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Role of Alcohol in the Induction of Hepatic Injury by Chronic Consumption of Medicated Wine

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ABSTRACT

Kinmen Benefit Life medicated wine (KBLMW), a medicated wine with unified formulas proclaimed by the Committee on Chinese Medicine and Pharmacy (Department of Health, Executive Yuan, Taiwan), was made of 17 kinds of Chinese medicinal materials soaked in 25% alcohol. KBLMW has been widely used as conventional or complementary medicines for health regimen. In the present study, Wistar rats were administered once daily via gastric tube with KBLMW, de-alcoholic KBLMW (0% alcohol/vol) or 25% alcohol at 2.08 mL/kg/day for 12 consecutive weeks, to clarify the role of alcohol in the induction of hepatic disorders by chronic consumption of KBLMW at the recommended intake amount. At the end of this treatment, the liver fibrosis in rats receiving 25% alcohol was found, featuring increase in serum levels of alanine transaminase and aspartate aminotransferase, as well as hepatic hydroxyproline contents. Comparing with the normal group, values of these serum indexes in KBLMW-fed rats elevated moderately but lower than those of 25% alcohol-fed rats. Also, the stage of liver fibrosis in KBLMW-treated rats was less advanced than that in 25% alcohol-treated group. In addition, the liver function parameters in rats receiving de-alcoholic KBLMW were close to those of normal group and liver fibrosis was not observed. The results suggest that alcohol is related to the induction of hepatic injury by chronic consumption of medicated wine even at the recommended daily intake amount. This finding can be used as the safety reference guide for clinical medicated wine drinking.

Key words: medicated wine, alcohol, Chinese medicinal materials, liver fibrosis

INTRODUCTION

Chinese medicine has been applied to medicine for centuries and is now being gradually accepted worldwide. In the West, people tend to take a single herb in therapies and try to identify the active ingredients and therapeutic mechanisms, whereas in Chinese medicine it is common to use mixtures of various medicinal herbs, which contain a range of pharmacologically active ingredients working additively/synergistically, to treat patients or to reduce harmful side effects of some chemical compounds[1,2]. Among them, medicated wine, which is usually made by soaking the fresh or dried Chinese medicinal materials, in solutions containing more than 20% of alcohol, has been used as conventional or complementary medicines for health regimen as well as disease prevention. Actually, chronic alcohol consumption leads to several metabolic disorders including hepatic diseases[3], which are initiated by reactive oxygen species generation and lipid peroxidation as found in liver, leading to cellular damage[4]. Thus, heavy drinkers and alcoholics tend to have liver cell damage and cirrhosis, and some may eventually develop hepatocellular carcinoma, which is unfortunately, and very often, a fatal malignancy without cure[5]. Unlike the case of heavy alcohol abuse, the safety evaluation for chronic consumption of medicated wine is not yet fully understood.

Kinmen Benefit Life medicated wine (KBLMW), one kind of the unified formulas of medicated wine proclaimed by the Committee on Chinese Medicine and Pharmacy, Department of Health, Executive Yuan, Taiwan, is made of 17 kinds of Chinese medicinal materials soaked in 25% alcohol (Table 1). The prescription is commonly used to increase blood circulation to relieve stasis, strengthening "spleen" as well as supplementing "qi" to reinforce body's immunological function. The recommended intake amount of medicated wine for people is usually 10~20 mL per administration, 2 or 3 times daily. Thus, this formula was used in this study to evaluate the safety for chronic administration of medicated wine at the recommended daily intake amount through examination of liver function and histopathology, in an effort to reveal the role of alcohol and formula in the

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Table 1. The Chinese medicinal materials contained in Kinmen benefit life medicated wine

