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Hepatoprotective effect of Gan Kang Yuan against chronic liver injury induced by alcohol

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ABSTRACT

Ethnopharmacological relevance: Gan Kang Yuan (GKY) is a compound medicine formulated on the basis of traditional Chinese medicine (TCM). It was composed of *Herba Cistanchis* (Roucongrong), *Radix Puerariae* (Gegen), *Radix Astragali* (Huangqi), *Fructus Schisandrae* (Wuweizi) and *Radix Glycyrrhizae* (Gancao).

Aim: The purpose of this study is to research the hepatoprotective effect of GKY against liver injury induced by alcohol, and to elucidate the mechanism of hepatopretective effect.

Materials and methods: Hepatoprotective activity of GKY was researched both in vivo and vitro. In vitro, effect of GKY on the survival rates of HepG2 cells were assessed. In vivo research, ICR mice were oral administrated with alcohol (Er Guo-tou white spirit, 56%, 6 mL/kg, once per day) for 31 days to establish liver injury model. Meanwhile, positive group or experimental groups were treated with bicyclol (300 mg/kg) or GKY (200, 600, 1800 mg/kg). Serological indexes including aspartate and alanine transaminases (AST, ALT), γ-glutamyl transpeptidase (γ-GTP), total bilirubin (TBil), total cholesterol (TCHO) and serum triglyceride (STG) were estimated. Hepatic indicators including superoxide dismutase (SOD), glutathione S-transferase (GST), glutathione (GSH), glutathione peroxidase (GSH-Px), malondialdehyde (MDA), and liver triglyceride (LTG) were analyzed. Histopathologic changes of liver tissue were observed.

Results: The survival rates of HepG2 cell were observably promoted by GKY. Alcoholic treatment drastically altered the serum indexes and liver indicators of model animals, while these alteration were significantly ameliorated by GKY ($p < 0.05$, 0.01 or 0.001) in experimental group. The microvesicular steatosis and necrosis in hepatic histopathology induced by alcoholic treatment also were notably attenuated by GKY administration.

Conclusions: These findings indicated that GKY possessed hepatoprotective property against liver injury induced by ethanol. GKY significantly promoted activities of relative enzymes and suppressed the contents of MDA and LTG, which might be the mechanism of hepatoprotective effect of GKY.

1. Introduction

Alcohol is a worldwide consumed beverage and food additive. Each year, alcohol abuse contributes nearly 2.5 million deaths (Rehm et al., 2013; Vasiljevik et al., 2013). In human body, alcohol is metabolized in liver. Thus, liver is an organ susceptible to alcohol (Nieto, 2012). Pathological evidences revealed that excessive drinking is one of leading causes for the hepatic disease and many other diseases (Edenberg and Foroud, 2013; Gao and Bataller, 2011). Alcohol induced chronic liver injury is a multistep progression, usually develops from alcoholic steatosis to alcoholic hepatitis, and finally deteriorate to alcoholic cirrhosis (Seth et al., 2008). Despite the modern medical science has been highly developed, chronic liver injury induced by alcohol is still a worldwide healthy problem. Thus, finding effective natural medicine for those alcohol consumers to prevent or slow down the progression of alcoholic liver injury will be a beneficial strategy.

Traditional Chinese medicine (TCM) is a medical practice system, which played an irreplaceable role in the Chinese health care system

Abbreviations: ALT, alanine aminotransaminase; AST, aspartate aminotransferase; DMSO, Dimethyl sulfoxide; FBS, fetal bovine serum; GKY, Gan Kang Yuan; GSH, glutathione; GSH-Px, glutathione peroxidase; GST, Glutathione S-transferase; LTG, serum triglyceride; MDA, malondialdehyde; MTT, 3-4, 5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide; γ-GTP, γ-glutamyl transpeptidase; PhGs, phenylethanoid glycosides; SOD, superoxide dismutase; STG, serum triglyceride; TBil, total bilirubin; TCHO, total cholesterol; TCM, traditional Chinese medicine

