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Protective effects of Kangxian ruangan capsule against nonalcoholic fatty liver disease fibrosis in rats induced by MCD diet

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ABSTRACT

Kangxian ruangan (KXRG) capsule is a classical formula containing various herbals that play a vital role of replenishing spleen and warming Yang. Traditional Chinese medicine believes that insufficiency of the spleen, damp-heat, and phlegm and stasis are the key factors to nonalcoholic fatty liver disease (NAFLD). The objective of this study was to investigate the effects of KXRG capsule on NAFLD fibrosis rats induced by MCD diet. The liver functions (ALT, AST and GGT) and levels of blood lipids (CHOL and TG) in each treatment group rats were significantly decreased, especially those in H-KXRG group. At the same time, the KXRG capsule alleviated the inflammatory response, histopathological changes and liver fibrosis of NAFLD fibrosis rats. In addition, the apoptosis of liver cells induced by diet was obvious via TUNEL staining. However, KXRG capsule reversed that negative change. Moreover, the levels of pro-apoptotic proteins (Caspase 3, 8, 9 and Bax) were reduced by exposure to KXRG capsule, except that the anti-apoptotic proteins (Bcl-2 and Bcl-XL) were elevated. In conclusion, KXRG relieved the progression of NAFLD fibrosis via maintaining the balance of TNF-α/IL-10 further relieving the inflammatory reaction, and regulating the balance of Bcl-2/Bax or Bcl-XL/Bax in a positive direction further activating damaged hepatocytes.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD), a major cause of liver disease worldwide [1], is now recognized as a chronic process liver disease characterized by inflammation, fat accumulation and hepatocyte dysfunction in liver [2,3]. An authoritative research from the American Association for the Study of Liver Diseases showed that global prevalence of NAFLD is 25.24% with highest prevalence in the Middle East and South America and lowest in Africa with a sample size of 8,515,431 from 22 countries from 1989 to 2015 [4]. The severity of NAFLD is evident. At present, the pathogenesis of NAFLD has not been determined, but a multiple strike theory revealed that NAFLD is caused by the combination of diet [5], genetics [6] and intestinal microenvironment [7]. Importantly, it is also closely linked to obesity and the metabolic syndrome [8]. And NAFLD predisposes susceptible individuals to cirrhosis [9], hepatocellular carcinoma [10], and cardiovascular disease [11] without treating on time. Over-inflammatory response is one of the typical pathological changes of NAFLD [12]. Previous studies showed that inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) [13], interleukin-1β (IL-1β), IL-6 [14] and IL-10 [15] are involved in the NAFLD followed by liver injury. Thus, suppressing inflammatory response effectively is one of the best ways to treat NAFLD.

Furthermore, a number of studies showed that hepatic fibrosis plays an important role in occurrence and development of NAFLD [16,17]. It is not only a common approach to development of all chronic liver diseases, but also the only way to further develop liver cirrhosis [18] and even liver cancer [19]. The complexity of this disease has been a major impediment to the development of appropriate metrics of its effective therapies. Current best treatment for NAFLD has not yet been defined. Nutritional counselling or diet prescription to reduce body weight, coupled with increased physical exercise, was standard care and remains the first line of treatment. Drug treatment may be added, to address specific pathogenic targets. For example, insulin sensitizing agents are the most promising drugs. Both metformin and thiazolidinediones reduce aminotransferase levels, reduce liver fat, and improve

Abbreviations: KXRG, Kangxian ruangan; MCD, methionine and choline deficient; NAFLD, Nonalcoholic fatty liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; CHOL, cholesterol; TG, triglycerides; HA, Hyaluronic acid; LN, laminin; C IV, type IV collagen; PC III, type III procollagen; HSCs, Hepatic stellate cells; TCM, traditional Chinese medicine; CHM, Chinese herbal medicine; TUNEL, Terminal deoxynucleotidy transferase dUTP nick end labeling; H&E, Haematoxylin and Eosin

