Hepatoprotective Effect of Qushihuayu Formula on Non-Alcoholic Steatohepatitis Induced by MCD Diet in Rat

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Research

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Abstract

Background:

NASH is developed from NAFLD and there is no proven pharmaceutical therapy for patients. Traditional Chinese medicine formula of Qushihuayu(QSHY) shows strong advantage because of their various bioactive compounds in curing NAFLD and NASH.

Methods:

Male Wistar rats with six weeks old received methionine-choline- supplemented diet (MCS) diet for 8 weeks as the blank control. Another 7 rats received MCD diet for 6 weeks first and MCS&MCD (1:1) mixture diet for the last 2 weeks as the model group. QSHY Pre-treatment, low dosage, medium dosage and high dosage groups were received the same diet as the model group. Except for pre-treatment group (1 week in advanced of other groups), all QSHY treatment groups received QSHY formula by gavage every day since the MCD diet started.

Results:

QSHY formula decreased the serum ALT and AST levels, lipid droplets, inflammation foci, FAS and α-SMA protein expression than MCD diet group. MAPK pathways phosphorylation are markedly depressed by QSHY formula. Moreover, QSHY formula enhanced PPAR-γ and p-p65 translocating into nucleus. The administration of QSHY increased hepatic mRNA levels of Transcription Factor 1 alpha (HNF1A), Hepatocyte Nuclear Factor 4 alpha (HNF4A) and Forkhead box protein A3 (FOXA3) which play a pivotal role in Hepatic stellate cell (HSCs) reprogramming.

Conclusion:

These findings suggest that QSHY formula exerts a hepatoprotective effect against steatosis and fibrosis presumably via depressed MAPK pathways phosphorylation, reinforcement of PPAR-γ and p-p65 translocating into nucleus and enhanced HSCs reprogramming.

What Is Already Known About This Subject?

1. Curcumin, the major active compound in Curcuma longa L in QSHY formula, has effect on NASH by regulating PPAR-γ expression.
2. There is no proven pharmaceutical therapy for patients with NASH.

What does this study add?

1. Optimized MCD diet induced NAFLD and NASH model in Wistar rat.
2. Obvious therapeutic effects of QSHY formula on MCD diet induced NAFLD and NASH model in Wistar rat.
3. Multi-effectiveness of QSHY formula on hepatic steatosis, inflammation, fibrosis and cell reprogramming.

**Background**

The rising trend in percentage of obesity is different in developed countries from developing countries. For children's and adolescent's BMI, the rising trend have plateaued in many high-income countries, albeit at high levels, but have accelerated in parts of Asia. For women in United States, class 3 obesity and the prevalence of overall both showed a significant linear trend between 2005 and 2014. However, urbanization in many Asian countries has led to the over-nutrition and sedentary lifestyle in the past two decades, setting the stage for the epidemic of obesity.

With the growing obesity epidemic, non-alcoholic fatty liver disease (NAFLD), a common cause of liver disease, became worldwide prevalence and continues to increase [1]. Nonalcoholic fatty liver disease (NAFLD) defines a spectrum of histological abnormalities, from simple fatty liver to nonalcoholic steatohepatitis (NASH), as these conditions have insulin resistance. NAFLD shares common pathophysiology with other metabolic syndrome that may explain why they frequently coexist. As a result, the patients who suffered from obesity and type II diabetes have high risk to have NAFLD[2].

Although most of NAFLD patients have simple steatosis, 7%-30% develop chronic hepatic inflammation (non-alcoholic steatohepatitis, NASH) associated with cirrhosis, portal hypertension and hepatocellular carcinoma but the causes of progression from NAFLD to NASH is still unclear.

Traditional Chinese Medicine (TCM) is wide studied in NASH therapy. TCM treatment in NASH can improve insulin resistance-a key process in NASH[3]. Furthermore, a review had reported that the liver, spleen and kidney oriented TCM treatment is significantly better than the western medicine treatment in terms of the recovery rate, total effective rate, and liver function. Furthermore, all these effects came with no serious adverse reactions[4].

