PHARMACOLOGY AND CELL METABOLISM

Saponins from Panax japonicus Protect Against Alcohol-Induced Hepatic Injury in Mice by Up-regulating the Expression of GPX3, SOD1 and SOD3

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Abstract — Aims: The purpose of this study was to investigate the possible mechanism(s) of saponins from Panax japonicus (SPJ) on alcohol-induced hepatic damage in mice. Methods: SPJ were identified by high performance liquid chromatography-evaporative light scattering detection-mass spectrometry (LC–ELSD–MS). Non-toxic concentrations of SPJ were assayed on alcohol-induced hepatic injury in male ICR mice and human hepatic cells. The protective effects were evaluated by biochemical values, histopathological observations and the relative gene expression. Results. In vitro, SPJ showed significant hydroxyl radical scavenging capacity. In vivo, SPJ (50 mg/kg) could rectify the pathological changes of aspartate transaminase, alanine transaminase, malondialdehyde, reduced glutathione (GSH), glutathione peroxidase (GPX), catalase (CAT) and superoxide dismutase (SOD) caused by alcohol metabolism to normal levels except for hepatic GSH and CAT. In hepatic cells, the results were in agreement with foregoing results determined in mice after pretreatment of SPJ (100 µg/ml). RT–PCR results showed that CAT, GPX and SOD mRNA decreased by alcohol metabolism were reversed, in which GPX3, SOD1 and SOD3 could return to a normal level, but CAT, GPX1 and SOD2 mRNA were still evidently lower than the control. Histopathological observations provided supportive evidence for biochemical analyses. Conclusions: SPJ plays an important role in the protection of the structure and function of hepatic mitochondria and karyon by directly scavenging reactive oxygen species/free radicals and up-regulating the expression of antioxidant enzymes (SOD, GPX and CAT), especially to GPX3, SOD1 and SOD3.

INTRODUCTION

Alcoholic liver disease (ALD), the common consequence of prolonged and heavy alcohol intake, is one of the most common causes of chronic liver disease in the world (Diehl, 2002); it encompasses a broad spectrum of morphological features ranging from steatosis with minimal injury to more advanced liver damage, including steato-hepatitis and fibrosis/cirrhosis (Yip and Burt, 2006). It is now well accepted that the progression of ALD to a more severe disease is a multifactorial process that involves a number of genetic, nutritional and environmental factors (Day, 2006). Among the mechanisms implicated in the pathogenesis of ALD, free-radical-mediated oxidative stress has received growing interest. Oxidative stress associated with alcohol toxicity is mainly caused by reactive oxygen species (ROS) generated by the mitochondrial respiratory chain, the ethanol-metabolizing cytochrome P450 (CYP2E1) of hepatocytes, the NADPH oxidase of Kupffer cells and liver-infiltrating granulocytes. These free radicals are capable of damaging many cellular components such as DNA, proteins and lipids (Albano, 2008). To counteract the oxidative damages, the body offers different defense mechanisms collectively known as antioxidants. Many types of antioxidants are employed, and these antioxidants can trap harmful forms of oxygen and prevent them from damaging cells. Antioxidants enter the bloodstream and act directly to protect cells of the organism from the invasion of ROS. In addition, some antioxidants can also stimulate the immune system and/or increase the activity of detoxifying enzymes (Xin et al., 2005).

Recently, a large number of reports suggest that both American and Asian ginseng have diverse components and multifaceted pharmacological functions (Lee et al., 2005; Kim et al., 2007). Among the diverse constituents of ginseng, saponins have been found to be the major components responsible for its biochemical and pharmacological actions (Lee et al., 1999). In previous studies, saponins isolated from the root of Panax grandiflorum and Panax notoginseng showed protective effects against acute ethanol-, acetalaminophen- and CCl4-induced hepatotoxicity in mice and inhibit the progress of hepatic fibrosis in rats (Lee et al., 2001, 2008; Khanal et al., 2009; Peng et al., 2009). In addition, the saponins isolated from P. grandiflorum and P. notoginseng also reportedly have potent antioxidant effects, such as superoxide radical scavenging activity, inhibit ROS production by tert-butyl hydroperoxide in hepatocytes and in the liver (Lee et al., 2004a; Zhao et al., 2006) and increases the hepatic glutathione peroxidase (GPX), superoxide dismutase (SOD) activities and the reduced glutathione (GSH) level (Voces et al., 1999). Panax japonicus (PJ), in which the saponins are said to make up the active major constituents, is a medicinal plant widely used in folk and traditional medicine for its anti-ulcer and anti-oxidation activities (Yamahara et al., 1987). Although we have already reported that PJ could prevent alcohol-induced gastric ulcer and have antioxidant effects in vivo (Li et al., 2007), the physiological functions and the protective mechanisms of saponins from PJ on chronic ethanol-induced liver injury are not clear. In the present study, we investigated the protective effect of saponins from PJ on ethanol-induced mice hepatic injury and the potential mechanisms of hepatoprotection involved.

