Thymoquinone modulates the expression of sepsis-related microRNAs in a CLP model

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Abstract. Sepsis is a clinical syndrome common in critical care settings. In the present study, the therapeutic effect of thymoquinone (TQ) on the expression of sepsis-related microRNAs (miRNAs/miRs), levels of inflammatory markers, organ dysfunction and mortality were investigated in a cecal ligation and puncture (CLP) rat model. A single dose of TQ (1 mg/kg) was administered to animals 24 h after CLP and the mortality rate was assessed up to 7 days following the induction of sepsis. In addition, blood samples were collected at different time points and the expression levels of miRNAs (i.e. miR-16, miR-21, miR-27a and miR-34a) were examined, along with the levels of inflammatory cytokines (i.e. TNF-α, IL-1α, IL-2, IL-6 and IL-10) and sepsis markers (i.e. C-reactive protein, endothelial cell-specific molecule-1, VEGF, procalcitonin and D-dimer). Liver, kidney and lung tissues were also collected for further histological examination. Treatment with TQ significantly downregulated the miRNA expression levels, as well as the levels of inflammatory cytokines and early-stage sepsis biomarkers by 30-70% at 12-36 h ($P<0.05$). Furthermore, CLP model rats treated with TQ exhibited an ~80% increase in survival rate compared with that in the untreated CLP group. In addition, TQ induced the preservation of organ function and structure. In conclusion, the present study demonstrated a promising therapeutic effect of TQ against the sequelae of sepsis.

Introduction

Sepsis is a potentially lethal condition that is commonly encountered in intensive care units (ICUs) (1-4). Septic shock is a subset of sepsis that is associated with a greater risk of mortality than sepsis alone (5). The usual host reaction is complex, aiming to detect and control pathogen incursion, and to initiate immediate tissue repair. The body activates both cellular and the humoral immunity, which release massive quantities of pro-inflammatory and anti-inflammatory mediators, leading to systemic inflammatory response syndrome (SIRS) (6,7). Aggravation of these mechanisms can cause a series of events that may lead to endothelial injury, tissue hypoperfusion, intravascular coagulation, multiple organ dysfunction syndrome and possibly death (8).

Despite improvements in critical care medicine, sepsis and septic shock continue to be among the leading causes of death and a serious challenge to clinicians and scientists (1,9). Some of the most important approaches in sepsis investigation consist of focusing on agents that may modify systemic inflammation; however, these efforts have not had much success. Another important feature of sepsis is cellular apoptosis, which can lead to organ failure (10-13). Parenchymal cells of the lung and the liver, as well as intestinal epithelial cells have been reported to exhibit higher levels of apoptotic death in animal models of sepsis following microvascular dysfunction and tissue hypoxia compared with non-septic animals (14,15). Unlike genomic DNA, which is static, RNA expression can dynamically change between healthy and diseased states, and thus can provide real-time information regarding cellular function. miRNAs have been recognized as a class of gene expression regulators that serve significant roles in normal cell function and in disease development, including the pathogenesis of cardiovascular disorders, cancer and inflammation (16). miRNAs are 19-24 nucleotide-long, endogenous, non-coding RNAs that function as post-transcriptional suppressors of gene expression by interfering with target mRNA translation or stability; notably, miRNAs affect multiple target genes (17). There is growing evidence that miRNA dysregulation corresponds with the clinical symptoms of sepsis (18). Increases in the levels of circulating miRNAs originating from lipopolysaccharide (LPS)-stimulated monocytes and monocyte-derived dendritic cells during sepsis have also been reported (19,20). In addition, the pathophysiological responses associated with sepsis, such as inflammation, shock or even ileus in the CLP model may contribute to the upregulation of circulating miRNAs. It has also been shown that miRNA dysregulation can correspond to clinical symptoms and severity of sepsis (18,21,22). Therefore, miRNAs could be potential therapeutic targets for sepsis management. In the present study, the expression

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levels of miRNAs (miR-16, miR-21, miR-27a and miR-34a) were examined, shortlisted from a group of miRNAs which have been shown to upregulate/downregulate during sepsis in multiple studies (16,18,23-25).

Thymoquinone (TQ) has been reported to possess notable immunomodulatory effects, as well as other pharmacological properties, including anti-inflammatory activity, improvement of microvascular function and regulation of endothelial nitric oxide synthase (26,27). In the present study, it was hypothesized that TQ could regulate miRNA expression levels, protect organs and improve survival in sepsis. Furthermore, changes in sepsis markers, such as cytokines, C-reactive protein (CRP), VEGF, endothelial cell-specific molecule-1 (ESM-1 or Endocan-1), procalcitonin (PCT) and D-dimer, were detected in a cecal ligation and puncture (CLP) model (28).

Materials and methods

Animals and CLP model. A total of 60 male Wistar albino rats (*Rattus norvegicus*), weighing 200-220 g, age, 8-10 weeks, were used in the present study. The animals were housed in filter-top cages in a temperature-controlled environment (23±2˚C, 40-60% humidity with a 12-hour light/dark cycle) and were provided access to standard rat chow and water *ad libitum*. The animals were allowed to acclimate for 1 week before the experiments were conducted. The present study was approved by the Ethics Committee of the College of Pharmacy, King Saud University (Riyadh, Saudi Arabia; approval no. KSU-SE-19-17).

