; Cell Signaling Technology, Inc.), anti-JNK (cat. no. 9252; 1:1,000; Cell Signaling Technology, Inc.), anti-p-JNK (cat. no. 4668; 1:1,000; Cell Signaling Technology, Inc.), anti-p38 (cat. no. 8690; 1:1,000; Cell Signaling Technology, Inc.) and anti-p-p38 (cat. no. 4511; 1:1,000; Cell Signaling Technology, Inc.). GAPDH was used as the loading control. Following this, membranes were probed with horseradish peroxidase-conjugated goat anti-rabbit IgG H&L (cat. no. ab6721; 1:5,000; Abcam) for 2 h at room temperature. PVDF membranes were subsequently exposed to X-ray film and visualized using an enhanced chemiluminescence detection system, GE ECL Start (GE Healthcare, Chicago, IL, USA). Lab Works Image Acquisition and Analysis software PLUS 4.1 (UVP, LLC, Phoenix, AZ, USA) was used to quantify the band intensities.

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation of three independent experiments. Statistical analysis was performed using SPSS software (version 22.0; IBM Corp., Armonk, NY, USA). Statistical differences were determined via one-way analysis of variance and Dunnett's post-hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**TFs enhance the viability of GES-1 cells injured by ethanol.** After 24 h of culture, cell morphologies of GES-1 gastric mucosa epithelial cells were observed by optical microscopy, when cells attained 70% confluence. Cells were observed regular polygonal or fusiform cell morphologies, and uniform interstitials (Fig. 1A). Once the cells had reached 70% confluence, the CCK-8 assay was performed to determine the effect of the administration of differing concentrations of TFs (20, 40 and 80  $\mu\text{g/ml}$ ) on the cell viability of GES-1 cells treated with 0.5 mol/l ethanol for specific time intervals (0, 6, 12, 24 and 48 h). The results revealed that the cell viability of the EtOH group was significantly suppressed at all time-points compared with the control group ( $P < 0.01$ ; Fig. 1B). Furthermore, the results of the CCK-8 assay revealed that TFs enhanced cell viability following treatment with ethanol in a dose-dependent manner compared with the EtOH group ( $P < 0.05$ ; Fig. 1B).

The CFSE assay was performed to further investigate the potential TF-induced promotion of GES-1 cell proliferation in cells treated with ethanol (0.5 mol/l) for 24 h. The results of the flow cytometry analysis demonstrated that the cell proliferation ability (reflected by M1 values) was significantly inhibited (decreased M1 values) in the EtOH group compared with the control group ( $P < 0.01$ ); however, this effect was significantly reversed (increased M1 values) following treatment with TF in a dose-dependent manner ( $P < 0.05$ ; Fig. 1C and D).

**TFs attenuate oxidative stress induced by ethanol in GES-1 gastric mucosa epithelial cells.** As an indicator of oxidative stress, ROS levels were detected as a marker of oxidative stress. DCTH-DA fluorescence detection was performed and the results revealed that the EtOH group exhibited significantly

Table I. Primer sequences used for reverse transcription-quantitative polymerase chain reaction.

Gene	Sequence	
	Forward	Reverse
GAPDH	CCATCTTCCAGGAGCGAGAT	TGCTGATGATCTTGAGGCTG
Caspase-3	TGAGCCATGGTGAAGAAGGA	TCGGCTCCACTGGTATTTT
Bax	AACATGGAGCTGCAGAGGAT	CCAATGTCCAGCCCATGATG
Bcl-2	TTCTTTGAGTTCGGTGGGGT	CTTCAGAGACAGCCAGGAGA

Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X.

