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### Article

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Semen Hoveniae extract protects against acute alcohol-induced liver injury in mice

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### **Running title**

Semen Hoveniae relieves acute alcohol-induced liver injury

## **Abstract**

The protective effects of Semen Hoveniae extract (SHE) from *Hovenia dulcis* Thunb. (Rhamnaceae) on the acute alcohol-induced liver injury were investigated *in vivo* using mice as test models. In the present study, SHE (150, 300, 600 mg/kg/d) were given to mice by intragastric administration for four days. Mice were gavaged with 60% ethanol 10 ml/kg after the last dose of the extract. Six hours after alcohol administration, liver injury was evaluated by biochemical examination. Lipid peroxidation and the activity of antioxidants were measured by spectrophotometric method. In mice, administration of SHE significantly decreased the activities of alanine amino transferase (ALT) and aspartate transaminase (AST) in serum. Administration of SHE also protected against alcohol-induced alcohol dehydrogenase (ADH) elevation in mice. Concurrently, there was an augmentation in the activities of antioxidant enzymes such as superoxide dismutase (SOD), glutathione-S-transferase (GST) and glutathione (GSH), and it also facilitated the alcohol metabolism. Acute toxicity test showed that a single dose of oral SHE up to 22 g/kg did not result in any death or toxic side effects of mice in 14 days' observation. These results demonstrate that SHE could protect against acute alcohol-induced liver injury without any toxic side effects. Therefore, Semen Hoveniae has the potential to develop a clinically useful agent which could protect the liver from alcohol-induced injury.

**Keywords:** Semen Hoveniae; Liver damage; Gastric metabolism

## **Introduction**

Alcoholic liver disease encompasses a broad spectrum of morphological features ranging from minimal injury to advanced liver damage (Arteel et al., 2003). Liver disease in the alcoholic is due to ethanol hepatotoxicity linked to its metabolism and the resulting production of toxic acetaldehyde.

Ethanol is mainly metabolized in the liver through three major pathways with different subcellular locations: alcohol dehydrogenase (ADH) in the cytosol, aldehyde dehydrogenase (ALDH) in the mitochondria, and microsomal ethanol oxidizing system in the endoplasmic reticulum (Lieber, 1997). All of them lead to over production of reactive oxygen species, including superoxide, peroxide, and hydroxyl radical, which can cause complete degradation of lipids, proteins, and DNA (Wu & Cederbaum, 2003). Additionally, alcohol exposure impairs enzymatic and non-enzymatic mechanisms that protect cells against reactive oxygen species, such as superoxide dismutase (SOD) and glutathione (GSH) (Wu & Cederbaum, 2003). Enhanced reactive oxygen species production and compromised antioxidant activity result in oxidative stress, which has been demonstrated to play a pivotal role in alcohol-induced liver injury (Cederbaum, 2001; Dey & Cederbaum, 2006).

Recently, a potential measure for the prevention of liver injury due to alcohol could resort to plants that are rich in flavonoids, which are exceptionally efficient antioxidants and radical scavengers (Bors et al., 1999). Different classes of flavonoids are produced in plants and are regularly ingested within the diet (Haslam, 1998; Aoa et al., 2009). It has been shown that the extract of green tea, a substance rich in the

procyanidin epigallocatechin gallate, protects the liver against necrosis in the enteral model of alcohol-induced injury (Arteel et al., 2002). Semen Hoveniae, the seed of the *Hovenia dulcis* Thunb. (Rhamnaceae), was found to show neuroprotective (Li et al., 2005) and hepatoprotective (Hase et al., 1997) effects. But the protective activity of Semen Hoveniae against acute alcohol-induced liver injury has not been studied, as far as we know.

The main objective of this study was to assess the effects of Semen Hoveniae extract on alcohol-induced liver injury. The levels of the hepatic ADH, SOD, GSH and glutathione-S-transferase (GST) were measured, and serum aspartate transaminase (AST) and alanine amino transferase (ALT) concentrations were also investigated. Additionally, the blood alcohol content, hepatic triglyceride (TG) level and the content of alcohol metabolite-malondialdehyde (MDA) were also measured.