Qushihuayu (QSHY) formula contains *Curcuma longa* L., *Gardenia jasminoides*, *Hypericum japonicum Thunb.ex Murry*, *Artemisia capillaris Thunb* and *Polygonum cuspidatum Sieb.et Zucc*. The chemistry includes curcuminoids and sesquiterpenoids as components, which are known to have anti-oxidative, anti-carcinogenic, and anti-inflammatory activities. Results from T Nishiyama’s research indicated that both curcuminoids and sesquiterpenoids exhibit hypoglycemic effects via PPAR-gamma activation as one of the mechanism[5]. Artemisia capillaris Thunb, aqueous extraction, have effect on lipopolysaccharide-induced inflammatory response by preventing NF-κB activation in rat liver[6]. Gardenia jasminoides also have hepatoprotective effect in bile duct ligated rats and hepatic stellate cells[7]. Total aqueous extract of Hypericum japonicum can decrease AST and ALT levels in serum in CCl4-induced liver injury in mice[8]. Huzhang, the root of *Polygonum cuspidatum Sieb. et Zucc.*, is widely used in anti-cancer, anti-inflammatory [9]and anti-oxidative due to the major bioactive compound resveratrol and polydatin.
Major approaches to NASH induction can be classified as follows: (1) Genetic approach; (2) nutritional approach; (3) a combination of genetic factors with others such as nutritional factors, oxidative stress, and drugs[10]. MCD diet is one of the widely-used methods to build NASH model. As early as 3 days after feeding MCD diet, the mice may develop hepatic inflammation. Severe pericentral steatosis may occur by 1 to 2 weeks and necroinflammation may develop after 2 weeks. Oxidative stress can be observed from 3 weeks after intake of the MCD diet. But the development and severity of MCD induced NASH in rodents may depend on the gender, strain, and species used. It is slower in Sprague-Dawley rats than in other rodents to develop steatohepatitis induced by MCD diet. The MCD diet induced NASH model is one of the best established models to study the evolution of inflammation, oxidant stress and fibrotic changes because this model is easily established and severer than other nutritional models[11].

The aim of our research was to investigate the hepatoprotective effect of QSHY formula in MCD diet induced non-alcoholic fatty liver disease (NAFLD) and Non-alcoholic steatohepatitis (NASH) in rat model.

**Methodology**

**Animal**

6 weeks-old male Wistar rats (200–280 g), were housed on 12 hours light/12 hours dark cycling. The environment was well controlled by temperature and humidity with free access to food and water. Rats were received either methionine-choline-deficient (MCD) diet or methionine-choline-supplemented (MCS) diet as normal diet. MCD diet and MCS diet were supplied by Trophic Animal Feed High-Tech Co., Ltd., Nantong, China. Treatment was sustained for 8 weeks. All animal experiments were performed according to the protocols approved by Animal Research Ethics Committee of University of Macau (UMARE-001-2017). All rats were divided into 7 groups randomly as showing in Table 1. Food intake were measured every week. All rats were killed for blood collection and tissue sampling after 8 weeks treatment.

**Table 1**

| Low dosage group | Medium dosage group | High dosage group | MCD group |
|------------------|---------------------|-------------------|-----------|
| Pre-treatment    | Normal diet         | MCD diet          | MCS : MCD=1:1 |
| 0 week           | 1st week            | 6th week          | 8th week  |
| 1st week         | 7th week            |                   |           |
|......             |                     |                   |           |

**QSHY formula**

QSHY formula was provided by Shanghai Sunrise Traditional Chines Medicine Co. Ltd., Shanghai, China. The powder of the formula was dissolved in water freshly and kept in 50°C water bath before gavage. Three dosages of QSHY formula (Low: 0.29 g/kg, medium: 0.57 g/kg, high: 1.14 g/kg, pre-treatment: 0.57 g/kg) were gavage to rats everyday.
Biochemical analysis

Blood were collected from tail vein into 1.5 ml EP tube every two weeks after fasting overnight. Then the blood were centrifuged at 3000 rpm at 4 ℃. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol (TCHO) and triglycerides (TG) were detected to show the levels of liver injury.