MATERIALS AND METHODS

Materials

PJ were collected from a natural protective area that were grown for >3 years in Qing-liang Mountain (Lin-An, China, Batch 0607/01), as authenticated by Dr. Lin Hong, Department of Botany, Zhejiang Academy of Agricultural Science. Alcohol (56%, wt./vol) was purchased from Hongxing Brewery, China. Protein concentration, aspartate transami-
nase (AST), alanine transaminase (ALT), malondialdehyde (MDA), catalase (CAT), GSH, GPX and SOD assay kits were obtained from Nanjing Jiancheng Bioengineering Company (Nanjing, China). RPMI 1640 were purchased from Sigma-Aldrich (St. Louis, MO, USA). RevertAid™ First-Strand cDNA Synthesis Kits were purchased from Fermentas (Lithuania). All other chemicals were of the highest purity available and were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of samples

The total saponins were extracted according to the method of Xiang and Zhai (2008) with some modification. Briefly, PJ root powder (~1 g) was defatted with 10 ml ether and then extracted three times with 10 ml of methanol by sonication for 3 h at room temperature. The mixture was filtered and evaporated to dryness using a rotary evaporator at 40°C. The residue was then redissolved in distilled water and further successively extracted using saturated n-butanol by water, 1% (w/w) NaOH and saturated water by n-butanol three times at room temperature, respectively. After centrifugation, the combined supernatants were evaporated to dryness. The residue was then dissolved in 10 ml of methanol and filtered through a 0.45-μm membrane before being used for the LC–MS analyses.

LC–ELSD–MS analysis

An LC system consisting of a Waters 600 pump equipped with a gradient controller, an automatic sample injector (Agilent 1200, USA) and an ELSD (Sedex 85, France) was used. The separation was performed on a ZORBAX SB-C18 (150 mm×2.1 mm, 5 μm) column temperature was kept at 35°C using a column heater–cooler (Agilent 1200 Series). A binary mobile phase consisting of 0.1% formic acid in water (A) and acetonitrile (B) was used for the separation. All solvents were filtered through a 0.45-μm filter prior to use. The flow rate was kept constant at 0.6 ml/min for a total run time of 70 min. The system was run with a gradient program: 0–20 min, 17–20% B; 20–21 min, 20–24% B; 21–30 min, 24–29% B; 30–40 min, 29–33% B; 40–45 min, 33–41% B; 45–57 min, 41–55% B; 57–58 min, 55–90% B; 58–63 min, 95% B; 63–64 min, 95–17% B; and 64–70 min, 17% B. The sample injection volume was 10 μl. Detection was ELSD: temperature of the nebulizer, 40°C; the nebulizer gas (nitrogen) adjusted to 2.5 bar; gain, 1; and impactor, off.

The MS (micrOTOF Q, Bruker Daltonics, Germany) conditions were as follows: collision energy (ampl), 30–50 eV; collision gas, Ar; HV capillary voltage, 3.5 kV; nebulizer/ drying gas N₂, 6.0 l/min; temperature, 220°C; pressure of nebulizer gas, 0.6 bar; flow rate, 0.6 ml/min; and scan range, m/z 50–3000 in negative ion mode. All the operations and the acquisition and analysis of data were controlled by HyStar (version 3.2) software (Bruker Daltonics).

Animals and experimental design

Male ICR mice (25±2 g) were obtained from Zhejiang Academy of Medical Sciences. Animals were housed in plastic cages under controlled conditions of 12 h light/12 h dark cycle, 60% humidity and 25±1°C. After 1 week of aclimatization, the mice were randomized into five groups. Each group contained 10 animals, and five animals were kept in one cage. The standard pellet diet and water were provided ad libitum. Group 1 was the control, receiving 0.25 ml 0.9% saline. ALD was induced in the rest of the groups (Groups 2–5) by daily gastric intubations of 14.2 ml/kg body weight of 56% alcohol for 30 days. Saponin powder of PJ (SPJ) (12.5, 25 and 50 mg/kg b.w.) were dissolved in 0.9% saline and given to Groups 3–5, respectively, by gavage 1 h prior to alcohol treatment. The duration of the experiment was 30 days. At the end of experiment, the mice were fasted overnight, anesthetized with pentobarbital sodium (40 mg/kg b.w.) and sacrificed by decapitation. Then blood and tissue were immediately collected, processed and used for various biochemical estimations. In the present study, all animal use procedures were in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People’s Republic of China on 14 November 1988.

Sample preparation

Blood samples were collected in tubes and allowed to clot. The sera were collected by centrifugation at 3000×g for 10 min at 4°C and stored at −80°C until analysis. The liver was also rapidly removed and rinsed in cold 0.9% saline. A portion of the liver was fixed for histopathology, while another portion was homogenized in cold 0.9% saline. Homogenization procedure was performed as quickly as possible under completely standardized conditions. The homogenates were centrifuged at 12,000×g for 5 min at 4°C and kept on ice until assayed.

