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Original Article

Effects of Shenfu Injection (参附注射液) on Inflammatory Response during Post-Resuscitation Myocardial Dysfunction after Cardiac Arrest in Swine*

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ABSTRACT **Objective:** To investigate whether Shenfu Injection (SFI, 参附注射液) can alleviate post-resuscitation myocardial dysfunction by inhibiting the inflammatory response. **Methods:** After 8 min of ventricular fibrillation and 2 min of basic life support, 24 pigs were randomly divided into 3 groups (*n*=8), which were given intravenous bolus injections of SFI (1.0 mL/kg), epinephrine (EP, 0.02 mg/kg) and normal saline (SA), respectively. The animals were sacrificed at 24 h after restoration of spontaneous circulation (ROSC), and serum interleuking-6 (IL-6) and tumor necrosis factor-α (TNF-α) levels were measured by enzyme-linked immunosorbent assay (ELISA); expressions of Toll-like receptor 4 (TLR4)/nuclear factor kappa B (NF-κB) mRNAs and proteins were determined by RT-PCR and Western blot, respectively. **Results:** Compared with the EP and the SA groups, the ultrastructure of myocardial cells were slightly damaged and the systolic function of the left ventricle was markedly improved in the SFI group at 24 h after ROSC (*P*<0.05). In addition, compared with the EP and SA groups, the SFI group also showed significantly reduced levels of serum IL-6 and TNF-α, protein and mRNA levels of myocardial NF-κB and TLR4 (*P*<0.05). **Conclusions:** Activation of TLR4/NF-κB signaling pathway may be involved in the pathological mechanisms of post-resuscitation myocardial dysfunction. SFI may block NF-κB-mediated inflammatory response by reducing the activity of NF-κB and the level of TNF-α, thus playing a protective role in post-resuscitation myocardial dysfunction.

KEYWORDS Shenfu Injection, Chinese medicine, cardiopulmonary resuscitation, post-resuscitation myocardial dysfunction, TLR4/NF-κB

Although tremendous efforts have been made in scientific research to improve the survival rate of cardiac arrest (CA), and restoration of spontaneous circulation (ROSC) is achieved after cardiopulmonary resuscitation (CPR) in 20% to 40% of patients, only 1.4% to 17% of patients are discharged alive and post-resuscitation myocardial dysfunction occurs at 72 h after ROSC in most of patients.[1] The main mechanism involves myocardial ischemia-reperfusion injury,[2] cell apoptosis,[3] calcium regulation disorder and immune dysfunction.[4,5] During CA and CPR, the body experiences strong stress responses and produces a large amount of cytokines and inflammatory mediators, including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), to directly act upon the myocardial cells and impair heart function.[5] Toll-like receptor 4 (TLR4), the most common member of the Toll-like receptor family, is the endogenous receptor of lipopolysaccharide (LPS). TLR4 can activate the downstream inflammatory pathways after LPS stimulation, leading to release of inflammatory mediators including TNF-α and IL-6, and consequent injury, necrosis and apoptosis of myocardial cells.[6]

Shenfu Injection (SFI, 参附注射液) is a famous traditional Chinese medicine preparation and the main components are ginsenoside and aconitine, which can stimulate myocardium, increase the contractility of myocardium, and enhance the blood flow velocity

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of the coronary artery. Our previous animal studies have confirmed that SFI significantly improved tissue perfusion and oxygen metabolism, alleviated ischemia reperfusion, increased cardiac output, and inhibited the apoptosis of myocardial cells, playing a protective role in post-resuscitation myocardial dysfunction.[7,8] Whether SFI exerts such protective function by modulating the TLR4/nuclear factor kappa B (NF-κB) signaling pathway has not yet been demonstrated. Therefore, the present study established the 8-min CA model of miniature pigs and observe protein and mRNA levels of TLR4/NF-κB in myocardial cells after CPR, with or without intravenous administration of SFI.

