Protective role of thymoquinone in sepsis-induced liver injury in BALB/c mice

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Abstract. Sepsis increases the risk of developing liver injury. Previous studies have demonstrated that thymoquinone (TQ) exhibits hepatoprotective properties *in vivo* as well as *in vitro*. The present study aimed to investigate the underlying mechanisms of the protective effects of TQ against liver injury in septic BALB/c mice. Male BALB/c mice (age, 8 weeks) were randomly divided into four groups, namely, the control, TQ (50 mg/kg/day) treatment, cecal ligation and puncture (CLP), and TQ + CLP groups. CLP was performed following gavage of TQ for 2 weeks. At 48 h post-CLP, the histopathological alterations in the liver tissue (LT) and plasma levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were assessed. The present study evaluated microtubule-associated protein light chain 3 (LC3), sequestosome-1 (p62) and beclin 1 protein expression by western blotting and immunostaining, as well as interleukin (IL)-6, IL-1β, IL-10, monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) mRNA expression by RT-qPCR. The results of the present study indicated that administration of TQ to mice reduced the histological alterations caused by CLP in LT. TQ inhibited the plasma levels of ALT, AST and ALP in the CLP group. TQ significantly inhibited the elevation of p62, IL-1β, IL-6, MCP-1 and TNF-α levels as well as increased the LC3, beclin 1 and IL-10 levels in LT. PI3K expression in the TQ + CLP group was significantly decreased compared with that in the CLP group. TQ treatment effectively modulated the expression levels of p62, LC3, beclin 1, PI3K and proinflammatory cytokines, and may be an important agent for the treatment of sepsis-induced liver injury.

Introduction

Sepsis is a major cause of mortality and critical illness in intensive care units and is defined as a life-threatening organ dysfunction caused by a dysregulated host response against infection that triggers systemic inflammatory response syndrome (1,2). Among the organ failures involved in sepsis, hepatic dysfunction is a prominent complication that contributes to an increased risk of development of multiple organ failure and is a strong independent predictor of mortality (3,4).

Clinically, the survival outcome of patients with sepsis remains unimproved although several clinical trials employ novel agents to modulate the hyperreactive inflammatory response, and a wide spectrum of effective antibiotics are available against sepsis (5,6). Previous studies have demonstrated a close interaction between cecal ligation and puncture (CLP)-induced septic organ failure and cell dysfunction due to autophagy (7-9). Autophagy is a well-conserved intracellular lysosomal degradation process in which specific enzymes degrade the damaged or abnormal cellular components and recycle the redundant or inefficient components in cells (3,10). Autophagy exhibits an inducible response towards stress, including tissue injury, oxidative stress, infection and protein aggregation (3). Several studies have indicated that autophagy is activated after performing CLP in animal models of sepsis (7,11). Furthermore, multiple studies revealed that autophagy serves an important role in liver physiology and pathology following CLP (12,13). Various clinical experiments have suggested that novel strategies that focus on cell survival, in which an important role is served by autophagy, may improve the survival outcome in patients with septic shock (14,15). Therefore, further investigations are required to develop novel therapeutic interventions against sepsis.

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Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CLP, cecal ligation and puncture; IHC, immunohistochemical; IL, interleukin; LC3, microtubule-associated protein light chain 3; LT, liver tissue; MCP-1, monocyte chemoattractant protein-1; p62, sequestosome-1; TNF-α, tumor necrosis factor-α; TQ, thymoquinone; WBA, western blot analysis

Key words: sepsis, thymoquinone, liver injury, autophagy, BALB/c mice

Several studies investigated herbal medicines with an increasing interest in pre-clinical and clinical studies (16-18). Multiple studies support the high efficiency of medicinal plants in the prevention and cure of a wide range of diseases (16,19). Thymoquinone (2-isopropyl-5-methylbenzo-1,4-quinone; TQ), an agent extracted from the *Nigella sativa* (black cumin) plant, is used in Middle and Far Eastern countries as a traditional medicine to treat a wide range of pathological conditions (20). According to traditional medicine, TQ exhibits various types of therapeutic properties, including antioxidative, anti-inflammatory, immunomodulatory and antimicrobial activities (21). However, the effect of TQ on CLP-induced septic liver injury remains unclear. Therefore, in the present study, a murine model that simulates human peritonitis was established by performing CLP in order to understand the effect of TQ on CLP-induced septic liver injury.

