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Original Article

# Calycosin attenuates triglyceride accumulation and hepatic fibrosis in murine model of non-alcoholic steatohepatitis via activating farnesoid X receptor

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ABSTRACT

*Background:* Non-alcoholic steatohepatitis (NASH) represents the more severe end of hepatic steatosis and is associated with progressive liver disease. Calycosin, derived from the root of Radix Astragali, has been demonstrated to have favorable efficacy on acute liver injury.
*Purpose:* The present study was to investigate the hepatoprotective effect of calycosin on attenuating triglyceride accumulation and hepatic fibrosis, as well as explore the potential mechanism in murine model of NASH.
*Study design:* The C57BL/6 male mice were fed with methionine choline deficient (MCD) diet for 4 weeks to induce NASH and treated with or without calycosin by oral gavage for 4 weeks.
*Methods:* The body weight, liver weight and the liver to body weight ratios were measured. Serum ALT, AST, TG, TC, FFA, MCP-1 and mKC levels were accessed by biochemical methods. H&E staining and Oil red O staining were used to identify the amelioration of liver histopathology. Immunohistochemistry of a-SMA, Masson trichrome staining and Sirius red staining were used to identify the amelioration of hepatic fibrosis. The quantitative real-time-PCR and Western blot were applied to observe the expression changes of key factors involved in triglyceride synthesis, free fatty acid $\beta$-oxidation and hepatic fibrosis.
*Results:* Calycosin significantly inhibited body weight loss induced by MCD diet, decreased the ALT and AST activities, MCP-1 and mKC in a dose-dependent manner. The H&E and Oil red O staining indicated calycosin effectively improved hepatic steatosis, improved the degree of triglyceride accumulation. Masson trichrome and Sirius red staining indicated that calycosin treatment remarkably attenuated the degree of hepatic fibrosis. Immunohistochemistry of a-SMA demonstrated that calycosin attenuated hepatic fibrosis by inhibiting hepatic stellate cell activation. Further, calycosin inhibited the expression of SREBP-1c, FASN, *ACC* and *SCD1* involved in triglyceride synthesis, promoted the expression of PPARa, CPT1, *Syndecan-1* and *LPL* involved in free fatty acid $\beta$-oxidation. The above effects of calycosin were attributed to FXR activation.
*Conclusion:* Calycosin attenuates triglyceride accumulation and hepatic fibrosis to protect against NASH via FXR activation.



*Abbreviations:* a-SMA, a-smooth muscle actin; ALT, alanine aminotransferase; AST, aspartate transaminase; ACC, acetyl-coenzyme A carboxylase; CPT1, carnitine palmitoyltransferase 1; CDCA, chenodeoxycholic acid; FASN, fatty acid synthase; FFA, free fatty acid; FXR, farmesoid X receptor; H&E, hematoxylin and eosin; HSC, hepatic stellate cell; Mkc, mouse keratinocyte-derived chemokine; MCD, methionine choline deficient; MCP-1, monocyte chemotactic protein 1; MCS, methionine choline sufficient; NASH, non-alcoholic steatohepatitis; PPARa, peroxisome proliferator activated receptor $\alpha$; SCD1, stearoyl-coenzyme A desaturase 1; Shp, small heterodimer partner; SREBP-1c, sterol regulatory element-binding protein 1c; TC, total cholesterol; TG, total triglyceride; TIMP-1, tissue inhibitor of metalloproteinases 1.

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## Introduction

Non-alcoholic steatohepatitis (NASH) is characterized by hepatocyte damage, inflammation and fibrosis, as well as hepatocyte ballooning degeneration or hydropic degeneration (Hashimoto et al., 2015). It has been reported that non-alcoholic fatty liver disease (NAFLD) has become the most common liver disease with about 20–30% of general population worldwide (Lin et al., 2016), while 2–10% are NASH patients (Birerdinc and Younossi, 2015). NASH may be induced from an intricate interaction of nutritional, genetic and immunologic factors that stimulate multiple parallel hits, eventually causing disordered lipid metabolism and hepatic fibrosis (Imajo et al., 2013). NASH could deteriorate and develop into irreversible cirrhosis, liver failure, and even hepatocellular carcinoma, which becomes the second-leading cause of death in those patients presently (Farrell and Larter, 2006; Khan and Newsome, 2016). In addition, NASH may increase the risk of hepatocellular carcinoma. Although it has not yet attested whether simple steatosis must increase the risk of hepatocellular carcinoma, it is conjectured that NASH is the common risk factor of diabetes and hepatocellular carcinoma (Heeboll et al., 2015). Nowadays, the treatment for NASH involves diet and life intervention, medicines including metformin, statins and fibrates their utilization are limited due to their potential adverse side effects. The “anti-NASH” drugs have no direct anti-fibrotic effect and there is still none approved specifically for NASH (Ratziu et al., 2015). Therefore, novel specific drugs for treatment of NASH with little or no side effects are urgently needed.

