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Tianhuang formula ameliorates non-alcoholic fatty liver diseases in type 2 diabetic mice through CRLS1-ATF3/ChREBP pathway

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ABSTRACT

Objective: Tianhuang Formula (THF) is a hospital formula summarized by Professor Jiao Guo's 30 years of clinical experience. Some studies have shown that it can alleviate dyslipidemia in the body. The purpose of this study is to confirm whether THF can improve non-alcoholic fatty liver diseases (NAFLD) in type 2 diabetic mice induced by high-fat diet (HFD)/streptomycin (STZ) and to clarify its potential mechanism.

Methods: After induction of diabetes, mice were administrated with THF (60 mg/kg or 120 mg/kg) once daily for 10 weeks. Blood glucose (FBG), glucose tolerance, and insulin resistance (IR) were assayed by oral glucose tolerance test (OGTT) and insulin tolerance test (ITT). Blood lipids, alanine transaminase (ALT), and aspartate transaminases (AST) were detected. Serum fasting insulin (INS) and adiponectin (APN) levels were measured using ELISA. Histological changes in liver and pancreatic islets were observed by H&E staining, followed by Oil Red O staining for liver lipid quantification and periodic acid-Schiff (PAS) staining to detect glycogen accumulation. Western blotting detected the levels of fatty cardiolipin synthase 1 (CRLS1), transcription factor activator 3 (ATF3), and carbohydrate-responsive element binding protein (ChREBP) in the liver. The mRNA transcripts of hepatic inflammatory factors, lipogenesis and lipolysis-related genes, and gluconeogenic enzyme-phosphoenolpyruvate carboxykinase (PEPCK), CRLS1, ATF3, and ChREBP mRNA levels were evaluated by RT-qPCR.

Results: THF restored impaired glucose tolerance and insulin resistance, respectively. There was an improvement in HFD/STZ-induced liver and islet damage, high serum HDL-C and ANP levels, and a significant decrease in FBG, total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), FFA, INS, ALT, and AST, and lipid droplet counts in the T2DM mice treated with THF. CREBBP binding protein ATF3 mediated the insulin resistance signaling pathway, which regulated glucose and lipid metabolism in the liver. THF upregulated CRLS1, and ChREBP downregulated the expression of downstream ATF3 in the liver. RT-qPCR analysis also systemically indicated that THF suppressed the pathway and key regulators related to inflammation, lipid accumulation, and gluconeogenesis.

Conclusion: Our findings demonstrated that THF ameliorated lipid profile and attenuated liver steatosis in T2DM mice through CRLS1-ATF3/ChREBP pathway activation.

1. Introduction

Diabetes mellitus (DM) is a syndrome of metabolic disorders, including blood vessel abnormalities, infarction, hepatopathy, nerve disorder, retinopathy, and nephropathy caused by islet dysfunction, insulin resistance, etc., accompanied by various complications.[1–3] It is characterized by persistent hyperglycemic status and is a chronic debilitating disease that has become one of the most rapidly escalating epidemics in the world. For reasons like urbanization, population aging, and obesity, the number of patients with diabetes continues to grow rapidly,[4] and there are about 463 million adults with diabetes worldwide, which is expected to reach 578.4 million by 2030 and 700.2 million worldwide in

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2045 according to the latest report of the International Diabetes Federation (IDF).

Patients with type 2 diabetes mellitus (T2DM), which is caused by a relative deficiency in insulin secretion or insulin resistance, are at a higher risk (35–80%) of developing hepatic steatosis compared to individuals without diabetes.[5] The liver is a key organ in maintaining lipid and energy homeostasis and excessive lipid is stored in the liver as fat droplets, eventually leading to inflammation, insulin resistance (IR), and diabetes. Another important metabolic consequence of diabetes is abnormal triglyceride deposition in hepatocytes, further promoting hepatic steatosis. Non-alcoholic fatty liver disease (NAFLD) occurs in up to 70% of DM patients.[6] It suggested that liver-focused treatments might reduce the risk of developing T2DM.[7]

The regulation of fatty acid oxidation is one of the central processes in hepatic lipid metabolism, at least partially, by some key transcription factors such as transcription factor activator 3 (ATF3). A member of the ATF/cAMP responsive element binding protein (CREB) family of transcription factors, ATF3 regulates the expression of crucial genes related to metabolic diseases, including obesity, dyslipidemia, and diabetes.[8] The ERK pathway regulates the expression of ATF3. Intensive research has proven that ATF3 is critical in regulating systemic homeostasis in essential metabolic tissues, especially in the liver.[9] CDP-alcohol phosphatidyl transferase class-I family member cardiolipin synthase 1 (CRLS1) is localized to mitochondria and is highly expressed in the liver.[10] Recent work by Sustarsic et al. declared that CL depletion in brown and beige fat suppressed glucose uptake and CRLS1 adipose levels positively correlated with insulin sensitivity.[11] One report recently suggested that CRLS1 improves nonalcoholic steatohepatitis (NASH) by inhibiting ATF3 activity.[12] Carbohydrate-responsive element binding protein (ChREBP) is a transcription factor that plays a crucial role in the homeostasis of carbohydrate and lipid metabolism[13] and is mainly expressed in lipogenic organs such as the liver, β cells of the pancreas, intestines, and adipose tissues. Excessive carbohydrate intake leads to activation of ChREBP, leading to hepatic triglyceride accumulation.[14] Although increasing evidence has demonstrated that ChREBP is involved in the pathogenesis of hepatic steatosis and IR, the role of liver ChREBP in IR development has been controversial. Glucagon promotes glucose production in response to low nutrient availability via protein-kinase-A-mediated CREB activation.

On the other hand, when energy levels increase with food consumption, ChREBP orchestrates the transcriptional response to nutrient availability that shifts metabolism toward utilization and energy storage.[15] Considering the close involvement of ChREBP with ATF3, we hypothesized that ATF3 may be essential for the function of ChREBP during the progression of hepatic steatosis. Nevertheless, the specific roles of CRLS1-ATF3/ChREBP in hepatocytes and related underlying mechanisms associated with the glucose metabolism disorder remain unexplored.

