Original Article

Quzhi Formula Alleviates Nonalcoholic Steatohepatitis by Impairing Hepatocyte Lipid Accumulation and Inflammation via Bip/eIF2α Signaling

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Abstract

Background and Aims: The Quzhi formula, a Chinese medicine compound prescription, relieves nonalcoholic steatohepatitis (NASH) symptoms. This study aimed to explore the mechanism of the Quzhi formula against NASH. ***Methods:*** A choline-deficient, L-amino acid-defined, high-fat diet induced a NASH mouse model and a free fatty acid-induced mouse hepatocyte cell model were used to evaluate the function of Quzhi formula *in vivo* and *in vitro*. Network pharmacology and molecular docking technology were performed to uncover the possible protective mechanisms of the Quzhi formula against NASH. Key factors in liver lipid metabolism and endoplasmic reticulum (ER) stress pathway were evaluated to verify the mechanism. ***Results:*** The positive contribution of the Quzhi formula on NASH was confirmed *in vivo* and *in vitro*. Abnormal accumulation of lipid in the liver and inflammatory responses were significantly decreased by the Quzhi formula. Network pharmacological analysis and Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis showed that the Quzhi formula protected against NASH by regulating ER stress and inflammatory responses, which was enhanced by further molecular docking analysis. In addition, mechanism exploration showed that Quzhi formula mainly reduced ER stress by downregulating Bip/ eIF2α signaling. ***Conclusions:*** The Quzhi formula protected against NASH by inhibiting lipid accumulation, ER stress, and inflammatory responses, which supports the potential use of Quzhi formula as an alternative treatment for NASH.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH), a severe subtype, is the predominant chronic liver disease worldwide.[1] NASH can progress to cirrhosis and hepatocellular carcinoma (HCC) which has a poor prognosis.[2] There are no approved pharmacological therapies to prevent or treat this condition.[3] Aberrant lipid metabolism and unfolded protein response (UPR) induce endoplasmic reticulum (ER) stress and inflammation that are distinguishing features of NASH.[4] Targeting these biology process may provide new insight of treatment of NASH.

Herbs are widely used to treat chronic liver disease as complementary and alternative medicine, especially in China, Japan, and other Asian countries.[5] Traditional Chinese medicine (TCM) includes various herbal prescriptions that have been proven effective in the treatment of NAFLD.[6] The Quzhi formula is one such prescription. It consists of *Polygonum cuspidatum*, *Cassiae semen*, and *Crataegi fructus*. These three herbs contain active substances including quinones, stilbenes, and flavonoids, which beneficial in hyperlipemia, and inflammation,[7] and have antidiabetes, anti-inflammatory, and hepatoprotective activity.[8,9] Given the pathogenesis of NASH, the Quzhi formula is an optimal treatment. Our previous clinical study demonstrated that the Quzhi formula was safe and effective for the treatment of NASH, and improved NASH symptoms including liver dysfunction, triglycerides,

and fibroscan results.[10] However, the number of ingredients and multipath pharmacological action make it difficult to elucidate the pharmacological effects of Chinese medicines in detail. The underlying mechanisms of action of Quizhi formula against NASH needs more exploration.

In this study, we confirmed the positive effect of Quzhi formula on NASH in a diet-induced mouse model. Network pharmacology and molecular docking technology were then used to clarify the relationships of the medication ingredients, the potential targets, and the possible mechanisms of the Quzhi formula. Ae also validated the Quzhi formula effect on steatosis, inflammation, and ER stress to intervene in NASH. Overall, our findings of Quzhi formula function mechanisms provide a promising therapeutic approach for NASH.

Methods

Ethical approval

All mouse experiments were approved by the Animal Care and Use Committee of Shanghai Jiao Tong University (Approval number: A2021107) and performed following the institutional guidelines and protocols.

Experimental animals

Six-week-old male C57BL/6 SPF mice were purchased from SIPPR/BK Experimental Animal Co., Ltd. (Shanghai, China), maintained under specific pathogen-free conditions at 22–25°C, 60% humidity, a 12 h dark-light cycle, and free access to water and a normal diet. After a week of acclimatization, the mice were randomly assigned to three groups: a normally fed control group, nonalcoholic steatohepatitis group (NASH group), or a Quzhi formula (QZF) group. After 3 weeks of feeding a choline-deficient, l-amino acid-defined, high-fat diet (CDAHFD; #CD-HF60, Dyets, Wuxi, China)[11,12] or a normal diet, daily gavage of saline in the control and NASH groups or the QZF formula (8.3331 g/kg/day was begun. After 4 weeks of administration of the Quzhi formula, the mice were euthanized, and plasma and liver tissues were collected. The liver weight index (%) was calculated as liver weight/body weight × 100.

Liver histology

Liver tissue was fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, cut into 5 μm sections. and stained with hematoxylin-eosin (HE) for histological evaluation.

Oil Red O staining

Frozen sections (6 μm) were fixed in 4% paraformaldehyde for 0.5 h and stained by Oil Red O solution for 0.5 h. The stained hepatocytes were rinsed with 60% isopropanol and counterstained with hematoxylin. After washing with distilled water, the stained lipid droplets within cells were observed with an inverted microscope.