Under aseptic conditions, the rats (n=10/group) were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). The animal was placed on a surgical tray 5 min after injection, its abdomen was shaved and an ~1-cm cut was made into the abdomen. The cecum was exposed, ligated and punctured at two points with a 21G needle; in the sham group, the cecum was not punctured. Subsequently, the cecum was placed back into the body and the incision was sutured. At 1 h following CLP, each animal received 1 ml sterile saline intraperitoneally for resuscitation, and the animals were allowed *ad libitum* access to food and water. At 24 h after CLP, a single intramuscular dose of TQ (1 mg/kg; prepared in 10% DMSO) was administered in the thigh muscles of the hind limbs to the animals in the treatment group (CLP + TQ). The sham and CLP control groups were treated only with 10% DMSO. For the survival study, animal mortality was assessed up to 7 days after TQ treatment. In another set of experiments, blood samples (0.5 ml) were collected in sterile tubes from the tail vein prior to TQ administration, and 12, 24 and 36 h post-treatment. The blood was centrifuged (3,000 x g, 4˚C for 10 min) immediately after collection, and plasma and serum were stored at -80˚C. The animals were subsequently euthanized with ketamine (100 mg/kg) and xylazine (10 mg/kg). The death of the animals was confirmed by lack of movement, absence of heartbeat and respiration over a sufficient period of time. The liver, kidney and lung tissues were harvested, dissected and stored in formalin for histopathological examination.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from the serum samples using miRNeasy kit (Qiagen, Inc.) according to the manufacturer's protocol. The quality and quantity of total RNA were checked using a spectrophotometer. To remove any DNA contamination, the total RNA was treated with DNase enzyme from a DNase 1 kit (Millipore Sigma). Total RNA, including miRNA, was reverse transcribed into cDNA using MystiCq microRNA cDNA Synthesis Mix (Millipore Sigma) according to the manufacturer's protocol. Prior to miRNA quantification by RT-qPCR, conventional PCR was run for all the primers used in the present study to check amplification specificity. Primer details are presented in Table I. In conventional PCR, all the primers produced only one amplification band corresponding to their expected amplicon size (data not shown). qPCR was subsequently performed using SYBR Green (Roche Molecular Diagnostics) according to the manufacturer's protocol; U6 small nuclear ribonucleoprotein was used as a reference gene. For each miRNA, the reaction mixture consisted of 10 μl 2X SYBR Green master mix, 0.25 μl each reverse and forward primer, 1 μl cDNA and 8.5 μl nuclease-free water. The following thermocycling conditions were used: 40 cycles of denaturation at 94˚C for 4 min, annealing at various temperatures as provided in Table I, and extension at 72˚C for 15 sec. To confirm the product specificity, melting curve analysis was performed. The relative expression levels of the different miRNAs were determined using the $2^{-\Delta\Delta Cq}$ method (29). Relative expression levels of the miRNAs detected in the present study are presented as fold change.

Biomarkers and biochemical analyses of plasma. ELISA was used to estimate the concentrations of inflammatory cytokines, including TNF-α (cat. no. PRTA00), IL-1α (RRA00), IL-2 (SR2000), IL-6 (SR6000B), and IL-10 (SR1000), according to the manufacturer's protocols (R&D Systems, Inc.). A quantitative sandwich ELISA was used to assess the levels of sepsis biomarkers, including CRP (EK0978), and VEGF (EK0540; all Boster Biological Technology, Pleasanton, CA, USA) and ESM-1 (MBS762527; MyBioSource, Inc. San Diego, CA, USA), according to the manufacturer's protocols. The concentration of PCT (CSB-E13419r) was measured using a specific ELISA kit (CUSABIO, Houston, TX, USA). Similarly, D-dimer (CSB-E12984r-1) levels were determined using an ELISA kit (Cosmo Bio Co., Ltd.); changes in color were measured using a spectrophotometer at 450 nm and were quantified using constructed standard curves. Pertinent biochemical parameters, such as alanine transaminase (ALT, aspartate transaminase, (AST) alkaline phosphatase (ALP), serum creatinine and blood urea nitrogen (BUN), were measured using colorimetric methods (HUMAN Diagnostics Worldwide, Wiesbaden, Germany).

Histopathological examination. Liver, kidney and lung tissues were sliced into small pieces and fixed in 10% formalin for 24 h at room temperature. The fixed tissues were then embedded in paraffin. Subsequently, the tissues were cut into 4-5-μm sections, deparaffinized and rehydrated with methanol as previously described (30). Morphological changes induced by sepsis with or without TQ treatment were assessed by evaluating the organ tissues using H&E staining; tissues were observed under a light microscope as previously described (31).

Table I. List of primers used for PCR.

Gene	Primer sequence (5'-3')	Annealing temperature (˚C)	Expected size (bp)
U6	F: CTCGCTTCGGCAGCACA	55	89
	R: AACGCTTCACGAATTTGCGT		
miR-16	F: CCGCTCTAGCAGCACGTAAA	60	82
	R: CCCTGTCACACTAAAGCAGC		
miR-21	F: GTACCACCTTGTCGGGTAGC	55	82
	R: ATGTCAGACAGCCCATCGAC		
miR-27a	F: CCTGTGGAGCAGGGCTTAG	60	73
	R: GCGGAACTTAGCCACTGTGA		
miR-34a	F: TGGCAGTGTCTTAGCTGGTT	56	81
	R: AACGTGCAGCACTTCTAGGG		

F, forward; miR, microRNA; R, reverse.

Statistical analysis. All of the data analysis was carried out using GraphPad Prism version 6 (GraphPad Software, Inc.) and SPSS 20.0 software package (IBM Corp.). Variables are presented as the mean ± SEM. Animal survival was assessed using Kaplan-Meier analysis and log-rank test. For the miRNA expression analysis, the relative expression of the different miRNAs was determined by the $2^{-\Delta\Delta Cq}$ method, normalized with ΔCq=Average Cq_{miRNA}-Average Cq_{U6}. The relative expression levels of miRNAs are presented as fold change, calculated from the mean Cq values for each group. Differences in the miRNA expression, biochemical parameters and inflammatory biomarkers between the groups were evaluated using one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

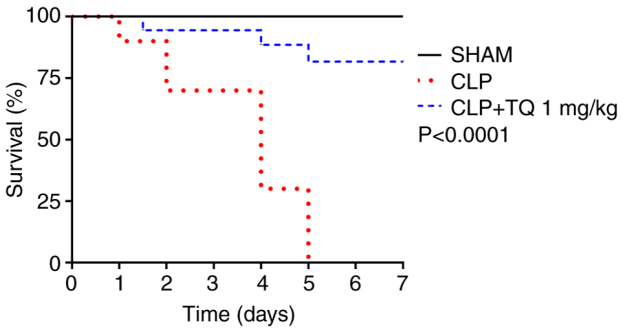

Figure 1. Kaplan-Meier survival plot following CLP and TQ treatment. TQ was administered 24 h after sepsis induction. n=10 rats/group; P<0.0001 vs. CLP. CLP, cecal ligation and puncture; TQ, thymoquinone.

