대한침구의학회지 제32권 제1호 (2015년 3월) : 13-22
The Acupuncture Vol. 32 No. 1 March 2015 : 13-22
pISSN 1229-1137 eISSN 2287-7797
http://dx.doi.org/10.13045/acupunct.2015002

Original Article

Effect of *Ganoderma Lucidum* Pharmacopuncture on Chronic Liver Injury in Rats※

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[Abstract]

Objectives : Alcohol-related liver disease is a major cause of morbidity and mortality worldwide. The present study was undertaken to determine whether *Ganoderma lucidum* pharmacopuncture(GLP) could protect against chronic liver injury induced by ethanol intoxication in rats.

Methods : Sprague-Dawley rats were divided into 4 groups: normal, control, normal saline pharmacopuncture(NP), and GLP, with 8 animals in each. Each group, except normal, received ethanol orally. The NP and GLP groups were treated daily with NP and GLP respectively. The control group was not treated. All rats except the normal group were intoxicated for 4 weeks by oral administration of EtOH(6 g/kg BW).

Two acupuncture points were used: *Qimen*(LR_{14}) and *Taechung*(LR_3). Body weight, histopathological analysis, liver function, activities of antioxidant enzymes, and immunohistochemistry were assessed.

Results : GLP reduced the histological changes due to chronic liver injury induced by EtOH and significantly reduced the increase in the alanine aminotransferase(ALT) and aspartate aminotransferase(AST) enzymes. It significantly reversed the superoxide dismutase(SOD) and the catalase activities(CAT). It also significantly decreased BAX and increased Bcl-2 immunoreactivity expression.

Conclusions : This study showed the protective efficacy of GLP against EtOH-induced chronic liver injury in SD rats by modulating ethanol metabolizing enzymes activity, attenuating oxidative stress, and inhibiting mitochondrial damage-mediated apoptosis.

Key words : *Ganoderma Lucidum pharmacopuncture*; Chronic liver injury; Aminotransferase; Superoxide dismutase; Catalase; BAX

Received : 2015. 01.20.
Revised : 2015. 02.04.
Accepted : 2015. 02.06.
On-line : 2015. 03.20.

※ This work was supported by Dong-Eui University(2014AA412)

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Ⅰ. Introduction

Alcohol-related liver disease is a major cause of morbidity and mortality worldwide, and the clinical syndrome of alcoholic liver disease(ALD) carries a particularly poor prognosis, such as liver cirrhosis or hepatocellular carcinoma[1,2].

It is well known that heavy consumption of alcohol is associated with liver damage[3]. The alcohol dehydrogenase(ADH) enzyme, which is largely expressed in hepatocytes, is responsible for the majority of ethanol catabolism that oxidizes ethanol to acetaldehyde[4].

Ethanol administration causes accumulation of reactive oxygen species(ROS), including superoxide, hydroxyl radical, and hydrogen peroxide[5]. ROS, in turn, cause lipid peroxidation of cellular membranes, and protein and DNA oxidation, which results in hepatocyte injury[6,7].

Apoptosis is a biological process by which cells are eliminated during proliferation and differentiation without releasing harmful substances into their environment. Alcohol-induced liver injury plays a central role in apoptotic cell death[8]. The bcl-2 family is a mitochondrial membrane protein, which consists of both proapoptotic BAX and antiapoptotic Bcl-2 members. Both BAX and Bcl-2 functions are thought to be associated with the mitochondrial membrane. BAX causes cytochrome c release, caspase-3 activation and apoptotic cell death. Bcl-2 prevents these changes that take place in the mitochondria[9,10].

Pharmacopuncture, or herbal acupuncture, is a new form of therapy derived from combinations of two traditional therapeutic methods, herbal medicine and acupuncture therapy,[11]. Pharmacopuncture with different types of herbs is effective at treating various disease[12].

