Targeting Sirt1 in a rat model of high-fat diet-induced non-alcoholic fatty liver disease: Comparison of Gegen Qinlian decoction and resveratrol

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Abstract. The present study aimed to explore the mechanism of action of Gegen Qinlian decoction (GGQLD) in experimental non-alcoholic fatty liver disease (NAFLD). A total of 30 rats were randomly divided into five groups: The chow, model, high- and low-dose GGQLD (GGQLD-H and GGQLD-L, respectively) and resveratrol (Resl) groups, and were treated with saline, GGQLD and Resl when a model of high-fat diet (HFD)-induced NAFLD was established. Blood lipid and liver enzymes were detected following treatment for 8 weeks and liver tissue pathology was observed using Oil Red O and haematoxylin and eosin staining. Furthermore, the liver protein and mRNA expression of sirtuin (Sirt)1, peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α and nuclear factor-κ-light-chain-enhancer of activated B cells (NF-κB) were measured using western blotting and reverse transcription-quantitative polymerase chain reaction. Compared with the chow group, the model group demonstrated significantly increased serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (P<0.01). GGQLD doses and Resl attenuated the elevated serum ALT and AST levels. GGQLD-L and Resl significantly decreased serum low-density lipoprotein cholesterol levels (P<0.01). The GGQLDs and Resl groups revealed an evident improvement in Sirt1 protein and mRNA expression. Although GGQLD and Resl significantly decreased NF-κB gene expression compared with the model group (P<0.01), the effect on NF-κB protein expression was not significant. Furthermore, the PGC-1α gene and protein expression in the HFD rat group slightly decreased compared to the levels in the chow group, but the decrease was insignificant. However, an evident increase in PGC-1α mRNA expression was observed in the GGQLD-H group compared with the model group (P<0.01). Histological staining revealed that GGQLD and Resl decreased the lipid droplets in hepatocytes and normalized steatosis in rats fed with a HFD. The results indicated that GGQLD treatment may be a potent strategy for managing NAFLD by managing lipid metabolism and inflammatory and histological abnormalities by triggering the Sirt1 pathway.

Introduction

Non-alcoholic fatty liver disease (NAFLD) consists of a spectrum of illnesses ranging from simple hepatic steatosis to non-alcoholic steatohepatitis (NASH) and irreversible cirrhosis (1). This syndrome has become the most common cause of liver disease (2). Indeed, NAFLD is currently recognized as an aetiological factor in numerous conditions previously labelled as cryptogenic cirrhosis (3). To date, the pathogenesis and progression of NAFLD are not fully understood, while current therapeutic treatment methods require improvement (1,4). Initial theories of the pathogenesis of NAFLD/NASH were based on the ‘2-hit’ hypothesis. Briefly, the ‘first hit’ involves hepatic triglyceride (TG) accumulation or steatosis, while the ‘second hit’ is usually linked to mediators, including inflammatory cytokines and oxidative stress (1). However, there is an increasing recognition of the function that free fatty acids (FFA) have in directly promoting liver injury, leading to the modification of this theory (1). Additionally, a ‘third-hit’ has been included to reflect the inadequate hepatocyte proliferation (1).

In the progression of chronic liver injury, the development of fibrosis/cirrhosis depends on the efficacy of hepatocyte
regeneration (5). Hence, cell death with impaired proliferation of hepatocyte progenitors represents the proposed ‘third hit’ in NAFLD pathogenesis (5). Furthermore, hepatic TG accumulation may occur as a result of increased fat synthesis and delivery, as well as decreased export and/or oxidation of fat (6). Additionally, inflammatory cytokines, including tumour necrosis factor (TNF)-α, interleukin (IL)-6 and IL-8, mediate steatohepatitis in patients with NAFLD (7) and may be regulated by the peroxisome proliferator-activated receptor (PPAR)-γ. PPAR-γ may be activated by the PPARγ coactivator (PGC)-1α following sirtuin (Sirt)-1 activation (8).