At present, the main methods of treating diabetes include insulin injection or oral hypoglycemic drugs, such as metformin and acarbose. Patient compliance, however, has gradually declined due to hypoglycemia, hepatic and renal dysfunction, gastrointestinal reactions, and other adverse effects in the clinic.[16] Furthermore, long-term application of hypoglycemic agents will reduce the sensitivity of the islet receptor in patients with DM and eventually deteriorate the control condition.[17]In traditional Chinese medicine (TCM), the mildness, multiple targets, and fewer adverse effects have gradually attracted attention for the treatment of lipid and glucose metabolism diseases,[18] which can intervene and regulate diabetes from a holistic perspective.[19] While significant progress has been made in identifying therapeutic targets and therapeutic strategies, no approved effective T2DM with NAFLD drugs are available. Studies searching for effective medicinal plants or bioactive compounds for T2DM with NAFLD are therefore of increasing interest. Tianhuang formula (THF), a TCM consisting of *Panax notoginseng* and *Coptis chinensis* has long been used to treat obesity and glycolipid metabolism.[20] However, whether THF ameliorates hepatic steatosis in diabetic conditions and its mechanism has not been fully elucidated.

This study we verified the effect of THF attenuated glucose and lipid metabolism disorders in a high-fat diet (HFD)/streptozocin (STZ)-induced T2DM mice, and analyzed whether part of the mechanism may be mediated through the CRLS1-ATF3/ChREBP signaling pathway. These data shed light on the role of the CRLS1-ATF3/ChREBP in non-alcoholic fatty liver diseases in T2DM and provide experimental basis for the clinical application of THF.

2. Materials and methods

2.1. Preparation of THF powder and HPLC analysis

Herbs in THF *[P. notoginseng and C. chinensis]* were provided by Zhixin Chinese Herbal Medicine Co., Ltd. (Guangzhou, China). THF was obtained from the Institute of Guangdong Metabolic Diseases Research Center of Integrated Chinese and Western Medicine, Guangdong Pharmaceutical University. It was prepared as follows: *P. notoginseng* (400 g) and *C. chinensis* (400 g) were extracted triply with 70% ethanol at 80 °C under reflux, each time for 2h. The extract was concentrated in a rotary evaporator to remove ethanol and then dissolved in water. The extract obtained was vacuum-dried at 60 °C. The quality control of THF was carried out based on the methodology determined in our pre-laboratory,[20] which contains eight main active ingredients, namely ginsenoside Rg1, ginsenoside Rb1, ginsenoside Rd, ginsenoside Re, Panax ginseng saponin R1, berberine, flavonoids, and palmatine.

2.2. Animals and experiment design

All the animal experiments were approved by the Animal Ethical Committee of Guangdong Pharmaceutical University (SPF2017310). C57BL/6J (14–18g) male specific pathogen-free (SPF) mice were obtained from the Guangdong Medical Laboratory Animal Center. The animal quality certificate number was 44007200077257. All mice were housed in a temperature-controlled room at (24 ± 2)°C with a humidity of 60%–70% and 12 h of light and darkness alternated. Standard solid food and water were provided during the experiment. All the mice were put on a one-week "adaptive feeding" pattern before the experiment began.

All mice were randomly divided into two groups: the normal control group and the model group. Mice in the normal group were fed a standard diet, and all mice in the other groups were provided with an HFD (Research Diet, D12492, 60% calories from fat) during the entire experimental period. At the end of the 8-week HFD diet in the model group, mice fasted for 12 h each night and intraperitoneally (i.p.) injected with STZ (40 mg/kg BW, dissolved in ice-cold 0.1 mM citrate buffer, pH 4.4) for 4 days to induce T2DM,[21] the molding rate of T2DM for this experiment was around 92%. The control group received an injection of citrate buffer during this time. After the model was successfully established, the T2DM group was randomly divided into the following 4 groups according to the fasting blood glucose (FBG): model group (distilled water), Met group (250 mg/kg metformin), low dose THF group (THF-L, 60 mg/kg), and high dose THF group (THF-H, 120 mg/kg). At 10 weeks, animals were given intragastric administration of each drug once a day. Body weights were measured weekly during the intervention. After dosing, blood was collected, and mice were sacrificed. The liver was rapidly separated, weighed, and snap-frozen at −80 °C for future biochemical analyses.

2.3. Oral glucose tolerance tests (OGTT) and insulin tolerance tests (ITT)

After 8 weeks of dosing, OGTT and ITT were carried out as previously described.[22] Briefly, during the last week of treatment, after overnight fasting for 12 h, mice were gavaged with 20% glucose. The blood samples were obtained from the tails of each mouse at intervals of 0, 15, 30, 60, 90, and 120 min after injection. ITT was performed on a different day.

After obtaining baseline glucose levels, mice were fasted for 6 h and injected i.p. with insulin (0.5 U/kg). Glucose levels in tail blood were measured at 0, 15, 30, 60, 90, and 120 min. Measurement results were used to calculate the area under the curve (AUC).

2.4. Biochemical analysis

Hepatic triglycerides (TG), serum TG, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), alanine transaminase (ALT), and aspartate transaminase (AST) levels were measured by commercial kits (Nanjing, Jiancheng, China). ELISA measured serum fasting insulin (INS) and adiponectin (APN) levels. The evaluation of insulin resistance by homeostasis model (HOMA-IR) was calculated using the formula: HOMA-IR = [fasting plasma glucose (mmol/L) * fasting plasma insulin (mU/L)]/22.5.

2.5. Histology

Liver and pancreatic tissues were fixed in 4% neutral-buffered formalin for 24 h. Tissues were then embedded in paraffin following dehydration in graded ethanol series (70–100%). The paraffin-embedded specimens were then sectioned into sections 5 μm thick and stained with hematoxylin and eosin (H&E). Slide examination was performed under a light microscope (magnification: × 400; Eclipse E200-LED, Nikon, Tokyo, Japan). Slides were imaged using a digital slide scanner. Liver cryosections (5 μm thick) were incubated with 10% formalin for 30 min at room temperature for Oil Red O staining and then stained with fresh Oil Red working solution for 20 min. Hepatitis C was performed using a commercially available kit. The sections were washed with water, counterstained with hematoxylin dye for 1 min, and then mounted under a microscope to visualize the lipid deposition. According to the method in the literature, the periodic acid Schiff staining (PAS) was used to observe glycogen deposits in the livers.[23]

2.6. Western blotting

Liver tissues (40 mg) were ground, and protein was extracted. BCA kits from Beyotime Biological Technology (Shanghai, China) were used to qualify for total protein. The protein samples were then denatured using protein loading buffer SDS-PAGE. Protein samples were separated via gel electrophoresis and transmembrane to polyvinylidene fluoride (PVDF) for 2 h. The PVDF membrane was washed using Tris-buffered saline with 1% Tween-20 (TBST), then sealed with 5% skim milk at room temperature for 1 h. The PVDF was washed with TBST and incubated with primary antibodies against CRLS1 (A12388, ABclonal, USA), ChREBP (#58069, Cell Signaling Technology, USA), ATF3 (ab207434, Abcam, USA) and β-actin (Beyotime Biological Technology, Shanghai, China) at 4 °C overnight. After washing with TBST, the samples were incubated with HRP-labeled goat anti-rabbit IgG (H+L) (Beyotime Biological Technology, Shanghai, China) at room temperature for 1 h.

