## Original Research Article

# Schisandra chinensis extract ameliorates myocardial ischemia/reperfusion injury via TLR4/NF-κB/MyD88 signaling pathway

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## Abstract

**Purpose:** To investigate the effects of Schisandra chinensis extract (SCE) on myocardial ischemia-reperfusion (I/R) injury and to elucidate its underlying mechanism of action.
**Methods:** A rat model of myocardial I/R injury was used. Ischemia was induced by occluding the left anterior descending artery for 30 min and the myocardium was then reperfused for 2 h in Sprague-Dawley rats. Triphenyltetrazolium chloride (TTC) staining was used to measure myocardial infarct size, while the levels of inflammatory cytokines were evaluated by enzyme-linked immunosorbent assay (ELISA). Western blot assay was conducted to determine protein levels.
**Results:** TTC staining showed that myocardial I/R injury was ameliorated after SCE treatment. Serum creatine kinase (CK), lactate dehydrogenase (LDH), and malondialdehyde (MDA) levels decreased, whereas superoxide dismutase (SOD) activity increased after SCE treatment. Moreover, serum interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) expression levels were reduced after SCE treatment. Furthermore, SCE treatment remarkably downregulated the protein expression levels of Toll-like receptor 4 (TLR4), nuclear factor-kappa B (NF-κB), and myeloid differentiation factor 88 (MyD88).
**Conclusion:** SCE may exert protective effects against myocardial I/R injury by downregulating TLR4-mediated NF-κB/MyD88 signaling pathway. However, this needs to confirmed in clinical studies.

**Keywords:** Schisandra chinensis, TLR4/NF-κB/MyD88, Inflammasome, Myocardial ischemia-reperfusion injury

## INTRODUCTION

Myocardial infarction is one of the cardiovascular diseases with high morbidity and mortality in developed and developing countries [1]. Previous studies have shown that prolonged reperfusion can lead to severe alterations in myocardial ultrastructure, remodeling, contractile and diastolic dysfunction, and myocardial damage [2]. Mitochondria are believed to control cell fate, including cell death [3]. The mitochondrial permeability transition hole is activated and translocated by cytochrome C release, which promotes apoptosis via Bcl-2 family members [4].

During ischemia/hypoxia, mitochondria are susceptible to damage, which results in increased oxidative stress and apoptosis of cardiomyocytes [5]. Therefore, how to prevent the induction of oxidative stress and apoptosis of cardiomyocytes have become a major challenge in the treatment of myocardial infarction [6].

Previous studies have demonstrated that the NF-kappa B (NF-κB)/Toll-like receptor 4 (TLR4) signaling markedly promotes myocardial ischemia and plays a key role in I/R –induced myocardial damage [7]. A TLR4 deficiency was recently shown to relieve symptoms of myocardial damage and inflammation after I/R induction [8]. The TLR4 activation was shown to participate in the activation of NF-κB and control the proinflammatory cytokines expression levels, in addition, Interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF-α) are thought to be released by myeloid differentiation factor 88 (MyD88), thereby enhancing the immune response and increasing endothelial cell permeability [9]. These investigations totally indicated the important role of TLR4/NF-κB/MyD88 signaling pathway-mediated inflammation in the progress of myocardial infarction.

The most abundant and active ingredient of *Schisandra chinensis* extract (SCE) is Schisandrin B, a traditional Chinese medicine [10]. Previous research has shown that SCE possesses hepatoprotective, anti-inflammatory, antioxidant and anticancer pharmacological effects. *Schisandra chinensis* extract (SCE) has been widely used to treat vascular injury, cardiovascular and cerebrovascular diseases, liver disorders, and various nerve diseases [11]. However, the mechanism by which SCE elicits its cardioprotective effects is still unknown and needs further investigation. Based on the previous studies, the mechanism of SCE-mediated cardioprotection may be closely associated with TLR4/NF-κB/MyD88 pathway-mediated inflammation. Thus, resistance to ischemia elicited by SCE was evaluated and its molecular mechanism was investigated.

# EXPERIMENTAL

## Reagents

*Schisandra chinensis* extract (SCE) was provided by Selleckchem (Shanghai, China). Thermo Fisher Scientific (Thermo Fisher Scientific, CA, USA) provided 2,3,5-Triphenyl tetrazolium chloride (TTC, ≥ 99 % purity) and Enzyme-linked immunosorbent assay kits (ELISA). Santa Cruz (Santa Cruz, USA) provided

the NF-κB, TLR4, and MyD88 proteins, GAPDH, antibodies specific for horseradish peroxidase.

