

ORIGINAL RESEARCH ARTICLE

Yinqi Tiaozhi Decoction ameliorates hepatic steatosis in metabolic dysfunction-associated fatty liver disease by regulating the miR-122-5p/FOXO3 axis to modulate lipid metabolism and oxidative stress

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Abstract

Introduction: Metabolic dysfunction-associated fatty liver disease (MAFLD) lacks targeted pharmacotherapies.

Objective: This study investigates whether Yinqi Tiaozhi Decoction (YQTZD) alleviates MAFLD via the miR-122-5p/forkhead box O3 (FOXO3) axis.

Methods: MAFLD was modeled using oleic acid-treated AML12 cells and rats fed a choline-deficient high-fat diet. The effects of YQTZD or YQTZD-containing serum on lipid accumulation, oxidative stress, inflammation, glucose metabolism, and miR-122-5p/FOXO3 expression were evaluated, and the role of miR-122-5p was further examined using mimic transfection.

Results: YQTZD-containing serum (YQTZD-CS) dose-dependently enhanced AML12 cell viability, reduced lipid accumulation (total cholesterol and triglycerides), and mitigated oxidative injury, as reflected by changes in superoxide dismutase, malondialdehyde, and glutathione peroxidase levels. MiR-122-5p downregulation and FOXO3 upregulation were observed alongside these protective effects, while miR-122-5p mimic transfection abolished YQTZD-CS's protection. In choline-deficient high-fat diet-induced MAFLD rats, YQTZD improved body weight, glucose tolerance, and insulin sensitivity, while histopathological assessments confirmed marked attenuation of hepatic steatosis. It also reduced serum lipids, suppressed hepatic pro-inflammatory mediators (interleukin [IL]-6, tumor necrosis factor alpha, and IL-1 β), and mitigated oxidative stress, alongside elevated glutamine synthetase activity and glutamine levels. Crucially, YQTZD consistently suppressed miR-122-5p and upregulated FOXO3 *in vivo*. Thus, YQTZD mitigates hepatic steatosis, oxidative damage, and inflammation in MAFLD, mechanistically linked to miR-122-5p downregulation-mediated FOXO3 upregulation.

Conclusion: This study underscores the translational promise of YQTZD and provides

a mechanistic foundation for its future integration into clinical management of MAFLD.

Keywords: Metabolic dysfunction-associated fatty liver disease; Yinqi Tiaozhi Decoction; Oxidative stress; Lipid deposition; miR-122-5p; FOXO3

1. Introduction

Metabolic dysfunction-associated fatty liver disease (MAFLD), formerly referred to as non-alcoholic fatty liver disease (NAFLD), is a hepatic pathology characterized by diffuse macrovesicular steatosis of hepatocytes and intrahepatic lipid accumulation, excluding cases attributable to excessive alcohol consumption or other well-established hepatotoxic factors. Disease progression in MAFLD follows a well-defined trajectory. It originates as simple steatosis (non-alcoholic fatty liver [NAFL]), may evolve into non-alcoholic steatohepatitis (NASH), and can subsequently culminate in severe outcomes, including liver fibrosis, cirrhosis, and hepatocellular carcinoma.^{1,2} At present, this condition represents a serious global health concern, consistently ranking as a predominant cause of hepatic complications across diverse populations. Epidemiological surveys indicate that the global incidence of MAFLD is rising annually, with an increasing propensity to affect younger populations, and currently afflicts 32.4% of the world's population.³ In China, the prevalence rate stands at 29.6%, positioning this condition as the nation's leading chronic hepatic disorder.⁴ Contemporary medical research suggests that dietary factors, pharmacological agents, and genetic predispositions may contribute to the development of MAFLD. Although the precise pathophysiological mechanisms remain incompletely understood, MAFLD is recognized as associated with metabolic disturbances, insulin resistance, excess adiposity, redox imbalance, endoplasmic reticulum stress, and intestinal microbiota disturbances.⁵⁻⁷ However, the complex pathophysiology and phenotypic heterogeneity of MAFLD contribute to its multifactorial etiology. At present, formal regulatory approval for targeted pharmacotherapies against MAFLD has not yet been secured. While certain therapeutic modalities can delay disease progression and reduce complications, a complete cure remains unavailable, and the risk of disease persists.⁸⁻¹⁰

The traditional Chinese medicine (TCM) field has extensively researched MAFLD and achieved favorable clinical outcomes. Owing to its holistic approach, syndrome differentiation, treatment, and multi-pathway and multi-target actions, TCM demonstrates unique advantages in the management of MAFLD, showing broad application prospects.¹¹ According to TCM theory, fatty liver is caused

by excessive activity, dietary irregularities, and emotional disturbances, leading to dysfunction of the liver, spleen, and kidneys. This internal disorder promotes the stagnation and conglomeration of multiple pathological elements, including deranged qi, static blood, excessive phlegm, retained dampness, and pathogenic heat, which ultimately become trapped and congested in the liver collaterals.^{12,13} The core pathogenesis of fatty liver involves stagnation of liver qi and the invasion of turbid pathogens into the liver, manifested as the interaction between liver stagnation and spleen deficiency or kidney deficiency, and the binding of heat with turbid toxins. Therefore, the treatment of MAFLD should adhere to the fundamental principle of clearing the liver and resolving turbidity. Throughout the treatment process, it is imperative to continuously regulate the flow of qi and invigorate blood circulation while simultaneously harmonizing the spleen and kidneys. Yinqi Tiaozhi Decoction (YQTZD) is derived from Qinggan Xiaozhi Decoction, an empirical formula developed by Professor Sun, a nationally recognized outstanding clinical practitioner of TCM.¹⁴ YQTZD comprises 12 medicinal herbs, including *Artemisiae Scopariae Herba*, *Lycii Fructus*, *Crataegi Fructus*, *Coicis Semen*, *Eupatorii Herba*, and *Radix Glycyrrhizae et Rhizoma*. For the treatment of MAFLD, YQTZD incorporates additional herbs into the original formula, including *Citri Sarcodactylis Fructus*, *Curcumae Rhizoma*, *Alismatis Rhizoma*, and *Pogostemonis Herba*, which are intended to soothe the liver, strengthen the spleen, and promote qi circulation. Furthermore, *Patriniae Herba* is added to clear heat and detoxify, while *Benincasae Semen* is included to clear heat and eliminate dampness. Collectively, the formula soothes the liver qi, resolves turbidity and filth, clears stagnant heat, and eliminates accumulation. Clinical studies have shown that a plus-or-minus formula based on YQTZD has significant efficacy in treating fatty liver.^{15,16} Previous research has confirmed that YQTZD can significantly reduce blood and liver lipid levels in rat models of fatty liver, inhibit lipid accumulation, alleviate oxidative stress, and improve liver pathological damage.¹⁷⁻¹⁹ Despite its observed therapeutic promise, the signaling pathways through which YQTZD alleviates MAFLD remain largely unclear.

In recent years, microRNAs (miRNAs/miRs) have gained recognition as a prominent category of epigenetic modulators, with emerging evidence implicating them in the pathogenesis of MAFLD.^{20,21} Among them, miR-122 is the most abundant liver-specific miRNA and participates in various physiological processes related to liver function and pathology.^{22,23} Of particular interest, circulating levels of miR-122-5p were markedly elevated in pediatric MAFLD patients, suggesting a significant role for this miRNA in driving the progression of MAFLD and its associated metabolic abnormalities, which may, in turn, heighten susceptibility to cardiovascular complications.²⁴ In our previous research, suppressing miR-122-5p may represent an effective therapeutic strategy for MAFLD, and we further identified forkhead box O3 (FOXO3) as a downstream target through which miR-122-5p exerts its ameliorative effects on intrahepatic lipid accumulation, inflammatory responses, and oxidative damage.^{25,26} Consequently, we hypothesize that YQTZD may attenuate hepatic steatosis and oxidative injury in MAFLD by upregulating FOXO3 expression through miR-122-5p suppression.

This investigation aims to assess the therapeutic efficacy and mechanisms of YQTZD in a rat model of MAFLD. The working hypothesis will be rigorously tested across molecular, cellular, and *in vivo* levels, with the ultimate objective of providing a theoretical foundation for YQTZD in MAFLD.

