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Original Research Article

Angelica sinensis polysaccharide ameliorates myocardial ischemia-reperfusion injury in rats by inhibiting TLR4/NF-κB

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Abstract

Purpose: *To evaluate the ameliorative effect of Angelica sinensis polysaccharide (ASP) in myocardial ischemia-reperfusion injury (MIRI) rats through Toll-like receptor 4 (TLR4)/Nuclear factor κB (NF-κB) pathway.*
Methods: *The MIRI rat model was established. Sprague-Dawley rats were randomized to sham, MIRI, and ASP low-dose (50 mg/kg) and high-dose (100 mg/kg) groups, and a model of hypoxia-reoxygenation (H/R) injury was established. In in vitro studies, H/R cardiomycetes (derived from neonatal cell culture) were randomized to control, H/R, H/R + ASP low dose (5 μg/mL), H/R + ASP high dose (10 μg/mL), and ASP high dose + TLR4 activator groups.*
Results: *After intragastric administration of ASP for 4 consecutive weeks, creatine kinase isoenzyme (CK-MB) and lactate dehydrogenase (LDH) were reduced after ASP treatment in MIRI rats ($p < 0.05$). Both in vivo and in vitro, ASP reduced TNF-α, IL-6, and IL-1β expressions ($p < 0.05$), and alleviated inflammatory response. Apoptosis was inhibited by ASP, which also increased Bcl2, reduced Bax and cleaved caspase-3 expressions, while TLR-4, p-IκBα, and p-p65 were decreased.*
Conclusion: *The cardioprotective effect of ASP on MIRI results in the inhibition of TLR4/NF-κB pathway. Thus, this study broadens the current body of knowledge on the pharmacological prevention of MIRI and the therapeutic potential of ASP.*

Keywords: *Myocardial ischemia-reperfusion injury, Angelica sinensis polysaccharide, TLR4/NF-κB, Inflammation, Apoptosis*

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INTRODUCTION

Myocardial ischemia-reperfusion injury (MIRI) means the myocardium is damaged in the case of ischemia, and when normal perfusion is restored, its tissue damage is aggravated. The ultrastructural, functional, and electrophysiological characteristics of the myocardium are further damaged after reperfusion, leading to a further progression of the disease. MIRI causes irreversible damage to cardiomyocytes and reduces cell viability [1]. Sudden decreased blood pressure and disturbance of the heart rhythm are the clinical manifestations of MIRI [2].

Therefore, it is necessary to explore the pathogenesis of MIRI for its treatment.

At present, studies show that the pathogenesis of MIRI is related to oxidative stress, intracellular calcium ion overload, endothelial cells inflammatory response, cell apoptosis, mitochondrial damage, myocardial energy metabolism disorder, etc, and some interactions between signals may also be involved [3]. Inflammation is the main pathological cause of MIRI, which will bring about a series of pathological reactions [4]. Cardiomyocytes and vascular endothelial cells have high levels of toll-like receptor 4 (TLR4) expression, which is associated with the development and occurrence of cardiovascular illnesses. It activates the inflammation and the immune system in the body by regulating the NF-κB pathway [5]. When the body is subjected to various physiological or pathological stimuli, the Activated TLR4 initiates downstream signal cascades through intracellular transduction, and then activate it. The activated NF-κB enters into the nucleus binding to target sequence, that promotes the inflammatory cytokine tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β) expressions and aggravates the cardiac tissue damage [6].

Myocardial ischemia-reperfusion triggers cell death programs, which include cell apoptosis, autophagy and necrosis. Apoptosis is connected with the Caspase signal transduction cascade, such as Caspase-9 and Caspase-3 activation. Research suggests that the balanced steady state of the Bcl-2 and Bax is disrupted during ischemia. Bcl-2 family genes are proved to be associated with the regulation and control of cell apoptosis and Bcl-2 subfamily genes include antiapoptotic gene (Bcl-2) and proapoptotic gene (Bax) [7]. The studies have demonstrated that Bcl-2 overexpression reduced the occurrence of apoptotic that myocardial ischemia and reperfusion (I/R)-induced [8].