Histopathological analysis

Steatohepatitis was observed by histopathological section. In all experiment group, 6-µm-thick sections of the liver samples which were fixed by 4% Paraformaldehyde (PFA) and embedded by paraffin, were due to hematoxylin and eosin staining (H&E) for analyzing the degree of inflammation and hepatic steatosis.

mRNA extraction and real-time Polymerase Chain Reaction (RT-PCR)

Livers were weighted in 2 mL tube and TRIZOL (Invitrogen, 100 mg:100 mL) were added into tubes. RNA extraction protocols followed by the instruction. RNA concentration and quality were measure by Nanodrop. 2 ng RNA were used in cDNA synthesize using a cDNA Reverse Transcription Kit. The primer for qPCR was shown in Table 2. The threshold cycle (Ct), the cycle number at which the amounts of amplified genes of interest reached a fixed threshold, was determined. Relative expression of the RT-PCR product was calculated by using the comparative $2^{-\Delta\Delta Ct}$ method. The endogenous control β-actin was used for normalization.

| Target genes | Forward sequence 5'-3' | Reverse sequence 5'-3' |
|--------------|------------------------|-----------------------|
| PPAR-γ       | CCCTGGCAAAGCATTTGTAT   | ACTGGCACCCTTGAAAAATG  |
| β-actin      | AGCCATGTACGTAGCCATCC   | CTTCAGCTGTGGGTGGTGAA  |
| FOXA3        | GACTCATGCCAAACCACCTT   | TCATTGAAGGACACGCGAGT  |
| HNF1A        | CAGCCAAACCCATTCACATC   | GCCATCTGGGTGGATAAA    |
| HNF4A        | AAATGTGCAGGTGTTGACCA   | CACGCTCCTCCTGAAGAATC  |

Western blotting

Protein expression was detected and measured by Western blotting assays. The liver samples were homogenized with RIPA containing phosphate and protease inhibitors using a homogenizer for 5 min and then incubated for 20 min on ice. Then the lysed samples were centrifuged at 12,000 g. The supernatant were collected and protein concentration was measured with Invitrogen BCA Protein Assay Kit. Proteins were denatured by boiling at 99℃ for 5 min with loading buffer. The denatured proteins were separated by using 12% SDS-PAGE. After that, proteins were transfer from the gel to polyvinylidene fluoride membranes. 5% milk diluted with TBST buffer was used to block the nonspecific binding site for 1 h at room temperature. Primary antibodies were then applied and incubated at 4℃ overnight. After
incubation, the membranes were washed three times for 5 min each with TBST buffer and then incubated with appropriate HRP-conjugated secondary antibody for 60 min at room temperature. The protein bands were visualized with chemiluminescent reagents and quantified using Image J software. β-actin was used as reference protein.

Nuclear protein extraction protocols followed the guide of Beibo company which were used in our research.

Statistical analysis

Data are presented as means ± SD. One-way ANOVA followed by Kruskal-Wallis’s multiple comparison tests were used to compare the differences between the groups. *P*-value < 0.05 was considered as statistically significant.

Results

Bodyweight, ratio of liver weight to bodyweight, Serum level of TC, TG ALT and AST

The body weights (Fig. 1A) and the ratio of liver weight to body weight (Fig. 1B) showed no significant changes with QSHY treatment group. Additionally, QSHY formula increased the serum level of TC (Fig. 1C) and TG (Fig. 1D). In order to assess the liver function, the serum levels of ALT and AST enzymes were determined. As they could be observed in Fig. 1E and Fig. 1F, at the second week and sixth week, the serum level of ALT decreased significantly comparing High group with MCD diet group. At second week, the serum level of AST decreased significantly when comparing High group with MCD diet group as shown in Fig. 1F. However, the serum level of AST increased significantly when comparing Low group, Medium group with MCD diet group at fourth week and eighth week. But at sixth week, the serum level of AST decreased significantly in QSHY treatment group compared with MCD diet group. ALT is chemical that the liver uses to make glycogen. AST is found in a variety of tissues, including the liver, brain, pancreas, heart, kidneys, lungs, and skeletal muscles. If any of these tissues are damaged, AST will be released into the bloodstream[12, 13]. While increased AST levels are indicative of a tissue injury, it is not specific to the liver per se[14, 15]. By contrast, ALT is found primarily in the liver. Any elevation of the ALT is a direct indication of a liver injury, whether minor or severe. For the pretreatment group, the serum level of ALT and AST were all decreased indicating that pretreatment showed more effective in our research.