2. Materials and methods

2.1. Preparation of Yinqi Tiaozi Decoction

Yinqi Tiaozi Decoction is composed of *Artemisiae Scopariae Herba* (40 g), *Lycii Fructus* (30 g), *Curcumae Rhizoma* (24 g), *Coicis Semen* (60 g), *Benincasae Semen* (60 g), *Alismatis Rhizoma* (40 g), *Pogostemonis Herba* (30 g), *Eupatorii Herba* (30 g), *Citri Sarcodactylis Fructus* (30 g), *Crataegi Fructus* (30 g), *Patriniae Herba* (40 g), and *Radix Glycyrrhizae et Rhizoma* (12 g). The initial step involved immersing 426 g of raw YQTZD plant materials (Department of Pharmacy, The Eighth Affiliated Hospital, Southern Medical University, China) in water (eight volumes relative to the herb weight), decocting, and simmering for 30 min before filtration. The residue was further decocted with 6× the volume of water for 20 min, then filtered. After pooling the two filtrates, we heated the mixture to concentrate it to a density of 5 g/mL. This procedure yielded 85.2 mL of a five-fold concentrated stock, which was stored at 4 °C until needed for later experiments.

2.2. Chemical composition analysis of Yinqi Tiaozi Decoction

A non-targeted liquid chromatography (LC)–tandem mass spectrometry (MS/MS)-based metabolomic workflow was established to characterize the chemical constituents of YQTZD. In brief, 100 µL of the decoction was mixed with 300 µL of methanol, vortexed for 30 min, and then centrifuged at 13,800× *g* for 10 min (Microfuge 22R Centrifuge, Beckman Coulter, United States of America [USA]). The clear supernatant was collected and injected onto an ACQUITY™ Premier HSS T3 column (1.8 µm, 2.1 × 100 mm; Waters Corporation, USA) maintained at 45 °C. Separation was carried out at a flow rate of 0.3 mL/min using a mobile phase composed of water with 0.1% formic acid (solvent A) and neat methanol (solvent B), following a 16-min linear gradient (U3000, Thermo Fisher Scientific, USA). Mass spectrometry data were acquired in data-dependent acquisition (DDA) mode (Q Exactive, Thermo Fisher Scientific), covering both positive and negative ionization polarities. Full-scan mass spectra were recorded across the range of *m/z* 150–1,500 with a resolution of 70,000, and the top 10 most abundant precursor ions from each scan were selected for subsequent MS/MS fragmentation. Raw data files were processed using MS-DIAL version 4.70 (RIKEN Center for Integrative Medical Sciences, Japan), which performed peak detection, deconvolution, retention-time alignment, and compound annotation by matching against combined spectral databases (MassBank, ReSpec, and Global Natural Products Social Molecular Networking [GNPS], totaling 14,951 reference records). The total ion chromatograms (TIC) are presented in Figure S1.

2.3. Animal experiments

A total of 31 male Sprague–Dawley rats, specific-pathogen-free (SPF) grade, aged 6–8 weeks, were sourced from Chengdu Dashuo Experimental Animal Co., Ltd. (China). These animals were housed in an SPF-grade barrier facility that adhered to standardized environmental parameters, including an ambient temperature range of 20 °C–26 °C, a relative humidity maintained between 30% and 70%, and a diurnal cycle of 12 h of illumination followed by 12 h of darkness. All husbandry practices and experimental procedures were conducted in compliance with institutional guidelines for laboratory animal care.

Following a seven-day acclimation period, 25 rats were assigned to five groups (*n* = 5/group) using a random grouping principle: control, model, low-dose YQTZD (L-YQTZD), high-dose YQTZD (H-YQTZD), and positive control. The required sample size was estimated with G*Power 3.1.9.7 (Franz Faul, Christian-Albrechts-

Universität Kiel, Germany) via an *F*-test. Adopting Cohen's *f* criteria, we set the effect size at $F = 0.88$, $\alpha = 0.05$, and power = 0.80, yielding a total sample size of 25 animals. The sample size was also guided by the 3R principles, aiming to minimize animal use. The MAFLD rat model was induced by feeding a specific choline-deficient and high-fat diet (CD-HFD) for 15 weeks.²⁷ The control cohort received standard chow and drinking water *ad libitum* throughout the entire 23-week period (15 weeks of model construction followed by 8 weeks of treatment). The model, L-YQTZD, H-YQTZD, and positive control cohorts were fed a CD-HFD throughout the 23-week study period. After 15 weeks of CD-HFD feeding, the L-YQTZD group received YQTZD daily via oral gavage at 0.5 times the clinical equivalent dose (9.51 g/kg/day) for 8 weeks while continuing the CD-HFD; the H-YQTZD group followed the same treatment schedule but received YQTZD at 2 times the clinical equivalent dose (38.04 g/kg/day); and the positive control group received atorvastatin (C17058552, Macklin, China) daily by oral gavage at 1.05 mg/kg/day for 8 weeks while continuing the CD-HFD. Atorvastatin was dissolved in 0.9% saline. Both the CD-HFD (SPF grade; XTMRCD60) and the normal diet were supplied by Jiangsu Synergy Medical Bioengineering Co., Ltd. (China).

Weekly body weight was recorded for all animals. At the conclusion of the study, glucose tolerance testing (GTT) and insulin tolerance testing (ITT) were carried out sequentially. For GTT, after a 12-h overnight fast, rats received an intraperitoneal glucose bolus (15% solution, 2 g/kg). Blood glucose was sampled from the tail vein at 0, 15, 30, 60, and 120 min post-injection. Following a three-day washout, ITT was performed under similar fasting conditions, with an intraperitoneal insulin injection (1 U/kg), and glucose readings were obtained at the identical time intervals. After treatment, rats were anesthetized with isoflurane. Once loss of consciousness was confirmed and absence of pain reflex was verified (no response to toe pinching with forceps), blood was drawn via the abdominal aorta. After verifying cessation of heart and respiratory rates, the livers were excised and preserved at -80°C for later assays.

2.4. Hematoxylin and eosin staining

Rat hepatic tissue sections (5- μm slices) were incubated with hematoxylin (H9627, Sigma-Aldrich, USA) for 10 min, and counterstaining was performed with eosin (YE2080, Hefei Bomei Biotechnology Co., Ltd., China) for 3 min. Following dehydration, clearing, and mounting with neutral gum, tissue sections were imaged using a BA210 Digital microcamera system (Mike Audi Industrial Group Co., Ltd., China). Hepatocyte fatty degeneration and inflammatory cell infiltration were evaluated. Lesion

severity was assessed using a graded scale from 0 to 4. The scores corresponded to normal, minimal, mild, moderate, and severe, respectively.

2.5. Western blotting

Rat liver samples were rinsed thoroughly with ice-cold 1×PBS and manually minced into fine pieces on an ice-cooled surface. These minced tissues were transferred into pre-chilled grinding tubes containing two or three stainless-steel beads, followed by the addition of ice-cold lysis buffer. Mechanical homogenization was performed using a refrigerated high-speed tissue disruptor (KZ-III-F, Servicebio, China) for 2–5 min until complete tissue disintegration, confirmed by the absence of macroscopic clumps. The resulting lysate was kept on ice for 30 min to enhance protein solubilization, then centrifuged at $12,000\times g$ for 10 min at 4°C . The clarified supernatant was carefully aspirated and immediately aliquoted for long-term storage at -80°C . Total protein concentration was quantified using a commercial assay kit (BL521C, Biosharp, China). Prior to electrophoresis, samples were denatured by boiling at 95°C for 10 min and flash-frozen at -80°C . Polyacrylamide gels were cast using an automated gel-forming system (G2180-2L, Servicebio), and proteins were separated through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Electrophoresis onto polyvinylidene fluoride (PVDF) membranes was carried out in a semi-dry transfer apparatus (JY-SCZ4+, Beijing Junyi Electrophoresis, China). Membranes were blocked for 2 h at room temperature with 5% non-fat dry milk dissolved in Tris-buffered saline with Tween 20 (TBST), then incubated overnight at 4°C with primary antibodies targeting β -actin (1:50,000; AC026, Abclonal, China), interleukin-1 beta (IL-1 β ; 1:1,000; ab254360, Abcam, USA), IL-6 (1:2,000; DF6087, Affinity Biosciences, China), and tumor necrosis factor alpha (TNF- α ; 1:2,000; A24214, Abclonal). After three 10-min washes in TBST, membranes were probed with horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulin G (IgG) secondary antibody (1:8,000; AS014, Abclonal) for 2 h under gentle agitation. Three further TBST washes were performed before detection. Chemiluminescent signals were generated in the dark using an enhanced ECL substrate (BL520B, Biosharp) and visualized using a Tanon 5200 Multi chemiluminescence imaging system (V2.0 software; Shanghai Tanon Life Science Co., Ltd., China). Densitometric analysis was conducted using Gel-Pro Analyzer 4.0 software (Media Cybernetics, Inc., USA), and relative protein abundance was expressed as integrated optical density (IOD) normalized to β -actin. Representative Western blotting images were shown in Figure R1.