2.7. RNA extraction and quantitative real-time PCR

PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real TIME) and TB GreenR premix Ex TaqTM II (Tli RnaseH Plus) were from Takara Medical Technology (Dalian, China). Primer sequences were designed and synthesized by Sangon Biotech (Shanghai, China) (https://www.sangon.com/), shown in Supplementary Table 1. We extracted the RNA from 25 mg of liver tissues, then reverse-transcribed it into cDNA using quantitative real-time (qRT-PCR) kits. The primers were centrifuged at 12000 r/min at 4 °C for 1 min and diluted to 10 μM. The 10 μL reaction system was prepared using the TB Greenr Premix Ex Taq™ II kit for amplification, then centrifuged at 2200 r/min for 2 min and loaded into the PCR instrument.

2.8. Statistical analysis

Quantitative data were shown as mean±SEM. The statistical analyses were performed and plotted using GraphPad Prism 9.4.1 (458, USA). Student's T-test was used to compare differences between the two groups. Data sets that involved more than two groups were assessed by one-way ANOVA followed by Tukey's multiple comparisons test, and statistical significance is reported in the figures. Results were considered statistically significant when $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$.

3. Results

3.1. THF improved glucose metabolism and insulin sensitivity in diabetic mice

Compared with the Control group, the food intake of the Model group was significantly reduced (Fig. 1A). HFD/STZ-induced T2DM mice were treated with THF for 10 weeks, the FBG was decreased considerably (Fig. 1B), and their glucose tolerance was evaluated by OGTT (Fig. 1C). Compared with the Control group, the Model group had the highest blood glucose level at each time point. Conversely, THF or Met-treated mice had significantly lower glucose levels compared to untreated T2DM mice regardless of dosage. Similar trends were also observed in the AUC results, indicating that THF improved glucose metabolism in diabetic mice (Fig. 1D). The insulin resistance was measured by ITT, which showed that glucose levels in the Control group significantly decreased 30 min after insulin injection and recovered close to normal within 60 min (Fig. 1E). Compared with untreated T2DM mice, blood glucose levels were markedly reduced after THF treatment. Similar trends were again observed in the AUC results, indicating that THF restored peripheral insulin sensitivity in T2DM mice (Fig. 1F). Using HOMA-IR measurements, we found that HFD combined with STZ injection significantly increased IR in the Model group (Fig. 1H). Both THF and Met showed significant improvements in insulin sensitivity (Fig. 1G). Collectively, THF not only restored impaired glucose tolerance but also improved IR in T2DM mice induced by HFD/STZ.

3.2. THF improved the function and structure of liver and pancreatic in T2DM mice

The mice's body weights were shown in Fig. 2A. After HFD/STZ induction, the Model group had gained significantly more weight than the Control group. The liver of the Model group mice exhibited hypertrophy, but there was no significant in the liver index (Fig. 2B and C). Biochemical markers of liver injury like AST and ALT were also analyzed (Fig. 2D and E). In the Model group, serum ALT and AST levels were significantly higher, which was attenuated by THF therapy. This indicated that THF improved liver injury in T2DM mice induced by HFD/STZ. H&E staining (Fig. 2F) showed that THF had improved in liver morphological change. In the Model group, severe damage to the liver lobules was observed, and increased periportal and interstitial fibrous connective tissue was seen. Many fatty degenerated liver cells occur around central veins. This morphological change against diabetic liver steatosis was significantly moderated by THF treatment since the liver of the THF group had a much more complete hepatic lobule structure, with its liver cells being more organized, and there was less infiltration of inflammatory cells.

Microscopic examination of pancreatic tissues of the Model group showed variable-sized pancreatic islets, atrophy, and reduction of its cellular components with areas of necrosis (Fig. 2F). Vacuolation and mononuclear cells with focal areas of degeneration, congestion, and necrosis were seen in some pancreatic tissues. After THF and Met treatment, the pancreatic morphologies resembled those of the Control group. The histological findings reflect the improved impact of THF on pancreatic islet number and regions.

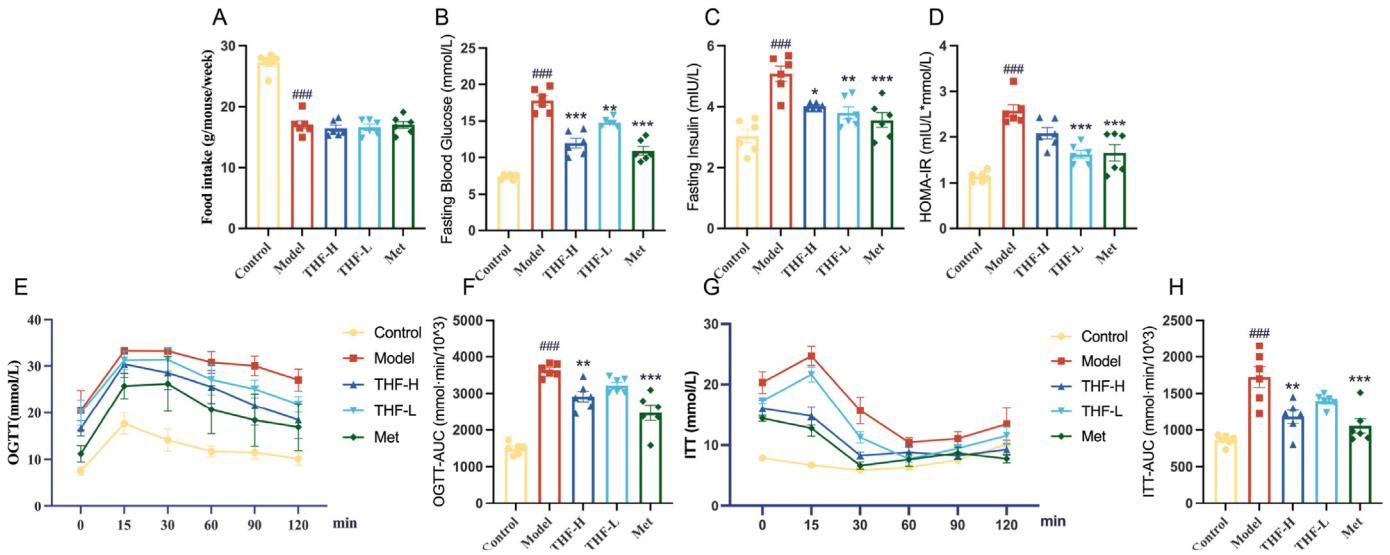

Fig. 1. THF improved glucose metabolism and insulin sensitivity in T2DM mice. **(A)** Food intake. **(B)** FBG. **(C–D)** OGTT results of the mice. **(E–F)** ITT results of the mice. **(G)** INS levels. **(H)** HOMA-IR of the mice. Data were presented as means ± SEM (n=6 per group). $^{###}P<0.001$ compared to the Control group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to the Model group.

