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Quercetin exerts cardiovascular protective effects in LPS-induced dysfunction in vivo by regulating inflammatory cytokine expression, NF-κB phosphorylation, and caspase activity

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Abstract

Impaired myocardial contractile function, one of the well-documented features of sepsis, contributes greatly to the high rate of mortality. Quercetin is widely accepted as a potential antioxidant and free radical scavenger. Epidemiologic studies have suggested that an increase in the intake of dietary Quercetin can reduce the risk of cardiac disease. However, presently there is no report yet on the influence of Quercetin on LPS-induced myocardial dysfunction in vivo. Cardiovascular protective effects of Quercetin on LPS-induced sepsis in mice were measured after intragastric administration, using normal saline as a positive control. Quercetin pretreatment significantly alleviated LPS-induced cardiac abnormalities in mice. The histopathologic findings in the present study justify the findings reported from the biochemical analyses. Our observation from the present research work reveals that Quercetin suppressed the production of proinflammatory cytokines at different levels, such as TNF-α and IL-1β, and inhibits the activation of I-κB phosphorylation, whereas the total content was not affected. Apoptotic pathways are related to Quercetin protection in the development of myocardial dysfunction. In conclusion, our findings demonstrate the adjuvant potentials of Quercetin for clinical sepsis treatment.

Keywords Myocardial dysfunction · LPS · Quercetin · Nuclear factor I-κBα · Apoptotic pathway

Abbreviations

LPS	Lipopolysaccharide
EF	Ejection fraction
NF-κB	Nuclear factor-κB
FS	Fractional shortening
BCA	Bicinchoninic acid
OD	Optical density
TNF-α	Tumor necrosis factor-α
IL-1β	Interleukin-beta

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Introduction

Sepsis, a systemic inflammatory response syndrome to infection, is initiated by pathogen-associated molecular patterns, such as LPS from gram-negative bacteria [1, 2], and is defined as a life-threatening organ dysfunction. It is hypothesized that the hypoinflammatory response may function as a homeostatic protective mechanism. However, like the disorganized, destructive hyperinflammatory response, the hypoinflammatory response may also be excessively severe and harmful and prolonged hypoinflammatory responses have been associated with an increased risk of death due to unresolved organ failure [3–5]. Indeed, there are approximately 750,000 cases of severe sepsis each year in the United States alone, resulting in over 200,000 deaths [6]. Myocardial dysfunction is a common complication of severe sepsis in patients and 40% of patients with sepsis develop cardiac dysfunction characterized by biventricular dilatation, decreased ejection fraction (EF), and

reduced response to fluid administration [7, 8]. It is reported that cardiovascular function was closely related to the mortality of patients with septic shock, while myocardial dysfunction occurs in approximately half of the patients with septic shock [9, 10]. Therefore, it is essential to take effective measures to prevent cardiac vascular disorder during sepsis.

Quercetin (3,5,7,30,40-pentahydroxyflavone, a bioflavonoid) is present in a variety of consumable foods including apples, berries, onions, tea, and Brassica vegetables. Quercetin belongs to the flavonoid family and has phenolic groups in structure which confer antioxidant capacity [11, 12]. Therefore, Quercetin, a typical dietary flavonoid, is a potential antioxidant and free radical scavenger. Studies have confirmed that Quercetin also produces many other beneficial effects, including antiviral and anti-inflammatory effects and neuroprotective activities [13–15]. Moreover, epidemiologic studies have suggested that an increase in the intake of dietary Quercetin can reduce the risk of cardiac diseases, some tumors, neurodegenerative diseases, and aging.

Several studies indicated that Quercetin plays a protective role for the myocardium from injury, not only when given before ischemia (preconditioning) [16, 17] but also when administered during reperfusion (postconditioning) in an isolated rat heart model [18]. However, no data about the protection of Quercetin against LPS-induced cardiac dysfunction in vivo emerged. Moreover, the molecular mechanism underlying Quercetin-mediated cardioprotection is unknown.

However, some research works reported that deregulated immune response induced by LPS is characterized by the overproduction of tumor necrosis factor-α (TNF-α), interleukin-1 beta (IL-1β), and nitric oxide (NO) [19, 20]. TNF-α, IL-1β, and NO accumulation in myocardial cells are known to be a crucial step in the development of septic heart depression. Some previous studies also suggested that LPS-induced cardiac dysfunction is mediated by myocardial apoptosis as well as nuclear factor-κB (NF-κB)-modulated overproduction of multiple proinflammatory mediators, which are TNF-α, IL-1β, and NO [8, 21, 22].

In the light of these data, we hypothesized that Quercetin might have a protective effect on LPS-induced myocardial dysfunction in vivo and apoptotic pathways are closely associated with cardioprotection. Thus, the purpose of this study was to determine whether treatment with Quercetin improves myocardial dysfunction and dysmorphia and the alteration of caspase 3/7 activity in a mouse model of endotoxemia.