Pharmaceutical name	Composition (mg)
Equus Asinus Colla Corii	2.6
Hominis Placenta	5.2
Chinemys Reevesii Gelatinum Plastrum	2.6
Gekko Gecko	5.2
Cervi Nippon Colla Cornu (Gelatin)	2.6
Rubus Chingii Fructus	2.6
Hippocampus Histrix	10.4
Ligustrum Lucidum Fructus	5.2
Polygonatum Sibiricum Rhizoma	5.2
Rehmannia Glutinosa Radix Conquita	5.2
Panax Ginseng Radix	0.5
Cynomorium Songaricum Herba	5.2
Cinnamomum Cassia Cortex	0.5
Citrus Reticulata Pericarpium	1.6
Cervus Pantotrichum Comu	1.0
Lycium Barbarum Fructus	5.2
Angelica Sinensis Radix	5.2

Mixture of Chinese medicinal materials was soaked with 1 mL of 25% alcohol following the principals of Chinese medicated wine from Committee on Chinese Medicine and Pharmacy.

pathogenesis of alcoholic liver disease.

MATERIALS AND METHODS

I. *Materials*

KBLMW was made of authentic Chinese medicinal materials of highest qualities soaked in 25% alcohol following the principals of Chinese medicated wine by Committee on Chinese Medicine and Pharmacy at Han Sheng Pharmtech, Inc. (Pingtung City, Pingtung County, Taiwan) under internationally certified Good Manufacturing Practices guidelines. The experienced botanists and chemists in the supplier performed macroscopic and microscopic examinations as well as thin-layer chromatography and high-performance liquid chromatography to authenticate the plants, plant parts used, and processed raw herbs. The reference specimens were deposited at the herbarium of supplier. De-alcoholic KBLMW (0% alcohol/vol), the product including Chinese medicinal materials of KBLMW but not alcohol, was obtained from KBLMW dealcoholized at Han Sheng Pharmtech, Inc. by vacuum distillation. Standard rat chow was from Purina Mills, LLC (St. Louis, Missouri, USA). Diagnostic kit for the determination of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were purchased from Roche Diagnostics Corp. (Indianapolis, Indiana, USA). Chloramines T solution was from Sigma-Aldrich, Inc. (St. Louis, Missouri, USA). The kit for protein assay was from Bio-Rad Laboratories (CA, USA). All chemicals were of analytical grade.

II. *Animals and Protocol*

Male Wistar rats aged 8 weeks were obtained from the Animal Center of the Medical College, National Cheng Kung University. They were kept in a temperature-controlled room (25 ± 1°C) and on a 12:12 light-dark cycle (light on at 06:00 AM). After normal feeding for a week, these rats were randomly assigned into four groups: (1) normal group (n = 10), (2) KBLMW-fed group (n = 10), (3) de-alcoholic KBLMW-fed group (n = 10), and (4) 25% alcohol-fed group (n = 10). A metabolism coefficient of 6.25 was employed to convert the recommended daily intake amount of KBLMW for people (20 mL/day) into rats, assuming that average body weight of an adult is 60 kg[5]. All animals were administered once daily via gastric tube. KBLMW-fed group was given KBLMW at 2.08 mL/kg/day, de-alcoholic KBLMW-fed group was fed with de-alcoholic KBLMW prepared in distilled water at 2.08 mL/kg/day, rats given 25% alcohol at 2.08 mL/kg/day was the alcoholic liver disease model group, and the normal control group were given an equal volume of distilled water at 2.08 mL/kg/day. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act. At the end of 12-week treatment, all the rats were anaesthetized and sacrificed. Blood sample and liver tissue specimens were collected. Plasma was separated by centrifugation at 4°C and kept at -20°C for assay. Liver tissue was fixed for histopathology.

III. *Serum ALT and AST Assay*

ALT and AST were evaluated in samples of serum obtained at the end of treatment. The activity was determined spectrophometrically with an automatic analyzer (HG-310, HITACHI, Japan) using commercially available kits according to instructions.