Materials and methods

Maintenance of animal models. All animal experiments were approved by the Animal Studies Committee of the Affiliated Zhongshan Hospital of Dalian University. Male BALB/c mice (n=48; weight, 18.8±1.3 g; age, 8 weeks) were purchased from Beijing Vital River Lab Animal Technology Co., Ltd. All mice were housed under constant conditions, including 23-25˚C temperature, 40-60% humidity and a 12-h light/dark cycle with *ad libitum* access to food and water.

Development of a murine model of sepsis. To induce polymicrobial sepsis, a murine model was established by performing CLP according to a previously described method (22). The mice were anesthetized with sodium pentobarbital (50 mg/kg; intraperitoneal injection). After the peritoneum was surgically opened and the bowel was exposed, a section (two-thirds) of the cecum was tied and punctured once with a 21-gauge needle. A gentle pressure was applied at the perforation site to extrude a small amount of feces, which were placed in the peritoneal cavity. Finally, the laparotomy site was stitched. Sham-operated (control and TQ group) mice underwent the same procedure that involved surgical opening of the peritoneum and exposing the bowel; however, the ligation and needle perforation of cecum were not performed. Male BALB/c mice (n=48; age, 8 weeks) were randomly divided into four groups (n=12/group), namely, the control, TQ (50 mg/kg/day; Sigma-Aldrich; Merck KGaA), CLP and TQ + CLP groups. In the TQ + CLP group, CLP was performed following gavage of TQ for 2 weeks. Mice exhibiting signs of humane endpoints were immediately sacrificed. Before the sacrifice of animals at 48 h post-CLP, the mortality rates of CLP group and TQ + CLP group were 16.67 and 8.33% respectively, and no mice died in the control and TQ groups. At 48 h post-CLP, all mice that survived were sacrificed, and blood samples were obtained from their inferior vena cava, collected in serum tubes and stored at -80˚C until further use. The coronal sections of liver tissues (LTs) were fixed in 10% formalin for 30 min at room temperature, dehydrated in 75% ethanol overnight then embedded in paraffin for histological evaluation. The remnants of LTs were snap-frozen (-80˚C) and used to perform mRNA or immunoblotting analyses. All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals (23). The present

study was approved by the Animal Studies Committee of the Affiliated Zhongshan Hospital of Dalian University.

Serum analysis. The concentrations of alanine aminotransferase (ALT; cat. no. G0050), aspartate aminotransferase (AST; cat. no. G0073) and alkaline phosphatase (ALP; cat. no. G0040) in the serum were estimated using ELISA kits according to the manufacturer's protocols (Shanghai Westang Bio-Tech Co., Ltd).

Hematoxylin and eosin (H&E) staining. Sections (4-μm thickness) were serially cut to perform the morphometric analysis of LTs. These sections were stained with hematoxylin for 15 min and eosin for 5 min at room temperature to perform histological analysis.

Morphological and IHC analyses. IHC analysis was performed using a histone simple stain kit (cat. no. 414341F; Nichirei Corporation) according to the manufacturer's protocol. Paraffin-embedded sections were deparaffinized using xylene and rehydrated in the descending ethanol series. These sections (4-μm thickness) were blocked with 3% H_2O_2 in methanol for 15 min at room temperature to inactivate the endogenous peroxidases then incubated at room temperature for 1 h with primary antibodies against sequestosome-1 (cat. no. 18420-1-AP; p62; rabbit anti-p62 antibody; 1:200 dilution; Wuhan Sanying Biotechnology) and microtubule-associated protein light chain 3 (cat. no. 14600-1-AP; LC3; rabbit anti-LC3 antibody; 1:200 dilution; Wuhan Sanying Biotechnology). The secondary antibody from Histone Simple Stain kit (cat. no. 414341F; Nichirei Corporation) was placed on the tissue for 30 min at room temperature. Histological examination was performed under a light microscope (magnification, x400; Olympus BX43; Olympus Corporation).

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from the LTs using ISOGEN reagent (Nippon Gene Co., Ltd.) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from total RNA using a first-strand cDNA synthesis kit (SuperScript VILO cDNA synthesis kit; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The specific gene expression levels were quantitatively analyzed by performing qPCR using fluorescent SYBR Green technology (Light Cycler; Roche Molecular Diagnostics). β-actin cDNA was amplified and quantified in each cDNA preparation to normalize the relative expression of target genes. The thermocycling conditions were as follows: 95˚C for 3 min; 95˚C for 15 sec; 60˚C for 15 sec; 72˚C for 1 min (35 cycles); and 72˚C for 10 min. The relative expression of genes was determined using the $2^{-\Delta\Delta Cq}$ method (24) with normalization to 36B4 expression. The primer sequences used in the present study are listed in Table I.