Ganoderma lucidum(Curtis)(*Youngji, Lingzhi, Reishi*) is a well-known medicinal mushroom particularly in China, Japan, and Korea. Many works have been carried out on the efficacy of *Ganoderma lucidum.* Several studies have demonstrated that various extracts of *Ganoderma lucidum* interfere with the cell cycle progression, induce apoptosis, and suppress angiogenesis in human cancer cells to act as anti-cancer agents[13]. It has also been found to inhibit platelet aggregation, to lower blood pressure, cholesterol, blood sugar[14], and to treat acute gastric ulcer[15].

The present study was undertaken to determine whether *Ganoderma lucidum* pharmacopuncture could treat chronic liver injury in rats.

Ⅱ. Materials and methods

A. Preparation of solution

500 g *Ganoderma lucidum* caps grown in South Korea were washed thoroughly with distilled water, cut into pieces, and were submerged into 4 L of 25 % alcohol for 10 hours at room temperature to be extracted. The alcohol extract was condensed by rotary evaporator to 500 ml, got rid of impurities with 0.22 μm filter, underwent sterilization, and was stored under the temperature of 20 ℃. The extract was dissolved in ethanol before administration, and diluted with 5 % DW(dextrose water, JW Pharmaceutical) to keep the final concentration of *Ganoderma lucidum* 10 %.

B. Animals and treatment

SD Rats were divided into 4 groups of 8 animals each: normal, control, NP(normal saline pharmacopuncture) and GLP(*Ganoderma lucidum* pharmacopuncture). Normal group received distilled water orally once daily for 28 days. Control, NP and GLP groups received ethanol(5 g/kg, 20 % w/v p.o.) for 28 days[16]. NP and GLP group were daily treated with injection of normal saline and *Ganoderma lucidum* extract respectively. The 2 local acupoints were used: *Qimen*(LR_{14}), *Taechuang*(LR_3). A phramacopuncture syringe(29 gauge × 8 mm, 1 mL, disposal, insulin-

 http://dx.doi.org/10.13045/acupunct.2015002

injection syringe from HWAJIN Co. Busan, Korea) was used, and the amount of injection was 1 mL for each animal. Control groups received no treatment.

C. Histopathological analysis

All the animals were humanely sacrificed using ether. Immediately after killing the rats, small pieces of liver were harvested and washed with ice-cold saline. Tissue fragments were then fixed in a 10 % neutral buffered formalin solution, embedded in paraffin and used for histopathological examination. Sections of 5 ㎛ thickness were cut, deparaffinized, hydrated and stained with hematoxylin and eosin(H&E).

The hepatic sections were examined in blind fashion in all treatments.

D. Assessment of liver function

Blood of approximately 3~4 mL was collected and allowed to clot for 30 min at room temperature. The serum was separated by centrifugation for 15 min, and used for the determination of marker enzymes such as AST, ALT.

E. Antioxidant enzyme activity assays

SD rats were intoxicated by ethanol(5 g/kg, 20 % w/v p.o.) or distilled water(normal group) for 4 weeks. After sacrifice, the liver of rats rapidly excised and homogenized in 50 mM potassium phosphate buffer for determining the biochemical parameters. After centrifugation at 10,000 g for 10 min at 4 ℃, supernatant was used for biochemical determination of antioxidant enzyme activities.

F. Immunohistochemistry

The cellular localizations of BAX and Bcl-2 were examined by immunohistochemistry. Liver tissue was embedded in paraffin. Thin sections(5 ㎛) were deparaffinized in xylene and then hydrated by washing them in descending grades of ethanol. Endogenous peroxidase activity was blocked by incubating the sections in a solution of 3 % hydrogen peroxide in Tris buffered saline contain 0.1 % Tween-20(TBS-T) for 30 min. Sections were incubated in 10 % normal goat serum for 2 h at room temperature to block non-specific binding, followed by incubation with BAX(1 : 100 in 10 % NGS, Santa Cruz Biotechnology) or Bcl-2 antibody(1 : 100 in 10 % NGS, Santa Cruz Biotechnology) at RT for overnight. After washing with TBS-T, sections were incubated with biotinylated mouse IgG secondary antibody(Vector Laboratories, Burlingame, Canada) for 30 min at 37 ℃, followed by incubation for 30 min at 37 ℃. Diaminobenzidine(DAB) was used as a chromogen. Sections were counterstained with hematoxylin and then mounted in Canada Balsam. Images were captured using an Olympus DP controller and imaged under a microscope. BAX and Bcl-2-immunostained cells were counted under a microscope and the results were analyzed.