III. *Collagen Measurement by Hydroxyproline Determination in Liver Tissues*

Hydroxyproline assays were performed as previously described with slight modification[6]. Liver tissue (0.3 g) was homogenized in 6 N HCl and hydrolyzed at 110°C for 18 hr. Aliquots (25 μL) were dried at 60°C. The sediment was dissolved in 1.2 mL of 50% isopropanol and incubated with 200 mL of 0.56% chloramines T solution in acetate citrate buffer (pH 6.0). After incubation for 10 min at room temperature, 1 mL of Ehrlich's reagent was added and the mixture was incubated at 50°C for 90 min. After cooling, the absorbance was measured at 558 nm (BECMAN,

DU530, USA). Hepatic protein concentration was determined by the BioRad protein dye binding assay. The amount of hydroxyproline was expressed as μg/mg protein.

IV. *Pathological Evaluation*

Liver specimens were preserved in 4% buffered paraformaldehyde, dehydrated in a graded alcohol series, embedded in paraffin and cut into 5 μm thick sections which were placed on plain glass slides. The paraffin-embedded tissue was sectioned for staining with hematoxylin-eosin and Masson trichome. Fibrosis was graded according to the previous method as follows[7]: grade 0 = normal liver, grade 1 = increase of collagen without formation of septa, grade 2 = formation of incomplete septa from portal tract to central vein (septa that do not interconnect with each other), grade 3 = complete but thin septa interconnecting with each other, so as to divide the parenchyma into separate fragments, and grade 4 = as grade 3, except with thick septa (complete cirrhosis). To avoid sampling error, all biopsies were obtained from the same liver lobe and these semi-quantitative grading were performed by the observer without knowledge of sample treatment under a light microscope.

V. *Statistical Analysis*

Data are expressed as the mean ± SEM for each group of animals in Tables. Statistical differences among groups were determined by using two-way repeated-measures ANOVA. The Dunnett range post-hoc comparisons were used to determine the source of significant differences where appropriate. A P-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

I. *Rat Condition*

During the first week of the experimental period, when the rats were given 25% alcohol, they became excited and ran around the cage. After that they could not walk, and at last fell into a sound sleep, while there was minor syndromes in KBLMW-fed groups. Actually, the behavior in rats fed with 25% alcohol did not show in de-alcoholic KBLMW-fed group. It seems that the chance to produce acute toxicity is very high in individual even taking alcoholic solution at micro- to moderate amount, but Chinese medicinal materials in KBLMW posses the ability to alleviate the toxicity.

As experiment proceeded to 10 weeks, spoor time changed from 1~2 hr to about 5 hr in 25% alcohol-fed rat. Similar to the previous study[8], the body weight gain was significantly lower in the rats fed 25% alcohol at 10th week than the other groups for the same period. Furthermore, the body weight of 25% alcohol-fed rats fell obviously and maintained cachexia at the 12th week as compared to that of KBLMW-fed group. However, these metabolic derangements did not appear in rats receiving 12-week of de-alcoholic KBLMW administration. Although the concentration of alcohol is the same, the chance for chronic toxicity induction in 25% alcohol-fed rats is higher than that in KBLMW. Thus, the potential clinical therapeutic value for individuals with alcoholic liver injury warrants further attention.

II. *Serum Levels of ALT and AST*

Serum levels of ALT and AST are indexes to describe liver functions. Most of ALT is present in the cytoplasm of liver cell and will be discharged into blood when degeneration, hyperpermeability and necrosis of liver cells occur. The increase of ALT level in serum thus reflects the degree of liver cell injury[9]. Indeed, it has been shown that the activities of AST and ALT were significantly raised in rats fed with alcohol for 10 week[8]. As shown in Table 2, rats receiving a 12-week of 25% alcohol administration has a marked increase in serum levels of ALT and AST, as compared to those in normal group. After 12 weeks of KBLMW consumption, all the indexes in rats were elevated as compared to those of normal group, but the values were still lower than those of 25% alcohol-fed rats relatively. It was noteworthy that each index in de-alcoholic

Table 2. Changes in the level of serum ALT, AST and hepatic hydroxyproline in rats receiving a 12-week treatment with Kinmen Benefit Life medicated wine (KBLMW)

