



## Research Paper

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# Shikonin from Zicao prevents against non-alcoholic fatty liver disease induced by high-fat diet in rats

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### ABSTRACT

Shikonin, a natural naphthoquinone extracted from the traditional Chinese herbal Zicao, was reported to exert beneficial effects in several medical fields. However, there are few reports concerning its effects on the non-alcoholic fatty liver disease (NAFLD) and the related mechanisms. In this study, we aim to investigate whether shikonin prevents NAFLD. After feeding high-fat diet (HFD) for 10 weeks, Sprague-Dawley rats received different doses of shikonin (5 mg/kg/day, 10 mg/kg/day and 20 mg/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 decreased accompanied by hepatic injury and lipid accumulation in HFD-fed rats. Shikonin treatment attenuated the aforementioned biochemical and histopathological changes. Similarly, HFD-induced the increase of hepatic TC and TG levels were also ameliorated after shikonin treatment. Furthermore, shikonin observably mitigated HFD-induced 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 decrease of proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) 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 $\gamma$  and MMP-9/TIMP-1 axis, suggesting that shikonin may be a potential therapeutic agent for the treatment of NAFLD.

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### INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common form of liver disease not due to excess alcohol consumption and is considered as the hepatic manifestation of metabolic syndrome (Marchesini et al., 2001; Marchesini and Marzocchi, 2007; Ray, 2013). Clinical researches demonstrate that NAFLD develops from simple steatosis and subsequently contributes to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis, leading to

hepatocellular carcinoma (Fabbrini et al., 2010; Song et al., 2013). About 10 to 29% of NASH patients are likely to develop cirrhosis and liver cancer in a decade (Angulo, 2002).

Furthermore, previous studies indicate that NASH and NAFLD confer an increased risk of cardiovascular disease (Anstee et al., 2013; Scorletti et al., 2011). Unfortunately, there is no effective pharmacological agent currently

available for the treatment of NAFLD, although, some methods 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 the treatment of carbuncles, burns, measles, sore throats and macular eruptions (Chen et al., 2002; Kim et al., 2014; Papathanasiou et al., 1999) for many years.

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 used to ameliorate rat obesity induced by high-fat diet (HFD) through attenuating lipid dysregulation and inflammation (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 prevent 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  $\gamma$  (PPAR $\gamma$ ), matrix-metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinase-1 (TIMP-1) and GAPDH were obtained from Santa Cruz Biotechnology Inc. (CA, USA).

### **Animal**

Fifty male Sprague-Dawley rats (8-weeks old, 200 to 250 g, Jackson Laboratories, CA, USA) 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 the 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 5 mg/kg/day, 10 mg/kg/day and 20 mg/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 0.1 ml/100 g physiological saline for 12 weeks. All experimental protocols for animals were performed according to the Institutional Animal Care and Use Committee of Zhejiang Hospital.

### **Determination of serum biochemistry**

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

### **Histopathological examination**

Following the 12-weeks treatment of shikonin, rats were sacrificed and the livers fixed in 4% buffered paraformaldehyde, embedded in paraffin and cut into 5- $\mu$ m slides using a microtome (SLEE, Mainz, Germany). Slides were stained with hematoxylin-eosin, oil Red O and massontrichrome. All slides were examined under an Olympus light microscope (CKX41, Tokyo, 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  $\times$  g for 15 min at 4°C. The level of TC and TG in the supernatant was determined according to the same method in plasma measurement. The protein concentrations of liver tissues were quantified by the Enhanced BCA Protein Determination Kit (Beyotime Institute of Biotechnology, Shanghai, China). Hepatic TC and TG levels were normalized to the content of total proteins of each sample.

### **Western blotting**

Rat livers were lysed in RIPA buffer (Beyotime Institute of Biotechnology) containing 1% phosphatase and protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentrations were examined by the Enhanced BCA

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

Parameters	Control	NAFLD	SKN-L	SKN-M	SKN-H
Body weight (g)	348.67±6.57	431.82±10.24	403.56±9.64	371.25±10.71	357.35±9.88
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, alanine aminotransferase; AST, aspartate aminotransferase. \* $p < 0.05$  versus control group; # $p < 0.05$  versus NAFLD group, n=10 rats per group.