2.6. Preparation of Yinqi Tiaozhi Decoction-containing serum

Six male Sprague–Dawley (SD) rats were randomly assigned to the control or YQTZD treatment group ($n = 3/\text{group}$). The YQTZD group received oral administration of the herbal decoction at a dose of 95.1 g/kg/day, divided into two equal doses administered 12 h apart, for a total of 3 days. In contrast, the control group was administered an equivalent volume of sterile 0.9% sodium chloride solution via oral gavage under identical conditions. Following induction of anesthesia with isoflurane, whole blood was drawn directly from the abdominal aorta using sterile syringes. Blood samples were first kept at 4 °C for 30 min to facilitate clot formation. Serum was then isolated through centrifugation at $3,000\times g$ for 10 min. The harvested supernatant was first heat-treated at 56 °C for 30 min to inactivate complement, thereby avoiding nonspecific immune interference in later functional tests, and then filter-sterilized using a 0.22- μm pore-size membrane. Portions of this processed Yinqi Tiaozhi Decoction-containing serum (YQTZD-CS) were snap-frozen and maintained at -80°C pending further analysis.

2.7. Cell culture and grouping

The murine hepatocyte cell line AML12 (iCell-m003) was purchased from Cellverse Bioscience Technology Co., Ltd. (China) and cultured in AML-12-specific medium (iCell-m003-001b, iCell Bioscience Inc., China).

To study the effects of YQTZD in oleic acid (OA)-induced AML12 cells, cells were divided into control, model, high-dose YQTZD-CS (H-YQTZD-CS), middle-dose YQTZD-CS (M-YQTZD-CS), and low-dose YQTZD-CS (L-YQTZD-CS) groups. Lipid accumulation was induced by 24-h incubation with 1 mM OA. Subsequently, the medium was replaced with the following treatments: the control and model groups received 20% blank serum in 70% base medium supplemented with 10% fetal bovine serum (FBS); H-YQTZD-CS group received 70% base medium + 10% FBS + 20% YQTZD-CS; M-YQTZD-CS group received 80% base medium + 10% FBS + 10% YQTZD-CS; and L-YQTZD-CS group received 85% base medium + 10% FBS + 5% YQTZD-CS.

To study miR-122-5p/FOXO3 signaling underlying the effects of YQTZD, cells were divided into control, model, YQTZD-CS, YQTZD-CS + negative control (NC) mimic, and YQTZD-CS + miR-122-5p mimic groups. Cells were pre-transfected for 12 h, followed by OA-induced lipid accumulation (1 mM, 24 h). Then, except for the control and model groups, which received 20% blank serum, other groups were treated with 20% YQTZD-CS for 24 h.

2.8. Cell transfection

Transfection was carried out using the riboFect™ CP Transfection Kit (Ribobio, China) with minor modifications. Briefly, AML-12 cells were harvested, counted, and resuspended in complete culture medium. Then, the suspension was plated and allowed to adhere overnight. After incubation, the pre-formed transfection complexes were diluted into 1,863 μL of fresh, serum-containing medium, yielding a total volume of 2 mL per well. This final transfection solution was carefully overlaid onto the adherent cells, and the plate was gently rocked back and forth to achieve uniform distribution. Cells were then returned to a humidified incubator at 37 °C with 5% CO_2 for further incubation and functional analysis.

2.9. Cell Counting Kit-8 assay

After transfection, AML-12 cells were seeded onto 96-well plates (5×10^4 cells) and incubated at 37 °C with 5% CO_2 . Following the first incubation, each well received 110 μL of Cell Counting Kit-8 (CCK-8; BS350B, Biosharp) working solution. A microplate reader (ELx800, BioTek Instruments, USA) was used to measure the absorbance at 450 nm after a further 2 h incubation at 37°C with 5% CO_2 .

2.10. Biochemical detection

The assay kits for total protein (TP; A045-2-1), total cholesterol (T-CHO; A111-2-1), and triglycerides (TG; A110-2-1) were obtained from Nanjing Jianjian Bioengineering Institute (China), whereas the kits for glutamine synthetase (GS; ZC-S0650) and glutamine (Gln; ZC-S0974) were sourced from Shanghai ZCIBIO Technology Co., Ltd. (China). All measurements were performed following the instructions provided by the respective manufacturers. A SpectraMAX Plus384 microplate reader (Molecular Devices, USA) was used to quantify cellular levels of these five parameters at 595, 510, 500, 540, and 450 nm, respectively.

2.11. Enzyme-linked immunosorbent assay

Superoxide dismutase (SOD), malondialdehyde (MDA), and glutathione peroxidase (GSH-Px) enzyme-linked immunosorbent assay (ELISA) kits were procured from Shanghai ZCIBIO Technology Co., Ltd., with catalog numbers ZC-38036, ZC-55718-J, and ZC-38196 for mouse assays, and ZC-36451, ZC-55718-J, and ZC-36648 for rat assays, respectively. All analytical determinations were performed in line with the vendor's established protocols. Briefly, 100- μL portions of each sample and standard, along with the blank control groups, were transferred in duplicate to the designated microplate wells, followed by a 37 °C incubation phase of 1–2 h. Next, 100 μL of HRP-conjugated secondary antibodies were added to the wells, and the plate

was incubated at 37 °C for a further 60 min. A subsequent addition of 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) chromogen was made, and the plate was then shielded from ambient light for 15–30 min to enable color formation. The addition of 50 µL of terminating buffer stopped the enzymatic reaction. Ultimately, absorbance readings were acquired at 450 nm using a microplate reader.

2.12. Oil Red O staining

After transfection and treatment, AML-12 cells were fixed with Oil Red O fixative for 20 min, followed by two distilled water washes. A 30-s rinse with 60% isopropanol preceded complete removal of the staining solution. Subsequent washes with 60% isopropanol continued until the interstitial space appeared transparent. Cells were then incubated with Oil Red O buffer for 15 min before microscopic examination (DMI1, LEICA, Germany).

2.13. Real-time fluorescence quantitative polymerase chain reaction

AML12 cells and rat tissues were used, and total RNA was extracted using the Molpure® Cell/Tissue Total RNA Kit (19221ES50, YEASEN, China). Subsequently, the purified RNA was reverse transcribed into complementary DNA (cDNA). Amplification reactions were established and performed on a Quantstudio™ 3 real-time quantitative polymerase chain reaction (RT-qPCR) instrument (Thermo Fisher, USA). Complete sequences of the target genes were retrieved from the National Center for Biotechnology Information (NCBI) databases. Primer Premier 5 software (PREMIER Biosoft International, USA) was utilized to design species-specific oligonucleotides for each target, which were then synthesized by Shanghai Sangon Biotech Co., Ltd (China) and purified via UltraPAGE precast protein gel (ACE Biosciences, China). The primer sequences are listed below:

- (i) *Actb* (mouse): CTACCTCATGAAGATCCTGA CC (forward), CACAGCTTCTCTTTGATGTCAC (reverse);
- (ii) *Foxo3* (mouse): TCACTGTATTCAGCTAGTG CAA (forward), ATGATGGACTCCATGTACACATT (reverse);
- (iii) *Actb* (rat): GGGAAATCGTGCGTGACATT (forward), GCGGCAGTGGCCATCTC (reverse);
- (iv) *Foxo3* (rat): TGCCAGCCTGTCGCCTTC (forward), GCCAGCCCATCGTTCAGATTTC (reverse);
- (v) *Il1b* (rat): AAAATGCCTCGTGCTGTCTGACC (forward), TTGTCGTTGCTTGTCTCTCCTTGTAC (reverse);
- (vi) *Il6* (rat): AGAGACTTCCAGCCAGTTGC (forward), AGCCTCCGACTTGTGAAGTG (reverse);
- (vii) *Tnf* (rat): CTGTGCCTCAGCCTCTTCTC (forward), ACTGATGAGAGGGAGCCCAT (reverse).

The miR-122-5p level was measured using stem-loop RT-qPCR. cDNA synthesis was carried out. The thermal cycling for reverse transcription consisted of incubation at 42 °C for 60 min, followed by enzyme inactivation at 70 °C for 10 min. For the subsequent PCR amplification, a 20-µL reaction system was prepared. The amplification protocol started with an initial denaturation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 2 s, 60 °C for 20 s, and 70 °C for 10 s. Finally, the relative abundance of miR-122-5p was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.14. Data analysis

All statistical evaluations were carried out utilizing SPSS version 21.0 (IBM Corp., USA). Results are presented as means ± standard deviations (SD). For intergroup comparisons involving more than two groups, one-way analysis of variance (ANOVA) was applied, followed by the least significant difference (LSD) test when variances were homogeneous, or by Tamhane's T2 procedure when variances were unequal. A probability value below 0.05 was considered statistically significant.

