Combination Therapy with Taurine, Epigallocatechin Gallate and Genistein for Protection against Hepatic Fibrosis Induced by Alcohol in Rats

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This study was to investigate the possibility of enhancing the anti-fibrotic effect by using a combination therapy with taurine, epigallocatechin gallate and genistein in a rat liver fibrosis model induced by alcohol, and to explore its underlying mechanism. Hepatic fibrosis was induced by intragastric administration with various amount of alcohol (5.0–9.5 g/kg) within 24 weeks in rats. The model group received alcohol only, and treatment groups received the corresponding drugs plus alcohol respectively, while the normal control group received an equal volume of saline. The antifibrotic effects of combination therapy were assessed directly by hepatic histology, and indirectly by measurement of serum biochemical markers, the fibrosis markers and related key cytokines/proteins. The results showed that combination therapy could significantly improve the liver function, as indicated by decreasing levels of alanine transaminase, aspartate transaminase, alkaline phosphatase, γ-glutamyltransferase, interleukin-6 and tumor necrosis factor-α. Moreover, combination therapy could effectively suppress the serum levels of fibrosis markers and hepatic hydroxyproline content, inhibit collagen deposition and reduce the pathological tissue damage. Research on mechanism showed that combination therapy was able to markedly reduce lipid peroxidation and recruit the anti-oxidative defense system, and inhibit the expression of B-cell lymphoma 2, α-smooth muscle actin, transforming growth factor β1, and small mothers against decapentaplegic homolog 3 proteins. Our results showed that combination therapy is effective in attenuating hepatic injury and fibrosis in the alcohol-induced rat model. The improved efficacy of the combination therapy with its good safety profile could represent a new protective approach for liver fibrosis.

Key words combination therapy; taurine; genistein; epigallocatechin gallate; liver fibrosis

Alcohol consumption is a leading cause of illness and death from liver disease throughout the world.1,2) Typically, alcohol-induced liver injury, which presents initially as acute inflammation then progresses to fatty liver, alcoholic hepatitis and ultimately to fibrosis and cirrhosis.3) Alcoholic liver fibrosis, a result of alcoholic liver injury, is characterized by excessive accumulation of extra-cellular matrix (ECM) in liver. Many studies have shown that liver fibrosis is reversible.4,5) However, cirrhosis, the end-stage consequence of fibrosis, is generally irreversible.6) Thus, efforts to understand fibrosis and discover medications for treatment of fibrosis seem to be of great urgency.

As we have known that liver fibrosis is triggered by a series of events involved with complicated factors, which requires varying medication. It is obvious that monotherapy with either tyrosine kinase inhibitor or antioxidant that has sole therapeutic target has limited effect and can not meet the criterion. Besides, the single use of drug at high dose would cause overwhelming side effect. Therefore, it is still a challenge to find more effective therapy with less adverse effect for hepatic fibrosis.

Superior to monotherapy, combination treatment is an effective multidiug medication therapy for some diseases because of the additive or synergistic effect of each medicine and relief from side effects, which may shed light on the remedy of liver fibrosis. Combination of drugs targeting different sites of action and molecules and intervening at different phase of fibrosis may provide potentially powerful therapy for liver fibrosis.

It has been reported that taurine, epigallocatechin gallate (EGCG) and genistein, which are different in therapeutic target, have effect on liver fibrosis. Taurine can promote apoptosis of hepatic stellate cells (HSCs) by restraining the expression of transforming growth factor β1 (TGF-β1) and blocking TGF-β1/Smad pathway.7–9) EGCG has remarkable antioxidative effect which can suppress the secretion of collagen.10) As tyrosine kinase inhibitors, genistein can demote the proliferation of liver sinusoidal endothelial cells.11) It is hypothesized that combination treatment of the three drugs-taurine, EGCG and genistein that have different therapeutic target and different onset in combinational manner may be promising and attractive for the treatment of liver fibrosis. Our pilot study revealed that the combination therapy had an obvious protective effect against carbon tetrachloride (CCl4) induced liver fibrosis in rats by inhibiting TGF-β1, collagen I (COL I) and collagen III (COL III) expression and regulating the expression of TGF-β1, matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of matrix metalloproteinase-2 (TIMP-2). In addition, combination therapy was more effective than monotherapy.12–14) The results would lend valuable reference for the investigation of combination therapy for alcohol-induced hepatic fibrosis.

