Inhibitory effects of *Schisandra chinensis* on acetaminophen-induced hepatotoxicity

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Abstract. *Schisandra chinensis* is a well-known traditional medicinal herb. Acetaminophen is a commonly used over-the-counter analgesic and overdose of acetaminophen was the most frequent cause of acute liver failure. However, no studies have demonstrated the role of *Schisandra chinensis* in acetaminophen-induced acute liver failure to the best of our knowledge. In this study, an acute liver injury model was established in mice using acetaminophen. The protective role of *Schisandra chinensis* was detected by histopathological analysis, and measurement of the serum transaminase levels and hepatic Cyp activity levels in the mouse model. Subsequently, hepatocytes were isolated from the livers of the mouse model. The cell cycle, apoptosis, mitochondrial membrane potential and reactive oxygen species were determined using flow cytometry. Cell proliferation and 26S proteasome activity were determined using spectrophotometry. *Schisandra chinensis* was found to resist acetaminophen-induced hepatotoxicity by protecting mitochondria and lysosomes and inhibiting the phosphor-c-Jun N-terminal kinase signaling pathway. These findings provide a novel application of *Schisandra chinensis* against acetaminophen-induced acute liver failure.

Introduction

Acetaminophen is a commonly used over-the-counter analgesic and overdose of acetaminophen was the most frequent cause of acute liver failure worldwide in 2008 (1). Acute acetaminophen intoxication results in centrilobular hepatic necrosis involving N-acetyl-p-benzoquinoneimine (NAPQI) and cytochrome P450 (Cyp) (2). Current treatment protocols recommend an initial dose of 150 mg/kg N-acetylcysteine (NAC), infused over a period of 1 h, followed by decreasing quantities of NAC infused over the subsequent 20 h (3). However, the optimal treatment for acetaminophen toxicity remains unclear (4).

Schisandra chinensis is a deciduous woody vine found in northwestern China, far eastern Russia and Korea (5). As a well-known traditional medicinal herb and food additive (5), *Schisandra chinensis* is used for its antioxidant, tonic and sedative effects (6,7). In addition to its adaptogenic properties, it is also a hepatoprotectant (8,9).

Lysosomes have long been recognized as the 'suicide bags' of cells (10). Studies have indicated that lysosomal disruption occurs subsequent to numerous types of cellular stresses in hepatocytes and other cell types (11-13). A breakdown of lysosomes may result in cell death by necrosis, which is associated with an increase in cytosolic acidification (14). Kon *et al* (15) found that mobilization of chelatable iron from lysosomes was key in acetaminophen hepatotoxicity. The formation of reactive oxygen species (ROS) increases following acetaminophen exposure, and agents that augment antioxidant defenses and scavenge ROS protect against acetaminophen toxicity *in vitro* and *in vivo* (16). In the present study, the mechanism and effect of *Schisandra chinensis* on acetaminophen-induced hepatotoxicity and liver failure in mice was evaluated by observing the extent of lysosomal disruption and ROS release.

Materials and methods

Preparation of Schisandra chinensis. Dried *Schisandra chinensis* fruits (500 g), provided by Zhixin Pharmaceutical Company (Guangdong, China), were authenticated by the pharmacist at The First Hospital of China Medical University (Shenyang, China) and macerated in 70% ethanol for 30 min at room temperature. The fruits were then refluxed three times (for 1 h each) with 70% ethanol. The combined extract was filtered and condensed by rotary evaporation (Rotary evaporator, Shyarong Biochemical Instruments Inc., Shanghai, China) under reduced pressure. The condensed extract was then freeze-dried to obtain a powder, which was placed in a desiccator at room temperature until use.

Animals and treatments. Wild-type C57BL/6 male mice (aged 7-9 weeks, weighing 20-25 g) were obtained from Charles River Laboratories, Inc. (Wilmington, MA, USA). All animals

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were maintained on food and water *ad libitum* and housed in microisolation cages. All experiments with animals were approved by and performed according to the guidelines of the China Medical University Ethics Committee (Shenyang, China). The mice were fasted overnight prior to administration of acetaminophen (300 mg/kg, intraperitoneal) or phosphate-buffered saline (PBS) control. A number of the mice with acute liver failure were then treated with *Schisandra chinensis* 3 h after the acetaminophen treatment (50 mg/kg, intraperitoneal).

