

Effects of shikonin from *Zicao* on high-fat diet-induced nonalcoholic fatty liver disease in rats

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Abstract: In this study, we aim to investigate whether shikonin prevents against NAFLD. After feeding high-fat diet (HFD) for 10 weeks, Sprague-Dawley rats were received different doses of shikonin (5mg/kg/day, 10mg/kg/day and 20mg/kg/day) by gavage for the last 12 weeks of a total of 22 weeks of a HFD. Our results showed that total cholesterol (TC), triacylglycerol (TG), low-density lipoprotein cholesterol, aspartate aminotransferase and alanine aminotransferase were significantly increased, while high-density lipoprotein cholesterol was decrease, accompanied by hepatic injury and lipid accumulation in HFD-fed rats. Shikonin treatment attenuated the above biochemical and histopathological changes. Similarly, HFD-induced the increase of hepatic TC and TG levels were also ameliorated by shikonin treatment. Furthermore, shikonin observably mitigated HFD-induced the liver fibrosis and the increase of plasminogen activator inhibitor type 1, connective tissue growth factor, collagen III and IV expression. Additionally, shikonin markedly inhibited HFD-induced the decrease of proliferator-activated receptor γ (PPAR γ) and matrix metalloproteinases-9 (MMP-9) expression and the increase of tissue inhibitor of metalloproteinases-1 (TIMP-1) expression in liver tissue. This study demonstrates that shikonin ameliorates hepatic lipid dysregulation and fibrosis through PPAR γ and MMP-9/TIMP-1 axis, suggesting that shikonin may be a potential therapeutic agent for the treatment of NAFLD.

Keywords: Nonalcoholic fatty liver disease, lipid accumulation, liver injury, fibrosis, shikonin.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), characterized by accumulation of lipids within hepatocytes, is the most common forms of liver disease not due to excess alcohol consumption and is consider as the hepatic manifestation of metabolic syndrome (Marchesini *et al.*, 2001; Ray 2013; Marchesini and Marzocchi 2007). Clinical researches demonstrate that NAFLD develops from simple steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis and even hepatocellular carcinoma (Fabbrini *et al.*, 2010; Song *et al.*, 2013) Approximately 10%-29% of patients with NASH will develop to cirrhosis and liver cancer within a 10-year period (Angulo 2002). Furthermore, previous studies indicate that NASH and NAFLD confer an increased risk of cardiovascular disease (Scorletti *et al.*, 2011; Anstee *et al.*, 2013). Unfortunately, there is no effective pharmacological agent currently available for the treatment of NAFLD, although some methods have been suggested for treating NAFLD include exercise, rational diet and medicines (fibrate, statins and metformin) (Harrison and Day 2007; Neuschwander-Tetri *et al.*, 2010). Thus, it is extremely important to identify effective regimens for the treatment of NAFLD.

Zicao, a traditional Chinese herbal plant, belongs to the Boraginaceae perennial herbs and has been widely used for many years for the treatment of burns, carbuncles, measles, macular eruptions and sore throats (Papathanasiou *et al.*, 1999; Chen *et al.*, 2002; Kim *et al.*,

2014). Shikonin is one of the main natural naphthoquinone derivatives of *Zicao*. Later studies about cancer researches revealed that shikonin inhibited tumor growth in lung and prostate cancers by attenuating VEGF-induced angiogenesis (Gaddipati *et al.*, 2000; Lee *et al.*, 2008). Notably, previous studies showed that another naphthoquinone derivative of *Zicao*, acetylshikonin, was effectively to ameliorate rat obesity induced by high-fat diet (HFD) through attenuating lipid dysregulation and inflammation (Su *et al.*, 2016; Su *et al.*, 2016), raising the intriguing possibility that *Zicao* may be beneficial for the treatment of NAFLD. In the present study, we aim to investigate the potential therapeutic effects of shikonin in preventing against hepatic lipid dysregulation and injury in a model of HFD-induced NAFLD rats.