Angelica sinensis polysaccharide (ASP) has a wide range of pharmacological actions as a water-soluble active substance of *Angelica sinensis*. It inhibits the invasion and metastasis of human liver cancer to slow tumors progression. ASP is able to alleviate immune colon injury in rats, and this protective mechanism is related to immune regulation and promotion of wound repair. Studies have shown that it has a beneficial role in cerebral ischemia-reperfusion injury [9] . However, the role of ASP in MIRI is still uncertain. This study aimed to investigate the influence of ASP on both MIRI rats and myocardial cell hypoxia-reoxygenation (H/R) culture in *in vitro* cell experiments, and also to probe into the regulation of ASP on TLR4/NF-κB pathway, a crucial signaling pathway which mediates immune inflammation and cell apoptosis.

EXPERIMENTAL

Animals and ethical approval

SPF-grade Sprague Dawley (SD, 250 – 280 g) were selected. The rats and the feed were purchased from the Zhejiang Experimental Animal Center. The room they were kept in was quiet, with a humidity of 55 % at the temperature of 23 ± 2 ℃. All the experimental protocols were approved by the Institutional Animal Care and Use Committee of Zhejiang Center of Laboratory Animals (approval no. ZJCLA-IACUC-20030043) and the International Guidelines 2.0 were followed in all procedures [10].

Establishment of MIRI procedure and animal grouping

Adult SD rats were anesthetized, and the MIRI model was established by means of ligation of the left anterior descending (LAD) for 30 min and reperfusion for 120 min. The adult SD rats were randomized to 4 groups: Sham, MIRI, and ASP low and high dose groups with 15 rats in each group. For 4 consecutive weeks, the rats in the low/high dose ASP group were dosed 50/100 mg/kg ASP once a day respectively while the Sham or MIRI group rats were offered the same amount of water instead.

Determination of inflammatory factors

The levels of inflammatory cytokines and tumor necrosis factors were assayed using the ELISA kit (Boster, China) in both the rat serum and the culture supernatant. The absorbance at 450 nm was measured.

Determination of creatine kinase CK-MB and lactate dehydrogenase (LDH) activity

After abdominal aorta intubation, collected blood was centrifuged to obtain serum. The LDH and CK-MB levels were assayed using the appropriate kits (Biosino, China).

Hypoxia-re-oxygenation (H/R) cell culture model

The culture for neonatal rat cardiomyocytes was prepared according to the previous method [11], and the cardiomyocytes of 1-day-old SD rats were treated with hypoxia/reoxygenation.

Cardiomyocytes of the newborn rat were separated from the rat heart, and then the ventricles were digested with 0.1% type II collagenase. The cardiomyocytes were then resuspended in DMEM and incubated with 37 ℃ in 5 % CO_2. These cells were treated with corresponding processing when they covered 70 - 80 % of the culture flask. The incubator was filled with gas mixtures containing 1 % O_2, 5 % CO_2 and 94 % N_2 to generate hypoxia conditions. During hypoxia, the cells grown in DMEM without serum and glucose for 6 h to simulate the energy deprivation., and then reoxygenated for 2 h. Five groups were used in the experiment: control, H/R, H/R + ASP low dose (5 μg/mL), H/R + ASP high dose (10 μg/mL), ASP high dose + TLR4 activator group (LPS, Beyotime, Shanghai, China).

Western blot

Protein was extracted from the cells and protease inhibitors were added to the supernatant. Protein concentrations were decided by using BCA method, and separated using SDS-PAGE (10 %). The polyvinylidene difluoride membrane (PVDF, Roche, Switzerland) was transferred. The primary antibodies incubated on the membrane and the internal control protein was determined with β-actin (1: 2000) for 2 h at 37 ℃. The PVDF incubated with secondary antibody was washed three times, then the PVDF was visualized using ECL kit.