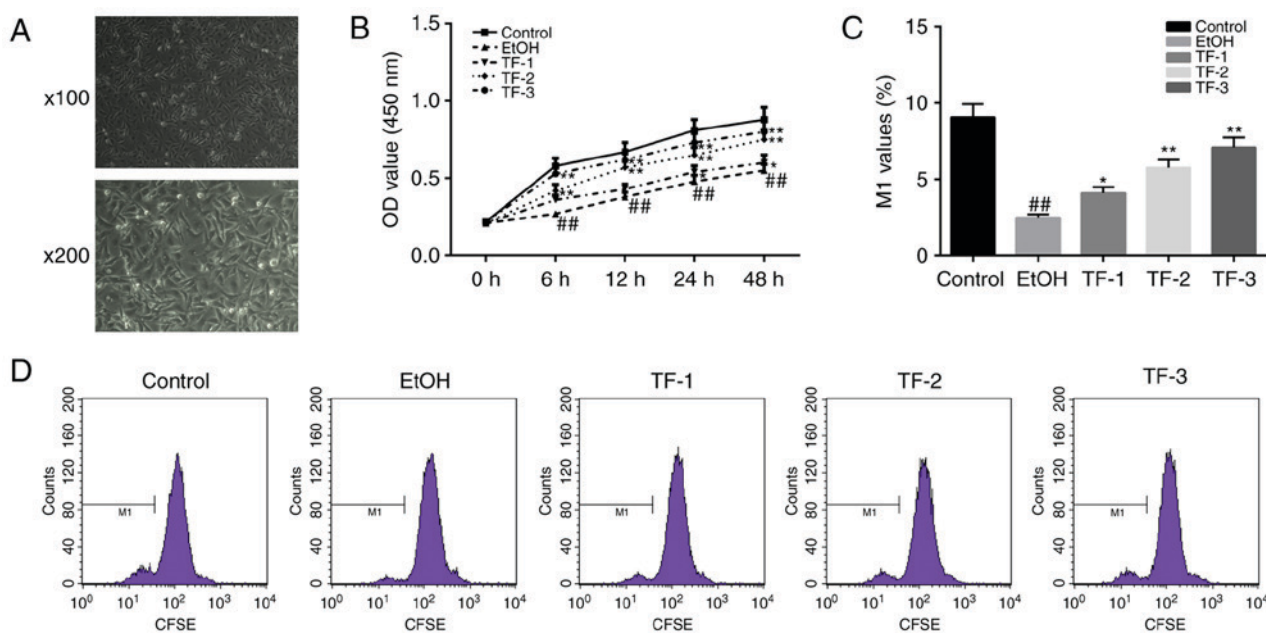


Figure 1. TFs promote the cell viability of ethanol-treated GES-1 gastric mucosa epithelial cells. (A) GES-1 cells were cultured and cell morphologies were observed by optical microscopy (magnification, x100 and x200). (B) A Cell Counting Kit-8 assay was performed to investigate the effect of 20, 40 and 80  $\mu\text{g/ml}$  TFs on the cell viability of ethanol-treated GES-1 cells at various time intervals. A CFSE assay was subsequently performed to determine the effect of TF administration on the cell proliferation of GES-1 cells treated with ethanol for 12 h. (C) M1 values obtained following the CFSE proliferation assay were statistically compared among the groups. (D) Representative flow cytometry graphs following the CFSE assay in different cell groups.  $^{##}P<0.01$  vs. control group;  $^{*}P<0.05$  and  $^{**}P<0.01$  vs. EtOH group. TFs, theaflavins; CFSE, carboxyfluorescein diacetate succinimidyl ester; EtOH group, GES-1 cells treated with 0.5 mol/l ethanol only; TF-1 group, GES-1 cells treated with 20  $\mu\text{g/ml}$  TFs prior to 0.5 mol/l ethanol treatment; TF-2, GES-1 cells treated with 40  $\mu\text{g/ml}$  TFs prior to 0.5 mol/l ethanol treatment; TF-3, GES-1 cells treated with 80  $\mu\text{g/ml}$  TFs prior to 0.5 mol/l ethanol treatment; OD, optical density.

enhanced ROS levels compared with the control group ( $P<0.01$ ; Fig. 2A and B). Furthermore, treatment with TFs reversed this effect in a dose-dependent manner, compared with the EtOH group ( $P<0.05$ ; Fig. 2A and B).

In addition, the levels of other common oxidative stress-associated factors, including SOD, GSH and MDA, were investigated. The activity of SOD in the EtOH group was significantly suppressed compared with the control group ( $P<0.01$ ; Fig. 2C). By contrast, the levels of MDA in the EtOH group were significantly increased compared with the control group ( $P<0.01$ ; Fig. 2D). Furthermore, the activity of the GSH/GSSG redox potential was significantly suppressed in the EtOH group compared with the control group ( $P<0.01$ ; Fig. 2E). However, treatment with TFs reversed the effects of ethanol treatment on the levels of ROS, SOD, MDA and GSH/GSSG in a dose-dependent manner (Fig. 2).