MATERIALS AND METHODS

Materials

Shikonin (purity>98%) was purchased from Wuhan Tianzhi Biotechnology Co. Ltd (Wuhan, China). Kits for determining serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were purchased from Jian Cheng Biological Engineering Institute (Nanjing, China). Antibodies against proliferator-activated receptor γ (PPAR γ), matrix-metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinase-1 (TIMP-1) and GAPDH were purchased from Santa Cruz Biotechnology Inc. (CA, USA).

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Animal

Fifty male Sprague-Dawley rats (8-week old, 200-250g) were obtained from Jackson Laboratories (CA, USA). All rats were housed in cages at controlled temperature and humidity with free access to water and the diet corresponding to their assigned treatment group. All rats were randomized to five groups (each group n=10): normal control group (control), HFD group (NAFLD), low-dose shikonin treatment group (SKN-L), medium-dose shikonin treatment group (SKN-M), high-dose shikonin treatment group (SKN-H). The rats in control group were fed with a standard diet, and the other four groups were fed with a HFD (45% kcal fat, D12451, Research Diets, Inc., NJ, USA). 10 weeks later, the rats in SKN-L group, SKN-M group and SKN-H group were intragastrically administrated with shikonin at a dose of 5mg/kg/day, 10mg/kg/day and 20mg/kg/day for another 12 weeks (a total of 22 weeks of a HFD), respectively. In HFD group, rats were induced by intragastric administration of 0.1ml/100g physiological saline for 12 weeks. All animal procedures of this study were approved by the Institutional Animal Care and Use Committee and carried out in strict accordance with the Institutional Animal Care and Use Committee of Zhejiang Hospital.

Determination of serum biochemistry

Blood samples were collected from the abdominal vena cava at the end of the experiments, and centrifuged for 10 min at 3000 × g to obtain serum. TC, TG, HDL-C, LDL-C, AST and ALT levels in serum were determined with commercially available kits according to the instructions of the kits.

Histopathological examination

Following the 12-week treatment of shikonin, rats were sacrificed and the livers were fixed in 4% paraformaldehyde, dehydrated with ethanol and xylene, embedded in paraffin, and cut into 5-μm slides on a microtome (SLEE, Mainz, Germany). Slides were stained with hematoxylin-eosin, oil Red O and masson trichrome. All slides were analyzed under an optical microscope (CKX41, Olympus, Japan).

Determination of biochemistry in liver tissues

At the end of the experiment, rat livers were harvested. Liver homogenates were prepared in a 10-fold volume (v/w) anhydrous alcohol, followed by centrifugation at 12000 × g for 15 min at 4°C. The supernatant was collected for TC and TG determination according to the same method in plasma measurement. The protein concentrations of liver tissues were quantified by the Enhanced BCA Protein Assay Kit (Beyotime, Jiangsu, China). Hepatic TC and TG levels were normalized to the content of total proteins of each sample.

Western blotting

The rat livers were lysed in RIPA buffer (Beyotime) containing 1% protease inhibitor cocktail (Roche,

Mannheim, Germany). The homogenate was centrifuged at 12000×g for 10 min at 4°C and then the supernatant was harvested. Protein concentrations were analyzed using the Enhanced BCA Protein Assay Kit. Equal amounts of protein (50μg) were subjected to SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). The membranes were subsequently treated with the appropriate antibodies against the following proteins: PPARγ, MMP-9, TIMP-1 and β-actin (dilution 1:1000). Blots were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (dilution 1:2000). The signals were determined using ECL kit (Beyotime) and the intensity of the protein bands was analyzed by Image J software (Version 1.41, NIH, MD, USA).

