Gastrodin Protects against Ethanol-Induced Liver Injury and Apoptosis in HepG2 Cells and Animal Models of Alcoholic Liver Disease

Yong Zhang, Can Wang, Bin Yu, Jian-Dong Jiang, and Wei-Jia Kong*

*Department of Virology, Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College; and State Key Laboratory of Bioactive Natural Products and Function, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College; Beijing 100050, China.

Received October 12, 2017; accepted February 26, 2018

This study aims to investigate the protective effects of gastrodin (GSTD), a natural compound isolated from the root of Gastrodia elata Bl., on ethanol-induced liver injury and apoptosis in HepG2 cells and animal models. For in vitro studies, GSTD was used to pre-treat the cells for 4h followed by 600 mm of ethanol co-administration for 24h. Alcoholic liver disease (ALD) of Sprague–Dawley (SD) rats was induced by chronic ethanol-feeding plus a single dose (5g/kg) of acute ethanol administration, GSTD at different doses were co-administered for 8 weeks. For acute liver injury experiment of ICR mice, GSTD (100 mg/kg/d) was pre-treated for 3d followed by ethanol administration (5g/kg) for 3 times. The results showed that GSTD protects HepG2 cells from ethanol-induced toxicity, injury, and apoptosis significantly. Co-administered with ethanol, GSTD prevented the loss of mitochondrial membrane potential, reduced the release cytochrome c from mitochondria, and inhibited the activation of caspase-3 in HepG2 cells. In SD rats induced by chronic ethanol-feeding, GSTD significantly restored liver function and ameliorated pathological changes of the liver. In rat liver, GSTD greatly suppressed the activation of caspase-3 and inhibited hepatocellular apoptosis. In ethanol-induced acute liver injury of ICR mice, GSTD reduced liver acetaldehyde and suppressed the up-regulation of alcohol dehydrogenase (ADH) and CYP2E1 significantly. Our results demonstrate that GSTD is efficacious in protecting liver cells from ethanol-induced injury and apoptosis; it may be useful for the development of novel agents for the treatment of ALD in the future.

Key words gastrodin; cytotoxicity; alcoholic liver disease; mitochondrial apoptotic pathway; alcohol dehydrogenase; acetaldehyde

Alcohol abuse has been a serious social problem in the western world for a long time. In recent years, with the rapid development of economy and change of lifestyle, the amount of alcohol consumption increased greatly in China. Chronic intake of excessive ethanol will cause injury of the liver, which is a major organ for the metabolism and detoxification of ethanol. Now in China, with the increase of the population of alcoholics, alcohol becomes the second cause of chronic liver disease, which is next only to the hepatitis viruses.

The natural course of alcoholic liver disease (ALD) includes alcoholic fatty liver, steatohepatitis, fibrosis and cirrhosis. If there are no effective interventions, ALD will lead to hepatocellular carcinoma and cause mortality of the patients eventually.

Gastrodin (GSTD), a low molecular weight (286.3) compound, is one of the main bioactive components of Gastrodia elata Bl., an ancient Chinese medicinal plant. GSTD and Gastrodia elata Bl. are now widely used in clinic to treat nerve disorders. GSTD was proved to have multiple pharmacological activities, for example, it was reported to lower blood pressure, improve heart function and regulate immune functions.

GSTD also had beneficial effects in liver diseases. For example, our previous results showed that GSTD alleviated hepatic steatosis and inflammation in animals with nonalcoholic fatty liver disease (NAFLD). In addition, GSTD was reported to increase the viability of ethanol-treated L02 liver cells, which suggested that it might have beneficial effects against ALD.

However, the activities and mechanisms of GSTD in protecting liver cells from ethanol-induced damage were not studied in detail previously, and the efficacies of GSTD in animals with ALD were unknown. In this work, we use ethanol to induce liver injury in HepG2 cells and animal models, and our results prove that GSTD protects liver cells from ethanol-induced injury and apoptosis significantly.