G. Statistical analysis

Data were analyzed by unpaired Student's t-test or one-way analysis of variance and were expressed as means ± S.E. A p-value of 0.05 or less was considered significant.

III. Results

A. Body weight

The body weight of each rat was daily measured. And, before sacrifice, the final body weight was measured. Rats from the normal group followed a normal pattern of growth and attained a normal weight gain over 8 weeks. But EtOH treated rats suffered growth retardation and had lower weight than normal groups. NP and GLP group gained more weight

than those of control group, but not as much as those attained in Normal group. There was little difference of weight gain rate between NP and GLP group(Fig. 1).

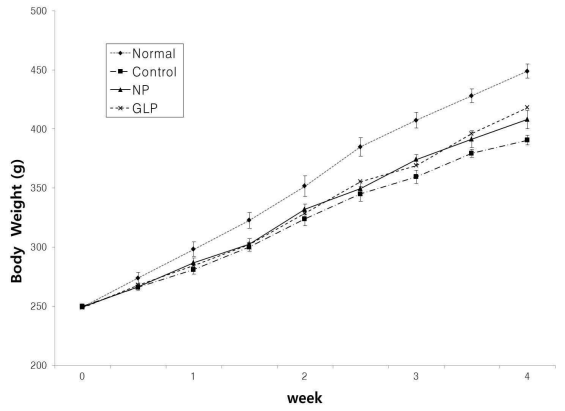

Fig. 1. Body weight changes of male SD rats during 4 weeks
Compared with normal group, mean body weight of control, NP and GLP group was decreased for whole 4 weeks.
There were no obvious differences among 3 groups.

B. Histopathological findings of liver tissue in chronic injury

Liver sections from normal group showed normal histology(Fig. 2A), while liver sections from control group showed severe microvesicular steatosis and hepatocellular swelling, exhibiting significantly enhanced hepatocyte degeneration compared with Normal group.

In control group, mainly microvesicular steatosis was evident. The focal necrosis of hepatocytes and perivascular fibrosis were also noticed(Fig. 2B). On the other hand, the pathological changes of the liver were recovered in NP and GLP groups. NP and GLP group showed mild microvesicular steatosis.

Importantly, the recovery of GLP group was more obvious than NP group(Fig. 2C, D).

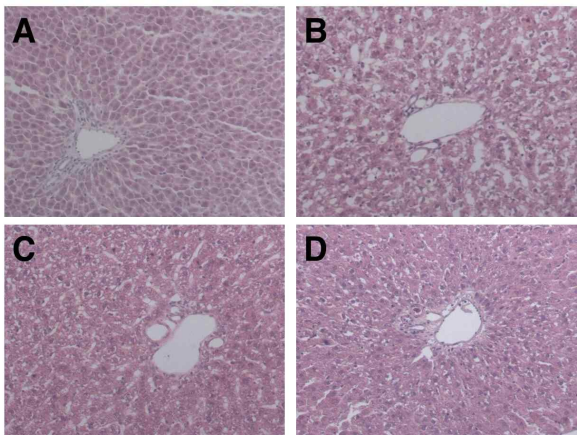

Fig. 2. Liver sections stained with hematoxylin and eosin(H&E)
In control group(B), severe microvesicular steatosis was showed comapred to Normal group(A).
On the other hand, the pathological changes of the liver were recovered in NP and GLP groups(C, D).

C. Liver functions

The plasma ALT and AST activity of control group were significantly higher than normal group. NP group had no significant effect in both parameters. But, the activities of these enzymes in GLP group were significantly reduced compared with control group(Fig. 3, 4).