## Animals

The Animal Experiment Center of Guangzhou Medical University (Guangzhou, China) provided adult male Sprague-Dawley rats (n=50, 220 – 250 g). Prior to the experiments, the rats were housed for 7 days under standard laboratory conditions. The rats were given drinking water and feed. All performance were approved by Jinhua Municipal Central Hospital, Zhejiang, China (approval no. JMC2017062) and conducted following the Laboratory Animal Care and Use Guidelines of National Institutes of Health.

## Drug treatment

SD rats were divided into (n = 10 rats/group) the sham group, I/R group, I/R + 50 mg SCE/kg group, I/R + 50 mg SCE/kg group and I/R + 200 mg SCE/kg group. SCE was administered intragastrically once per day for 7 days prior to surgery. Surgery was performed as described in previous studies [12]. Myocardial reperfusion was performed for 2 hours after 30 min of ischemia. Continuous monitoring was performed using a standard limb lead electrocardiogram. Finally, the rats were sacrificed by decapitation, the hearts and blood samples were obtained for morphological and biochemical tests and for biochemical assays, respectively.

## Measurement of myocardial infarct size

Rat hearts were stored for 15 min at -18 °C, and five uniform tissue samples were then cut parallel to the line below the heart ligature and the coronary sulcus. Tissue samples were cultured for 15 min in TTC for pathological examination in the dark at 37 °C. Image-Pro software (Version 4.1, X-Ray Scan, LP, USA) was used to analyze the tissue staining.

## Determination of serum CK, LDH, MDA, and SOD expression levels

Serum was collected by centrifugation of whole blood for 15 min at 4,000 rpm. Prior to analysis, samples were stored at -80 °C. CK, LDH, MDA, and SOD expression levels were evaluated by commercial ELISA kits.

## ELISA of inflammatory cytokines

The serum IL-1β, IL-6, and TNF-α expression levels were evaluated using ELISA kits. The absorbance at 450 nm of per well was read with

a spectrophotometer, and the content of per well was calculated from the standard curve.

## Western blotting analysis

Proteins were examined via western blotting by using the monoclonal anti-TLR4, anti-MyD88, anti-p-IKKβ, anti-p-IKKα, anti-p-NF-κB, and anti-p-IκBα proteins (1:1000). GADPH (1:5000) was served as a loading control. The secondary antibody of horseradish peroxidase (HRP)-labeled (1:1000) was used and cultured for one hour at 25 °C. Quantify band density using the LICOR Odyssey infrared imaging system (LICOR Bio-science, Nebraska, USA).

## Statistical analysis

GraphPad 7.0 software was used to analyze the data. All data were repeated as independent experiment triple times and expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) analysis was used for analyzing the differences among experimental groups, $p < 0.05$ of data was regarded as significantly.

# RESULTS

## Effects of SCE on the size of myocardial infarct in rats with I/R injury

TTC staining showed that the presence of irreversible ischemic injury to the myocardium greatly affected the extent of I/R injury. I In I/R group, the infarct size was increased as compare to the sham group ($p < 0.001$, Figure 1). However, SCE (50, 100, and 200 mg/kg) treatment significantly reduced infarct size (all $p < 0.05$) in a dose-dependent manner. These results indicate that SCE relieves myocardial ischemia in rats with I/R injury.

## Effects of SCE on serum CK, LDH, MDA, and SOD levels in rats with I/R injury

To confirm whether SCE attenuated the amount of myocardial damage induced by I/R, animals were administered intragastrically with SCE (1 mL/100 mg) once per day for 7 days before surgery and reperfused for 2 h after 30 min of ischemia. Serum CK, LDH, MDA, and SOD levels, key indicators of myocardial damage, were measured 2 h after reperfusion. Myocardial I/R increased CK, LDH, and MDA levels in serum (all $p < 0.01$), whereas the SOD activity was significantly decreased ($p < 0.05$) (Figure 2). In contrast, SCE (50, 100, and 200 mg/kg) treatment lowered CK (all $p < 0.05$), LDH (all $p < 0.05$), and MDA (all $p < 0.05$) expressions as compared to I/R injury group. Additionally, serum