MATERIALS AND METHODS

Reagents and Kits GSTD (purity $\geq$98%) (lot number: 20150812) was purchased from Zhejiang Cheng Yi Pharmaceutical Co., Ltd. (Dongtou, Zhejiang, China), ethanol was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, U.S.A.) (purity $\geq$99.8%, for cell experiments) and Sinopharm Chemical Reagent Beijing Co., Ltd. (Beijing, China) (AR, for animal experiment), thiazolyl blue tetrazolium bromide (MTT) was purchased from Sigma-Aldrich Co. LLC, and the Lactate Dehydrogenase (LDH) Assay Kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Fetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium (DMEM) were purchased from Gibco-Invitrogen (Grand Island, NY, U.S.A.). The Cell and Tissue Triglyceride (TG) Assay Kit was purchased from Applygen Technologies Inc (Beijing, China), serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), TG, albumin (ALB), and total protein (TP) assay kits were purchased from Beijing Strong Biotecnologies, Inc (Beijing, China). The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI)
Cell Apoptosis Detection Kit was purchased from Beijing TransGen Biotech Co., Ltd. (Beijing, China); the One Step TdT-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) Apoptosis Assay Kit, DNA Ladder Extraction Kit, and Mitochondrial Membrane Potential Assay Kit with JC-1 were purchased from Beyotime Biotechnology (Shanghai, China); the CaspASE<sup>TM</sup> Assay System Colorimetric was purchased from Promega (Beijing) Biotech Co., Ltd. (Beijing, China). Reagents required for RNA isolation and real-time RT-PCR were purchased from Promega (Beijing) Biotech Co., Ltd. The Mammalian Protein Extraction Reagent and Bis(2-ethylhexyl) succinate (BCH) Protein Assay Kit were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, U.S.A.). Monoclonal or polyclonal antibodies against cytochrome c, caspase-3, and β-actin (ACTB) were purchased from the Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.), and those against CYP2E1 and alcohol dehydrogenase (ADH) were from Abcam (Shanghai, China). The Cell Mitochondria Isolation Kit and the Glutathione (GSH)/Oxidized Glutathione (GSSG) Assay Kit were purchased from Beyotime Biotechnology. The Blood Ethanol (ALC) Assay Kit was purchased from Beijing BioLab Science and Technology, Ltd. (Beijing, China), the Alcohol-Dehydrogenase Assay Kit was purchased from Megazyme (Bray, Wicklow, Ireland), the Tissue ADH Assay Kit was from Nanjing Jiancheng Bioengineering Institute, the Tissue Microsome Isolation Kit and the CYP2E1 Activity Fluorescence Assay Kit were from Shanghai GenMed Science and Technology, Ltd. (Shanghai, China).

**Cell Culture and Treatment** HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) +10% FBS and appropriate antibiotics at 37°C in a carbon dioxide incubator as described previously. GSTD was dissolved in sterile saline to make a stock solution of 100 mM and was diluted with culture medium to desired concentrations in our experiment. One day before experiment, cells were seeded onto 96-well plates (2 x 10<sup>4</sup>/well), 24-well plates (1 x 10<sup>5</sup>/well), or 6-well plates (4 x 10<sup>5</sup>/well) and cultured for 24 h to reach about 70–80% confluence.

To determine the concentration of ethanol used in the experiment, HepG2 cells were treated with increasing concentrations of ethanol for 24 h and cells viabilities were assayed. In the following experiments, cells were left untreated or pre-treated with GSTD at 125, 250 or 500 M for 4 h; then, cells were again left untreated or treated with ethanol at a concentration of 600 M for 24 h. The plates were sealed with Parafilm (Bemis Company, Inc., Oshkosh, WI, U.S.A.) during ethanol treatment.

**Cellular MTT and LDH Assay** After treatment, culture media were removed, cells were subjected to MTT staining and the absorbances were read on a Multilabel Plate Reader (PerkinElmer, Inc., Waltham, MA, U.S.A.) as described previously. The viabilities of cells were calculated as percentages of control cells, which were left untreated and designated as 100. For the measurement of LDH release, culture media were moved to microcentrifuge tubes and centrifuged at 500 g for 5 min, LDH concentrations in the supernatants were determined by a commercially available kit according to the supplier's protocol, the absorbances were read at 450 nm.

**Cellular TG, ALT and AST Assay** After treatment, cells were harvested; intracellular TG was determined by a commercially available kit and normalized to protein content of the sample. Meanwhile, culture supernatants were harvested for the measurement of ALT and AST levels by commercially available kits according to the supplier's protocols.

**Flow Cytometry** After treatment, cells were detached with 0.25% trypsin (Gibco-Invitrogen), washed twice with cold phosphate buffer saline (PBS), and centrifuged at 500 x g for 5 min at 4°C. For one well of a 6-well plate, cells were resuspended in 100 µL of cold 1% Annexin V Binding Buffer, then, 5 µL of Annexin V-FITC and 5 µL of PI were added and mixed gently. The tubes were incubated at room temperature for 15 min in the dark, then, 400 µL of cold 1% Annexin V Binding Buffer was added and mixed gently, cell apoptosis was analyzed in a BD LSR II Flow Cytometer (BD Biosciences, San Jose, CA, U.S.A.).

**TUNEL and DNA Fragmentation Assay** TUNEL assay was performed according to the supplier’s protocol. Briefly, cells (in 24-well plates) were washed with PBS, fixed in 4% paraformaldehyde for 30 min, and treated with the Enhanced Immunostaining Permeabilization Buffer (Beyotime Biotechnology) for 5 min. After washing with PBS, 50 µL of TUNEL assay solution which contained TdT and Cysteine 3 (Cy3) labeled-dUTP was added and incubated at 37°C for 1 h in the dark. After washing with PBS for 3 times, 50 µL of Anti-fluoride Mounting Medium (Beyotime Biotechnology) was added, cells were observed under a Olympus BX53 Semi-Motorized Fluorescence Microscope (Olympus Corporation, Tokyo, Japan) (excitation wavelength 550 nm, emission wavelength 570 nm) and photographed.

For evaluation of DNA fragmentation, cells were harvested and DNA was isolated by a commercially available kit which was based on the method of phenol–chloroform extraction and ethanol precipitations. Samples were subjected to 1.5% agarose gel electrophoresis, stained with GelRed (Biotium, Inc., Fremont, CA, U.S.A.) and photographed.