Cell culture

C57BL/6 mouse primary hepatocytes were isolated by an improved classic two-step collagenase perfusion technique.[13] Mouse hepatocyte AML12 (#CRL-2254, ATCC, Shanghai, China) cells were purchased from the American Type Culture Commission and cultured at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (#S811-001, Lonsera, Shanghai, China) and 100 units of penicillin-streptomycin (#15-140-122, Gibco, Waltham, MA, USA). To develop a NASH model, the hepatocytes were stimulated with 0.75 mM free fatty acids (FFAs; #SYSJ-KJ006, Kunchuang Biotechnology, Xi'an, China) with oleic acid and palmitic acid 2: 1 for 24 h. The cells were incubated with various concentrations of Quzhi formula extract (1.25 μg/ml) for 24 h.

Statistical analysis

Results were reported as means ± standard deviation. Graphpad Prism 9.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for the statistical analysis. Statistical significance was determined by Student's *t*-test for between-group comparisons. One-way analysis of variance was used for multiple group comparisons. Differences were significant at $p<0.05$, $p<0.01$, $p<0.001$, and $p<0.001$. Additional materials and methods were described in Supplementary File 1.

Results

Quzhi formula alleviated hepatic steatosis in NASH mice

A rapidly induced NASH mouse model was developed with a CDAHFD to assess treatment effectiveness of Quzhi formula. (Fig. 1A). Although Quzhi formula treatment did not affect CDAHFD-induced bodyweight loss (Fig. 1B, left panel), the liver weight (Fig. 1B, middle panel) and liver weight index (Fig. 1B, right panel) significantly decreased in QZF group. A representative image (Fig. 1C) shows that the liver from the QZF group was smaller than one from the NASH group.

Serum levels of aspartate aminotransferase (AST) were significantly higher in the NASH group than those in the control group, and Quzhi formula treatment reversed that condition (Fig. 1D), which indicates that Quzhi formula inhibited steatohepatitis in the liver. Increased serum low-density lipoprotein cholesterol (LDL-C) and decreased serum high-density lipoprotein cholesterol (HDL-C) characteristic of NASH. Quzhi formula treatment significantly reduced the elevated serum LDL-C in CDAHFD-induced NASH and increased serum HDL-C (Fig. 1E, left and middle panels). The LDL-C/HDL-C ratio was significantly decreased in Quzhi formula-treated NASH mice (Fig. 1E, right panel)

H&E staining of liver sections showed obvious damage of hepatocyte structure with vacuolation, severe steatosis, inflammation, and hepatocellular hypertrophy that was alleviated in mice treated with the Quzhi formula (Fig. 1F). Oil red O staining revealed accumulation of red lipid droplets in CDAHFD-induced NASH livers. The Quzhi formula significantly ameliorated lipid accumulation in liver cells (Fig. 1G). Overall, the results reveal that Quzhi formula alleviated hepatic steatosis in NASH model mice.