Group	ALT (U/L)		AST (U/L)		Hydroxyproline (μg/mg protein)	
	Before	After	Before	After	Before	After
Normal	48.3 ± 4.7^{d}	50.9 ± 6.4^{d}	158.7 ± 7.8^{d}	159.2 ± 9.7^{d}	9.7 ± 3.1^{d}	9.9 ± 3.7^{d}
25% alcohol	49.2 ± 6.1^{d}	276.3 ± 15.3^{b}	159.1 ± 9.2^{d}	512.3 ± 16.7^{b}	9.8 ± 3.4^{d}	27.9 ± 4.2^{b}
KBLMW	49.4 ± 5.6^{d}	$192.8 \pm 10.1^{b,c}$	157.9 ± 7.4^{d}	$329.4 \pm 14.3^{b,c}$	9.8 ± 2.6^{d}	$16.1 \pm 3.5^{a,c}$
De-alcoholic KBLMW	48.7 ± 4.5^{d}	51.6 ± 7.2^{d}	158.2 ± 8.3^{d}	161.5 ± 10.2^{d}	9.9 ± 2.8^{d}	10.3 ± 2.9^{d}

All animals were administered with the indicated prescription at 2.08 mL/kg/day once daily via gastric tube for 12 consecutive weeks. Values (mean ± SEM) were obtained for each group of 10 animals. Rats received distilled water treatment as the same manner was taken as normal control. $^{a}P < 0.05$ and $^{b}P < 0.01$ represents the level of significance compared to the values of normal control before treatment, respectively. $^{c}P < 0.05$ and $^{d}P < 0.01$ compared to the values of 25% alcohol-treated rats at the end of treatment, respectively.

KBLMW-fed group was the same as that in normal group, indicating the Chinese medicine have hepatoprotective activities against alcoholic toxicity.

II. *Hydroxyproline Content in Liver Tissues*

The excessive accumulation of extracellular matrix proteins, including collagen, occurs in chronic liver diseases[10]. Hydroxyproline, a major component of the protein collagen, is produced by hydroxylation of proline, and helps provide stability to the triple-helical structure of collagen by forming hydrogen bonds[11]. The alteration of hydroxyproline levels in the liver is considered as an index of collagen metabolism and provides valuable information about the biochemical and pathologic events of hepatic fibrosis[12]. Therefore the hydroxyproline assay was used as a method for collagen measure.

In this study, we observed that liver hydroxyproline level in 25% alcohol-fed rats was much higher than those of the normal controls at the end of a 12-week treatment (Table 2). Liver hydroxyproline level in KBLMW-fed group was also higher than that of normal group, but the value was significantly lower than that of 25% alcohol-fed rats (Table 2). Interestingly, the liver hydroxyproline level of rats receiving a 12-week treatment with de-alcoholic KBLMW has no significant difference from the control group (Table 2). Thus, Chinese medicinal materials in KBLMW possibly displayed their hepatoprotective effects through their effects against hydroxyproline formation.

III. *Histological Changes under an Optical Microscope*

When the liver is damaged, it can initiate regenerative actions[13], thus increasing the weight of liver. If it is heavily damaged, however, liver fibrosis and cirrhosis can result in liver atrophy[14]. Therefore, the change in weight of liver cannot predict directly the pathological processes in chronic liver injuries. Histopathologic examination was thus performed to prove that the fibrosis was caused by alcohol but not the Chinese medicinal materials of KBLMW.

At the end of treatment, the liver of control rats had no appreciable alterations (Figure 1A, Table 3). In 25% alcohol-fed rats group, the margin of liver was uneven with more fibrous tissues formed and extended into the hepatic lobules to separate them incompletely and a large amount of inflammatory cells infiltrated in the intralobular and the interlobular regions. The liver structure was disordered with some displacement of central veins, and there were more necrotic and degenerated liver cells as compared with the control (Figure 1B, Table 3). In KBLMW-treated groups, the pathological changes of liver was rather milder, showing less fibrous tissue proliferation and inflammatory cell infiltration in the interlobular space, where only micro- and moderate steatosis were found (Figure 1C, Table 3). As expected, steatosis could nearly not be seen in de-alcoholic KBLMW -treated groups (Figure 1D, Table 3).