Materials and methods

Ethics statement

All the animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), approved by the Institutional Animal Care and Use Committee of Zoucheng People's Hospital and the Institutional Animal Care and Use Committee of Qingdao University, and the Guide for the Care and Use of Laboratory Animals according to the regulation in the People's Republic of China. All animals were provided by Shanghai Experimental Animal Center, People's Republic of China. All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Animal housing and procedure

In this study, healthy adult male BALB/c mice (6–8 weeks old) weighing between 22 and 25 g were obtained from an experimental animal center. All animals had access to standard chow and water and housed under standard laboratory conditions with a 12-h light–dark cycle at room temperature (24 ± 2 °C) during the experimental period. Then the mice were randomly divided into four groups of five animals each. The negative control group received LPS and vehicle (0.2 mL/10 g body weight), the positive control group received standard chow and water, test group-3 received LPS only, and test group-4 received LPS and Quercetin.

Experimental design

Distilled water (0.1 mL/10 g body weight) and Quercetin (50 mg/kg; Sigma; St Louis, MO) were administered intragastrically once a day for 3 consecutive days. One hour after intragastric treatment on the third day, normal saline (0.2 mL/10 g body weight) or LPS (*Escherichia coli*, 055: B5; Sigma Chemical Co; 20 mg/kg, 0.2 mL/10 g body weight) was injected intraperitoneally. Animals were treated in compliance with the Guiding Principles in the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Echocardiographic examinations

Cardiac functions were tested immediately 12 h after LPS administration. M-mode and Doppler echocardiography with a 30-MHz-center frequency RMV 707 scan head were performed in accordance with the previously described methods [23, 24]. Two-dimensional guided M-mode tracings at the papillary muscle level were used to digitally measure left ventricular EF and fractional shortening (FS). E/A ratio, one of the important indicators of the assessment of left ventricular diastolic function, was also calculated. Septic animals took a hunched position, with piloerection, a bloated abdomen, and reduced interest in their surroundings. Customized needle electrodes were inserted subcutaneously in the limbs,

and standard lead II ECGs were recorded with LabChart software 7.0 using a sampling rate of 1 kHz.

Enzyme-linked immunosorbent assay

After the injection of LPS or normal saline for 1 or 4 h, the mice were sacrificed by cervical dislocation. Hearts were dissected, weighed, and homogenized thoroughly on ice in a lysis buffer. The homogenates were centrifuged at 3500 rpm using a cool centrifuge. The supernatants were collected and stored at − 80 °C for biochemical analysis. The levels of TNF-α and IL-1β protein were determined using a bicinchoninic acid (BCA) protein assay kit according to the manufacturer's protocol (Enzo Life Science, Switzerland).

Determination of nitric oxide by Griess assay

NO production was also determined by the NO oxidation products present in the supernatants (nitrate and nitrite) reacting with Griess reagent. Nitric oxide was measured by the colorimetric assay based on the Griess reaction. Briefly, Griess reagent [1% sulfanilamide, 0.1% naphthylethylenodiamine in 5% phosphoric acid (HCL)] was added to an equal volume of the supernatants; in the presence of nitrite, this reagent forms a violet color, and the absorbance at 546 nm was measured after 10 min. The NO_2^- concentration was determined with reference to standard curve of sodium nitrite.

Western immunoblotting

The method of heart collection was the same as previously described, which were later homogenized thoroughly on ice in a cold split buffer RIPA, of which one mixture includes phosphatase inhibitor. The homogenates were centrifuged at 12,000×*g* and 4 °C for 30 min and the supernatants were immediately stored in the freezer at − 80 °C after concentration determination using the BCA protein assay kit and fair distribution into fresh tubes. Samples were added to the same level with the filler of loading buffer and boiled for 5 min. Proteins (30 µg) from each sample were loaded onto 4–20% polyacrylamide gels and separated by electrophoresis. Separated proteins were then transferred to polyvinylidene difluoride membranes with a Bio-Rad electrotransfer apparatus. After blocking, the membranes were exposed to the primary antibodies overnight at 4 °C. The primary antibodies used were anti-I-κBα (1:1000; Cell Signaling Company), anti-p-I-κBα (1:1000; Cell Signaling Company), and anti-β-actin (1:3000; Abcam Company). After washing three times with TBS-T, the membranes were incubated in a dilution of secondary monoclonal antibody for 1 h at room temperature and ECL western blotting Kit. Reactive bands were visualized on an X-ray film between the developing time of 20 and 60 s. However, β-actin was used as the control. The optical density of each protein band was determined using the Gel-Pro analyzer 4.0 software.