Biochemical estimations

Total protein in all samples, MDA, AST, ALT, GSH levels, activities of SOD, GPX and CAT were estimated in the serum and liver using assay kits.

Histopathological examination

For histopathological study, the tissues of three animals from each group were separated, and a portion of the tissues were separated and stored in 10% formalin. They were later sectioned using a microtome, dehydrated in graded alcohol, embedded in paraffin and stained with hematoxylin and eosin (H&E), and then viewed using a Leica DM2500 (Germany) microscope. The other portions were fixed in 2.5% glutaraldehyde. They were dehydrated in graded alcohol, embedded in Sturr, colored with uranyl acetate and Reynolds, sectioned using a microtome and observed using a JEOL 1230 (Japan) transmission electron microscope.

Measurement of cell growth, viability and biochemical estimations

Normal human liver cells (L-02) were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, and grown in RPMI 1640 containing 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. SPI was dissolved with 0.1% DMSO and adjusted to final concentrations with culture medium before use.
cells were chilled on ice and incubated with 2 U of RNase inhibitor (20 U/μl) for 10 min in a thermocycler (ABI 9700 PCR, USA). Finally, the reaction mixture was incubated at 37°C for 10 min, 42°C for 60 min and 70°C for 5 min and chilled on ice for at least 1 min. Subsequently, 4 μl of 5× reaction buffer, 1.0 μl RiboLock™ ribonuclease inhibitor (20 U/μl) and 2.0 μl dNTP (10 mM) were mixed into 1 μl of total RNA, 1.0 μl of oligo-(dT)18 (0.5 μg/μl) and 7.0 μl of DEPC-treated water at denatured at 70°C for 5 min and chilled on ice for at least 1 min. Subsequently, 4 μl of 5× reaction buffer, 1.0 μl RiboLock™ ribonuclease inhibitor (20 U/μl) and 2.0 μl dNTP (10 mM) were mixed and incubated at 37°C for 5 min. After incubation, 1.0 μl M-MLV Reverse Transcriptase (200 U/μl) was added and incubated at 37°C for 10 min, 42°C for 60 min and 70°C for 10 min in a thermocycler (ABI 9700 PCR, USA). Finally, samples were chilled on ice and incubated with 2 U of Rnase H for 20 min at 37°C before amplifying the target DNA. cDNA was quantified and assessed for purity using a UV spectrophotometer, and the concentration was measured at 260 nm; the A260/A280 relation was calculated to determine cDNA quality.

The PCR primers of CAT, GPX1, GPX3, SOD1, SOD2, SOD3 and β-actin were synthesized by Shanghai Shenenergy Biocolor BioScience and Technology. The sequences of the primers used in this study are shown in Table 1. PCR was carried out in 20 μl of reaction mixture containing 10 μl of TaKaRa 2× PCR Master Mix, 0.5 μl×10 μM of each forward and reverse primer, template cDNA and PCR-grade water up to a final volume of 20 μl in a Bio-Rad iQ5 96-well plate. An initial activation at 95°C for 2 min was followed by an amplification target sequence of 40 cycles of 95°C for 30 s, 59°C for 30 s and 72°C for 30 s. Polymerase chain reaction of β-actin chosen as an internal control was carried out in the same tubes as for the genes.

### Quantitative real-time RT–PCR analysis

Total RNA of the L-02 cells was extracted using TRIzol reagent according to the supplier’s instruction. RNA was quantitated by optical density measurement at 260 and 280 nm using a spectrophotometer, and integrity was confirmed by running 4 μl of RNA on a 1.2% agarose gel.

RT was performed from total cellular RNA using ReverTra Ace™ First-Strand cDNA Synthesis Kit for RT–PCR; a total volume of 20 μl reaction mixture containing 4.0 μg of total RNA, 1.0 μl of oligo-(dT)18 (0.5 μg/μl) and 7.0 μl of DEPC-treated water at denatured at 70°C for 5 min and chilled on ice for at least 1 min. Subsequently, 4 μl of 5× reaction buffer, 1.0 μl RiboLock™ ribonuclease inhibitor (20 U/μl) and 2.0 μl dNTP (10 mM) were mixed and incubated at 37°C for 5 min. After incubation, 1.0 μl M-MLV Reverse Transcriptase (200 U/μl) was added and incubated at 37°C for 10 min, 42°C for 60 min and 70°C for 10 min in a thermocycler (ABI 9700 PCR, USA). Finally, samples were chilled on ice and incubated with 2 U of RNase H for 20 min at 37°C before amplifying the target DNA. cDNA was quantified and assessed for purity using a UV spectrophotometer, and the concentration was measured at 260 nm; the A260/A280 relation was calculated to determine cDNA quality.