Active ingredients of the Quzhi formula

To investigate the mechanism of action of the Quzhi formula against NASH, we performed key active compounds analysis. A total of 187 compounds were retrieved from the TCMSP and BATMAN-TCM databases for the Quzhi formula,
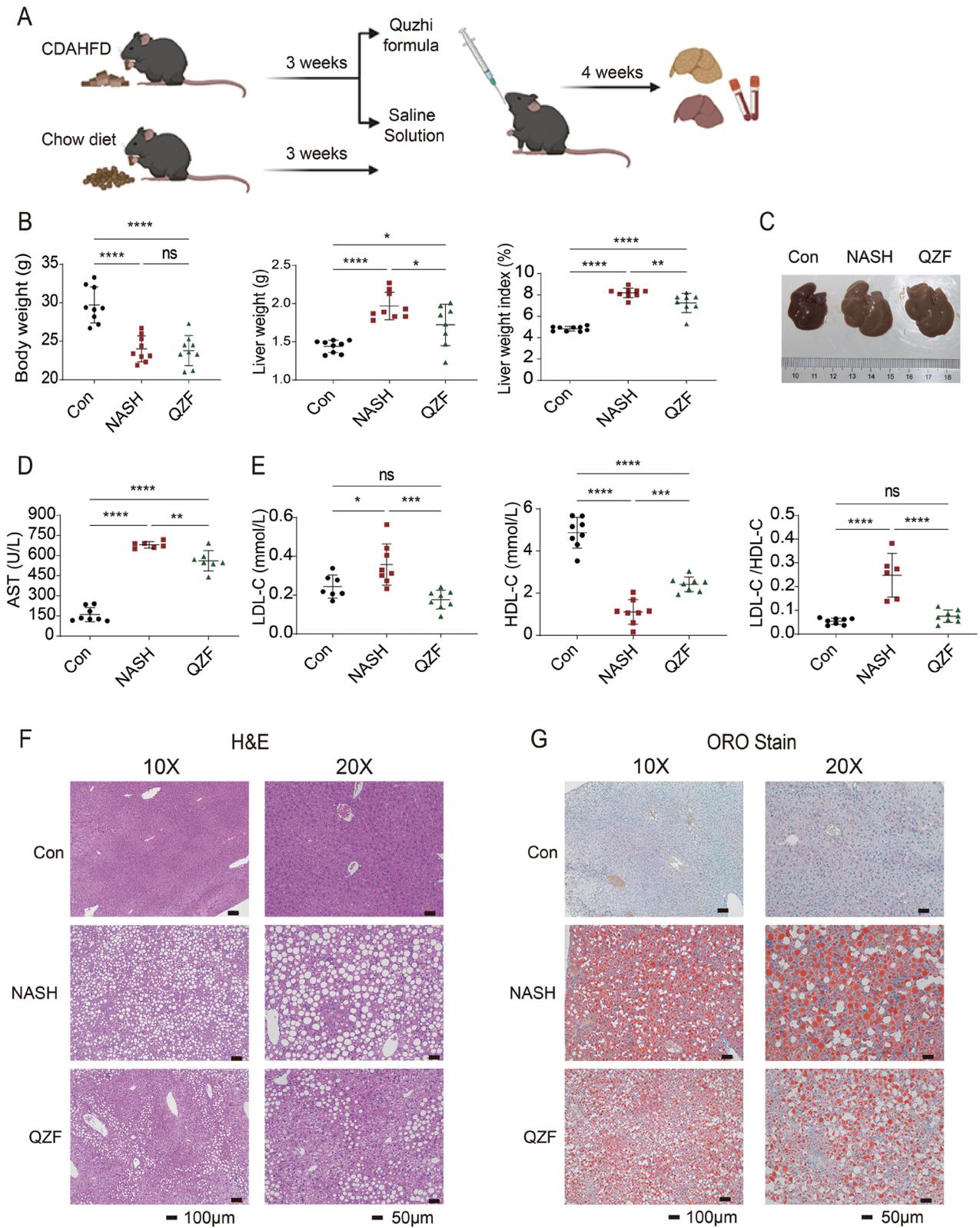
Fig. 1. Quzhi formula alleviation of hepatic steatosis in NASH mice. (A) Diagram of the experimental design. (B) Body weight, liver weight, and liver weight index were assessed in mice (*n*=6–9). (C) Representative mouse liver images. (D) Serum AST, (E) LDL-C, HDL-C, and LDL-C/HDL-C levels in mice (*n*=6–9). (F) Representative hematoxylin and eosin and (G) Oil Red O staining of liver sections. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. CDAHFD, choline-deficient, l-amino acid-defined, high-fat diet; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; NASH, nonalcoholic steatohepatitis; QZF, Quzhi formula.
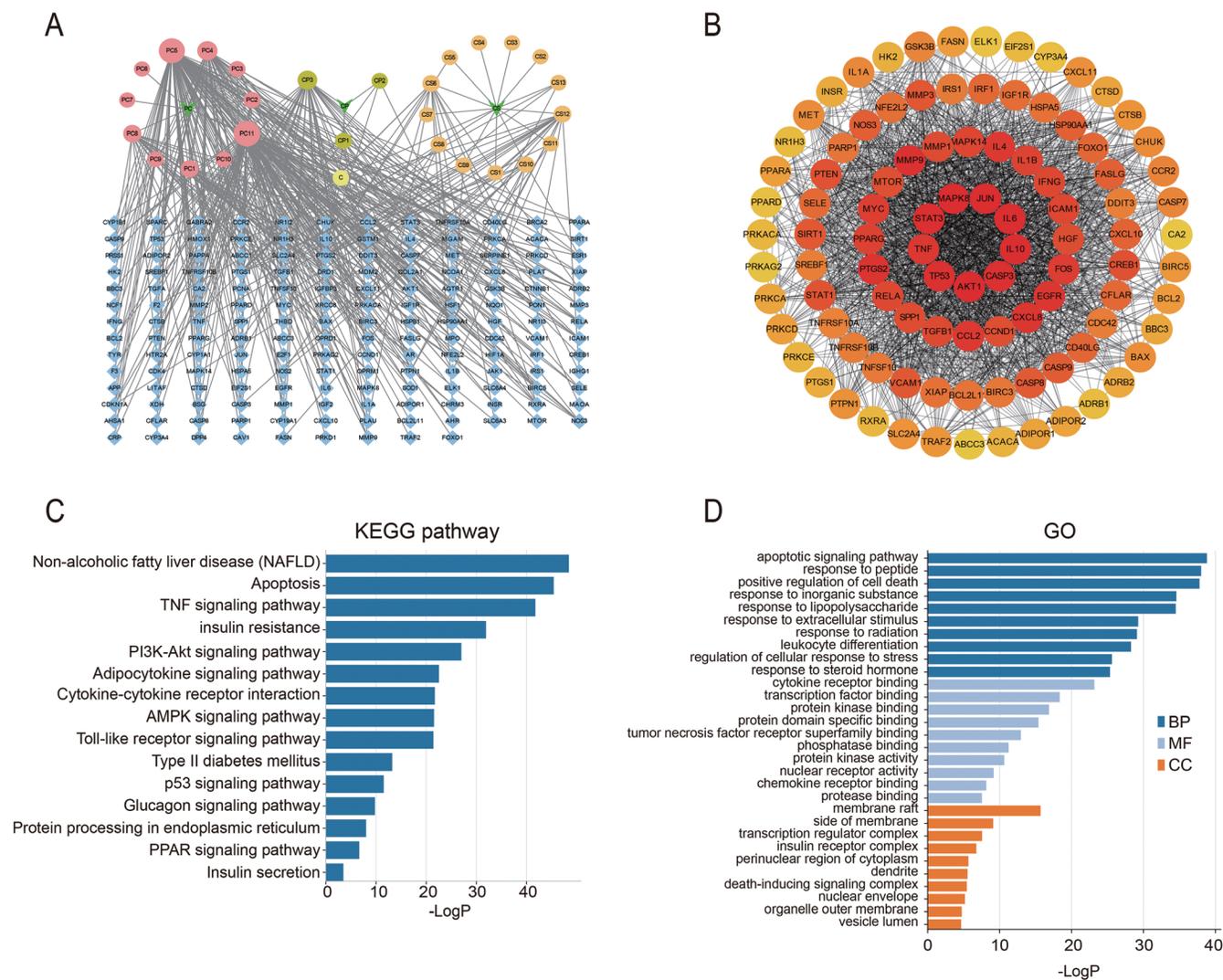
Fig. 2. Network pharmacology analysis of the Quzhi formula for NASH treatment. (A) Herb-compound-target network of Quzhi formula. The network includes three herbs, 28 compounds, and 165 proteins, forming 426 edges. Node size was positively associated with node degree. (B) PPI network of the identified targets. The darker and closer to the center of the node indicates a more advanced RANK and a closer relationship between the proteins. (C) KEGG pathway analysis of the targets. (D) GO function analysis of the targets. C, rhein; CP, *Crataegus pinnatifida*; CP1, suchilactone; CP2, linoleyl acetate; CP3, ursolic acid. CS, *Cassiae semen*; CS1, aurantio-obtusin; CS10, 9,10-dihydroxy-7-methoxy-3-methylene-4H-benzo[g]isochromen-1-one; CS11, obtusin; CS12, aloe-emodin; CS13, cassiaside A; CS2, campest-5-en-3beta-ol; CS3, CLR; CS4, rubrofusarin-6-beta-gentiobioside; CS5, gluco-obtusifolin; CS6, stigmasterol; CS7, rubrofusarin; CS8, toralactone; CS9, quinizarin; PC, *Polygonum cuspidatum*; PC1, physovenine; PC10, polydatin; PC11, resveratrol; PC2, 6,8-dihydroxy-7-methoxyxanthone; PC3, (+)-catechin; PC4, luteolin; PC5, quercetin; PC6, physciondiglucoside; PC7, torachrysone-8-O-beta-D-(6'-oxayl)-glucoside; PC8, beta-sitosterol; PC9, picralinal,