QSHY formula improves liver injuries induced by MCD diet

As shown in Fig. 2 and Table 2, no pathological changes were observed in the MCS diet group and histologically livers appeared normal. However, the MCD diet led to grade 5 liver steatosis and lobular inflammation. The QSHY treated rats liver displayed less ratio of steatosis and fewer inflammation foci.
Table 1

Summary of histopathological lesions in the liver

| Group       | Steatosis | Inflammatory cells |
|-------------|-----------|--------------------|
| MCS Diet    | 0 ± 0     | 0 ± 0              |
| MCD Diet    | 4 ± 0###  | 2.29 ± 0.49###     |
| Pre-treatment | 3 ± 0.53*** | 1 ± 0.53***      |
| Low         | 2.875 ± 0.33*** | 1.38 ± 0.52**    |
| Middle      | 2.75 ± 0.46*** | 1 ± 0.53***      |
| High        | 2.625 ± 0.52*** | 0.25 ± 0.46***   |

1 Means±SD are shown (* P < 0.05 vs MCD diet, **P < 0.01 MCD diet, ***P < 0.001 MCD diet, ### P < 0.001 vs MCS diet)

2 These values are averaged from the grading score of steatosis, which was graded 0–4 based on the average percent of fat-accumulated hepatocytes per field at × 100 magnification under H&E staining (Grading 0 = < 5%, 1 = 5 ~ 25%, 2 = 26 ~ 50%, 3 = 51 ~ 75%, 4 = > 75%)

3 Overall assessment of all inflammatory foci per field at × 200 magnification under H&E staining, which was graded 0–3 (Grading 0 = 0, 1 = < 2, 2 = 2–4, 3 = > 4)

**QSHY formula decreased FAS protein expression without effects on SREBP-1c protein expression**

Fatty acid synthase (FAS) and Sterol regulatory element-binding transcription factor 1 (SREBP-1c) have important function in lipid metabolism. As shown in Fig. 3, no significant changes in SREBP-1c expression was observed with QSHY treatment except pre-treatment group, whereas obvious changes were observed in FAS. SREBP-1c transduces the insulin signal but it also participates to the hepatic steatosis observed in humans and related to alcohol consumption and hyperhomocysteinaemia which is insulin-independent SREBP-1c activation[16]. FAS expression is regulated by SREBP1c at the transcriptional level[17]. QSHY formula can only affect FAS protein expression without influence on SREBP-1c protein expression.

**QSHY formula decreased α-SMA protein expression**

Results of western blot analysis for α-SMA (Fig. 3D), a biomarker for fibrosis, indicated that QSHY formula decreased α-SMA protein expression obviously comparing with MCD diet group.

**The effect of QSHY formula on MAPKs pathway**
To investigate the potential mechanism of QSHY formula’s effect on NASH and NAFLD, MAPKs pathway including p38, ERK and JNK were all measured and results were shown in Fig. 4. High dosage of QSHY formula showed strong effect on suppressing p38, ERK and JNK phosphorylation.

**QSHY formula affected PPAR-γ and p-p65 translocated into nucleus**

The expression level of PPAR-γ in mRNA and protein were both measured (Fig. 5). Real-time PCR analysis showed that QSHY formula increased PPAR-γ mRNA expression level comparing with MCD group (Fig. 5D). PPAR-γ protein expression level were increased in nucleus and decreased in whole cell relatively with QSHY treatment (Fig. 5A-C). These results indicated QSHY formula can enhanced PPAR-γ mRNA expression and translocated into nucleus. The expression of phospho-NF-kappaB p65 (p-p65) in nucleus was decreased significantly by QSHY formula suggesting its effect on enhancing NF-κB pathway activation.