## **Materials and methods**

### ***Preparation of Semen Hoveniae extract***

Semen Hoveniae were purchased from Tong-Ren-Tang Pharmaceutical Group (Shanghai) and identified as the seeds of *Hovenia dulcis* by Professor Lu-Ping Qin (The School of Pharmacy, Second Military Medical University, Shanghai, China). A voucher specimen was deposited with the number SY3225 in the Department of Pharmacognosy, School of Pharmacy, Second Military Medical University. The seeds (1 kg) were pulverized in a motor driven grinder to prepare the extract. After refluxing extraction with 8 L 75% (v/v) analytical reagent alcohol twice for 1 h each

time, the extract was filtered and then the solvent was evaporated to dryness under reduced pressure in a rotary evaporator. The extractum was freeze-dried for *in vivo* evaluation. The yield of Semen Hoveniae extract (SHE) was 10.78 %.

### ***Animals and treatments***

Ninety male kunming mice, obtained from Shanghai Si-Lai-Ke Experimental Animal Ltd. (Shanghai China), with an initial body weight of  $20 \pm 2$  g, were used in this study. They were housed in a regulated environment ( $22 \pm 2^{\circ}\text{C}$ ) with a 12 h dark and 12 h light cycle (08:00-20:00, light), and free access to food and tap water. All animal treatments were strictly in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals, and the experiments were carried out with the approval of the Committee of Experimental Animal Administration of the University.

Forty animals were randomly divided into four groups of ten each. One of the groups served as control and the other three groups were administrated with SHE (150, 300, 600 mg/kg d) for four consecution days. Animals in the model group received an equal volume of vehicle as control. The animals were fasted for 12 h before the last administration of the drugs. All animals were treated orally with 60% ethanol (10 ml/kg) after the last dose of the drugs. SHE and alcohol were administered using intragastric tube. One hour later, animals were sacrificed and blood samples were collected and heparinized for the measurement of the alcohol level.

Another fifty animals were also randomly divided into five groups with ten mice

in each group. In the test groups, mice were given SHE 150, 300, 600 mg/kg/d by gavage for four consecutive days, and the control were treated with an equal volume of water. The animals were fasted for 12 h before the last administration of the drugs. All animals were treated orally with 60% ethanol (10 ml/kg) after the last dose of the drugs except mice in normal control group. Six hours later, animals were decapitated and blood was collected for the test of serum ALT and AST level. The livers were immediately removed and cleaned in 0.9% sodium chloride (4°C), then were cut into small pieces and stored at -80°C for biochemical assays.

#### ***Ethanol assay***

Blood alcohol concentrations were determined using blood samples collected one hour after the administration of alcohol, with an enzymatic alcohol test kit (Sigma Diagnostics). Ethanol concentration was determined by measuring absorbance at 366 nm resulting from the reduction of NAD<sup>+</sup> to NADH by alcohol dehydrogenase (Bergmeyer, 1998).

#### ***Serum measurements and hepatic triglyceride assay***

Those blood samples collected 6 h after the administration of alcohol were kept at room temperature for 1 h. The serum samples were separated by centrifugation at  $1500 \times g$  for 15 min, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured spectrophotometrically using an automated analyzer (Bayer-Opera) to assess the hepatic functions.

Liver tissue was homogenized in 0.9% NaCl (1:19) and hepatic TG contents were measured by the colorimetric enzymatic method using commercial kits

purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

### ***Hepatic lipid peroxidation assay***

The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously (Beuge & Aust, 1978). Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  and results are expressed as nmol MDA/g liver.