3. Results

3.1. Chemical composition analysis of Yinqi Tiaozhi Decoction

This comprehensive analysis identified 994 compounds in YQTZD. The TIC is shown in Figure S1. These compounds encompass a wide range of chemical structural types,

including flavonoids (quercetin, kaempferol, and their glycosides), alkaloids, triterpenoid saponins, organic acids, amino acids and their derivatives (e.g., phenylalanyl-isoleucine), fatty acid glycosides, and terpenoids and coumarins. Among the detected constituents, many are recognized for their biological activities, including modulation of lipid profiles, alleviation of oxidative injury, and inhibition of pro-inflammatory mediators. For example, flavonoids (e.g., quercetin and kaempferol) and certain alkaloids have been widely reported to modulate hepatic lipid deposition and enhance antioxidant capacity. This diverse array of bioactive components collectively forms the multi-component, multi-target material basis of YQTZD. We hypothesized that the concerted effects of these active components in YQTZD could attenuate hepatic fat accumulation, mitigate oxidative damage, and suppress inflammatory cascades in MAFLD. A comprehensive list of all identified constituents is available in Table S1.

3.2. YQTZD-CS improves lipid deposition, alleviates oxidative stress, and regulates the miR-122-5p/FOXO3 axis in oleic acid-induced AML12 cells

According to the CCK-8 results, AML12 cell viability was markedly diminished following OA exposure. However,

when YQTZD-CS was administered, a dose-dependent improvement in cellular viability was observed, with the most significant response occurring at the highest concentration (both $p < 0.01$; Figure 1A). Moreover, YQTZD-CS dose-dependently attenuated the OA-induced elevation of TG and T-CHO levels (both $p < 0.05$; Figure 1B and 1C). As depicted in Figure 1D and 1E, the model group exhibited a markedly higher percentage of lipid droplets relative to the control group, indicating substantial steatosis. Relative to the model control group, treatment with YQTZD-CS at all three dosage levels (low, medium, and high) resulted in a marked attenuation of intracellular lipid deposition (all $p < 0.001$). In parallel, exposure of AML12 cells to OA elicited a pronounced suppression of both SOD and GSH-Px enzymatic activities and induced a conspicuous elevation in MDA concentrations, while YQTZD-CS at all three doses effectively reversed these alterations, suggesting a suppressive effect of YQTZD on oxidative stress (all $p < 0.05$; Figure 1F–H). RT-qPCR

analysis demonstrated that OA upregulated miR-122-5p expression and downregulated *Foxo3* messenger RNA (mRNA) levels. Conversely, administration of low-, medium-, and high-dose YQTZD-CS elicited a marked reduction in miR-122-5p levels, coupled with a significant upregulation of *Foxo3* mRNA expression (all $p < 0.05$; Figure 1I and 1J). Collectively, YQTZD effectively ameliorates OA-induced lipid deposition and oxidative injury while improving AML12 cell viability. These effects appear to be partially attributable to the suppression of miR-122-5p expression, which in turn promotes FOXO3 upregulation.

3.3. Overexpression of miR-122-5p reversed the protective effect of YQTZD-CS on lipid deposition and oxidative stress damage in oleic acid-induced AML12 cells

The present subsection offers additional confirmation that YQTZD counteracts OA-elicited lipid accumulation

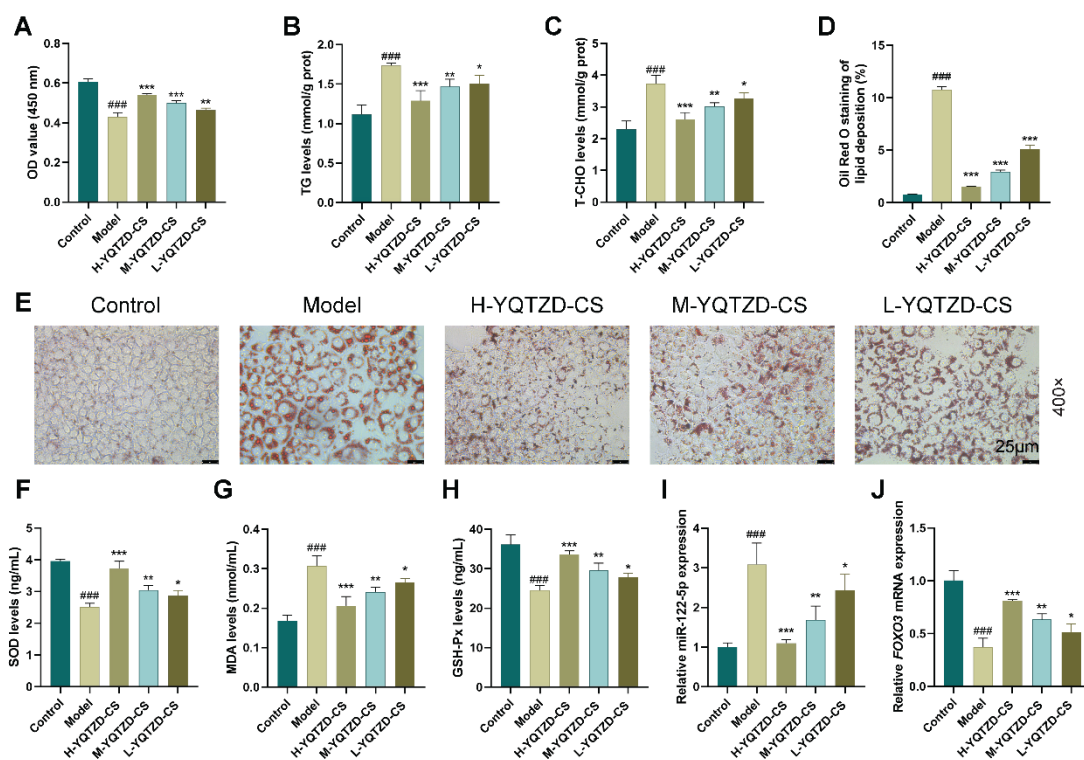


Figure 1. YQTZD-CS improves lipid deposition and oxidative stress, and modulates the miR-122-5p/FOXO3 axis in OA-induced AML12 cells. OA (1 mM)-induced AML12 cells were treated with YQTZD-CS at high (20%), medium (10%), and low (5%) doses for 24 h. (A) CCK-8 assay for cell viability of AML12 cells. (B) TG and (C) T-CHO levels were detected using a microplate reader. (D) Quantification of the Oil Red O-stained area and (E) representative pictures of Oil Red O staining of AML12 cells. Scale bars: 25 μ m; magnification: 400 $\times$. (F) SOD, (G) MDA, and (H) GSH-Px levels were detected using ELISA kits. RT-qPCR analysis of (I) miR-122-5p and (J) *Foxo3* mRNA expression in AML12 cells. Notes: $^{###}p < 0.001$ vs. control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. model group.

Abbreviations: CCK-8: Cell Counting Kit 8; ELISA: Enzyme-linked immunosorbent assay; FOXO3: Forkhead box O3; GSH-Px: Glutathione peroxidase; MDA: Malondialdehyde; mRNA: Messenger RNA; OA: Oleic acid; OD: Optical density; RT-qPCR: Real-time quantitative polymerase chain reaction; SOD: Superoxide dismutase; T-CHO: Total cholesterol; TG: Triglyceride; Yinqi Tiaozhi Decoction-CS: YQTZD-containing serum.

and oxidative damage in AML12 cells by acting through the miR-122-5p/FOXO3 pathway. To investigate this mechanism, cultures were transfected with a mimic targeting miR-122-5p, then incubated with OA, and finally exposed to YQTZD-CS. According to CCK-8 assay results, YQTZD-CS attenuated the OA-induced decline in cell viability. However, miR-122-5p overexpression reversed this beneficial action (both $p < 0.001$; Figure 2A). In parallel, YQTZD-CS suppressed miR-122-5p and enhanced *Foxo3* mRNA, while the miR-122-5p mimic upregulated miR-122-5p and downregulated *Foxo3* (both $p < 0.05$, Figure 2B and 2C). Additionally, Oil Red O staining revealed pronounced accumulation of lipid droplets (Figure 2D and

2E), and OA significantly increased intracellular TG and T-CHO concentrations (Figure 2F and 2G), mimicking features of hepatic lipid metabolic disorders. YQTZD-CS intervention reduced lipid droplet number (Figure 2D and 2E) and markedly lowered TG and T-CHO concentrations (Figure 2F and 2G), demonstrating its capacity to alleviate lipid deposition. However, these improvements were significantly reversed by miR-122-5p mimic transfection (both $p < 0.01$). Furthermore, YQTZD-CS significantly counteracted the OA-induced reductions in SOD and GSH-Px activities, and concurrently suppressed the increase in MDA concentrations (all $p < 0.001$). Moreover, the oxidative-stress-mitigating action of YQTZD-CS was