METHODS

Preparation of Experimental Animals

Wuzhishan miniature pigs (30 pigs) from Chinese Academy of Agricultural Sciences, male inbred, 12–14 months old, weighing 30±2 kg, were randomly divided into a CPR group of 24 h and a sham operation group of 6 h using a random number table. Intramuscular injection of ketamine at 20 mg/kg combined with ear vein injection of propofol at 2 mg/kg was administered to induce anesthesia, with an initial dose of intravenous bolus injection of 0.25 mg remifentanil, followed by continuous intravenous infusion at 30–50 μg/(kg•h) for analgesia. During the anesthesia maintenance phase, an initial dose of intravenous bolus injection of 3% pentobarbital sodium was given at 30 mg/(kg•h), followed by continuous intravenous infusion at 8 mg/(kg•h). Orotracheal intubation was performed to mechanical ventilation, under the control of a CO_2SMO plus respiratory monitor. Inhaled oxygen concentration was 21%, with ventilation frequency of 12 times/min. Tidal volume (VT) was initially set at 8 mL/kg, and the ventilator was adjusted to make the end tidal carbon dioxide ($PETCO_2$) maintained at 35–40 mm Hg. A 7F angiography catheter was inserted into the right femoral vein through a sheath, and a bipolar temporary pacing electrode was placed to the right ventricle. A 5F Outlook catheter was inserted into the aortic arch through the right femoral artery, to which a pressure transducer and a PICCO monitor were connected to measure the aortic pressure. Cardiac output was measured using thermal dilution method.[7]

Procedures

The endocardial electrical stimulation method was employed. Intraventricular lead was connected to medical program-controlled stimulator (GY-600A; Kaifeng Huanan Equipment Co., Ltd., China), and esophageal output S1S2 (300/200 ms) model was chosen. Continuous electrical stimulus at 8:1 ratio and step size of 10 ms was commenced until occurrence of ventricular fibrillation (VF). The criteria of VF were a rapid drop of the arterial blood pressure and a VF waveform in ECG.[9] Eight minutes after VF, chest compressions was performed at a frequency of 100 times/min. CPR group was randomly divided into 3 groups according to a random number table, namely, a Shenfu (SFI) group, a epinephrine (EP) group and a saline (SA) group. Animals in 3 groups were given SFI (1.0 mL/kg), EP (0.02 mg/kg) and SA, respectively, which were prepared in 20 mL solutions and injected into the central vein. If no ROSC occured, defibrillation was performed at 120 J, followed by continuous chest compressions for 2 min. If there's still no spontaneous circulation, a second defibrillation was performed at 150 J. There was a 6-min interval following the 2-min CPR to analyze the rhythm of the heart, and another 2-min CPR was performed according to the requirements of each group and the next defibrillation was prepared. CPR quality was monitored by a HeartStart MRx Monitor/Defibrillator with Q-CPR, and operators were switched after every 2-min CPR to avoid fatigue that may affect the quality of CPR. The criteria of ROSC included systolic pressure remained ⩾80 mm Hg for at least 10 min. If ROSC was not achieved after 30 min of resuscitation, failure of CPR and animal death were announced.[7] For the animals in the sham group (n=6), the same procedure was performed, including anesthesia, mechanical respiration, and catheter indwelling, but without defibrillation and compression. Including heart rate (HR), cardiac output (CO), mean arterial pressure (MAP), were measured continuously, and the values at baseline and 30 min, 2, 4, and 6 h after ROSC were recorded.

Histopathology

Some myocardial specimens were preserved in 4% paraformaldehyde to observe pathologic changes for light microscope and transmission electron microscopy (TEM).

The slides were deparaffinized with xylene twice for 5–10 min, followed by absolute ethanol for 5 min, 90% ethanol for 2 min, 70% ethanol for 2 min and distilled water for 2 min. Then, the slides were stained in hematoxylin solution for 15 min, and flushed with tap water for 10 min, then stained in eosin for 1 min

(time can be adjusted according to the staining results and experimental requirements), and finally washed twice with 70% ethanol. Dehydration, clearing and mounting: The slides were dehydrated twice with 95% ethanol for 2 min, cleared twice with xylene for 5 min, and then mounted with neutral balsam. Nuclei should be blue, while cytoplasm should be pink or red through microscopic observation.