D. Liver antioxidant enzyme activities

EtOH treatment for 4 weeks significantly decreased

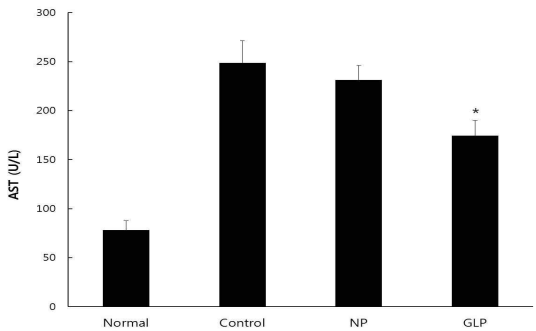

Fig. 3. The effect of GLP on chronic EtOH-induced changes in plasma AST
The plasma AST was increased in all chronic EtOH-treated groups.
While, the plasma AST of GLP group was significantly restored compared with control group.
Data were expressed as means ± SE.
$*$: $p < 0.05$ compared to control group.

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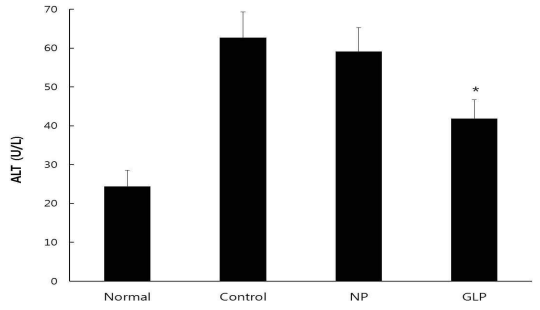

Fig. 4. The effect of GLP on chronic EtOH-induced changes in plasma ALT

The plasma ALT was increased in all chronic EtOH-treated groups. While, the plasma ALT of GLP group was significantly restored compared with control group.
Data were expressed as means ± SE.
* : $p < 0.05$ compared to control group.

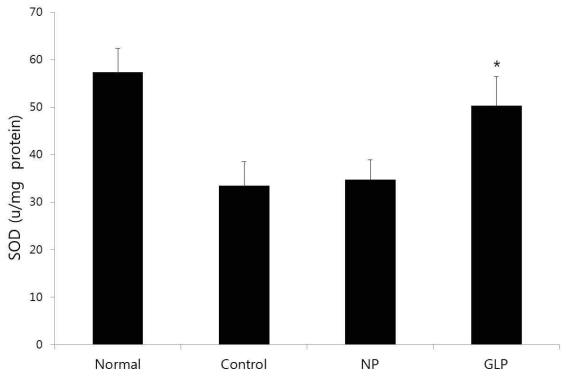

Fig. 5. Total SOD activities in the chronic liver injury

SOD activity was decreased in all EtOH-treated groups. While, SOD activity was significantly higher in GLP group. No significant difference was found in SOD activity between control and NP group.
Data were expressed as means ± SE.
* : $p < 0.05$ compared to control group.

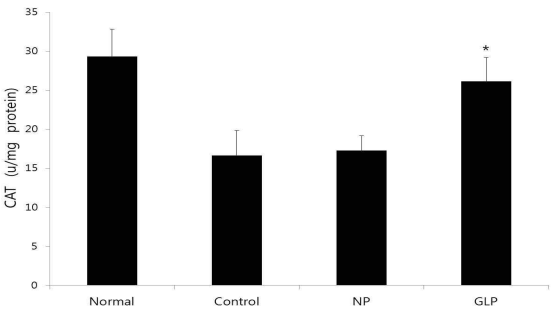

Fig. 6. Total CAT activities in the chronic liver injury

The CAT activity was decreased in all EtOH-treated groups. No significant difference was found in CAT activity between control and NP group. Whereas, CAT activity of GLP group was significantly recovered compared to control group.
Data were expressed as means ± SE.
* : $p < 0.05$ compared to control group.

hepatic antioxidant enzyme activities as SOD, CAT. GLP significantly ameliorated SOD and CAT activity. The treatment with *Ganoderma lucidum* pharmacopuncture significantly reversed all EtOH-induced decrease in antioxidant enzyme activities of SOD, CAT(Fig. 5, 6).