including 62 for *Polygonum cuspidatum*, 68 for *Cassiae semen*, and 57 for *Crataegus pinnatifida*. A total of 24 compounds were screened for oral bioavailability (OB) ≥30% and drug-like properties (DL) ≥0.18, and four compounds were added by literature review, for a total of 28 compounds (Supplementary Table 1).

Herb-compound-target network and protein-protein interaction (PPI) network

With the aim to explore potential targets of the 28 compounds, we exploited herb-compound-target network and PPI network calculation, and 287 targets were obtained by converting the active ingredient targets obtained from the TCMSP database into gene symbols. A total of 2,554 NASH-related targets were obtained in the GeneCards and OMIM databases, and one target was deleted after checking the UniProt database for homo sapiens. After intersecting ingredient and disease targets, 165 potential targets of the Quzhi formula for NASH were obtained. An herb-compound-target network was constructed to identify the relationship between herbs, bioactive compounds, and their potential targets, which included 196 nodes and 426 edges (Fig. 2A). Rhein is a component of *Polygonum cuspidatum* and *Cassiae semen*. The top 100 potential targets of MCC were selected for the network graph, including 100 nodes and 1,797 edges (Fig. 2B). Nodes represent proteins and edges represent protein-protein relationships. The darker and closer to the center of the node indicates a more advanced RANK and a closer relationship between the proteins. Among them, AKT1, IL6, JUN, IL10, TP53, CASP3, and TNF-α were highly connected in the network (Supplementary Table 2), which