Immunohistochemistry

Cardiac left ventricular myocardium was rapidly harvested and washed with ice-cold normal saline to be fixed with 4% formaldehyde solution in phosphate-buffered saline and later embedded in paraffin. Then they were sliced into 5-mm-thick sections with the use of a microtome. After dewaxing and rehydration, the pretreatment of sections with 3% H_2O_2 at 37 °C for 10 min was performed to block endogenous peroxidase activity. After antigen retrieval using 10 mM sodium citrate buffer (pH 6) for 10 min in the microwave oven, the sections were blocked with 5% bovine serum albumin for 2 h at room temperature and incubated with primary antibody overnight 4 °C, processing for horseradish peroxidase labeling secondary antibody. The above sections were also stained with hematoxylin and eosin for microscopic evaluation. After dehydration and coated with the resinous medium, the sections were covered with coverslips. The quantification of cells in the stained areas was analyzed by a blinded observer.

Statistical analysis

All data are shown as mean ± SEM. Differences between groups were determined using analysis of variance and an unpaired Student's *t* test; $P < 0.05$ was considered statistically significant.

Results

Ameliorative effect of intragastric administration of Quercetin on LPS-induced myocardial dysfunctions

The functions of the left ventricles were observed by echocardiography under light isoflurane anesthesia using a 16-bit Power Lab system (Fig. 1). Our observation from this present research work reveals a statistically significant difference ($P < 0.05$) within the experimental groups. Echocardiography data also showed that LPS administration induced significant dysfunction of the left ventricular contractile with decreases in EF and FS in comparison with the negative control group-1 that received the vehicle only (Fig. 2). Pretreatment with Quercetin significantly attenuated changes induced by LPS on left ventricular EF and FS (Fig. 2a, b). This implies that Quercetin can ameliorate LPS-induced myocardial systolic dysfunction. E/A ratio, another important indicator used in the assessment of the left

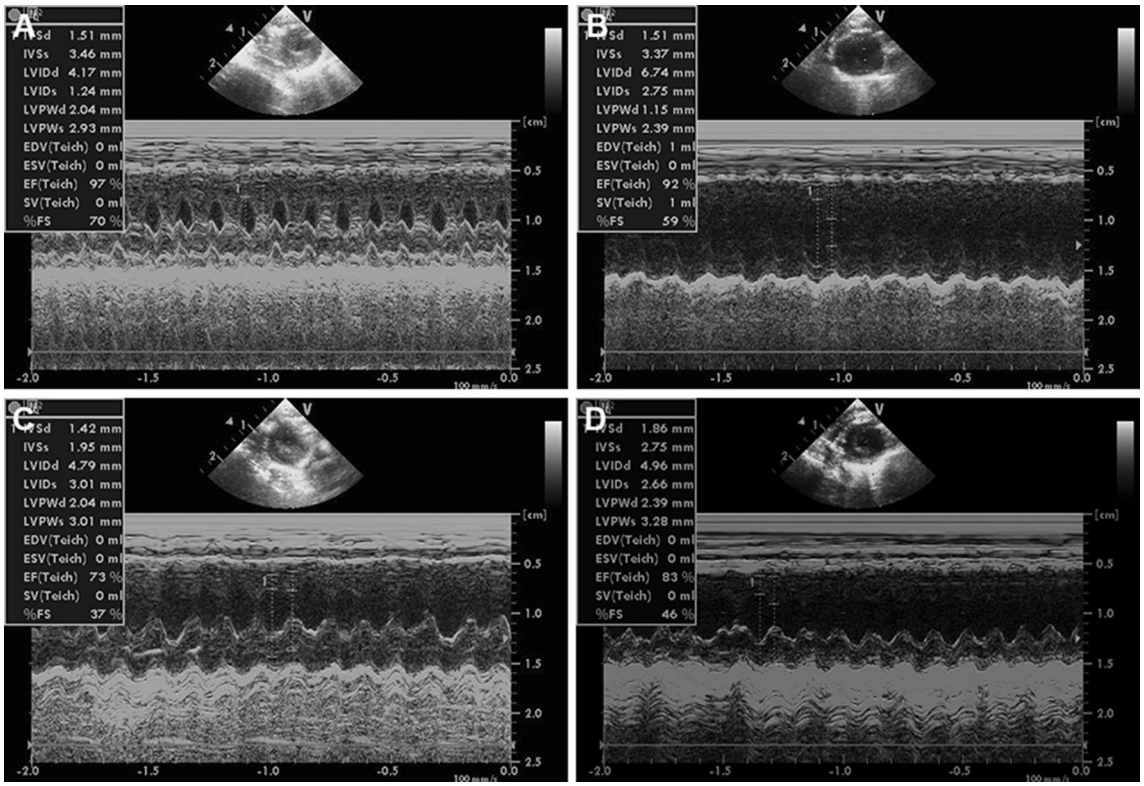

Fig. 1 M-mode echocardiography measurements obtained from mice at 12 h after administration of normal saline or LPS. **a–d** respectively represent the control, LPS, Quercetin + LPS, and vehicle + LPS groups

ventricular diastolic function, is calculated from Doppler-derived mitral inflow measurements. This scientific method has been reported to be highly sensitive, accurate, and reliable. Our observation from this research work indicates that LPS administration significantly lowered E/A ratio (Fig. 2c). Quercetin pretreatment exhibited a significant increase in E/A ratio in comparison with test group-3 treated with LPS alone. However, there was no significant difference in E/A ratios between the LPS and LPS+ vehicle groups.