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### Anti-free-radical activities assay of SPJ in vitro

The hydroxyl radical and superoxide radical assay were measured according to the method of Luo and Fang (2008). Samples were dissolved in distilled water at 0 (control), 12.5, 25, 50, 100, 200, 400, 800 and 1600 μg/ml. In the hydroxyl radical assay, the sample solution (0.1 ml) was mixed with 0.6 ml of reaction buffer (0.2 M phosphate buffer pH 7.4, 2.67 mM deoxyribose and 0.13 mM EDTA), 0.2 ml of 0.4 M ferrous ammonium sulfate and 0.05 ml of 2.0 mM ascorbic acid, and 0.05 ml of 20 mM H2O2 was then added to the reaction solution. The reaction solution was incubated for 15 min at 37°C and then 1 ml of 1% thioarbituric acid and 1 ml of 2.0% trichloroacetic acid were added to the mixture. The mixture was boiled for 15 min and cooled on ice. The absorbance of the mixture was measured at 532 nm. In the superoxide radical assay, 0.1 ml of the sample solution was mixed with 1 ml of 16 mM Tris–HCl (pH 8.0) containing 557 μM NADH, 1 ml of 16 mM Tris–HCl (pH 8.0) containing 45 μM PMS and 1 ml of 16 mM Tris–HCl (pH 8.0) containing 108 μM NBT. After 5 min of incubation at 25°C, the absorbance was measured at 560 nm. Percent inhibition of hydroxyl radical (superoxide radical) was calculated as (1–absorbance of sample/absorbance of control)×100%.

### Statistical analysis

Results are reported as means±SD. ANOVA was used to evaluate the difference between multiple groups. If significa...
cance was observed between groups, a Duncan’s multiple range test was used to compare the means of two specific groups by a commercially available statistics software package (SPSS for Windows, v. 12.0, Chicago, IL, USA), with $P < 0.05$ considered as significant.

RESULTS

Composition of the saponins
The LC–ELSD chromatogram of the crude extract from the root of PJ is given in Fig. 1. Six major peaks were separated and detected. Tentative identification of the saponins was achieved by comparing their retention times with those of the authentic standards; consequently, peaks 1–6 were identified to be ginsenosides Rg1, Re, Rf, f3, Rg2 and Rd, the contents of which were 12.23, 16.24, 16.95, 7.51, 8.53 and 11.47%, respectively (Fig. 2, Table 3). Further identification of the structures of these six compounds by LC–ELSD–MS source collision-induced dissociation (CID) experiments was attempted, and the results are shown in Fig. 3 (Rg1 for example; other dates not shown) and Table 3.

The LC–MS of peak 1 gave $m/z$ 799 as the deprotonated molecular ion $[M–H]^–$, which confirmed the molecular mass to be 800. Further experiments in the CID of the $m/z$ 799 ($[M–H]^–$) produced two main fragment ions at $m/z$ 637

<table>
<thead>
<tr>
<th>Num</th>
<th>Saponins</th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>Molecular mass</th>
<th>Molecular formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Rg₁</td>
<td>H</td>
<td>Glc-O-</td>
<td>Glc-</td>
<td>800</td>
<td>C₄₂H₇₂O₁₄</td>
</tr>
<tr>
<td>2</td>
<td>Re</td>
<td>H</td>
<td>Rha-Glc-O-</td>
<td>Glc-</td>
<td>946</td>
<td>C₄₈H₈₂O₁₈</td>
</tr>
<tr>
<td>3</td>
<td>Rf</td>
<td>H</td>
<td>Glc-Glc-O-</td>
<td>H</td>
<td>800</td>
<td>C₄₂H₇₂O₁₄</td>
</tr>
<tr>
<td>4</td>
<td>f3</td>
<td>H</td>
<td>HO-</td>
<td>Ara-Glc-</td>
<td>770</td>
<td>C₄₁H₇₀O₁₃</td>
</tr>
<tr>
<td>5</td>
<td>Rg₂</td>
<td>H</td>
<td>Rha-Glc-O-</td>
<td>H</td>
<td>784</td>
<td>C₄₂H₇₂O₁₃</td>
</tr>
<tr>
<td>6</td>
<td>Rd</td>
<td>Glc-Glc-</td>
<td>H</td>
<td>Glc-</td>
<td>946</td>
<td>C₄₈H₈₂O₁₈</td>
</tr>
</tbody>
</table>

Abbreviations: Glc, β-D-glucose; Ara, arabinose; Rha, rhamnose.

Fig. 2. Structures of saponins in *P. japonicus* identified in this paper.
Table 3. The ESI–MS and corresponding CID data in the negative ion mode (m/z values) of peaks 1–6 in LC profile

<table>
<thead>
<tr>
<th>Peak identification</th>
<th>$t_r$ (min)$^a$</th>
<th>Content (%)</th>
<th>[M+AcO]$^b$ (m/z)</th>
<th>[M−H]$^c$ (m/z)</th>
<th>CID (m/z)$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(5) Ginsenoside Rg2</td>
<td>32.087</td>
<td>8.53</td>
<td>843</td>
<td>783</td>
<td>637[M−Rha-H]−, 475[M−Glc-Rha-H]</td>
</tr>
</tbody>
</table>

$^a$Retention time.
$^b$Molecular adduction.
$^c$Deprotonated molecular ion.
$^d$CID fragment ions.

and 475 (Table 3, Fig. 3). The compound corresponding to peak 1 was therefore identified as ginsenoside Rg1 by comparing its retention time with that of the standard, and by congruent mass spectral data. Peaks 2–6 were also determined with this method.