Myocardial specimens were fixed with 1.5%–3% glutaraldehyde for 1–2 h, then they were fixed with 1% starving acid for 1–2 h. After that the specimeds were washed with 0.1 mol/L PBS twice for 5–10 min, and were dehydrated by 50%, 70%, 90% ethanol each time (5–10 min/each time), respectively. The samples were then dehydrated by 100% ethanol for three times (5–10 min/time), and by propylene oxide for 10 min at room temperature. Following that, they were embedded by Epon812, and were polymerized at 40 ℃ for 2 h, at 60 ℃ for 4 h, at 80 ℃ for 10 h, respectively. Semi thin section was first stained by toluidine blue for 1 min, then stained by uranium acetate and lead citrate for 15 min each time, and finally observed under the electron microscopy (H-7650; HITACHI, Tokyo, Japan).

Enzyme Linked Immunosorbent Assay for Serum Cytokine Levels

The 2 mL vein blood was centrifuged at 35,000 × *g* for 10 min at 4 ℃, centrifuged blood was collected and immediately frozen at –80 ℃ for enzyme linked immunosorbent assay (ELISA, Sunbio Biotech Co. Ltd., China), Microtitration plates were coated overnight with 50 μL of a 10 μg/mL solution of Biotin anti-TNF-α and IL-6 in carbonate buffer pH 9.6. After blocking and washing, the sample was incubated for 30 min at room temperature. Each well was added with 50 μL color liquid and stop solution, gently mixed for 30 s and incubated for 15 min at room temperature. The absorbance at 450 nm was measured after 30 min using an ELISA plate reader. Cytokine concentrations were obtained from a standard curve. Duplicate readings were obtained for all samples and the means were calculated.

Western Blot Analysis of TLR4 and NF-κB

A 100-mg frozen heart sample was homogenized and then centrifuged at 12,000 r/min for 10 min at 4 ℃. A total of 100 μg of protein was loaded onto 10% SDS-PAGE gel in each sample. Western blotting was performed with the membranes blocked for 2 h with 5% non-fat milk and then incubated with the primary antibodies (diluted overnight at 4 ℃): TLR4 and NF-κB, 1:200 (AbcamBiotechnology, UK); and GAPDH, 1:250 (Santa Cruz Biotechnology, USA). Blots were blocked and incubated at 4 ℃ overnight with the specific primary antibody. The immunoreactive bands were visualized on film and scanned. The data were analyzed by Image Pro Plus (version 4.1, Media Cybernetics). The quantitative data from Western blot bands were expressed as the target protein OD/GAPDH OD ratio.

Reverse Transcriptase-PCR Assay for TLR4 and NF-κB

Total RNA was extracted from 50 to 100 mg of heart tissue according to the protocol described for the BioEasy SYBR Green Ⅰ Real-Time PCR Kit Manual (Bo Ri Technology Co., Ltd., China). The primer sequences of the expected PCR products were as follows: for TLR4, Forward primer: 5'-GGGTCACTTCTGTTCACG-3' and reverse primer: 5'-GATGTTGTCAGG-GATTTT-3'; for NF-κB, forward primer: 5'-ATTTCGTTTCCGTTATGTG-3', and reverse primer: 5'-CTGAGGGTAGGACTTCTTG-3'. Preincubation was performed at 94 ℃ for 2 min, followed by amplification at 94 ℃ for 30 s, 72 ℃ for 60 s, and finally, during slow heating up, 72 ℃ for 10 min. After the amplification, melting curve analysis with a temperature gradient from 65 ℃ to 94 ℃ was recorded every 0.5 ℃. Relative quantification is generally calculated with the $2^{-\triangle\triangle CT}$ formula by the comparative Ct method, the copy number of the target gene $2^{-\triangle\triangle CT}=2^{-(\triangle CT\ target\ gene\ -\ \triangle CT\ GAPDH\ gene)}$.

Statistical Analysis

The experimental data were analyzed by SPSS 17.0 (SPSS Inc, Chicago, Ⅲ, USA). The results are expressed as mean ± standard deviation ($\bar{x} \pm s$), and Student's *t* test was used for comparisons between two groups. Differences at different time points within groups were compared with repeated-measures analysis of variance (ANOVA). A two-tailed value of $P<0.05$ was considered significant.