**RNA Extraction and Real-Time RT-PCR** After treatment, cells were harvested for isolation of total RNA by using a commercially available kit according to the supplier’s protocol. For reverse transcription, 1 µg of total cellular RNA was used as template in a 20 µL reaction system as described before. The reactions were conducted at 25°C for 5 min, 42°C for 1 h, followed by 70°C for 15 min; the cDNAs were frozen at −20°C. Real-time PCR was performed in an ABI 7900 High-Throughput Real-Time PCR System (Applied Biosystems, Foster City, CA, U.S.A.) with gene specific primers (Table 1). The reaction condition was as following: 95°C for 2 min, followed by 40 cycles of 95°C 15 s + 60°C 1 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primers</th>
<th>Reverse primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>caacctgaacccctcaacagttg</td>
<td>actcaactcctaaagacagc</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>acttgagctgcggcc</td>
<td>getggagttgctgacgc</td>
</tr>
<tr>
<td>p53</td>
<td>ctcggcgtctgctgctgac</td>
<td>tttaaagctggttgctgac</td>
</tr>
<tr>
<td>GAPDH</td>
<td>aggcctctagctgacac</td>
<td>gccctaaatcctgacac</td>
</tr>
<tr>
<td>Rat</td>
<td>gggcctgctgtgcacacac</td>
<td>caaagctgctgctgctgac</td>
</tr>
<tr>
<td>Bax</td>
<td>gggcctgctgtgcacacac</td>
<td>caaagctgctgctgctgac</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>gcgtgctgctgctgctgctgac</td>
<td>gcgtgctgctgctgctgctgac</td>
</tr>
<tr>
<td>p53</td>
<td>ctgctgctgctgctgctgctgac</td>
<td>tcgctgctgctgctgctgctgac</td>
</tr>
<tr>
<td>ACTB</td>
<td>tggctgctgctgctgctgctgac</td>
<td>ggtgctgctgctgctgctgctgac</td>
</tr>
</tbody>
</table>

Table 1. Primers for Real-Time RT-PCR (5’ to 3’)

Vol. 41, No. 5 (2018)  
671
an internal control for relative quantification of target genes, and their mRNA levels were plotted as fold of control cells, which was defined as 1.

**Measurement of Mitochondrial Membrane Potential**
The working solution for 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide (JC-1) staining was prepared by using JC-1, water, and staining buffer according to supplier's protocol. For one well of a 6-well plate, cells were washed once with PBS after treatment, then, 1 mL of culture medium and 1 mL of the JC-1 working solution were added and mixed well. The plates were incubated at 37°C for 20 min, then, the supernatants were discarded, cells were washed 2 times with staining buffer, and 2 mL of culture medium was added. By using appropriate excitation and emission wavelengths, cells were observed under a fluorescence microscope for the detection of red and green fluorescence and photographed.

**Mitochondria Isolation and Western Blot** After treatment, cells were harvested, washed once with cold PBS, and treated with the Mitochondria Isolation Reagent which contained phenylmethanesulfonyl fluoride (PMSF). After incubation for 15 min on ice, cells were homogenized in a glass homogenizer and centrifuged at 11000 g for 10 min at 4°C. The supernatants were moved to new tubes and centrifuged at 11000×g for 10 min at 4°C. The precipitates (mitochondria) were lysed by adding the Mitochondria Lysis Buffer which contained PMSF; the supernatants were centrifuged again at 12000×g for 10 min at 4°C, and the resulting supernatants were collected as cytoplasmic proteins without mitochondria. In parallel experiments, cells were harvested after treatment, total cell proteins were extracted as described before.14)

The protein concentrations of the above samples were quantified, about 30 µg protein of each sample was subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electric transfer as described before.14) After appropriate blocking, the membranes were incubated with specific monoclonal or polyclonal antibodies and subsequently appropriate horseradish peroxidase (HRP)-labeled secondary antibodies (Beyotime Biotechnology) to detect the levels of corresponding proteins in mitochondria, cytoplasm, or whole cell lysates. After development of signals,14) the blots were quantified, and the levels of target proteins were normalized to that of ACTB and plotted as fold of control cells.

**Caspase-3 Activity Assay** After treatment, cells were harvested, washed once with cold PBS, and resuspended in the Cell Lysis Buffer supplied by the kit. The cells were lysed by 3 times of freeze-thaw, and each time after thawing, cells were incubated on ice for 15 min. After that, samples were centrifuged at 15000×g for 20 min at 4°C, and the supernatants were collected. The assays were performed in 96-well plates in a 100 µL of reaction system which contained the Caspase Assay Buffer (32 µL), dimethyl sulfoxide (DMSO, Sigma-Aldrich Co. LLC) (2 µL), dithiothreitol (DTT, Sigma-Aldrich Co. LLC, 100 mM) (10 µL), sample (10 µL), the colorimetric substrate (p-nitroaniline (pNA) labeled-DEVD (Asp-Glu-Val-Asp), 10 mM) (2 µL), and water. The plates were incubated at 37°C for 4 h, and the absorbances at 405 nm were read by a plate reader. Caspase-3 activities of the samples were calculated according to the supplier’s protocol, normalized to protein contents, and presented as pmol of pNA released per h per mg of protein.