MTT assay

After being incubation in a 96-well culture plate with 0.5 × 10^4 cells/well, cells were cultured with ASP for 48 h, and 10 μL MTT (5 mg/mL) was added to per well. After then, 150 μL DMSO (Sigma, USA) was added. Absorbance was measured spectrophotometrically at 570 nm.

Statistical analysis

Statistical Package for the Social Sciences (SPSS), version 10.0, was used to process the data. Data are expressed as mean ± standard deviation (SD). Student' s *t*-test was used to determine differences between groups and $p < 0.05$ was considered statistically significant.

RESULTS

Effect of ASP on CK-MB and LDH in serum

The MIRI model rats was established, and serum content of LDH and CK-MB were determined for MIRI rats treated with low and high dose of ASP, respectively (Figure 1). The content of LDH and CK-MB in MIRI group rats serum were higher than that of Sham group. The LDH and CK-MB in ASP low and high-dose group decreased compared to MIRI, and ASP high-dose group were less than those in ASP low-dose. The results showed that ASP alleviated myocardial injury in MIRI rats, and that effect was dose-dependent.

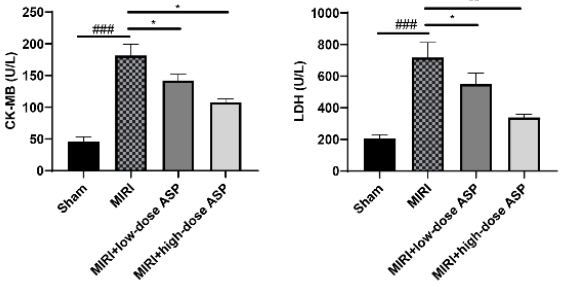

Figure 1: Effects of ASP on the levels of CK-MB and LDH. $^{###}P < 0.001$ vs. sham, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ *vs.* MIRI

ASP improved inflammatory response in MIRI rats

Compared with the rats in the sham group, the levels of TNF-α, IL-6, IL-1β in the MIRI group were significantly increased. In comparison to MIRI group rats, inflammatory factor secretion was reduced in the low-dose ASP-treated rats, and in comparison to low-dose ASP treated rats, inflammatory factor secretion was reduced in the high-dose ASP-treated rats (Figure 2). These results suggest that in MIRI rats, ASP reduced inflammatory response.

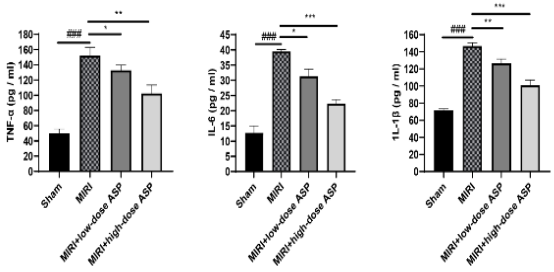

Figure 2: The effect of ASP on the secretion of TNF-α, IL-6 and IL-1β in serum. $^{###}P < 0.001$ versus sham. $*P < 0.05$, $**p < 0.01$, $***p < 0.001$ versus MIRI

Effect of ASP on myocardial apoptosis in MIRI rats

Compared to sham group, Bax and cleaved caspase-3 proteins in MIRI rats were up-regulated, while t Bcl2 was down-regulated, which promoted myocardial apoptosis. With low-

dose ASP treatment, the cleaved caspase-3 and Bax were reduced significantly, and the Bcl2 was up-regulated. With high-dose ASP treatment, the cleaved caspase-3 and Bax were further diminished, meanwhile the Bcl2 was enhanced, which inhibited myocardial apoptosis (Figure 3). These results suggest that ASP alleviated myocardial apoptosis in MIRI group rats.