Western blot analysis (WBA). Proteins were extracted from the LT using radioimmunoprecipitation assay buffer (cat. no. P0013B; Beyotime Institute of Biotechnology). Protein concentration was detected with a bicinchoninic acid Protein Assay kit (Beyotime Institute of Biotechnology). Protein samples (20 μg per lane) were separated by SDS-PAGE

Table I. Primer oligonucleotide sequences.

Gene	Primer sequences (5'-3')
TNF-α	F: TCTCATGCACCACCATCAAGGACT
	R: ACTTGCAGAACTCA
IL-6	F: TACCAGTTGCCTTCTTGGGACTGA
	R: TAAGCCTCCGACTTGTGAAGTGGT
IL-1β	F: TGCCCCTTTTGACAGTGAT
	R: TGTGCTGCTGCGAGATTTGA
MCP-1	F: ACTGAAGCCAGCTCTCTCTTCCTC
	R: TTCCTTCTTGGGTCAGCACAGAC
IL-10	F: GACAACATACTGCTAACCGACT
	R: ATCACTCTTCACCTGCTCCAC
β-actin	F: CGATGCCCTGAGGGTCTTT
	R: TGGATGCCACAGGATTCCAT

F, forward; R, reverse; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-α.

(10% gel), and proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon; EMD Millipore). PVDF membranes were blocked in TBS with 0.1% Tween-20 containing 5% skimmed milk for 2 h at room temperature, incubated in primary antibody diluent (cat. no. P0023A; Beyotime Institute of Biotechnology) and gently agitated overnight at 4˚C. Primary antibodies against p62 (cat. no. 18420-1-AP; 1:1,000 dilution), LC3 (cat. no. 14600-1-AP; 1:1,000 dilution), beclin 1 (cat. no. 11306-1-AP; 1:1,000 dilution), PI3K (cat. no. 20584-1-AP; 1:1,000 dilution) all from Wuhan Sanying Biotechnology, and anti-β-actin (cat. no. 4970; 1:1,000 dilution; Cell Signaling Technology, Inc.) were utilized in the present study. Membranes were then further incubated with horseradish peroxidase-conjugated goat anti-rabbit Immunoglobulin G (cat. no. 7074; 1:1,000 dilution; Cell Signaling Technology, Inc.) for 1 h at room temperature. WBA was performed as three independent experiments. The protein levels were expressed as a specific protein/β-actin ratio to minimize the loading variations. Protein bands were visualized by Enhanced Chemiluminescence kit (Beyotime Institute of Biotechnology). The relative chemiluminescent signal intensity was semi-quantified using ImageJ software version 1.48 (National Institutes of Health).

Statistical analysis. All experiments were repeated three times independently. All data are presented as the mean ± standard error of the mean. Statistical analysis was performed using SPSS software version 23.0 (IBM Corp.). Inter-group variation was assessed by performing one-way analysis of variance and subsequent Tukey's test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Metabolic characterization. The metabolic characteristics of the four groups of BALB/c mice subjected to various treatments are summarized in Fig. 1. The body weight was recorded

48 h post-operation did not vary significantly among the four groups. The CLP group exhibited a significant increase in the ALT, AST and ALP levels compared with all other groups; however, these levels were significantly decreased in the TQ + CLP group. There was no significant difference with respect to the aforementioned parameters between the control and TQ groups.

TQ reduces histopathological damage in LT. H&E staining was performed to evaluate the histopathological damage in LT (Fig. 2) and the results for the control and TQ groups revealed that hepatocytes were in regular shape and contained a large spheroidal nucleus. Contrastingly, the CLP group exhibited a marked liver injury demonstrated by hepatic strand disorganization, zonal necrosis, mononuclear cell infiltrations, centrilobular swelling, and sinusoidal and centrilobular congestions compared with the other groups. However, the administration of TQ to mice prevented the degenerative alterations in the hepatic structure induced by CLP.

TQ increases LC3 and beclin 1 expression and decreases p62 expression in LT. IHC staining was performed to evaluate the expression levels of LC3 and p62 in LTs (Fig. 3A). The TQ + CLP group exhibited a marked increase in LC3 expression and a decrease in p62 expression in the LT compared with the CLP group. WBA was performed to semi-quantify LC3, beclin 1 and p62 levels (Fig. 3B and C). The present study revealed that the expression levels of LC3 and beclin 1 were significantly increased and the expression levels of p62 were significantly decreased in the TQ + CLP group compared with the CLP group (Fig. 3B and C).