RESULTS

Modeling and Resuscitation Outcomes

Six animals were successfully resuscitated with a ROSC rate of 83.3% in each of the 3 groups. CPR time to ROSC, defibrillation frequency and defibrillation energy in the EP and SFI groups were significantly different from that of the SA group, while

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no significant differences were found between the SFI group and the EP group in terms of the ROSC rate, CPR time to ROSC, defibrillation frequency and defibrillation energy (Table 1).

Table 1. Resuscitation Outcome in Swine among Groups ($\bar{x} \pm s$)

Group	*n*	Number of shock	Energy of shock (J)	Time to ROSC (min)
SA	6	5.51 ± 2.52	795.00 ± 375.68	10.00 ± 3.79
EP	6	2.75 ± 1.66*	312.70 ± 134.28**	6.01 ± 2.17**
SFI	6	2.61 ± 1.03*	332.51 ± 168.39**	5.00 ± 1.69**

Notes: SA, saline; EP, epinephrine; SFI, Shenfu Injection; ROSC, restoration of spontaneous circulation. *P<0.05, **P<0.01 *vs*. SA (one-way repeated-measures ANOVA)

Hemodynamics

As shown in Figure 1, differences in MAP and CO between the CPR group and the sham group were not statistically significant at the baseline (P>0.05), and MAP in the SA group, however, was significantly higher than that of the EP and SFI groups after ROSC (P<0.05). On the contrary, MAP in the SFI group was not different from that of the EP group at 30 min and 2 h after ROSC, respectively, and MAP in the SFI group was significantly increased compared with that of the EP group at 6 h after ROSC (P<0.05). Similarly, CO in the CPR group was significantly decreased compared with that of the sham group after ROSC (P<0.01). CO in the SFI and EP groups was significantly higher than the SA group at 30 min and 2 h after ROSC (P<0.05), and CO of the SFI group was significantly higher than the EP group at 6 h after ROSC (P<0.05).

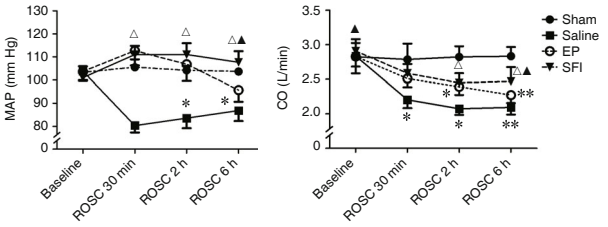

Figure 1. Comparison of Hemodynamics and Oxygen Metabolism among Groups (n=6, $\bar{x} \pm s$)

Notes: SFI, Shenfu Injection; ROSC, restoration of spontaneous circulation; MAP, mean aortic pressure; CO, cardiac output; 1 mmHg=0.133kPa; *P<0.05, **P<0.01 *vs*. sham (one-way repeated-measures ANOVA); △P<0.05 *vs*. saline; ▲P<0.05 *vs*. EP (student's *t* test).

Histopathology

Myocardial cells of the sham group showed normal structure under light microscope (Figure 2A). Massive swelling, necrosis of myocardial cells, and ruptured myocardial fibers were found in the CPR group (Figures 2B and 2C). Level of myocardial cell necrosis in the SFI group was significantly reduced (Figure 2D). While Funder electron microscopy, the sham group showed intact myocardial ultrastructure, neatly arranged myofilaments, clear Z- and M-line of sarcomere, normal nuclear structure, and normally-shaped mitochondria (Figure 2E). Mitochondria in the myocardial cells of the CPR group was swelling, with vacuolar degeneration and damaged cristae and outer membranes, and Z- and M-line of sarcomere was ruptured (Figure 2F and 2G). Destruction of myocardial cells in the SFI group was significantly reduced with partial nuclear chromatin condensation, and mitochondrial swelling and vacuolation was also significantly reduced. The Z- and M-line of sarcomere remained visible (Figure 2H).