**Animal Experiment** Before experiment, the protocols were reviewed and approved by the Ethics Committee of the Institute of Medicinal Biotechnology, the experiments were conducted and animals were cared for according to relevant guidelines of the Chinese Academy of Medical Sciences (CAMS).

Male Sprague–Dawley (SD) rats, weighting about 103±10.7 g, were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). During the 5d of accommodation period, the rats were fed with a regular rodent diet and housed as described before.11) During the next 5d, rats were fed with a control Lieber–DeCarli liquid diet (TROPHIC Animal Feed High-Tech Co., Ltd., Nantong, Jiangsu, China) which contained 35% kcal fat, 47% kcal carbohydrate and 18% kcal protein for accommodation.

ALD of the rats was replicated according to the Gao-Binge model.17,18) Some rats were continued fed with the control liquid diet and used as control group, while the others were fed with a 5% (v/v) ethanol containing-Lieber–DeCarli liquid diet (TROPHIC Animal Feed High-Tech Co., Ltd.) which contained 35% kcal fat, 19% kcal carbohydrate, 18% kcal protein and 28% kcal ethanol.17,18) Among the ethanol-fed rats, some were left untreated (ethanol group); the others were orally treated with GSTD at doses of 50, 100, or 200 mg/kg/d (dissolved in saline) at the same time of ethanol-feeding. There were 10 rats in each group, except that in the ethanol group, 1 rat died during the experiment (n=9).

The ethanol-feeding and GSTD administration lasted for 8 weeks, body weights of the rats were measured 2 times a week. The intake amounts of control and ethanol containing-liquid diets were recorded and fresh diets were replaced at 4 p.m. everyday.

On the last day of experiment, single dose of ethanol (diluted with water to 31.5%) at 5 g/kg was orally administered to the ethanol-fed rats in the early morning.17,18) Nine hours later, blood samples were collected through retro-orbital puncture, sera were isolated for the determination of ALT, AST, TG, ALB, and TP by using kits. The rats were sacrificed, their livers were harvested and weighed, and the liver index was calculated as described before.11)

A part of liver tissue was excise from the same site and fixed in 10% formalin. The tissues were subjected to paraffin embedding, histological section (4 µm), and hematoxylin and eosin (HE) staining. Pathological changes of the liver were evaluated and scored by steatosis (0–3, 0 for <5% of liver cells, 1 for 5–33%, 2 for 34–66%, 3 for >66%), portal inflammation (0–4, 0 for none, 1 for minimal, 2 for mild, 3 for moderate, 4 for severe), and tissue destruction (0–4, according to the number of necrotic foci under a 20×field, 0 for none, 1 for <2, 2 for 2–3, 3 for 4–5, 4 for >5). Liver tissue sections were also used for TUNEL staining after dewaxing and proteinase K (Beyotime Biotechnology) treatment according to the supplier’s protocol.

The rest of liver tissues were quickly frozen in liquid nitrogen. After homogenization, hepatic levels of TG, GSH, GSSG, and caspase-3 activities were assayed by kits and normalized to protein contents; the ratios of GSH/GSSG were calculated. Total RNAs and proteins were extracted from liver tissues for real-time RT-PCR and Western blot analysis of target genes (Table 1) or proteins.

In another animal experiment, ethanol-induced acute liver
injury of ICR mice (male, 21.5±1.10 g, Beijing Vital River Laboratory Animal Technology Co., Ltd.) was replicated as described before with modifications. Briefly, the mice were fed with a regular rodent diet and were divided into 3 groups with 10 mice each, which were the control group, ethanol group, and ethanol+GSTD group, respectively. GSTD at 100 mg/kg/d was orally administered to the ethanol+GSTD group of mice for 3 d, 1 h after the last administration, 5 g/kg of ethanol (diluted with water to 31.5%) was orally administered to the mice except the control group. The mice were treated with ethanol every 12 h for 3 times.

One hour after the last ethanol administration, 0.2 mL of blood was collected through retro-orbital puncture, and serum was isolated for the assay of ethanol concentration by using a kit. Then, 9 h later, 0.8 mL of blood was collected through retro-orbital puncture; serum was isolated for the assay of ethanol concentration as well as ALT and AST levels. The mice were then sacrificed and their livers were harvested and stored in liquid nitrogen. After homogenization, liver total proteins were extracted for Western blot analysis of the expression levels of target proteins. Hepatic acetaldehyde contents and ADH activities were determined and normalized to proteins according to the suppliers’ protocols which were based on the enzymatic methods. Liver microsomes were isolated by using ultracentrifugation according to the protocol; the activity of CYP2E1 was determined by using a fluorogenic substrate method according to the supplier’s protocol and presented as pmol of product produced per min per mg of protein.