Results

Effect of TQ on animal survival and biochemical estimation. Animals that underwent CLP started displaying signs of infection including lethargy, piloerection, huddling and a decrease in water and food uptake within 12 h of surgery. As shown in Fig. 1, 24 h post-CLP, death started to occur in the CLP groups but significantly decreased when TQ was administered in the CLP+TQ group. The mortality rate reached 100% in the rats in the untreated CLP group at 5 days, whereas animals in the TQ treatment group exhibited an increase in survival rates of up to 80%; no deaths were reported in the sham group during this time. Results from the biochemical analyses used to assess organ function showed a significant increase in liver enzymes (i.e. ALT, AST and ALP) in the CLP group, which were reduced within 12 h of TQ treatment. Similarly, TQ administration significantly improved the kidney function as shown by a reduction in serum creatinine and BUN compared with that in the CLP control (Table II).

Effect of TQ on miRNA and biomarker estimation. The expression levels of miR-16, miR-21, miR-27a and miR-34a were significantly increased at 12 and 24 h after CLP compared with the sham group (Fig. 2). Treatment with TQ significantly downregulated the miRNA expression levels by 40-80%. Similarly, the concentrations of IL-1α, IL-2, IL-6, IL-10 and TNF-α, were significantly increased in the CLP group compared with those in the sham group, and TQ treatment resulted in a significant reduction in the levels of the inflammatory cytokines in a time dependent manner and effect of TQ seems to diminish by the 36 h (Figs. 3 and 4A; P<0.05).

The effect of CLP on sepsis biomarkers CRP, VEGF and ESM-1 was also determined. CLP resulted in a notable increase in the concentrations of the sepsis biomarkers, which was reversed by TQ administration (Fig. 4B-D; P<0.05). TQ treatment also mitigated the increased levels of D-dimer and PCT in septic model animals (Fig. 5A and B), which was consistent with its antiseptic effect.

Histopathology observations. The histological evaluation (Fig. 6) of rat renal tissues from the sham group showed normal architecture of the renal cortex, renal glomeruli and renal tubules (Fig. 6Aa). By contrast, animals having undergone the CLP procedure exhibited deterioration of the renal cortex and medullary tubules, and some interstitial hemorrhages with mononuclear cell infiltration (Fig. 6Ab). In addition, epithelial tubular necrotic areas and cellular atrophy were observed. CLP model animals treated with TQ exhibited reduced deterioration of the renal cortex, renal glomeruli and renal tubules (Fig. 6Ac); in addition, reduced dilatation of renal glomeruli and cortical tubules was observed. No signs of deterioration in

Table II. Comparison of biochemical parameters between different experimental groups.

Parameter	Group	Time after TQ, h			
		0[a]	12	24	36
ALP, IU/l	Sham	118.79±4.73	131.85±5.94	129.41±5.37	121.32±5.18
	CLP	292.66±6.94[b]	358.25±12.20[b]	388.56±18.20[b]	418.74±10.68[b]
	CLP + TQ	278.08±5.57[b]	191.12±4.99[b,c]	236.01±5.50[b,c]	260.44±6.10[b,c]
AST, IU/l	Sham	121.45±3.76	129.19±4.65	119.79±3.26	124.52±4.84
	CLP	238.89±5.13[b]	275.14±7.64[b]	289.04±8.86[b]	320.86±2.96[b]
	CLP + TQ	224.35±8.38[b,c]	157.76±6.48[b,c]	177.35±7.59[b,c]	187.49±6.86[b,c]
ALT, IU/l	Sham	29.66±1.94	34.87±2.49	31.76±2.09	27.06±2.78
	CLP	53.37±2.01[b]	58.15±1.62[b]	62.42±1.82[b]	69.32±1.39[b]
	CLP + TQ	51.31±0.58[b]	38.12±1.55[c]	41.02±1.08[b,c]	46.51±1.27[b,c]
Creatinine, mg/dl	Sham	1.30±0.12	1.27±0.22	1.32±0.43	1.30±0.59
	CLP	2.15±1.22	2.39±0.84[b]	2.66±1.02[b]	3.13±0.60[b]
	CLP + TQ	2.18±0.51[b]	1.58±0.65[c]	1.75±.22[b,c]	1.91±0.78[b,c]
BUN, mg/dl	Sham	49.07±0.65	51.98±0.77	53.69±0.52	52.85±0.61
	CLP	91.16±0.38[b]	101.95±0.65[b]	125.85±0.76[b]	129.37±0.35[b]
	CLP + TQ	95.87±0.20[b,c]	67.65 ±0.66[b,c]	74.51±0.42[b,c]	81.75±0.91[b,c]

[a]Time 0 h represents the time point 24-h post-sepsis induction; [b]P<0.05 vs. Sham; [c]P<0.05 vs. CLP. ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BUN, blood urea nitrogen; CLP, cecal ligation and puncture; TQ, thymoquinone.