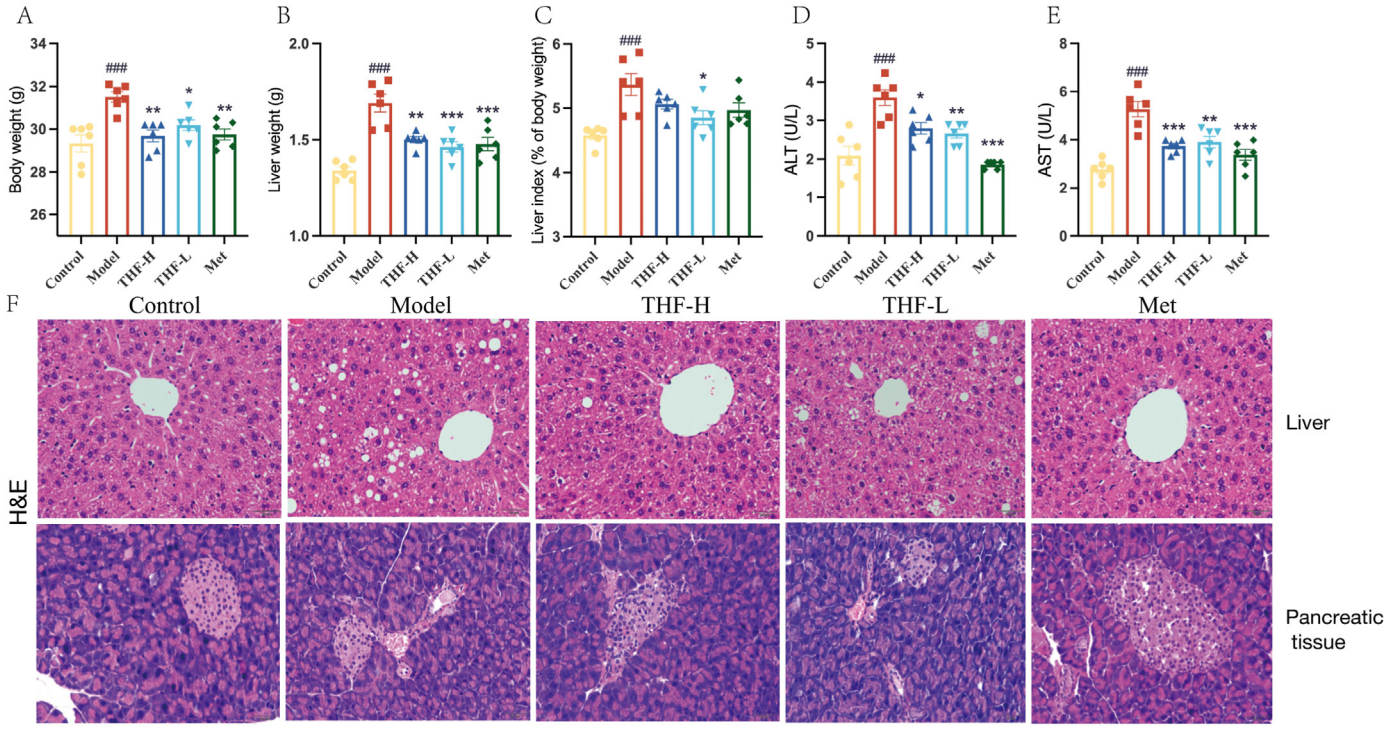

Fig. 2. THF improved deteriorated the function and structure of liver and pancreatic tissue in T2DM mice. **(A)** Body weight. **(B–C)** Liver weight and liver index. **(D–E)** The levels of ALT and AST in serum. **(F)** H&E staining of liver and pancreatic tissue in different groups of mice (n=3, Scale bar = 50 μm). Data were presented as means ± SEM (n=6 per group). $^{###}P<0.001$ compared to the Control group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to the Model group.

3.3. THF regulated lipid metabolism disorder in T2DM mice

The serum TC, LDL-C, and TG levels of the Model group mice significantly increased compared to the Control group mice (Fig. 3A–C), while the TC, TG, and LDL-C levels decreased in THF and Met groups. We observed the opposite pattern of change in HDL-C and APN levels (Fig. 3D and E). In T2DM mice, THF treatment effectively improved lipid metabolism disorder. Liver lipid profiling also showed that TG levels in the livers of mice significantly increased in the Model group, which was attenuated by THF treatment (Fig. 3F). To examine further the effect of THF on hepatic lipid accumulation, Oil Red O staining was performed (Fig. 3G), no histopathological changes were observed in the liver of the Control group. In contrast, the Model group had a large amount of lipid deposition. Hepatic steatosis was not entirely obliterated by THF treatment, but lipid droplet size was decreased regardless of the dose. Moreover, as presented in the PAS staining results, THF prevented the reduction of hepatic glycogen synthesis caused by the HFD/STZ induced. Collectively, THF significantly attenuated hepatic lipid accumulation in T2DM mice.