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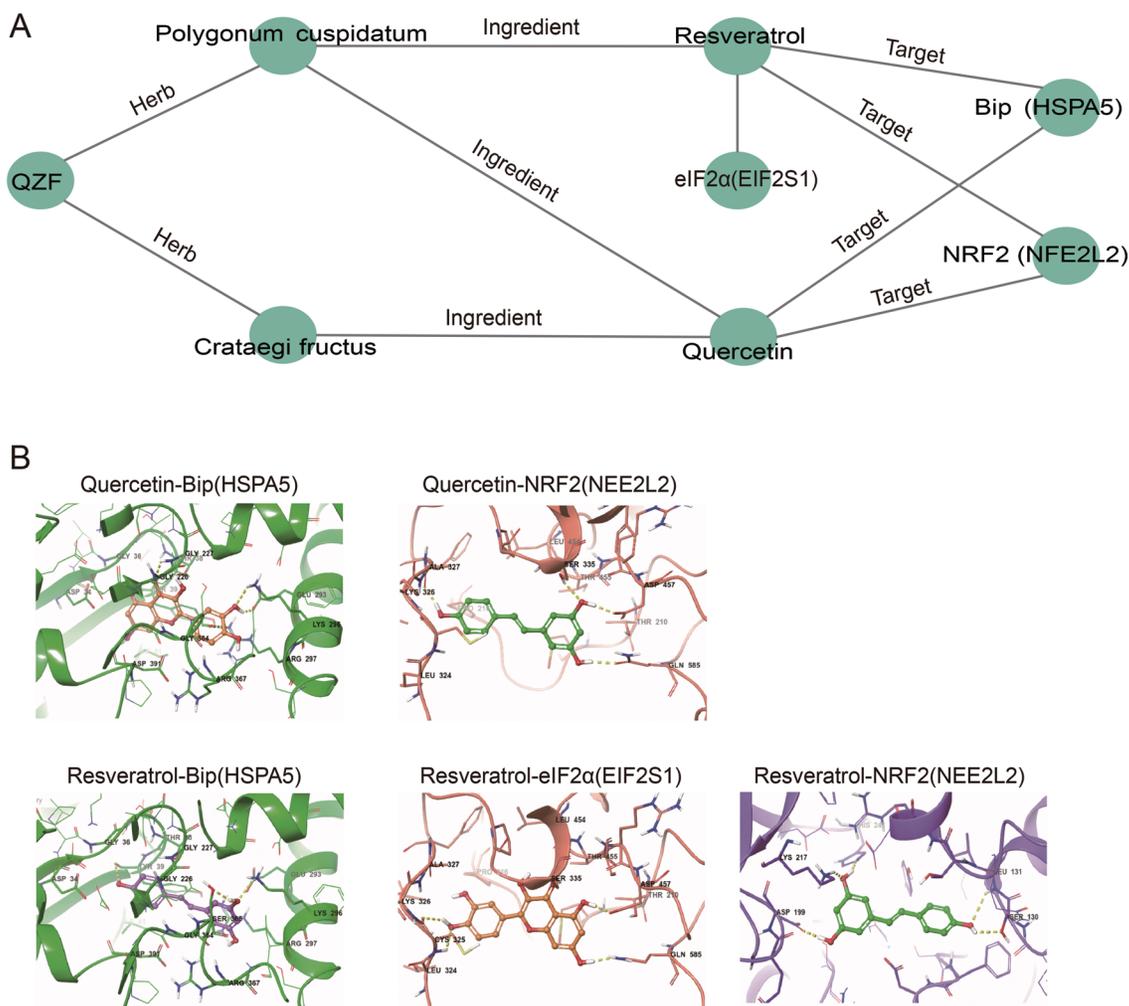
Fig. 3. Molecular docking. (A) Docking analysis of active pharmaceutical ingredients of Quzhi formula and core genes of NASH. (B) Resveratrol and quercetin have multiple binding sites with Bip, eIF2α, and NRF2.

suggested that the targets might be the potential key targets of the Quzhi formula compounds for the treatment of NASH.

We conducted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis on 100 potential targets and screened out 157 pathways based on the threshold of $p \leq 0.01$. Excluding pathways not related to NASH, the top 15 pathways were identified by p-value, suggesting that the Quzhi formula protected against NASH by regulating inflammatory response, ER stress, and insulin resistance (Fig. 2C). In addition, the Metascape database was used to perform gene ontology (GO) function analysis on 100 potential targets. There were 2,116 GO terms acquired based on a p-value ≤ 0.01, including 1,951 of biological progress, 57 cellular components, and 108 molecular functions. The enrichment results showed that apoptotic signaling pathway, cytokine receptor binding, and membrane raft were the most important biological functions (Fig. 2D).

Molecular docking

According to the KEGG analysis, we identified 30 potential targets associated with inflammation, ER stress, and insulin resistance. The top 10 compounds in terms of degree were docked with 30 potential target proteins (Fig. 3A). Generally, when the binding force is below -5.5 kJ/mol, bioactive compounds are considered to have a superior binding activity to the target. Further molecular docking results showed that resveratrol and quercetin have multiple binding sites with Bip, eIF2α, and NRF2 (Fig. 3B). It is suggested that the active ingredients of the Quzhi formula may be involved in the regulation of ER stress by directly binding to Bip, eIF2α, and NRF2.

Effect of Quzhi formula on Bip/eIF2α signaling and inflammation and lipid-related gene expression in liver tissue

To verify the upper calculated conclusion, we detected the changes of ER stress pathways in Quzhi formula-treated mouse NASH model livers. Western blot results showed that the expression of Bip and eIF2α proteins were markedly increased, compared with the control group, which were significantly downregulated after the Quzhi formula treatment (Fig. 4A). In addition, expression of the inflammation and lipid-related targets identified from network pharmacology