**The effect of QSHY formula on HNF1A, HNF4A and FOXA3 mRNA expression**

HNF1A, HNF4A and FOXA3 play a pivotal role in HSCs reprogramming[18]. These three genes mRNA expression had been tested using Real-time PCR (Fig. 5G-I). Pre-treatment of QSHY formula enhanced these three genes mRNA expression and high dosage of QSHY only increased HNF4A mRNA expression obviously. All these results suggesting that QSHY formula improved liver fibrosis partly by enhancing HSCs reprogramming.

**Discussion**

As the worldwide popular of obesity, its related diseases including type II diabetes disease, NAFLD, NASH, hyperglycemia and so on. NASH is characterized by sever inflammation and steatohepatitis and there is no established therapy for NASH. TCM due to their multiply active compounds and multiple drug targets become the potential therapy for NASH.

Optimizing MCD diet induced NASH model experiment were done ahead of QSHY formula treatment. According to present results including serum level of TC, TG, ALT, AST and H&E staining, we found that even though the rat received MCD diet for 6 weeks and then changed to MCS diet in the last two weeks, hepatic steatosis and inflammation were not severe. Therefore, considering the limitation of the MCD model of inhibiting the lipid exporting from the liver, a mixture diet which was made up with the ratio of MCS: MCD was 1:1 was applied in the last two weeks.

In our research, QSHY formula decreased serum level of ALT and AST, hepatic fat content, size of lipid droplet, the number of inflammation foci and α-SMA expression. We discuss some probable mechanism below according our present results.
FAS catalyzes the synthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA[19]. FAS is responsible for producing lipids in the liver for exporting to metabolically active tissues or storage in adipose tissue while MCD diet can block the lipid exportation[20]. This may explain all rats received MCD diet showed lower expression of FAS. But after changing MCD diet to mixture of MCS & MCD diet, QSHY formula enhanced FAS expression that may partly explain the reduced lipid synthesis in liver. Increased interest has focused on FAS as a potential target for the diagnosis and treatment of metabolic syndrome[21, 22].

α-SMA positive cells are normal appeared in vascular walls of the central vein and portal tract but they are also observed in fibrotic area. α-SMA is a good marker of myofibroblast and its appearance in liver mesenchymal cells seems closely related to the process of hepatic fibrosis[23]. Hepatic fibrosis is a symbol of NASH and QSHY formula inhibited hepatic α-SMA protein expression suggesting its anti-fibrosis effect. Chronic inflammation is a major causes of most kinds of fibrosis including liver fibrosis[24, 25]. The mitogen-activated protein kinase (MAPK) signaling pathways (ERKs: extracellular-signal-regulated kinases; JNKs: Jun-amino-terminal kinases; P38/SAPKs: stress-activated protein kinases) are activated by a variety of extra and intracellular stimuli including cytokines, growth factors, and hormones. These pathways play critical roles in the regulation of many cellular processes including proliferation, differentiation, the stress response, motility, growth, differentiation, survival, and death. QSHY formula suppressed ERK, P38 and JNK phosphorylation in our research. An excess of non-ester fatty acids (NEFAs) existed lipotoxicity induced excessive ROS which overactivated the hepatic JNK and p38MAPK pathway and then impaired insulin signaling pathway in patients with NASH[26]. In vitro research in transformed human proximal tubular epithelial cells from Niculescu-Duvaz I suggested that TGF-β1 induced fibronectin secretion appeared to be reliant on the p38 MAPK pathway[27]. QSHY formula therapy in our research effectively suppressed ERK, JNK and P38 proteins phosphorylation that may partly explained the possible mechanism of its hepatoprotective effect.

Transcription factors of the nuclear factor κB (NF-κB)/Rel (p65) family play a important role in inflammatory and immune responses[28]. After NF-κB translocating into nuclear, its downstream genes expression such as IL-6, TNF-α, MCP1 will be increased. Detecting the phosphorylated p65 (p-p65) in nuclear is widely used to investigate whether the NF-κB pathway is activated. QSHY formula suppressed p-p65 translocated into nuclear obviously suggesting that the effect of QSHY formula on NASH may be partly via suppressing NF-κB pathway activation.

