Protective Role of Thymoquinone in Hyperlipidemia-Induced Liver Injury in LDL-R-/- Mice

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Research

Abstract

Background: Hyperlipidemia, a heterogeneous group of disorders characterized by elevated levels of plasma lipids, causes serious health problems and can lead to fatty liver and nonalcoholic fatty liver disease. Thymoquinone (TQ), a major active component of *Nigella sativa*, can exert a vast array of biological effects. Various studies have reported that TQ protects against liver injury, and we previously reported that TQ reduces cardiac damage in mice fed a high-cholesterol diet (HD). However, few studies have evaluated the effects of TQ on hyperlipidemia-induced liver injury. The aim of this study was to investigate the possible protective effects of TQ against liver injury in hyperlipidemia-induced LDL-R$^{-/-}$ mice.

Methods: Eight-week-old male LDL-R$^{-/-}$ mice were randomly divided into three groups based on diet: normal diet (ND group, which was the control), high cholesterol diet (HD group), and HD mixed with TQ (HD + TQ group). All mice were fed for 8 weeks. Blood samples were obtained from the inferior vena cava, collected in serum tubes, and stored at −80 °C to investigate the serum lipoprotein profile. Longitudinal sections of liver tissues were fixed in 10% formalin and embedded in paraffin for histological evaluation. The remainder of the liver tissues was snap-frozen in liquid nitrogen for mRNA and immunoblotting analyses.

Results: TQ administration significantly reduced liver histological alterations caused by hyperlipidemia. TQ mitigated hyperlipidemia-induced liver injury, as indicated by the suppression of increases in metabolic parameters (total cholesterol, triglyceride, and low-density lipoprotein-cholesterol), hepatic biochemical parameters (alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase), pyroptosis indicators (nucleotide-binding oligomerization domain-like receptor 3, interleukin [IL]-1β, and IL-18), a macrophage marker (CD68-positive cells), and phosphatidylinositide 3-kinase levels induced by the HD.

Conclusions: These results indicate that TQ is a potential therapeutic agent for liver injury caused by hyperlipidemia.

Background

Modern living environments and excessive energy intake have led to a great increase in the number of individuals with metabolic diseases, such as hyperlipidemia, diabetes, hypertension, and cardiovascular diseases (1). The consumption of a high-fat diet usually leads to hyperlipidemia, a heterogeneous group of disorders characterized by elevated levels of plasma lipids (2), which causes serious health problems, including atherosclerosis, coronary artery disease, cerebrovascular disease, acute pancreatitis, and nonalcoholic fatty liver disease (3–5). Moreover, hyperlipidemia and its complications are responsible for approximately 50% of deaths worldwide (6). In China, the situation is extremely serious, and the number of patients affected by dyslipidemia has reached 160 million (6).

The liver plays a vital role in the regulation of systemic lipid homeostasis (7). Because liver regulated the biosynthesis and metabolism, packing and export within lipoproteins, and reuptaked via surface receptors for very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL).(8) Hyperlipidemia indicates abnormally levels of lipids or lipoproteins in the blood due to abnormal fat metabolism or function, it may result in solid organ injury, including damage to the liver and kidneys (9,10). Also, long-term hyperlipidemia may cause hepatic diseases, including hepatic steatosis and liver injury (11), by the dysregulation of lipid metabolism, including lipid synthesis, fatty acid oxidation, and lipoprotein uptake and secretion, in the liver (12). Moreover, several studies have recently revealed the relationship between pyroptosis, an inflammasome-activated programmed cell death, and a high-fat diet (13–15). Nucleotide-binding oligomerization domain-like receptor 3 (NLRP3), the best-studied canonical inflammasome (16), plays a vital role in liver diseases, including ischemia/reperfusion injury, drug-induced hepatotoxicity, and fibrosis (17–19). The NLRP3 inflammasome is a molecular platform, activated by cellular damage, that promotes the maturation and secretion of proinflammatory cytokines, such as interleukin (IL)-1β and IL-18 (15, 20).

Currently, the aim of hyperlipidemia treatments is to increase the levels of anti-atherogenic lipoproteins, such as high-density lipoprotein, or lower those of low-density lipoprotein (LDL), total cholesterol (TC), and triglycerides (TGs) (21). Lipid-lowering drugs, such as 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins), effectively lower the levels of TC and LDL cholesterol (LDL-c). However, adverse reactions to statins, including myopathy and rhabdomyolysis, limit its use (22,23). Moreover, the hepatotoxicity of statins has been reported (24). Thus, studies are being conducted to pursue safer therapy methods.