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(Cao et al., 2015; Cheung, 2011). Actually, compound medicine (prescription or fufang in Chinese) is the most widely used clinical practice of TCM. In compound medicine, several botanical, animal or mineral medicines were formulated on the basis of TCM prescription (Lin et al., 2012). A special therapy of quaternity, which was named monarch, minister, assistant and guide (Jun, Chen, Zuo and Shi in Chinese), was highly respected by TCM formulation (Yan, 2013). Briefly, monarch medicine plays a major therapeutic role in the treatment of the main symptom or disease. Minister medicine is used for either strengthening the efficiency of monarch medicine in treatment of main symptom, or curing the accompanying symptoms. Assistant medicine is used for strengthening the therapeutic effect of monarch or minister medicine, treating the secondary accompanying symptoms, or eliminating the toxicity of the monarch and minister drug. The guide medicine is used to reconcile the effects of various medicine (Zhang et al., 1992). Sheng Nong's herbal classic (Sheng Nong Beng Cao Jing) recorded that a basic property of monarch medicine should be no side effects for long term usage (Shang, 2008).

TCM believed that the liver and kidney were consanguineous, liver injury always induced kidney deficiency. Cistanchis pill (Congrong Wan), a famous TMC formula recorded in many classical medicine books, was used for nourishing liver and kidney (Zhu et al., 1959; Sun et al., 1955; Jiao et al., 1962a, 1962b; Jin et al., 1981). The composition of Cistanchis pills showed little change in different prescription, but the Herba Cistanchis (Roucongrong), Radix Astragali (Huangqi), Fructus Schisandrae (Wuweizi) were the basic component of the formula. Herba Cistanchis, the stems of *Cistanche deserticola* Y. C. Ma, were commonly used in TCM (Chinese Pharmacopoeia Commission, 2010a). Herba Cistanchis is beneficial for the kidney and liver, mild and invigorate medicine, no side effects for long term usage (Ni et al., 2005). The modern pharmaceutical research express that it has hepatoprotective activity (Xiong et al., 1999, 1998). Radix Astragali, the roots of *Astragalus membranaceus* (Fisch.) bunge, can be used to treat the alcoholic macula and creeping chill after drunk (Li et al., 2006a). Published reports claimed that Radix Astragali possessed hepatoprotective effects (Chu et al., 2010; Sun et al., 2012). Fructus Schisandrae, the ripe fruit of *Schisandra chinensis* (Turcz.) Baill., has five flavor, salty and sour in flavor, attributive to the liver and kidney meridians; pungent and bitter in flavor, attributive to the heart and lung meridians; sweet in flavor, attributive to the spleen and stomach meridians. It also can be used for detoxify the alcoholism (Li et al., 2006b; Liu et al., 2009). The modern pharmaceutical research showed that Fructus Schisandrae has hepatoprotective ability (Li et al., 2016; Sung et al., 2014; Wang et al., 2014). Radix Puerariae pulvis (Gegen San) was an antialcoholismic formula, used for curing excessive drinking and hangover (Zhang et al., 1991). The fundamental component of the formula were Radix Puerariae (Gegen) and Radix Glycyrrhizae (Gancao). Radix Puerariae, the roots of *Pueraria lobata* (Willd.), can be used to treat drunken stupor and vomiting (Chen, 1995). It also reported that Radix Puerariae has the protective effect against the CCl_4-induced hepatotoxicity (Hwang et al., 2007). In order to develop an antialcoholismic and hepatoprotective medicine, we reconciled two prescriptions together to form a novel compound medicine (named as Gan-Kang-Yuan). GKY was composed of Herba Cistanchis, Radix Puerariae, Radix Astragali, Fructus Schisandrae and Radix Glycyrrhizae. In the formula of Gan-Kang-Yuan, Herba Cistanchis was mild and invigorate medicine, no side effects for long term usage (Ni et al., 2005). Thus the Herba Cistanchis was selected as monarch medicine. Radix Puerariae, Radix Astragali and Fructus Schisandrae were selected as the minister medicines. Radix Puerariae is cool in nature (Chinese Herbalism Editorial committee, 1999a). Radix Astragali and Fructus Schisandrae are all warm in nature. Radix Glycyrrhizae, the roots of *Glycyrrhiza uralensis* Fisch. or *G.* grabra L., possesses neutralization property and reconciliation effect (Chinese Herbalism Editorial committee, 1999b), thus it was selected as assistant and guide medicine. The present study will focuses on the investigation of the hepatoprotective effect of this formulated medicine (GKY) on the chronic liver injury induced by alcohol.

