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Original Article

CGplus, a standardized herbal composition ameliorates non-alcoholic steatohepatitis in a tunicamycin-induced mouse model

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ABSTRACT

Background: The prevalence of Non-alcoholic fatty liver disease (NAFLD) including non-alcoholic steatohepatitis (NASH) has increased by 15–39% worldwide, but no pharmaceutical therapeutics exists.
Hypothesis/Purpose: This study investigated anti-hepatosteatotic effect of CG^{plus} (a standardized herbal composition of *Artemisia iwayomogi, Amomum xanthioides, and Salvia miltiorrhiza*) and its underlying mechanisms in a tunicamycin-induced NASH model.
Methods: C57/BL6J male mice were orally administrated CG^{plus} (50, 100, or 200 mg/kg), dimethyl dimethoxy biphenyl dicarboxylate (DDB, 50 mg/kg) or distilled water daily for 5 days. 18 h after a single injection of tunicamycin (*ip,* 2 mg/kg), the parameters for hepatic steatosis and inflammation were measured.
Results: Pretreatment with CG^{plus} significantly attenuated the accumulation of triglycerides and total cholesterol as well as lipid peroxidation, evidenced by quantitative and histopathological analyses in liver tissues. The elevations of serum aspartate transaminase, alanine transaminase and lactate dehydrogenase were significantly ameliorated by CG^{plus}. Also, it normalized the altered activities of pro- (TNF-α, IL-1β and IL-6), anti-inflammatory (IL-10) cytokines and lipid metabolism-related molecules in protein and gene expression analyses.
Conclusion: Our data present experimental evidence for the potential of CG^{plus} as an herbal therapeutic against NAFLD and NASH. Its underlying mechanisms may involve the modulations of pro- and anti-inflammatory cytokines, but further study is required especially for the actions of CG^{plus} on lipid metabolisms.

Introduction

Recently, fatty liver disease, especially nonalcoholic fatty liver disease (NAFLD), has been recognized as the initial stage of various chronic liver diseases. The prevalence of NAFLD in western societies ranged from 15 to 39% (Tiniakos et al., 2010) and approximately 25–30% in South Korea (Oh et al., 2016). NAFLD presents a histopathological feature of excessive triglycerides accumulation and can involves wide range of disorders, including liver tissue inflammation, liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) . NAFLD is also a risk factor for conditions related to metabolic syndrome including obesity, type 2 diabetes, and dyslipidemia (Musso et al., 2008).

Non-alcoholic steatohepatitis (NASH), an advanced stage of NAFLD, is a condition characterized by the coexistence of fat accumulation and hepatic tissue inflammation (Kwon et al., 2012). NASH develops through a complex process involving an imbalance between lipogenesis and lipolysis, which is followed by harmful events, including an inflammatory response (McCullough, 2004). Therefore, therapeutic approaches toward treating NASH mainly focus on one of the following two steps; reversing the abnormal accumulation of triglycerides in hepatocytes or suppressing hepatic inflammation (Sahebkar et al., 2014). However, the mechanisms underlying their pathogenesis still remain

Abbreviations: ACADL, acyl-CoA dehydrogenase long chain; AMPK, AMP-activated protein kinase; ANOVA, analysis of variance; ALT, alanine transaminase; AST, aspartate transaminase; DDB, dimethyl dimethoxy biphenyl dicarboxylate; H&E, hematoxylin and eosin; HCC, hepatocellular carcinoma; HMGCR, HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; HNE, hydroxynonenal; HPLC, high-performance liquid chromatography; HSL, hormone-sensitive lipase; IL-1β, interleukin-1 β; IL-6, interleukin-6; IL-10, interleukin-10; LDH, lactate dehydrogenase; MDA, malondialdehyde; NASH, Non-alcoholic steatohepatitis; NAFLD, Non-alcoholic fatty liver disease; pAMPK, phosphorylated AMP-activated protein kinase; Plin2, adipose differentiation-related protein; SREBP-1, sterol regulatory element-binding protein 1; TNF-α, tumor necrosis factor alpha; TG, triglycerides; TC, total cholesterol; TKM, traditional Korean medicine

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uncertain. To date, healthy lifestyle modification is the only clinical suggestion to overcome NAFLD or NASH as no pharmacological therapeutics exists for treating them (Dowman et al., 2011).

Meanwhile, medicinal herbal plants or phytochemicals have recently attracted interest as therapeutics for NAFLD and NASH (Jadeja et al., 2014). We previously reported that an herbal formula consisting of *Artemisia iwayomogi* Kitamura, *Ammomum xanthioides* Wallich, and *Saviae miltiorrhiza* Bunge exerted anti-haptofibrotic effects in a chemically-induced animal model (Kim et al., 2016). This herbal drug (called CG^{plus}) was formulated based on traditional Korean medicine (TKM), and standardized as an herbal drug treating NAFLD including NASH in clinics since 2015.

In the present study, we investigated the hepatoprotective activity of CG^{plus} against NASH and explored the underlying mechanisms responsible for its effects using a tunicamycin-induced mouse model.

Material and methods

Reagents and chemicals

Diphenyl dimethyl bicarboxylate (DDB) was obtained from Lancaster Co. (Lancashire, England). Mayer's hematoxylin, tunicamycin, quercitrin, quercetin-dehydrate, scopoletin, potassium chloride (KCl), acetic acid, bicinchoninic acid solution, bovine serum albumin, copper(II) sulfate pentahydrate, skim milk, phosphoric acid, radioimmunoprecipitation assay (RIPA), and sodium phosphate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Rosmarinic acid, salvianolic acid B and tanshinone IIA were obtained from Ministry of Food and Drug Safety (Chungbuk, Rep. of Korea). Ethanol, n-butanol and ethyl acetate were obtained from Samchun Chemical (Daejeon, Rep. of Korea). The pAMPK, AMPK, SERBP-1c, Pan-actin, and secondary horseradish peroxidase (HRP)-conjugated antibodies used for western blotting were obtained from Abcam (Cambridge, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA); the doublecortin (DCX), biotinylated secondary antibodies, and avidin-biotin peroxidase complex used for immunohistochemical staining were obtained from Abcam (Cambridge, MA, USA), Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Vector Laboratories (Burlingame, CA, USA).