Thymoquinone (TQ; 2-isopropyl-5-methylbenzo-1,4-quinone; $C_{10}H_{12}O_2$; Fig. 1), a major active component of *Nigella sativa*, can exert a vast array of biological effects (25). A number of pharmacological activities of TQ have been investigated, including antioxidant, antitumor, antidiabetic, and anti-inflammatory properties, as well as lipid-lowering effects (25). More recently, the protective effects of TQ on ethanol and anti-tuberculosis drugs induced liver injures have been demonstrated (26, 27). In addition, the effect of TQ on hyperlipidemia induced-cardiac has been confirmed (28,29). Additionally, TQ causes few adverse effects and has a low degree of toxicity (25).Despite the high number of studies that have reported the health-promoting properties of TQ, few have evaluated the effects of TQ on hyperlipidemia-induced liver injury. Thus, the aim of the present study was to explore the effects of TQ on hyperlipidemia-induced liver injury in LDL-R$^{-/-}$ mice.

Methods

Animal experiments

LDL-R$^{-/-}$ mice were purchased from Beijing Vital River Lab Animal Technology Co., Ltd. (Beijing, China). All mice were housed on a room under uniform conditions (12/12-h light/dark cycle, 24–26°C). All mice (8-weeks-old, weight, 25.47 ± 1.66 g) were randomly divided into three groups based on diet: normal diet

(ND group, n = 8), high-cholesterol diet (HD group, n = 8), and HD + TQ by gavage (100 mg/kg/d; Sigma-Aldrich, St. Louis, MO, USA) (HD + TQ group, n = 8). The HD contained 1.5% cholesterol and 15% fat. The experimental diet was purchased from Shanghai Slac Laboratory Animal Co., Ltd. (Shanghai, China). Each group were fed the respective diet for 8 weeks. After 8 weeks, all mice were weighed and then euthanized with a high dose of pentobarbital (100 mg/kg, intraperitoneally), and lack of respiration and heartbeat was used as an indicator of mouse death. Longitudinal sections of the livers were fixed in 10% formalin and embedded in paraffin for histological evaluation. All animal experiments was approved by the Ethical Committee of Zhongshan Hospital, affiliated with Dalian University of China.

Serum lipoprotein profile

Blood samples were collected and serum was prepared by centrifugation at 1006 x g for 10 min at 4 °C. Serum Total Cholesterol (TC), Low-density Lipoprotein Cholesterol (LDL-c), Triglyceride (TG), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were detected using Phosphatase Assay Kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions.

Histological analysis

Longitudinal sections of the livers were fixed in 10% formalin and embedded in paraffin for histological evaluation. Paraffin-embedded liver tissues (LTs) were cut into 5-µm-thick cross-sections and deparaffinized via immersion in xylene (three times, 5 min each) and rehydrated in a descending alcohol series (100, 90, 80 and 70% alcohol; 5 min each). Hematoxylin and eosin (H&E) staining was used to visualize the lesion area. According to the manufacturer's instructions (Zsbio, Beijing, China), Immunohistochemical staining was performed using an antibody against CD68 (rabbit anti-CD68 antibody, 1:200; Proteintech, Wuhan, China). National Institutes of Health (NIH) ImageJ software version 1.8.0 was used for quantification.

RNA isolation and real-time reverse transcription polymerase chain reaction (RT-qPCR)

Total RNA was isolated from LT and complementary DNA (cDNA) was synthesized using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen, Beijing, China) according to the manufacturer's protocol. Gene expression was analyzed quantitatively by qPCR using TransStart Top Green qPCR SuperMix kit (TransGen). β-Actin cDNA was amplified and quantitated in each cDNA preparation to normalize the relative expression of the target genes. Primer sequences are listed in Table 1.

Table 1
Primer oligonucleotide sequences

Gene	Primers
NLRP3	F: 5'- CTGCGGACTGTCCCATCAAT-3'
	R: 5'- AGGTTGCAGAGCAGGTGCTT-3'
IL-18	F: 5'-ATGGCTGCTGAACCAGTAGAAG-3'
	R: 5'- CAGCCATACCTCTAGGCTGGC-3'
IL-1β	F: 5'-TGCCACCTTTTGACAGTGAT-3'
	R: 5'-TGTGCTGCTGCGAGATTTGA-3'
β-Actin	F: 5'-CGATGCCCTGAGGGTCTTT-3'
	R: 5'-TGGATGCCACAGGATTCCAT-3'

Abbreviations: NLRP3, nucleotide-binding and oligomerization domain-like receptor 3; IL-18, interleukin-18; IL-1β, interleukin-1β

Western blotting (WB)

Proteins were extracted from LTs using radioimmunoprecipitation assay buffer (P0013B; Beyotime, Shanghai, China). Samples were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, and proteins were transferred to polyvinylidene fluoride membranes (Immobilon, Millipore, Billerica, MA, USA). Membranes were blocked in Tris-buffered saline with 0.1% Tween-20 containing 5% skim milk and then incubated with primary antibodies against NLRP3 (rabbit anti-NLRP3 antibody, 1:1000; Boster, Wuhan, China), IL-18 (rabbit anti-IL-18 antibody, 1:1000; Proteintech), IL-1β (rabbit anti-IL-1β antibody, 1:1000; Arigo, Hamburg, Germany), phosphatidylinositide 3-kinase (PI3K; Rabbit anti-PI3K, 1:1000; Proteintech), and anti-β-actin (1:1000; Proteintech) in primary antibody diluent (P0023A; Beyotime) overnight at 4°C with gentle shaking. Membranes were then incubated with the secondary antibody for 1 h (anti-rabbit Ig-G, 1:1000; Cell Signaling Technology, USA) at room temperature. This analysis was carried out independently three times. Protein levels are expressed as protein/β-actin ratios to minimize loading differences. The relative signal intensity was quantified using NIH ImageJ software.