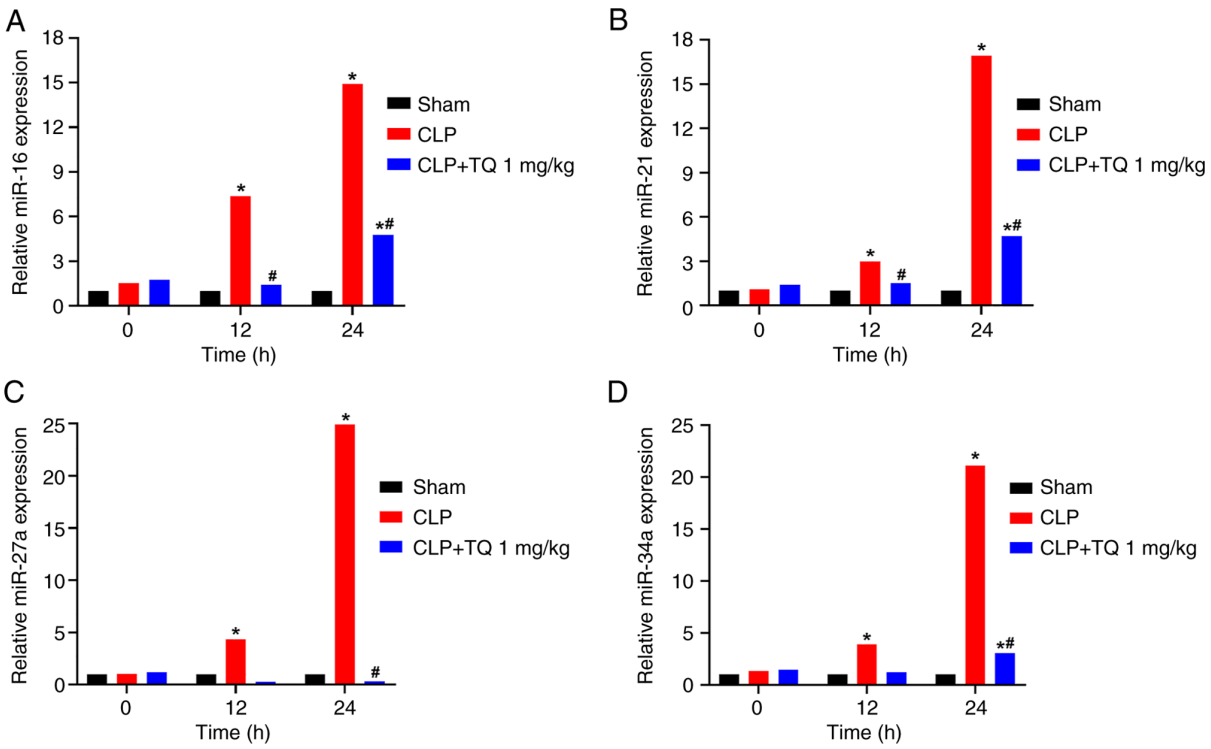

Figure 2. Fold change in the expression levels of (A) miR-16, (B) miR-21, (C) miR-27a and (D) miR-34a in response to CLP-induced sepsis measured at different time points following treatment with TQ. n=6 rats/group. *P<0.05 vs. Sham; #P<0.05 vs. CLP. CLP, cecal ligation and puncture; miR, microRNA; TQ, thymoquinone.

cellular organelles and no inferential hemorrhages were found (Fig. 6Ac).

Similarly, the rat liver from the sham group exhibited normal liver composition with a prominent nucleus, well preserved cytoplasm, central vein and a compact arrangement of hepatocytes without any fatty lobulation (Fig. 6Ba). By contrast, animals in the untreated CLP group exhibited acute cellular swelling, congestion of the central vein, medium

