Shenfu injection attenuates lipopolysaccharide-induced myocardial inflammation and apoptosis in rats

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[ABSTRACT] Shenfu injection (SFI), a Chinese medicinal product, shows potent efficacy in treating sepsis. The aim of the present study was to clarify the protective effects of SFI against lipopolysaccharide (LPS)-induced myocardial inflammation and apoptosis. Experiments were carried out in Sprague-Dawley (SD) rats treated with LPS or LPS + SFI, and in H9C2 cardiomyocytes. The sepsis-associated myocardial inflammation and apoptosis was induced by the intraperitoneal injection of LPS (20 mg·kg–1). SFI attenuated the increased expression of tumor necrosis factor (TNF)-α and interleukin (IL)-1β induced by LPS both in serum and heart. In LPS group, cell viability was reduced, and reversed after SFI administration. LPS treatment increased the expression levels of cleaved-caspase 3 and Bax, and those of Bcl2 and Bcl2/Bax. These two trends were reversed by SFI administration. The expression levels of phosphorylated mitogen-activated protein kinase kinase (p-MEK) and phosphorylated extracellular regulated protein kinases (p-ERK) were increased by LPS, and reversed by SFI. MEK inhibitor U0126 attenuated the apoptosis induced by LPS. These results indicate that SFI could treat LPS-induced cardiac dysfunction. In conclusion, SFI attenuates the inflammation and apoptosis induced by LPS via down-regulating the MEK and ERK signaling pathways.

[KEY WORDS] Shenfu injection; Lipopolysaccharide; Inflammation; Apoptosis; Mitogen-activated protein kinase kinase; Extracellular regulated protein kinases

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Introduction

Sepsis is the main cause of death in non-cardiac intensive care unit[1–3]. Sepsis induced myocardial dysfunction (SIMD) is one of the major predictors of mortality of sepsis. It is present in more than 40% of cases of sepsis and its appearance can increase the mortality rate up to 70%[3–4]. Nearly 30 years of research on SIMD have not been sufficient to elucidate its pathophysiology and many of treatment strategies are still in experimental phase[6]. The administration of lipopolysaccharide (LPS) to laboratory animals has been widely used to study the mechanisms of septic cardiomyopathy[15–17]. Inflammatory cytokines can directly or indirectly cause cardiac dysfunction in sepsis[5–8]. LPS markedly produces inflammatory cytokines in both the serum and cardiac tissues of mice, which in turn impairs the mice' hearts[7]. Cardiac apoptosis is a major process involved in sepsis induced myocardial dysfunction. LPS promotes apoptosis and the activation of caspase-8, 9 and 3[9].

Shenfu injection (SFI) is prepared from red ginseng (steamed roots of Panax ginseng) and aconite (processed lateral roots of Aconitum carmichaeli) by using countercurrent extraction and macroporous resin adsorption chromatography. Shenfu decoction is a classic formula for replenishing Qi and warming Yang in traditional Chinese medicine and SFI is widely used clinically to treat various shocks and improve cardiac function for more than two decades[9–10]. Shenfu formula can increase the left ventricular ejection fraction, improve the hemodynamic index of heart failure rats, decrease the positive rate of myocardial cells detected by the TUNEL, and suppress the expression of caspase 3[11]. Mi-
togen-activated protein kinases signaling pathway is involved in the apoptosis in the heart, LPS increases the level of phosphorylated extracellular regulated protein kinases (p-ERK) in the heart [50]. The present study was designed to determine how SFI regulates the myocardial inflammation and apoptosis in LPS-challenged septic rats and reveal the signaling pathways involved in these processes.

Materials and Methods

Animals and LPS-treatment

Experiments were conducted in male Sprague Dawley (SD) rats (weighing 250–300 g) at Nanjing University Model Animal Research Center. The rats were housed in a temperature-controlled room on a 12–12 h light-dark cycle with free access to standard chow and tap water. Sepsis-associated inflammation and apoptosis were induced by intraperitoneal injection of LPS (20 mg·kg⁻¹, Sigma, MO, USA). SFI (10 mL·kg⁻¹, Yaan Sanjiu Pharmaceutical Co., Ltd., Sichuan, China) or saline (10 mL·kg⁻¹) was intraperitoneally injected 6 h after LPS treatment. Samples were harvested 12 h after LPS administration.

Culture of H9C2 cells

The cell line of rat myoblast (H9C2) was cultured in DMEM supplemented with 10% fetal bovine serum (Nanjing Bio-Channel Biotech Co., Ltd.), 100 U·mL⁻¹ penicillin, and 100 μg·mL⁻¹ streptomycin for 48 h at 37 °C in humidified air containing 5% of CO₂. Cells were seeded into a 6-well plate (2 × 10⁴ cells/well).