Statistical analysis

All data are presented as the mean ± SEM. Statistical analysis was performed using SPSS software version 23.0 (SPSS Inc., Chicago, IL, USA). Inter-group variation was measured by one-way ANOVA and subsequent Tukey's test. Significance was set at $P < 0.05$.

Results

Metabolic characterization

The metabolic parameters of the LDL-R$^{-/-}$ mice are summarized in Fig. 2. Body weight did not vary among the three groups. Mice in the HD group exhibited significantly higher TC, TG, and LDL-c levels than those in the ND group; however, these levels were significantly decreased in the HD + TQ group.

TQ reduced serum ALT, AST, and ALP levels of mice in the HD group

The hepatic biochemical parameters of the LDL-R$^{-/-}$ mice are summarized in Fig. 3. Mice in the HD group exhibited significantly higher ALT, AST, and ALP levels than those in the ND group; however, these levels were significantly decreased in the HD + TQ group.

TQ altered histopathological and immunohistochemical staining in the LT of mice in the HD group

H&E staining showed that the LTs of mice in the ND group had normal histology. In contrast, the LTs of mice in the HD group showed liver injury, evidenced by hepatic lobule disorder, focal necrosis, swelling of liver cells, and widespread distribution of lipid droplets. However, the administration of TQ prevented the degenerative changes in hepatic structure induced by HD. Immunohistochemical staining with anti-CD68 antibody showed that the LTs of mice in the HD + TQ group exhibited a reduced accumulation of CD68-positive cells compared with that of mice in the HD group (Fig. 4).

TQ reduced expression of pyroptosis-related genes in the LT of mice in the HD group

qPCR showed that the expression of pyroptosis-related genes, *NLRP3*, *IL-1β*, and *IL-18*, was upregulated in the HD group compared with the ND group; however, this upregulation was attenuated in the HD + TQ group (Fig. 5).

TQ decreased the expression of pyroptosis-related proteins in the LT of mice in the HD group

WB showed that the LTs of mice in the HD + TQ group had significantly lower NLRP3, IL-1β, and IL-18 expression levels than that of mice in the HD group (Fig. 6a and b).

TQ reduced PI3K expression in the damaged LTs

WB showed a higher PI3K expression level in the HD group than in the ND group. However, this increased expression was significantly suppressed in the HD + TQ group (Fig. 7).

Discussion

The results of this study illustrated that TQ has a protective effect against liver injury via the suppression of increases in metabolic parameters, hepatic biochemical parameters, pyroptosis indicators, a macrophage marker (CD68-positive cells), and PI3K levels resulting from HD-induced hyperlipidemia.

Among the metabolic parameters, the levels of TC, TG, and LDL-c were higher in the HD group than in the ND group. These results are consistent with those of Zhang et al. (30). Interestingly, these increases were significantly suppressed by TQ, which is in agreement with the results of Ragheb et al. in a HD rabbit model (31).

The effects of TQ on hyperlipidemia-induced liver injury were further investigated by the measurement of hepatic biochemical parameter (AST, ALT, and ALP) levels. The results of this study showed that serum AST, ALT, and ALP levels were significantly higher in the HD group than in the ND group, as reported by Li et al. in high-fructose-fed Kunming mice (32). Furthermore, the levels of these parameters were significantly reduced by TQ, as reported previously during diazinon- and anti-tuberculosis drug-induced hepatic injury in murine models (26, 33).

Histologically, according to H&E staining, TQ protected mice against hepatic lobule disorder, focal necrosis, swelling of liver cells, and widespread distribution of lipid droplets caused by hyperlipidemia. Moreover, liver injury induced by hyperlipidemia is usually associated with an increase in the number of macrophages. Macrophage-derived foam cells release cytokines that recruit more macrophages to lesions and influence lipid deposition (34). CD68, which is used as a marker to identify macrophages, is present in liver tissue damaged by hyperlipidemia (35). In the present study, we performed immunohistochemical staining to measure the number of macrophages in the liver tissue. Significantly more CD68-positive cells were observed in the HD group than in the ND group. However, the accumulation of CD68-positive cells in LT was significantly reduced by TQ.