According to traditional Chinese medicine theory, "alcohol is pungent and hot". It has been documented that dampness, heat and gore are the major pathogenesis of alcoholic liver disease. In regard to theories, most components in medicated wine can invigorate blood circulation, remove blood stasis, cool the blood, treat carbuncles and tranquilize the disturbed mind by nourishing the blood. It could be considered that Chinese medicinal materials with the unique function in clearing dampness and heat of liver to eliminate the gore of the blood might have hepatoprotective property. Actually, drugs in KBLMW can also expel pathogenic factors from the exterior to reduce fever, sooth the depressed liver and invigorate the spleen-yang; thus, it is reasonable that hepatic injury was not observed in de-alcoholic KBLMW groups at the end of 12-week treatment. The results definitely demonstrate that even at the recommended daily intake amount, alcohol must be of pathophysiological importance in the process of hepatic injury in person with the habit of drinking medicated wine.

Actually, alcohol is essential for the production of Chinese medicinal materials since it is the best solvent to extract and concentrate the active ingredients, to preservative the active constituents of most Chinese medicinal materials. Therefore, not only the recommended daily intake of medicated wine, but also the content of alcohol

Table 3. Pathological observation of liver condition in rats receiving a 12-week treatment with Kinmen Benefit Life medicated wine (KBLMW)

Group	Score of hepatic fibrosis					Average
	0	1	2	3	4	
Normal control	10	0	0	0	0	0^{d}
25% alcohol	0	0	0	3	7	3.7 ± 0.5^{b}
KBLMW	0	1	6	3	0	$2.2 \pm 0.3^{a,c}$
De-alcoholic KBLMW	10	0	0	0	0	0^{d}

All animals were administered with the indicated prescription at 2.08 mL/kg/day once daily via gastric tube for 12 consecutive weeks. Values (mean ± SEM) were obtained for each group of 10 animals. Rats received distilled water treatment as the same manner was taken as normal control. $^{a}P < 0.05$ and $^{b}P < 0.01$ represents the level of significance compared to the values of normal control, respectively. $^{c}P < 0.05$ and $^{d}P < 0.01$ compared to the values of 25% alcohol-treated rats, respectively.