TQ reduces PI3K expression in the damaged LT. To investigate the effect of TQ on the regulation of the PI3K signaling pathway, the PI3K level was assessed in the treatment groups by performing WBA (Fig. 4A). The present study revealed that PI3K expression was higher in the CLP group compared with the control group. Additionally, the TQ + CLP group exhibited significantly decreased PI3K expression compared with the CLP group (Fig. 4B).

TQ reduces the expression levels of interleukin (IL)-6, IL-1β, monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor α (TNF-α) but increases IL-10 expression in the LTs of the CLP group. To examine the involvement of inflammatory factors at the gene expression level in the LTs obtained from mice, the expression levels of IL-6, IL-1β, MCP-1, IL-10 and TNF-α were quantified using RT-qPCR (Fig. 5). IL-6, IL-1β, MCP-1 and TNF-α expression were upregulated in the CLP group; however, this upregulation was attenuated in the TQ + CLP group. By contrast, IL-10 expression was downregulated in the CLP group; however, this was upregulated in the TQ + CLP group.

Discussion

To the best of our knowledge, TQ affects autophagy in myocardial ischemia-reperfusion (I/R) injury in Langendorff-perfused rat hearts and exhibits anti-inflammatory activities in rheumatoid arthritis rat model (25,26). However, limited
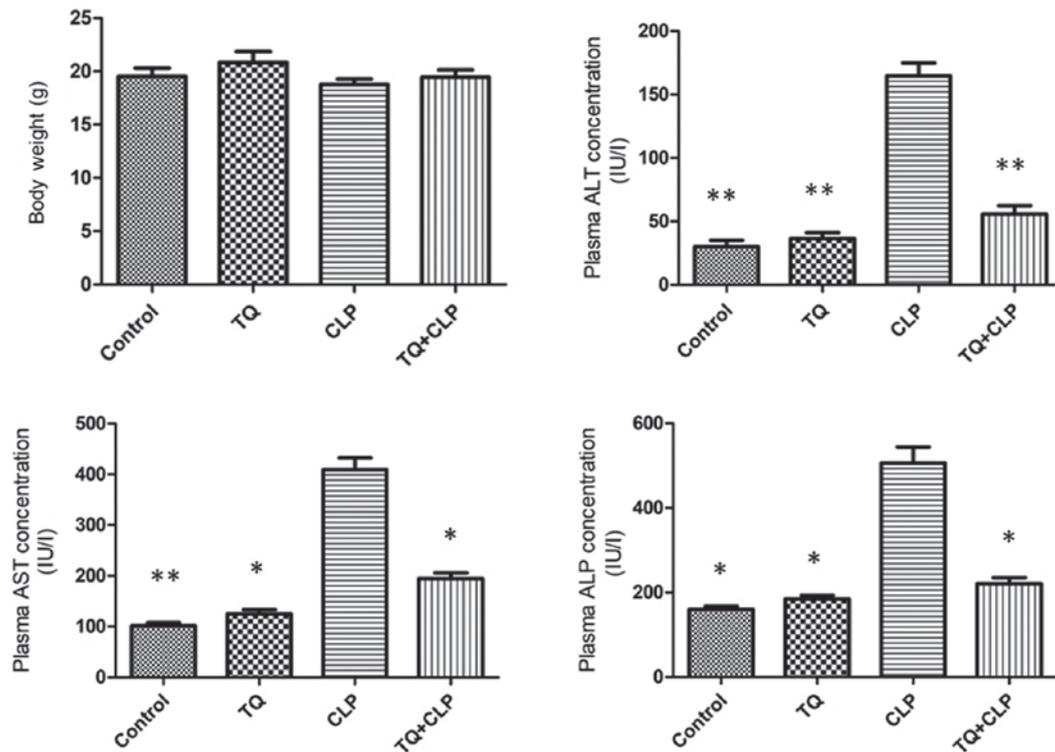
Figure 1. Metabolic data of the four groups of BALB/c mice after various treatments. Body weight, and ALT, AST and ALP levels are presented. Data are represented as the mean ± SEM; n=10-12 per group. *P<0.05 and **P<0.01 vs. CLP group. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CLP, cecal ligation and puncture; TQ, thymoquinone.
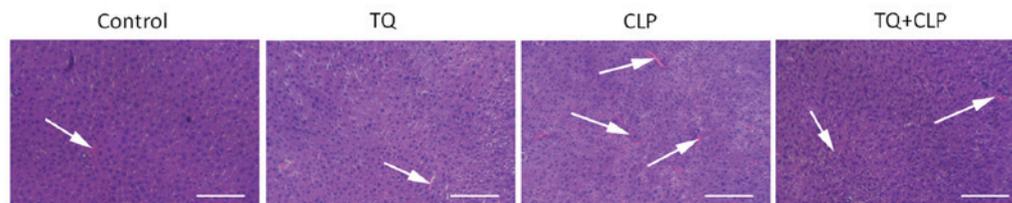