Protein Determination Kit. 50 µg of protein were separated by 8 to 19% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). The membranes were treated with the appropriate antibodies against the following proteins: PPAR $\gamma$ , MMP-9, TIMP-1 and  $\beta$ -actin (dilution 1:1000). After incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies (dilution 1:2000), the signals were determined using an ECL kit (Beyotime Institute of Biotechnology) and quantified by ImageJ software (ImageJ, Version 1.41, NIH, MD, USA).

### Real-time quantitative PCR

Total RNA was isolated from liver tissues using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's protocols. Complimentary DNA was synthesized through reverse transcription of RNA using the SuperScript 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 by the Shanghai Biological Engineering Technology Services Co. Ltd. (Shanghai, China): plasminogen activator inhibitor type 1 (PAI-1) sense 5'-AGCTTTGTGAAGGAGGACCG-3' and antisense 5'-CAGGGATGCAGACCCCAAAT-3'; connective tissue growth factor (CTGF) sense 5'-GCCCGCCTAGTCTCACAC-3' and antisense 5'-GTCACGCTCCGTACACAGTT-3'; collagen III sense 5'-ACGTAAGCACTGGTGGACAG-3' and antisense 5'-GGAGGGCCATAGCTGAACTG-3'; collagen IV sense 5'-GGGGTCGGGCTGGGAGTGAT-3' and antisense 5'-GCTGGCCGTCCATACCCGTG-3'; PPAR $\gamma$ sense, 5'-GAGGATCCCCGGGGTACCGGATGACCATGGTTGACACAG-3' and antisense, 5'-ACATTCCACAGTTAGCTAGCTAAGCATAGTCTGGGACATCAT AAGGGTA-3'; MMP-9 sense, 5'-TTTGACAGCGACAAGAAGTGG-3' and antisense, 5'-TCCCATCCTTGAACAAATACA-3'; TIMP-1 sense, 5'-TCTTCTGCAATTCCGACCTC-3' and antisense, 5'-CGGGCAGGATTCAGGCTAT-3'; GAPDH sense, 5'-GCCATCGTCACCAACTGGGAC-3' and antisense, 5'-CGATTTCCCGCTCGGCCGTGG-3'. Target gene expression ( $2^{-\Delta\Delta Ct}$ ) was normalized to endogenous GAPDH expression

### Statistical analysis

All parameters are expressed as the mean ± SD. Data analysis was performed with SPSS 19.0 software (Analytical Software, Chicago, IL, USA). The results were statistically analyzed by one-way ANOVA with Tukey's multiple comparison test. The criterion for significance was  $p < 0.05$ .

## RESULTS

### Effects of shikonin on body weight and biochemical parameters

After 22 weeks of a HFD, the HFD-treated rats gained a higher body weight than the control rats, which 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 serums TC, TG, HDL-C and LDL-C levels were examined. Table 2 shows that the serums TC, TG and LDL-C levels in HFD-fed rats were remarkably higher than those of the control group. However, 12-weeks treatment with shikonin was associated with reduced levels of the aforementioned biochemical parameters. Moreover, the level of serum HDL-C in HFD-fed rats was significantly elevated as compared with control rats and this increase was dramatically inhibited by shikonin treatment.