### ***Hepatic biochemical assays***

Some pieces of liver were homogenized at 4°C in 10 ml of 0.1 M phosphate buffer, pH 7.4, containing 1% Bovine serum albumin (BSA). BSA was added to the buffer to protect ADH from protease attack by serving as an alternate substrate. The homogenate was centrifuged at 20,000 g for 20 min and the supernatant fraction was then centrifuged for 1 h at 100,000 g. The 100,000 g supernatant (cytosol) was used for ADH assays after a dialysis overnight against 0.1 M phosphate buffer, pH 7.4. All steps were carried out at 4°C. ADH activity was determined at room temperature at 340 nm using an ATI Unicam UV/VIS Spectrometer (Aasmoe et al., 1998). Hepatic GSH content was determined as described by Ellman (1959).

Liver sample (0.5 g) was homogenised in 10 ml ice-cold 0.01 M phosphate 0.15 M KCl buffer at pH 7.4. The homogenate was centrifuged at 10,000 g for 15 min at 4°C. The supernatant was used as the source of liver GST. All samples were kept on ice prior to use. GST activity was determined by a modification of the method use by Habig et al. (1974), using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. Hepatic



SOD activity was assayed by its ability to inhibit the auto oxidation of hematoxylin into hematin following the method of Martin et al. (1987). The results were expressed as unit SOD/mg protein.

### ***Statistical analysis***

The data were analyzed using a SPSS 11.0 statistical package. The data for multiple comparisons were performed by one-way ANOVA followed by Dunnett *t*-test. A value of  $P < 0.05$  was considered statistically significant and all results are presented as the mean  $\pm$  s.e.m.

## **Results**

### ***Effects of SHE on blood alcohol content in mice***

After administration of a same volume of alcohol, the concentrations of blood alcohol in mice were measured. As showed in Table 1, SHE significantly decreased the blood alcohol level at 300 and 600 mg/kg but not at the low dose, 150 mg/kg. To speak exactly, the elimination of alcohol in the mice treated with SHE was faster than the model mice.

### ***Effects of SHE on the levels of serum ALT, AST and hepatic TG***

Acute alcohol-induced liver injury was indicated by elevated serum ALT, AST and hepatic TG. The result reported in Table 2 shows the activities of serum AST, ALT and hepatic TG in control and experimental mice. It also indicates that ethanol administration significantly increased the activities of the above parameters in model mice after administration of alcohol 6 h later. Treatment with SHE remarkably reduced the elevation of serum ALT (150, 300, 600 mg/kg), AST (300, 600 mg/kg)

and accumulation of hepatic TG (300, 600 mg/kg) in a dose-dependent manner.

#### ***Effects of SHE on hepatic lipid peroxidation***

As described in Table 3, the content of MDA, an ethanol metabolite, a type of aldehyde adduct were found remarkably increased in the model mice compared to the control mice. Then, the middle and high doses of SHE (300, 600 mg/kg) decreased the MDA level, as evidenced by closing to normal level.

#### ***Effects of SHE on the levels of hepatic ADH, SOD, GST and GSH***

ADH is involved in the major pathway for ethanol metabolism in liver under normal physiological conditions. The activity of ADH increased in the model mice comparing to that of the normal mice. Administration of SHE (300, 600 mg/kg) significantly protected against alcohol-induced ADH elevation except the low dose of SHE (Table 3).

Table 3 also represents the levels of non-enzymatic antioxidant, GSH status in livers. The levels of GSH were significantly reduced in alcohol treated mice when compared with control rats. Administration of SHE (150, 300, 600 mg/kg) significantly restored the levels of non-enzymatic antioxidants in tissues.

The activities of antioxidant enzymes namely SOD and GST in liver were given in Table 3. A significant decrease in the activities of enzymatic antioxidants was observed in alcohol treated mice when compared with control mice. Administration of SHE (150, 300, 600 mg/kg) along with alcohol significantly reversed these functional markers towards normal in a dose-dependent manner.