A

B

C
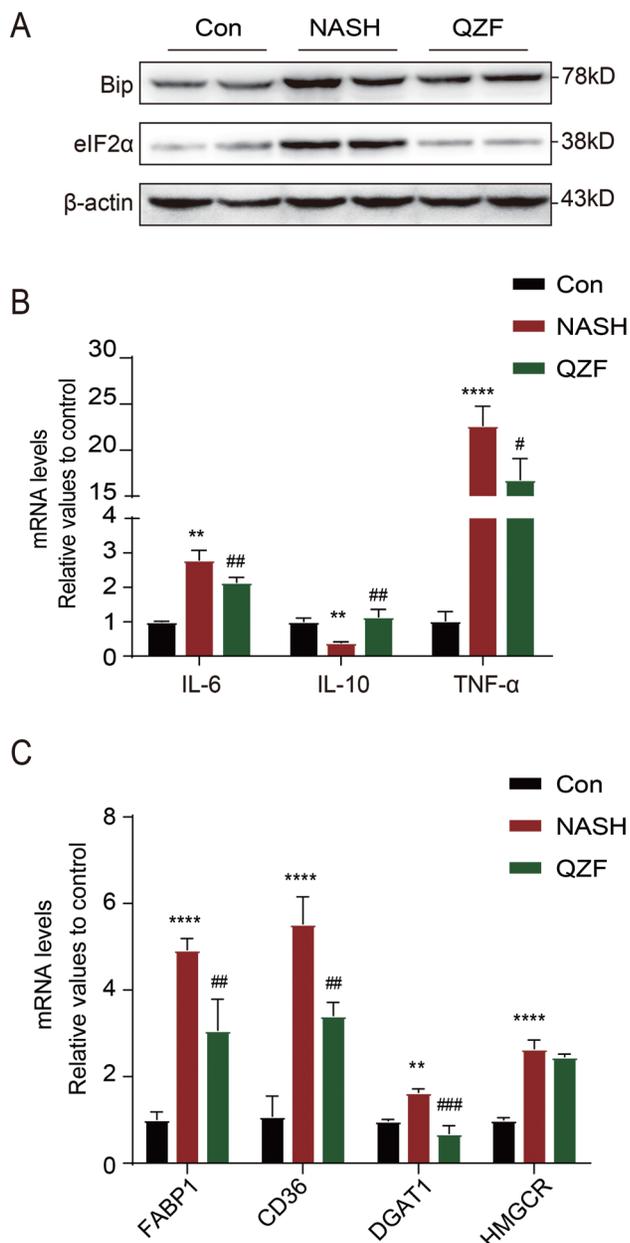
Fig. 4. Administration of Quzhi formula inhibits inflammation-related gene expression via Bip/eIF2α signaling, and downregulates lipogenic gene expression in mice fed with CDAHFD. (A) Western blot analysis of hepatic protein levels of Bip and eIF2α. (B) Hepatic mRNA expression of inflammatory response related cytokines, including IL6, IL10, and TNF-α. (C) Hepatic mRNA expression of the lipid-related genes, including *FABP1, CD36, DGAT1*, and *HMGCR*. *$P<0.05$, **$p<0.01$, ***$p<0.001$, ****$p< 0.0001$; #$p<0.05$, ##$p<0.01$, ###$p<0.001$, and ####$p<0.0001$. CD36, cluster of differentiation 36; DGAT1, diacylglycerol acyltransferase 1; FABP1, fatty acid-binding protein 1; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase. IL-10, interleukin-10; Abbreviation: IL-6, interleukin-6; TNF-α, tumor necrosis factor alpha.

were assayed. qRT-PCR showed that IL6 and TNF-α were significantly increased, and IL10 was significantly decreased in the NASH group compared with the control group, and the Quzhi formula treatment significantly decreased the expression of IL6 and TNF-α and increased the expression of IL10 in the QZF group (Fig. 4B). At the same time, the Quzhi formula improved hepatic lipid metabolism in NASH

mice, including downregulation of FABP1, CD36, and DGAT1 (Fig. 4C).

Effect of Quzhi formula on FFA-induced steatohepatitis in vitro

To further explore the mechanism of Quzhi formula on NASH, we performed *in vitro* detection on FFA-induced steatohepatitis in hepatocytes. The effect of different concentrations of Quzhi formula on the viability of mouse hepatocytes was assessed by CCK-8. Quzhi formula did not have significant toxicity at the tested concentrations of 0, 0.625, 1.25, 2.5, and 5 µg/ml (Fig. 5A). The Quzhi formula significantly downregulated the expression of Bip and eIF2α protein, which were increased after FFA treatment (Fig. 5B). Oil Red O staining showed significant lipid droplet accumulation in FFA-treated hepatocytes, but the Quzhi formula significantly reduced lipid accumulation (Fig. 5C). The Quzhi formula significantly also reduced inflammation-related gene expression *in vitro*, including downregulation of IL6 and TNF-α and upregulation of IL10 (Fig. 5D). Quzhi formula also increased the expression of lipid-related genes, such as *FABP1, CD36, DGAT1*, and *HMGCR* (Fig. 5E).

Discussion

TCM, indispensable sources for providing therapeutic candidates for treating NASH, have garnered increased attention. Multicompound composition and multitarget activities are the main advantages of TCM and the main challenges for pharmacological mechanism research.[14] Herein, we applied network pharmacology to investigate the intervention and impact of the Quzhi formula on NASH from a holistic perspective, revealing the complex relationship between drug, disease, target, and pathway, and explored the effects and potential mechanisms of the Quzhi formula on NASH in *in vivo* and *in vitro* experiments.

We successfully induced NASH in mice by CDAHFD, which exhibited distinct features of hepatic steatosis and inflammation. Quzhi formula treatment significantly improved the liver weight index and hepatocyte injury markers (AST) in serum and alleviated hepatic steatosis and hepatic lipid accumulation. As an epidemic metabolic disease, patients with NASH usually experience disturbance of lipid metabolism, which was also observed in mice fed with CDAHFD, including increased LDL-C decreased lower HDL-C. However, Quzhi formula treatment significantly reduced elevated serum LDL-C and increased serum HDL-C. NASH pathohistological features were blocked by Quzhi formula treatment in mouse liver hepatocytes cultured with FFA.

A total of 28 active ingredients of the Quzhi formula were screened by network pharmacological analysis, among which polydatin, cassiaside A, and ursolic acid were identified by HPLC analysis. It has been demonstrated that polydatin prevents NASH and fibrosis by inhibiting oxidative stress and inflammation.[15] Previous studies have found that ursolic acid inhibits the development of NASH by increasing lipid β-oxidation and attenuating ER stress.[16] Ursolic acid has also been found to be a naturally occurring LXRα and PXR antagonist for inhibition of hepatic steatosis.[17] The role of cassiaside A remains to be confirmed. No studies have reported cassiaside A as a potential drug candidate for the prevention of NASH.