Real-time quantitative PCR

Total RNA was extracted from liver tissues using Trizol reagent (Invitrogen, CA, USA) according to manufacturer's instructions. Complimentary DNA was synthesized through reverse transcription of RNA using the Super Script III First-Strand Synthesis system (Qiagen, CA, USA). Real-time quantitative PCR was performed using Fast SYBR® Green Master Mix Kit (Applied Biosystems, CA, USA) with an ABI Prism 7300 Fast Real-Time PCR system (Applied Biosystems). The specific primer sequences were synthesized and provided by the Shanghai Biological Engineering Technology Services Co. Ltd. (Shanghai, China): plasminogen activator inhibitor type 1 (PAI-1) sense 5'-AGCTTTGT GAAGGAGGACCG-3' and antisense 5'-CAGGGATGC AGACCCCAAAT-3'; connective tissue growth factor (CTGF) sense 5'-GCCCCCTAGTCTCACAC-3' and antisense 5'-GTCACGCTCCGTACACAGTT-3'; collagen III sense 5'-ACGTAAGCACTGGTGGACAG-3' and antisense 5'-GGAGGGCCATAGCTGAAGT-3'; collagen IV sense 5'-GGGGTCGGGCTGGGAGTGAT-3' and antisense 5'-GCTGGCCGTCCATACCCGTG-3'; PPARγ sense, 5'-GAGGATCCCCGGGGTACCGG ATGACCATGGTT GACACAG-3' and antisense, 5'-ACATTCCACAGTTAGCTAGCTAAGCATAGTCTGGG ACATCATAAGGGTA-3'; MMP-9 sense, 5'-TTTGACA GCGACAAGAAGTGG-3' and antisense, 5'-TCCCATC CTTGAACAAATACA-3'; TIMP-1 sense, 5'-CCTTCTG CAATTCCGACCTC-3' and antisense, 5'-CGGGCAGG ATTCAGGCTAT-3'; GAPDH sense, 5'-GCCATCGTCAC CAACTGGGAC-3' and antisense, 5'-CGATTTCCCGCT CGGCCGTGG-3'. Reaction conditions: denaturation: 95°C, 1 min; denaturation: 95°C, 15s; annealing: 60°C, 30 s; extension: 72°C, 30s, with a total of 40 cycles. Target gene expression ($2^{-\Delta\Delta C_t}$) was normalized to endogenous GAPDH expression.

STATISTICAL ANALYSIS

All parameters are expressed as the mean ± SD. Data analysis was conducted with SPSS 19.0 software package (Analytical Software, USA). The results were statistically

Table 1: Body weight, ALT and AST in control and experimental groups.

Parameters	control	NAFLD	SKN-L	SKN-M	SKN-H
body weight (g)	348.7±6.6	431.8±10.2*	403.6±9.6 [#]	371.3±10.7 [#]	357.4±9.9 [#]
ALT (IU/L)	27.10±3.20	89.80±5.40*	76.50±4.10 [#]	61.20±3.20 [#]	50.40±4.30 [#]
AST (IU/L)	14.90±3.80	62.80±4.80*	52.40±3.70 [#]	41.20±3.60 [#]	28.40±2.90 [#]

Data are represented as mean ± SD. ALT, alanineamino transferase; AST, aspartate aminotransferase. * $p < 0.05$ versus control group; [#] $p < 0.05$ versus NAFLD group, n=10 rats per group.

Table 2: The serum lipid profiles in control and experimental groups.

Parameters	control	NAFLD	SKN-L	SKN-M	SKN-H
TC(mmol/L)	1.31±0.14	2.73±0.24	2.34±0.18	2.01±0.13	1.67±0.15
TG(mmol/L)	0.91±0.04	1.63±0.14*	1.34±0.09 [#]	1.21±0.08 [#]	1.11±0.07 [#]
HDL-C(mmol/L)	1.11±0.09	0.73±0.06*	0.84±0.07 [#]	0.91±0.06 [#]	0.96±0.06 [#]
LDL-C(mmol/L)	0.53±0.04	0.78±0.07*	0.71±0.06 [#]	0.66±0.05 [#]	0.61±0.04 [#]

Data are represented as mean ± SD. TC, total cholesterol; TG, triglycerides; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; * $p < 0.05$ versus control group; [#] $p < 0.05$ versus NAFLD group, n=10 rats per group.

analyzed by one-way ANOVA, followed by Tukey's multiple comparison test. The criterion for significance was $p < 0.05$.