Hepatotoxicity verification. Blood samples were collected by cardiac puncture 12 h after the end of treatment. The samples were analyzed for serum alanine transaminase (ALT) and aspartate transaminase (AST) (Beijing Gersion Bio-Technology Co., Ltd., Beijing, China). Briefly, the values of the serum ALT and AST activities were derived according to the 'absorptivity micromolar extinction coefficient' of NADH at 340 nm and were expressed in terms of unit per liter. Pyruvate is reduced to lactate by lactate dehydrogenase with the simultaneous oxidation of NADH to NAD, which was monitored by measuring the rate of decrease in absorbance at 340 nm.

Histopathological analysis. Liver tissue samples were collected following the blood collection. The samples were fixed with 10% formaldehyde in PBS for 24 h, dehydrated in a graded ethanol series, embedded in paraffin and sliced at a thickness of 5 μm. The paraffin sections were stained with hematoxylin and eosin for histopathological analysis.

Measurement of hepatic glutathione levels. Samples of liver (50 mg) were minced in ice-cold 5% metaphosphoric acid (1:10), homogenized and then centrifuged at 3,000 x g for 10 min at 4˚C. The supernatants were filtered through a 0.2-μm syringe filter, and the reduced glutathione (GSH) and oxidized glutathione disulfide (GSSG) were quantified using the respective colorimetric assay kits (Beyotime Institute of Biotechnology, Beijing, China).

Measurement of hepatic Cyp activity. To prepare the microsomes, liver samples (1 g) were homogenized at 4˚C in two volumes (w/v) 10 mM Tris-base (pH 7.4) containing 1.5% KCl using a Teflon-glass homogenizer (DuPont, Wheaton, NJ, USA). The homogenates were centrifuged at 1,000 x g (10 min, 4˚C), and then the supernatants were collected and centrifuged at 12,000 x g (20 min, 4˚C) to remove cellular debris, followed by centrifugation at 100,000 x g (1.5 h, 4˚C). The microsomes were resuspended in homogenization buffer containing 0.5 mM phenylmethanesulfonylfluoride and centrifuged at 100,000 x g (90 min, 4˚C). The pellets were resuspended in 0.25 M sucrose containing 10 mM Tris-base (pH 7.4) and stored at -80˚C. The levels of Cyp2e1, Cyp1a2 and Cyp3a activity were measured according to the methods of Gardner *et al* (17). To assess Cyp isoform specificity, enzyme activity levels were measured following the addition of either 1 μM of the Cyp1a2 inhibitor, rutaecarpine, or of the Cyp3a inhibitor, ketoconazole.

Mice hepatocytes. According to the methods of Qian *et al* (18), hepatocytes were isolated from overnight-fasted wild-type C57BL/6 male mice by collagenase digestion and plated on type 1 collagen-coated 24-well microtiter plates, 6-cm culture dishes or glass bottom Petri dishes in Waymouth's medium MB-752/1 (HiMedia Laboratories, Mumbai, India) supplemented with 2 mM L-glutamine, 10% fetal calf serum, 100 nM insulin, 100 nM dexamethasone, 100 U/ml penicillin and 100 μg/ml streptomycin. Cell viability was identified as >90% by trypan blue exclusion, according to the manufacturer's instructions (Beyotime Institute of Biotechnology). After 4 h, the hepatocytes were placed in hormonally defined medium consisting of RPMI-1640 supplemented with 240 nM insulin, 2 mM L-glutamine, 1 μg/ml transferrin, 0.3 nM selenium, 1.5 μM free fatty acids, 100 U/ml penicillin and 100 μg/ml streptomycin.

Table I. Antibodies used in the western blot analysis.

Antibody	Santa Cruz (sc) catalog number	Dilution
anti-JNK	sc-7345	1:200
anti-p-JNK	sc-293136	1:200
anti-Bax	sc-7480	1:200
anti-Bcl-xL	sc-8392	1:200
anti-Bcl-2	sc-783	1:200
anti-P-Bcl-2	sc-16323	1:200
anti-β-actin	sc-47778	1:1000

JNK, c-Jun N-terminal kinase; Bcl, B-cell lymphoma; Bax, Bcl-2-associated X protein

Cell growth inhibition assays. The cells were plated in 96-well plates (1,500 cells/well) and allowed to attach overnight. Subsequently, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma-Aldrich, Carlsbad, CA, USA; final concentration, 0.5 mg/ml) was added to the wells and the cells were incubated for 4 h. Absorbance was measured at 550-560 nm by using a microplate reader (Bio-Rad, Hercules, CA, USA).