In the present study, we evaluated the therapeutic effects of combination therapy on alcohol-induced liver fibrosis in rats. Further, the underlying mechanism was investigated by histopathological analysis, measurement of serum enzymes and identification of related key cytokines/proteins. This research may provide new insight into the remedy of alcohol-induced hepatic fibrosis.

Colchicine is an alkaloid agent that has been widely used in clinical practice for the treatment of acute gout.15) Long-term
coli and anesthetized with ketamine hydrochloride (Ketalar®): 5.0 g/kg/d from 1 to 4 weeks; 7.0 g/kg/d from 5 to 8 weeks. Group II served as alcohol-induced liver fibrosis and was given the same volume of saline.

Animals and Treatments
Male Wistar rats, weighing 180±10 g, were provided by the Experimental Animal Center of Guanxi Medical University (Guangxi, China). The research was conducted according to protocols approved by the institutional ethical committee of Guanxi Medical University (approval No. 2011030801). All rats were housed under controlled conditions with temperature of 25±2°C, relative humidity of 60±10%, room air changes 12–18 times/h, and a 12-h light/dark cycle.

After a period of one week, the animals were divided into six groups of 15 rats per group. Rats in Group I served as normal control and was given the same volume of saline. Animals in Groups II–VI were given intragastric alcohol infusion to induce liver fibrosis. The concentration and amount of alcohol was increased gradually according to the previous study: 1.25 mg/kg; 2.5 mg/kg; 5.0 mg/kg; 10 mg/kg; and 20 mg/kg, respectively.

Estimation of Antioxidant Enzyme and Lipid Peroxidation Levels
Liver was homogenized in 50 mM phosphate buffer, pH 6.0 containing 0.5% hexadecyl trimethyl ammoniumbromide. The supernatant obtained after centrifugation was mixed with 10 nm phosphate buffer (pH 6.0) and 1 mL of 1.5 mM o-dianisidine hydrochloride containing 0.2 mM H₂O₂. The change in absorbance at 450 nm of each sample was recorded.

MATERIALS AND METHODS

Chemicals
Taurine (2-amino ethane sulfonic acid) and genistein (4’,5,7-trihydroxyisoflavone) were purchased from Sigma Co. (U.S.A.); epigallocatechin gallate (EGCG) was got from Wuhan Boster Bio-engineering Co., Ltd. (Sichuan, China); colchicine was purchased from Xiamen Sanland Chemical Co., Ltd. (Fujian, China); alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyltransferase (GGT), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) kits were gained from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China); total hyaluronic acid (HA), laminin (LN), type III collagen terminal peptide (PC-IIINP) kits were obtained from Beijing Furui Bioengineering Research Company (Beijing, China). Interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) kits were purchased from Wuhan Boster Bio-engineering Co., Ltd. (Wuhan, China).

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- Group III: colchicine 0.5 mg/kg;
- Group IV: (taurine 12.5 mg+EGCG 1.875 mg+genistein 1.25 mg)/kg;
- Group V: (taurine 25 mg+EGCG 3.75 mg+genistein 2.5 mg)/kg;
- Group VI: (taurine 50 mg+EGCG 7.5 mg+genistein 5 mg)/kg.

At the end of 24 weeks, animals were put on an overnight fast, and anesthetized with ketamine hydrochloride (30 mg/kg body weight (b.w.), intravenously (i.v.)) prior to sacrifice. Serum samples were collected into heparinized tubes (50 μL). Liver samples were dissected out and washed immediately with an ice-cold saline to remove as much blood as possible. One part of the liver samples was immediately stored at −80°C for future analysis, another part was excised and fixed in 10% formalin solution for histopathologic analysis.

Analysis of Serum Markers
Serum levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT) were measured by using commercially available kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) according to the manufacturer’s instructions.

Assay of Plasma Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α) and Liver Myeloperoxidase (MPO) Activity
Plasma IL-6 and TNF-α were assayed by sandwich enzyme linked immunosorbent assay method following the protocol provided by the manufacturers. MPO activity was measured according to the method of the previous study.

Tissue was homogenized in 50 mM phosphate buffer, pH 6.0 containing 0.5% hexadecyl trimethyl ammoniumbromide. The supernatant obtained after centrifugation was mixed with 10 mM phosphate buffer (pH 6.0) and 1 mL of 1.5 mM o-dianisidine hydrochloride containing 0.2 mM H₂O₂. The change in absorbance at 450 nm of each sample was recorded. MPO activity was expressed as μmol of the oxidized product formed/min/mg protein using the extinction coefficient of 10062 m⁻¹ cm⁻¹. Protein content was determined by the method of Shaker using bovine serum albumin as standard.