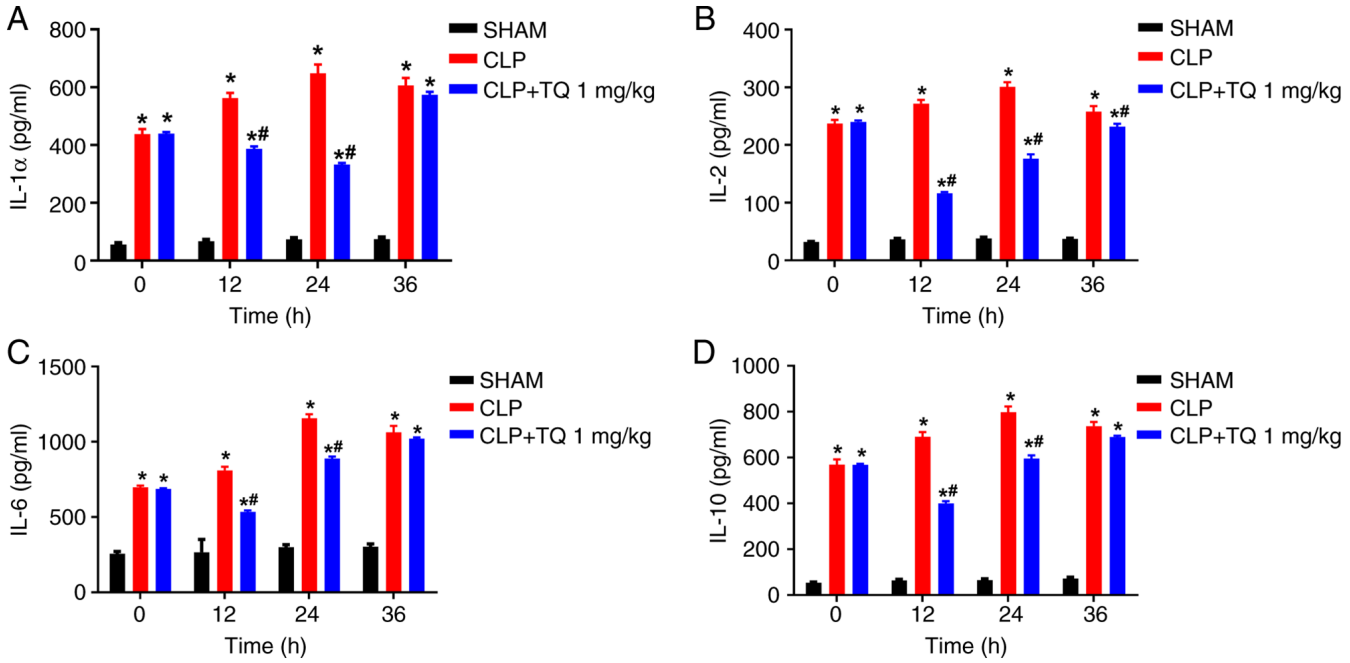

Figure 3. Plasma levels of the inflammatory cytokines (A) IL-1α, (B) IL-2, (C) IL-6 and (D) IL-10 in response to CLP-induced sepsis measured at different time points following treatment with TQ. Data are presented as the mean ± SEM. n=10 rats/group; *P<0.05 vs. Sham; #P<0.05 vs. CLP. CLP, cecal ligation and puncture; TQ, thymoquinone.

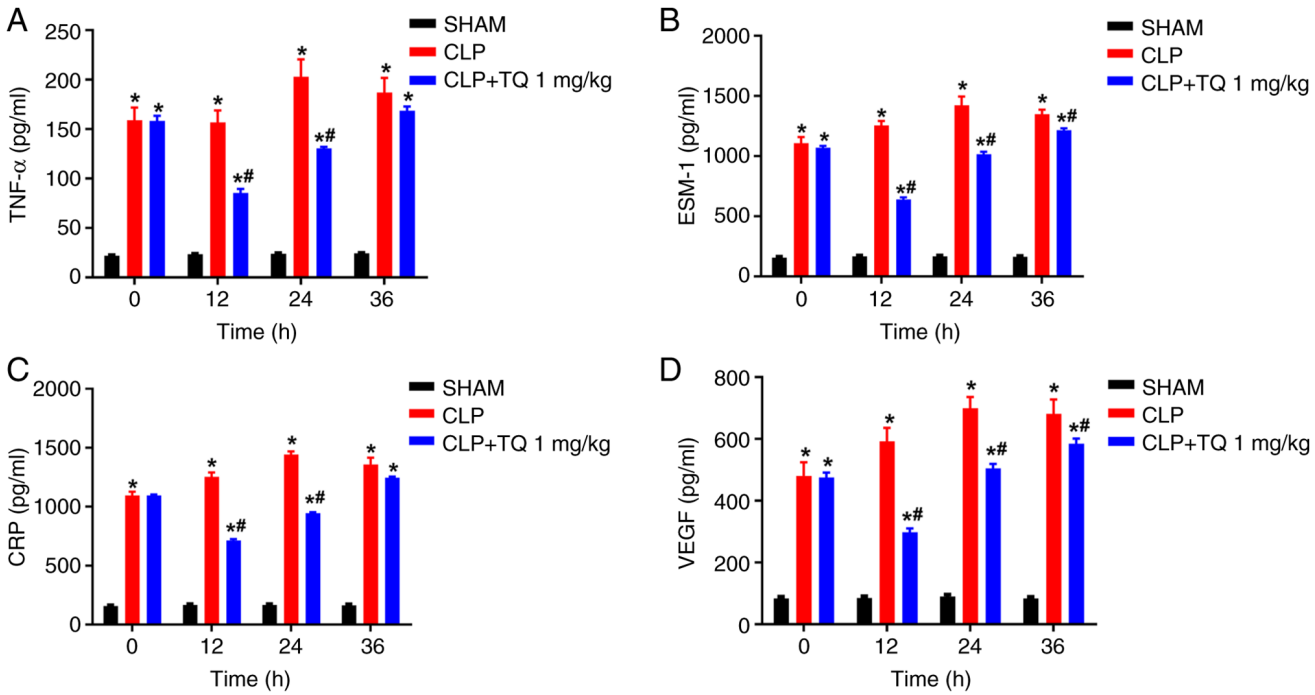

Figure 4. (A) TNF-α, (B) ESM-1, (C) CRP and (D) VEGF plasma levels in response to CLP-induced sepsis measured at different time points following treatment with TQ. Data are presented as the mean ± SEM. n=10 rats/group; *P<0.05 vs. Sham; #P<0.05 vs. CLP. CLP, cecal ligation and puncture; CRP, C-reactive protein; ESM-1, endothelial cell-specific molecule-1; TQ, thymoquinone.

centrilobular necrosis of hepatocytes and apoptotic bodies with sinusoidal dilatation (Fig. 6Bb). An acute massive focal infiltration of mononuclear cells in the portal area, and sinusoidal infiltration in the central zones with vacuolization and steatosis were also evident (Fig. 6Bb). CLP animals treated with TQ displayed distinct features, such as pentagonal or hexagonal lobules, with central veins and borderline hepatic triads or tetrads, engrained in connective tissues (Fig. 6Bc). A mild degree of centrilobular necrosis of hepatocytes was observed but no apoptotic bodies were seen, whereas slight sinusoidal dilatation and mild congestion of central vein were detected. A small number of mononuclear cells and sinusoidal