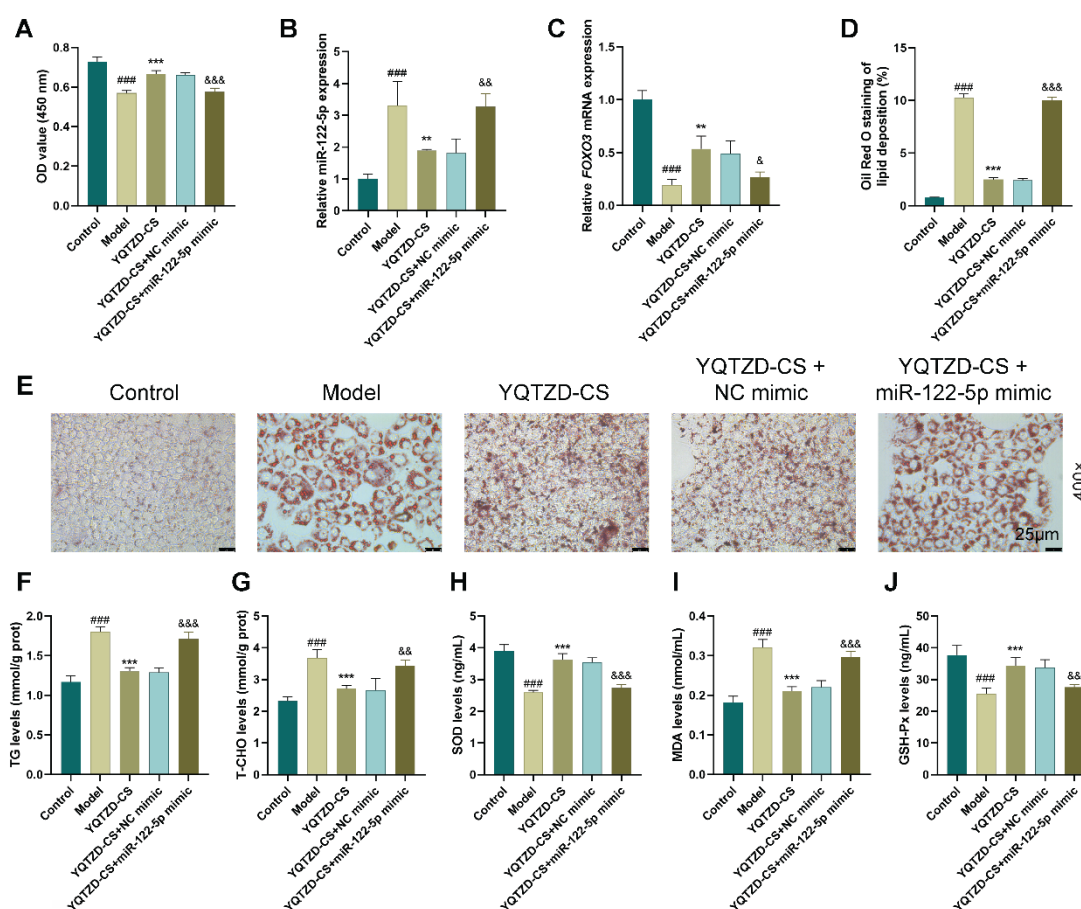


Figure 2. Overexpression of miR-122-5p reverses the protective effect of YQTZD-CS on lipid deposition and oxidative stress damage in OA-induced AML12 cells. After AML12 cells were transfected with miR-122-5p mimic or NC mimic for 12 h, OA (1 mM) was added, and AML12 cells were treated with 20% YQTZD-CS for 24 h. (A) CCK-8 assay was used to assess cell viability in AML12 cells after transfection. RT-qPCR analysis of (B) miR-122-5p and (C) *Foxo3* mRNA expression following AML12 cell transfection with miR-122-5p mimic. (D) Quantification of Oil Red O-stained areas and (E) representative pictures of Oil Red O staining of AML12 cells after transfection. Scale bars: 25 μ m; magnification: 400 $\times$. (F) TG and (G) T-CHO levels were detected using a microplate reader. (H) SOD, (I) MDA, and (J) GSH-Px levels of AML12 cells after transfection were detected using ELISA kits. Notes: ^{###} $p < 0.001$ vs. control group; ^{**} $p < 0.01$, ^{***} $p < 0.001$ vs. model group; [&] $p < 0.05$, ^{&&} $p < 0.01$, ^{&&&} $p < 0.001$ vs. YQTZD-CS + NC mimic group.

Abbreviations: CCK-8: Cell Counting Kit 8; ELISA: Enzyme-linked immunosorbent assay; GSH-Px: Glutathione peroxidase; MDA: Malondialdehyde; mRNA: Messenger RNA; NC: Negative control; OA: Oleic acid; OD: Optical density; RT-qPCR: Real-time quantitative polymerase chain reaction; SOD: Superoxide dismutase; T-CHO: Total cholesterol; TG: Triglyceride; YQTZD-CS: Yinqi Tiaozhi Decoction-containing serum.

abolished when cells were transfected with the miR-122-5p mimic ($p < 0.01$). In summary, YQTZD ameliorates lipid deposition and oxidative stress in hepatocytes by regulating the miR-122-5p/FOXO3 pathway, while miR-122-5p overexpression largely abolishes these beneficial effects.

3.4. YQTZD improves liver oxidative damage, lipid deposition, and inflammation in CD-HFD-induced MAFLD rats through the miR-122-5p/FOXO3 axis

In vivo, rats fed on CD-HFD for 15 weeks showed a significant increase in body weight compared with the starting point ($p < 0.05$; Figure 3B). This observation confirms the successful induction of the MAFLD model. After eight weeks of treatment with either low- or high-dose YQTZD or atorvastatin, body weight significantly decreased (all $p < 0.05$; Figure 3C). In all experimental groups, blood glucose levels peaked at 15 min after glucose administration and then gradually declined. In comparison to the model cohort, animals receiving either low- or high-dose YQTZD or atorvastatin showed a faster decline

in blood glucose after the peak value was reached, whereas glucose levels in the model group remained persistently elevated (all $p < 0.05$; Figure 3D). ITT revealed that, following insulin injection, blood glucose concentrations in MAFLD rats receiving low- or high-dose YQTZD or atorvastatin decreased rapidly (all $p < 0.05$; Figure 3E). Furthermore, the area under the curve (AUC) values derived from both GTT and ITT were significantly reduced in the groups receiving YQTZD (either low or high dose) or atorvastatin, as compared with the model group (all $p < 0.05$; Figure 3F and 3G), collectively indicating that YQTZD effectively ameliorates glucose intolerance and insulin resistance in MAFLD rats.

To determine whether YQTZD could alleviate the disorder of hepatic lipid metabolism induced by CD-HFD, this work performed pathological staining of liver sections. Figure 4A shows that YQTZD improved steatosis in rat liver. Hematoxylin and eosin (H&E) staining results showed that high-dose YQTZD treatment significantly reduced