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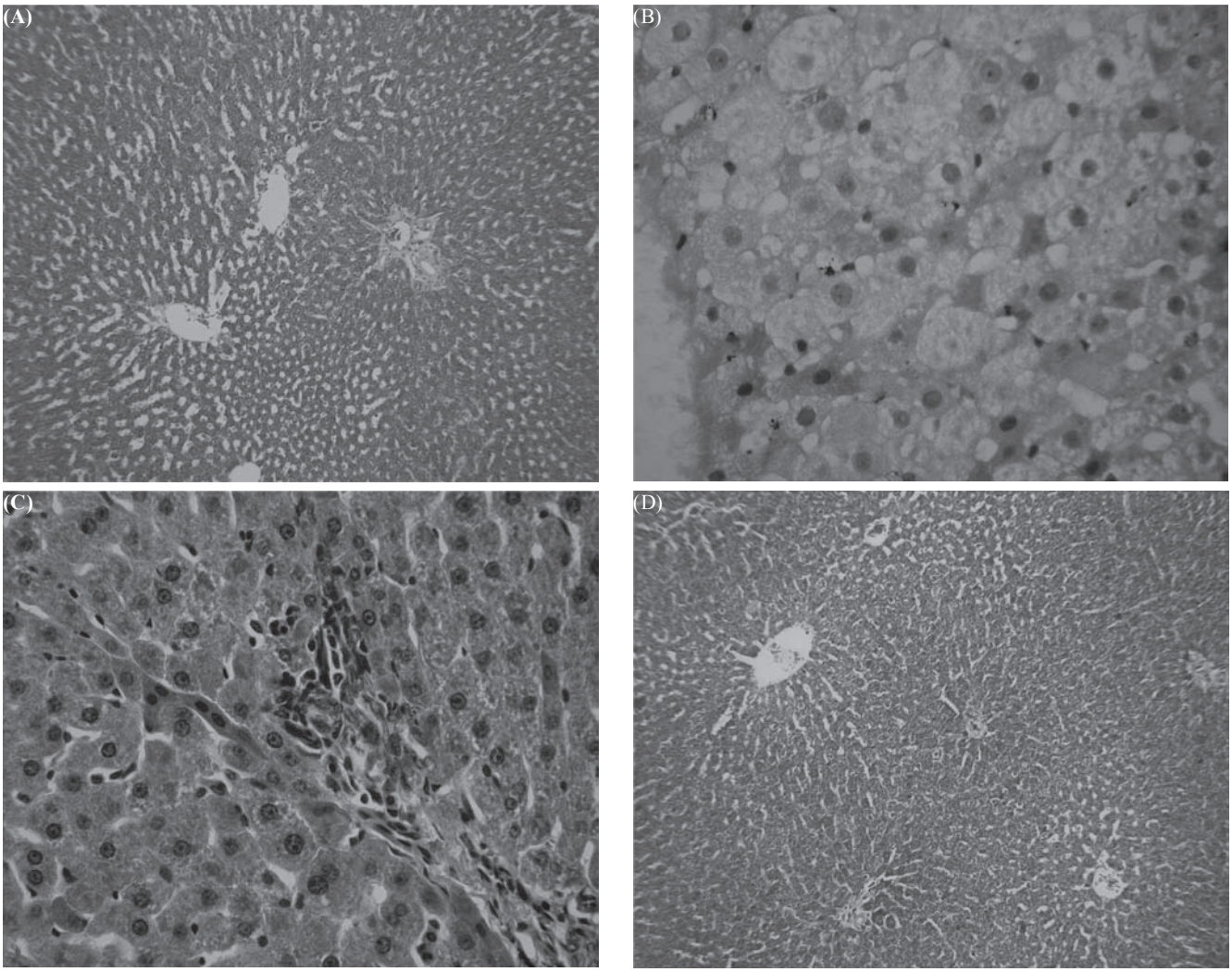

Figure 1. Analysis of liver pathology of each group under light microscope (original magnification, ×200). All animals were administered with the indicated prescription at 2.08 mL/kg/day once daily via gastric tube for 12 consecutive weeks. (A) normal control group; (B) 25% alcohol-fed group; (C) KBLMW-fed group; (D) de-alcoholic KBLMW-fed group.

in the preparation of medicine, needs to be modified in order to preserve the benefit in health regimen and to avoid liver injury as possible. In addition, the real mechanism of alcohol-induced hepatic cells injury is still not completely clear. Some researchers found that alcohol could activate lipid peroxidation, leading to liver injury[4,8]. Among the components of KBLMW, Lycium barbarum fructus is well known for nourishing the liver, and in turn, improving the eyesight[15]. It has also been reported that ginsenoside Rb1, one of the active compounds of Panax ginseng, has hepatoprotective effect on tert-butyl hydroperoxide-induced liver injury[16]. Whether the cytoprotective effects of Chinese medicinal materials in KBLMW mainly attributable to antioxidant and free radical scavenging properties remained to be clarified.

Although Chinese medicinal materials has been employed in the treatment of liver diseases[17,18], hepatic impairment resulting from the use of conventional drugs is widely acknowledged[19]. In fact, there is little awareness of the potential hepatotoxicity of herbal preparations and other botanicals, many of which are believed to be harmless and are commonly used for self-medication without supervision. Research is needed to further demonstrate the safety and efficacy of the medicated wine.

CONCLUSIONS

The obtained data showed that alcohol is related to the induction of hepatic injury due to chronic consumption of medicated wine even at the recommended daily intake dose. It can be considered as the reference of safe strategy for the application of medicated wine in clinic.

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