Estimation of Antioxidant Enzyme and Lipid Peroxidation
Liver tissues were washed with normal saline to remove any blood and clots, and then were homogenized on ice with Tris–HCl (5 mMol/L containing 2 mMol/L ethylene-diaminetetraacetic acid (EDTA), pH7.4). Homogenates were centrifuged at 10000×g for 15 min at 4°C. The supernatants were used immediately for the assays of SOD and GSH-Px. All of these enzymes were determined by using commercially available kits (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) according to the manufacturer’s instructions. The results were presented as the units for SOD or GSH-Px per milligram of liver tissue. Lipid peroxidation in the liver was determined by measuring the level of malondialdehyde (MDA), an end product of lipid peroxidation, using a thiobarbiturate method.

The level of hepatic MDA was expressed as μmol/g protein.

Determination of Serum Levels of Hyaluronic Acid (HA), Type III Precollagen (PCHII) and Laminin (LN)
Serum levels of HA, PCIII and LN were determined by radioimmunoassay (RIA) using commercially available kits (Beijing Furui Bioengineering Research Company, Beijing, China) according to the manufacturer’s instructions.

Measurement of Hydroxyproline Content
Hydroxyproline content in liver samples was determined according to the method described previously.

Livers were homogenized and hydrolyzed in 6 M HCl for measurement of hydroxyproline content. Protein concentration was determined by the Bradford reagent (Bio-Rad) using bovine serum albumin as the standard. Results were normalized by protein concentration.

Pathological Examination
A portion of the liver tissue that was instantly fixed in 10% phosphate buffered formalin, was processed by routine histology procedures and then embedded in paraffin. Tissue sections (5 μm) were stained with haematoxylin and eosin (H&E) for histopathological examination.

Reverse Transcription-Polymerase Chain Reaction
(RT-PCR) Analysis for B-Cell Lymphoma 2 (bcl-2)  Total RNA of the hepatic tissues was extracted using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) according to the supplier’s instruction. RNA was quantitated by optical density measurement at 260/280nm using a spectrophotometer (THERMO SCIENTIFIC Co., U.S.A.), and integrity was confirmed by running 4 µL RNA on a 1.5% agarose gel.

Reverse transcription was performed in 20 µL of reaction mixture containing nuclease-free water (5.80 µL), 10× buffer (2 µL), deoxyribonucleotide triphosphate (dNTP) (1 mm), MgCl₂ (5 mm), 4 µL of RNA of total RNA, ribonuclease inhibitor (1 U/µL), oligo (25 µg/µL) and avian myeloblastosis virus reverse transcriptase (0.75 U/µL) at 42°C for 60 min. Then, heating at 95°C inactivated reverse transcriptase for 5 min. Resulting reverse transcription products were stored at −80°C until used.

The PCR primers of bcl-2 and β-actin were synthesized by Sangon Biotech (Shanghai) Co., Ltd., China. The sequences of the primers used in this study were bcl-2 sense primer: 5'-CAC CCC TGG CAT CTT CTC CT-3'; bcl-2 antisense primer: 5'-GGT GAC GCT CCC CAC ACA-3' (349 bp product); β-actin sense primer: 5'-TAA AGA CCT CTA TGC CAA CAC AGT-3'; β-actin antisense primer: 5'-CAG GAT GGA GGG GCC GGA CTC ATC-3' (260 bp product).

Polymerase chain reaction was carried out in 20 µL of reaction mixture, which contained 2 µL of 10× Taq buffer with KCl, dNTP (2 mm), 0.6 µL (10 pmol/µL) of each specific PCR primer, Taq DNA polymerase (0.1 U/µL), MgCl₂ (2.5 mm), cDNA 2 µL, nuclease-free water (10.8 µL). PCR procedure was used as follows: predenaturation for 60s at 94°C, 1 cycle; 94°C for 15s, 60°C for 30s, 72°C for 60s, 30 cycles; and 8 min of extra-elongation at 72°C. PCR of β-actin chosen as an internal control was carried out in the same tubes as for the genes. The final products were identified by electrophoresis in 1.5% agarose gel. The PCR signal intensities were measured by scanning the gels using the Smartview software, and bcl-2 densitometric measurement was normalized with the internal control β-actin.