Effects of Quercetin on morphological changes of cardiac left ventricular myocardium in mice challenged by LPS

Previous experimental studies have shown an association between sepsis and structural myocardial changes [23–27]. An abnormal distribution of blood flow among circulatory beds and increases of cardiac afterload lead to extra stress on the myocardium. Finally, it results in decreasing left ventricular ejection fraction (LVEF), ventricular dilation, increasing cardiac output, and descending systemic vascular resistance [27]. In this study, we also observed the improvement of cardiac dysfunction caused by Quercetin. Therefore, it is necessary to perform histological evaluation of the myocardium.

The histopathologic findings in the present study justify the data obtained from the biochemical analysis. In the control groups, the structure of cardiac muscle fibers was normal with clear striation and no inflammatory cell infiltration found in heart tissue (Fig. 3a). However, the photomicrographic sections of test group-3 treated with LPS alone showed that the interspace among cardiac muscle fibers broadens and also showed infiltration of inflammatory cells (Fig. 3b). A distortion of myocardial cell congestion, edema, and cell lysis were observed. Nevertheless, all these typical histological lesions of cardiomyocyte were attenuated by Quercetin pretreatment as shown in the cardiomyocyte photomicrography (Fig. 3c). The results reveal that the heart tissues in the Quercetin group had better architecture and morphology than those with both positive and negative control groups (Fig. 3c).

Quercetin pretreatment induces different changes in TNF-α, IL-1β, and NO levels in myocardial tissue

We further investigated the effects of Quercetin on the production of these inflammatory mediators. The concentrations of IL-1β, TNF-α, and NO levels in the heart tissues were measured at 1 and 4 h after administration of LPS

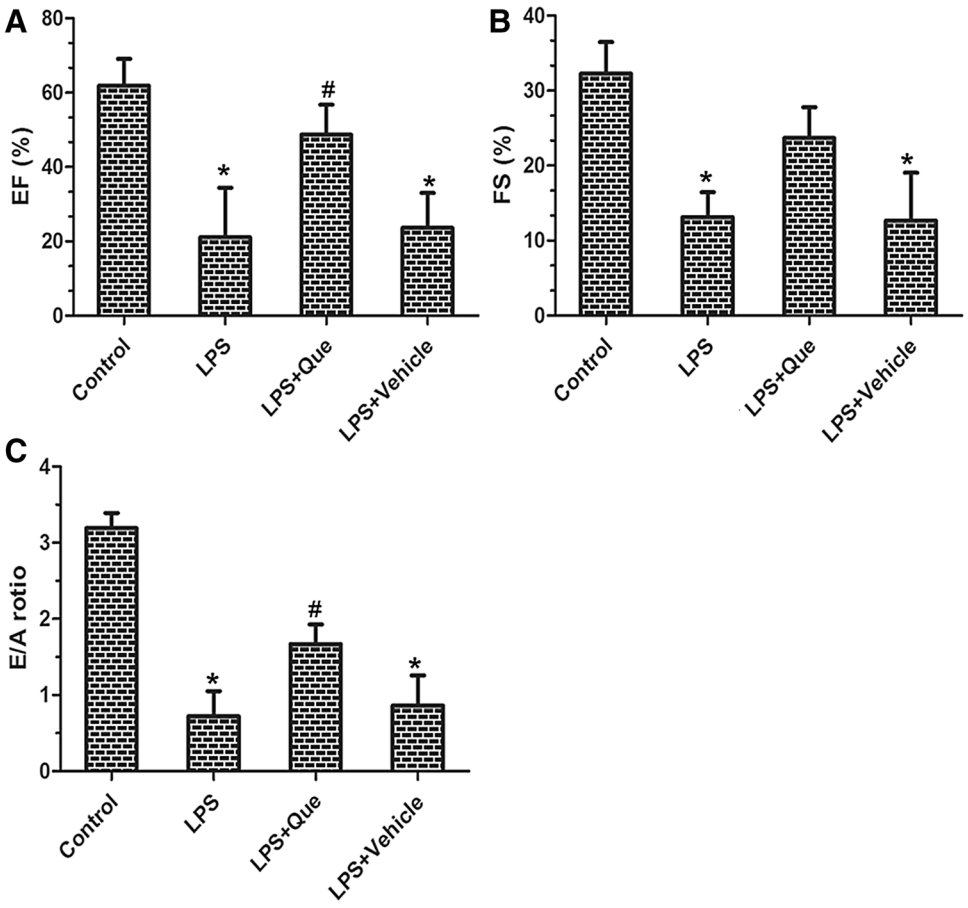

Fig. 2 Cardiac function parameters including the left ventricular EF (**a**), FS (**b**), and E/A ratio (**c**) in mice at 12 h after intraperitoneal injection with normal saline or LPS (20 mg/kg) in different groups. $n = 12$. $^{*}P < 0.05$ and $^{\#}P < 0.05$ compared with the control and LPS groups, respectively