RESULTS

Effects of shikonin on body weight and biochemical parameters

At the end of the experimental treatment, the HFD-treated rats gained a higher body weight than those of control rats, which was gradually decreased by shikonin treatment in a dose-dependent manner. Administration with shikonin significantly decreased the levels of serum ALT and AST in HFD-fed rats (table 1). To investigate the effect of shikonin on lipid profiles, the serum TC, TG, HDL-C and LDL-C levels were examined. As shown in table 2, the serum TC, TG and LDL-C levels in HFD-fed rats were higher than control rats ($P < 0.05$ for TG and LDL-C). However, 12-week treatment with shikonin was associated with reduced levels of the above biochemical parameters. Moreover, the level of serum HDL-C in HFD-fed rats was lower than in control rats, and this decrease was dramatically inhibited by 59.5% after shikonin treatment at the dose of 20mg/kg/day.

Shikonin ameliorated HFD-induced liver injury and lipid accumulation

The histopathological examination of the liver tissue samples certified the results obtained from the biochemical tests. Histopathological findings showed disordered arrangement of the hepatic lobules and fatty degeneration of the hepatocytes in HFD-fed rats compared with control rats. However, these changes were noticeably attenuated by shikonin (fig. 1A). Similarly, oil red O staining revealed that the shikonin treatment also decreased the deposition of lipid droplets in hepatocytes induced by HFD (fig. 1B). Consistent with the changes of plasma lipids, the hepatic TC and TG levels were both increased after HFD treatment, while shikonin

administration was associated with reduced hepatic TC and TG levels (fig. 1C and D).

Shikonin treatment attenuated HFD-induced liver fibrosis

Masson trichrome staining showed increased deposition of collagen fibrils in liver tissues of HFD-fed rats compared with control rats. However, shikonin diminished the deposition of collagen in a dose-dependent manner (fig. 2A). To further confirm the effect of shikonin on liver fibrosis, we examined the expression of PAI-1, CTGF, collagen III and IV, which are well known as important factors of hepatic fibrosis. Real-time quantitative PCR showed that HFD-induced the increase of PAI-1, CTGF, collagen III and IV expression were significantly inhibited after shikonin challenge. These results suggest that shikonin can effectively suppress liver fibrosis in NAFLD rats (fig. 2B-F).

Effects of shikonin on hepatic PPAR γ , MMP-9 and TIMP-1 expression

To elucidate the potential mechanism by which shikonin attenuated lipid accumulation and liver fibrosis during NAFLD, the expression of PPAR γ , MMP-9 and TIMP-1 were examined. Western blotting revealed that HFD insult significantly decreased the expression of PPAR γ and MMP-9, while increased the expression of TIMP-1. The above changes of protein expression were all inhibited after shikonin treatment (fig. 3A-C). To further confirm these findings, we measured the mRNA expression of PPAR γ , MMP-9 and TIMP-1, which were consistent with the above results (fig. 3D-F).

DISCUSSION

The aim of the present study was to investigate the effects of shikonin on HFD-induced NAFLD. NAFLD is considered as a critical hepatic manifestation of metabolic syndrome. The HFD model has been widely used to