Figure 2. Histopathological damage in the liver tissue in the four groups of BALB/c mice subjected to various treatments. Arrows exhibited hepatic strand disorganization, zonal necrosis, centrilobular swelling, and sinusoidal and centrilobular congestions. Scale bar, 100 μm. CLP, cecal ligation and puncture; TQ, thymoquinone.

information is available regarding the effect of TQ on CLP-induced septic liver injury. Therefore, the present study investigated the effect of TQ on CLP-induced septic liver injury using a murine model of CLP-induced polymicrobial sepsis. The present study demonstrated that TQ ameliorated septic liver injury as indicated by the regulation of hepatic enzymes (AST, ALT and ALP), autophagy indicators (LC3, p62 and beclin 1), the PI3K signaling pathway and inflammatory factors (IL-6, IL-1β, IL-10, MCP-1 and TNF-α).

In the present study, no significant variation was observed regarding the increment in body weight among the four groups of mice. The sepsis-induced liver damage could further be investigated by assessment of liver cell integrity markers (AST and ALT) and cholestasis parameters (ALP) (27). The serum levels of hepatic enzymes primarily mirror the degree of liver injury (28) and have been used in the present study as a diagnostic marker for hepatotoxicity. The present study indicated that the serum levels of AST, ALT and ALP were significantly increased in the CLP group compared with in the control group. The TQ group exhibited a prominent reduction in

these hepatic biochemical parameters compared with the CLP group. These hepatic biochemical parameters clearly reflected the damage of hepatotoxicity (ALT and AST) and cholestasis (ALP) (27) that was alleviated by TQ. Previous studies indicated that treatment with TQ led to similar results regarding the serum levels of AST, ALT and ALP during lead-induced hepatic toxicity and alcoholic liver injury induced by chronic-plus-binge ethanol feeding in murine models (29,30). Additionally, according to the histological evidence of liver injury, as assessed by performing H&E staining, treatment of murine models with TQ protected mice against the liver damage caused by CLP-induced sepsis.