Statistical Analysis For in vitro results, values are mean±standard deviation (S.D.) of at least 3 separate experiments, for the animal experiment, values are mean±S.D. of 9 or 10 rats in each group. The SPSS 13.0 software was used for statistical analysis. After validation of the test for homogeneity of variance, differences among studied groups were examined by one-way ANOVA followed by multiple comparisons. *p<0.05 was considered to be statistically different.

RESULTS

GSTD Prevents Ethanol-Induced Toxicity and Injury in HepG2 Cells First, we determined the appropriate concentration of ethanol used in our experiments. As shown in Fig. 1A, ethanol administration resulted in a reduction of cell viability dose-dependently. When the concentration of ethanol reached 600 mM in culture medium, cell viability declined to about 51.3% (p<0.01 vs. control cells) after 24 h, and we chose...
this concentration for the subsequent experiments.

To evaluate the protective effects of GSTD on ethanol-induced damage, HepG2 cells were pre-treated with GSTD, and then, ethanol were added, cell viability was determined by MTT staining. As shown in Fig. 1B, co-administration of GSTD with ethanol increased the viability of HepG2 cells in a dose-dependent manner, which was in agree with a previous report.\(^\text{11}\) When the concentration of GSTD in the culture medium reached 500 \(\mu\text{M}\), cell viability restored greatly \((p<0.01 \text{ vs. ethanol alone})\), which was near to baseline level of control cells.

LDH leakage is another classical indicator of cytotoxicity. As shown in Fig. 1C, ethanol treatment induced a significant LDH release into the culture medium \((p<0.001 \text{ vs. control cells})\). In accordance with the restoration of cell viability, pre-treatment and co-administration of GSTD greatly reduced the level of LDH in the culture medium \((p<0.05 \text{ or } p<0.01 \text{ vs. ethanol alone})\).

Ethanol treatment induced hepatic steatosis, as indicated by a significant TG accumulation in HepG2 cells (Fig. 2A) \((p<0.01 \text{ vs. control cells})\). However, co-administration of GSTD prevented intracellular TG accumulation greatly \((p<0.05 \text{ or } p<0.01 \text{ vs. ethanol alone})\).

Administration of ethanol caused a massive release of aminotransferases from HepG2 cells. As shown in Fig. 2B, compared to control cells \((p<0.001)\), the levels of ALT and AST increased for about 4.23-and 3.80-fold in the supernatant of cells treated with ethanol, respectively. For comparison, GSTD at 125, 250 and 500 \(\mu\text{M}\) prevented the elevation of ALT and AST in the culture supernatant significantly and dose-dependently \((p<0.05 \text{ or } p<0.01 \text{ vs. ethanol alone})\).

GSTD Suppresses Ethanol-Induced Apoptosis and Mitochondrial Apoptotic Pathway in HepG2 Cells

Ethanol-induced apoptosis was evaluated by Annexin V-FITC/PI double staining and flow cytometry (Fig. 3A). As compared to control cells (7.18%), administration of ethanol caused a great increase of the percent of early apoptotic and late apoptotic cells (36.7%). GSTD suppressed cell apoptosis dose-dependently (Fig. 3A and data not shown). When 125 \(\mu\text{M}\) of GSTD was used to pre-treat the cells and co-administered with ethanol, the percent of apoptotic cells declined to about 23.0%.

Fig. 3. Effects of GSTD on Ethanol-Induced Cell Apoptosis

HepG2 cells were treated as described in Fig. 1B. (A) After treatment, cells were harvested for Annexin V-FITC/PI double staining, cell apoptosis was analyzed by flow cytometry. Q4: viable cells, Q3: early apoptosis, Q2: late apoptosis, and Q1: primary necrosis. (B) In parallel experiments, cells were fixed in 4% paraformaldehyde and subjected to TUNEL assay, which was observed under a fluorescence microscope (×100) and photographed. (C) Cell DNA was extracted, subjected to 1.5% agarose gel electrophoresis, stained with GelRed, and photographed. M: DNA marker, lane 1: control, lane 2: ethanol+GSTD 125 \(\mu\text{M}\), lane 3: ethanol+GSTD 250 \(\mu\text{M}\), lane 4: ethanol+GSTD 500 \(\mu\text{M}\), and lane 5: ethanol. (D) The mRNA expression levels of apoptosis-related genes were analyzed by real-time RT-PCR with GAPDH as an internal control (upper panel); the ratio of Bax/Bcl-2 was calculated and presented as fold of control cells (lower panel). Images in A–C are representative results of at least 3 separate experiments. Values are mean±S.D. of 3 separate experiments in D. **\(p<0.01\), ***\(p<0.001\) vs. that of control cells; \(^p<0.05\, **p<0.01\) vs. that of cells treated with ethanol alone.