### **Discussion**

The results of the present study demonstrated that acute alcohol administration caused liver injury as evidenced by the elevation of serum ALT, AST and hepatic TG levels, which reflected early biochemical and pathological changes in alcoholic liver disease. ALT and AST are the reliable markers for liver function (Gross et al., 2009). It is established that AST can be found in liver, cardiac muscle, skeletal muscle, kidney, brain, pancreas, lungs, leukocytes and erythrocytes whereas ALT is present in liver (Rej et al., 1997). The increased levels of serum enzymes such as AST and ALT indicate the increased permeability and damage and/or necrosis of hepatocytes (Goldberg & Watts, 1965). In our study, we found that a large quantity of ethanol consumption at one time caused a significant increase in the activities of AST and ALT, which could lead to severe damage to tissue membrane. Pretreatment with SHE decreased activities of these enzymes in mice which indicated its hepatoprotective effect.

Binge drinking causes fatty liver, which represents the early stage of alcoholic liver disease, and is usually reversible. There are multiple mechanisms underlying ethanol-induced development of fatty liver. Ethanol administration promotes fatty-acid synthesis; increases expression of some mRNAs, which promotes TG synthesis; and decreases expression of some mRNAs, which leads to inhibition of fatty acid oxidation. Additionally, results of several studies have identified accumulation of lipids in the liver to be the onset of ALD (Teli et al., 1995; Wanless & Shiota, 2004). In the present study, the hypothesis that SHE protects against early alcohol-induced fat accumulation was first tested in a model of acute alcohol

ingestion.

Alcohol dehydrogenase (ADH) is an important enzyme that oxidizes alcohol at a faster rate to ease out alcohol concentration. An increase in ADH activity is followed by a decrease in ethanol concentration. Nevertheless we observed in this study that SHE decreased the activity of hepatic ADH, accompanied by the decrease of blood alcohol content. Actually alcohol metabolism is a complex process. Ingested ethanol is partly oxidized in the upper digestive tract by gastric ADH and the remaining is absorbed through the portal bloodstream into the liver and metabolized by hepatic ADH (Livy et al., 2003). The metabolism by gastric ADH means less ethanol is available for entry into the systemic bloodstream, resulting in lower blood ethanol content in the gavaged animals. Some plant extracts can facilitate ethanol metabolism in the gastrointestinal tract (Matsuda et al., 2002; Tinoco et al., 2009). The investigation of Tinoco et al (2009) indicated that the hexane extract from *Laurus novocanariensis* leaves significantly enhanced gastric ADH activity in ethanol-treated rats and reduced hepatic ADH activity, followed by the decrease of blood alcohol content, which is in accordance with our study. Another probable reason for our results is that SHE might have increased glycine levels, which decreased blood ethanol concentration by stimulating ethanol metabolism in the stomach (Iimuro et al., 1996).

MDA, an ethanol metabolite, was generally considered to be responsible for ALD. Perivenous adducts of the acetaldehyde product of ethanol metabolism and the MDA, product of lipid peroxidation, appear to precede necrosis and fibrosis (Niemelä

et al., 1995). Acute alcohol intoxication has been associated with lipid peroxidation in both human beings (Meagher et al., 1999) and rodents (Shaw et al., 1990; Lang et al., 2009). SHE attenuate the toxicity of MDA on liver by depressing the hepatic MDA content in mice, which indicated its effect on eliminating this toxicity ethanol metabolite in human beings.

The pathogenesis of alcohol-induced liver disease involves the adverse effects of ethanol metabolites and also oxidative tissue injury. The role of oxidative stress in the development of alcoholic liver disease has been suspected since the early 1960s by Diluzio et al. (1964) and Diluzio and Hartman (1967), who observed that alcohol administration promoted the oxidative breakdown of cell membranes. Our results confirmed the involvement of oxidative stress in acute alcohol-induced liver injury, and both the compromised non-enzymatic antioxidant GSH and enzymatic antioxidants, including SOD and GST, were restored by the treatment of SHE.

Non-enzymatic antioxidants, such as GSH, play an excellent role in protecting the cell from lipid peroxidation. The depleted level of GSH in alcohol toxicity may be due to scavenging of toxic radicals and inhibition of the synthesis and increased rates of turnover (Lieber, 1997). The active constituents such as flavanols and triterpene glycosides were found in *Hoveniae Semen Seu Fructus* (Yoshikawa et al., 1995), which could be responsible for the reversal of antioxidant levels in tissues of alcohol fed mice treated with SHE.