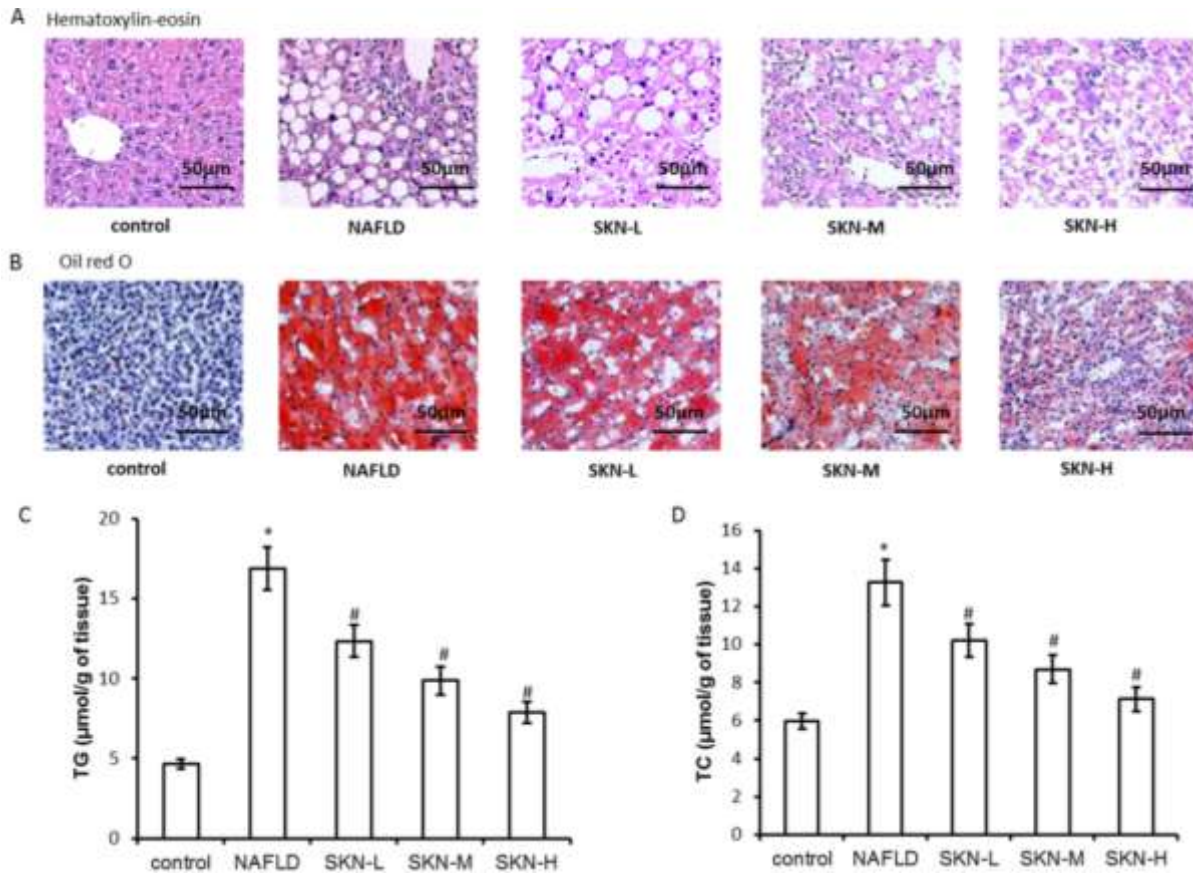
### Shikonin ameliorated HFD-induced liver injury and lipid accumulation

The histopathological examination of the liver tissue samples certified the results obtained from the biochemical tests. Histopathological findings showed obvious hepatic lobules disarrangement, inflammatory cell infiltration and hepatocyte ballooning in HFD-fed rats as compared with control rats. These changes were noticeably attenuated by shikonin (Figure 1A). Similarly, oil red O staining revealed that the shikonin treatment also decreased the deposition of lipid droplets in hepatocytes induced by HFD (Figure 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

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

Parameters (mmol/L)	Control	NAFLD	SKN-L	SKN-M	SKN-H
TC	1.31±0.14	2.73± 0.24	2.34±0.18	2.01±0.13	1.67±0.15
TG	0.91±0.04	1.63±0.14*	1.34±0.09#	1.21±0.08#	1.11±0.07#
HDL-C	1.11±0.09	0.73±0.06*	0.84±0.07#	0.91±0.06#	0.96±0.06#
LDL-C	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.