Fig. 4. Effects of GSTD on Ethanol-Induced Mitochondrial Apoptotic Pathway

HepG2 cells were treated as described in Fig. 1B. (A) Mitochondrial membrane potential was determined by JC-1 staining, cells were observed under a fluorescence microscope (×100), and the aggregate and monomer forms of JC-1 were detected by red or green fluorescence, respectively. Images are representative results of 3 separate experiments. (B) Mitochondrial, cytoplasmic, and total cell proteins were extracted as described in the Materials and Methods. The samples were subjected to SDS-PAGE and Western blot for the detection of cytochrome c (Cyto c), caspase-3, and cleaved caspase-3 (left panel), and their protein levels were normalized to that of ACTB and plotted as indicated (right panel). (C) Cellular caspase-3 activities were assayed by the chromogenic substrate method. Values are mean±S.D. of 3 separate experiments in B and C. **\(p<0.01\) vs. that of control cells; \(^p<0.05\, **p<0.01\) vs. that of cells treated with ethanol alone.
The inhibitory effect of GSTD on ethanol-induced HepG2 cell apoptosis was verified by the TUNEL assay. As shown in Fig. 3B, a large number of cells were stained red after ethanol treatment, and 125 µM of GSTD greatly reduced the number of TUNEL-positive cells.

As the positive TUNEL staining is an index of DNA breakage, we extracted the cellular DNAs in order to observe DNA fragmentation. As shown in Fig. 3C, agarose gel electrophoresis clearly showed that DNA ladder occurred after ethanol treatment; co-administration of GSTD at 125, 250 and 500 µM reduced DNA fragmentation by different extents.

The expression levels of apoptosis-related genes were analyzed by real-time RT-PCR (Fig. 3D, upper panel). After ethanol treatment, the mRNA expression levels of proapoptotic genes such as Bax and p53 increased significantly (p<0.01 or p<0.001 vs. control cells), while that of Bel-2, an antiapoptotic gene, decreased significantly (p<0.01 vs. control cells). As a result, the ratio of Bax/Bcl-2 elevated greatly after ethanol treatment (p<0.001 vs. control cells) (Fig. 3D, lower panel). GSTD down-regulated the levels of Bax and p53, increased levels of Bcl-2, and reduced the ratio of Bax/Bcl-2 dose-dependently in HepG2 cells (p<0.05 or p<0.01 vs. ethanol alone) (Fig. 3D).

In JC-1 staining (Fig. 4A), ethanol treatment resulted in a significant loss of the mitochondrial mitochondrial membrane potential in HepG2 cells, as indicated by the reduction of JC-1 aggregates (red fluorescence) and the significant increase of JC-1 monomers (green fluorescence). In contrast, GSTD restored mitochondrial membrane potential greatly (Fig. 4A and data not shown). When 125 µM of GSTD was used to pre-treat the cells and co-administered with ethanol for 24h, it effectively increased the red fluorescence and reduced the green fluorescence.

After ethanol treatment, the reduction of mitochondrial membrane potential was translated into a significant release of cytochrome c from mitochondria into cytoplasm (Fig. 4B, p<0.01 vs. control cells). GSTD prevented the release of cytochrome c and reduced its level in cytoplasm significantly and dose-dependently (p<0.05 or p<0.01 vs. ethanol alone) (Fig. 4B).

As the release of cytochrome c from mitochondria was associated with the activation of caspase-3 and initiation of cell apoptosis,20) we determined caspase-3 activation by Western blot and enzyme activity assay. As shown in Fig. 4B and 4C, ethanol exposure greatly increased the level of cleaved caspase-3 (17 kd), an active form of caspase-3, and enhanced its enzyme activity accordingly (p<0.01 vs. control cells). In accordance with the suppressing effect on cytochrome c release, GSTD decreased the level of cleaved caspase-3 (Fig. 4B) and inhibited its activity (Fig. 4C) significantly (p<0.05 or p<0.01 vs. ethanol alone).

GSTD Ameliorates Liver Injury and Apoptosis in a Rat Model of ALD ALD of the rats was induced by chronic ethanol-feeding plus a single dose of acute ethanol administration. As shown in Table 2, when compared to the control group (p<0.05, p<0.01 or p<0.001), serum ALT and AST levels increased significantly, but the levels of ALB and TP decreased significantly in the model group of rats, indicating liver injury and impairment of liver function. GSTD dose-dependently ameliorated liver injury and restored liver function, as indicated by the significant reduction of serum ALT, AST, and the significant increase of ALB and TP levels after treatment (p<0.05, p<0.01 or p<0.001 vs. ethanol group). When the dose of GSTD reached 200 mg/kg/d, these parameters almost restored to baseline levels of control group.

The volumes of liquid diet intake and endpoint body weights (Table 2) of the rats were not statistically different among the studied groups. Ethanol treatment caused a significant increase of liver weight and liver index of the rats, which was prevented by 100 or 200 mg/kg/d of GSTD administration. In addition, the rats developed hypertriglyceridemia and hepatic steatosis after ethanol treatment, as indicated by the elevation of serum and liver TG levels (p<0.01 or p<0.001 vs. control group). Co-administration of GSTD at 50, 100 or 200 mg/kg/d reduced serum and liver TG significantly (p<0.05, p<0.01 or p<0.001 vs. ethanol group).