**Effect on the contents of MDA, GSH and activities of AST, ALT, GPX, CAT and SOD in mice**

Tables 2 and 4 showed that administration of alcohol produced severe liver damage as indicated by markedly increased AST and ALT activities and MDA in serum. However, on SPJ administration, there was a reverse trend in these values towards normal.

In Table 2, it can also be seen that the antioxidants such as GSH, GPX, CAT and SOD were significantly deteriorated by alcohol administration. When pretreated with SPJ, all the levels could return to normal (50 mg/kg b.w.) in serum. In liver determination, as depicted in Table 4, the administration of SPJ significantly increased the activities of GPX and SOD, and the final values were restored to normal at a dose of 50 mg/kg b.w. after 30 days. The levels of CAT and GSH were also significantly elevated compared with alcohol treatment mice, but still evidently lower than the control.

**Histopathological studies**

Histopathological analysis showed that alcohol induced hepatocyte and hepatic cord degeneration, focal necrosis and vascular congestion in mouse livers (Fig. 4B) (H&E, ×200). SPJ pretreatment significantly prevented alcohol-induced abnormalities in a dose-dependent manner (Fig. 4C–E). Under low magnification through TEM (×15K), histopathological ex-
amination of the liver cells of alcohol-treated mice evidently revealed degenerative changes such as swelling of the mitochondria, thinning of the cytoplasm (Fig. 5B, arrow a) as well as disruption of the nuclear membrane and scattered nucleoli (Fig. 5B, arrow b) in comparison with the control (Fig. 5A). The severe hepatic lesions induced by alcohol were remarkably ameliorated by SPJ, and the cell structure was restored to nearly normal at a dose of 50 mg/kg b.w. (Fig. 5E). Through higher magnification electron micrograph (×60K; Fig. 6), we found that the structure of hepatic mitochondria from ethanol-administered mouse deteriorated, in which the mitochondria were swollen and cristae vague and partly dissolved (Fig. 6B). In SPJ (50 mg/kg b.w.)-treated mice, a part of mitochondria returned to normal, while another portion of mitochondria still showed with little distorted cristae (Fig. 6E).

Protective effects of SPJ on ethanol-induced hepatic L-02 cell injury

In order to further observe the protective effects of SPJ on alcohol-induced liver injury, SPJ was added to culture medium of hepatic L-02 cells at the time of cell plating and maintained for 24, 48, 72 and 96 h. On treatment with ethanol (150 mmol/l), the cell proliferation was inhibited; the viable cell number was evidently lower than the control (P<0.01) after cultivation for 24 h (Fig. 7). Treatment with SPJ (100 μg/ml) showed a significant protective effect on L-02 cells, and the viable cell number was near normal.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDA (nmol/mg protein)</th>
<th>GSH (mmol/mg protein)</th>
<th>GPX*</th>
<th>CAT (U/g protein)</th>
<th>SOD (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.1±0.2</td>
<td>95.2±7.4</td>
<td>345.2±32.9</td>
<td>69.6±11.3</td>
<td>108.9±11.9</td>
</tr>
<tr>
<td>Alcohol (A, 14.2 ml/kg)</td>
<td>2.6±0.2**</td>
<td>68.1±2.4***</td>
<td>164.3±28.1**</td>
<td>11.6±3.8**</td>
<td>67.2±6.2**</td>
</tr>
<tr>
<td>SPJ (12.5 mg/kg)+A</td>
<td>2.4±0.4*</td>
<td>75.3±5.7****</td>
<td>210.9±29.6***</td>
<td>10.9±5.2**</td>
<td>76.6±6.9****</td>
</tr>
<tr>
<td>SPJ (25 mg/kg)+A</td>
<td>2.1±0.3***</td>
<td>79.4±3.9****</td>
<td>270.2±38.5***</td>
<td>41.0±4.9****</td>
<td>79.4±10.5****</td>
</tr>
<tr>
<td>SPJ (50 mg/kg)+A</td>
<td>2.0±0.3***</td>
<td>83.8±4.3****</td>
<td>332.9±30.9***</td>
<td>39.7±6.2****</td>
<td>101.1±9.4****</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD; n=10.

*Micromoles of GSH reduced per minute per milligram of protein.