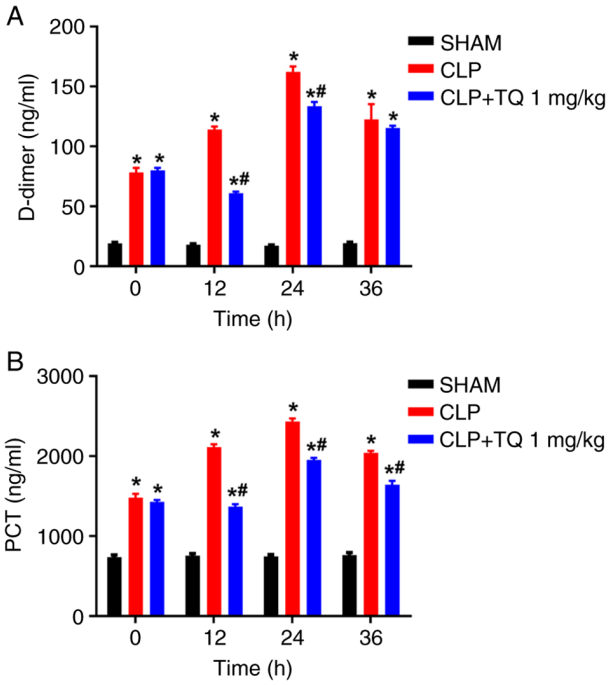

Figure 5. Effect of TQ on (A) D-dimer and (B) PCT levels in response to CLP-induced sepsis measured in plasma at different time points. Data are presented as the mean ± SEM. n=10 rats/group; *P<0.05 vs. Sham; #P<0.05 vs. CLP. CLP, cecal ligation and puncture; PCT, procalcitonin; TQ, thymoquinone.

intrusion in the central zones with less vacuolization and steatosis was detected but without signs of edematous tissue (Fig. 6Bc).

Lung tissues from animals in the sham group displayed normal structure and composition, with a well-preserved alveolar space and pulmonary interstitium, and no inflammatory cell infiltration into the alveolar cavity (Fig. 6Ca). Evaluation of lung tissues of the animals in the CLP group showed acute edema, emphysema and pulmonary interstitial hyperemia, resulting in impaired alveolar architecture with penetrations of mononuclear cells causing alveolar congestion and hyperemia in the pulmonary capillaries (Fig. 6Cb). By contrast, tissues from CLP model animals treated with TQ displayed limited inflammatory cell infiltration and restoration of the normal alveolar structure (Fig. 6Cc).

Discussion

Sepsis is a detrimental condition with a high mortality rate in ICUs; therefore, scientists and clinicians have focused on identifying new therapeutic modalities to combat its negative outcomes (32,33). The CLP animal model has been extensively used to study new approaches for sepsis management (34). The constant source of bacteria in this model is the punctured cecum and endotoxin, which is the main constituent of the external membrane of bacterial cell walls, activates several pathophysiological events of gram-negative sepsis (35). The overproduction of reactive oxygen species (ROS) produced under these conditions has been implicated in tissue injury (36). Subsequently, CLP triggers pathological changes in the lung, liver and kidney tissues, signifying varying degrees of organ injury. In the present study, it was demonstrated that treatment of CLP model animals with TQ improved animal survival by up to 80% over a period of 7 days. This coincided with a significant reduction in the expression levels of specific miRNAs, levels of circulating inflammatory cytokines and early-stage sepsis biomarkers, as well as preservation of liver, lung and kidney functions.

Among the circulating miRNAs that have been reported to be upregulated following CLP, miR-16 serves a vital role in sepsis. Notably, deletion of miR-16 in myeloid cells has been shown to significantly decrease *Escherichia coli*-associated mortality in several sepsis models (37). Consistently, miR-16 overexpression can decrease both phagocytosis and production of mitochondrial ROS. Additionally, lack of miR-16 has been reported to enhance secretion of cytokines and chemokines from bone-marrow-derived macrophages at the early stages of infection (38). Furthermore, Gao and Yu (39) identified IκB kinase-β (IKKβ) as a direct target of miR-16, the expression of which was negatively regulated by miR-16 at the mRNA and protein expression levels, indicating that miR-16 may suppress the inflammatory response by inhibiting the IKKβ/NF-κB signaling pathway. Notably, bacterial infection-induced miR-16 upregulation may cause significant organ damage and cell death. Another study demonstrated a positive correlation between circulating miR-16 in serum and death of patients infected with sepsis (23). Therefore, it may be concluded that miR-16 has a crucial role in tissue damage and cell death in sepsis and associated SIRS.

Another miRNA that is highly expressed in different immune cells, including monocytes, macrophages, T and B lymphocytes, and dendritic cells, is miR-21 (25). Numerous studies have shown that different inflammatory stimuli, such as LPS, lipids (prostaglandin E_2 and resolvin A1) and cytokines, can trigger miR-21 expression (25,40,41). miR-21 has also been shown to be upregulated in acute sepsis and sustained in late sepsis in patients and mice (41-43). McClure *et al* (44) demonstrated that administration of miR-21 antagomir to BALB/c mice improved animal survival and decreased the bacterial load following CLP. Determination of the functions of miR-21 have been difficult owing to its multiple mRNA target interactions, as well as its complex regulation in response to extracellular signals (25). Furthermore, miR-21 has been associated with numerous key processes of inflammation, such as detection and response to homeostatic disturbances throughout the body as well as coordinating these responses appropriately, thus serving a dynamic function in inflammatory responses (25,45). Differing from other mediators, the presence of miR-21 is not restricted to pro-inflammatory or immunosuppressive states; it can act as a crucial signal to mediate the homeostasis between both states (25,45).

miR-27a is another miRNA that has been reported to serve an important role in the regulation of inflammatory responses in sepsis (24). Inhibition of miR-27a has been shown to downregulate the expression levels of TNF-α and IL-6 by reducing the phosphorylation levels of NF-κB p65 subunit and through the inhibition of its DNA-binding activity (24). Moreover, miR-27a neutralization has been suggested to upregulate peroxisome proliferator-activated receptor γ, possibly inhibiting the production of monocyte inflammatory