(Fig. 4). Immediately 1 h after LPS administration, the concentrations of both IL-1β and TNF-α in test group-4 pretreated with Quercetin were statistically significantly lowered when compared with test group-3 treated with LPS alone (Fig. 4). However, 4 h later, the concentration of the induced IL-1β increased four times as much as that observed at 1 h in all the groups (Fig. 4). The concentration of IL-1β at the 4th hour after LPS administration was significantly lower in test group-4 pretreated with Quercetin in comparison with the test group-3 treated with LPS alone (Fig. 4). Our observation implies that Quercetin inhibited LPS-induced IL-1β overproduction in the heart. The result further indicates that TNF-α, the most important mediator of sepsis-induced myocardial dysfunction, significantly decreased slowly at the 4th hour after LPS administration. The results indicate that the test group-4 pretreated with Quercetin accelerated this tendency. There was no significant difference between the concentrations of myocardial TNF-α and IL-1β in groups treated with LPS + Vehicle and LPS alone. Besides, our study witnessed a rapid LPS-induced increase in the level of NO at 4 h after LPS administration, which was significantly inhibited by Quercetin pretreatment.

Quercetin inhibits the activation of phosphorylation of I-κBα mediated by LPS

TNF-α and IL-1β are in response to signaling by receptors of the Toll-like receptor (TLR) family which can activate NF-κB through I-κB phosphorylation mediated by multiple signal transduction cascades. I-κBα phosphorylation represents a key event for the initiation of TNF-α and IL-1β gene transcription, and cardiac I-κBα overexpression not only inhibits TNF-α production but also prevents myocardial dysfunction after LPS challenge [19]. It was essential for us to decide whether Quercetin affects the phosphorylation of I-κBα in LPS-administered mice. The total protein concentrations in the heart were respectively measured for each group. The levels/concentrations of total I-κBα and phosphor-I-κBα were measured by Western blotting after a quantitative analysis (Fig. 5A, B). Observation from this research work indicates that there was an increased phosphorylation of I-κBα in cardiac tissue after LPS treatment. However, Quercetin significantly inhibited LPS-provoked phosphorylation of I-κBα without any significant change in the content of total I-κBα in the mouse heart.