E. BAX & Bcl-2

To investigate the role of GLP to mitochondrial regulation in EtOH-induced chronic hepatocyte apoptosis, we carried out an immunohistochemical analysis of BAX and Bcl-2. GLP significantly decreased Bax expression compared with control group. Moreover, GLP significantly increased Bcl-2 expression compared with the control group(Fig. 7, 8).

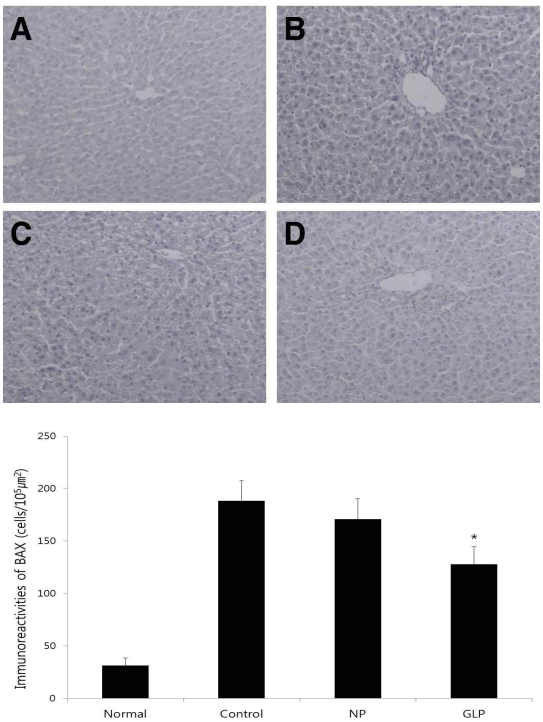

Fig. 7. Immunohistochemical staining of BAX proteins in EtOH induced chronic liver injury

The increased level of BAX protein was observed in the control group(B).
Whereas, the expressions of BAX protein in NP(C) and GLP(D) group were decreased compared with control group.
The reduction of BAX immunoreactivity was significant in GLP group(D).
Magnification : 100 ×.
* : $p < 0.05$ compared to control group.

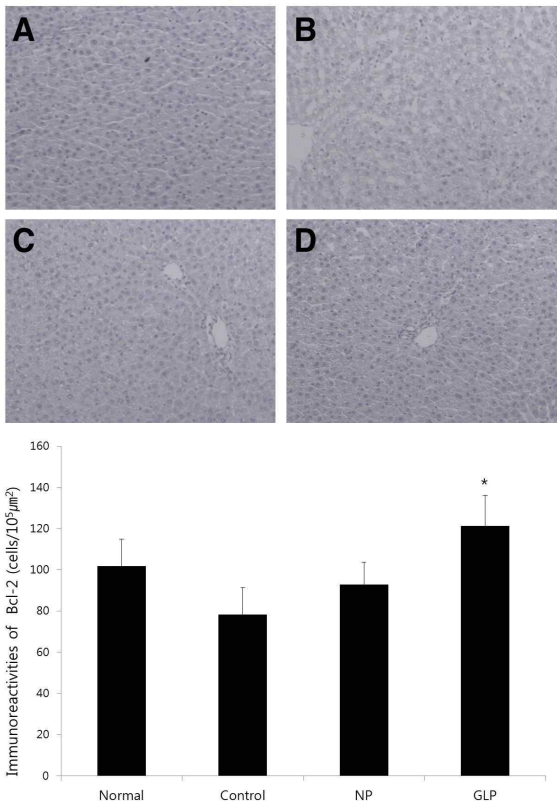

Fig. 8. Immunohistochemical staining of Bcl-2 proteins in EtOH induced chronic liver injury
Bcl-2 expressions of control(B) and NP(C) group were weakly observed and the expressions of Bcl-2 protein in GLP group(D) were significantly increased compared with control group.
Magnification : 100 ×.
* : $p < 0.05$ compared to control group.