Annexin V-fluroescein isothiocynate (FITC) and propidium iodide (PI) double staining. Following the manufacturer's instructions (Apoptosis Detection kit; KeyGen Biotech Co., Ltd., Nanjing, China), the cells were washed and resuspended in binding buffer prior to incubation in FITC-labeled Annexin V and PI for 10 min. The suspensions were immediately analyzed by a FACSCalibur machine (BD Biosciences, Baltimore, MD, USA).

Cell cycle analysis. Cells were collected and centrifuged at 1,500 x g for 5 min, and the pellet was resuspended in 100 μl PBS at a density of $1x10^6$ cells/ml. Cold ethanol (900 μl, 70%) was added to the mixture for 1 h on ice. The cells were collected by centrifugation at 1,500 x g for 5 min. The pellet was then resuspended in 100 μl PBS containing RNase A (0.2 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) and maintained at room temperature for 30 min. The cells were recovered by centrifugation and the pellets were resuspended in 350 μl PBS containing PI (50 μg/ml; KeyGen Biotech Co., Ltd.) and analyzed by flow cytometry using the FACSCalibur machine.

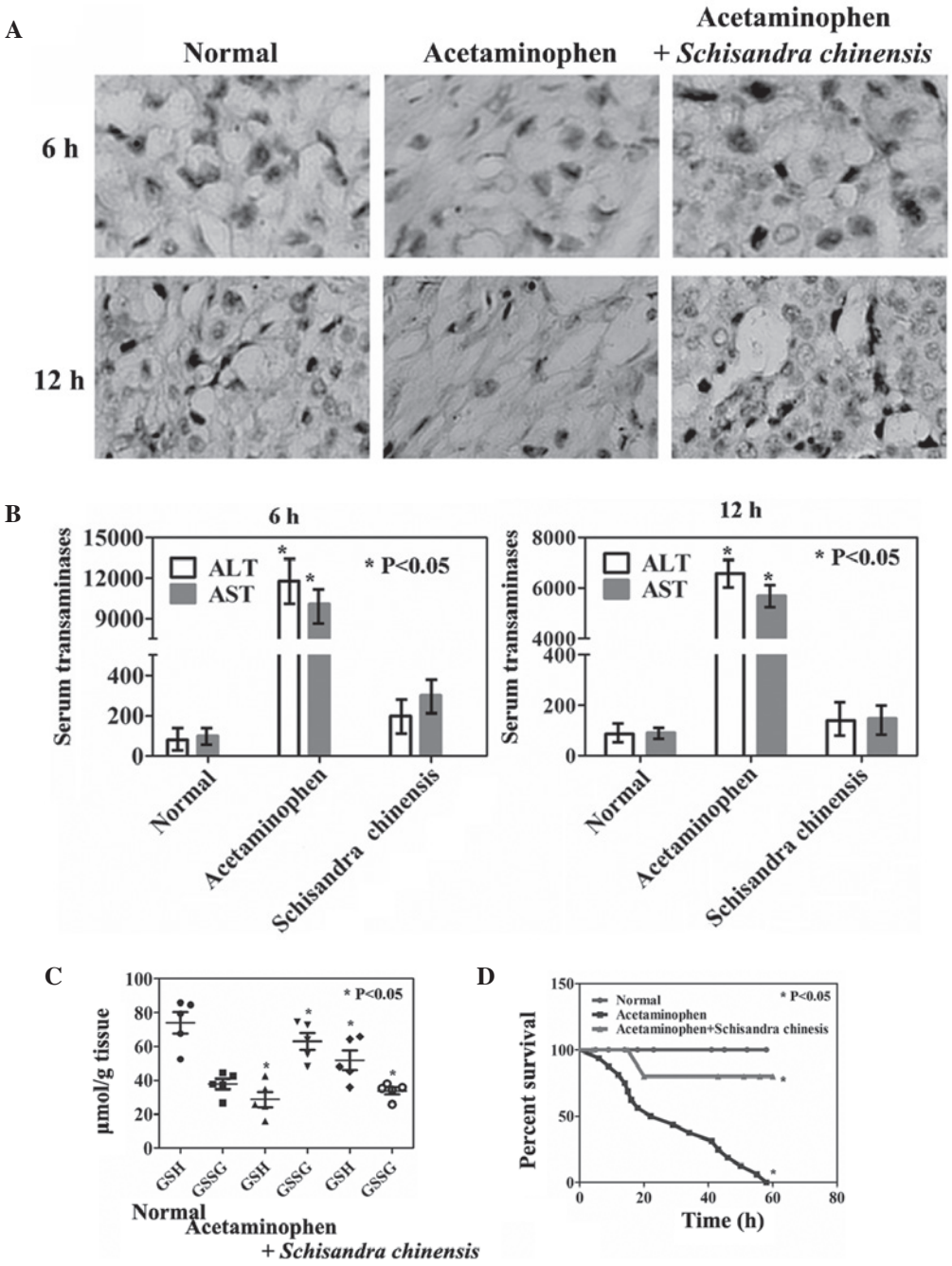

Figure 1. Effects of *Schisandra chinensis* on acetaminophen-induced hepatotoxicity *in vivo*. Mice were fed a standard diet and divided into three groups: Normal, untreated mice; Acetaminophen, mice treated with acetaminophen; and Acetaminophen + *Schisandra chinensis*, mice treated with acetaminophen and *Schisandra chinensis*. (A) Liver sections were prepared 12 h after treatment and stained with hematoxylin and eosin. (B) Serum was collected at 6 or 12 h after treatment and analyzed for alanine transaminase (ALT) and aspartate transaminase (AST). *P<0.05, compared with the normal group. (C) Liver samples collected 12 h after treatment were analyzed for glutathione (GSH) and glutathione disulfide (GSSG) content. *P<0.05 acetaminophen group vs. the normal group and *Schisandra chinensis* group vs. acetaminophen group. (D) Kaplan-Meier curves for the cumulative survival rates of the mice. *P<0.05, acetaminophen group vs. the normal group and *Schisandra chinensis* group vs. acetaminophen group. All values are presented as the mean ± standard error of the mean.