Peroxisome proliferator-activated receptor-gamma (PPAR-γ) is a transcription factor which regulates lipid metabolism and inflammatory responses. PPAR-γ deficient (PPAR-γ +/-)mice fed the MCD diet developed more severe steatohepatitis than wild-type mice and PPARγ activation suppressed hepatic lipoperoxide [29]. The content of PPAR-γ in nucleus was obviously increased in QSHY formula treatment group indicating QSHY formula may enhance PRAR-γ nuclear translocation to improve NASH in our research.
Hepatic stellate cells (HSCs) are resident mesenchymal cells that keep features of resident fibroblasts and pericytes and comprise 15% of total resident cells in normal human liver. Quiescent HSCs are activated then they transdifferentiate into proliferative, migratory, and contractile myofibroblasts, manifesting pro-fibrogenic transcriptional and secretory properties. This process can be reversed and forkhead box protein A3 (FOXA3), transcription Factor 1 (HNF1A), Hepatocyte Nuclear Factor 4 alpha (HNF4A) play important roles in HSCs reprogramming process\[30\]. QSHY formula enhanced these three genes mRNA expression indicating the anti-fibrosis effect of QSHY formula may be involved reinforced HSCs reprogramming.

**Conclusion**

These findings suggest that QSHY formula exerts a hepatoprotective effect against steatosis and fibrosis presumably via depressed MAPK pathways phosphorylation, reinforcement of PPAR-γ and p-p65 translocating into nucleus and enhanced HSCs reprogramming. These pathways perform a probable therapeutic target for steatosis and NASH in drug development.

**Abbreviations**

ANOVA Analysis of variance  
ALT Alanine aminotransferase  
AST aspartate aminotransferase  
BMI Body Mass Index  
Ct Threshold cycle  
EMG Electromyography  
ERK Extracellular-signal-regulated kinases  
FASN Fatty Acid Synthase coding gene  
FOXA3 Forkhead box protein A3  
HNF1A Transcription Factor 1 alpha  
HNF4A Hepatocyte Nuclear Factor 4 alpha  
HSCs Hepatic stellate cells  
JNK Jun-amino-terminal kinases  
MAPK Mitogen-activated protein kinase
MCD methionine-choline-deficient diet
MCS methionine-choline-supplemented diet
NAFLD Nonalcoholic fatty liver diseases
NASH Non-alcoholic steatohepatitis
NF-κB Nuclear factor κB
OCT Optimal cutting temperature compound
p38 P38 mitogen-activated protein kinases
p-p65 Phosphorylated p65
PPAR-γ Peroxisome proliferator-activated receptor-gamma
PGC-1α Peroxisome proliferator-activated receptor gamma co-activator 1-alpha
qPCR Quantitative polymerase chain reaction
QSHY Qushihuayu
RT-PCR Real-time polymerase chain reaction
SD. Standard Deviation
SREBP1-c Sterol regulatory element binding transcription factor 1 c
TCM Traditional Chinese Medicine
TCHO total cholesterol and
TG total triglycerides

Declarations

- Ethics approval and consent to participate
  Yes

- Consent to publish
  Yes

- Availability of data and materials
Yes

- Competing interests

Simon Ming-Yuen Lee declares that he has no conflict of interest.

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- Authors’ Contributions

Qingping Lan: Study conception and design; Acquisition of data; Analysis and interpretation of data; Drafting of manuscript.

Zhitao Ren: Study conception and design; Acquisition of data; Analysis and interpretation of data; Drafting of manuscript.

Yan Chen: Acquisition of data; Analysis and interpretation of data.

Guozhen Cui: Study conception and design.

Cheong Choi: Drafting of manuscript.

Hon Ho Yu: Study conception and design.

Simon Ming-Yuen Lee: Study conception and design; Drafting of manuscript; Critical revision.

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