*TFs inhibit cell apoptosis in GES-1 cells injured by ethanol.* Annexin V-FITC/PI double-staining assay and flow cytometry were performed to determine the apoptosis rates exhibited by the control, EtOH, TF-1, TF-2 and TF-3 cell groups. The results demonstrated that the apoptosis rate was significantly increased in the EtOH group compared with the control group ( $P<0.01$ ) and that treatment with TFs significantly reversed this effect in a dose-dependent manner ( $P<0.05$ ; Fig. 3A and B).

Furthermore, the expression levels of apoptosis-associated factors were investigated by RT-qPCR and western blot analyses. The results demonstrated that the mRNA and protein levels of proapoptotic factors (caspase-3 and Bax) were significantly enhanced in the EtOH group compared with the control group ( $P<0.01$ ), while the mRNA and protein levels of the anti-apoptotic protein Bcl-2 were significantly suppressed in the EtOH group compared with control group ( $P<0.01$ ;

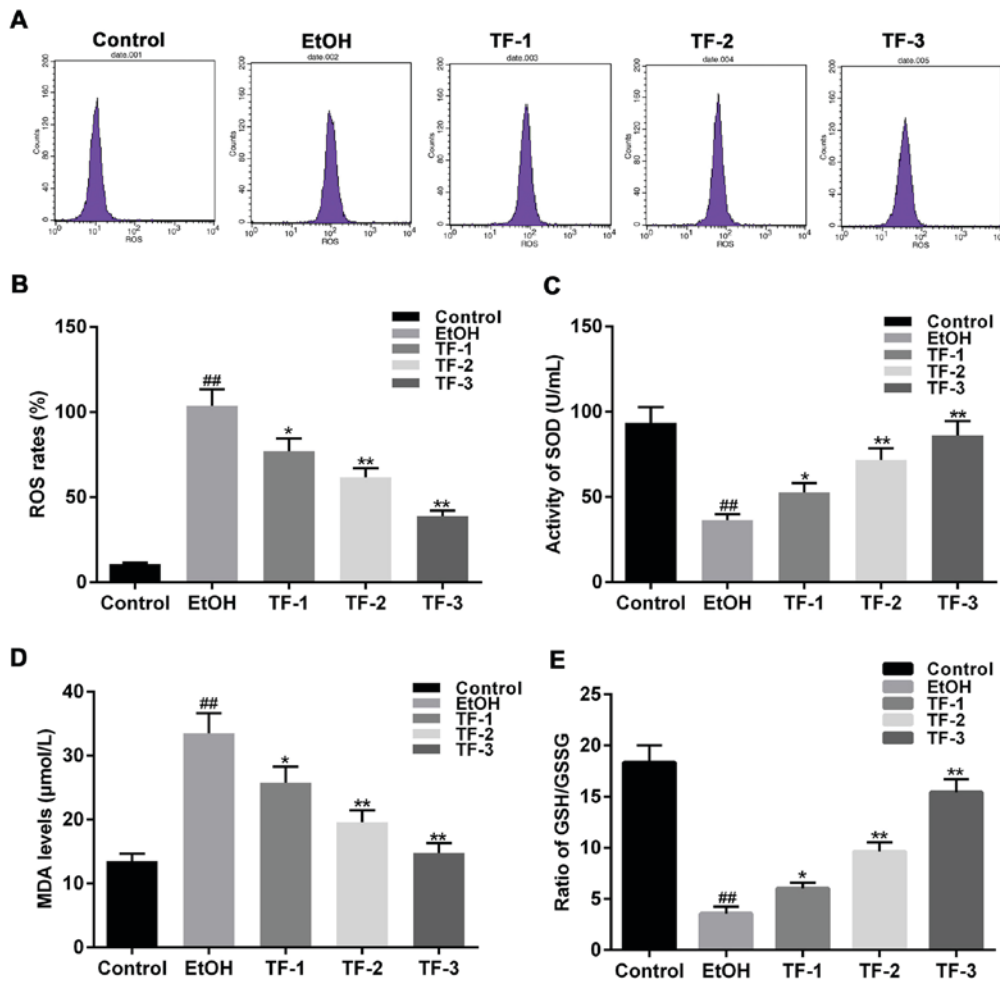


Figure 2. TFs attenuate oxidative stress induced by ethanol in GES-1 gastric mucosa epithelial cells. ROS levels were investigated via administration of DCTH-DA to GES-1 cells following treatment with 0.5 mol/l ethanol. (A) Representative flow cytometry graphs for each cell group following the addition of DCTH-DA. (B) ROS levels were quantified by flow cytometry and statistically compared among the groups. Levels of (C) SOD, (D) MDA and (E) GSH/GSSG were determined using commercial kits. <sup>##</sup>P<0.01 vs. control group; <sup>\*</sup>P<0.05 and <sup>\*\*</sup>P<0.01 vs. EtOH group. TFs, theaflavins; ROS, reactive oxygen species; DCTH-DA, dichlorofluorescein-diacetate; SOD, superoxide dismutase; MDA, malondialdehyde; GSH, glutathione; GSSG, glutathione disulfide; EtOH group, GES-1 cells treated with 0.5 mol/l ethanol only; TF-1 group, GES-1 cells treated with 20 µg/ml TFs prior to 0.5 mol/l ethanol treatment; TF-2, GES-1 cells treated with 40 µg/ml TFs prior to 0.5 mol/l ethanol treatment; TF-3, GES-1 cells treated with 80 µg/ml TFs prior to 0.5 mol/l ethanol treatment.

Fig. 3C-E). However, treatment with TFs significantly reversed the effects of ethanol administration in a dose-dependent manner (P<0.05; Fig. 3C-E).