SOD levels were restored after SCE treatment as compared to theI/R group ($p < 0.05$). These results clearly show that SCE protects the heart from I/R injury.
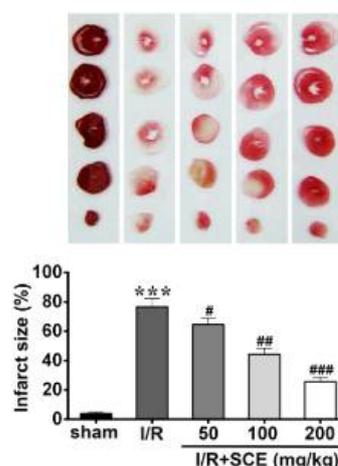


**Figure 1:** Effect of SCE on infarct size in rats with myocardial damage induced by I/R. Infarct regions (white stained) and viable area (red stained). The infarct sizes were analyzed by TTC staining (n = 10). Data are shown as mean ± SD; $^{***}p < 0.001$ *vs.* sham group; $^{#}p < 0.05$, $^{##}p < 0.01$, or $^{###}p < 0.001$ *vs.* I/R group
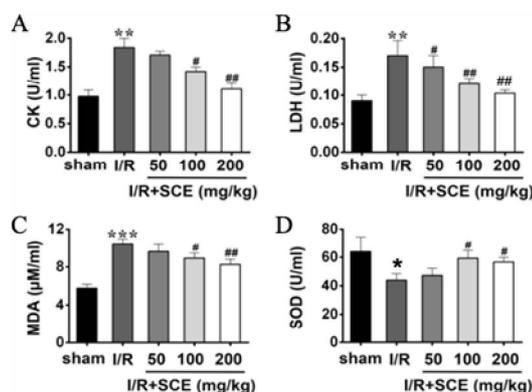


**Figure 2:** Effects of SCE on serum CK (A), LDH (B), MDA (C), and SOD (D) levels. N=10 in each group. Results are shown as the means ± SD. $^{*}P < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$ *vs.* sham group; $^{#}p < 0.05$ or $^{##}p < 0.01$ *vs.* I/R group

## Effect of SCE on serum IL-1β, IL-6, and TNF-α expression levels in rats with I/R injury

IL-1β, IL-6, and TNF-α are key indicators of myocardial I/R injury. IL-1β, IL-6, and TNF-α levels were evaluated to confirm the effects of SCE on the inflammatory response in rats after I/R injury. As depicted in Figure 3, markedly higher serum IL-1β, IL-6, and TNF-α expression levels were observed, indicating exposure to I/R injury. However, compared to the I/R group,

treatment with SCE (50, 100, and 200 mg/kg) markedly decreased serum IL-1β, IL-6, and TNF-α levels. These data indicate that reducing the inflammatory factors expression may mediate the cardioprotective effects of SCE.
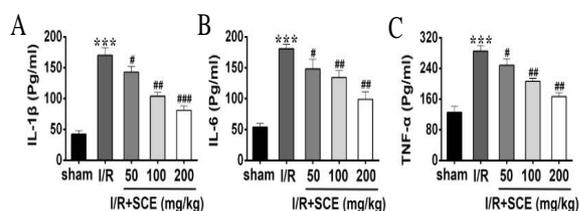


**Figure 3:** Effect of SCE on serum levels of IL-1β (A), IL-6 (B), and TNF-α (C). N=10 in each group. Results are shown as the means ± SD. ***$p < 0.001$ *vs.* sham group; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ *vs.* I/R group

**Effects of SCE on TLR4, NF-κB, and MyD88 expression levels in heart tissues of rats with I/R injury**

TLR4, NF-κB, and MyD88 protein levels were evaluated by western blot analysis to examine the possible molecular mechanisms. In the I/R group, protein levels of TLR4, p-IKKβ, MyD88, p-NF-κB, p-IKKα and p-IκBα were upregulated as compared to the sham group (Figure 4). In contrast, TLR4 and MyD88, and the phosphorylation of IKKβ, IKKα, IκBα, NF-κB protein expressions were markedly downregulated in a dose-dependent manner by SCE (50, 100, and 200 mg/kg) administration. These data indicate that SCE inhibits the myocardial damage induced by myocardial I/R via the NF-κB/TLR4/MyD88 pathway *in vivo*.
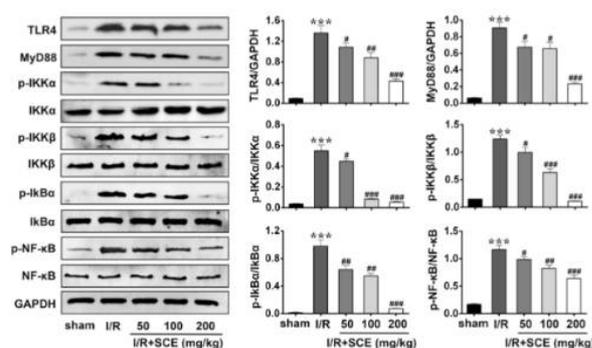