ELISA Analysis of IL-6 and TNF-α Levels

As shown in Figure 3, serum IL-6 and TNF-α levels were significantly increased throughout the study

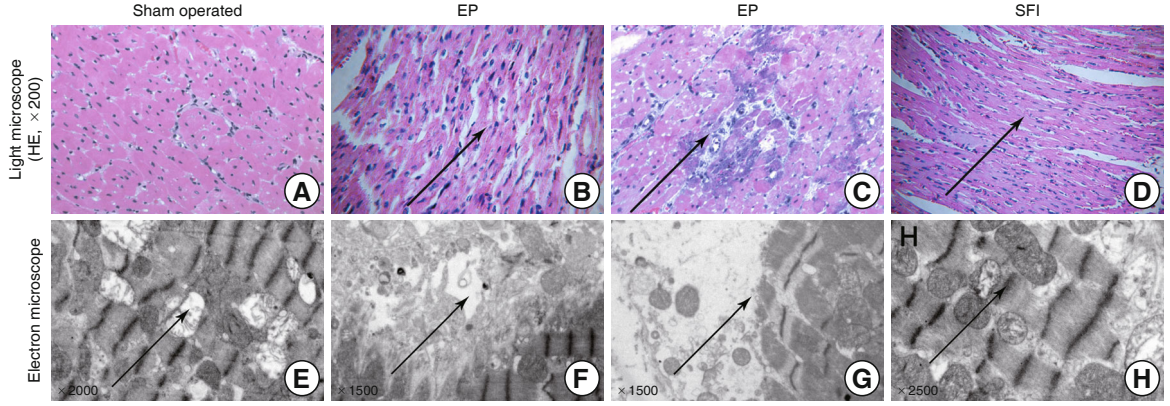

Figure 2. Cytoplasmic Ultrastructure and Histopathology Photographs of the Myocardium

Notes: (A) normal architecture of cardiomyocytes: (B, C, F and G) myofibril organelles were extensively damaged, and the myocardium exhibited progressive, severe deterioration (arrows): (E) normal mitochondrial architecture (arrows): (D and H) mitochondrial architecture of cardiomyocytes exhibited little intracellular damage in the SFI group at 24 h after cardiac resuscitation (arrows)

time points after ROSC compared with baseline values in all three resuscitation groups (P<0.05 or P<0.01). However, IL-6 and TNF-α were significantly decreased in the SFI group compared with the SA and EP group at 6 h (P<0.05 or 0.01); they were lower in the SFI group than in the SA and EP groups at 24 h after successful resuscitation (P<0.05).

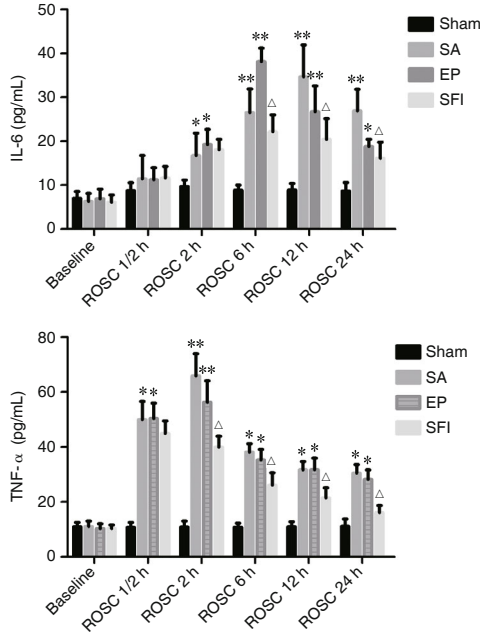

Figure 3. ELISA Analysis of IL-6, TNF-α Levels in Serum of Pig at 24 h after ROSC (n=6, $\bar{x} \pm s$)

Notes: *P<0.05, **P<0.01 *vs*. sham group, △P<0.05 *vs*. EP group

Protein Expressions of TLR4 and NF-κB

As shown in Figure 4, Western blot analysis showed that the protein expressions of TLR4 and NF-κB (P<0.05 or 0.01) progressively increased in the SA, EP and SFI groups compared with the sham-operated group. The levels of TLR4 and NF-κB were significantly decreased in the SFI group than in the EP group at 24 h after ROSC (P<0.05).

mRNA Levels of TLR4 and NF-κB

The copy number of the TLR4 and NF-κB gene in the SA, EP, and SFI groups significantly increased compared with that in the sham-operated group (P<0.05 or P<0.01). Furthermore, the mRNA expression of TLR4 and NF-κB in the SFI group was reduced compared with the EP and SA group at 24 h after ROSC (P<0.05).