Determination of mitochondrial membrane potential (MMP). MMP was analyzed using the fluorescent dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) according to the manufacturer's instructions (KeyGen Biotech Co. Ltd.). Briefly, cells were plated on a six-well culture plate. Following treatment for 24 h, the cells were washed twice with PBS, harvested and incubated with 20 nM JC-1 for 30 min in the dark. MMP was then analyzed using the FACSCalibur machine.

Quantification of cellular ROS. Cells ($5x10^5$) were cultured in 12-well tissue culture plates overnight, and then cotreated with drugs and 2',7'-dichlorofluorescin diacetate (DCF-DA), an ROS-sensitive dye. Following treatment, the cells were harvested and suspended in PBS. The relative fluorescence intensities of the cells were quantified using the FACSCalibur machine.

26S proteasome activity assay. The 26S proteasome function was assayed as described previously (19). The assay was based

Table II. Effects of *Schisandra chinensis* on the levels of Cyp activity in mouse liver microsomes.

	Treatment group		
Enzyme	Normal	Acetaminophen	Acetaminophen + *Schisandra chinensis*
Cyp2e1	1.07±0.04	1.04±0.03	1.12±0.06
Cyp1a2	33.8±1.5	145.2±27.8	42.7±2.2[a]
Cyp1a2 + rutaecarpine	1.5±0.1	5.6±0.3	1.8±0.2[a]
Cyp3a	5.9±0.8	8.3±0.5	6.4±0.7
Cyp3a + ketoconazole	1.08±0.08	1.04±0.05	0.98±0.06

[a]P<0.05, compared with the acetaminophen treatment group. Cyp, cytochrome P450. All values are presented as the mean ± standard error of the mean.

on the detection of the fluorophore 7-amino-4-methylcoumarin (AMC) following cleavage from the labeled substrate Suc-LLVY-AMC (Boston Biochem Inc., Cambridge, MA, USA). This fluorogenic proteasome substrate was added to the cell lysate at a final concentration of 80 μM in 1% dimethylsulfoxide. Adenosine triphosphate-dependent cleavage activity was monitored continuously by detection of free AMC using a microplate reader (Bio-Rad) at 380/460 nm at 37˚C.

Western blot analysis. Cell extracts were resolved on SDS-PAGE and then transferred to nitrocellulose membranes. These membranes were developed and visualized with electrochemiluminescence (Pierce, Waltham, MA, USA). The primary antibodies used are listed in Table I.