*TFs alleviate oxidative stress via MAPK pathways in GES-1 cells treated with ethanol.* The phosphorylation levels of ERK, JNK and p38 were investigated by western blot analyses in control, EtOH, TF-1, TF-2 and TF-3 cell groups. The results demonstrated that the protein levels of p-ERK (Fig. 4A and B), p-JNK (Fig. 4C and D) and p-p38 (Fig. 4E and F) were significantly increased in EtOH groups compared with the control group (P<0.01). However, treatment with TFs significantly attenuated this effect in a dose-dependent manner compared with the EtOH group (P<0.05; Fig. 4). The total levels of ERK, JNK and p38 did not exhibit any significant differences between the control, EtOH, TF-1, TF-2 and TF-3 cell groups (Fig. 4).

## Discussion

It is well established that chronic alcohol consumption is one of the most important risk factors for the induction of digestive

tract diseases (37). Chronic, excessive alcohol consumption may lead to the induction of various acute digestive tract diseases, including gastritis, gastric ulcers and gastrorrhagia, as a result of subsequent gastric mucosa injury, particularly via oxidative stress and apoptosis (38,39). Tea polyphenols, which are predominantly extracted from green tea, have been widely investigated and have been revealed to exhibit antioxidative properties (40,41). TFs, polyphenolic compounds that are the major beneficial component of black tea, have also been demonstrated to exert antioxidative effects and thus require further investigation (42-44).

In the present study, GES-1 gastric mucosa epithelial cells were used to construct an oxidative stress cell model using ethanol treatment to induce injury. The viability of GES-1 cells was significantly suppressed following treatment with ethanol (0.5 mol/l) after 6, 12, 24 and 48 h, while the levels of oxidative stress and apoptosis were significantly enhanced following treatment with 0.5 mol/l ethanol. However, the results demonstrated that treatment of GES-1 cells with TFs attenuated the effects of ethanol administration on levels of oxidative stress, apoptosis and cell viability in a dose-dependent manner.