Chinese herbal medicine (CHM), which has been traditionally used in China and other parts of Asian countries for thousands of years, is now spreading worldwide (9). A specific and basic feature of Chinese medicine is the use of formulas containing several herbs (herbal cocktail) to ameliorate a series of abnormal symptoms associated with a particular disease. Gegen Qinlian decoction (GGQLD), a classical formula from the Treatise on Febrile Diseases, is one of the well-known traditional Chinese medicines and consists of Kudzu root, Rhizoma coptidis, Scutellaria baicalensis Georgi and honey-fried liquorice root (10). Furthermore, it is widely used to treat diarrhoea (11). In previous studies, it was revealed that GGQLD improved diabetes (12,13) and had an anti-inflammatory effect based on its constituents (14-16). Furthermore, glucose and lipid metabolism disorder and inflammation are important in NAFLD. A previous study demonstrated that GGQLD therapeutically managed NAFLD by improving the PPARγ-mediated regulation of lipids and suppression of inflammation (17). However, the mechanisms by which GGQLD triggers PPARγ are unknown, including whether it has a potential Sirt1 agonist activity. Therefore, following the guidelines of the traditional Chinese medicine theory of ‘same treatment for different diseases’ and based on the ameliorating effects of PPARγ in NAFLD management (17), it was hypothesized that GGQLD could improve NAFLD as a Sirt1 agonist. In the present study, this hypothesis was investigated.

Materials and methods

Preparation of GGQLD and resveratrol (Resl). GGQLD granules were provided by the Department of Pharmacy of Dongfang Hospital, Beijing University of Chinese Medicine (Beijing, China). The granules consisted of the following ingredients of the Gegen Qinlian formula: Kudzu root, Rhizoma coptidis, Scutellaria baicalensis Georgi and main liquorice at 24, 9, 9 and 6 g, respectively. Resl was purchased from Zelingyiyi Co., Ltd., (Nanjing, China).

Animals and treatment. A total of 30 male Sprague-Dawley rats (7 weeks old; weight, 200±20 g) were supplied by Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animal experimental procedures were approved by the Animal Ethics Committee of Beijing University of Chinese Medicine under the guidelines issued by Regulations of Beijing Laboratory Animal Management (18). The rats were maintained on a 12-h light/dark cycle under a constant temperature (22±2°C) and humidity of 50±10% with ad libitum access to standard chow diet (control group) or a high-fat diet (HFD; 34% fat, 19% protein and 47% carbohydrate by energy composition; n=6 per group) for 8 weeks to induce NAFLD. Additionally, the GGQLD granules and Resl were dissolved in 100 ml of distilled water, and stored at 2-8°C until further use. The five groups of animals (n=6) were treated orally for 8 weeks as follows: GGQLD low- and high-dose (GGQLD-L and GGQLD-H) groups treated with 5.04 and 10.08 g/kg/day GGQLD, respectively; Resl group, treated with 400 mg/kg/day Resl; and the HFD model and control groups were treated with 10 ml/kg/day saline for 8 weeks from the beginning. The GGQLD and Resl groups were administered the respective treatments at the beginning of the HFD feeding, and the control received the standard chow diet.

Determination of metabolic parameters and liver enzymes. At the end of the treatment, the animals were anaesthetized using 4% chloral hydrate (Sinopharm Chemical Reagent Co. Ltd, Shanghai, China) after a 12-h overnight fast and blood samples were collected from the abdominal aorta of rats. The fasting serum TGs (Jing 20142401132), total cholesterol (TC; Jing 20162400910), high-density lipoprotein cholesterol (HDL-C, Jing 20152400950) and low-density lipoprotein cholesterol (LDL-C, Jing 20142400518) as well as fasting serum alanine aminotransferase (ALT, Jing 20142401158) and aspartate aminotransferase (AST, Jing 20142401158) were analysed using ELISA kits (BioSino Biotechnology and Science, Inc., Beijing, China).

Histological analysis. Paraffin sections of the liver tissues were fixed with 10% formaldehyde solution for 48 h at room temperature, and the tissue slices (thickness, 4 µm) were prepared for hematoxylin staining for 2 min at room temperature. Hematoxylin and eosin (H&E) were purchased from Beijing Zeping Bioscience & Technologies Co., Ltd. (Beijing, China). The Oil Red O (ORO; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) staining was performed with freshly frozen liver tissues (-196°C, liquid nitrogen) for 3 min at room temperature. The H&E- and ORO-stained slides were visualized using a light microscope in order to investigate architecture of the liver and hepatic lipid droplets. The liver tissue samples from 3-5 rats from each group were prepared, stained and the images were subsequently captured using an Olympus digital camera (BX40; Olympus Corporation, Japan) and the NIS Element SF 4.00.06 software (Nikon Corporation, Tokyo, Japan).