Western blotting

Heart tissues or cultured cells were sonicated in RIPA lysis buffer and homogenized. The debris was removed and the supernatant was obtained by centrifugation at 12 000 g for 10 min at 4 °C. The protein was separated by electrophoresis, transferred to PVDF membrane, and probed with primary antibodies against cleaved-caspase 3, Bax, Bcl2, MEK, p-MEK, ERK, p-ERK (Cell Signaling, Danvers, MA, USA); and with GAPDH (Abcam, MA, USA) as an internal control. Images were analyzed using Image-Pro Plus software.

Terminal deoxyribonucleotidyl transferase (TdT)-labeling (TUNEL) assay

Heart samples were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned into 4-cm-thick slides. Apoptosis was determined by the terminal deoxyribonucleotidyl transferase (TdT) labeling (TUNEL) assay using an in situ cell death detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Images were obtained using microscope under a magnification of 200 × and analyzed using a quantitative digital image analysis system (Image-Pro Plus 6.0).

TNF-α and IL-1β assay

Samples were frozen in liquid nitrogen soon after harvesting, and stored at −70 °C before use. The levels of TNF-α and IL-1β were determined by specific enzyme immunoassay kits (USCN Life Science Inc., TX, USA) in accordance with manufacturer’s instructions.

Immunohistochemistry

Samples were fixed with 4% paraformaldehyde and incubated with a blocking solution consisting of 10% bovine serum albumin for 1 h. The sections were incubated with MEK, p-MEK, ERK, or p-ERK (Cell Signaling, Danvers, MA, USA) at 4 °C overnight, and then washed for three times in phosphate-buffered saline (PBS). The sections were incubated with biotinylated goat anti rabbit Ig for 2 h and then stained with DAB according to the manufacturer’s instructions (Abcam, MA, USA). All sections were covered with mounting medium and observed under a light microscope (Olympus, Tokyo, Japan).

Cell viability assay

Cell viability was assessed by a modified MTT assay. Cells (1 × 10⁵ cells/mL) were seeded into 96-well plates and incubated for 24 h. Then, the cells were exposed to SFI or LPS at various concentrations. Next, 20 μL of 5 mg·mL⁻¹ MTT (Sigma, MO, USA) was added to each well and the cells were incubated for another 4 h at 37 °C. Then, the medium was then replaced by 150 μL dimethyl sulfoxide (DMSO) to dissolve the precipitate. The optical density was measured in a microplate reader (Bio-Rad, CA, USA) at 490 nm.

Flow cytometric analysis of apoptotic cells

Cell apoptosis was determined using Annexin V-FITC/propidium iodide (PI) kit (Merck, Germany). The cells were exposed to SFI, LPS, LPS + SFI, MEK inhibitor U0126 (1 μmol·L⁻¹, Selleckchem, TX, USA), or U0126 + LPS. SFI was added into the cells 12 h before LPS treatment, and U0126 added into the cells 2 h before LPS treatment. The cells were collected and washed with PBS twice, and then resuspended in binding buffer at a concentration of 1 × 10⁶ cells/mL. Then, 5 μL annexin V-FITC and 5 μL PI were added. The cells were incubated for 15 min in the dark. After staining, the quantification of apoptotic cells was analyzed by flow cytometry (BD Biosciences, CA, USA).

Statistical analyses

Data was presented as mean ± standard error of mean (SEM). Using GraphPad Prism 4.0 (GraphPad software Inc., CA, USA), statistical difference between multiple groups was evaluated by one-way analysis of variance (ANOVA) and t test. P < 0.05 was considered statistically significant.

Results

Effects of SFI on LPS-induced inflammation in rats

LPS treatment increased the levels of TNF-α and IL-1β in rat serum. Treatment with SFI inhibited the increase of TNF-α and IL-1β protein levels (Fig. 1 A). SFI inhibited the increase of TNF-α and IL-1β protein levels induced by LPS in the heart (Fig. 1B).

Effects of SFI on LPS-induced apoptosis in rats

According to the results of Tunnel staining, LPS increased the percent of positive cells in the heart, and SFI reduced the percent of positive cell. LPS treatment increased the expression levels of cleaved-caspase 3 and Bax, and reduced those of Bcl2 and Bcl2/Bax. However, SFI treatment...
reversed the expression of cleaved-caspase 3, Bax, Bcl2 and Bcl2/Bax (Fig. 2).