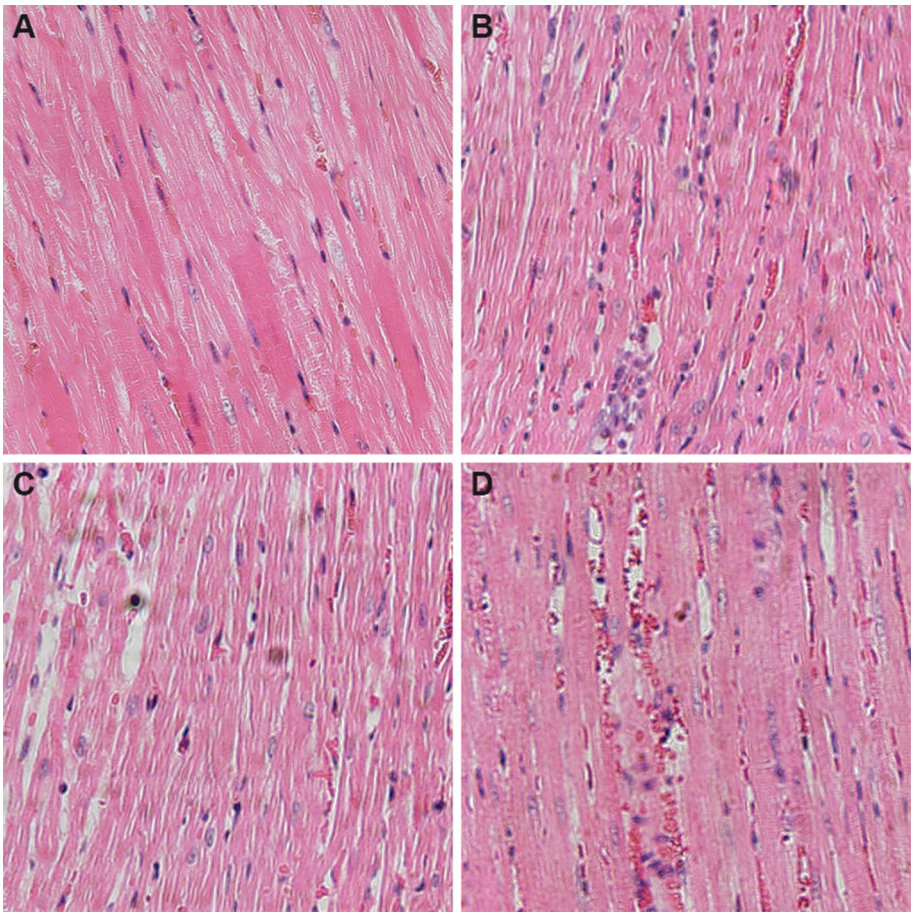

Fig. 3 Morphological effect of Quercetin pretreatment on LPS-induced cardiac muscular tissue determined by hematoxylin–eosin staining. **a–d** respectively represent the control (**a**), LPS-treated mice (**b**), Quercetin + LPS (**c**), and vehicle + LPS (**d**) groups

Apoptosis of cardiomyocyte and cardiac caspase 3/7 activity associated with Quercetin protection in myocardial cells

In accordance with other studies, Quercetin has been proved to provide cardioprotection against damage from sepsis. However, the mechanisms behind the protective properties of Quercetin in cardiac cells are poorly understood. Oxidative stress and inflammation are widely known to activate additional biochemical cascades leading to cell death. Because it has been demonstrated that LPS-induced apoptosis may directly induce myocardial dysfunction [28], we further investigated whether the effects of Quercetin on the myocardium are involved in apoptosis or not. The method of TUNEL staining which detects apoptosis was used to display/detect apoptotic cardiomyocytes in our study. TUNEL-positive cells in the control mice were negligible. However, a marked appearance of apoptotic cardiomyocytes was observed in the test group-3 LPS-treated mice (Fig. 6a–d). In contrast, the number of TUNEL-positive cells was significantly reduced in the Quercetin-treated group compared with the saline group. Caspase-3/7 levels in the heart were low in the control group and increased significantly in the LPS-treated group at 2 h after LPS administration. No significant difference was found between test group-3 (LPS-treated group) and negative group-1 (vehicle group). However, there were visible declines in Quercetin-treated test group-4 which were statistically significant compared with test group-3 (LPS-treated group).

Discussion

The present study showed that the functional and morphological outcomes were improved by Quercetin and thus provide further evidence that this flavonoid is a potential therapeutic pretreatment for clinical sepsis patients.

Myocardial dysfunction is recognized as an important factor contributing to the high mortality of septic patients [29]. Nonspecific myocardial changes in function were also observed in the present study. Our observation indicates that Quercetin supplement in vivo significantly reversed the LPS-induced depression in the left ventricle, including EF, FS, and E/A ratio in mice. These findings suggest that Quercetin exerts its positive effects on endotoxin-induced sepsis, especially heart dysfunction. Although we observed that cardiac function was depressed, it remains controversial whether the myocardial contractility dysfunction is secondary to

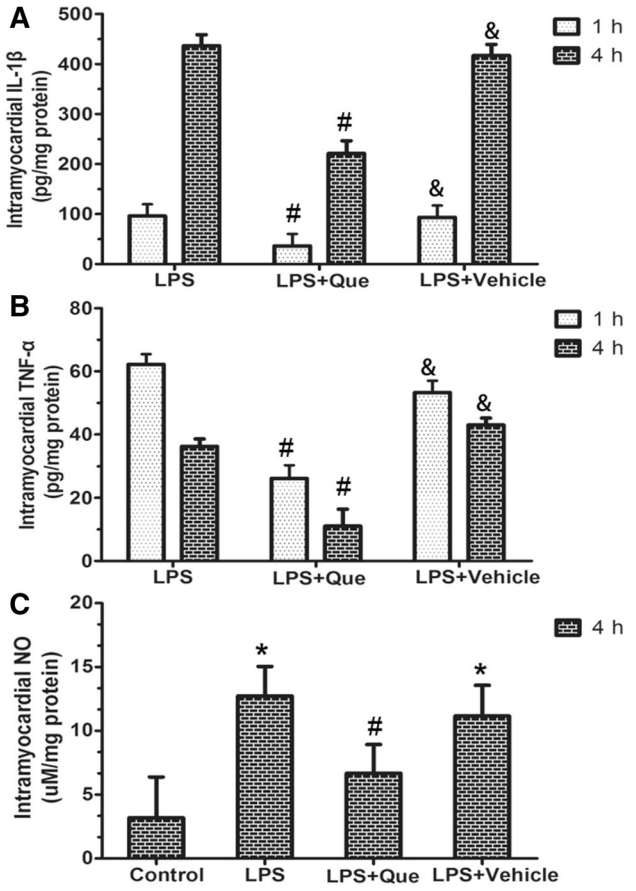

Fig. 4 Effect of Quercetin supplementation on the production of TNF-α, IL-1β, and NO spontaneously provoked by LPS in the mouse heart supernatants. The contents of TNF-α and IL-1β were observed separately at 1 and 4 h after LPS administration, and NO formation was just examined at 4 h after normal saline or LPS challenge. $n=12$. $^{*}P<0.05$ and $^{\#}P<0.05$ compared with the control and LPS groups, respectively