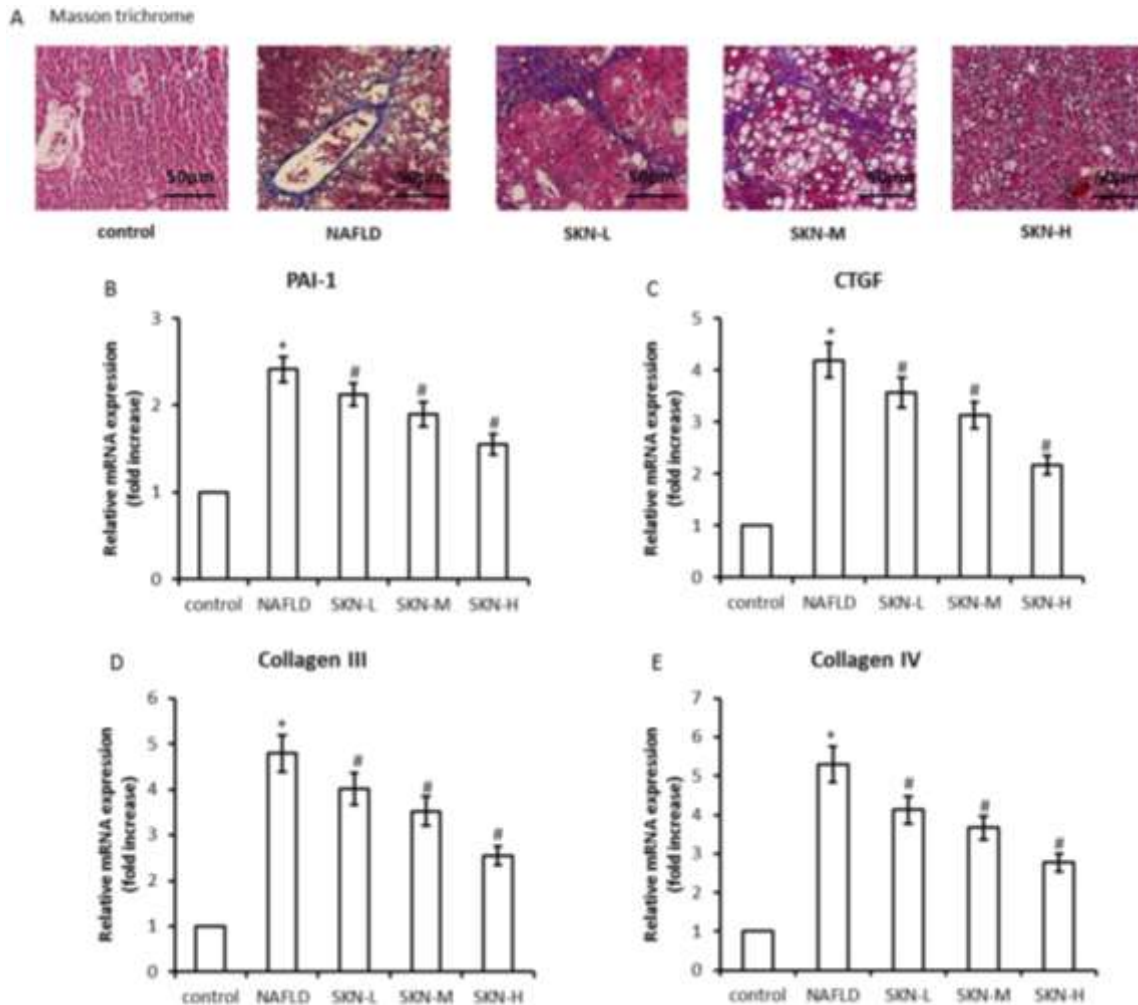
**Figure 1:** Effect of shikonin on HFD-induced liver injury and lipid accumulation. (A and B) NAFLD was induced by feeding rats for 22 weeks with HFD. After feeding HFD for 10 weeks, the rats were administrated with different doses (5mg/kg/day, 10mg/kg/day and 20mg/kg/day) of shikonin (SKN) for the 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×). (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.

with reduced hepatic TC and TG levels (Figure 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 (Figure 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 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 (Figure 2B



**Figure 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.

to F).