**P<0.01 vs normal.

***P<0.01 vs alcohol.

Fig. 4. Effect of SPJ on alcohol-mediated experimental liver damage, H&E staining, magnification, ×200. (A) Control. (B) Alcohol (56% wt./vol, 14.2 ml/kg b.w.). (C) SPJ (12.5 mg/kg b.w.)+alcohol. (D) SPJ (25 mg/kg b.w.)+alcohol. (E) SPJ (50 mg/kg b.w.)+alcohol.
Fig. 5. Micrographs of uranyl acetate and Reynolds colorated transmission electron microscopy sections of mouse hepatic karyon, TEM: ×15K. (A) Control. (B) Alcohol (56% wt./vol, 14.2 ml/kg b.w.). (C) SPJ (12.5 mg/kg b.w.)+alcohol. (D) SPJ (25 mg/kg b.w.)+alcohol. (E) SPJ (50 mg/kg b.w.)+alcohol.

Fig. 6. Micrographs of uranyl acetate and Reynolds colorated transmission electron microscopy sections of mouse hepatic mitochondria, TEM ×60K. (A) Control. (B) Alcohol (56% wt./vol, 14.2 ml/kg b.w.). (C) SPJ (12.5 mg/kg b.w.)+alcohol. (D) SPJ (25 mg/kg b.w.)+alcohol. (E) SPJ (50 mg/kg b.w.)+alcohol.
Effect of SPJ on lipid peroxidation and antioxidant enzyme activity in L-02 cells

To elucidate the protective mechanisms of SPJ on alcohol-induced hepatic cell injury, the intracellular MDA, SOD, CAT and GPX levels of L-02 cells were measured. After treatment with ethanol (150 mmol/l) for 48 h, the MDA level in L-02 cells was increased and the antioxidant enzymes (SOD, GPX and CAT) were evidently decreased compared with the control. However, the SPJ evidently presented inhibitory ability on MDA in L-02 cells (Table 5). Meanwhile, the SOD, GPX and CAT activities were increased, in which the SOD and GPX activities were restored to normal after treated with SPJ (100 μg/ml), but the CAT was still lower than the control (Table 5).

Expression of CAT, GPX1, GPX3, SOD1, SOD2 and SOD3 mRNA in L-02 cells

In order to clarify the reason why the SOD and GPX could return to a normal level, but the CAT could not in L-02 cells, the CAT, GPX1, GPX3, SOD1, SOD2 and SOD3 mRNA were detected by quantitative real-time RT–PCR. As shown in Figs. 8–10, the expression of all the antioxidant enzymes was remarkably decreased with ethanol treatment (150 mmol/l) for 48 h. When pretreated with SPJ (100 μg/ml), these down-regulated tendencies of all the antioxidant enzymes were reversed, in which GPX3, SOD1 and SOD3 were restored to normal level, but the CAT and GPX1 mRNA levels evidently were still lower. The results of the RT–PCR studies (Fig. 8–10) provided supportive evidence for biochemical analyses (Tables 2, 4 and 5).

Radical scavenging activity

Antioxidant activities measured by hydroxyl radicals (OH) and superoxide anion radical (O2−) scavenge assays are shown in Fig. 11. Hydroxyl radicals, generated by the reaction of iron–EDTA complex with H2O2 in the presence of ascorbic acid, attack deoxyribose to form products that, upon heating with 2-thiobarbituric acid under acidic conditions, yield a pink tint. Added hydroxyl radical scavengers compete with deoxyribose for the resulted hydroxyl radicals and diminish tint formation. Figure 11a shows that SPJ had a higher scavenging hydroxyl radical effect than Vc, and their scavenging effects increased with increasing concentration. Scavenging effect of SPJ was 3.37–59.47% at the amount of 12.5–1600 μg/ml, respectively, and that of vitamin C was ∼3.10–31.79%.

Superoxide radicals were generated in a PMS/NADH system being assayed in the reduction of NBT. Figure 11b shows that the SPJ has no significant scavenging activities. At the amount of 12.5–1600 μg/ml, the effect on scavenging superoxide of SPJ was just 1.01–6.87%. Although the scavenging activity of vitamin C for superoxide radical was 0.89–15.98%, the radical scavenging activity indicated concentration dependence.

Table 5. Effect of SPJ on the levels of MDA, SOD, GPX and CAT in the different treated hepatic cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MDA (nmol/ml)</th>
<th>SOD (U/ml)</th>
<th>GPX *</th>
<th>CAT (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.05±0.08</td>
<td>59.36±4.85</td>
<td>170.2±9.9</td>
<td>32.1±4.2</td>
</tr>
<tr>
<td>Ethanol (E, 150 mmol/l)</td>
<td>1.27±0.05*</td>
<td>31.71±4.94</td>
<td>87.1±7.1*</td>
<td>10.9±5.1*</td>
</tr>
<tr>
<td>SPJ (25 μg/ml)+E</td>
<td>1.21±0.08</td>
<td>51.56±4.05</td>
<td>105.3±10.6***</td>
<td>12.89±4.3*</td>
</tr>
<tr>
<td>SPJ (50 μg/ml)+E</td>
<td>1.15±0.03***</td>
<td>66.03±3.79***</td>
<td>135.4±9.0***</td>
<td>22.6±4.3***</td>
</tr>
<tr>
<td>SPJ (100 μg/ml)+E</td>
<td>1.09±0.01***</td>
<td>68.17±4.08***</td>
<td>166.7±8.1***</td>
<td>25.3±3.2***</td>
</tr>
</tbody>
</table>

Values are mean±SD; n=4.