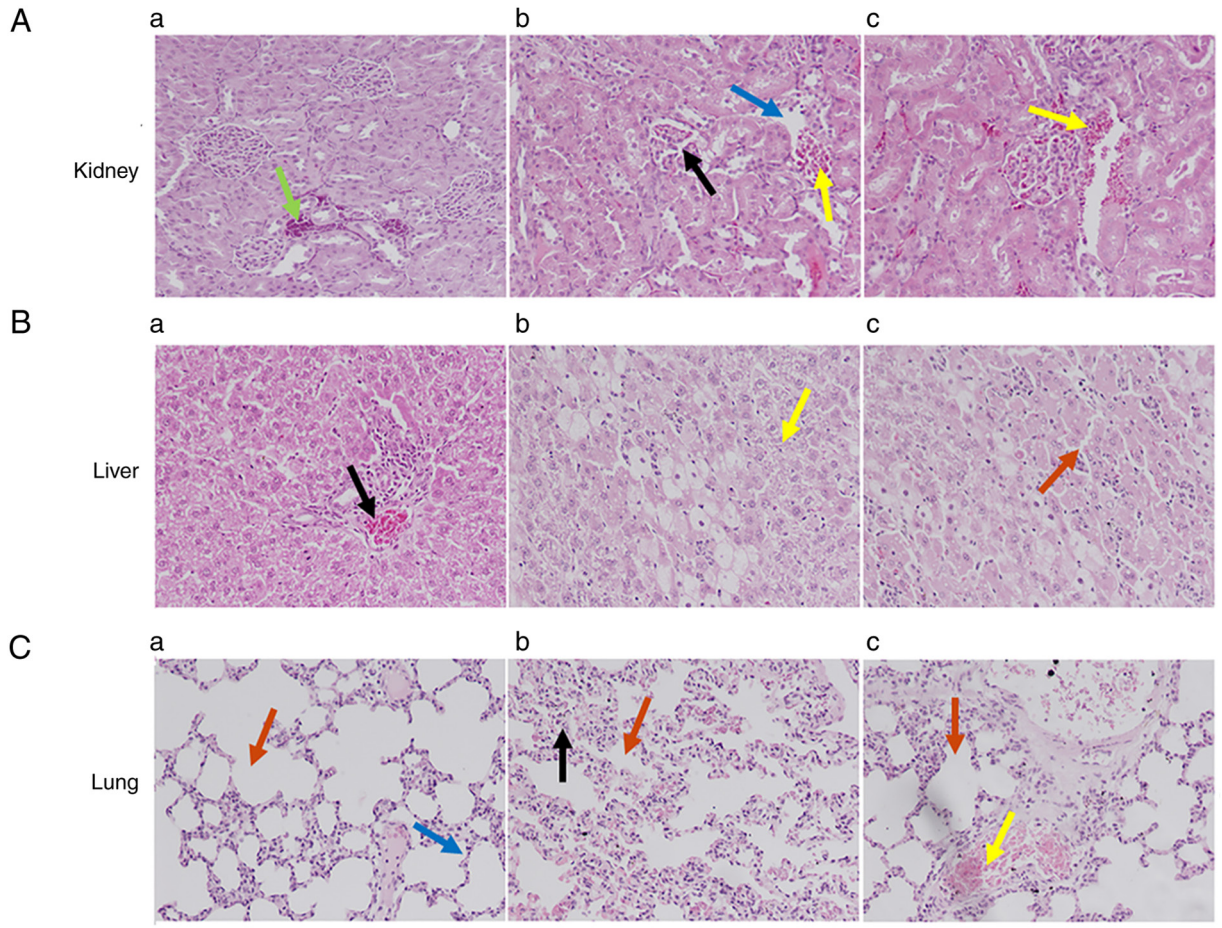

Figure 6. Kidney, liver and lung tissues were stained with hematoxylin and eosin, and images were captured after 36 h of treatment at a magnification of 200X. (A) TQ improved the histopathological alterations in the renal tissues of rats with CLP-induced acute renal injury. (Aa) Normal renal tissue. (Ab) CLP-induced acute renal injury. (Ac) CLP-induced acute renal injury treated with TQ. Green arrows indicate distal convoluted tubules; black arrows indicate glomerulus; yellow arrows indicate infiltration of inflammatory cells; and blue arrows indicate vacuolization of renal tissue. (B) TQ improved the histopathological alterations in the liver tissues of rats with CLP-induced acute liver injury. (Ba) Normal hepatic tissue. (Bb) CLP-induced acute hepatic injury. (Bc) CLP-induced acute hepatic injury treated with TQ. Black arrows indicate central vein; yellow arrows indicate infiltration of inflammatory cells; and orange arrows indicate vacuolization. (C) TQ improved the histopathological alterations in the lung tissues of rats with CLP-induced acute lung injury. (Ca) Normal lung tissue. (Cb) CLP-induced acute lung injury. (Cc) CLP-induced acute lung injury treated with TQ. Orange arrows indicate alveoli; blue arrows indicate alveolar septa; black arrows indicate emphysema and hemorrhagic alterations; and yellow arrows indicate infiltration of inflammatory cells.

cytokines and activating macrophages to boost innate defense against pathogens, thereby downregulating TNF-α expression, relieving inflammation and increasing the survival rate of patients with sepsis (46,47).

A previous study has reported that accumulation of ROS and reduced antioxidant enzyme activities increase oxidative stress and serve important roles in the progression of sepsis, leading to mitochondrial dysfunction and organ failure (48). miR-34a regulates oxidative stress and autophagy through the inhibition of silent information regulator T1 (SIRT1) and autophagy gene 4B signaling. Differential miR-34a expression has also been suggested as a potential prognostic biomarker, in addition to providing insight into the mechanisms of endothelial dysfunction of septic shock. Notably, miR-34a can target and inhibit BCL-2 and SIRT1, which are important negative regulators of endothelium apoptosis and cellular senescence (49,50). Therefore, increased circulating miR-34a levels in response to cytokine stimulation may contribute to septic shock-induced endothelial dysfunction through its effects on apoptosis and senescence.

Collectively, targeting these miRNAs, particularly after the initial phase of infection, may provide a novel therapeutic and/or diagnostic tool for sepsis. The present study demonstrated that CLP-induced experimental sepsis resulted in a time-dependent upregulation in the expression levels of these selected miRNAs, which was subsequently mitigated by treatment with TQ.