**Figure 4:** Effects of SCE on TLR4, NF-κB, and MyD88 protein levels (n = 10). Data are expressed as mean ± SD. ***$p < 0.001$ *vs.* sham group; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ *vs.* I/R group

## DISCUSSION

Here, I/R increased myocardial infarct size in rats, and this effect was attenuated by SCE treatment. SCE treatment also reduced the levels of creatine kinase (CK), lactic dehydrogenase (LDH), and malondialdehyde (MDA), but increased SOD activity. Moreover, the serum levels of IL-1β, IL-6, and TNF-α were suppressed by SCE. Western blot analysis showed that SCE also decreased the TLR4, NF-κB, and MyD88 expressions in the heart from rats with I/R injury. Taken together, SCE may protect against myocardial damage induced by myocardial I/R via the NF-κB/TLR4/MyD88 pathway.

CK and LDH, released from damaged cells, are important serum enzyme biomarkers for myocardial infarction [13]. In our study, compared to the I/R + 50 and 100 mg/kg SCE groups, the I/R + 200 mg/kg SCE group showed lower levels of CK and LDH, indicating that higher concentration of SCE may have better protective effects. The destruction of cellular macromolecules, separation of protein modifications, lipid peroxidation, and DNA damage are induced by oxidative stress. Previous studies have shown that MDA inhibits mitochondrial complex I- and complex II-linked respiration and reduces mitochondrial membrane potential, leading to mitochondrial dysfunction [14]. According to current results, MDA levels were reduced and SOD activity was restored by SCE, which largely contributed to less mitochondrial oxidative damage. Combined with the results on myocardial infarction, it seems that the protective roles of SCE against I/R –induced myocardial damage was through reducing mitochondrial oxidative damage via regulating MDA and SOD activities.

There is increasing evidence showing that myocardial I/R induces the proinflammatory cytokines (IL-1β, IL-6, and TNF-α) expressions, indicating that inflammation is an important manifestation of I/R injury [15]. Stroke triggers inflammatory responses via several factors, such as the presence of debris, necrotic cells and reactive oxygen species, and many other factors that have yet to be identified [16]. Induction of neuronal apoptosis and production of inflammatory chemokines by IL-1β have also been observed [17]. During the pathogenesis of cerebral ischemia, IL-6 is involved in neuronal apoptosis and the mediation of inflammatory cytokines [18]. Previous research has shown that TNF-α inhibits myocardial contractility and causes a decline in blood pressure, possibly increasing myocardial infarct size because of adjustments in ventricular remodeling [19]. Consistently, the current study showed that treatment with SCE markedly decreased inflammation of the heart by suppressing the production of inflammatory cytokines induced by I/R in rats.

Several studies have shown that the pathogenesis of myocardial damage induced by myocardial I/R is closely associated with the TLR4/NF-κB pathway [20]. The adaptor molecule of MyD88 and related downstream events are induced by the activation of TLR4 [21]. Nuclear factor kappa-B (NF-κB) is commonly regarded as a mediator of both energy failure and inflammation [22]. In addition, NF-κB is responsible for the generation of proinflammatory cytokines, which are key regulators of inflammatory signaling pathways [23]. IKK-β and IKK-α are major components of IκBα, which inhibits NF-κB-p65 and is controlled by IκB kinase (IKK) [24]. Upon activation, the degradation and phosphorylation of IκBα activates the NF-κB subunit, p65 [25]. NF-κB activation then leads the initiation of transcription and expression of IL-1β, IL-6, and TNF-α. These proinflammatory cytokines are known to exert pivotal effects on various immune processes. These results demonstrate that during the myocardial damage induced by myocardial I/R, TLR4 drives the activation of NF-κB, MyD88, and other inflammatory factors.

## CONCLUSION

The findings of this study demonstrate the cardioprotective roles of SCE in I/R-induced myocardial injury in rats. The molecular mechanism involves inflammation mediated via TLR4/NF-κB/MyD88 inflammasome pathway. The clinical application of SCE in this regard needs to be explored.

## DECLARATIONS

### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. YL designed all the experiments and revised the paper. BX and XL achieved the experiments. YL and XX wrote the paper.

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