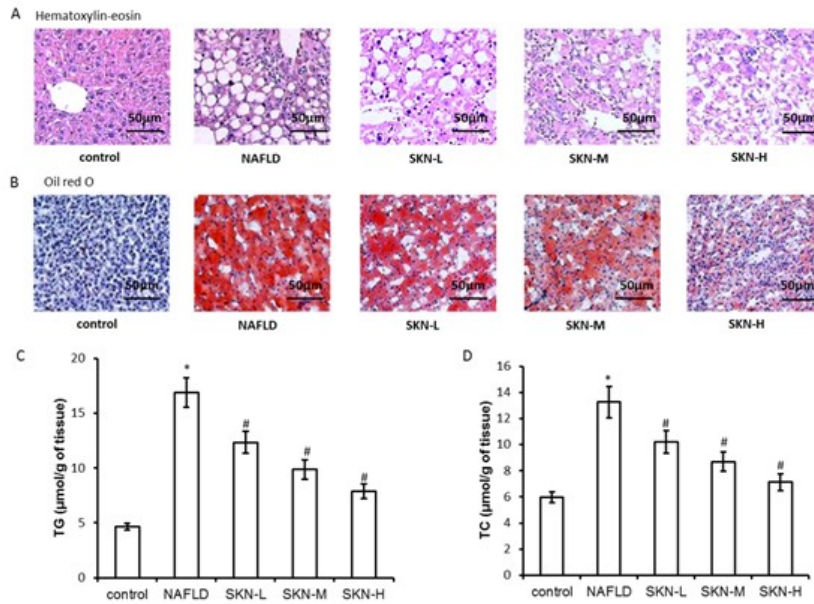


Fig. 1: Effect of shikonin on HFD-induced liver injury and lipid accumulation. (A and B) NAFLD was induced by feeding rats for 22 weeks with HFD. After feeding HFD for 10 weeks, the rats were administrated with different doses (5mg/kg/day, 10mg/kg/day and 20mg/kg/day) of shikonin (SKN) for the last 12 weeks of a total of 22 weeks of HFD. Hematoxylin-eosin staining (A) and oil red O staining (B) of liver sections (magnification, 200 \times). (C and D) TG (C) and TC (D) of liver tissues after HFD or treatment with different doses of shikonin. * $p < 0.05$ versus the control group; # $p < 0.05$ versus the NAFLD group, n=6 rats per group.

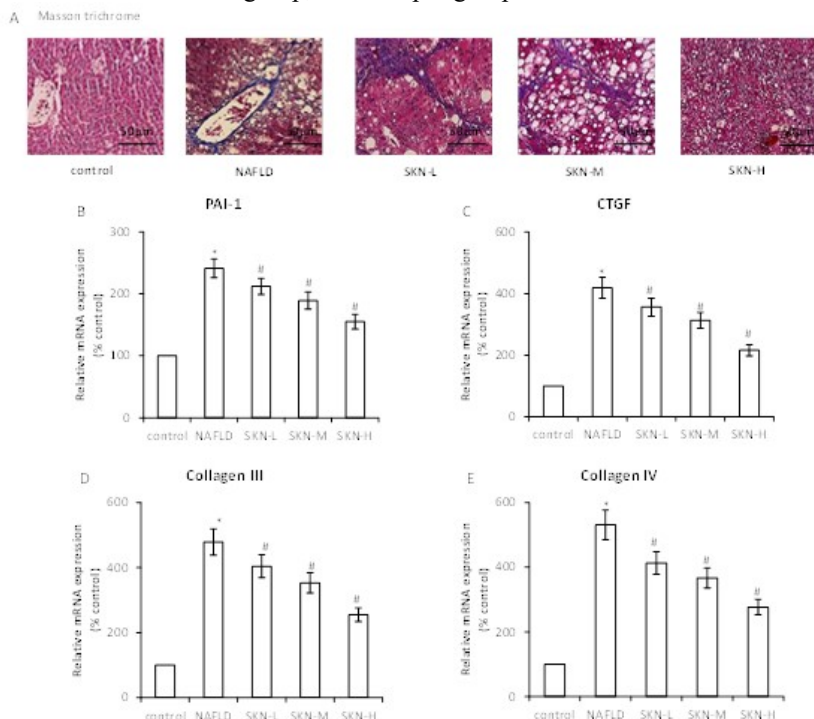


Fig. 2: Effect of shikonin treatment on HFD-induced liver fibrosis. (A) Masson trichrome staining of liver sections (magnification, 200 \times). Representative images of liver tissues isolated from control group, NAFLD group, SKN-L group (5mg/kg/day), SKN-M group (10mg/kg/day) and SKN-H group (20mg/kg/day) are provided. (B-E) Quantitative PCR showed increased PAI-1 (B), CTGF (C), collagen III (D) and collagen IV (E) mRNA expression in the liver tissues from HFD-induced NAFLD rats, which were inhibited after the treatment of shikonin. * $p < 0.05$ versus the control group; # $p < 0.05$ versus the NAFLD group, n=8 rats per group.