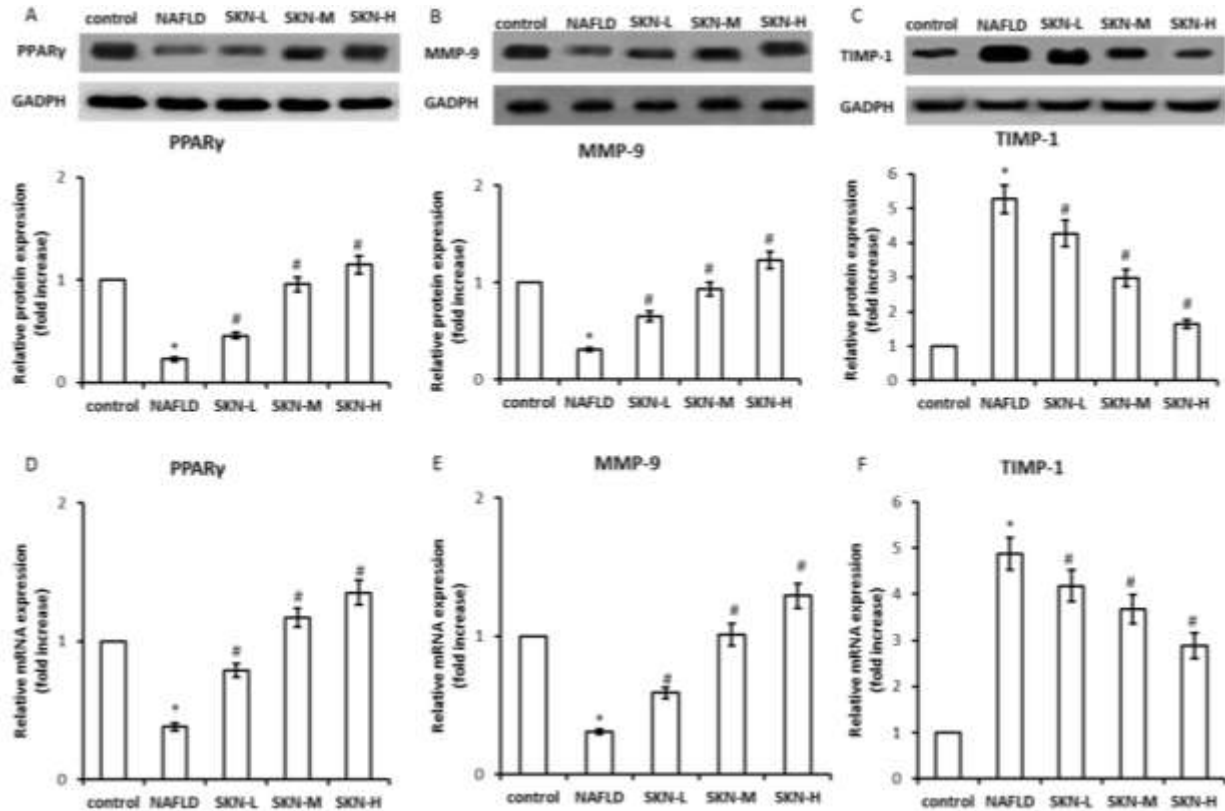
### Effects of shikonin on hepatic PPAR $\gamma$ , MMP-9 and TIMP-1 expression

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

consistent with the earlier mentioned results (Figure 3D to F).

### DISCUSSION

The present study aimed to investigate the effects of shikonin on HFD-induced NAFLD in rats. The HFD model was widely used to induce NAFLD in multiple studies (Kucera and Cervinkova, 2014; Silva et al., 2014). Therefore, in this 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



**Figure 3:** Effects of shikonin on hepatic PPAR $\gamma$ , MMP-9 and TIMP-1 expression. (A-C) At the end of experimental period, the livers were isolated. The expression of PPAR $\gamma$  (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 $\gamma$  and MMP-9 protein expression and the increase of TIMP-1 expression. (D-F) The mRNA expression of PPAR $\gamma$  (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.

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 levels 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 vital 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 extracellular 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 matrix-metalloproteinases (MMPs), thus, further accelerates the progression of liver fibrosis (McMahon, 2001). Meanwhile, CTGF can induce extracellular matrix proteins expressions such as type III and IV collagen (Weston et al., 2003). In our study, shikonin treatment markedly inhibited the increase of PAI-1 and CTGF expression induced by HFD. As expected, the increased expression of type III and IV collagen were also reduced by shikonin administration.

PPAR $\gamma$  is a sequence-specific and ligand-dependent nuclear transcription factor, regulating lipid storage and cell differentiation in adipocytes and macrophages (Law et al., 2000). It was demonstrated that PPAR $\gamma$  is implicated in regulating insulin resistance, lipid metabolism and inflammation in liver steatosis (Lutchman, 2007; Nan et al., 2009). The agonists of PPAR $\gamma$  could enhance the insulin sensitivity and lipid accumulation in adipose, liver and skeletal muscle tissues (Mohammadi et al., 2014; Promrat et al., 2004; Rani and O'Driscoll, 2015). It was found that the hepatic PPAR $\gamma$  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 $\gamma$  in NAFLD group than in control group. However, the decrease of PPAR $\gamma$  expression was markedly restored after shikonin treatment, indicating the restoration of PPAR $\gamma$  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 (Hemmann et al., 2007; Tomita et al., 2006). Several MMPs have been suggested to be expressed in human liver, such as MMP-1, MMP-9, MMP-10 and MMP-11 (Garcia et al., 2006; Lichtinghagen et al., 2003). 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 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 CCl $_4$ -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.

In 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 $\gamma$  and MMP-9-TIMP-1 axis. Our findings indicate that shikonin may be a potential therapeutic drug candidate for NAFLD treatment.

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