Multiple cytokine biomarkers have been identified over the past few decades for the diagnosis and treatment of sepsis, of which TNF-α, IL-1, IL-2, IL-6 and IL-10 are some of the most important mediators of inflammation (51,52). TNF-α serves a role in apoptosis, cell survival, inflammation and immunity (53). IL-1 is a prototypical pro-inflammatory cytokine that aids in the stimulation of local and systemic responses. TNF-α and IL-1 have been demonstrated to augment inflammatory cascades through the stimulation of macrophages, which release pro-inflammatory cytokines, such as IL-6 and IL-8, as well as ROSs, reactive nitrogen species and lipid mediators that are vital in sepsis-induced organ failure (54,55). Results from the present study demonstrated that TQ treatment reduced

TNF-α and IL-1α levels during sepsis, which may explain its protective effects on CLP-induced sepsis. These results are consistent with our previous findings showing beneficial effects of TQ using a slightly different sepsis model (27,56).

IL-2 has varying and sometimes opposing functions during inflammation, contributing to both the initiation and the termination of the immune response (55). The increased plasma levels of IL-2 in response to CLP infection may act as a prognostic marker for septic shock (55). IL-2 is released from T and B lymphocytes, possibly contributing to the pathogenesis of sepsis (57). In the present study, TQ treatment significantly decreased the levels of IL-2 in septic animals. Another important cytokine mediator that enhances the production of acute phase reactants in the liver is IL-6 (58). IL-6 is rapidly produced in response to infections and tissue injuries, contributing to host defense by stimulating the acute phase responses, hematopoiesis and immune reactions. The present study results confirmed that TQ could regulate IL-6 concentration in a CLP model. IL-10 is a key anti-inflammatory cytokine, which is released in response to TNF-α and IL-1α (59-61). Notably, the present study also demonstrated that TQ reduced IL-10 levels at an early time point, which might be a consequence of the initial downregulation of the release of pro-inflammatory cytokines in septic animals.

Precise assessment of sepsis remains a challenge in most ICU settings; therefore, there is a high demand for accurate and early diagnostic biomarkers (62). An early diagnostic biomarker for sepsis must have a rapid turnaround time and be widely available for effective therapeutic potential. CRP, a well-known marker of inflammation, has been suggested to bind the phospholipid components of microorganisms, thereby enabling eradication by macrophages (63,64). During systemic inflammation of a microbial origin, the levels of CRP are increased, as observed in the present study. TQ treatment was able to decrease CRP levels in the CLP model rats, thus indicating its ability to ameliorate the levels of early phase reactant proteins.

VEGF was originally considered to be only a potent stimulator of endothelial permeability, but has since been reported to enhance proliferation and survival of endothelial cells (65,66). Previous studies have shown that a number of features of VEGF make it a strong candidate to control inflammation (66-68). An association between higher circulating levels of VEGF and severe human septic shock has also been reported (69,70). Notably, sepsis or septic shock is associated with a time-dependent surge in the circulating levels of VEGF (71,72). The present findings revealed that the circulating levels of VEGF were significantly increased following CLP, whereas TQ administration substantially decreased these levels. These findings suggested that TQ may also exert its effect on sepsis, at least partially, by controlling the production of VEGF which are in support of our previous finding using a different sepsis model (27,56).

Endotoxins, such as LPS, induce endothelial cell contraction and the development of intercellular gaps, thus increasing permeability of blood vessels (73,74). In addition, it has been demonstrated that ESM-1 can cause vascular responses in *in vivo* models of inflammation, as well as increase barrier permeability and the passage of leukocytes by upregulating cytokines during sepsis (75). ESM-1 release has been shown to be neutralized by ESM-1 antibodies in a CLP-induced mouse model of sepsis (75). The treatment of rats with TQ in the present study markedly reduced ESM-1 release, providing consistent evidence of its promising effect for the management of sepsis and septic shock (56).

PCT is a peptide precursor of calcitonin hormone that has been identified as a biomarker of bacterial infection (76,77). Moreover, it can be used in evaluating a suitable treatment response, determining severity of sepsis, and estimating morbidity and mortality rates (77-82). Similarly, increased D-dimer levels are another sign of systemic thrombosis, which has been identified as an effective predictor of mortality in severe sepsis (83-86). In the present study, CLP increased PCT and D-dimer levels in a time-dependent manner. Treatment with TQ reduced the levels of both markers, particularly at the 12-h time point, and improved the outcome of sepsis, including animal survival.

From a histopathological perspective, liver, kidney and lung tissue samples of the sham group had normal features, whereas rats subjected to CLP exhibited substantial histopathological alterations. For example, liver sections of the CLP group displayed penetration of inflammatory cells along with necrotic damage; this may be due to increased ROS and lipid peroxidation (12,87). TQ has been shown to attenuate ROS and lipid peroxidation, as well as organ injury caused by various agents (27,88). In the present study, TQ treatment improved both morphological and histological features of the tissue. TQ significantly mitigated kidney and lung injuries in CLP model rats. In particular, renal tissue of septic rats exhibited degeneration in the renal cortex and medullary tubules, which was ameliorated by TQ. Similarly, lung tissue exhibited damage to the alveolar structure with infiltration of mononuclear cells; these pathological changes were restored to normal alveolar architecture with TQ treatment.

In conclusion, the present study demonstrated the effects of TQ in sepsis management by reducing morbidity and mortality in a CLP model. These potential effects are thought to be due to immunomodulation and control of inflammatory status, including effects on the expression of relevant miRNAs, under septic conditions.

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Availability of data and materials

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

Authors' contributions

KMA and BLJ wrote the manuscript, and KMA designed the experiments. AA and MR carried out animal experiments and

analyzed the data. MUR and BLJ carried out the microRNA study. KMA, AA and BLJ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the College of Pharmacy, King Saud University (Riyadh, Saudi Arabia; approval no. KSU-SE-19-17).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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