Chen et al. established the formation and activation of the NLRP3 inflammasome in the liver of mice on the high-fat diet and, consequently, the release of IL-1β in nonalcoholic steatohepatitis (36). Additionally, Hendrikx et al. reported that the expression of *IL-18* and *IL-1β* is increased in hyperlipidemic mice (37). Importantly, the use of TQ has been shown to significantly inhibit NLRP3, IL-1β, IL-18 expression in cecal ligation and puncture-induced septic cardiac damage in mice(38). Moreover, Suguna et al. revealed that TQ suppresses the expression of *IL-1β*, *IL-18*, and *NLRP3* in high-fat diet-fed rats (39). In our study, the use of TQ also significantly reduced NLRP3, IL-1β, and IL-18 expression in the LTs of HD-fed mice, as shown by RT-qPCR and WB. These results indicate that TQ downregulates the expression of pyroptosis-related genes and proteins induced by hyperlipidemia.

In this study, to investigate the effect of TQ on the regulation of the PI3K signaling pathway, we performed western blotting to assess the expression of PI3K, a key signaling molecule that regulates several cellular functions, including proliferation, survival, adhesion, and migration, in the liver (40, 41). Hu et al. showed that PI3K expression significantly increases following aluminum overload in the murine liver (42). A recent study indicated that TQ significantly enhances the anti-tumor effects of cisplatin against gastric cancer via the inhibition of the PI3K/AKT signaling pathway (43). Additionally, Bai et al. established that TQ significantly inhibits PI3K phosphorylation in thioacetamide- and lipopolysaccharide-induced hepatic fibrosis and inflammation in male Kunming mice(44, 45). Wang et al. demonstrated that TQ inhibits lipopolysaccharide-induced PI3K phosphorylation in BV2 microglial cells (46). In our study, we

observed a higher PI3K expression level in the HD group than in the ND group. However, TQ reduced the HD-induced PI3K level. Taken together, TQ may inhibit PI3K signaling to protect against hyperlipidemia-induced liver injury.

Conclusion

In conclusion, we elucidated that TQ mitigates hyperlipidemia-induced liver injury via the suppression of the increases in metabolic parameters (TC, TG, and LDL-c), hepatic biochemical parameters (ALT, AST, and ALP), pyroptosis indicators (NLRP3, IL-1β, and IL-18), a macrophage marker (CD68-positive cells), and PI3K levels. These results provide insights into the role of TQ in hyperlipidemia-induced liver injury and indicate its potential for the development of new therapeutic interventions to treat liver injury.

Abbreviations

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HD, high-cholesterol diet; IL-1β, interleukin-1β; IL-18, interleukin-18; LDL-c, low-density lipoprotein cholesterol; LT, liver tissue; ND, normal diet; NLRP3, nucleotide-binding oligomerization domain-like receptor 3; PI3K, phosphatidylinositide 3-kinase; TC, total cholesterol; TG, triglyceride; TQ, thymoquinone, WB, western blotting.

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Animal Studies Committee of the Affiliated Zhongshan Hospital of Dalian University.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