DISCUSSION

Post-resuscitation myocardial dysfunction is

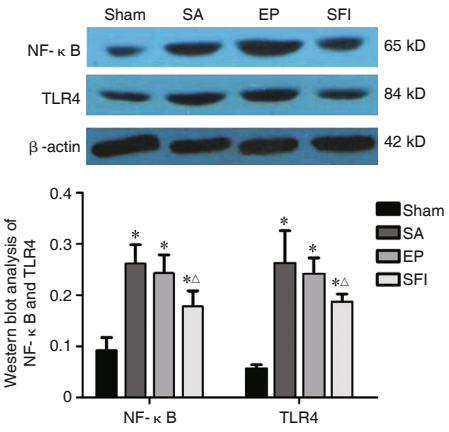

Figure 4. Quantification of TLR4 and NF-κB Protein Levels in Myocardial Tissue of Pig in Groups at 24 h after ROSC (n=6, $\bar{x} \pm s$)

Notes: *P<0.01 *vs*. sham group, △P<0.05 *vs*. EP group

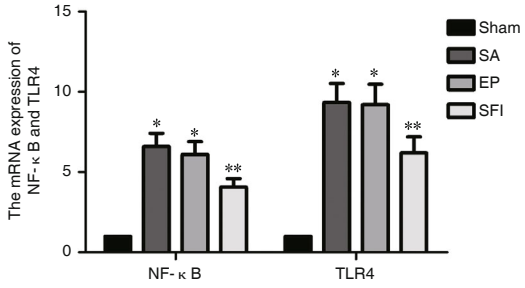

Figure 5. mRNA Expressions of TLR4 and NF-κB in Myocardial Tissue of Pig by RT-PCR (n=6, $\bar{x} \pm s$)

Notes: *P<0.01 *vs*. sham group, △P<0.05 *vs*. EP group

one of the main causes of death in patients with CA. Coronary artery blood flow was decreased or interrupted after the occurrence of CA, and global ischemia of the heart induces myocardial stunning. Early after ROSC, ischemia/reperfusion injury occurs in myocardial cells and hemodynamic disturbance is aggravated, leading to cardiogenic shock, accompanied by systemic low blood volume and vasomotor dysfunction.[10] Meanwhile, CA represents the most severe form of shock, during which oxygenated metabolites cannot be eliminated and a large amount of lactic acid deposits in myocardial cells, directly leading to myocardial damage. The underlying mechanism is similar to that of myocardial damage induced by septic shock.[11] The present study found that severe myocardial dysfunction occurred in miniature pigs within 6 h after ROSC, which was manifested in the considerably decreased MAP and CO, as well as serious damage of myocardial cell ultrastructure.

Unstable hemodynamics and microcirculatory disturbance due to post-resuscitation myocardial

dysfunction will cause persisted oxygen debt and subsequent activation of endothelial cells and release of inflammatory factors (such as TNF-α), leading to systemic inflammatory response.[11] TNF-α, one of the early proinflammatory cytokines, is a key cytokine involved in ischemia-reperfusion injury.[12] It induces the massive expression of various inflammatory mediators, such as TNF-α and IL-6, by activating neutrophils, and results in "cascade reactions", which further amplify TNF-α expression in a positive feedback manner, and TNF-α, in turn, activates NF-κB. This fuels a vicious circle of uncontrolled inflammatory cytokine release, which directly impair the myocardial cells, resulting in myocardial dysfunction.[6] In the present study, we found that the serum levels of TNF-α and IL-6 in miniature pigs in the CPR group were significantly increased at 24 h after ROSC, which was similar to the mechanism of myocardial dysfunction secondary to sepsis.