Preparation of CG^{plus} and quantitative analysis

A. iwayomogi, A. xanthioides, and S. miltiorrhiza were obtained from the Jeong-Seong Oriental Medicine Store (Daejeon, Republic of Korea). All three herbal plants (100 g each) were mixed and then boiled in distilled water for 90 min and concentrated for 120 min. After filtration and lyophilization, we obtained a final water extraction yield of 9.58% (W/W) and the extract was promptly stored at −70 °C until use.

To investigate the chemical composition of CG^{plus}, we next performed fingerprinting analysis using high-performance liquid chromatography (HPLC) equipment on CG^{plus} and, as a reference, its active components, including quercitrin, quercetin-dehydrate, rosmarinic acid, salvianolic acid B, scopoletin, and tanshinone IIA. Briefly, reference standard stock solutions of a total of six chemical compounds were prepared by dissolving them at a concentration of 250 μg/ml in 90% methanol, and stock solutions of CG^{plus} was prepared in methanol; the stock solutions were stored at −4 °C. The standard solutions were prepared at six concentrations by diluting the stock solutions. All calibration curves were made by assessing the peak areas in the range of 2.5–500 μg/ml. The linear curve of the peak area (y) versus concentration (x, μg/ml) for each component was used to calculate the contents of the main components in CG^{plus}.

Quantitative analysis was performed simultaneously using an 1100 series high-performance liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA) equipped with an auto-sampler (G11313A), column oven (GA1316A), binary pump (G1312), diode-array-detector (G1315B), and degasser (G1379A). The analytical column, a Kinetex C18 (4.6 × 250 nm, particle size 5 μm, Phenomenex, Torrance, CA, USA) was kept at 30 °C during the experiment. The data were acquired and processed by ChemStation software (Agilent Technologies, USA). The mobile phase consisted of 10% acetonitrile in distilled water (DW) with 0.05% formic acid (A) and 90% acetonitrile in water (B). The gradient flow was as follows: 0–30 min, 0–20% B; 30–50 min, 20–75% B; 50–60 min, 75–100% B. The analysis was conducted at a flow rate of 1.0 ml/min and detection wavelength of 280 nm. The injection volume was 10 μl. All aspects of the system operation and data acquisition were controlled using Mass Lynx software.

Identification of three main compounds by LC-MS/MS

For identification of rosmarinic acid, salvianolic acid B, and tanshinone IIA, the mass spectral analyses were performed using an LC-MS/MS system (Agilent 1200 Series HPLC system with AB Sciex Q-Trap 4000 system). The analytical column used was Waters X-Terra C18 column (5 μm, 50 mm × 2.1 mm). The mobile phase composed of methanol/0.1% (v/v) formic acid in water (80:20) at a flow rate of 0.5 ml/min. Mass spectrometer was operated in both positive (Tanshinone IIA) and negative ion mode (Salvianolic acid B and Rosmarinic acid). The scan results were performed using selected reaction monitoring (SRM) of the transitions of 358.9 → 160.9 m/z for rosmarinic acid (Liu et al., 2010), 717.1 → 518.9 m/z for salvianolic acid B (Xu et al., 2007), 295.1 → 277.0 m/z for tanshinone IIA (Hao et al., 2004; Liu et al., 2010). CGplus extract was prepared by weighing 0.2 g of dried powder, grinding, adding 5 ml of methanol, vortexing then filtering with 0.2 μm PTFE syringe filter and diluted 1000–2000 times with methanol (Fig. 1C).

Animals and experimental design

For the animal experiments, 48 specific pathogen-free male C57BL/6 J mice (6 weeks old, 17–20 g) were obtained from a commercial animal breeder (Daehanbio-link, Chung-buk, Rep. of Korea), and acclimated for l week before the experiment. All animals were housed in an environmentally controlled room kept at 22 ± 2 °C and 60 ± 5% relative humidity under a 12/12 h light/dark cycle. All mice were given access to distilled water (DW) and commercial standard chow diet (Daehanbio-link) with tap water ad libitum. After 7 days of acclimation, the mice were divided into the following six groups (n = 8 for each group): normal (DW without tunicamycin injection), control (DW and tunicamycin injection), CG^{plus} treatment groups (50, 100 or 200 mg/kg of CG^{plus} and tunicamycin injection), and positive control groups (100 mg/kg of DDB and tunicamycin injection). CG^{plus} and DDB were dissolved in DW. All mice were orally administrated CG^{plus} or DDB for 7 consecutive days before receiving a single injection of tunicamycin intraperitoneally (dissolved in normal saline at 2 mg/kg, *i.p.*). After the final administration of each drug to the mice, tunicamycin or normal saline was injected. All mice were kept under starvation conditions for 8 h before the tunicamycin or saline injection, and then they were sacrificed 30 h after the tunicamycin injection by the collection of whole blood via an abdominal vein under ether anesthesia. Liver tissue was removed, immediately weighed and fixed or stored in 10% neutral buffered formalin, RNAlater (Ambion, TX, USA) or in a deep freezer (−70 °C) for histopathological analysis, mRNA expression analysis, and biochemical parameter determination, respectively.

Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Daejeon University (approval No. DJUARB: 2016–017).