Authors' contributions

XZ designed this study and provided research funds, ZP designed this study and analyzed and interpreted the data. FW and QY helped to perform the experiments, FW provided research funds. ZP helped to perform the experiments and drafted the manuscript. YH analyzed and interpreted the data and helped to revise the manuscript. YW prepared the figures and helped to revise the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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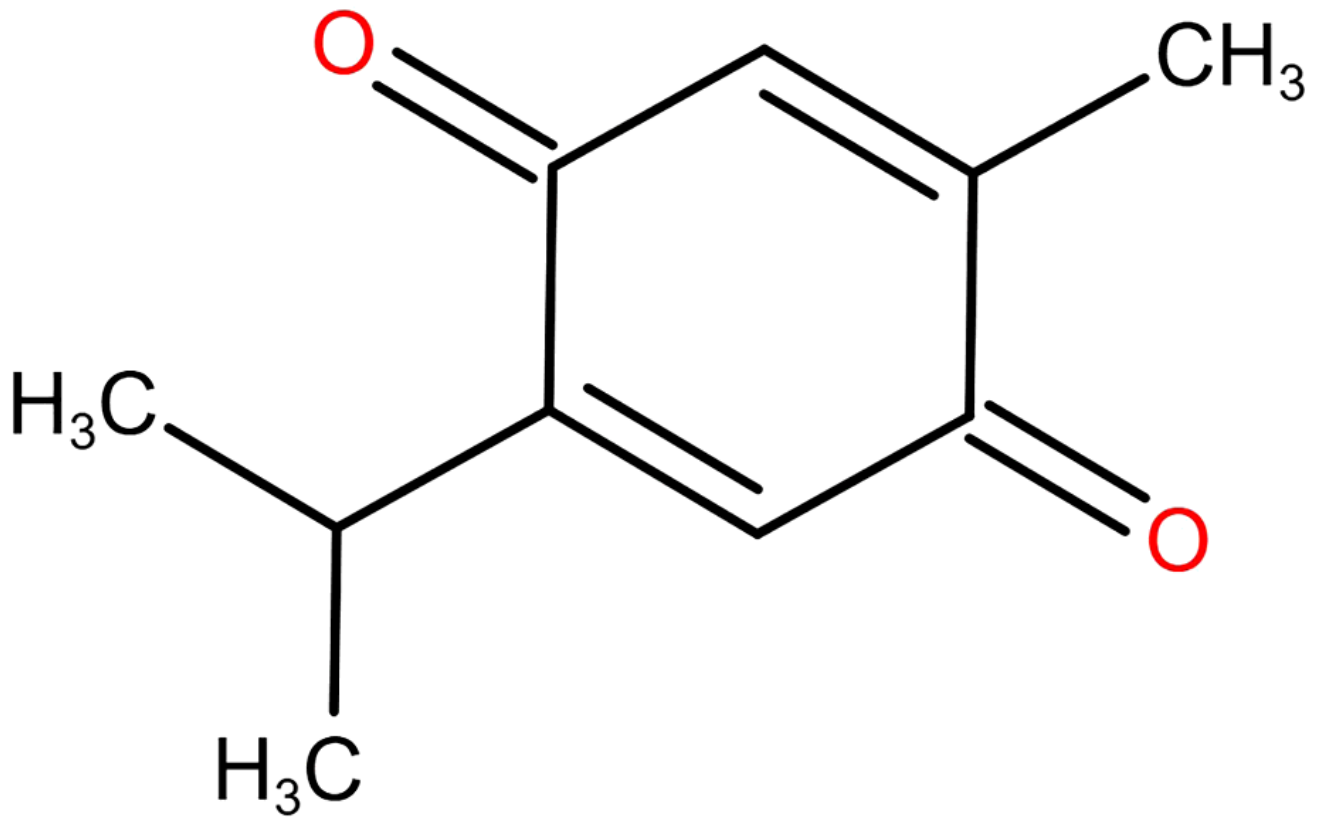
Figures

Figure 1

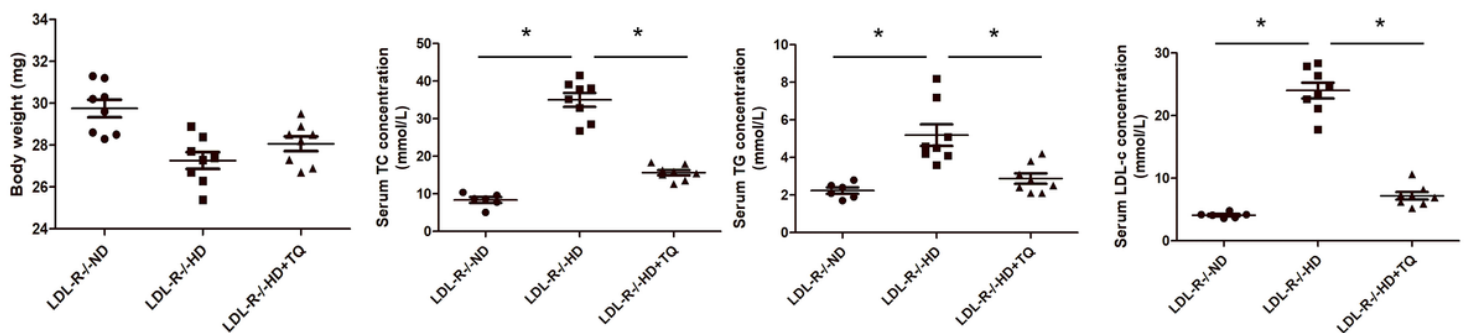Thymoquinone (2-isopropyl-5-methylbenzo-1,4-quinone).

Figure 2

Metabolic data of the LDL-R-/- mice after 8 weeks of feeding. Body weight and TC, TG, and LDL-c concentrations are shown. Data are shown as mean ± SEM; n = 6−8 per group. *P < 0.05 vs. HD group.