Statistical analysis. All values are presented as the mean ± the standard error of the mean. Student's paired t-test was used to identify statistically significant differences. Kaplan-Meier survival plots were generated and comparisons were made with log-rank statistics. P<0.05 was considered to indicate a statistically significant difference. Statistical analyses were performed using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA).

Results

Effects of Schisandra chinensis on acetaminophen-induced hepatotoxicity in vivo. The effects of a diet containing *Schisandra chinensis* on mice with acetaminophen-induced liver injury were analyzed. Hepatocellular cytoplasmic degeneration, bridging necrosis and severe congestion were observed in mice treated with acetaminophen (Fig. 1A). Compared with those of the untreated mice, the mice administered acetaminophen exhibited a rapid induction of hepatotoxicity with significant elevations in the levels of serum ALT and AST (Fig. 1B, P<0.05). Treatment with *Schisandra chinensis* appeared to inhibit acetaminophen-induced hepatotoxicity, as reduced levels of serum transaminases and marginal structural alterations in the liver were observed (Fig. 1A and B). Acetaminophen treatment alone resulted in a rapid reduction in the levels of GSH and an increase in the levels of GSSG. However, the levels of GSH in the acetaminophen and *Schisandra chinensis* combined treatment group showed a restored trend compared with those of the untreated group (Fig. 1C, P<0.05). No significant differences were identified in the levels of microsomal Cyp2e1 and Cyp3a activity among the mice in the different groups. However, the levels of Cyp1a2 were significantly suppressed by *Schisandra chinensis* administration compared with those in the acetaminophen-treated mice (Table II, P<0.05). Furthermore, a significantly different survival rate between the untreated group and the acetaminophen-treated group, as well as between the acetaminophen-treated group and the acetaminophen- and *Schisandra chinensis*-treated group in the Cox model was identified (Fig. 1D, P<0.05).

Effects of Schisandra chinensis on acetaminophen-induced hepatotoxicity in vitro. The proliferation of hepatocytes was inhibited by acetaminophen as revealed using the MTT assay (Fig. 2A, P<0.05). PI staining of cells revealed that acetaminophen-treated cells were arrested in the G_1 phase (Fig. 2B). Annexin V-FITC and PI double staining was performed to detect apoptotic cells. In the cells with acetaminophen treatment, the apoptotic ratio was 11-12-fold higher than that of the untreated cells (Fig. 2C, P<0.05). As shown in Fig. 2D, the red/green ratio, used to measure the MMP, in the normal cells (1.2% green, 98.8% red) was reversed following acetaminophen treatment (62.5% green, 37.5% red). The fluorescent dye DCF-DA was used to measure the ROS content in cells following acetaminophen treatment. As shown in Fig. 2E, acetaminophen treatment directly induced an increase in the fluorescence intensity of the cells (42.7%) when compared with that of the normal cells (13.4%, P<0.05). Furthermore, acetaminophen was found to inhibit proteasome activity in hepatocytes (Fig. 2F, P<0.05). Consistent with the results obtained *in vivo*, *Schisandra chinensis* protected hepatocytes against acetaminophen-induced apoptosis, ROS release and injury to mitochondria and proteasomes (Fig. 2).

Mechanism(s) of Schisandra chinensis-mitigated acetaminophen-induced hepatotoxicity in hepatocytes. The aforementioned results revealed the changes of mitochondria in hepatocytes. Due to these results, the changes were further analyzed; expression of Bax, Bcl-xL, Bcl-2 and p-Bcl-2 was