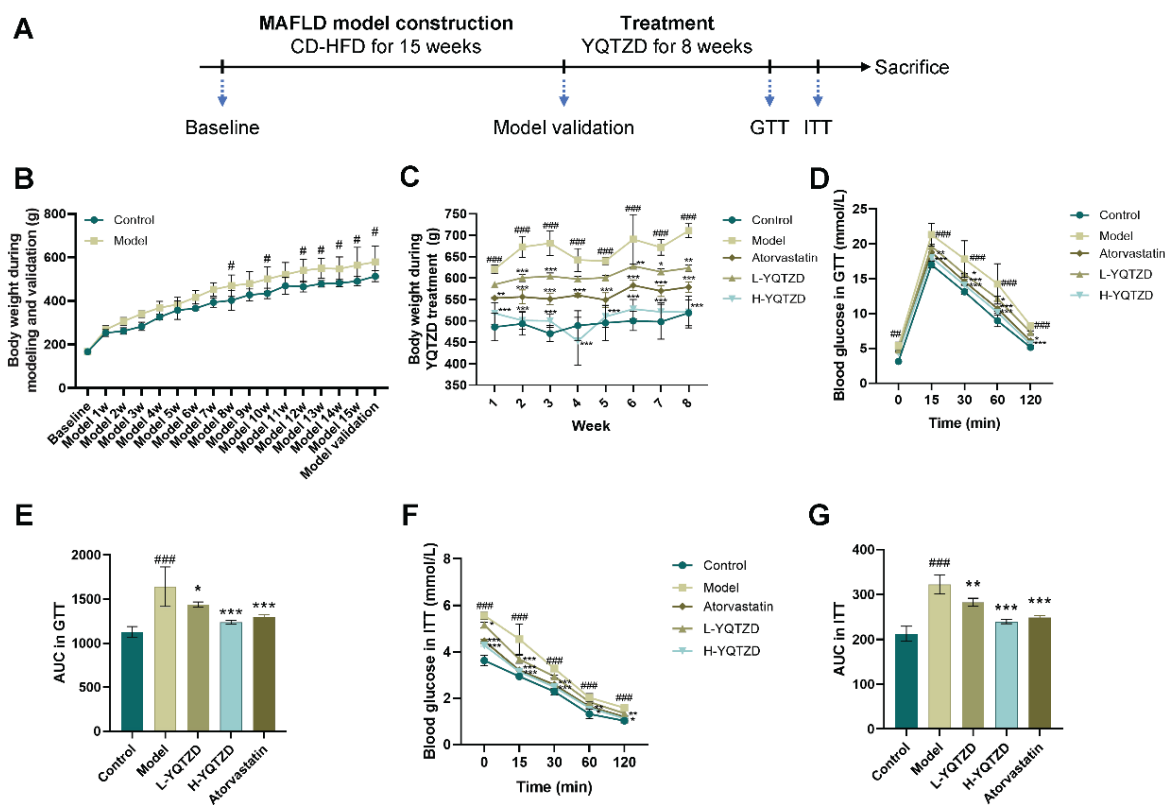


Figure 3. YQTZD improved glucose and insulin tolerance in MAFLD rats. (A) Study flowchart and timeline. (B) Body weight of rats in the control and model groups during the modeling and validation periods. (C) Body weight of rats in all groups during YQTZD treatment. (D) Blood glucose concentration was measured during GTT. (E) GTT area under the blood glucose concentration curve. (F) Blood glucose concentration was measured during ITT. (G) ITT area under the blood glucose concentration curve. Notes: * $p < 0.05$, *** $p < 0.001$ vs. control group; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. model group. Abbreviations: AUC: Area under the curve; CD-HFD: Choline-deficient and high-fat diet; GTT: Glucose tolerance test; ITT: Insulin tolerance test; MAFLD: Metabolic dysfunction-associated fatty liver disease; YQTZD: Yinqi Tiaozhi Decoction.

hepatocyte steatosis, inflammatory cell infiltration, and lipid droplet accumulation, effects comparable to those of the positive drug ($p < 0.001$; Figure 4B and 4D). YQTZD treatments also significantly reduced liver lipid deposition induced by CD-HFD, thereby alleviating liver injury in MAFLD rats (both $p < 0.001$, Figure 4C and 4E). In addition, YQTZD treatments significantly reduced TG and T-CHO levels (all $p < 0.01$, Figure 4F and 4G).

Hepatic injury is characterized by glutamine depletion, which in turn promotes inflammatory responses. Biochemical analyses revealed that YQTZD significantly reversed the CD-HFD-induced reductions in GS and

glutamine levels (both $p < 0.05$; Figure 5A and 5B). Western blotting and RT-qPCR further demonstrated that YQTZD markedly downregulated IL-6, TNF- α , and IL-1 β expression (all $p < 0.05$; Figure 5C–G). In short, YQTZD may be involved in glutamine metabolism, and it can reduce inflammatory factors and promote the repair of liver cells. Consistent with the cellular results, hepatic SOD and GSH-Px activities were markedly diminished by CD-HFD, whereas MDA concentration increased sharply; however, treatment with either low- or high-dose YQTZD or atorvastatin effectively counteracted these alterations (all $p < 0.05$, Figure 5H–J), suggesting the inhibitory effect

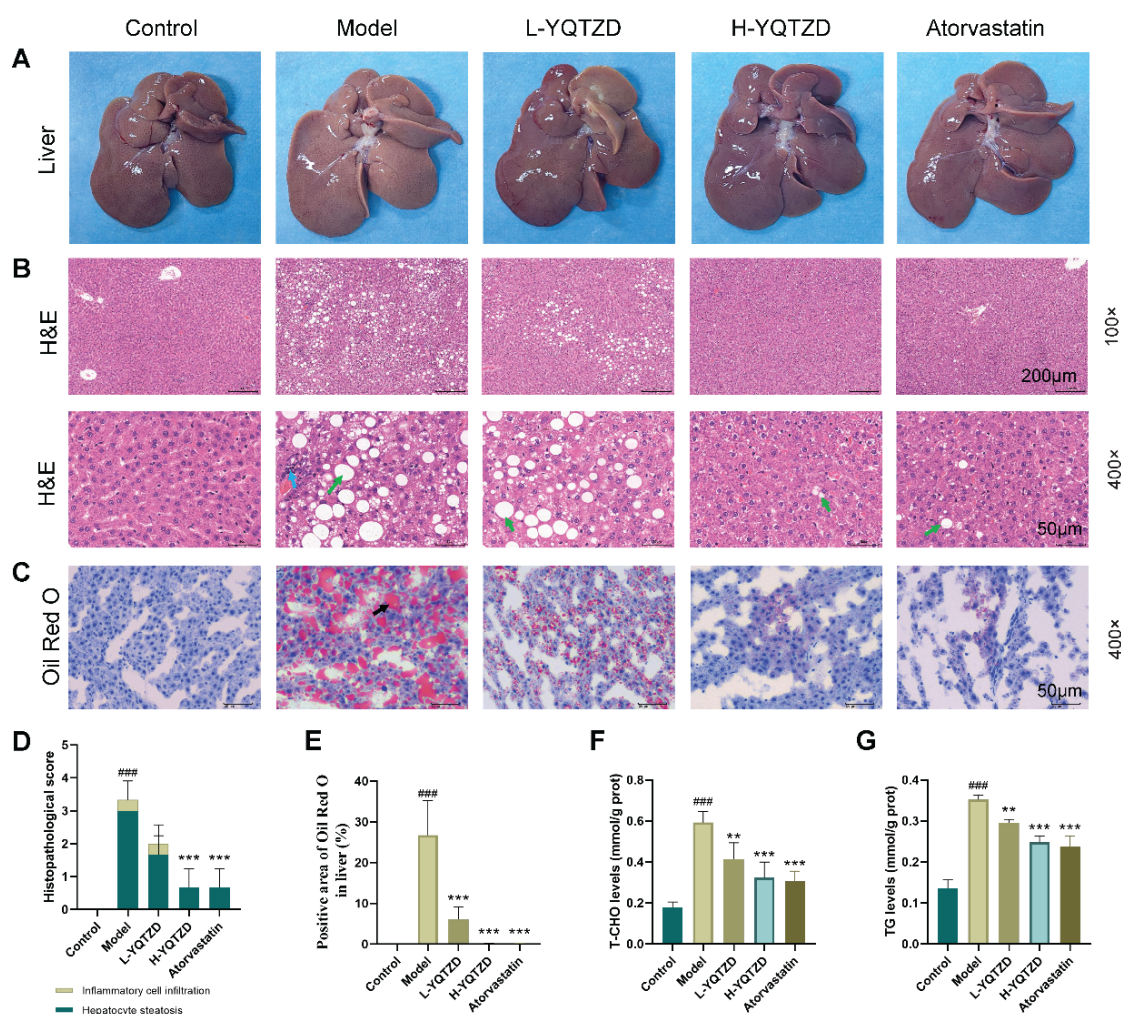


Figure 4. YQTZD alleviated hepatic lipid metabolism disorder in MAFLD rats. (A) Gross pathological examination of livers. (B) H&E staining of rat liver. Green arrows indicate hepatocyte steatosis, and blue arrows indicate neutrophils. Scale bars: (upper panels) 200, (lower panels) 50 μ m; magnification: (upper panels) 100 $\times$, (lower panels) 400 $\times$. (C) Oil Red O staining of liver tissue. Lipid droplets are stained red (black arrows). Scale bars: 50 μ m; magnification: 400 $\times$. (D) Histopathological score of H&E staining. (E) Positive area of Oil Red O staining. (F) T-CHO and (G) TG levels in liver tissues were detected using a microplate reader. Notes: *** $p < 0.001$ vs. control group; ** $p < 0.01$, *** $p < 0.001$ vs. model group.

Abbreviations: CD-HFD: Choline-deficient and high-fat diet; H&E: Hematoxylin and eosin; MAFLD: Metabolic dysfunction-associated fatty liver disease; T-CHO: Total cholesterol; TG: Triglyceride; YQTZD: Yinqi Tiaozhi Decoction.