Bip, a molecular chaperone in the ER, is a well-known marker of ER stress activation. Recently, ER stress has been identified as a key mechanism in many liver disorders, including NAFLD-NASH transition, by promoting inflammation, lipid biosynthesis, and apoptosis.[18] Chronic imbalance
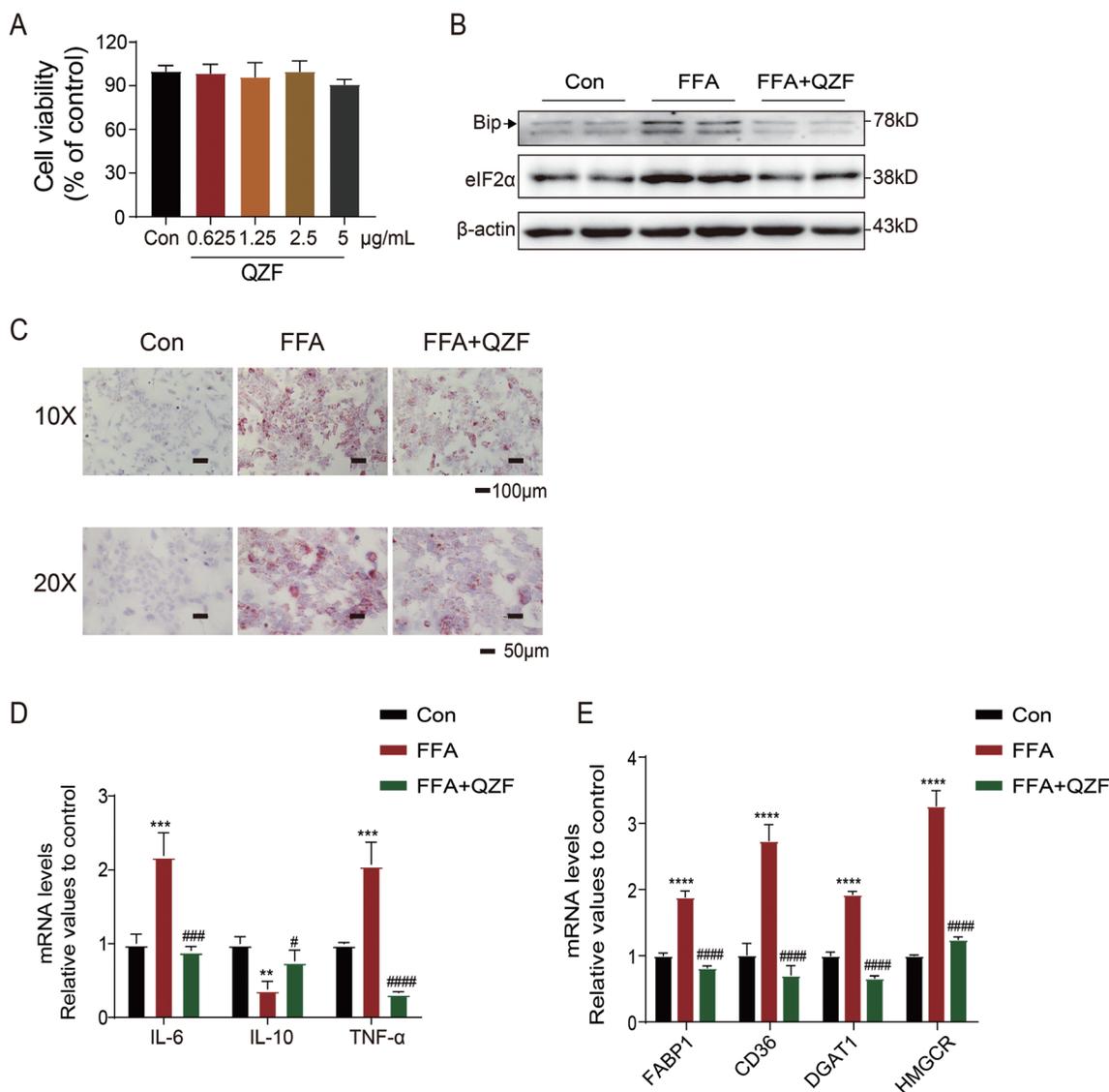
Fig. 5. Effect of Quzhi formula on FFA-induced steatohepatitis *in vitro*. (A) CCK8 assay of mouse hepatocytes, which were incubated in FFA with or without Quzhi formula for another 24 h. (B) Western blot assay of p of Bip and eIF2α protein expression (C) Oil Red O staining of hepatocytes. (D) Hepatocyte mRNA expression levels of inflammatory response related cytokines, including IL6, IL10, and TNF-α; (E) Hepatocyte mRNA expression of lipid-related genes, including *FABP1, CD36, DGAT1*, and *HMGCR*. *P<0.05, **p<0.01, ***p<0.001, ****p<0.0001; #p<0.05, ##p<0.01, ###p<0.001, and ####p<0.0001. FFA, free fatty acid.

of energy supply and demand that exposes hepatocytes to toxic lipids and accumulation of unfolded or misfolded proteins in the ER, causes intracellular stress, that induces a UPR.[19] It has been reported that Bip is positively correlated with hepatocytic apoptosis,[20] and the phospho-eIF-2α branch of the ER stress response is induced in both NAFLD and NASH patients.[21] As an important positive regulator of ER stress, eIF2α is an intermediate regulator of the PERK-eIF2a-ATF4 axis, which is primarily implicated in lipogenesis and steatosis regulation.[22] Quzhi formula treatment has shown its beneficial effect by decreasing UPR parameters, Bip and eIF2α, along with lipogenesis and inflammation associated markers following NAFLD development.