Immunoblot Analyses for α-Smooth Muscle Actin (α-SMA), Transforming Growth Factor β₁ (TGF-β₁) and Small Mothers against Decapentaplegic Homolog 3 (Smad3) Liver tissue was homogenized in a buffer containing 10mmol/L Tris–HCl (pH 7.4) and 1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate, 1 mmol/L phenylmethanesulfonyl fluoride (PMSF), and 5 mmol/L EDTA. The protein concentration of the tissue homogenate was determined by the previous study22) with bovine serum albumin (BSA) as a standard. Sixty micrograms of protein from liver homogenates was loaded per lane on 8% polyacrylamide gels and electrophoresed. Proteins were transferred to nitrocellulose membranes. The membranes were blocked overnight with buffer and then incubated with primary antibodies for 1 h using 1:500 dilution of mouse monoclonal anti-α-SMA (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.); or 1:1000 dilution of goat polyclonal anti-β-actin (Santa Cruz Biotechnology). The membranes were then washed three times in Tween in Tris-buffered saline (TTBS) for 15 min each and incubated with 1:5000 dilution of alkaline phosphatase conjugated goat anti-mouse immunoglobulin G (IgG) (Calbiochem Co., San Diego, CA, U.S.A.) as second antibody for 1 h. The protein was visualized with an enhanced chemiluminescence Western blotting detection kit (Amersham, Arlington Heights, IL, U.S.A.). The membranes were finally exposed to X-ray film for 1 min. The relative expression of various proteins was quantified by densitometric scanning using Image-Analysis system.

Liver tissue (100mg) was homogenized in 1 mL of ice-cold cytoplasmic extraction buffer (150 mm sodium chloride, 50 mm Tris, 1 mm EDTA, 1% nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS) and 1 µg/mL each of aprotinin, leupetin and peptatin). After incubation for 30 min on ice, the homogenate was centrifuged at 16000×g for 30 min at 4°C and the supernatant was transferred to a new tube and stored at −80°C. After measuring the protein content, the fraction containing 50 µg protein was electrophoresed through 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in Tris-glycine electrophoresis buffer (25 mm Tris, 192 mm glycine and 0.1% SDS, pH 8.3) and blotted onto a nitrocellulose membrane in the transferring buffer (380 mm glycine, 50 mm Tris, pH 8.3 and 20% methanol) at 80 mAh for 90 min. The membrane was pre-incubated in the blocking buffer (phosphate buffer saline (PBS) containing Tween-20 and 5% BSA) overnight at room temperature and then probed with primary antibody (1:1500 diluted in blocking buffer) overnight at 4°C. The membrane was washed three times with 0.1% Tween-PBS and then it was incubated with anti-rabbit IgG conjugated with horseradish peroxidase diluted to 1:1000 in the blocking buffer for 2 h at room temperature. Antibody-antigen complexes were detected with DAB as the substrate. The band was then scanned and the intensity of the protein was measured using densitometry software. β-Actin was used as the internal control. The values were normalized with those of β-actin and expressed as arbitrary units relative to the control.

Total protein was extracted from liver tissue and analyzed with bicinchoninic acid (BCA) protein concentration assay kit (Shanghai Haoran Bio Technologies Co., Ltd., China). Sample protein was separated by electrophoresis in 12% SDS-PAGE separating gel with Bio-Rad electrophoresis system. The primary antibodies (rabbit anti Smad3 antibody, 1:1000 dilution, MILLIPORE Inc., U.S.A.) were incubated at 4°C overnight. The corresponding horseradish peroxidase conjugated secondary antibodies (anti-rabbit IgG, 1:5000 dilution, Amyjet Scientific Inc., China) were incubated at room temperature. Immobilon™ Western Chemiluminescent HPR Substrate (MILLIPORE Inc.) and Quantity ONE (BIO-RAD) were employed for revealing and quantitative analysis of the blots. β-actin protein was used as the internal control.

Statistical Analysis Statistical analysis was performed using SPSS 11.5 for Windows. One-way analysis of variance (ANOVA) was used to compare the means among different groups; and Tukey test was used in the post hoc multiple comparisons. Data was presented as mean±S.E. A p-value <0.05 was considered to be statistically significant.