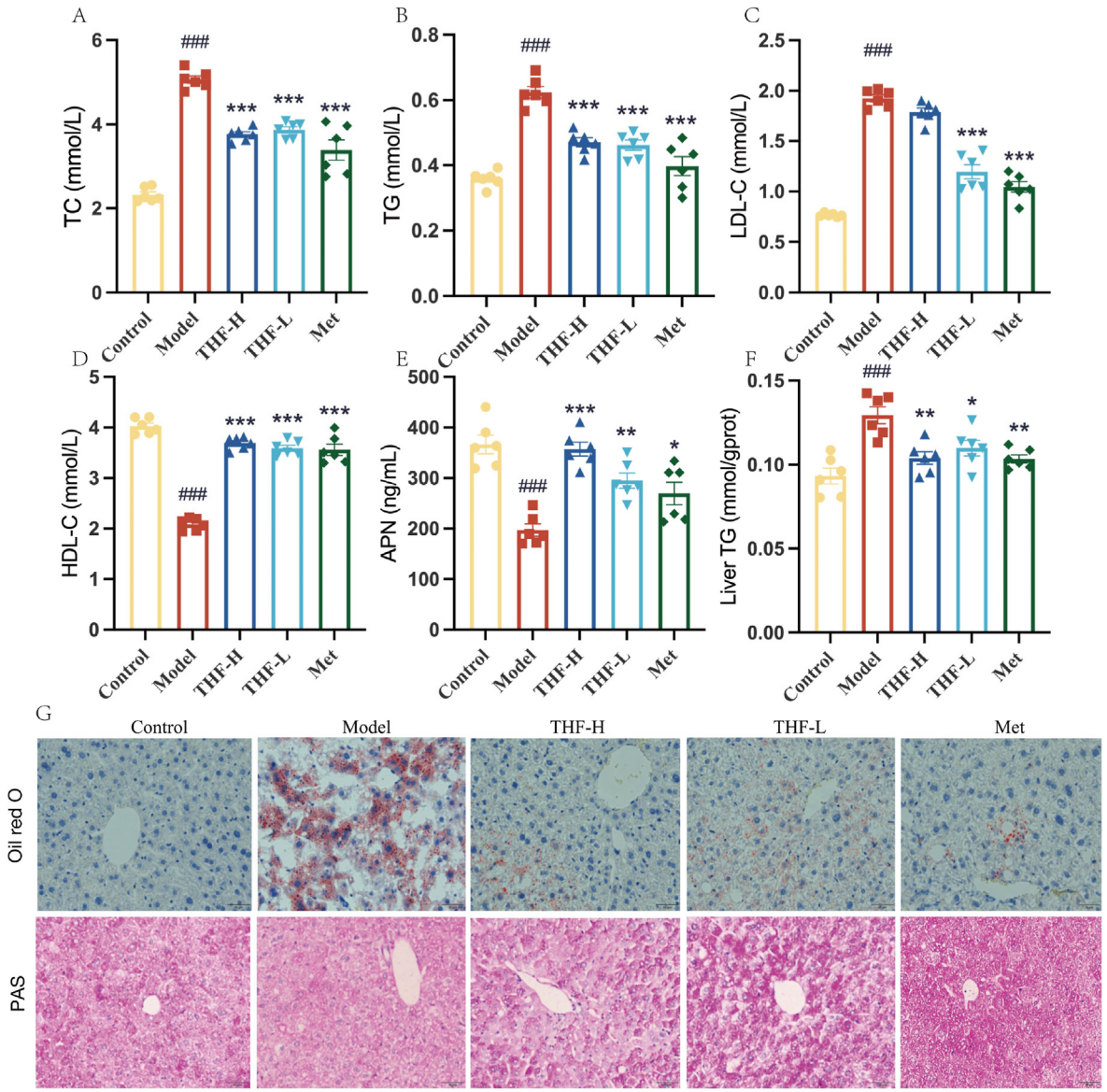

Fig. 3. Effects of THF on hepatic histopathological changes and lipid levels in T2DM mice. The levels of **(A)** TC, **(B)** TG, **(C)** LDL-C, **(D)** HDL-C, and **(E)** APN in serum. **(F)** TG level in liver. **(G)** The macroscopical photos of livers. Oil red O and PAS staining of liver sections in different groups of mice (n=3, Scale bar = 50 μm). Data were presented as means ± SEM (n=6 per group). $^{###}P<0.001$ compared to the Control group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to the Model group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. THF relieved inflammation infiltration, improved glucose metabolism, and reduced de novo lipogenesis in T2DM mice

Inflammation plays an essential role in the pathogenesis of diabetic hepatic injuries. To further confirm the effect of THF on inflammation, tissue inflammatory factors (TNF-α, IL-6, IL-1β, and MCP1) were assessed by qPCR. As shown in Fig. 4A–D, THF reduced IL-1β, IL-6, TNF-α, and MCP-1 levels in the liver of T2DM mice.To elucidate the mechanisms by which THF effectively ameliorated hepatic steatosis in T2DM mice, we assayed the gene expression of the key enzyme in gluconeogenesis, such as PEPCK. The results showed that THF treatment effectively reduced the gluconeogenic gene compared with the Model group (Fig. 4I). As was shown in Fig. 4E–H, compared with the control group, the key factor in lipogenesis genes (Fasn, Srebf1, Acaca, and Pparg) and the key factor in lipid uptake (Fabp1 and S1c27a1) were significantly upregulated, while the key gene in fatty acid oxidation including acyl-CoA oxidase 1 (Acox 1) was downregulated (Fig. 4J and K). There was no significant intra-group variation in carnitine palmitoyltransferase 1A (Cpt1a), PPARa, and Acadm (Fig. 4L-O). THF prominently reduced the expression in lipogenic genes and fatty acid uptake yet had little influence on fatty acid oxidation in T2DM mice. These data indicated that THF prevented hepatic steatosis, effectively improved glucose metabolism, and decreased de novo lipogenesis in T2DM mice.

3.5. The effects of THF were mediated by CRLS1-ATF3/ChREBP signaling pathway

To further investigate the mechanism of the protective effect of THF, CRLS1, ATF3, and ChREBP expression levels were analyzed in vivo, and a significant decrease in both CRLS1 and ChREBP mRNA and protein was seen in T2DM mice, while ATF3 increased. After administration of THF, the gene and protein expression of AFT3 was significantly decreased, and CRSL1 and ChREBP were increased considerably. These data indicated that THF could improve T2DM with NAFLD via the CRSL1-AFT3/ChREBP signaling pathway (Fig. 5A–G).