Western blotting of Sirt1, PGC-1α and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) p65. Proteins in 100 mg liver tissue homogenate were extracted using ice-cold tissue lysis buffer (WB0006; TDY Biotech Co., Ltd., Beijing, China) at 4°C in an electric tissue homogenizer at 250 x g for 5 sec, 5 times at 5 sec intervals, then incubated on ice for for 20 min. The supernatant was extracted by centrifugation at 12,000 x g for 20 min at 4°C. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Promega Corporation, Madison, WI, USA). Samples (30 µg) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were then incubated in blocking buffer (5% skimmed milk
Results

Effect of GGQLD on lipid metabolism and liver enzymes in HFD rats. All rats tolerated the experimental procedures well and no mortalities occurred during the study. The serum ALT and AST (36.10±3.92 and 425.85±13.84 U/l, respectively) concentrations in the HFD-fed rats were significantly higher than those in the chow-fed rats (19.27±3.36 and 298.10±5.13 U/l, respectively; P<0.01). Treatment with GGQLD-L and GGQLD-H significantly attenuated ALT levels (28.20±3.01 and 24.98±1.55 U/l, respectively; P<0.01) and AST (291.24±22.36 and 206.68±20.48 U/l, respectively; P<0.01) compared with the HFD-fed rats. Similarly, Resl also significantly reduced the ALT and AST levels (26.88±1.45 and 246.66±20.47 U/l, respectively) compared with rats fed with HFD (P<0.01; Fig. 1).

The HDL-C levels rose significantly in the GGQLD-H and Resl groups (1.90±0.10 and 1.30±0.11 mmol/l, respectively) compared with those in the HFD model group (0.95±0.04 mmol/l; P<0.01). Furthermore, GGQLD-H also decreased the LDL-C concentration (0.98±0.15 mmol/l) compared with the HFD group, but not significantly (P>0.05), whereas the decrease in the GGQLD-L and Resl groups (0.79±0.09 and 0.78±0.09 mmol/l, respectively) was significant compared to that of the HFD model group (1.07±0.20 mmol/l; P<0.01). Treatment with GGQLD-L, GGQLD-H and Resl significantly attenuated the elevated TC (1.94±0.39, 1.53±0.11 and 1.63±0.13 mmol/l, respectively) compared with that of the HFD group (2.81±0.79 mmol/l; P<0.05; Fig. 2). Treatment with GGQLD-L, GGQLD-H and Resl had no significant effect on serum TG levels compared with those in the HFD group.

Histopathological changes in rats. An increase in the number of H&E-positive hepatocytes was evident in the HFD rats compared to those in the chow group (Fig. 3A and B). The H&E-stained images also exhibited steatosis and infiltration with inflammatory cells in the intercellular substance (Fig. 3B). The treatment of HFD rats with GGQLD and Resl reduced the fat deposits in the liver (Fig. 3C-E) and the GGQLD-treated rats revealed histological features similar to the chow group with little steatosis (Fig. 3C and D).

Furthermore, few lipid droplets were detected in the liver sections from the chow group in ORO staining analysis (Fig. 4A). Compared to that of HFD-fed model rats (Fig. 4B), GGQLD and Resl markedly restrained the deposition of lipid droplets in hepatocytes (Fig. 4C-E).

GGQLD regulates Sirt1, PGC-1α and NF-κB (p65) expression in rats with HFD-induced NAFLD. The possibility of GGQLD having a regulatory effect on Sirt1, PGC-1α and NF-κB (p65) expression was investigated. As demonstrated in Fig. 5, the Sirt1 protein level was significantly decreased in the HFD group (P<0.05) compared with the chow group. Notably, both GGQLD groups significantly improved the Sirt1 level compared with the HFD group (P<0.01; Fig. 5). Resl also improved the Sirt1 level compared with the HFD group, however this difference was not significant. As demonstrated in Figs. 6 and 7, although the PGC-1α and NF-κB (p65) levels were improved by GGQLD-L, GGQLD-H and Resl, the effect was not significant (P>0.05 vs. HFD group).