Several studies demonstrated a close interaction between CLP-induced sepsis-associated organ failure and cell dysfunction caused by autophagy (3,31). Additionally, a previous study indicated that prominent autophagosomal accumulation was observed in the liver of patients who died of sepsis as well as in the early stages of experimentally-induced sepsis in mouse model (3). In the present study, autophagosome formation was determined by performing IHC analysis and verified using
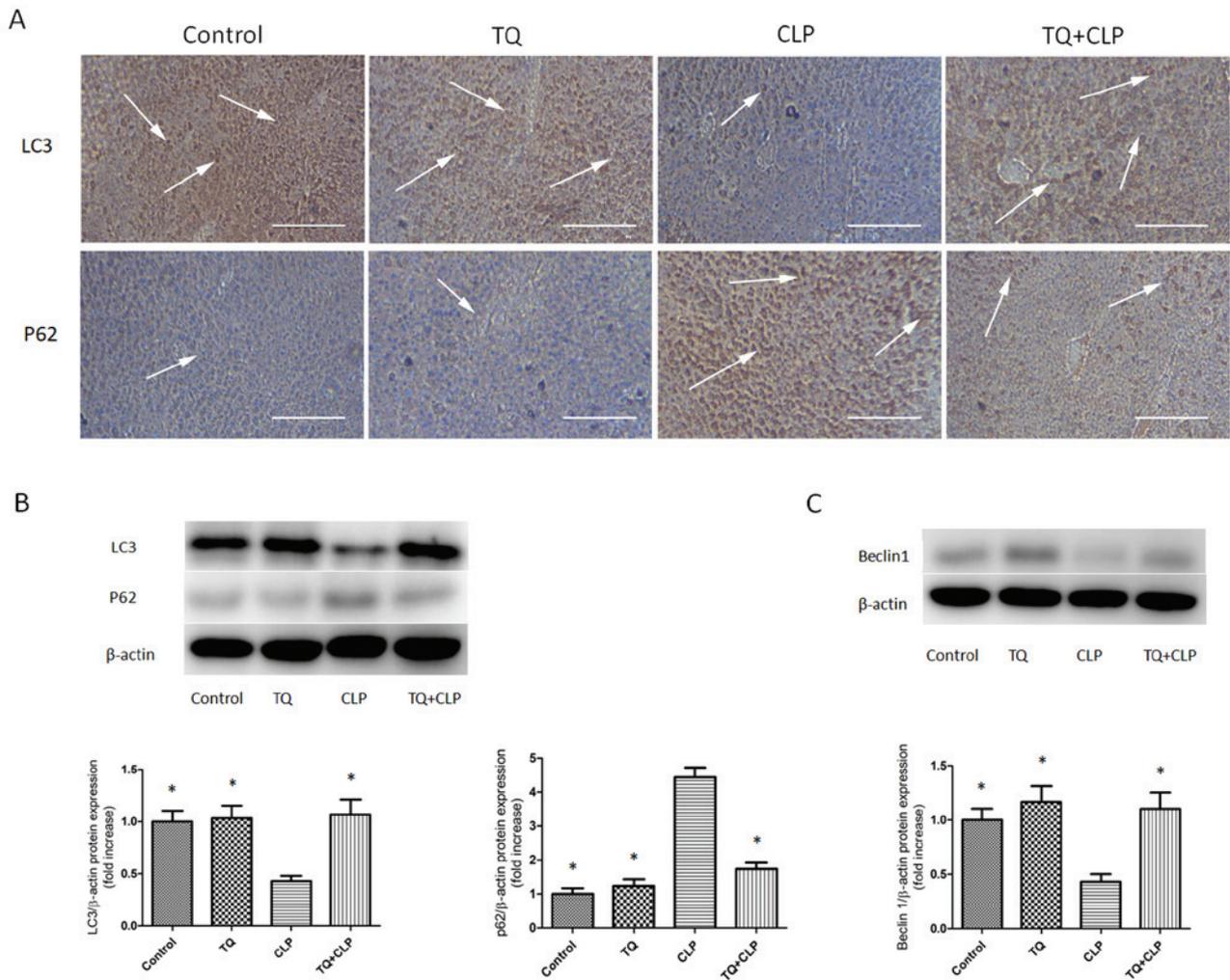
Figure 3. LC3, p62 and beclin 1 expression in LTs of the four groups of BALB/c mice following various treatments. (A) Representative images of immunohistochemical analysis to detect the LC3 and p62 levels in the LTs. Arrows indicate positively stained cells. Scale bar, 100 μm. Western blot analysis to detect the expression levels of (B) LC3 and p62, and (C) beclin 1 in the LTs. Data are presented as the mean ± SEM; n=3-4 per group. *P<0.05 vs. CLP group. CLP, cecal ligation and puncture; LC3, microtubule-associated protein light chain 3; LT, liver tissue; p62, sequestosome-1; TQ, thymoquinone.
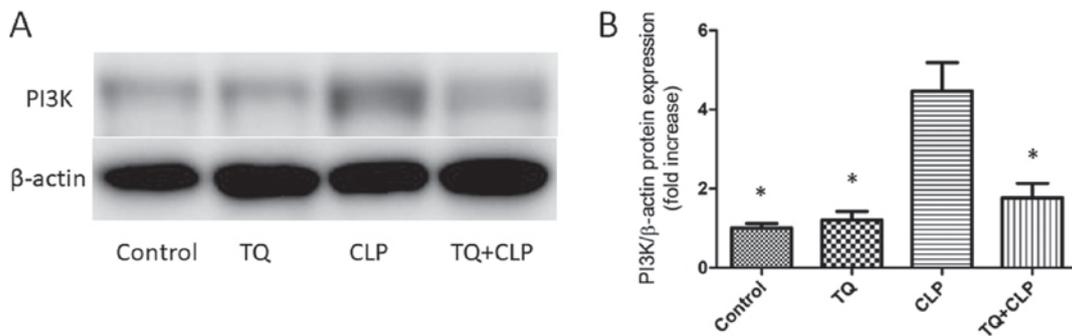

Figure 4. PI3K expression in the LTs of the four groups of BALB/c mice following various treatments. (A) Western blot analysis to detect the PI3K expression in the LTs. (B) Bar graph depicting the semi-quantification of PI3K expression. Data are presented as the mean ± SEM; n=3-4 in each group. *P<0.05 vs. CLP group. CLP, cecal ligation and puncture; LT, liver tissue; TQ, thymoquinone.