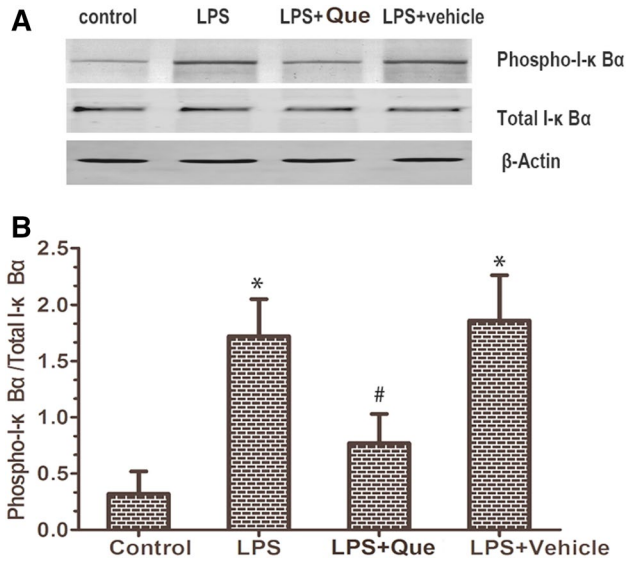

Fig. 5 Effect of Quercetin supplementation on the cardiac activation of I-κBα phosphorylation intermediated by LPS. From the upper row, representative immunoblot indicates phosphorylated and total I-κBα protein levels in the mouse heart. β-actin was used as a loading control

structural changes or not. All cardiomyocytes were regularly stained showing distinct cross-striations. In contrast, the septic tissue with greater infiltration of inflammatory cells showed irregularly disorganized cross-striations within the cardiomyocytes, while relatively regular cross-striations throughout the cardiac muscle cells were observed in the Quercetin group. Quercetin prevents cardiomyocytes from morphological changes and inflammatory cell infiltration. The agreement between the functional and morphologic changes justifies the cardioprotection of Quercetin from LPS-provoked damage. Morphologic changes are closely related to cardiac dysfunction.

Several studies indicated that Quercetin, when given before ischemia (preconditioning), protects the myocardium from I/R injury through its antioxidant and anti-inflammatory activities [16, 17], and Wu et al. also reported a protective role for Quercetin postconditioning against myocardial I/R injury in rats via activating the PI3K/Akt pathway [30].

Although the mechanism of Quercetin cardioprotection remains controversial, increasing evidence supports a critical role for the production of proinflammatory cytokines, such as TNF-α and IL-1β, in the development of myocardial dysfunction in endotoxemia [8, 21]. Several NF-κB-dependent genes including the cytokines TNF-α and IL-1β have been implicated in the pathogenesis of sepsis and associated with cardiac dysfunction in sepsis [31]. Phosphorylation of NF-κB entry into the nucleus and gene transcription and protein synthesis alters iNOS directly involving the production of NO. The equilibrium between IκB and IκB phosphorylation was possibly associated with the dysregulation. Therefore, we examined the effects of Quercetin on IκB phosphorylation at 30 min, TNF-α and IL-1β concentrations at 1 and 4 h, and NO production at 4 h after LPS challenge in the present study. Our results indicate that LPS-induced myocardial activation of IκB phosphorylation at 30 min significantly increases myocardial TNF-α and IL-1β proteins at 1–4 h after challenge [32]. Pretreatment with Quercetin in vivo can inhibit I-κB phosphorylation and reduce the generation of TNF-α, IL-1β, and NO and consequently improve myocardial function in mice after LPS challenge. NF-κB phosphorylation occurs in response to several signaling factors, including Akt, PKA, TNF-α, IKK, and MAPKs [33], which are also important clues for further study. In line with previous studies, endotoxin induces increases in caspase activity. Cultured myocytes isolated from endotoxin-treated rat hearts had increased effector caspase activity and caspases in ventricular myocytes are directly responsible for