RESULTS

Animal Vital Signs and Index of Liver At the end of the experimental period, no death was found in the normal control group, whereas there were seven deaths in the model group, five in the colchicines group, and six, four and two in the low, medium and high dosages combination drugs groups...
respectively. Irritability, aggression and weight loss of rats were present predominantly in the model group.

Rats in the medium and high dosages combination drugs groups weighted more than those in the model control group. Both liver weight and liver index (liver weight/body weight) in these groups were also significantly reduced compared with those in the model group (Table 1).

**Serum ALT, AST, ALP and GGT Activities** To evaluate the extent of liver injury in liver fibrosis, we carried out an analysis of serum ALT, AST, ALP and GGT activities. A significant increase in the activities of the four enzymes was observed in the model group compared with those of the normal control. Conversely, animals treated with colchicine and the medium, high dosages combination drugs exhibited significant decrease in the activities of the enzymes (Fig. 1).

**Plasma IL-6, TNF-α and Liver MPO Activities** The serum IL-6, TNF-α and liver MPO activities in the model group were significantly higher compared to those in the normal control group. Uprregulation was markedly inhibited after treatment with colchicine and combination drugs (Fig. 2).

**Antioxidant Enzyme and Lipid Peroxidation** Liver fibrosis induced by alcohol provoked a significant reduction of liver SOD, GSH-Px activities and a remarkable promotion of liver MDA content in model group compared with normal control. Instead, results showed that liver SOD and GSH-Px activities were obviously increased by treatment with colchicine and combination drugs, and liver MDA production level were markedly decreased after treatment (Fig. 3).

**Serum Levels of HA, LN, and PC III** Serum levels of HA, LN and PC III, indicators of liver fibrogenesis, increased significantly in model group. Treatment with colchicine and combination drugs effectively decreased the levels of the three markers (Fig. 4).

**Hepatic Hydroxyproline Content** Hepatic hydroxyproline content is also an index of liver fibrosis. In this study, elevated hydroxyproline level was observed in the model group. There were significant decrease in hepatic hydroxyproline content with colchicine and combination drugs administration (Fig. 5).

**Histopathological Findings** To assess histological changes, H&E staining of liver tissue sections from each group were examined. The normal control showed normal lobular architecture with central veins and radiating hepatic cords (Fig. 6 I). Typical pathological characters including marked fatty degeneration, portal inflammation and necrosis, obvious collagen deposition, perihepatocyte fibrosis and hepatocyte loosening in the model group demonstrated the successful establishment of liver fibrosis (Fig. 5 II). The groups treated

Table 1. Body Weight, Liver Weight and Liver Index of Experimental Animals (Mean±S.E.)

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Body weight (g)</th>
<th>Liver weight (g)</th>
<th>Liver index (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>15</td>
<td>178.9±14.5</td>
<td>479.7±68.3</td>
<td>9.76±1.63</td>
</tr>
<tr>
<td>II</td>
<td>8</td>
<td>176.4±12.8</td>
<td>312.5±40.1</td>
<td>12.02±1.75</td>
</tr>
<tr>
<td>III</td>
<td>10</td>
<td>180.5±13.7</td>
<td>348.2±43.5</td>
<td>10.51±1.69</td>
</tr>
<tr>
<td>IV</td>
<td>9</td>
<td>177.2±13.5</td>
<td>351.4±44.7</td>
<td>10.87±1.58</td>
</tr>
<tr>
<td>V</td>
<td>11</td>
<td>173.8±13.6</td>
<td>360.1±50.9</td>
<td>10.06±1.24</td>
</tr>
<tr>
<td>VI</td>
<td>13</td>
<td>174.9±12.4</td>
<td>428.5±54.6</td>
<td>9.85±1.13</td>
</tr>
</tbody>
</table>

Note: *p<0.05 compared with group I; **p<0.05 compared with group II. Liver index was liver weight/body weight. I: normal control group; II: model group; III: colchicines 0.5 mg/kg; IV: (taurine 12.5 mg+EGCG 1.875 mg+genistein 1.25 mg)/kg; V: (taurine 25 mg+EGCG 3.75 mg+genistein 2.5 mg)/kg; VI: (taurine 50 mg+EGCG 7.5 mg+genistein 5 mg)/kg.
with colchicines, and the medium and high dosages combination drugs respectively appear to relieve the pathological damages (Fig. 6 III, V and VI). However, the low dosage combination drugs did not show significant effect (Fig. 6 IV).