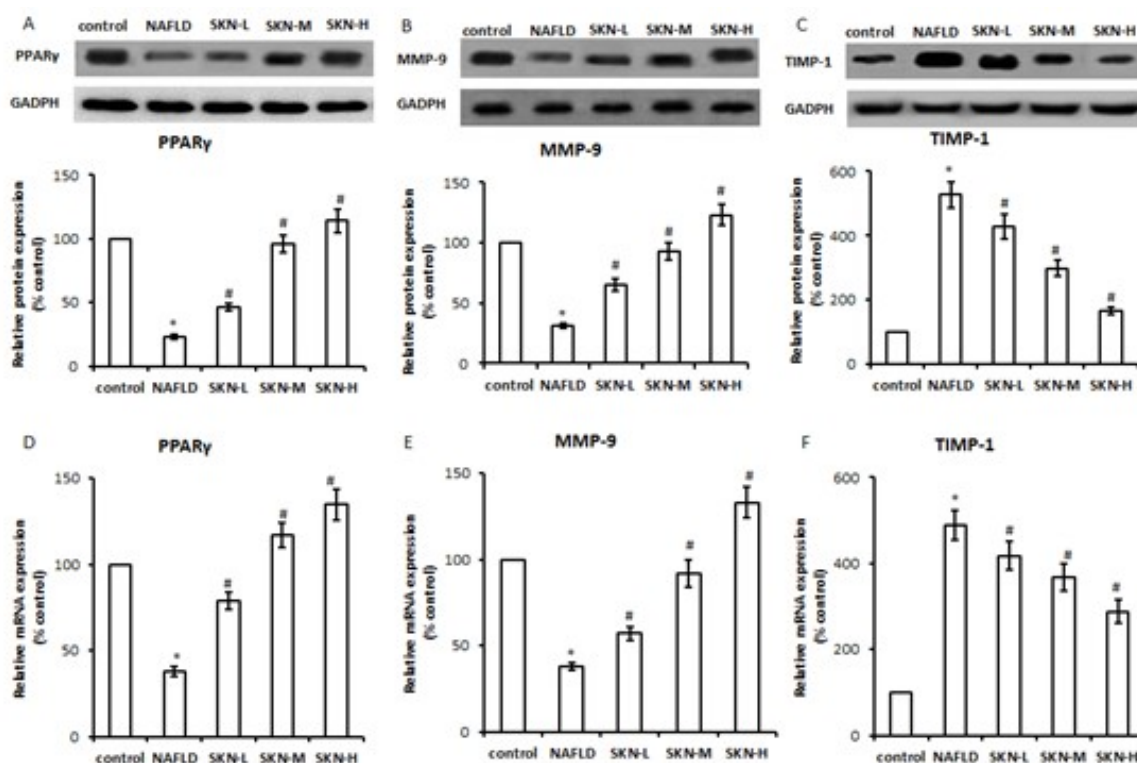


Fig. 3: Effects of shikonin on hepatic PPAR γ , MMP-9 and TIMP-1 expression. (A-C) At the end of experimental period, the livers were isolated. The expression of PPAR γ (A), MMP-9 (B) and TIMP-1 (C) expression in liver were determined by western blotting. Densitometric analysis showed that shikonin inhibited the decrease of PPAR γ and MMP-9 protein expression and the increase of TIMP-1 expression. (D-F) The mRNA expression of PPAR γ (D), MMP-9 (E) and TIMP-1 (F) were determined by quantitative PCR. Data are represented as mean \pm SD. * $p < 0.05$ versus control group; # $p < 0.05$ versus NAFLD group, $n = 6$ rats per group.

induce NAFLD in multiple studies (Silva *et al.*, 2014; Kucera and Cervinkova 2014). Therefore, in the present study, rat model of NAFLD was induced by feeding a HFD for 22 weeks. HFD induced a remarkable weight gain in NAFLD group and this increase was obviously prevented by shikonin in a dose-dependent manner. In addition to the weight gain, we also found abnormal serum biochemical parameters and dyslipidemia in HFD-fed rats, which were significantly attenuated by shikonin treatment. Additionally, HFD led to a higher level of serum ALT and AST, which indicated a capacity of chronic stress for hepatocyte injury. However, the increased levels of serum ALT and AST were dramatically inhibited in response to all the doses of shikonin. Furthermore, the hepatic function was also determined, as suggested by reduced hepatocyte ballooning, inflammatory cell infiltration, lipid accumulation, and hepatic TG and TC level after shikonin treatment. Collectively, our study demonstrates for the first time that shikonin prevents HFD-induced hepatic lipid accumulation and injury.