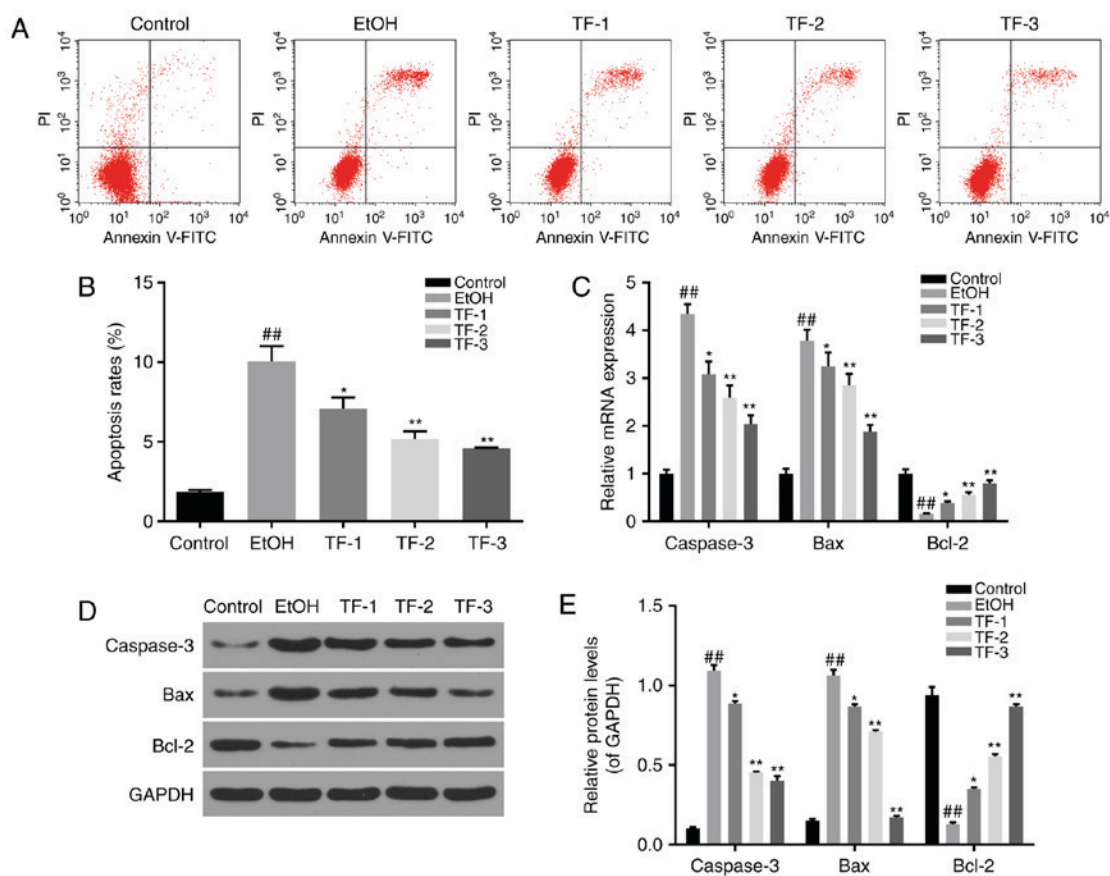


Figure 3. TFs suppress cell apoptosis in GES-1 cells treated with ethanol. Annexin V-FITC/PI double staining followed by flow cytometry was performed to measure apoptosis rates in the different cell groups. (A) Representative flow cytometry scatter plots following Annexin V-FITC/PI double staining, lower right + upper right quadrants were considered to indicate apoptotic cells. (B) Apoptosis rates determined by flow cytometry were statistically compared among the groups. (C) Reverse transcription-quantitative polymerase chain reaction was performed to measure the mRNA levels of apoptosis-associated factors, including caspase-3, Bax and Bcl-2. (D) Representative western blot bands obtained for the protein expression of caspase-3, Bax and Bcl-2 apoptosis-associated factors. (E) Densitometric analysis was performed to quantify and statistically compare the protein levels of caspase-3, Bax and Bcl-2 among the different cell groups.  $^{##}P < 0.01$  vs. control group;  $^{*}P < 0.05$  and  $^{**}P < 0.01$  vs. EtOH group. TFs, theaflavins; FITC, fluorescein isothiocyanate; PI, propidium iodide; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X; EtOH group, GES-1 cells treated with 0.5 mol/l ethanol only; TF-1 group, GES-1 cells treated with 20  $\mu$ g/ml TFs prior to 0.5 mol/l ethanol treatment; TF-2, GES-1 cells treated with 40  $\mu$ g/ml TFs prior to 0.5 mol/l ethanol treatment; TF-3, GES-1 cells treated with 80  $\mu$ g/ml TFs prior to 0.5 mol/l ethanol treatment.

The underlying molecular mechanisms of this process were investigated further by analyzing oxidative stress, apoptosis and MAPK pathways that are important for cell proliferation.