Ethanol induced oxidative stress in the liver, as indicated by the depletion of GSH, the elevation of GSSG, and the decline of GSH/GSSG ratio (Table 2) (p<0.01 or p<0.001 vs. control group). GSTD restored liver GSH and GSH/GSSG ratio dose-

<table>
<thead>
<tr>
<th>Control (n=10)</th>
<th>Ethanol (n=9)</th>
<th>Ethanol+GSTD (mg/kg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 (n=10)</td>
<td>100 (n=10)</td>
</tr>
<tr>
<td>Serum ALT (U/L)</td>
<td>46.2±3.21</td>
<td>111.2±18.2***</td>
</tr>
<tr>
<td>Serum AST (U/L)</td>
<td>135.8±11.6</td>
<td>225.5±34.7**</td>
</tr>
<tr>
<td>Serum ALB (g/L)</td>
<td>34.2±3.25</td>
<td>27.7±3.69*</td>
</tr>
<tr>
<td>Serum TP (g/L)</td>
<td>75.9±6.78</td>
<td>60.4±8.54*</td>
</tr>
<tr>
<td>Serum TG (mmol)</td>
<td>0.95±0.06</td>
<td>1.60±0.19**</td>
</tr>
<tr>
<td>Liquid diet intake (mL/rat/d) (week 8)</td>
<td>73.7±6.54</td>
<td>71.1±10.2</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>341.4±42.2</td>
<td>321.8±45.6</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>11.1±1.35</td>
<td>13.8±1.68*</td>
</tr>
<tr>
<td>Liver index (%)</td>
<td>3.25±0.51</td>
<td>4.21±0.62</td>
</tr>
<tr>
<td>Liver TG (µmol/mg)</td>
<td>0.12±0.01</td>
<td>0.43±0.05***</td>
</tr>
<tr>
<td>Liver GSH (nmol/mg)</td>
<td>2.68±0.33</td>
<td>1.57±0.24**</td>
</tr>
<tr>
<td>Liver GSSG (nmol/mg)</td>
<td>0.18±0.02</td>
<td>0.31±0.04**</td>
</tr>
<tr>
<td>GSH/GSSG</td>
<td>14.9±1.85</td>
<td>5.06±0.76***</td>
</tr>
</tbody>
</table>

Values are mean±S.D. of 9 or 10 rats in each group.*p<0.05, **p<0.01, ***p<0.001 vs. that of control group; †p<0.05, ‡p<0.01, ‡‡p<0.001 vs. that of ethanol group.
In histopathological examination, chronic intake of ethanol induced severe pathological changes in the liver (Fig. 5), such as steatosis, portal inflammation, and tissue destruction ($p < 0.001$ vs. control group), which were effectively improved by GSTD ($p < 0.05$, $p < 0.01$ or $p < 0.001$ vs. ethanol group). When 200 mg/kg/d of GSTD was used to treat the ethanol-fed rats, their liver pathological changes were largely restored.

Hepatocellular apoptosis in vivo was evaluated by TUNEL

assay by using liver sections of the rats. While a large number of apoptotic cells appeared in the liver of ethanol group, GSTD greatly reduced the number of TUNEL-positive cells in rat liver (Fig. 6A and data not shown). The hepatic expression levels of Bax and p53 mRNAs declined, while that of Bcl-2 increased after GSTD treatment, as a result, the ratio of Bax/Bcl-2 was significantly reduced by GSTD ($p < 0.05$, $p < 0.01$ or $p < 0.001$ vs. ethanol group) (Fig. 6B). The protein level of cleaved caspase-3 was determined by Western blot. As shown in Fig. 6C, while ethanol greatly induced caspase-3 cleavage ($p < 0.01$ vs. control group), co-administration of GSTD at 50, 100 or 200 mg/kg/d greatly reduced the level of cleaved caspase-3 in the liver ($p < 0.05$ or $p < 0.01$ vs. ethanol group). Accordingly, the ethanol-induced enzyme activity of caspase-3 was significantly suppressed by GSTD (Fig. 6D).

**GSTD Alleviates Ethanol-Induced Acute Liver Injury in Mice** To further study the protective effect of GSTD on ethanol-induced liver injury, ICR mice were pre-treated with GSTD and then ethanol were administered to induce acute alcohol intoxication. As shown in Fig. 7A, compared to the control mice ($p < 0.001$), the serum ethanol concentration increased for about 44.1-fold at 1 h after ethanol administration and returned to baseline after 10 h. GSTD had no effect on serum ethanol concentration (Fig. 7A), but effectively prevented the elevation of liver acetaldehyde (B). Liver total proteins were extracted for Western blot analysis of the levels of ADH and CYP2E1, which were normalized to ACTB and plotted as fold of control (C). Liver ADH activity (D) and microsome CYP2E1 activity (E) were also measured. Values are mean ± S.D. of 10 mice in each group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. that of control group; # $p < 0.05$ vs. that of ethanol group.

**DISCUSSION**

Now in clinic, in addition to abstinence, targeted therapies for ALD are still lacking. Commonly used treatments include nutritional support, liver protection therapy, glucocorticoid therapy, and so on. Here in the present study, we report that GSTD, a natural product with a long history of clinical ap-
plication, protects liver cells from ethanol-induced injury and apoptosis.