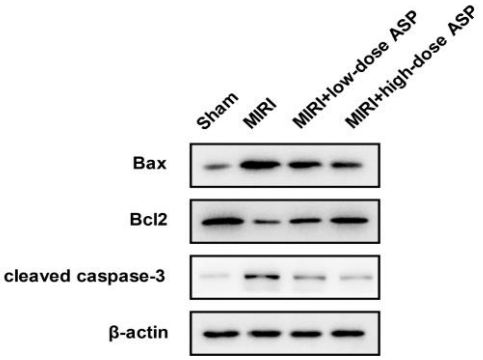

Figure 3: Effect of ASP on the apoptosis-related proteins

Effect of ASP on TLR4/NF-κB in MIRI rats

The TLR4/NF-κB pathway-related protein was assayed in MIRI rats (Figure 4). TLR-4, p-p65 and p-IκBα and expressions in the MIRI rats were clearly greater than the Sham rats. The levels of TLR-4, p-p65 and p-IκBα in the low-dose ASP were down-regulated compared to MIRI and were greater than the high-dose. These results indicated that the gene expressions involving the TLR4/NF-κB could be affected by ASP treatment.

Effect of ASP on inflammatory response and apoptosis in H/R cardiomyocytes

Cell viability in H/R group significantly reduced in contrast to control (Figure 5 A). The IL-6, IL-1β, and TNF-α were secreted significantly higher (Figure 5 B). Western blot results showed that Bcl-2 was reduced, while cleaved caspase-3 and Bax increased (Figure 5 C). TLR-4, p-IκBα, and p-p65 expressions were elevated. (Figure 5 D). There was increased cell activity and decreased secretion of inflammatory factors after ASP treatment. However, apoptosis was down-regulated and TLR4/NF-κB pathway-related proteins expression decreased. In cardiomyocytes treated with TLR4 activator (LPS), it was observed that cell viability decreased and the inflammatory factors secretion increased. Also, Bax, cleaved caspase-3, TLR-4, p-IκBα, and p-p65 expressions were increased. The result was consistent with that found in MIRI rats, indicating that ASP reduced inflammatory response and apoptosis of H/R cardiomyocytes *in vitro* by affecting TLR4/NF-κB pathway.