ROS are involved in cell oxidative stress mechanisms, predominantly via the induction of lipid peroxidation in the phospholipid membrane, the production of oxygen radicals and the induction of cell apoptosis (26,27). Antioxidant enzymes, such as SOD and GSH, eliminate ROS to maintain the balance of ROS generation and elimination during cell normal metabolism (45,46). Excessive accumulation of ROS leads to the destruction of this balance and induces oxidative stress and cell apoptosis (28). Consistent with previous research, the results of the present study revealed that ROS and MDA levels were significantly increased following treatment with ethanol, whereas the activities of SOD and GSH antioxidant enzymes were significantly decreased following treatment with ethanol. Furthermore, the results of the present study demonstrated that administration of TFs attenuated the effects of ethanol treatment in GES-1 cells in a dose-dependent manner.

Apoptosis, a form of programmed cell death, is important for normal cell survival. Bcl-2 family members and caspases are important regulators of mitochondria-mediated

apoptosis (47,48). Bcl-2 family members, which possess a BH homology domain, such as Bcl-2, Bax and Bcl-2-like 1, have been previously demonstrated to be regulators of the mitochondria membrane potential via competitive level variations of antiapoptotic factors (e.g. Bcl-2) and proapoptotic factors (e.g. Bax) (29). In addition, such factors modify the release of cytochrome *c* in order to regulate the activation of downstream caspase pathways (49). Caspases are a family of cysteine-aspartic proteases. Cell apoptosis in mammals is predominantly induced by caspases, some of which function as apoptosis activators and others function as apoptosis executioners (50). Caspase-3 is the most important executive factor in the apoptosis pathway (51). The present study demonstrated that treatment with TFs downregulated the expression levels of Bax and caspase-3, which were otherwise induced by ethanol injury in GES-1 cells. Furthermore, treatment with TFs upregulated the expression levels of Bcl-2, which were suppressed following treatment with ethanol alone. Therefore, TFs may protect GES-1 cells against ethanol injury via the regulation of cell apoptosis.

MAPK pathways have important roles in cell proliferation, differentiation, apoptosis and inflammation (52,53). Studies