WBA. The present study revealed that the expression levels of autophagy-specific proteins in the liver were altered in the CLP group compared with in the control group.

LC3, p62 and beclin 1 are three major proteins involved in autophagy (32). The levels of LC3 have been used to assess autophagosome formation (33). The results of the present study indicated that low expression levels of LC3 aggregation in the LT were observed at 48 h in the CLP group compared with in the control group. Additionally, several studies have indicated a time-course-dependent alteration in LC3 aggregation
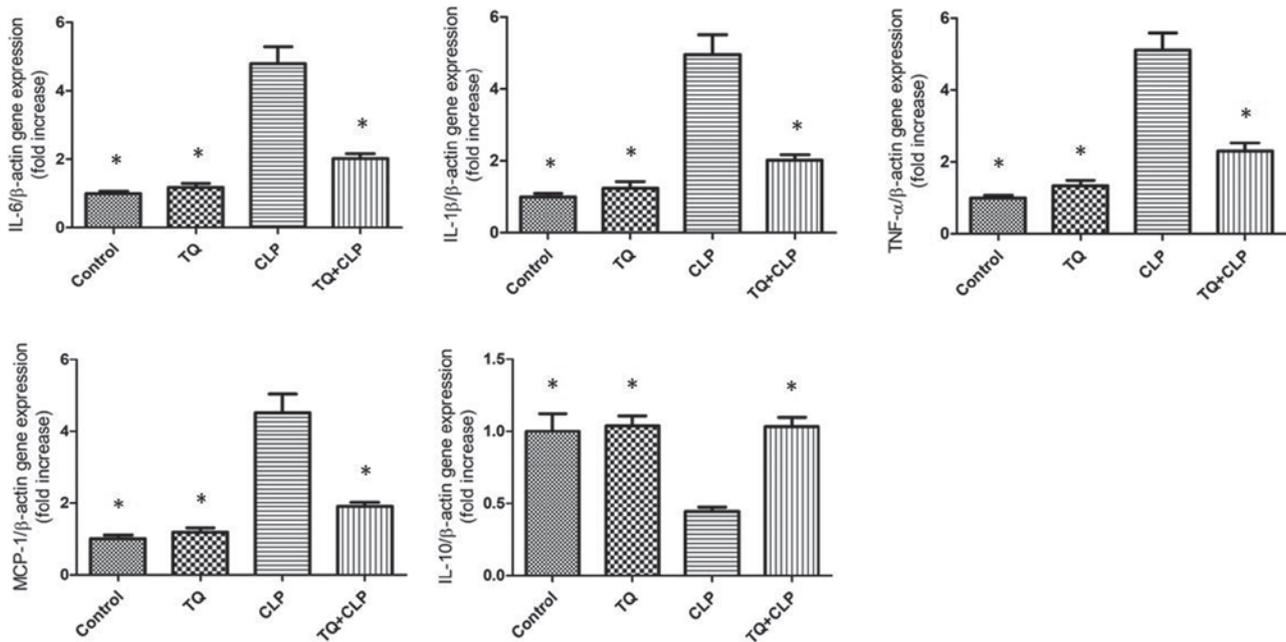
Figure 5. Expression levels of proinflammatory cytokines in the LTs obtained from the four groups of BALB/c mice after various treatments. Relative mRNA expression levels of IL-6, IL-1β, MCP-1, IL-10 and TNF-α in the LTs. Data are presented as the means ± SEM; n=6 in each group. *P<0.05 vs. CLP group. CLP, cecal ligation and puncture; IL, interleukin; LT, liver tissue; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor α; TQ, thymoquinone.