Validation of GGQLD effects on Sirt1, PGC-1α and NF-κB gene expression using RT-qPCR. To confirm the action of GGQLD on liver Sirt1, PGC-1α and NF-κB protein expression levels, their gene expression levels were also measured. Sirt1 gene expression in the HFD group significantly decreased compared with that of the chow group (P<0.05; Fig. 8). Notably,
GGQLD-L, GGQLD-H and Resl significantly increased Sirt1 gene expression compared with the HFD group (P<0.05; Fig. 8). As demonstrated in Fig. 9, PGC-1α gene expression in the HFD group decreased slightly compared with that of the chow group, but not significantly (P>0.05). However, a significant increase in PGC-1α gene expression was evident in the GGQLD-H group (P<0.01) compared with the HFD group. Furthermore, NF-κB gene expression in the HFD rat group increased significantly compared with that of the chow group (P<0.01; Fig. 10). Notably, GGQLD and Resl significantly decreased the NF-κB gene expression compared with the HFD group (P<0.01; Fig. 10).

**Discussion**

NAFLD, which is a multi-factorial disorder associated with a variety of genetic and environmental contributory factors, is considered to be the most common cause of liver disease (21). Whether the initial ‘2-hit hypothesis’ or ‘3-hit hypothesis’ is applied in determining the etiology, insulin resistance, oxidative stress and inflammatory cascades are believed to serve integral roles in the pathogenesis and progression (22). The presence of steatosis induced by the overaccumulation of FFA/TGs and cholesterol is closely associated with chronic hepatic inflammation (23), which...
is partly mediated by the activation of the inhibitor of the NF-κB kinase subunit β/NF-κB signalling pathway. In murine models of HFD-induced steatosis, increased NF-κB activity is associated with elevated hepatic expression of inflammatory cytokines, including TNF-α and activation of Kupffer cells (23). PPARγ is one of three subtypes of the PPARs. Its agonists stimulate adipose tissue to absorb and store FFAs, and thereby, inhibits liver fatty acid synthesis (24) by activating adenosine monophosphate (AMP)-activated protein kinase (AMPK). PPARγ not only has anti-inflammatory effects but also effectively improves insulin resistance (25). The activation of PPARγ in the immune system modulates...
the inflammatory response (26). Furthermore, PGC-1α is a key transcriptional regulator of energy homeostasis, and is important in modulating liver fatty acid oxidation (27). The induction of this coactivator has been documented in experimental models of obesity and diabetes (28). The activation of PGC-1α increased the expression of genes that are critical to gluconeogenesis, fatty acid oxidation, lipid transport and mitochondrial biogenesis (29-31).

PGC-1α is not only an important coactivator in liver biology, but may be activated by Sirt1. Sirt1, which is the receptor for Resl, targets numerous proteins, including PPARγ, PGC-1α, uncoupling protein 2 and NF-κB, which serve key roles in various metabolic disorders (32). Furthermore, they lead to the activation of mitochondrial fatty acid oxidation genes. At present, accumulating evidence has indicated an important role for Sirts (33). In total, seven Sirts (Sirt1-7) have been identified in mammals, of which the nuclear-located Sirt1 is the closest homologue of the Sir2 protein, based on a highly-conserved family of nicotinamide adenine

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**Figure 6.** Western blotting for PGC-1α in liver tissue. PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; HFD, high-fat diet; GGQLD-H, Gegen Qinlian decoction high dose; GGQLD-L, Gegen Qinlian decoction low dose; Resl, resveratrol.

**Figure 7.** Western blotting for NF-κB (p65) in liver tissue. NF-κB, nuclear factor-κ-light-chain-enhancer of activated B cells; HFD, high-fat diet; GGQLD-H, Gegen Qinlian decoction high dose; GGQLD-L, Gegen Qinlian decoction low dose; Resl, resveratrol.