The farnesoid X receptor (FXR; NR1H4), a ligand-activated transcription factor, belongs to the nuclear hormone receptor superfamily and is highly expressed in the enterohepatic system such as liver, intestine and kidney. $FXR^{-/-}$ mice livers exhibit serious liver injury and hepatic fibrosis, even spontaneously develop hepatocellular carcinoma, which is very similar to the end stage of NASH patients (Yang et al., 2007). Activation of hepatic FXR, which induces the expression of the FXR target gene small heterodimer partner (*Shp*). By regulating *Shp* downstream genetic expression, FXR modulates TG homeostasis (Desvergne and Wahli, 1999). The pharmacological modulation of FXR has been proposed as a potential therapeutic strategy for metabolic diseases involving the liver (Mazuy et al., 2015). In consideration of the novel regulation of lipid and glucose levels, FXR is also proposed as a potential drug target for treatment of hyperlipidemia (Beuers et al., 2015). Therefore an intriguing question arises whether FXR also is the drug target for attenuating TG accumulation and hepatic fibrosis.

Traditional Chinese medicines have developed new therapies for NAFLD based on its particular efficacy. Isoflavones have received increasing attention because of their potential effects on preventing fatty liver disease, adiposity, cardiovascular disease, atherosclerosis, hyperlipidemia and osteoporosis (Kim and Kang, 2012). Genistein and daidzein are reported to alleviate NAFLD and preventing the emergence of NASH by stimulating the hepatocyte and adipocyte antioxidative enzymes and attenuating oxidative stress (Qiu and Chen, 2015). Puerarin is also reported to prevent acute alcoholic liver injury by inhibiting oxidative stress (Zhao et al., 2010). Radix Astragali, the dry root of *Astragalus propinquus* Schischkin (Fabaceae), is known as Huangqi in Chinese. Calycosin (7,3′-dihydroxy-4′-methoxyisoflavone, PubChem CID: 5280448) is one of main bioactive compounds derived from the root of Radix Astragali. Its main medicinal properties are to improve TG metabolism, mitigate obesity and hyperlipidemia (Wang et al., 2014). Calycosin has been demonstrated to have favorable efficacy on hepatoprotective properties against acute liver injury in our previous study (Chen et al., 2015). To the best of our knowledge, the effect of calycosin on protecting against chronic liver disease such as NASH has not been reported previously.

Despite the important clinical significance, there are still no proven effective drugs or therapeutic strategies for treatment of NASH. Mice feeding with MCD diet is a well-established nutritional model of NASH. The MCD model of liver histological changes are similar to NASH patients, including hepatic steatosis and pericelluar fibrosis (Farrell and Larter, 2006; Ip et al., 2004). The present study was designed to investigate the hepatoprotective effect of calycosin on attenuating TG accumulation and hepatic fibrosis, and further elucidate the possible mechanism in the murine model of NASH.

## Materials and methods

### *Animals and diet*

Male 8-old-week C57BL/6 mice were purchased from the Experimental Animal Center of Dalian Medical University (Dalian, China; approval number: SCXK 2008–0002). All procedures involving animals were reviewed and approved by the Ethical Committee and the China National Institutes of Healthy Guidelines for the Care and Use of Laboratory Animals. The C57BL/6 mice were divided into 9 experimental groups ($n = 6$ per group), fed and treated as follows for 4 weeks: (1) normal group with a standard chow diet, (2) MCS group with a control chow diet (A02082003B, Research Diets, New Brunswick, USA), (3) MCD model group fed a MCD diet (A02082002B, Research Diets, New Brunswick, USA) with a daily oral gavage of vehicle, (4) mice fed a MCD diet with a daily oral gavage of 12.5 mg/kg calycosin, (5) mice fed a MCD diet with a daily oral gavage of 25 mg/kg calycosin, (6) mice fed a MCD diet with a daily oral gavage of 50 mg/kg calycosin, (7) mice fed a MCD diet with a daily oral gavage of 50 mg/kg CDCA, (8) mice fed a MCD diet with a daily injecting intraperitoneally with 10 mg/kg GS, (9) mice fed a MCD diet with a daily oral gavage of 50 mg/kg calycosin and injecting intraperitoneally with 10 mg/kg GS. The mice were injected intraperitoneally with 10 mg/kg of GS 4 h before administration of vehicle or calycosin every time. Throughout the experiment, mice were maintained in laboratory animal facilities with a standard 12 h light/dark cycle with ad libitum access to food and water. On the day of sacrifice, mice were anesthetized, organs were harvested and blood samples were collected from the portal vein. Tissue samples were either fixed in 4% paraformaldehyde solution for histological analysis or snap frozen in liquid nitrogen and stored at −80 °C

### *Chemicals and reagents*

Calycosin (purity > 98%, CAS No. 13,081,501, Must Biotechnology, Chengdu, China) was treated to mice by oral gavage. Guggulsterone (GS, purity > 98%) and chenodeoxycholic acid (CDCA, purity > 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### *Serum biochemistry measurements*

Serum was obtained from blood after the centrifugation (5000 RPM, 10 min) at 4 °C. Serum alanine aminotransferase (ALT) and aspartate transaminase (AST) activities, total triglyceride (TG), total cholesterol (TC), low density lipoprotein (LDL), very low density lipoprotein (VLDL) and free fatty acid (FFA) were detected by using commercial kits according to the manufacturer’s instructions from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Monocyte chemotactic protein-1 (MCP-1) and mouse keratinocyte-derived chemokine (mKC) detection kits were obtained from Meso Scale Diagnostics (Gaithersburg, MD, USA).