**Effects of SFI and LPS on cells viability in H9C2 cells**

The effects SFI and LPS on the cells’ viability were

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**Fig. 1** Shenfu injection (SFI) attenuated inflammation in sepsis induced by lipopolysaccharide (LPS) in rats (mean ± SEM, n = 6). (A) SFI (10 mL·kg⁻¹) inhibited the increases of tumor necrosis factor (TNF)-α and interleukin (IL)-1β in the serum induced by LPS. (B) SFI (10 mL·kg⁻¹) inhibited the increases of tumor necrosis factor (TNF)-α and interleukin (IL)-1β in the heart induced by LPS. *P < 0.05 vs saline group; #P < 0.05 vs LPS group

**Fig. 2** Shenfu injection (SFI) attenuated apoptosis in sepsis induced by lipopolysaccharide (LPS) in rats (mean ± SEM, n = 6). (A) SFI (10 mL·kg⁻¹) inhibited the increases of TUNEL positive cells in the heart induced by LPS. (B) SFI (10 mL·kg⁻¹) reversed the increases of cleaved-caspase 3 and Bax, and the decreases of Bcl2 and Bcl2/Bax in the heart induced by LPS. *P < 0.05 vs saline group; #P < 0.05 vs LPS group
identified using MTT assay. Nine doses of SFI (0.625, 1.25, 2.5, 5, 10, 20, 40, 80, 160 μg·mL$^{-1}$) did not exert a significant effect on cells’ viability (Fig. 3A). Seven doses of LPS (0.5, 1, 2, 4, 8, 16, 32 μg·mL$^{-1}$) reduced cells’ viability, but two doses (0.125 and 0.25 μg·mL$^{-1}$) did not (Fig. 3B). SFI (20, 40, 80 μg·mL$^{-1}$) hindered the decrease of cells’ viability induced by LPS (16 μg·mL$^{-1}$) (Fig. 3C). Then 16 μg·mL$^{-1}$ of LPS and 80 μg·mL$^{-1}$ of SFI were selected in the following experiments.

**Effects of SFI on LPS-induced inflammation in H9C2 cells**

TNF-α and IL-1β expression levels were increased in H9C2 cells after LPS treatment, and SFI inhibited the increase of TNF-α and IL-1β expression induced by LPS (Fig. 4).

**Effects of SFI on signaling pathways treated by LPS**

LPS increased p-MEK and p-ERK levels in the heart tissues, and SFI inhibited the increase of p-MEK and p-ERK expression induced by LPS (Figs. 5A and 5B). In H9C2 cells, p-MEK and p-ERK levels were increased by LPS treatment, and the increase of p-MEK and p-ERK levels were inhibited by SFI administration (Fig. 5C).

**Effects of MEK inhibitor U0126 on LPS-induced apoptosis in H9C2 cells**

As the flow cytometry assay showed, LPS promoted the apoptosis compared with PBS, and SFI attenuated this process. What’s more, MEK inhibitor U0126 inhibited the apoptosis induced by LPS (Fig. 6A). MEK inhibitor U0126 reversed the increase of cleaved-caspase 3 and Bax levels, and the reduction of Bcl2 and Bcl2/Bax levels induced by LPS (Fig. 6B).

**Discussion**

Sepsis is defined by consensus as a life-threatening organ dysfunction caused by a dysregulated host response to infection$^{[11]}$. Septic cardiomyopathy contributes to multi-organ failure due to insufficient vascular perfusion pressure and increases the mortality$^{[14]}$. The presence of cardiac dysfunction is associated with increased mortality in patients with sepsis: 28-day mortality of patients hospitalized for sepsis was 16% in absence and 47% in the presence of myocardial dysfunction$^{[13]}$. SFI is a commercial medicinal product approved by the China Food and Drug Administration and administered routinely in the treatment of sepsis shock, acute myocardial dysfunction, chronic congestive heart failure and postoperative recovery after surgery$^{[16-17]}$. The main active components in Shenfu Formula are ginsenosides and aconitum alkaloids$^{[18]}$. In recent years, clinical observations and pharmacological effects on SFI have been well investigated. Here, we studied whether Shenfu injection can descend LPS induced myocardial inflammation and apoptosis in rats. We also investigated the anti-apoptotic mechanisms of SFI. We demonstrated that MEK-ERK signal pathway might be involved in the anti-apoptotic effects associated with SFI.