ER stress-induced inflammatory responses (IL6, IL10, and TNF-α) lead to the progress of NASH.[4] Mice with CDAH-FD-induced NASH presented an imbalance of pro-inflammatory cytokines (IL6 and TNF-α) and anti-inflammatory cytokines (IL10) in the liver. Increased expression of IL6

and TNF-α, two important proinflammatory cytokines, play a central role in the promotion of liver inflammation and tumorigenesis in dietary and genetic obesity.[23] Our results showed that treatment with the Quzhi formula inhibited the abundance of inflammatory markers, including TNF-α and IL6.

Hepatic steatosis and NASH begin with the abnormal accumulation of hepatocyte lipids. Lipid accumulation in hepatocytes triggers lipotoxicity leading to chronic damage of the liver tissue and the initiation of liver inflammation, which is a marker of NASH.[24] Our results revealed that the key targets of Quzhi formula for NASH treatment involved fatty acid metabolism (DGAT1), which was demonstrated *in vivo* and *in vitro*. In addition, several other lipid-related genes, including *FABP1*, *CD36*, and *HMGCR* were also up-regulated by the Quzhi formula. *FABP-1* prevents the damaging effects of excess FFAs, facilitates intracellular FFA transport, and is an important ligand for PPAR-mediated
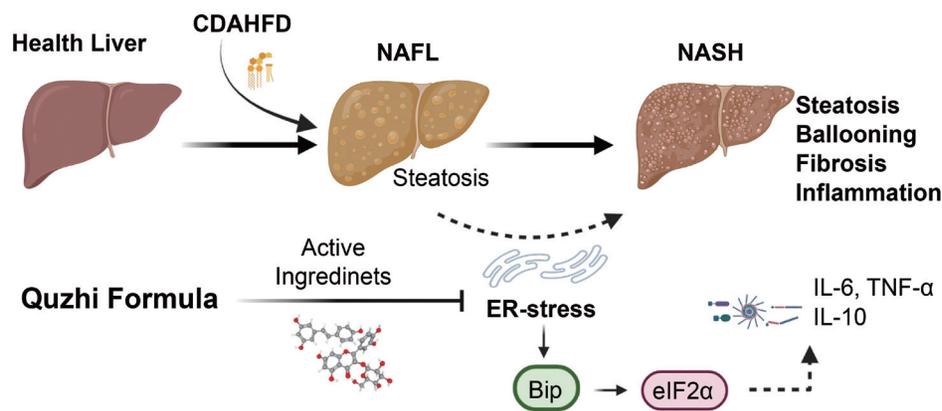
Fig. 6. Graphical model. Quzhi formula alleviates NASH by impairing hepatocyte lipid accumulation and endoplasmic reticulum stress-induced inflammatory response.

transcription.[25] It is well known that the fatty acid translocase CD36 plays a key role in increasing FFA uptake driving hepatosteatosis onset.[26] DGAT1 is one of the membrane-bound O-acyltransferase (MBOAT) superfamily, which mainly function on lipids acylation and triglyceride synthesis.[27,28] HMGCR is a rate-limiting enzyme in cholesterol synthesis in the liver.[29] Increased expression of *FABP1*, *CD36*, *DGAT1*, and *HMGCR* mRNA caused by high lipid levels indicates an increase in adipogenesis and cholesterol accumulation in the liver. While decrease in *FABP1*, *CD36*, *DGAT1*, and *HMGCR* expression caused by the Quzhi formula indicate that Quzhi formula protects against NASH via active chemicals that targeting these lipid-related genes, inhibiting the activity of transcription factors for lipid synthesis, thus inhibiting lipid accumulation.

Conclusion

The Quzhi formula treatment significantly improved liver weight index and serum markers of hepatocyte injury, alleviating hepatic lipid accumulation. NASH pathohistological features were blocked by the Quzhi formula in hepatocytes cultured with FFAs. Network pharmacology and *in vitro* and *in vivo* evidence in this study indicate that the Quzhi formula had a protective effect against NASH by attenuating lipid accumulation, inflammatory response, and ER stress. The results support the potential of the Quzhi formula to become an alternative treatment for NASH (Fig. 6). Diet and exercise should not be neglected even if the patient is on the Quzhi formula herbal treatment. We do not recommend it for NASH patients with chronic gastritis and diarrhea. In clinical practice, its efficacy for NASH patients with different TCM syndromes would need to be explored.

Funding

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (QZ, HFC, JXW, YLW), acquisition of data (YLW, TTS, HSC), analysis and interpretation of data (JXW, YLW, TTS), administrative, technical, or material support (QZ, JXW, YLW, TTS), drafting of the manuscript (QZ, JXW, YLW, TTS), study supervision (QZ). All authors reviewed and commented on the manuscript and approved the final version.

Data sharing statement

All data are available upon request.

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