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liver histology. Antioxidants have a specific place in non-alcoholic fatty liver in children, but their effectiveness is low. Therefore, it is very important to seek effective treatment for NAFLD. Recent clinical data place increasing importance on identifying fibrosis, as it is a strong indicator of hepatic disease-related mortality [20]. Hyaluronic acid (HA), laminin (LN), type IV collagen (C IV), and type III procollagen (PC III), are generally considered to be the four important indicators for clinical detection of liver fibrosis and are able to respond to the degree of liver fibrosis correctly [21]. Hepatic stellate cells (HSCs) are the cytological basis of liver fibrosis [22]. Under normal conditions, HSCs are located in the interstitium around the hepatic sinusoid, promoting hepatocyte interactions by releasing soluble inflammatory cytokines (such as TNF-α, IL-1β, and so on) and creating extracellular matrix (ECM) [23]. When the liver is damaged by a variety of stimuli, hepatocytes are prone to damage or even apoptotic but the HSCs are activated and more ECM is released, and the balance of damage can lead to liver fibrosis [24]. Hence, promoting the apoptosis of HSCs and further ensuring the activity of hepatocytes are considered to be another vital way to treat liver fibrosis.

It is well known that Chinese herbal medicine (CHM) has been traditionally used in the treatment of various illnesses for thousands of years in some Asian countries [25]. With the continuous development and urgent need of medical research, CHM has gradually become a growing focus of worldwide medical research in order to get a better therapeutic effect for recognized common diseases, including NAFLD [26]. The art of CHM is to dissect pharmacologically and therapeutically valuable herbal drugs from harmful and toxic ones and to develop combinations of medicinal plants as safe and efficient herbal remedies. A variety of natural compounds from CHM could target different pathological pathways of the diseases, and provide therapeutic effects through a range of actions [27]. Kangxian ruangan capsule, one of the well-known traditional CHM, is a classical formula containing *Artemisia capillaries* (20 g), *Salvia miltiorrhiza* (30 g), *Turtle shell* (10 g), *Panax notoginseng* (6 g), *Peach seed* (10 g), *Angelica sinensis* (10 g), *Curcuma zedoary* (10 g), *Parched pangolin scales* (6 g), *Ground beeltle* (10 g), *Rhizoma atractylodis macrocephalae* (10 g), *Coix seed* (20 g) and *Astragalus membranaceus* (10 g).

For the mechanism of NAFLD, traditional Chinese medicine (TCM) believes that insufficiency of the spleen, damp-heat, and phlegm and stasis are the key factors to NAFLD. Based on the pharmacological principles of CHM, Kangxian ruangan capsule has the functions of replenishing spleen and warming Yang. Therefore, judging from the dialectical thinking of TCM, we guess that it also possesses the same remarkable effects on NAFLD as the reported TCM (such as Fuzheng huayu recipe) and common western medicine (colchicine). In this study, rat model of NAFLD fibrosis was established by methionine and choline deficient (MCD) diet. The therapeutic effects of Kangxian ruangan capsule with various concentrations and other medications on model rats were evaluated and compared, which aims to provide more targeted natural medicine for the treatment of NAFLD.

2. Materials and methods

2.1. Preparation of medications

Kangxian ruangan capsule, one of the well-known traditional CHM, is a classical formula containing *Artemisia capillaries* (20 g), *Salvia miltiorrhiza* (30 g), *Turtle shell* (10 g), *Panax notoginseng* (6 g), *Peach seed* (10 g), *Angelica sinensis* (10 g), *Curcuma zedoary* (10 g), *Parched pangolin scales* (6 g), *Ground beeltle* (10 g), *Rhizoma atractylodis macrocephalae* (10 g), *Coix seed* (20 g) and *Astragalus membranaceus* (10 g). All herbals were purchased from Hubei Tianji Chinese Herbal Sliced Medicine Co., Ltd. (Wuhan, China). According to the traditional method of preparation of decoction, the decoction was concentrated and prepared to capsule by pharmacy department of Hubei provincial hospital of TCM (Different concentrations of concentrated solution: 0.046 g (crude drug) /mL; 0.092 g (crude drug) /mL; 0.184 g (crude drug) /mL). Fuzheng huayu capsule (No.230097) was purchased from Shanghai Huanghai Pharmaceutical Co., Ltd. (Shanghai, China). Colchicine (No.B14200011502) was purchased from Xishuangbanna banna pharmaceutical Co., Ltd. (Xishuangbanna, China).