Cardiac dysfunction can develop from sepsis in humans and rodents$^{[19-20]}$. The enrichment of inflammatory cytokines is a leading cause of myocardial contractile dysfunction, with bacterial endotoxin lipopolysaccharide (LPS) acting as the culprit$^{[20]}$. The inflammatory response is involved in most of heart disease. Administration of LPS can weaken the cardiovascular function of mice$^{[22]}$. The expression of IL-1β, IL-6, and TNF-α can be increased by LPS treatment$^{[23]}$. SFI can en-
hance the cellular immunity of patients with septic shock, showing its potential to treat septic shock. SFI counters inflammation through regulating the NF-κB signaling pathway. In this experiment, LPS increased the expression of TNF-α and IL-1β in rats’ myocardial tissue and H9C2 cells, and this process was inhibited by SFI. These results demonstrate that SFI inhibits LPS-induced inflammation.

Apoptosis, a tightly regulated cell deletion process, plays an important role in various cardiovascular diseases and con-
Contributes to the deterioration of left ventricular function in heart failure[26-27]. Sepsis-induced cardiac apoptosis is one of the major pathogenic factors in myocardial dysfunction[28]. As it enhances numerous proinflammatory factors, LPS is considered the principal mediator in this pathological process[29]. LPS, a gram-negative bacterial cell wall component, triggers apoptosis in cardiac myocytes by promoting the secretion of cytokines such as TNF-α, IL-6, IL-10, and interferon[30]. Studies have indicated that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The mitochondrial pathway is controlled by the Bcl2 protein family that activates caspase-3 when cytochrome c is released from damaged mitochondria in response to diverse stresses. In the Bcl-2 protein family, Bax and Bcl-2 have three “BH” (Bcl-2 homology) domains, Bax promotes the activation of apoptosis whereas Bcl2 inhibits apoptosis through binding with Bax. The balance of Bcl-2 and Bax determines whether the intrinsic apoptosis pathway is initiated[31]. Shenfu Formula reduces cardiomyocyte apoptosis in heart failure rats by regulating microRNAs[32]. SFI could prevent the ECV304 cells against H2O2 oxidative-stress by enhancing antioxidant enzyme activities, reducing the membrane lipid peroxidation, as well as upregulating antiapoptotic and downregulating apoptosis protein expressions[32]. In the present study, SFI treatment restrained LPS-increased expression levels of TUNEL-positive cardiomyocytes, and cleaved-caspase 3 and Bax. LPS reduced the Bcl2 expression and Bel-2/Bax ratio at the protein level in rats’ heart tissues, a process also reversed by SFI. Moreover, in H9C2 cells, SFI restored the cell viability damaged by LPS and antagonized LPS-induced changes of cleaved caspase 3, Bax and Bcl-2. These results suggest that SFI attenuates LPS-induced apoptosis.

Since cardiomyocyte loss is the major determinant of patient morbidity and mortality, fully understanding the regulatory mechanisms of apoptotic signaling is crucial. The generic mitogen-activated protein kinases (MAPK) signaling pathway is shared by four distinct cascades, including the extracellular signal-related kinases (ERK1/2), Jun amino-terminal kinases (JNK1/2/3), p38-MAPK and ERK5. Mitogen-activated protein kinases/extracellular signal regulated kinase (MAPK/ERK) pathway is reported to be associated with the cell proliferation, differentiation, migration, senescence and apoptosis[33]. ERK signaling pathway has bidirectional regulation on apoptosis, which may be related to cell types and diverse stimuli. ERKs protect cardiomyocytes from daunomycin-induced apoptosis, whereas p38 MAPK is involved in the induction of myocardial apoptosis[34]. Kawasaki et al. sugges-
tated nitric oxide (NO) can induce astrocyte apoptosis through activating MEK/ERK signaling pathway, and this effect can be blocked by U0126. Activation of the ERK1/2 and JNK MAP kinases contributes to, while activation of the p38 MAP kinase counteracts chlorpyrifos-induced apoptosis in cortical neurons. The cross-talk between MAPK cascades are not distinct and the certain mechanism involved need further research. Our study showed that LPS increased the expression levels of p-MEK and p-ERK, which was then inhibited by SFI treatment or inhibitor of MEK U0126. U0126 weakened the promotive effect of LPS on apoptosis, and affected the expression of cleaved caspase 3, Bax, and Bcl2. These results demonstrate that SFI fights against LPS-induced sepsis.

In conclusions, we discovered that SFI attenuates the inflammation and apoptosis induced by LPS. MEK-ERK pathway is involved in the LPS-induced myocardial apoptosis. Shenfu injection could protect myocardial apoptosis in rats though suppressing MEK/ERK phosphorylation.

Acknowledgments

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