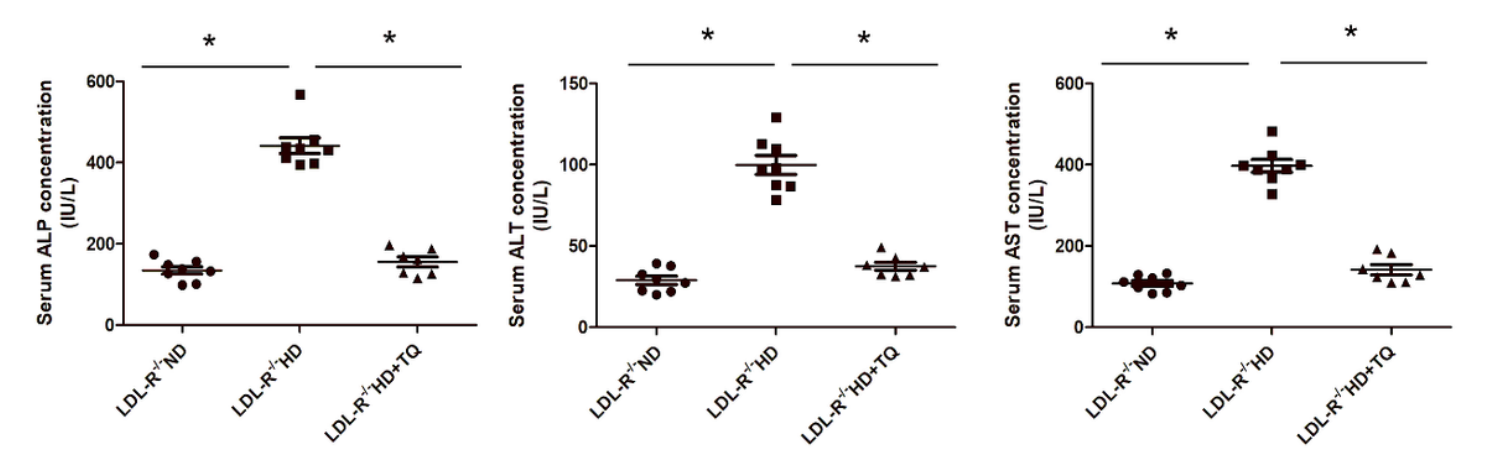Abbreviations: BW, body weight; TC, total cholesterol; TG, triglyceride; LDL-c, low-density lipoprotein cholesterol.