2.2. Rats and treatments

56 specific pathogen-free male Wistar rats (age: 7 ~ 8 weeks; weigh: 250–280 g) were purchased from the Animal Center of Hubei (No.42000600013965, Hubei, China). All experimental procedures were approved by the Animal Ethics Committee of Hubei provincial hospital of TCM. All rats were maintained under conditions of constant temperature (22 °C) and humidity (50% ± 15%) in a 12 h-light/dark cycle, with free access to drink deionized water and fed the irradiated disinfectant food. After one week of adaptive feeding, 56 rats were randomly divided into 7 groups (8 rats in each group): (1) Control group (CTRL); (2) Model group (MOL); (3) Fuzheng huayu group (FZHY); (4) Colchicine (COL) group; (5) Normal Kangxian ruangan (N-KXRG) group; (6) Middle Kangxian ruangan (M-KXRG) group and (7) High Kangxian ruangan (H-KXRG) group. In this study, rats model of NAFLD fibrosis were established by MCD diet. Rats in CTRL group were fed with methionine and choline supplement (MCS) fodder. But rats in other groups were fed with MCD fodder (Trophic Animal Feed High-tech Co., Ltd., Jiangsu, China). While building the NAFLD fibrosis model, the rats in each treatment group were gavaged with the corresponding drug for 8 weeks [FZHY group: 15 g/kg/d; COL group: 0.1 mg/kg/d; N-KXRG group: 0.92 g (crude drug) /kg/d; M-KXRG group: 1.84 g (crude drug) /kg/d; H-KXRG group: 3.68 g (crude drug) /kg/d]. Rats in CTRL and MOL group were gavaged with the same volume (2 mL/100 g/d) normal saline for 8 weeks. Details are at Fig. 1. In addition, the weight of each group of rats was weighed and recorded every two weeks.

2.3. Determination of biochemical indicators

After 8 weeks treatment, all rats were anesthetized using 60 mg/kg pentobarbital (intraperitoneal injection, No.P3761, Sigma-Aldrich, USA) after 12 h of fasting, and blood samples were collected from the abdominal aorta of rats. The serum was separated and collected through a centrifuge at 4 °C. With that, fasting serum alanine aminotransferase (ALT), fasting serum aspartate aminotransferase (AST), fasting serum gamma-glutamyl transpeptidase (GGT), fasting serum total cholesterol (CHOL) and fasting serum triglycerides (TG) were determined using commercial kits (ALT: No.C009-2; AST: No.C010-2; GGT: No.C017-1; CHOL: No.F002-1; TG: No.F001-1, Nanjing Jiancheng Biology Engineering Institute, Jiangsu, China) following the manufacturers' instructions.

2.4. Enzyme-linked immunosorbent assays (ELISA)

The levels of hyaluronic acid (HA), laminin (LN), IV-collagen (IV-C), procollagen-III (PC-III), TNF-α and IL-10 in serum were determined by corresponding commercial ELISA kits according to manufacturer's instructions (HA: No.MU30353; LN: No.MU30187; IV-C: No.MU30211; PC-III: No.MU30821; TNF-α: No.MU30030; IL-10: No.MU30055, Bioswamp, China). Absorbance was detected with a microplate reader (Bio-Rad, USA) at 450 nm. Finally, the value of various indicators was calculated by a standard curve.

2.5. Histopathological examination of liver

When the rats were sacrificed, the part of fresh liver tissue was removed and fixed with 4% paraformaldehyde for 24 h, embedded in paraffin, cut into 5 ~ 7 μm thick sections, and stained by Haematoxylin and Eosin (H&E) staining and Masson staining kit (No.PAB180023,