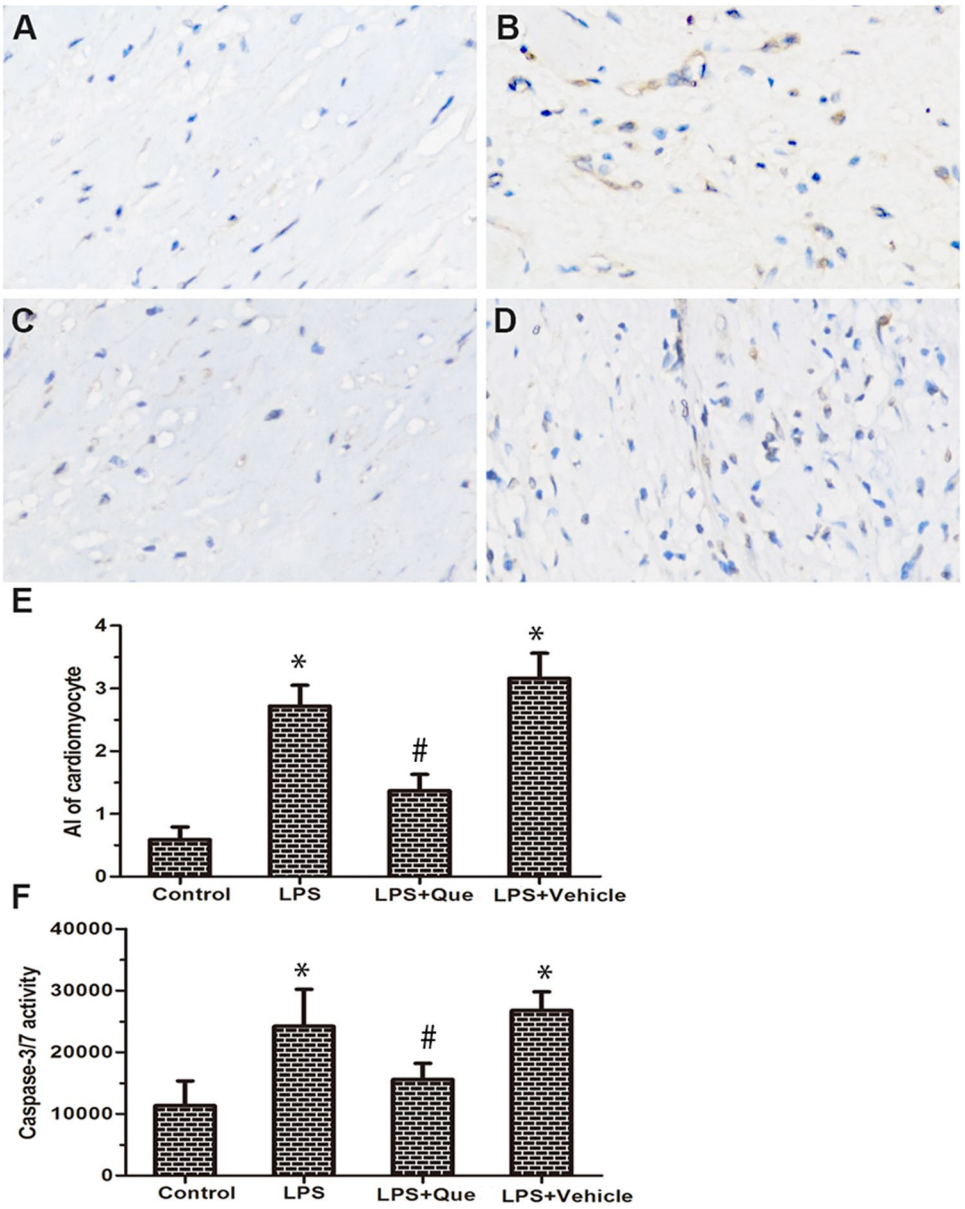

Fig. 6 Inhibition of Quercetin to LPS-induced cardiomyocyte apoptosis and cardiac caspase 3/7 activation. Representative immunohistochemical results of TUNEL-stained sections from the control (**a**), LPS-treated mice (**b**), Quercetin + LPS (**c**), and vehicle + LPS (**d**) groups. Quantification of TUNEL-positive cardiomyocytes (**e**) and cardiac caspase 3/7 activity detection (**f**) at 12 h after normal saline or LPS injection. $n = 8–10$. $^{*}P < 0.05$ and $^{\#}P < 0.05$ compared with the control and LPS groups, respectively

endotoxin-induced cardiac dysfunction [28]. Besides, some scientists have revealed that Quercetin attenuates cell apoptosis in focal cerebral ischemic rat brain via activation of the BDNF–TrkB–PI3K/Akt signaling pathway [34]. It was reasonable for us to decide whether Quercetin cardioprotection was involved in activation of apoptotic pathways. We further assessed the change of caspase 3/7 activity and found that pretreatment with Quercetin remarkably inhibited cardiomyocyte caspase 3/7 activation, which are likely to mediate Quercetin cardioprotection. Further understanding of the molecular mechanism of Quercetin inhibition of LPS-induced cardiomyocyte apoptosis will help us in designing Quercetin-based drugs.

Pretreatment with Quercetin can help improve the health state of the heart, both morphologically and functionally. In conclusion, this is the first identification of Quercetin's protective effects on the heart in LPS-induced sepsis by inhibiting myocardial apoptosis and declining I-κB phosphorylation level in the heart. These findings suggest that Quercetin treatment may be a potential and available agent for coping with septic cardiac dysfunction.

Author Contributions JY and CL designed the study. XW, XL, YY, and CL performed the experiments and collected the data. CL and JY analyzed and interpreted the experimental data. XW and JY prepared the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

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