Figure 3

Hepatic biochemical parameters of the LDL-R-/- mice after 8 weeks of feeding. ALT, AST, and ALP levels are presented. Data are shown as mean ± SEM; n = 8 per group. *P < 0.05 vs. HD group. Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
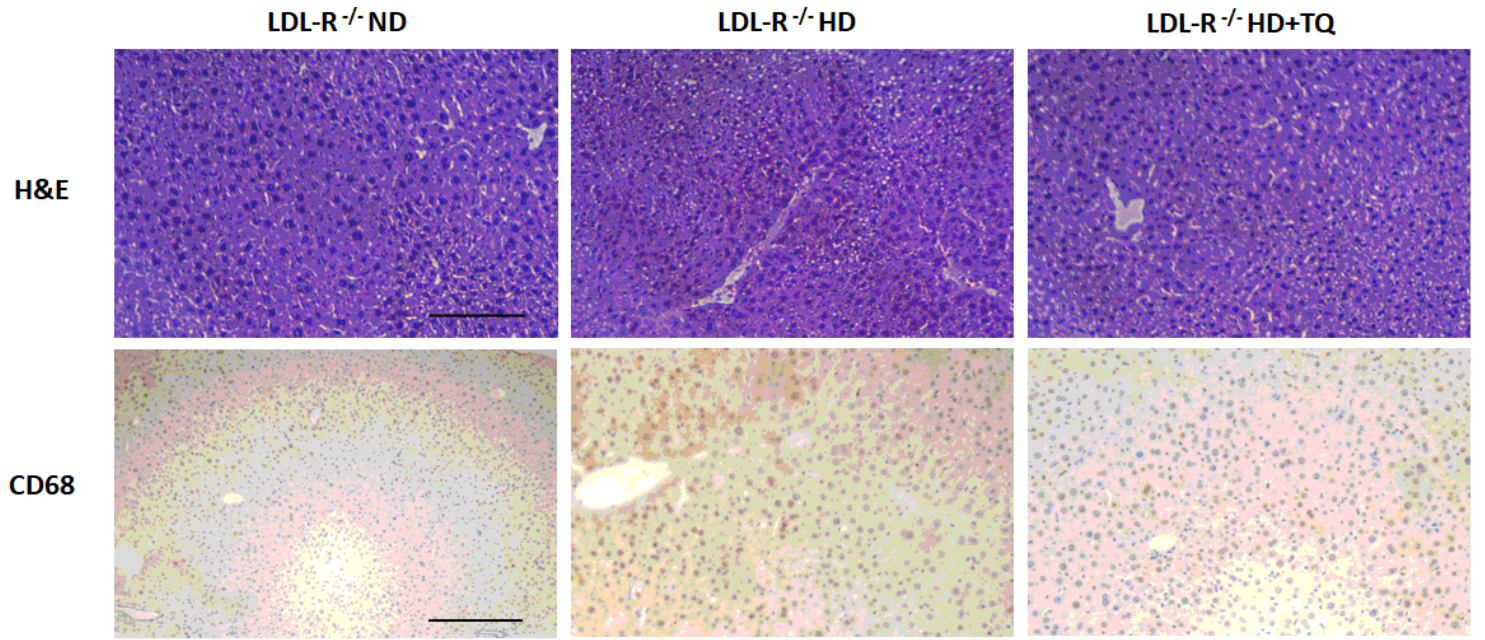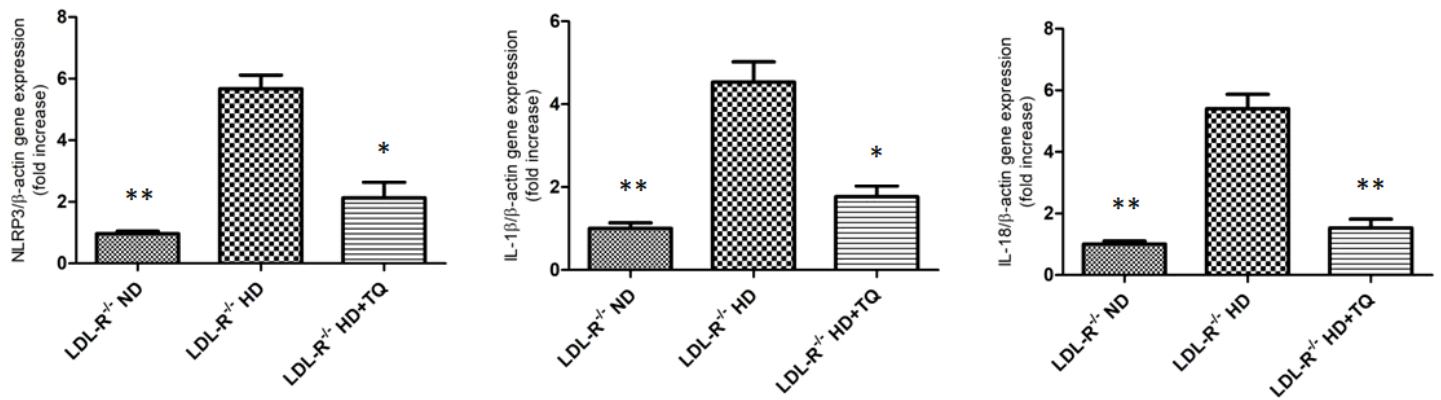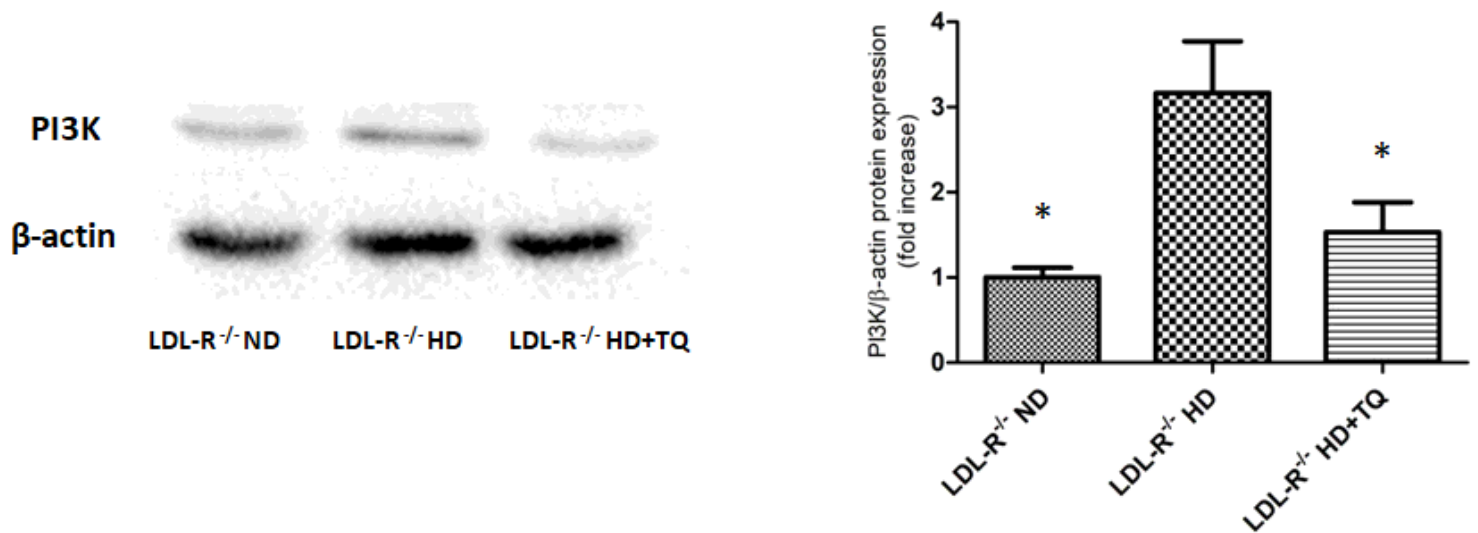