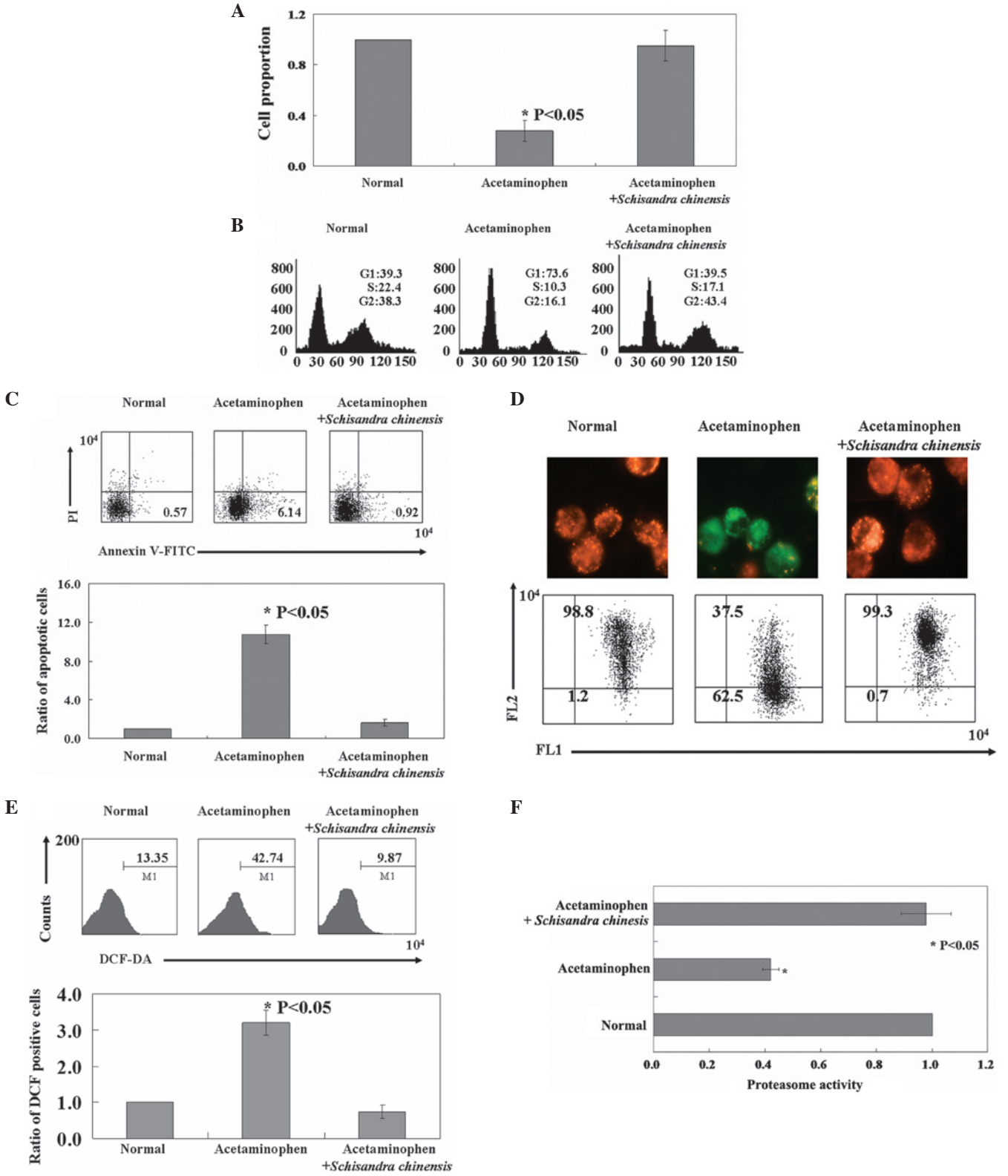

Figure 2. Effects of *Schisandra chinensis* on acetaminophen-induced hepatotoxicity *in vitro*. (A) The proliferation rate of hepatocytes was measured using the MTT assay. (B) Cells were stained with propidium iodide (PI) to analyze the cell cycle distribution of each cell type by flow cytometry. (C) The proportion of apoptotic cells was determined by double staining with Annexin-V/fluorescein isothiocyanate and PI. (D) The mitochondrial membrane potential was analyzed by immunofluorescence and flow cytometry. (E) The dichlorofluorescein (DCF)-positive cells (reactive oxygen species production) were detected with a FL1 signal detector (525 nm) using a FACSCalibur machine. (F) The effect of *Schisandra chinensis* on proteasomes was determined using a microplate reader. All values are presented as the mean ± standard error of the mean. *P<0.05, compared with the normal group.

detected using western blot analysis. As shown in Fig. 3 (lanes 1 and 2), a reduction in the Bcl-2 and Bcl-xl expression levels and an increase in Bax and P-Bcl-2 expression levels were identified in hepatocytes with acetaminophen treatment compared with those in the normal cells. The levels of these proteins were markedly reversed following *Schisandra chinensis* treatment