GSTD itself is low toxic, when its concentration in the culture medium is no more than 3386.5 μM, it has no significant influence on the growth of HL-7702 liver cells.23) In the present study, the concentrations of GSTD used to treat HepG2 cells are far lower than that one, suggesting that it is nontoxic to the cells. We found that when GSTD was used prior to or together with ethanol to treat HepG2 cells or animal models, it had detoxifying and liver protective effects, as indicated by the restoration of cell viability and ALB production, the inhibition of the release of aminotransferases and LDH, the amelioration of oxidative stress and proinflammatory response, and the improvement of liver pathological changes after treatment.

GSTD had no influence on serum concentration of ethanol after alcohol intake. However, it was able to reduce the level of acetaldehyde in the liver. In hepatocytes, ethanol is metabolized by a series of enzymes, which may exist in cytoplasm or organelles. ADH and CYP2E1 are 2 important enzymes responsible for the metabolism of ethanol and their expressions are able to be stimulated by ethanol.25) Up-regulation of ADH will cause an overproduction of acetaldehyde.23) While excessive CYP2E1 is associated with oxidative stress in the liver.22,23) GSTD may protect liver from injury through inhibition of ADH and acetaldehyde production after ethanol administration.

Our results also showed that GSTD inhibited hepatic TG accumulation induced by ethanol treatment, either in cultured cells or in rats. This effect was in accordance with our previous studies, in which GSTD activated the cellular AMP-activated protein kinase (AMPK) pathway and inhibited hepatic TG accumulation induced by oleic acid (OA) treatment22) or high-fat diet (HFD)-feeding.11)

In addition to alcohol, the liver protective effects of GSTD were verified in some other animal models of liver damage caused by different factors. For example, our previous results were verified in some other animal models of liver damage.22,23) Our results showed that GSTD not only reduces serum ALT and AST levels, restored liver morphology, and inhibited inflammatory cell infiltration in the liver.11) In liver damage of rats caused by vincristine, administration of GSTD greatly improved the general condition and locomotion of the rats; meanwhile, it effectively reduced serum ALT and AST levels, and protected liver cells from edema, inflammation, and necrosis in the pathological examination.25,26) In liver damage and fibrosis induced by bile duct ligation, GSTD significantly lowered serum ALT and AST, ameliorated oxidative stress and proinflammatory response in the liver, and improved liver pathological changes of the rats.27)

Due to the limitation of the animal models of ALD, liver fibrosis is not obvious in our animal experiment. However, GSTD was proved to reduce liver fibrosis in rats with NAFLD11) and in a rat model of liver fibrosis induced by bile duct ligation.27) It will be reasonable to infer that GSTD may also reduce liver fibrosis induced by ethanol in vivo, considering the potent activity of this compound in improving the liver pathological changes in our study.

One of the major findings in the current study is that GSTD suppresses ethanol-induced liver cell apoptosis through inhibiting the mitochondrial apoptotic pathway. The proteins of Bax and Bel-2 play important roles in regulating cell survival and apoptosis through modulating the mitochondrial apoptotic pathway.28-31) Elevation of the ratio of Bax/Bel-2 will promote the formation of Bax/Bax homodimer, which will result in an increase of the permeability of mitochondrial outer membrane and loss of mitochondrial membrane potential.28,29) Cytochrome c is then released and cell apoptosis will be initiated.28-30) Our results clearly showed that GSTD reduced the ratio of Bax/Bel-2 which was elevated by ethanol treatment, and then restored mitochondrial membrane potential, inhibited cytochrome c release, caspase-3 activation, and cell apoptosis.

In addition to the mitochondrial apoptotic pathway, ethanol also stimulates the extrinsic apoptotic pathway, which is mediated by death receptors like Fas and the Fas ligand (FasL).31) Fas and FasL are target genes of nuclear factor-B (NF-κB), and GSTD has been shown to suppress proinflammatory response through blocking the NF-κB pathway.33) It is possible that GSTD may also inhibit the extrinsic apoptotic pathway in preventing ethanol-induced hepatocellular apoptosis, which merits further investigation.

The antiapoptotic effect of GSTD was also observed in nerve cells,34-36) in which administration of GSTD down-regulated the protein expression levels of Bax, cleaved caspase-3, and p53, but up-regulated that of Bel-2 and protected nerve cells from apoptosis. Considering the well-known toxic effect of ethanol to the nerve system, GSTD may have beneficial effects on the nerve system if it is used to treat ALD in the future.

In conclusion, results from the present study indicate that natural product GSTD protects HepG2 cells and animals from ethanol-induced liver toxicity, injury and apoptosis effectively. The antiapoptotic effect of GSTD is associated with its inhibition of the mitochondrial apoptotic pathway, and GSTD suppresses the production of acetaldehyde, which may also contribute to its liver protective effect. GSTD may be developed as a potential novel agent for the treatment of ALD in the future.

Acknowledgment This work was supported by the CAMS Major Collaborative Innovation Project (No. 2016-I2M-1-011) and the National Mega-Project for Innovation Drugs (2014ZX09101005-008).

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

5) Zhan HD, Zhou HY, Sui YP, Du XL, Wang WH, Dai L, Sui F, Hua