2. Materials and methods

2.1. Reagents and chemicals

The Chinese herbal medicine of Herba Cistanchis, Radix Puerariae, Radix Astragali, Fructus Schisandrae and Radix Glycyrrhizae were provided by Hong Kui Cong Rong Group (Alashan, Inner Mongolia), and identified by Prof. Xiaodong Wang (Division of Biorefinery Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing, China).

Assay kits including alanine aminotransaminase (ALT), aspartate aminotransferase (AST), glutathione (GSH), glutathione peroxidase (GSH-Px), glutathione S-transferase (GST), malondialdehyde (MDA), γ-glutamyl transpeptidase (γ-GTP), total bilirubin (TBil) and superoxide dismutase (SOD) were purchased from Nanjing Jiancheng Bioengineering Institute. Total cholesterol (TCHO), liver triglyceride (LTG) and serum triglyceride (STG) were purchased from BioSino Bio-Technology & Science Inc. Dimethyl sulfoxide (DMSO) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen, Inc. Er Guo-tou white spirit was purchased from Beijing Red Star Co., LTD. Bicyclol was purchased from Beijing Union Pharmaceutical Factory. Aqueous solutions were prepared with ultra-pure water from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other reagents were of analytical grade.

2.2. Preparation of GKY and determination of active components

The powder (40 mesh) of Herba Cistanchis (3 kg) was ultrasonically (40 kHz and 1 kW) extracted twice with 30 L of aqueous ethanol solution (50%, v/v) at 60 °C for 30 min. The powder (40 mesh) of Radix Puerariae (2 kg) was ultrasonically (40 kHz and 400 W) extracted twice with 20 L of aqueous ethanol solution (70%, v/v) at 50 °C for 30 min. The Radix Astragali (2 kg) and Fructus Schisandrae (2 kg) were extracted with the same method of Radix Puerariae. Radix Glycyrrhizae (1 kg) was ultrasonically (40 kHz and 1 kW) extracted twice with 10 L of ammonia solution (1%, v/v) at 60 °C for 30 min. The extracts concentrated under reduced pressure (vacuum degree is about −0.1 MPa) at 60 °C with a rotary evaporator. Then the concentrated solution was centrifuged, supernatant was spray dried. The five kinds of powder were mixed (exact weight ratio of Herba Cistanchis, Radix Puerariae, Radix Astragali, Fructus Schisandrae and Radix Glycyrrhizae were 3:2:2:2:1) and made into capsules, the product was named as GKY.

According to the Pharmacopoeia of People's Republic of China, the active compounds of the five medicinal herbs were listed in the Table 1. So the eight active compounds in GKY were determined following the method of Pharmacopoeia of People's Republic of China. Besides, the moisture, total flavonoids and total ash were also determined.

2.3. Hepatoprotective activity of GKY in vitro

2.3.1. Cell culture and treatment

HepG2 cells (purchased from China Center for Type Culture Collection, Beijing, China) were cultured in DMEM containing inactivated FBS (10%), non-essential amino acid, streptomycin (0.1 μg/mL) and penicillin (100 IU/mL). The cells were incubated in humidified atmosphere of 5% CO_2 at 37 °C. The medium was changed according to its color. Cells were harvested at the exponential growth phase, seeded into 96-well plates (4×10^4 cells per well, 100 μL) and incubated for 24 h. Cells of negative group were treated with alcohol (final concentration was 3.5%, v/v) while naive group treated with tap water. Cells of positive group were treated with alcohol and bicyclol (final concentration was 200 μg/mL). Cell of the four experimental groups were treated with alcohol and GKY (final concentration of GKY was 0.11, 0.33, 1.00 and 3.00 mg/mL). All cells were cultured for 48 h.