Ⅳ. Discussion

Much progress has been made in understanding the pathogenesis of ALD, but there remains no effective therapy for it. Novel therapeutic targets that successfully correct the fundamental cellular disturbances resulting from excessive alcohol consumption are attractive. Accumulated evidence indicates that oxidative stress and steatosis are main pathological factors in the development of ALD[17,18].

In addition to pro-oxidants formation, antioxidants depletion caused by ethanol administration also results in oxidative stress[19]. Another major consequence of ethanol metabolism is lipid accumulation in liver. Ethanol metabolism changes the NAD/NADH ratio, which has important consequences on fuel utilization in the liver, favoring the synthesis of fatty acids and inhibiting the oxidation of fatty acid[20].

Herbal medicines have long been used as therapy of liver injury. And many herbal medicines are now being collected and examined in an attempt to identify possible sources of anti-liver injury. Natural compounds, because of their structural diversity, provide a good opportunity for screening for anti-liver injury agents[21].

In general, pharmacopuncture treatment is performed by injecting small amounts of extracted medicinal materials at acupuncture points or in affected areas in order to obtain combined efficacies of the acupuncture and herb. Although the first primitive trials with bee venom or herbal extractions were recorded in old medical books from the Han dynasty of China, acupuncture with injection started in the early 1950's in China and was referred to aqua-puncture. Recently, pharmacopuncture therapies in Korea have developed into quite a unique and systematic framework for the diagnosis and treatment of various diseases[22].

Many studies indicated that mushrooms are proving to be novel and rich sources of bioactive compounds. Among them, *Ganoderma lucidum*(靈芝) is a polypore mushroom that grows on the lower trunks of deciduous trees. This mushroom, as a traditional oriental medicine, has been widely used as a tonic in promoting longevity and health for thousands of years in Asian countries including China, Japan and Korea[23].

The pharmacological activities of *Ganoderma lucidum,* especially its intrinsic immunomodulating, and anti-tumor properties, have been well documented extensively[24]. *Ganoderma lucidum* has been shown to have anti-cancer, immunomodulatory and immunotherapeutic effects[14]. It was also revealed that *Ganoderma lucidum* induces hepatoprotective effects on acute liver injury[25]. In previous study we revealed the hepatoprotective effect of GLP on acute ethanol induced acute liver injury[26].

We chose *Qimen*(LR_{14}) and *Taechuang*(LR_3). *Qimen*

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(LR_{14}) can pacify the liver and regulate qi, so treat hepatitis, cholelithiasis, cholesystitis etc. *Taechuang* (LR_3) can soothe the liver and regulate qi, so treat liver function disorder, jaundice etc[27].

In the present study, we dealt with the protective effect of GLP on chronic EtOH-induced oxidative stress in rat liver, so added the evidence of GLP in alcoholic liver injury for the purpose of developing GLP as treatment method for ALD.

Chronic alcohol ingestion is known to be associated with defective gut motility that indirectly results in an elevated level of endotoxin in the liver[28]. Furthermore, the major metabolic product of alcohol, acetaldehyde activates hepatic stellate cells(HSCs), triggering inflammatory and fibrogenic signals, such as tumor necrosis factor(TNF), interleukins-6(IL-6) and transforming growth factor-1(TGF-1)[29]. Numerous strategies have been employed to develop antifibrotic therapies, including inhibition of hepatic stellate cells[30], interference in the secretion of extracellular matrix(ECM) and cytokines[31], and prevention of oxidation through use of antioxidants[32].

Apoptosis is the mechanism responsible for the physiological deletion of cells and appears to be intrinsically programed. This mode of programed cell death serves to balance mitosis in regulating animal development and tissue homeostasis, as well as mediate pathologic processes[33].