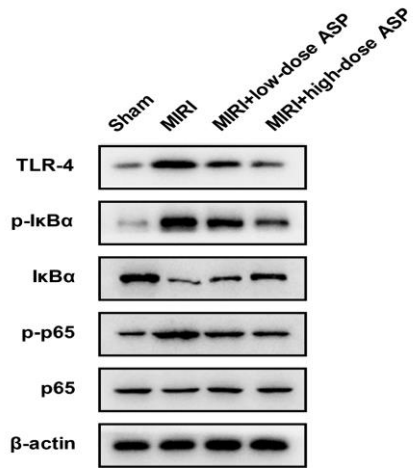

Figure 4: Effect of ASP on the TLR4/NF-κB

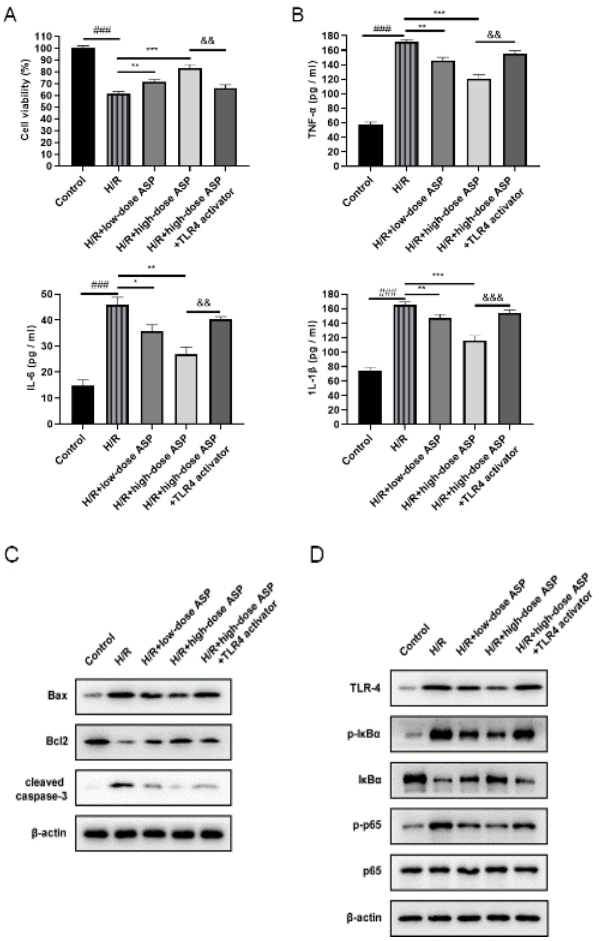

Figure 5: Effect of ASP on H/R cardiomyocytes on H/R cardiomyocytes. A. Cell viability. B. Inflammatory factors secretion. C. Cell apoptosis D. TLR4/NF-κB signaling pathway. $^{###}P < 0.001$ *vs.* Control. $^{*}P < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* H/R. $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ *vs.* H/R + high-dose ASP

DISCUSSION

MIRI causes cardiac dysfunction. Intracellular enzymes are released into the bloodstream during reperfusion after myocardial cells are damaged. CK-MB, as a characteristic isoenzyme in myocardial cells, mainly distributed in the myocardium, reflects the degree of myocardial cell damage [12]. LDH is widely present in myocardial tissue and is a commonly used index to evaluate the development of myocardial diseases [13]. It was found that after ASP treatment in MIRI rats, CK-MB and LDH reduced significantly, consistent with the pathological changes of myocardial tissue.

The temporary interruption of blood supply to organs causes ischemic injury, and the reperfusion injury resulted from a strong inflammatory response triggered by blood re-supply. NF-κB is involved in physiological processes, for example, processes, apoptosis, inflammation, and autoimmune diseases [14]. TLR4, a member of the Toll-like receptor family, triggers the viability of the NF-κB, which initiates the synthesis of inflammatory factors. As an indispensable upstream regulatory molecule of NF-κB, IκB binds to NF-κB p65 and thus deactivates the pathway. IκB kinase complexes (IKKs) activate IκB and induce the degradation and dissociation of IκB in p65; IκBα is the key regulatory molecule of IκB [15].

The downstream transcription factor NF-κB is activated under LPS stimulation and induces an increase in pro-inflammatory molecules cytokine molecules production [16]. Through treatment with high and low doses of ASP in MIRI rats, ASP reduced the degree of inflammation in rats and pro-inflammatory cytokine secretions were inhibited.

Studies have confirmed that TLR4 binds to ligands through many signal pathways in the process of signal transduction *in vivo*, activates NF-κB, and increases cardiomyocyte apoptosis. During myocardial ischemia, apoptosis of a massive range of damaged cardiomyocytes will gradually appear. Caspase activation is an essential part of apoptosis, and Bcl-2 family proteins are the main controllers. Bcl-2 is a proto-oncogene isolated from lymphoma and an inhibitor of apoptosis. When stimulated by apoptotic signals, Bax is transferred from the cytoplasm to mitochondria, where it interacts with Bcl-2 on the mitochondrial membrane to play a pro-apoptotic role [17].

In this study, ASP treatment was performed on MIRI rats, and it was found that the expression of inhibitory cell apoptosis factor Bcl-2 was significantly increased. Previous studies have shown that as for the rats with MIRI, when treated with *Salvia miltiorrhiza* polyphenolate, the area of myocardial infarction and cardiomyocytes apoptosis were reduced, the expression of the apoptosis-related protein Bcl2 was upregulated, and Bax and activated Caspase-3 were inhibited [18]. In the present study, ASP also played a substantial role in the myocardial apoptosis signaling pathway in the treatment of MIRI.

CONCLUSION

The cardioprotective effect of ASP on MIRI results in the inhibition of TLR4/NF-κB pathway. Thus, this study broadens the current body of knowledge on the pharmacological prevention of MIRI and the therapeutic potential of ASP.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jianhua Ye conceived and designed the study, and wrote the manuscript; Shenghui Shen and Xialing Dai collected and analyzed the data; Tianjie Zhang read and revised the manuscript critically. All authors read and approved the manuscript for publication.

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