The prevailing theory of NAFLD pathogenesis is commonly based on the ‘double-hit’ hypothesis. Lipid accumulation plays a critical role in the ‘first hit’, whereas

the ‘second hit’ is in combination with oxidative stress and inflammation that eventually leads to liver fibrosis (Day and James 1998). Another significant finding of this study was that shikonin could alleviate HFD-induced liver fibrosis. Masson trichrome staining showed lower deposition of collagen fibrils in liver in shikonin-treated HFD-fed rats than in HFD-fed rats alone. PAI-1 is an inhibitor of extra cellular matrix (ECM)-degrading enzymes which plays an important role in regulating fibrinolysis. Upregulation of PAI-1 expression inhibits the activity of the fibrinolytic system and MMPs, thus further accelerates the progression of liver fibrosis (McMahon 2001). Meanwhile, CTGF can induce extra cellular matrix proteins expressions such as type III and IV collagen (Weston *et al.*, 2003). In our study, HFD-induced the increase of PAI-1 and CTGF expression were significantly inhibited after shikonin treatment. As expected, the increased expression of type III and IV collagen were also reduced by shikonin administration.

PPAR γ is a sequence-specific and ligand-dependent nuclear transcription factor, related to the control of the lipid storage and the differentiation of adipocytes and macrophages (Law *et al.*, 2016). Previous studies have demonstrated that PPAR γ is implicated in regulating

insulin resistance, lipid metabolism and inflammation in liver steatosis (Lutchman *et al.*, 2007). The agonists of PPAR γ could enhance the insulin sensitivity and lipid accumulation in adipose, liver and skeletal muscle tissues (Promrat *et al.*, 2004; Mohammadi *et al.*, 2014; Rani and O'Driscoll 2015). It has been found that the hepatic PPAR γ protein and mRNA expression was decreased in insulin resistance accompanied by the development of NAFLD in HFD-fed rats (Zhao *et al.*, 2016). Consistently, we also found lower expression of PPAR γ in NAFLD group than in control group. However, the decrease of PPAR γ expression was markedly restored after shikonin treatment, indicating the restoration of PPAR γ expression may at least partially underlies the inhibitory effect of shikonin on hepatic lipid accumulation.

Fibrotic liver is well characterized by increased deposition of ECM. MMPs play an important role in the metabolism of collagen and ECM degradation (Tomita *et al.*, 2006; Hemmann *et al.*, 2007). Several MMPs have been suggested to be expressed in human liver, such as MMP-1, MMP-9, MMP-10 and MMP-11 (Lichtinghagen *et al.*, 2003; Garciade *et al.*, 2006). The activity of MMPs is regulated through the action of specific inhibitors, including the tissue inhibitor of metalloproteinases (TIMPs). Increased level of TIMP-1 can be observed in experimental and clinical subjects with liver fibrosis (Xu *et al.*, 2004). Moreover, the degree of collagen deposition and fibrosis is due to the balance between MMPs and TIMPs (Abraham *et al.*, 2005; Ries 2014). Here, in liver, MMP-9 expression was decreased and TIMP-1 expression was increased in liver of HFD-induced NAFLD rats. These results were consistent with the report that MMP-9 was also induced whereas TIMP-1 expression was inhibited in CCl₄-induced rat hepatic fibrosis model (Xie *et al.*, 2017). However, a markedly higher expression of MMP-9 was found in shikonin treatment groups than in NAFLD group. Moreover, HFD-induced TIMP-1 expression was dramatically down regulated after shikonin treatment. These results suggest that shikonin attenuates HFD-induced liver fibrosis by regulating the balance of MMP-9 and TIMP-1.

CONCLUSION

Our data demonstrate that shikonin exerts promising therapeutic effects on NAFLD rats induced by HFD. Shikonin effectively ameliorates HFD-induced lipid accumulation, liver injury and fibrosis through PPAR γ and MMP-9-TIMP-1 axis. Our findings indicate that shikonin may be a potential therapeutic agent for the treatment of NAFLD.

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