In addition, the toxicity profile of oral SHE was evaluated in mice in a single-dose acute toxicity test. Consecutive doses ranging from 1 to 22 g/kg were designed to assess the potential toxicity and lethal dose of oral SHE. However the

dose up to 22 g/kg was well tolerated, which was unassociated with any death or toxic side effects in 14 days' observation.

In summary, SHE evidently decreased the levels of serum AST, ALT and hepatic TG, ADH and MDA in acute hepatic injury mice induced by alcohol, reduced blood alcohol concentrations in acute alcoholism mice, and increased the levels of GSH, GST and SOD. The current data strongly indicates protective effects of SHE on the acute alcoholic liver injury mice, most likely through increasing the concentrations of antioxidants following alcohol exposure. Its function may be mediated by facilitating the alcohol metabolism and reducing the metabolite. However, its underlying mechanism of action on anti-hepatic injury induced by alcohol needs to be further investigated.

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Table1. The effects of SHE on blood alcohol content in mice

Group	Mice	Blood alcohol content (mg/dL)
Alcohol	10	394.26 ± 69.27
SHE (150 mg/kg) + Alcohol	10	343.72 ± 61.34
SHE (300 mg/kg) + Alcohol	10	315.83 ± 57.45*
SHE (600 mg/kg) + Alcohol	10	283.31 ± 63.42**

\* P<0.05 \*\* P<0.01 compared with the Alcohol group. Data are expressed as the mean ± s.e.m.

Table 2. The effects of SHE on the levels of serum ALT , AST and hepatic TG

Goup	Serum ALT level (U/L)	Serum AST level (U/L)	Hepatic TG level (mmol/L)
Control	51.80 ± 3.43	147.52 ± 7.41	2.56±0.06
Alcohol	74.40 ± 2.87ΔΔ	302.56 ± 20.00ΔΔ	2.97 ± 0.05ΔΔ
SHE (150 mg/kg) + Alcohol	61.80 ± 3.49*	263.88 ± 17.12	2.88±0.15
SHE (300 mg/kg) + Alcohol	52.70 ± 4.00**	198.63 ± 19.66**	2.66±0.10*
SHE (600 mg/kg) + Alcohol	49.20 ± 3.14**	188.74 ± 10.96**	2.54±0.05**

ΔP<0.05, ΔΔP<0.01 compared with the control group; \*P<0.05, \*\*P<0.01 compared with the alcohol group. Data are expressed as the mean ± s.e.m. Ten animals were used per group.

Table 3. The effects of SHE on some hepatic parameters

Goup	Hepatic MDA level (nmol/g)	Hepatic ADH level (umol/min.L)	Hepatic GSH level (nmol/g)	Hepatic GST level (nmol/min.mg)	Hepatic SOD level (U/mg)
Control	35.10±1.93	272.45±12.01	6.50±0.12	93.84±3.38	151.80±6.6
Alcohol	47.20±2.31ΔΔ	384.16±14.37ΔΔ	5.54±0.21ΔΔ	71.86±4.02ΔΔ	94.40±6.98ΔΔ
SHE (150 mg/kg) + Alcohol	44.50±1.08	335.81±17.53	6.85±0.15**	84.67±3.46*	131.80±9.50*
SHE (300 mg/kg) + Alcohol	36.40±1.88**	312.03±13.15**	7.90±0.18**	95.13±4.70**	142.70±10.33**
SHE (600 mg/kg) + Alcohol	32.70±1.87**	307.31±10.36**	8.74±0.22**	123.43±7.31**	149.20±6.31**

ΔP<0.05, ΔΔP<0.01 compared with the control group; \* P<0.05, \*\*P<0.01 compared with the alcohol group. Data are expressed as the mean ± s.e.m. Ten animals were used per group.