in CLP-induced sepsis. LC3 aggregation was significantly increased at 6-8 h and gradually decreased 12-24 h after CLP (34-36). In the present study, LC3 expression in the TQ + CLP group was higher compared with that in the CLP group at 48 h post-CLP. This indicated that pretreatment with TQ may increase the autophagosome formation in CLP-induced sepsis. As a traditional Chinese medicine that has been used for centuries, multiple studies support the positive effect of TQ in the prevention and cure of various diseases. A previous study reported that TQ dose-dependently increased intracellular LC3 expression in camptothecin-11-resistant LoVo colon cancer cells (37). Additionally, Chu *et al* (38) reported that TQ treatment increased the accumulation of autophagosomes in human squamous carcinoma cells. As for beclin 1, the present study indicated that pretreatment with TQ may increase expression of beclin 1 in CLP-induced sepsis. Wan *et al* (36) demonstrated that the expression of autophagy-associated proteins, including beclin 1, declined to basal levels by 12 h post-CLP. Liu *et al* (39) revealed that TQ increased beclin 1 gene and protein expression levels; this result was in accordance with the results of the present study. However, high p62 aggregation in the LT was observed in the CLP group compared with in the control group. Pretreatment with TQ after performing CLP reduced the p62 level compared with that in the CLP group. Previous studies have established that p62 is a multidomain adaptor protein that transports ubiquitinated proteins during autophagy. Consistent with the results of the present study, other studies suggest that p62 concentration is generally inversely associated with autophagosomal activity (40-42).

Generally, autophagy is a stringently regulated machinery that degrades and recycles the unnecessary or damaged components of a cell to maintain cellular homeostasis (43). In the present study, TQ was used to prevent CLP-induced septic liver injury *in vivo*.

In the present study, to investigate the effect of TQ on the regulation of the PI3K signaling pathway, PI3K expression was initially assessed by performing WBA. PI3K represents a key signaling molecule that regulates several cellular functions, including proliferation, survival, adhesion and migration in the liver (44,45). Previous studies suggested that the PI3K pathway is closely associated with autophagy. Li *et al* (46) reported that several microbial virulent factors induced autophagy by inactivating the PI3K/Akt/mTOR pathway, and Li *et al* (47) suggested that spironolactone promoted autophagy by inhibiting the PI3K/AKT/mTOR signaling pathway.

A recent study indicated that TQ significantly augmented cisplatin-induced antitumor effects in gastric cancer *in vitro* as well as *in vivo* through the inhibition of the PI3K/Akt signaling pathway (48). Furthermore, another study suggested that TQ inhibited PI3K phosphorylation in thioacetamide-induced hepatic fibrosis and inflammation (49). Upon lipopolysaccharide stimulation, the PI3K pathway activation serves to balance between proinflammatory and anti-inflammatory responses (50). Recknagel *et al* (51) demonstrated that PI3K signaling was spontaneously induced in a rat model with long-term fecal peritonitis. A significantly increased PI3K expression was also observed in the murine liver following aluminum overload (52). In the present study, high PI3K expression was observed in the CLP group compared with in the control group. Conversely, the TQ + CLP group exhibited a reduced PI3K level compared with the CLP group. Collectively, TQ may inhibit PI3K signaling to protect against CLP-induced septic liver injury.

The present study demonstrated that the mRNA expression levels of IL-6, IL-1β, MCP-1 and TNF-α in the liver were significantly upregulated in the CLP group compared with those in the control group. In the TQ pretreatment group, the expression levels of IL-6, IL-1β, MCP-1 and

TNF-α in the liver were reduced as a response against CLP. Inversely, IL-10 expression was downregulated in the CLP group; however, its expression levels were upregulated in the TQ + CLP group. Experimental studies indicated that TQ reduced the production of IL-6, IL-1β, MCP-1 and TNF-α in the blood and other tissues (53). Furthermore, a previous study reported that oral administration of TQ results in significantly increased expression levels of IL-10 in the collagen-induced arthritis in Wistar rats model (54). Additionally, Hsiao *et al* (55) reported that treatment using an inducer of autophagy diminished TNF-α-induced DNA fragmentation. Additionally, Giegerich *et al* (56) illustrated that autophagy-dependent pellino E3 ubiquitin protein ligase family member 3 degradation inhibited proinflammatory IL-1β expression.

In conclusion, the present study demonstrated that TQ assisted the mitigation of septic liver injury, as indicated by the upregulation of LC3, beclin 1 and IL-10 expression as well as the suppression of p62, PI3K, IL-6, IL-1β, MCP-1 and TNF-α expression. These findings provided novel insights into the role of TQ in sepsis-induced liver injury, and improve the possibility of developing novel therapeutic interventions to treat liver injury.

Acknowledgements

Availability of data and materials

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

Authors' contributions

ZP designed this study. FW and XL performed experiments. ZP, HZ, YZ and QY analyzed the data, interpreted the results of the experiments and prepared the figures. QL analyzed the data, interpreted the results of the experiments and prepared the figures. XL drafted the manuscript. ZP, QL and YZ contributed to the revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

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

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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