**Figure 8.** Reverse transcription-quantitative polymerase chain reaction results for Sirt1 expression in liver tissue. *P<0.05 vs. chow; †P<0.05 vs. HFD. Sirt1, sirtuin 1; HFD, high-fat diet; GGQLD-H, Gegen Qinlian decoction high dose; GGQLD-L, Gegen Qinlian decoction low dose; Resl, resveratrol.

**Figure 9.** Reverse transcription-quantitative polymerase chain reaction results for PGC-1α expression in liver tissue. *P<0.01 vs. chow; †P<0.01 vs. HFD. PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; HFD, high-fat diet; GGQLD-H, Gegen Qinlian decoction high dose; GGQLD-L, Gegen Qinlian decoction low dose; Resl, resveratrol.
lipogenesis and fatty acid β-oxidation, including the aforementioned AMPK, Sirt1 and PGC-1α (55). Resl, a deacetylase, is an indirect activator of Sirt1, which increases the intracellular cyclic AMP (cAMP) concentration by inhibiting cAMP phosphodiesterases that downregulate cAMP (56). Additionally, increased cAMP concentrations activate AMPK, which finally binds to the promoter of PGC-1α, demonstrating its important enzymatic role in regulating cellular energy homeostasis (57).

CHM has been demonstrated to exhibit an extremely beneficial effect on numerous diseases (58-60), and the formulations used have a relatively wide safety. Our previous study (14) demonstrated that GGQLD therapeutically managed NAFLD by improving PPARγ dysregulation, thereby regulating lipids and suppressing inflammation. In the present study, treatment with GGQLD for 8 weeks significantly lowered the liver hepatic aminotransferases (ALT and AST) to a level that was comparable to that of the normal chow-fed control group. Additionally, it was effective in impeding fat infiltration, which was evidenced by the decreased hepatic TC, LDL and lipid droplets. Although the pathogenic mechanisms of NAFLD are still under investigation, fat accumulation, particularly TG filtration into hepatocytes, is considered the first step in the development of NAFLD (1). Hence, lipid accumulation serves a confirmed vital role in NAFLD. The results of the biochemical and histological assays demonstrated that GGQLD-L and Resl reduced the serum LDL-C and TC, respectively. Additionally, the HDL-C levels were raised in the GGQLD-H group while the histological staining revealed that GGQLD-L, GGQLD-H and Resl decreased the lipid droplets in the hepatocytes and normalized the steatosis in HFD rats.

Sirt1, a regulator of PGC-1α that activates PPARγ, induces the transcription of metabolically relevant genes for the oxidation of mitochondrial fatty acids (8). This cascade has a negative regulatory effect on inflammatory processes. The present study demonstrated that GGQLD and Resl evidently improved the Sirt1 protein and gene expression level. Although GGQLD and Resl considerably and significantly decreased NF-xB gene expression, the protein expression demonstrated a declining trend. PGC-1α gene and protein expression in the HFD group was slightly decreased compared to that in the chow group but not significantly. However, an evident increase in the PGC-1α gene expression was observed in the GGQLD-H group compared with the chow and HFD groups. Notably, GGQLD increased the Sirt1 protein and gene expression, with effects that were comparable to those of Resl.

Thus, based on the data of the present study, GGQLD had a positive effect on NAFLD by improving the regulation of Sirt1, which has a critical role in lipid and inflammation regulation, thereby additional experimental evidence was provided to support its clinical use. Since the herbal content of GGQLD has been used for thousands of years in traditional medicine, it is considered relatively safe, reliable and tolerable. In summary, the present study explored GGQLD as a potential optional approach to treat NAFLD by managing lipid metabolism, inflammation and histological abnormalities via the Sirt1 pathway. Further experiments would focus on the intestinal immune response in NAFLD based on the involvement of the gut liver axis using systems biology and omics methods.

**Figure 10. Reverse transcription-quantitative polymerase chain reaction for NF-xB expression in liver tissue.** *P<0.01 vs. chow; †P<0.01 vs. HFD. NF-xB, nuclear factor-κ-light-chain-enhancer of activated B cells; HFD, high-fat diet; GGQLD-H, Gegen Qinlian decoction high dose; GGQLD-L, Gegen Qinlian decoction low dose; Resl, resveratrol.
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