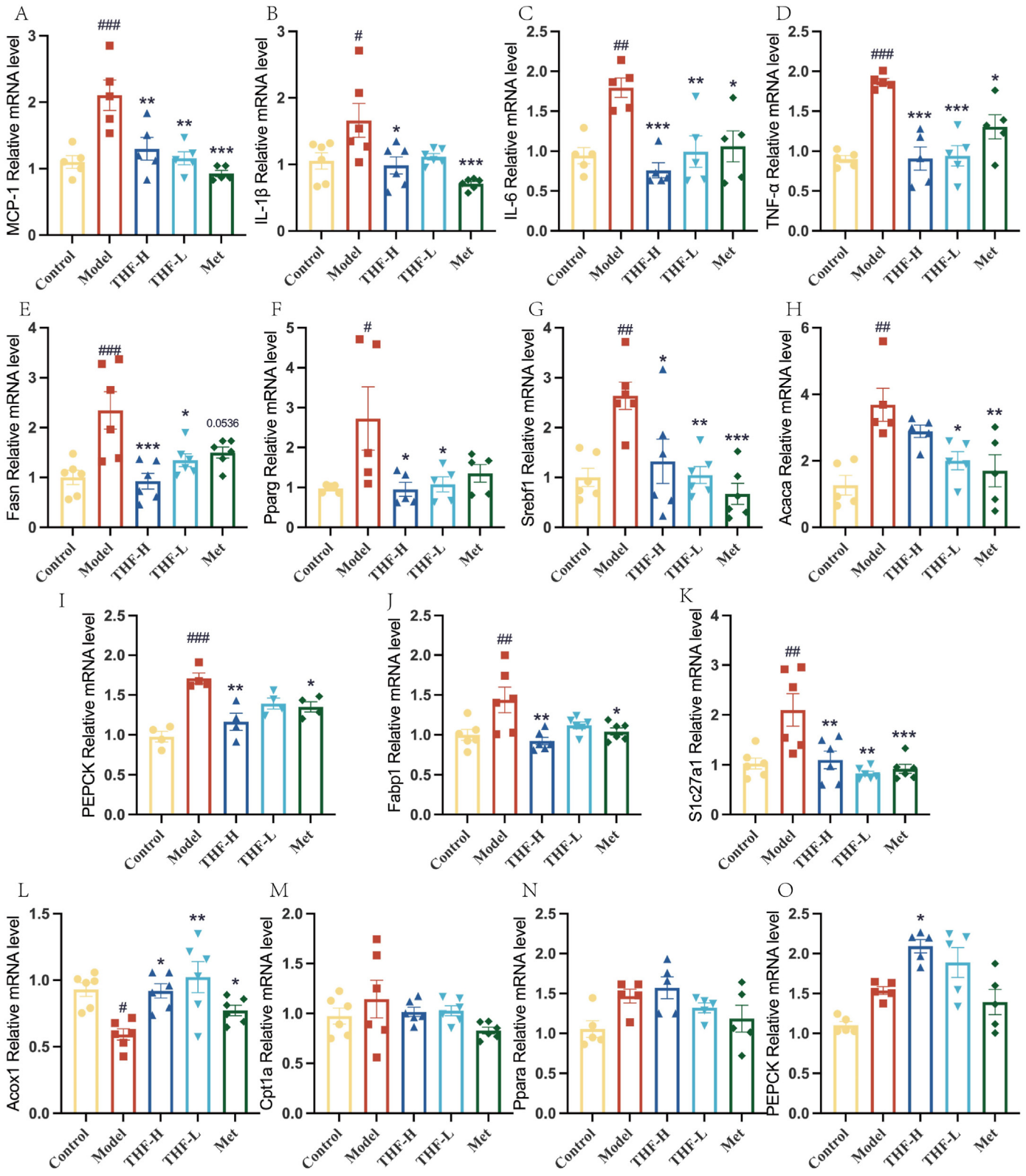

Fig. 4. THF relieved inflammation infiltration, improved glucose metabolism, and reduced de novo lipogenesis in T2DM mice. **(A–D)** The mRNA levels of inflammatory factors (TNF-α, IL-6, IL-1β, and MCP1) in the liver. **(I)** The key factor in gluconeogenesis (PEPCK) in the liver. **(E–H)** The mRNA levels of the key factor in lipogenesis (Fasn, Srebf1, Acaca, and Pparg) in the liver. **(J–K)** The mRNA levels of the key factor in fatty acid uptake (Fabp1 and S1c27a1) in the liver. **(L–O)** The mRNA levels of the key factor in fatty acid oxidation (Acox 1, Cpt1a, Ppara, and Acadm) in the liver. Data were presented as means ± SEM (n=4–6 per group). $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared to the Control group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to the Model group.

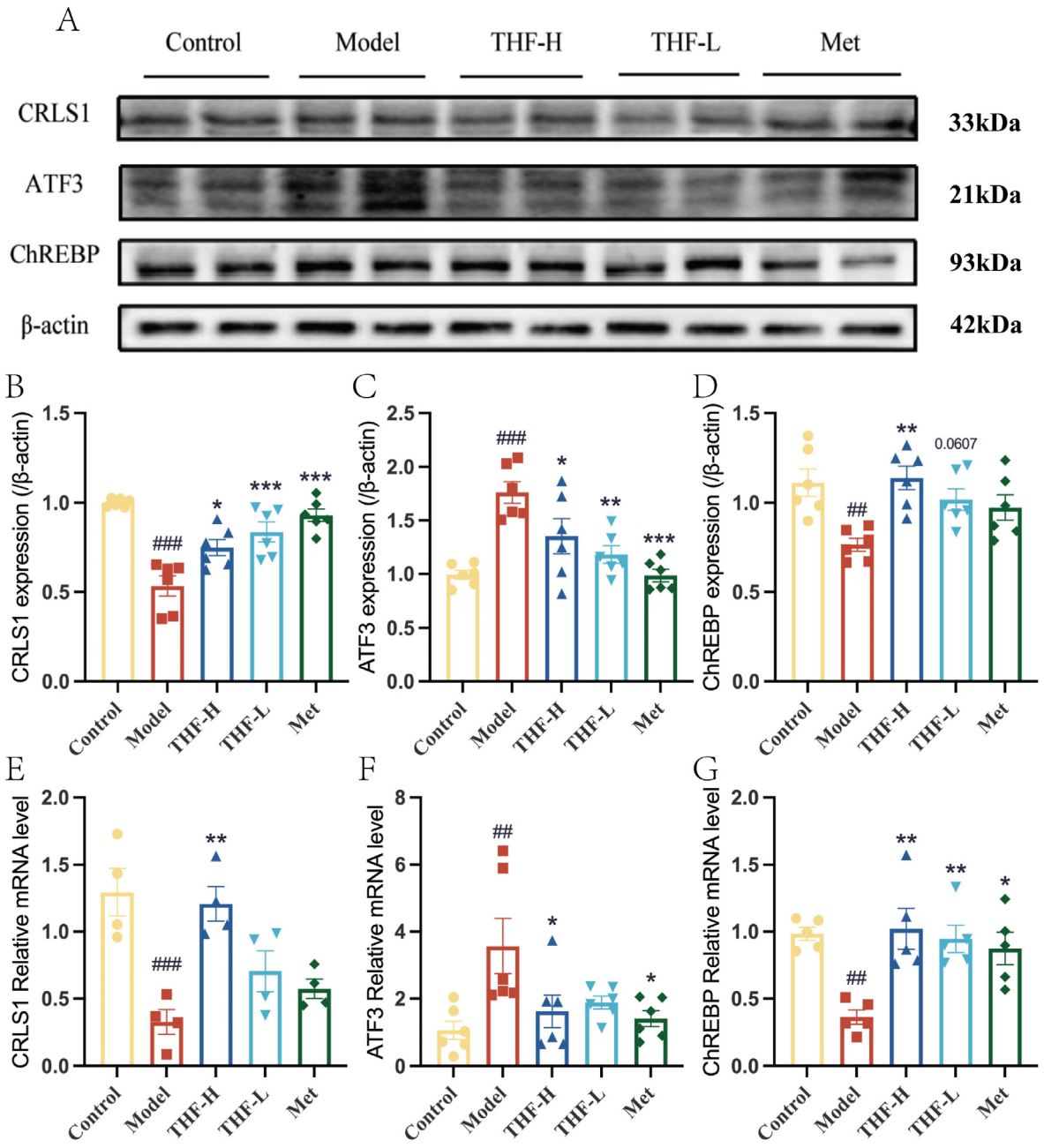

Fig. 5. The effects of THF were mediated by CRLS1-ATF3/ChREBP signaling pathway. **(A–D)** The protein levels of CRLS1, ATF3, and ChREBP in the liver (n = 3). **(E–G)** The mRNA levels of CRLS1, ATF3, and ChREBP in the liver. Data were presented as means ± SEM (n=4–6 per group). $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared to the Control group. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to the Model group.