Expression of bcl-2 mRNA in Liver

The result of semi-quantitative RT-PCR analysis of bcl-2 mRNA in the liver tissue of each group was shown in Fig. 7. It was obvious that the level of bcl-2 mRNA was higher in the model group than that in the normal control group, but decreased greatly after treated with colchicine, and the medium and high dosages combination drugs.

Expression of α-SMA

Hepatic stellate cells (HSCs) are activated in response to liver damage. They produce type I collagen leading to hepatic fibrosis and also express the intracellular microfilament protein α-SMA. Therefore, the effect of combination therapy on the expression of α-SMA in rats was determined by Western blot analysis. In alcohol-induced liver fibrosis model rats, expression of α-SMA increased significantly. However, treatment with colchicine and the medium and high dosages combination drugs significantly decreased the α-SMA expression in liver tissue (Fig. 8).

Immunoblotting for TGF-β1

Western blot analysis showed an increased expression of TGF-β1 in liver of animals fed with ethanol as compared to normal control. On the contrary the expression of TGF-β1 was significantly lower in the
liver tissue of rats after treatment with colchicine and the medium and high dosages combination drugs (Fig. 9).

Smad3 Level in Liver Tissue Smad3 in liver was increased significantly in the model group, and decreased obviously in colchicine and combination drugs treatment groups (Fig. 10).

DISCUSSION

Currently, liver fibrosis is mainly induced either by the ligation of common bile duct or by application of hepatotoxins such as CCl_{4}, thioacetamide or dimethylnitrosamine. In this study, experimental liver fibrosis was induced by ethanol infusion for 24 weeks in Wistar rats. It has been reported that hepatic histological changes in ethanol-induced fibrosis model were similar to those found in human alcoholic cirrhosis. Many studies have confirmed that the reduction in body weight was related to the toxicity of the ethanol because ethanol impaired the activation and utilization of nutrients due to malnutrition or malabsorption, while increased liver weight...
could be due to accumulation of lipids and collagen.26) In the present study, combination drugs could markedly improve animal diet and activity, reduce death ratio, increase body weight, and decrease the liver index compared with that in model group, which indicated that combination drugs could efficiently repair the injury originated from alcohol and retrieve nutrients leading to the improved vital signs.

Analysis of pathology highly demonstrated that ethanol administration caused damage to hepatic architecture and produced histological changes such as micro- and macro-vesicular fatty infiltration, Kupffer cell hyperplasia and sinusoidal dilatation in the liver. Besides, the increased serum levels HA, LN and PCIII as well as the hepatic hydroxyproline content, which are the indexes for liver fibrosis, also confirmed the hepatic fibrogenesis in rats. And treatment with colchicines and the combination drugs could markedly decrease pathologic characters of the liver tissues and the fibrosis markers. This indicated that the combination drugs was effective in the inhibition of collagen deposition in liver to alleviate the progression of hepatic fibrosis.

Moreover, analysis of serum enzymes is one of the most sensitive tests employed in the diagnosis of hepatic diseases. In the present study, we have observed increased activities of serum ALT, AST, ALP and GGT in ethanol-induced liver fibrosis in rats. This can be attributed to the damaged structural integrity of the hepatic cells. The enzyme ALP is located in the cytoplasm and will be released into circulation when injury involves organelles such as mitochondria.28) As a result, the establishment of liver fibrosis in this study caused both plasma membrane and organelle membrane damage. On administering colchicines and the combination drugs, we have observed decreased levels of serum ALT, AST, ALP and GGT. Together with the results of histopathology mentioned above, we hypothesize that the combination drugs may possess the ability of preserving the structural integrity of the liver from the adverse effects of ethanol and therefore reverse hepatic injury.

Further exploration of the underlying mechanism of drug synergy showed that the combination drugs played a role in reduction of proinflammatory nitric oxide-generated mediators, inhibition of lipid peroxidation and mediation of HSC activation. As essential processes for subsequent fibrogenesis, inflammatory responses are known to participate in the collagen synthesis and accumulation. Both TNF-α and IL-6 are implicated in the hepatic fibrogenic process. TNF-α stimulates the parenchymal cells to produce acute phase proteins that activate the fibrogenic process and IL-6 has a direct mitogenic effect on HSC.29) Besides, increase in liver MPO activity is suggestive of parenchymal infiltration of neutrophils and lymphocytes during fibrosis. During fibrosis, injured hepatocytes release reactive oxygen species and fibrogenic mediators (cytokines and chemokines) and recruit the mononuclear cells towards the site of injury.30) In this study, the levels of serum IL-6, TNF-α and MPO in the model group were significantly higher than those in the normal control group, revealing inflammatory reaction involved in alcohol induced liver injury. After treatment with colchicines and the combination drugs, upregulation of serum IL-6, TNF-α and MPO was markedly inhibited. This suggested that the combination drugs exert a therapeutic effect possibly through restricting the production and the release of inflammatory mediators.