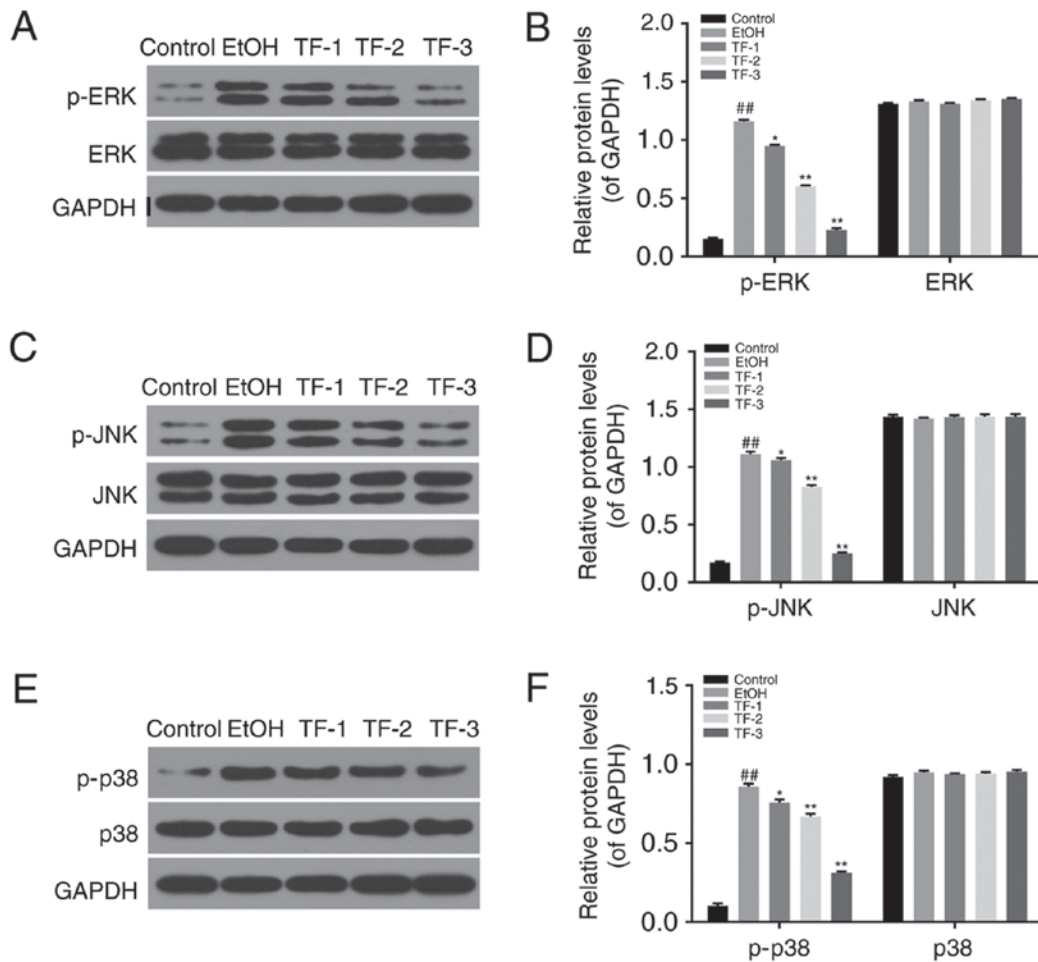


Figure 4. TFs attenuate oxidative stress in GES-1 cells treated with ethanol via MAPK pathways. Western blot analyses were performed to measure the phosphorylation levels of ERK, JNK and p38 MAPK proteins. (A) Representative western blot bands obtained for the protein expression of p-ERK and ERK. (B) Densitometric analysis was performed to quantify and statistically compare the protein levels of p-ERK and ERK. (C) Representative western blot bands obtained for the protein expression of p-JNK and JNK. (D) Densitometric analysis was performed to quantify and statistically compare the protein levels of p-JNK and JNK. (E) Representative western blot bands obtained for the protein expression of p-p38 and p38. (F) Densitometric analysis was performed to quantify and statistically compare the protein levels of p-p38 and p38. ##*P*<0.01 vs. control group; \**P*<0.05 and \*\**P*<0.01 vs. EtOH group. TFs, theaflavins; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; p38, p38 kinase; p-, phosphorylated-; EtOH group, GES-1 cells treated with 0.5 mol/l ethanol only; TF-1 group, GES-1 cells treated with 20  $\mu$ g/ml TFs prior to 0.5 mol/l ethanol treatment; TF-2, GES-1 cells treated with 40  $\mu$ g/ml TFs prior to 0.5 mol/l ethanol treatment; TF-3, GES-1 cells treated with 80  $\mu$ g/ml TFs prior to 0.5 mol/l ethanol treatment.

have indicated that extracellular signals are transferred between cells via the MAPK pathway in order to induce various cellular responses (54,55). ERK, JNK and p38 are important proteins in MAPK pathways (56). ERK is closely associated with cell viability and proliferation, while JNK and p38 are involved in apoptotic pathways and are more readily activated by stimuli in the extracellular environment, including oxidative stress, ultraviolet irradiation, high temperatures, ischemia reperfusion and inflammatory factors (57,58). The results of the present study revealed that treatment with ethanol upregulated the phosphorylation levels of ERK, JNK and p38, indicating the activation of associated MAPK pathways so as to induce oxidative stress and apoptosis in GES-1 cells. Furthermore, the results revealed that treatment with TFs protected GES-1 cells from ethanol-induced injury via downregulation of the phosphorylation of ERK, JNK and p38.

In conclusion, the results of the current study indicated that TFs may attenuate ethanol-induced oxidative stress and apoptosis in GES-1 gastric mucosa epithelial cells by increasing

the function of SOD and GSH in order to reduce ROS and MDA levels, regulating the activity of apoptosis pathways, such as via the upregulation of Bcl-2 expression and the downregulation of Bax and Caspase-3 expression levels, and by suppressing the activation of ERK, JNK and p38 MAPK pathways. The results of the present study indicate that TFs, a type of natural and health-benefitting properties, may represent a novel therapeutic agent for the treatment and/or prevention of diseases resulting from ethanol-induced gastric mucosa injury. However, the effect of TFs on gastric mucosa injury in a clinical setting requires further investigation.

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### Availability of data and materials

All data analyzed during this study are included in this published article.

### Authors' contributions

ZW and HL conducted all the experiments, HL also produced the manuscript, and HX made substantial contributions to the conception and design of the present study.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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