Figure 4

Histopathological changes in the LT of LDL-R-/- mice after 8 weeks of feeding. Scale bar = 100 μm. Accumulation of CD68-positive cells in the LT of LDL-R-/- mice after 8 weeks of feeding. Scale bar = 100 μm. Abbreviations: LT, liver tissue.
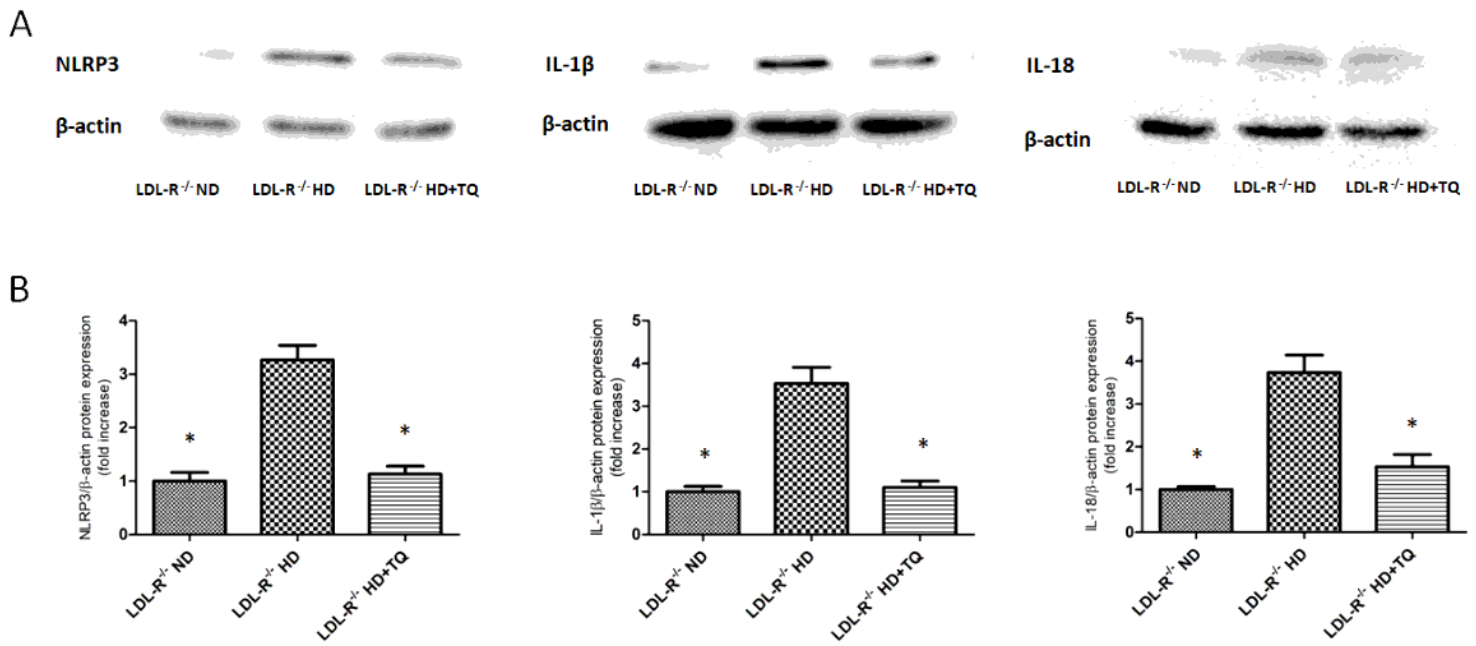
Figure 5

Expression of pyroptosis-related genes in the LT of LDL-R-/- mice after 8 weeks of feeding. qPCR measured the relative mRNA expression of NLRP3, IL-1β, and IL-18 in the LTs. Data are shown as mean ± SEM; n = 6 in each group. *P < 0.05, **P < 0.01 vs. HD group. Abbreviations: IL-1β, interleukin-1β; IL-18, interleukin-18; LT, liver tissue; NLRP3, nucleotide-binding oligomerization domain-like receptor 3.

Figure 6

Expression of pyroptosis-related proteins in the LT of LDL-R-/- mice after 8 weeks of feeding. a. WB measured NLRP3, IL-1β, and IL-18 expression levels in the LT. b. Bar graph depicts the quantification of NLRP3, IL-1β, and IL-18 expression levels. Data are shown as mean ± SEM; n = 3−4 in each group. *P < 0.05, vs. HD group. Abbreviations: IL-1β, interleukin-1β; IL-18, interleukin-18; LT, liver tissue; NLRP3, nucleotide-binding oligomerization domain-like receptor 3; WB, western blotting.

Figure 7

PI3K expression levels in the LT of LDL-R-/- mice after 8 weeks of feeding. WB measured the PI3K level in the LTs. Bar graph depicts the quantification of PI3K expression level. Data are shown as mean ± SEM; n = 3−4 in each group. *P < 0.05 vs. HD group. Abbreviations: LT, liver tissue; PI3K, phosphatidylinositide 3-kinase; WB, western blotting.