*P<0.01 vs normal.

**P<0.05.

***P<0.01 vs ethanol.
DISCUSSION

The purpose of this study was to investigate whether the effect of the prolonged administration of SPJ can attenuate the ethanol-induced hepatic injury in mice and the possible mechanisms of SPJ extract hepatoprotection. To our knowledge, the ethanol absorbed by gastrointestinal mucosa is almost exclusively metabolized in the liver. The obvious sign of hepatic injury is the leakage of cellular enzymes into plasma (Saravanan et al., 2006). In the present study, the increased activities of serum enzymes (AST and ALT) had been detected in alcohol-administered mice (Table 2), implying the increased permeability, damage and/or necrosis of hepatocytes. These results are in agreement with the results published by Saravanan et al. (2006). Because the enzyme ALT is located in the cytoplasm and the soluble enzyme AST mainly indwells in organelles such as mitochondria (Senthil et al., 2003), increased levels of AST and ALT suggested the damage of both hepatic cellular and mitochondrial membrane in alcohol-administered mice. On administering...
SPJ, transaminase activities (ALT and AST) in serum were reverted to the levels similar as the control; the results showed that SPJ may possess the capacity to protect the structural integrity of the hepatic cells and mitochondria from the damage induced by ethanol.

Lipid peroxidation has been implicated in the pathogenesis of hepatic injury by ethanol, which leads to membrane dysfunction (Bandyopadhyay et al., 1999). In the present study, increased MDA, a product of lipid peroxidation, observed in the liver of ethanol-administered mice indicated excessive formation of free radicals resulting in hepatic damage. Recent researches have shown that extracts of many ginseng varieties such as Korean ginseng (Jung et al., 2005), North American ginseng (Kitts et al., 2000), Panax notoginseng (Zhao et al., 2006; Peng et al., 2009) and Panax japonicus (Xiang and Zhai, 2008) have antioxidant properties. The hepatoprotective effect of Panax extracts against ethanol-induced liver injury possibly involves mechanisms related to free radical scavenging effects (Lee et al., 2004a, 2008; Khanal et al., 2009) in which saponins Rg2 showed a protective effect on human umbilical cord vein endothelial cells (VEC-304) from H2O2-induced cell apoptosis (Xin et al., 2005), and ginsenoside Rg1 could attenuate dopamine-induced apoptotic cell death through suppression of intracellular oxidative stress (Chen et al., 2003). So the pretreatment of SPJ prevented lipid peroxidation, which could be attributed at least partly to its free radical scavenging activity. This hypothesis was further proven in the determination of anti-free-radical activities in vitro; SPJ showed a significant scavenging hydroxyl radical effect at a dose of 100 μg/ml (Fig. 11a), and their scavenging effects increased with increasing concentration.

The formation of ROS/free radicals is a naturally occurring intracellular metabolic process. These harmful species are known to cause oxidative damage to a number of molecules in cells, including membrane lipids, proteins and nucleic acids (Cederbaum et al., 2001). When the generation of ROS in cells impairs antioxidant defenses or exceeds the ability of the antioxidant defense system to eliminate them, oxidative stress results. Therefore, removing the superoxide ion (O2−) and hydroxyl radical is probably one of the most effective defense mechanisms against a variety of diseases. SOD, CAT and GPX are part of the key enzymes that defend the contents of cells against oxidative stress. SOD generates hydrogen peroxide from the O2−, and CAT converts hydrogen peroxide to wa-

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**Fig. 10.** SOD1, SOD2 and SOD3 mRNA levels in L-02 cells after incubation with SPJ (25, 50 and 100 μg/ml) and ethanol (150 mmol/l) for 48 h. The results are corrected by the control gene β-actin and the values are given as mean ± SD. Each mean represents three separate experiments. Values displaying statistical differences compared with the DMSO control are identified with **P < 0.01 vs normal; ##P < 0.01 vs alcohol.