4. Discussion

Diabetes is associated with various liver diseases, and the pathophysiological links between hyperglycemia and hepatopathy are complex and unclear. Chronic inflammation, persistent hyperglycemia, increased visceral fat content, and IR are the leading causes of its pathogenesis, causing lipotoxicity in the liver and impaired hepatic function. The metabolism of glucose in hepatocytes is mediated by glycolysis to pyruvate, which is then used by de novo lipogenesis to synthesize fatty acids, TG, phospholipids, and cholesterol esters, which are stored in lipid droplets or secreted into the circulatory system.[24] The liver has been identified as a pivotal player in the regulation of glucose production via gluconeogenesis and glycogenolysis pathways.[25] A traditional Chinese medicine therapy of "Tiaogan Qishu Huazhuo", used to treat glucose and lipid metabolism diseases, including T2DM, was first put forward by *Prof.* Jiao Guo from the Guangdong Pharmaceutical University. Hepatic steatosis is a frequent complication of diabetes and is a regular coexisting condition with T2DM. An imbalance of hepatic lipid metabolism leads to hepatic triglyceride and IR accumulation, ultimately causing diabetes.[26] Hepatic dysfunction may, therefore, cause disturbances in glucose and lipid metabolism, accelerate the development of diabetes, and gradually develop dyslipidemia.

There are a series of drugs to treat T2DM and NAFLD, which cannot take into account the multiple pathogenic factors of T2DM combined with NAFLD to increase the risk of adverse (hepatic and extra-hepatic) clinical outcomes. For successful clinical treatment of T2DM with NAFLD, finding new approaches with few adverse effects. The traditional Chinese formula THF, based on the "TiaoGan QiShu HuaZhuo", a promising anti-dyslipidemia property, has shown beneficial effects in metabolic diseases.[20] Still, its underlying mechanisms have not been

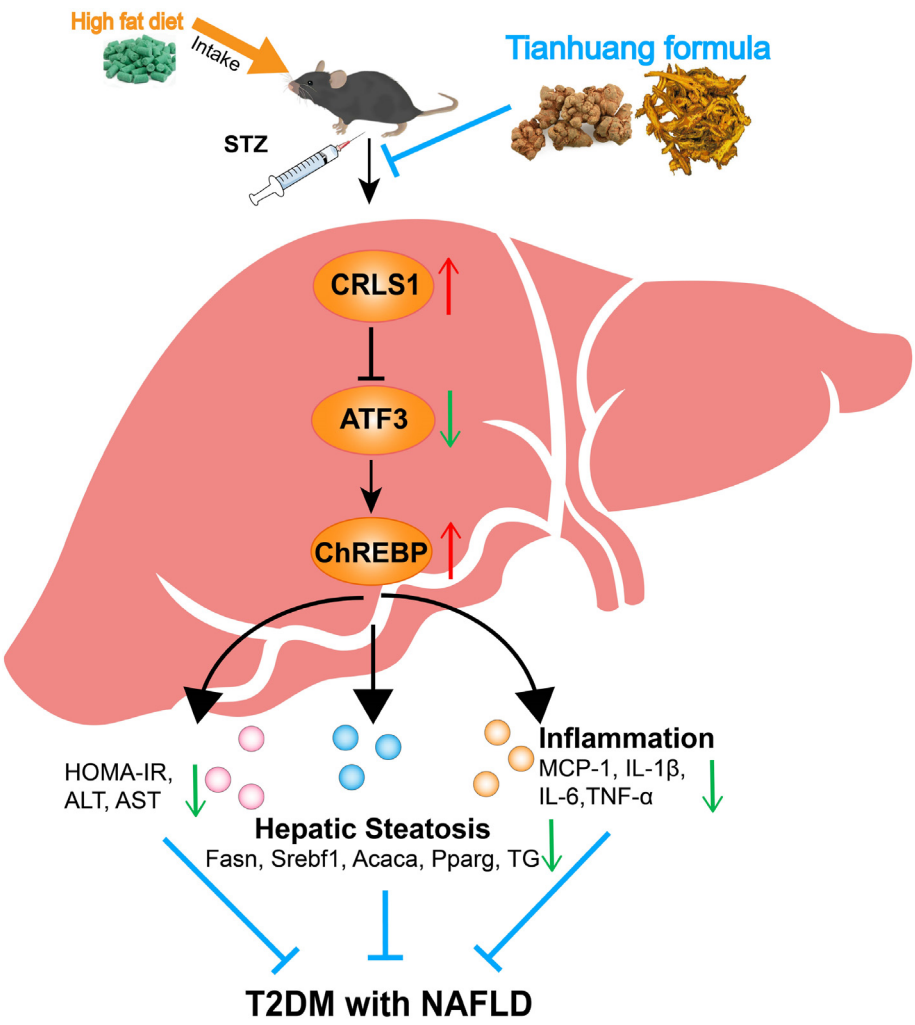

Fig. 6. A schematic illustration of THF attenuating non-alcoholic fatty liver diseases in type 2 diabetic mice.

adequately demonstrated. In our study, UPLC-Q/TOF-MS was used to analyze the composition of THF, composed of *Panax notoginseng* and *Coptis chinensis*. A total of 35 compounds, all saponins and alkaloids, were obtained. Saponins have anti-inflammatory,[27] neuroprotective,[28] anti-cancer[29] and other pharmacological activities. Alkaloids have pharmacological effects such as hypoglycemic,[30] anti-inflammatory,[31] and anticancer.[32] In the study, THF also proved its therapeutic effect on diabetic liver disease. The effect of THF was tested in a well-established T2DM mouse model, which was induced by feeding with HFD in conjunction with an intraperitoneal injection of STZ. After HFD, insulin resistance and glucose intolerance were induced by STZ treatment, which severely reduces pancreatic β-cell function. In addition to regulating glucose metabolism, THF was reported to improve hepatic lipid accumulation, impair glucose tolerance, and reduce fasting glucose.[20] In our study, plasma lipid profiles of T2DM mice treated with THF showed similar trends to those observed in the HFD/STZ-induced rats treated with polysaccharide boletus.[33]