NF-κB is a dimer composed of two subunits, P50 and P65. Under normal conditions, it is bound by the inhibitor of NF-κB (I-κB) to form an inactive trimer and is sequestered in the cytoplasm. When the body is stressed by infection, shock, and reperfusion, NF-κB dissociates from I-κB and translocates into nucleus, where it binds to specific promoters and regulate gene expression, and may lead to uncontrolled systemic inflammatory response and cell apoptosis.[13] Activated NF-κB can initiate and regulate various proinflammatory cytokines and cellular adhesion molecules, causing neutrophil adhesion and aggregation as well as cascade amplification of cellular inflammatory response.[14] TLR4, an important member of the TLR family, can specifically recognize LPS on the cell walls of gram negative bacteria and is considered as the major LPS receptor.[6] In the early stage after ROSC, the body experiences ischemia/reperfusion process and produces a large amount of metabolites such as free oxygen radicals and lactic acid, leading to systemic inflammatory responses and hypoimmunity. Therefore, the body is often associated with severe bacterial (gram negative bacteria) infection, which causes the release of LPS and intestinal translocation of LPS.[15] LPS binds to receptor CD14 on monocyte/macrophage membrane, and then to receptor TLR4 to transfer the signal into the cell. When TLR4 is activated, it interacts with NF-kB to produce large amounts of oxygen free radicals and releases massive inflammatory mediators.[16] Thereby the downstream TNF-α and other inflammatory factors are activated, directly leading to myocardial cell injury. Studies have shown that TLR4 is involved in myocardial dysfunction in ischemia/reperfusion.[17] It was found in the present study that the myocardial function of pigs was seriously impaired after ROSC, and the protein and mRNA levels of TLR4/NF-kB in myocardial cell of the CPR group were significantly higher than that of the sham group at 24 h after ROSC.

SFI is derived from a modified classic prescription of Chinese medicine "Shenfu Decoction (参附汤)" and the active ingredients include ginsenosides and n-desmethyl aconitine. Ginsenoside can also activate ribonuclease, which increases the biosynthesis of DNA and promotes the metabolism of sugar, protein and fat. This helps myocardial repair and myocardial cell energy supply, increases myocardial contractility, and improves myocardial function.[18] In addition, ginsenosides also prevent against stress, oxidation and ischemia and hypoxia. They may also eliminate free-radicals, prevent lipid peroxidation, reduce intracellular calcium overload, and stabilize the structure of cell membrane.[19] Studies have shown that SFI can protect the brain tissue by scavenging free oxygen radicals, inhibiting the inflammatory response, and reducing cell edema in ischemia-reperfusion injury.[20] In addition, Qiu Z, et al[21] suggested that SFI could modulate severe immune disturbances, maintain a proinflammatory/anti-inflammatory balance, and improve clinical outcomes. In our previous studies, SFI was found to inhibit inflammatory responses, regulate Th1/Th2 cell imbalances, and improve post-resuscitation myocardial immune dysfunction.[22] In addition, we also observed that SFI could improve immunity and inhibit inflammatory responses by improving the apoptosis of splenic T lymphocytes.[23] In the study by Wang, et al[24] rats with endotoxin-induced systemic inflammatory response syndrome were treated with SFI, and the results showed that SFI significantly reduced the activity of NF-κB and the levels of IL-6 and TNF-α, and alleviated the pathological damage of lung and liver.

We found in the present study that both serum IL-6 and TNF-α levels and TLR4/NF-κB expression in myocardial cells in the SFI group were significantly reduced, suggesting that SFI may reduce the activity of NF-κB and TNF-α levels by inhibiting the

inflammatory response, so as to block the inflammatory response and cell apoptosis regulated by NF-κB, and to reduce myocardial cell injury. This may serve as one of the main mechanisms underlying the preventive and therapeutic effects of SFI for post-resuscitation myocardial dysfunction. In addition, our results also suggested that SFI may enhance the balance of SIRS by inhibiting the overexpression of proinflammatory mediators, thereby reducing systemic inflammatory response and regulating immune function.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Author Contributions

Gu W and Li CS conceived and designed the experiments. Gu W, Zheng PY and Hou XM performed the experiments. Gu W and Hou XM analyzed the data. Hou XM contributed reagents/materials/analysis tools. Gu W and Li CS wrote the paper.

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