Oxidative stress and consequent lipid peroxidation have been currently considered to be involved in the generation of liver fibrosis. Chronic ethanol consumption significantly inhibits mitochondrial acetaldehyde dehydrogenase activity while the rate of ethanol oxidation to acetaldehyde is even enhanced, resulting in a striking increase of tissue and plasma acetaldehyde levels.31) These changes lead to cell injury and chronic liver injury, resulting in liver fibrosis. Oxidative stress parameters, including SOD, GSH-Px, and MDA were examined in this study, which showed declined tendency for the former two while increased for the latter one in the alcohol-treated rats. It was converse after administrated with colchicines and the combination drugs intragastrically, with significantly elevated activity of SOD and GSH-Px and markedly decreased MDA level. And this indicated that the combination drugs may inhibit lipid peroxidation and effectively recruit the anti-oxidative defense system in liver fibrosis.

Recently, it is widely believed that the most essential characteristic of hepatic fibrosis is HSC activation. HSCs activation accompanies phenotypic transformation into myofibroblast-like cells, upon which α-SMA that marks the transformation of HSC increases leading to actively secreted fibrillar collagens with the resultant deposition of fibrotic matrix.32) In this study, activation of HSCs was identified with increased expression of the activation marker α-SMA in alcohol treated groups, while treatment of colchicines and the combination drugs significantly reduced α-SMA expression. It was proposed that the combination drugs could prevent the initiation of fibrotic process, and inhibit the beginning of the excessive connective tissue component synthesis.

Mechanisms underlying the effect of combination therapy on HSC inactivation may have relationship with the modulation of TGF-β-Smad signaling pathway. TGF-β1 is one of the most important cytokines involved in the fibrotic and cirrhotic transformation of the liver, which was found to play roles by action on HSC. During a fibrogenic injury, expression of three forms of TGF-β is greatly upregulated in HSCs.33) Due to the pleiotropic fibrogenic effects of TGF-β1, strategies aimed at disrupting TGF-β1 synthesis and/or signaling pathways can markedly decrease fibrosis.34) In addition, TGF-β-Smad signaling pathway is the main pathway of TGF-β1,35) which transfers the stimulating signal from outside into the affected cells. Smads (such as Smad3) are TGF-β receptor substrates with a demonstrated ability to propagate signals.36) It has been reported that HSCs activation was driven by TGF-β1 and Smad3. The results of this study showed that colchicines and the combination drugs treatment significantly decreased the extraordinarily high level of TGF-β1 and Smad3, suggesting that the inhibitory effects of these drugs on liver fibrosis might be related to its action on HSC deactivation by the control of the production of TGF-β1 and Smad3.

Bcl-2 itself inhibits apoptosis in response to a wide variety of signals, and overexpression of bcl-2 can protect cardiac myocytes from apoptosis.37) It was found that colchicines and the combination drugs could down-regulate bcl-2 mRNA expression, indicating that the combination drugs reversing alcohol-induced hepatic fibrosis, at least in part, by inducing apoptosis of activated HSC through down-regulation of bcl-2 mRNA expression.

In brief, results from the present study demonstrated that
the combination drugs composed of three natural drugs produce satisfactory results in the treatment of alcohol induced liver fibrosis in rats, as proved by histological findings, vital signs, liver fibrogenesis indicators, and serum enzymes relating with liver diseases. The preliminary exploration of the underlying mechanisms indicated its protection against hepatic injury by radical scavenging action, antioxidant activity, as well as its ability to attenuate HSC activation. Further investigation showed that the effect of the combination drugs on HSC inactivation was associated with the modulation of TGF-β-Smad signaling pathway and induction of HSC apoptosis by down-regulating bcl-2 mRNA. All the findings supported that the combination drugs should be regarded as a new and promising treatment scheme, and should be useful for the prevention and treatment of liver fibrosis and even cirrhosis.

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