**Fig. 11.** (A) Scavenging effect of SPJ and Vc on hydroxyl radical (OH). (B) Scavenging effect of SPJ and Vc on superoxide anion radical (O2−).
ter and molecular oxygen, thus reducing their destructive effects. In addition, GPX reduces hydrogen peroxide and lipid peroxides at the expense of GSH to water and alcohols, respectively, to produce non-reactive molecules. These enzymes may act in concert to reduce the damaging effects of ROS and free radicals to levels that allow normal metabolic functions in cells (Cadenas et al., 1993). The levels of these antioxidants (SOD, GPX, CAT and GSH) help to scavenge superoxide ions and hydroxyl ions, respectively, and were significantly lower in alcohol-administered mice as compared with untreated alcoholic mice, which will result in the accumulation of these highly reactive free radicals leading to deleterious effects such as loss of cell membrane integrity and membrane function (Sheela et al., 1995). When pretreated with SPJ for 30 days, the values of SOD, GPX, CAT and GSH returned to normal in serum (Table 2). In liver (Table 4), the activities of SOD and GPX could also return to normal after 30 days of treatment. In contrast, although the levels of CAT and GSH were markedly enhanced, they were still obviously under normal. SPJ possesses antiradical activity against OH radicals but did not show significant O$_2^.$ radical scavenging activity in vitro (Fig. 11), so the observed restoration of the SOD and GPX activities in our study on treatment with SPJ may be due to the direct stimulatory effects; the decrease in the tissue lipid peroxidation on SPJ treatment can also be correlated with the elevated SOD and GPX activities.

In order to clarify this hypothesis, SPJ was added to a culture medium of hepatic L-02 cells in vitro. Cell proliferation was inhibited and viable cell number was evidently lower than the control after cultivation with ethanol (150 mmol/l) for 24 h. SPJ (100 μg/ml) showed a significantly protective effect on L-02 cells, and the viable cell number was not different from normal during 96 h of cultivation (Fig. 7). Furthermore, lipid peroxidation and antioxidant enzyme activities (SOD, GPX and CAT) measured in L-02 cells showed that intracellular MDA was increased and the antioxidant enzymes were evidently decreased after ethanol treatment. However, the SPJ evidently inhibited on MDA and increased the SOD, GPX and CAT activities, and the changes in intracellular MDA, SOD, CAT and GPX levels in L-02 cells were in agreement with foregoing results determined in vivo. The above results indicated that SPJ could directly protect against ethanol-induced oxidative damage to the liver. The hepatoprotective activity of SPJ could, at least partly, be due to the free radical scavenging property and directly enhanced antioxidant capacity of the liver. But the reason why the levels of hepatic CAT and GSH were markedly enhanced and were still obviously lower than normal still attracted our attention.

As antioxidant enzymes SOD, CAT and GPX play a critical role in the defense against oxidative stress, RT–PCR results indicated that SOD1, SOD2, SOD3, GPX1, GPX3 and CAT expression were evidently decreased in L-02 cells exposed to 150 mmol/l ethanol for 48 h (Figs. 8–10). So many approaches have been investigated in attempts to increase SOD, CAT and GPX to augment the demand levels required for defense against the ravages of oxidative stress. Insertion of extra copies of the SOD gene into various genomes has produced transgenic animals with highly improved resistance to oxidative stress (Parkes et al., 1998). Our data suggest that this result can also be achieved by up-regulating the expression of SOD, GPX and CAT genes that already exist in the genome of cells by treating L-02 cells with SPJ in vitro, but in which GPX3, SOD1 and SOD3 could return to a normal level, the CAT and GPX1 mRNA levels evidently were still under normal. The results of RT–PCR studies were in agreement with biochemical analyses in vivo and in vitro (Tables 2, 4 and 5), and may also provide supportive evidence for biochemical changes (Tables 2, 4 and 5).

Moreover, liver histology of ethanol-administered animals showed pathomorphological alterations; histopathological evaluation of the mice livers revealed that treatment with SPJ reduced the morphological changes produced by ethanol and greatly reverted the microanatomy of the liver to normal (Fig. 4E). To our knowledge, under normal physiological conditions the mitochondrion is the major source of ROS production in the hepatocyte (Boveris and Cadenas, 1997). It is also recognized that the disturbances in structure and function of hepatic mitochondrion are likely to be associated with increased ROS production and oxidative damage to the mitochondrion of liver from chronic ethanol consumers (Bailey et al., 1999). And oxidative mitochondrial damage represents a direct cause of hepatocyte death. As shown in Fig. 6, we found that hepatic mitochondria of ethanol-administered mice were severely destroyed (Fig. 6B) when the mice were fed SPJ at a dose of 50 mg/kg b.w.; the persistent damage disappeared and most of the mitochondria returned to normal, while a few portions of mitochondria still showed with little distorted cristae (Fig. 6E). These results also verified the foregoing hypothesis that SPJ possesses the capacity to preserve the structural integrity of the hepatic cells from the adverse effects of ethanol.

CONCLUSIONS

Results from the present study demonstrated that SPJ was effective in the treatment of alcohol-induced hepatic injury in mice. The primary mechanism of the protection could be that SPJ plays an important role in the protection of the structure and function of hepatic mitochondria and karyon by directly scavenging ROS/free radicals and up-regulating the expression of antioxidant enzymes (SOD, GPX and CAT), especially to GPX3, SOD1 and SOD3.

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Conflict of interest statement — None declared.

REFERENCES