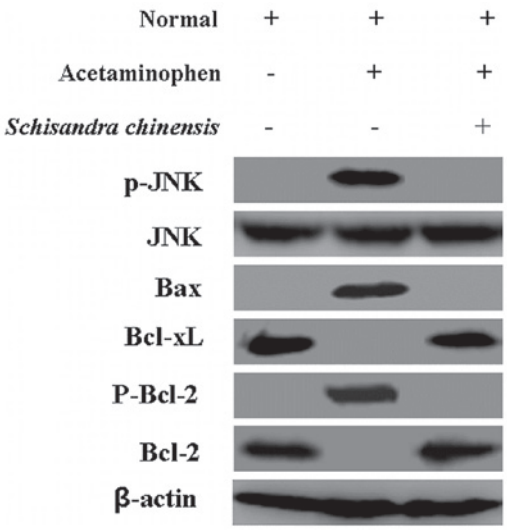

Figure 3. Changes in the levels of mitochondrial apoptosis-related proteins. Cellular protein was isolated from hepatocytes and western blot analysis was performed to detect c-Jun N-terminal kinase (JNK), B-cell lymphoma (Bcl)-xl, Bcl-2, Bcl-2-associated X protein (Bax), phosphor-JNK and phosphor-Bcl-2 using the respective specific antibodies. β-actin served as an internal control.

(Fig. 3, lanes 2 and 3). The levels of phosphor-c-Jun N-terminal kinase (p-JNK) were upregulated by acetaminophen treatment but the levels of total JNK did not change compared with those in the normal cells (Fig. 3, lanes 1 and 2). However, p-JNK was inhibited following *Schisandra chinensis* treatment (Fig. 3, lane 3). β-actin served as an internal control for all western blotting.

Discussion

Acetaminophen is a commonly used analgesic drug that in overdose results in hepatic necrosis and liver failure (1). In the present study, acute liver injury in mice was successfully established using acetaminophen, as detemined by the levels of the serum transaminases and histological changes. Elevations in ALT and AST levels are characteristic of acute acetaminophen overdose (20,21). The levels of ALT and AST were confirmed to be elevated in the mice in the present study following acetaminophen treatment compared with those in the untreated mice. GSH is key in scavenging ROS (22). Consistent with the results of previous studies (23,24), acetaminophen intoxication resulted in reduced GSH levels in mice compared with those in the control group.

Schisandra chinensis has been used in China, Korea and Japan to regulate various pathophysiological conditions, including hepatitis and cancer (6). In the present study, *Schisandra chinensis* was confirmed, with histological evidence, to inhibit acetaminophen-induced acute liver injury. For example, the levels of ALT and AST were reduced in mice following *Schisandra chinensis* treatment compared with those in the mice which only received acetaminophen. Hu *et al* (25) found that *Schisandra chinensis* inhibited the reduction in the levels of GSH and reduced the levels of GSSG, and similar effects were identified in the present study. Acetaminophen-induced hepatotoxicity involves oxidative stress, which is generated as a consequence of Cyp-mediated NAPQI formation and inflammatory cell production of ROS (26,27). In the present study, *Schisandra chinensis* inhibited the elevated levels of ROS induced by acetaminophen. Furthermore, the normal levels of hepatic Cyp1a2 activity were restored in mice following *Schisandra chinensis* treatment.

In the *in vitro* experiments, acetaminophen-induced apoptosis in hepatocytes was associated with changes in the MMP. Changes in the levels of Bcl-2 and Bax in hepatocytes were also identified following acetaminophen treatment compared with those in the normal cells. However, *Schisandra chinensis* may be able to treat acute liver injury though protection of the mitochondria. Disruption of lysosomes in hepatocytes following acetaminophen treatment has been observed in previous studies (13,15). Notably, *Schisandra chinensis* was also found to protect lysosomes in the present study.

Activation of hepatic JNK is recognized as a key event in the progression and exacerbation of acetaminophen toxicity, and inhibition of p-JNK has been shown to protect mice against acetaminophen-induced hepatotoxicity (28,29). In the present study, *Schisandra chinensis* was found to inhibit p-JNK expression levels compared with those in the acetaminophen-treated hepatocytes

In conclusion, the present study provides evidence that *Schisandra chinensis* positively inhibited acetaminophen-induced hepatotoxicity *in vivo* and *in vitro*. The collective results showed that *Schisandra chinensis* may protect mitochondria and lysosomes. Furthermore, *Schisandra chinensis* inhibited the p-JNK signaling pathway. These findings provide further support for the clinical application of *Schisandra chinensis* in the treatment and prevention of acetaminophen-induced hepatotoxicity.

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