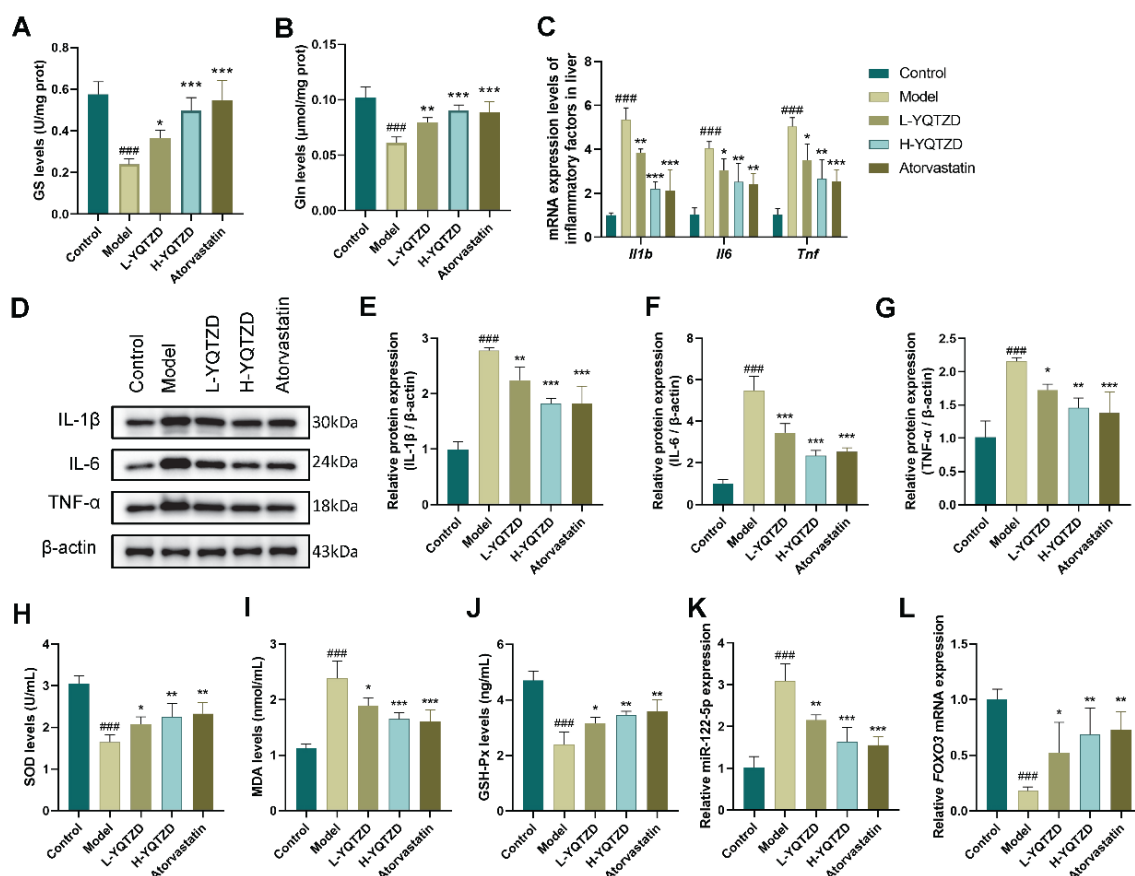


Figure 5. YQTZD improved glutamine metabolism, oxidative stress, and inflammation in MAFLD rats. (A) GS and (B) Gln levels were detected using ELISA kits. (C) mRNA expression of the inflammatory factors *Il1b*, *Il6*, and *Tnf* was detected using RT-qPCR. (D-G) Representative Western blotting images and quantification of IL-1β, IL-6, and TNF-α proteins. (H) SOD, (I) MDA, and (J) GSH-Px levels in rat liver tissues were detected using ELISA kits. (K & L) mRNA expression of (K) miR-122-5p and (L) *Foxo3* in rat liver tissues was detected using RT-qPCR. Notes: ###*p* < 0.001 vs. control group; **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs. model group.

Abbreviations: ELISA: Enzyme-linked immunosorbent assay; Gln: Glutamine; GS: Glutamine synthase; GSH-Px: Glutathione peroxidase; IL-1β: Interleukin-1 beta; MAFLD: Metabolic dysfunction-associated fatty liver disease; MDA: Malondialdehyde; mRNA: Messenger RNA; RT-qPCR: Real-time quantitative polymerase chain reaction; SOD: Superoxide dismutase; TNF-α: Tumor necrosis factor alpha; YQTZD: Yinqi Tiaozi Decoction.

of YQTZD on oxidative stress. In addition, CD-HFD increased miR-122-5p expression and decreased *Foxo3* mRNA expression in the liver; these alterations were significantly counteracted by administration of low- or high-dose YQTZD or atorvastatin (all *p* < 0.05, Figure 5K and 5L). In summary, administration of YQTZD potentially ameliorates disturbances in both glucose and lipid metabolism, while concurrently attenuating hepatic fat accumulation, oxidative injury, and inflammatory responses in MAFLD rats by downregulating miR-122-5p and upregulating FOXO3 *in vivo*, thereby providing liver protection.

4. Discussion

Metabolic dysfunction-associated fatty liver disease poses a

formidable and escalating global health challenge, with its pathogenesis intricately linked to genetic predisposition, lipid metabolism, and environmental factors. TCM, guided by its holistic philosophy and multi-targeted approach, presents a viable therapeutic strategy. Collectively, YQTZD exerts potent therapeutic efficacy against MAFLD. Furthermore, we uncover a previously unrecognized mechanistic axis in which YQTZD attenuates hepatic steatosis, oxidative damage, and inflammatory responses by regulating the miR-122-5p/FOXO3 pathway.

The MAFLD rat model was generated by feeding a CD-HFD diet, effectively recapitulating metabolic dysfunction-associated steatohepatitis, as evidenced by characteristic hepatic steatosis, inflammation, and fibrosis. Pathological evaluation, biochemical indices, and

inflammatory cytokine assays collectively confirmed the liver injury features in this model, verifying its successful establishment. Serum analysis showed significantly elevated T-CHO and TG levels, reflecting abnormal hepatic lipid metabolism. Histopathology revealed inflammatory cell infiltration, lipid accumulation, and steatosis in liver tissues. Measurements of inflammatory cytokines further indicate an aggravated inflammatory response. In short, CD-HFD effectively induced the MAFLD rat model, providing a solid foundation for subsequent investigations.

The defining characteristic of MAFLD is the overaccumulation of TG and T-CHO within hepatocytes, which leads to hepatic steatosis.²⁸ Our results demonstrate that YQTZD-CS reversed OA-induced lipid deposition in AML12 cells. Although the highest concentration of drug-containing serum (20%) in this study is at the upper end of the acceptable range in many serum pharmacology studies, the possibility that a high serum concentration or its constituent drug components may induce nonspecific cellular stress or cytotoxicity cannot be excluded. This anti-steatotic effect was further substantiated *in vivo*, as YQTZD treatment significantly improved liver histopathology in CD-HFD-treated rats, diminished inflammatory infiltration and lipid droplet accumulation, and reduced hepatic TG and T-CHO levels. Herbal medicines, including *Crataegi Fructus* and *Alismatis Rhizoma*, are recognized for their roles in promoting digestion and resolving dampness, directly addressing lipid metabolism. *Alisol* compounds were reported to modulate cholesterol synthesis and metabolism in the liver, acting as potent natural lipid-lowering agents.²⁹ Modern pharmacological studies have established that active constituents in *Crataegi Fructus*, such as flavonoids and triterpenic acids, inhibit key fatty acid synthesis enzymes (acetyl-CoA carboxylase [ACC], fatty acid synthase [FAS]) and activate AMP-activated protein kinase (AMPK), a central regulator of cellular energy balance, thereby suppressing lipogenesis.^{30,31} Similarly, *Coicis Semen* and *Benincasae Semen* have demonstrated efficacy in improving insulin sensitivity and lipid metabolism. *Curcumae Rhizoma* promotes qi and blood circulation, alleviating the qi stagnation and blood stasis characteristic of chronic MAFLD, thereby enhancing hepatic microcirculation and cellular function. Through multi-component synergy, YQTZD may thus cooperatively inhibit lipogenesis and facilitate hepatic lipid clearance.