Furthermore, THF administration also significantly reduced lipid accumulation in the liver of T2DM mice. The lipid metabolism was severely disordered, and the liver lesions were severe in the HFD/STZ-induced mice. In contrast, blood glucose and blood lipid contents of T2DM mice with THF administration for ten weeks tended to the Control group mice, liver and pancreatic islet lesions also attenuated, and the correlation coefficient of lipid metabolism was also improved. THF improved the body's ability to metabolize, promoted recovery of insulin function, and effectively reversed the disorder of glucose and lipid metabolism in T2DM mice. Anomalous hepatic TG accumulation is frequently associated with T2DM. Improvements in lipid metabolism halt fatty acid efflux from adipose tissue to the liver, thereby mitigate liver injury in diabetes.[34] Certain adipokines secreted by adipose tissue are implicated in NAFLD and T2DM pathogenesis. APN, as the most abundant adipokines, are known to be insulin sensitizers, improving glucose and lipid metabolism, and have anti-inflammatory and anti-atherosclerotic properties.[35] Since APN indicates of early risk prediction for T2DM and negatively correlates with the fibrosis stage, recombinant APN treatment can ameliorate mouse hepatopathy. Serum APN content in mice of T2DM was significantly reduced in our experiment, and THF could reverse it to some extent, indicating THF might increase APN level, thus reducing IR and promoting normalization of glucose metabolism. Collectively, our observations demonstrated that THF exerted protective effects on hepatic glucose and lipid metabolism in the HFD/STZ-induced T2DM mice. Metformin, a first-line drug in the clinical treating of T2DM, has demonstrated excellent glucose-lowering effects. In the study, we also found that metformin was highly effective in lowering glucose in T2DM, and there is no significant difference between THF and metformin. While THF was superior to metformin in serum APN levels, hepatic IL-6, TNF-α.

It is important to note that the liver is the central organ regulating the body's metabolic homeostasis.[36] Its functional deficiency will cause an imbalance of glucose and lipid ratio, leading to metabolic diseases such as IR, T2DM and NAFLD, even increasing cancer risk. ATF3 can function as an activator or transcriptional repressor[37] and plays a significant role

in the occurrence and development of these chronic diseases. Despite some studies that have been done on ATF3,[38] it remains unclear whether the physiological role of ATF3 is beneficial or detrimental to the occurrence of metabolic dysfunctions. Kim et al.[39] showed that ATF3 promotes the development of T2DM in human NAFLD subjects and may serve as a predictive biomarker in early hepatic steatosis-induced T2DM. It's been reported that enabling the ATF3 level will cause ectopic fat deposition in the liver, which can easily trigger the mechanism of an inflammatory response, thus promoting hepatic steatosis.[40] In our results, the expression of fatty acid synthesis and uptake-related genes such as Fabp1 and S1c27a1 in T2DM mice and the upregulation of gluconeogenesis genes such as PEPCK increased. In contrast, mRNA level of fatty acids β-oxidation was downregulated, further inducing the inflammatory indicators to increase, indicating that T2DM progressed to some extent. However, these performances were significantly improved after THF administration, indicating that THF has some capacity to regulate hepatic inflammation and glucose and lipid metabolism in T2DM mice.

Furthermore, ATF3 modulates the expression of key metabolic enzymes involved in gluconeogenesis, fatty acid oxidation, and oxidative phosphorylation in the liver through its functional interplay. Recently, a study showed that increasing hepatic CRLS1 protein expression could ameliorate IR symptoms in obese mice via transcriptional repression of ATF3 gene expression.[41] Our results showed that the mRNA expression of ATF3 was negatively correlated with the changing trend of CRLS1. ChREBP mRNA expression was significantly reduced in T2DM mice, consistent with Jois et al.,[42] suggesting that it can maintain hepatic insulin sensitivity, regulate systemic lipid metabolism by controlling lipogenesis, and regulate glycolipid balance. Our validation results showed that both the protein and gene expression levels of ATF3 were directly proportional to the expressions of inflammation-related factors, fatty acid synthesis and uptake-related factors, and gluconeogenesis-related factors, while those of CRLS1 and ChREBP gene, fatty acid β oxidation is inversely proportional, indicating that THF may partially improve IR in T2DM mice by activating the hepatic CRLS1-ATF3/ChREBP signaling pathway, further to inhibit gluconeogenesis and inflammation and stabilize liver metabolic function, thereby delaying the development of T2DM with NAFLD.

5. Conclusion

In conclusion, our study showed that THF could ameliorate nonalcoholic fatty liver diseases in type 2 diabetic mice, and the mechanism was related to the CRLS1-ATF3/ChREBP pathway. Our findings shed light on the molecular mechanisms of THF in alleviating diabetic hepatopathy and provided further evidence for developing its therapeutic potential (Fig. 6).

Ethics approval and consent to participate

All the animal experiments were approved by the Animal Ethical Committee of Guangdong Pharmaceutical University (GDPULACSPF2017079).

Author contribution

GJ and LD were responsible for the conception and design of the study; HY, ZY, XX, and LZ for the data collection, analysis, image processing, and writing the manuscript. All authors read and approved the final manuscript.

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Declaration of competing interest

The authors declare that they have no competing interests.

Abbreviations

ALT	Alanine transaminase
APN	Adiponectin
AST	Aspartate transaminase
ATF3	Activating transcription factor 3
AUC	Area under the curve
ChREBP	Carbohydrate responsive element binding protein
CRLS1	Adipose cardiolipin synthase 1
DH	Diabetic hepatopathy
FBG	Fasting blood glucose
FFA	Free fatty acids
HDL-C	High density lipoprotein
HFD	High-fat diet
HOMA-IR	Homeostasis model assessment of insulin resistance
i.p.	Intraperitoneally
INS	Fasting insulin
IR	Insulin resistance
ITT	Insulin tolerance test
LDL-C	Low density lipoprotein
OGTT	Oral glucose tolerance test
PAS	Periodic acid-Schiff
PEPCK	Phosphoenolpyruvate carboxykinase
STZ	Streptozocin
T2DM	Type 2 diabetes mellitus
TC	Total cholesterol
TG	Triglycerides
THF	Tianhuang formula
THF-H	High dose Tianhuang formula
THF-L	Low dose Tianhuang formula

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhip.2023.09.002.

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