EtOH-induced liver injury plays a central role in the apoptosis of the cell death[34]. Apoptotic cell death induced as a consequence of EtOH metabolism is dependent on oxidative stress. Ethanol consumption can lead to cell apoptosis in various tissues, ranging from liver, heart, spleen, stomach to brain[35], which has been confirmed in rats, mouse and human experiments. Therefore, alcoholic liver injury was prevented and cured by way of apoptotic mechanisms, and ultimately can be effectually improved. Recently, studies focused on mitochondrial injury occurring in connection with cell apoptosis are thought to be responsible for the development of alcoholic liver injury. Oxidation of acetaldehyde to acetate is achieved in the mitochondria by aldehyde dehydrogenase (ALDH), but excessive production of acetaldehyde via hepatic ADH and/or CYP2E1 generates ROS, which results in a deficiency in mitochondrial respiratory chain components and ATP production, GSH depletion and enhanced peroxidation of lipids, proteins and DNA[36] and eventually mitochondrial damage. Mitochondria play a central role in the regulation of cellular apoptosis through release of proteins into the surrounding cytosolic environment. In brief, oxidative stress, endoplasmic reticulum stress, mitochondrial damage and mitochondrial damage mediated endogenous pathway are involved in the occurrence and development of hepatocyte apoptosis in the mechanism of EtOH-induced liver injury. Animal experiments have shown that chronic ethanol treatment resulted in significant increases of the hepatocyte apoptosis[37].

EtOH-associated oxidative stress induces BAX transmigration into the mitochondria and demonstrated that ethanol administration leads to an increase in caspase-3 activity and that the induction of apoptosis was found to be linked to the metabolism of alcohol[38]. It was demonstrated that chronic alcohol exposure resulted in increased hepatocellular immunoreactivity for caspase-3, but there has been no report of acute alcohol exposure effect on caspase-3 immunoreactivity[39]. Bcl-2 is a mitochondrial membrane protein that prevents apoptotic cell death. It has been reported that the increased anti-apoptotic Bcl-2 expression reflects an adaptive response to alcohol related stress[40].

In the present study, to investigate the role of GLP to mitochondrial regulation in EtOH-induced chronic hepatocyte apoptosis, we carried out an immunohistochemical analysis of BAX and Bcl-2. As a results, it demonstrated that Bcl-2 expression was decreased in alcohol-induced liver injury[41,42]. Similarly, we found that Bcl-2 expression was decreased in chronic alcohol-induced liver injury in rats. But GLP decreased BAX immunoreactivity in chronic alcohol exposure for 4 weeks. Moreover, the treatment with GLP significantly increased Bcl-2 expression compared with the control group. In other words, GLP showed not only up-regulated the levels of Bcl-2 but also down-regulated the levels of BAX compared with ethanol-treated group.

In summary, these apoptotic changes are inhibited by the addition of antioxidants, GLP, suggesting that oxidative stress is involved in the release of cytochrome c that precedes apoptosis in hepatocytes exposed to ethanol. Therefore, GLP suppressed apoptosis by regulating mitochondrial damage-mediated endogenous pathway which could be one of important mechanisms of preventing alcoholic liver injury.

In summary, this study showed the protective efficacy of GLP against EtOH-induced chronic liver injury in SD rats by modulating ethanol metabolizing enzymes activity, attenuating oxidative stress, and inhibiting mitochondrial damage-mediated apoptosis. In the future, we hope the clinical study of GLP on ALD.

Ⅴ. Conclusion

1. GLP reduced the histological changes induced by EtOH treatment in chronic liver injury.
2. Both AST and ALT enzymes of GLP group was significantly reduced in chronic liver injury.
3. GLP significantly reversed all EtOH-induced decrease of antioxidant SOD and CAT enzyme activities in chronic injury.
4. GLP significantly decreased BAX and increased Bcl-2 immunoreactivity expression in chronic alcohol exposure.

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 http://dx.doi.org/10.13045/acupunct.2015002