Oxidative stress acts as a critical “second hit” that drives the transition from NAFL to the inflammatory and fibrotic stages of NASH. This pathological state stems from a perturbation in the equilibrium between reactive oxygen species production and endogenous antioxidant capacity, leading to lipid peroxidation, mitochondrial

dysfunction, and cellular injury. Our findings consistently indicate that YQTZD exhibits potent antioxidant activity. Notably, YQTZD-CS effectively reversed the decline in the antioxidative enzymes SOD and GSH-Px, while also diminishing the elevated MDA, a byproduct of lipid peroxidation. Moreover, these observations corroborate recent evidence indicating that alleviation of MAFLD is intimately associated with the simultaneous modulation of lipid metabolism, oxidative stress, and inflammatory pathways. Zhou *et al.*³² reported that suppressing *mmu_circ_0009303* counteracts MASLD through regulation of key lipogenic and oxidative proteins as well as via attenuation of oxidative stress and inflammation, which is mediated by the miRNA-182-5p/FOXO3 pathway, thereby emphasizing FOXO3's critical involvement in hepatic metabolic homeostasis. Several constituents of YQTZD are recognized for their antioxidant properties. For example, *Lycii Fructus*, which is rich in carotenoids, polysaccharides, and polyphenols, functions as an effective free radical scavenger.³³ Previous research has demonstrated that *Artemisiae Scopariae Herba* upregulates nuclear factor erythroid 2-related factor 2 (NRF2) expression, thereby promoting SOD and GSH-Px transcription.³⁴ By enhancing endogenous antioxidant defense and suppressing lipid peroxidation, YQTZD directly counteracts a central pathological mechanism in MAFLD progression, thereby helping to preserve hepatocyte integrity and function.

The progression of MAFLD is typically accompanied by the activation of inflammatory pathways. A marked suppression of pro-inflammatory cytokine levels was observed following YQTZD treatment in our studies. This anti-inflammatory effect is multifaceted. Firstly, by reducing lipid accumulation and oxidative stress, YQTZD eliminated two major triggers of inflammation. Secondly, specific components in YQTZD, such as polysaccharides from *Lycii Fructus*, chlorogenic acid from *Artemisiae Scopariae Herba*, and curcumin from *Curcumae Rhizoma*, possess well-documented anti-inflammatory properties.³⁵⁻³⁷ A particularly noteworthy finding is the effect of YQTZD on glutamine metabolism. CD-HFD feeding led to reductions in glutamine and GS, which were restored by YQTZD treatment. Glutamine serves as a precursor for glutathione synthesis and exerts anti-inflammatory effects by modulating immune cell responses.³⁸ Depletion of hepatic glutamine reserves is implicated in the pathophysiology of MAFLD, as it exacerbates oxidative stress and increases the susceptibility of the liver to inflammatory injury.^{39,40} The finding that YQTZD may directly influence enzymes involved in glutamine metabolism opens new avenues for future research into the mechanisms by which TCM modulates amino acid metabolism in MAFLD treatment.

Our findings provide robust, modern scientific validation of the YQTZD formulation from a TCM perspective. The core pathophysiology of MAFLD is considered to be liver qi stagnation and spleen deficiency, leading to the generation of phlegm-dampness in the body, which eventually transforms into turbid toxin that accumulates in the liver collaterals. The multi-target, multi-pathway nature of YQTZD aligns with the emerging systems pharmacology paradigm in herbal medicine research. Integrative multiomics approaches have demonstrated that herbal extracts can simultaneously modulate metabolomic, transcriptomic, and proteomic profiles to restore metabolic homeostasis in MAFLD.⁴¹ In this context, YQTZD's regulation of the miR-122-5p/FOXO3 axis may represent only one node within a broader network of molecular interactions through which the formulation exerts its holistic therapeutic effects. In YQTZD, *Artemisiae Scopariae Herba* clears and soothes the liver to promote qi movement, and *Alismatis Rhizoma* clears heat, drains turbidity, and promotes diuresis; together, they serve as the sovereign medicinal. *Curcumae Rhizoma* and *Crataegi Fructus* activate blood circulation, resolve stasis, and promote qi movement; *Coicis Semen*, *Benincasae Semen*, and *Eupatorii Herba* clear heat, eliminate dampness, and reduce turbidity; *Agastaches Herba* and *Citri Sarcodactylis Fructus* soothe the liver, regulate qi, and dispel turbidity; *Patriniae Herba* and *Radix Glycyrrhizae et Rhizoma* clear heat, resolve toxicity, and alleviate pain; these collectively serve as minister medicinals, assisting the sovereign medicinal in enhancing the actions of soothing and clearing the liver. *Lycii Fructus* serves as the assistant medicinal, nourishing yin and blood and supplementing the liver substance, thereby preventing the disease at its source. The entire formulation works synergistically to soothe liver qi, resolve turbidity and filth, clear stagnant heat, and disperse accumulation and stagnation. The oxidative stress and inflammatory responses we measured at the molecular level correspond to the TCM concepts of "turbid toxin" and "heat." The multi-target effects of YQTZD on lipid deposition, oxidative stress, inflammation, and glucose metabolism, mediated through key nodes such as miR-122-5p and FOXO3, provide a concrete biological basis for the holistic and syndrome-differentiation-based approach of TCM.

MiR-122-5p dysregulation is closely associated with liver diseases and lipid homeostasis. Our initial *ex vivo* experiments revealed that OA-elicited lipid accumulation correlated with elevated miR-122-5p levels, whereas YQTZD-CS reduced its expression. This finding is in agreement with human data showing increased serum miR-122-5p concentrations in individuals with MAFLD. Furthermore, miR-122-5p mimic largely abolished the

beneficial effects of YQTZD-CS on cellular survival, fat accumulation, and oxidative injury, substantiating the involvement of YQTZD in modulating miR-122-5p expression. FOXO3 is a well-established regulator of cellular metabolism and stress resistance. It promotes antioxidant defense, autophagy, and gluconeogenesis, while suppressing lipogenesis under certain conditions.^{42,43} During the progression from hepatitis to liver fibrosis and cirrhosis, the normal function of hepatocytes is impaired. FOXO3, a key transcription factor involved in cellular metabolism, apoptosis, and repair, may be downregulated by hepatitis-related pathological processes (oxidative stress and abnormal lipid metabolism). Furthermore, FOXO3 plays a significant role in inflammatory responses. Knockout or functional inhibition of FOXO3 exacerbates inflammatory responses; for example, FOXO3 deficiency in macrophages aggravates tissue inflammation.⁴⁴ Activation of FOXO3 or enhancement of its signaling pathway is expected to suppress inflammatory progression. Our data indicate an interaction between miR-122-5p and FOXO3. YQTZD downregulated miR-122-5p while upregulating *Foxo3* expression, thereby ameliorating inflammation and metabolic disorders and reducing liver tissue damage associated with the inflammatory state in MAFLD rats. YQTZD helps reduce inflammation by modulating the miR-122-5p/FOXO3 signaling pathway. However, since this pathway involves multiple mechanisms, further investigation is required to elucidate the intricate interactions within this network.

Despite the robust findings, our study has several limitations. Firstly, although we identified *Foxo3* as a target, the complete downstream gene network regulated by the miR-122-5p/FOXO3 cascade remains to be fully elucidated. We have noted that future studies will prioritize FOXO3 protein quantification via Western blot. Secondly, we focused on miR-122-5p and *Foxo3*, but it is highly probable that YQTZD, as a complex formulation, acts on multiple other pathways simultaneously. Investigating its effects on other key players in MAFLD, such as AMPK, sterol regulatory element-binding protein 1c (SREBP-1c), and peroxisome proliferator-activated receptor alpha (PPAR α), would provide a more integrated picture. Thirdly, the specific bioactive compounds within YQTZD responsible for modulating the miR-122-5p/FOXO3 axis are unknown. Future studies should aim to isolate and identify these active components. Furthermore, the simple CD-HFD may not fully simulate the metabolic syndrome associated with human MAFLD. It is acknowledged that the relatively limited number of samples constitutes a shortcoming of the present work. Accordingly, future investigations with broader studies will be necessary to confirm these results. Finally, translating these discoveries

into clinical practice would require rigorous randomized clinical trials to assess the effectiveness and tolerability of YQTZD in patients with MAFLD.

5. Conclusion

Collectively, YQTZD counteracts hepatic steatosis, oxidative insult, and pro-inflammatory processes by modulating the miR-122-5p/FOXO3 axis. It exhibits considerable therapeutic promise against MAFLD in both cellular and animal models, thereby furnishing an experimental rationale for its future development as a candidate anti-MAFLD agent.

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Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

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Ethics approval and consent to participate

The animal experiments were approved by the Laboratory Animal Ethics Committee of West China Hospital, Sichuan University (No. 20251031013). This study adheres to internationally accepted standards for animal research, following the 3Rs principle. The study was conducted in accordance with the local legislation and institutional requirements.

Consent for publication

Not applicable.

Availability of data

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Further disclosure

During the preparation of this manuscript, the authors used GeenMedical (<https://www.geenmedical.com/ai-polish>) for polishing and grammar improvement, only assisting, not leading, the creation. After its use, the authors thoroughly reviewed, verified, and revised all AI-assisted content to ensure accuracy and originality. The authors take full responsibility for the integrity and final content of the published article.

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