Pretreatment with geniposide mitigates myocardial ischemia/reperfusion injury by modulating inflammatory response through TLR4/NF-κB pathway

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ABSTRACT

Geniposide (GEN), a medical herb, is known for its therapeutic applications in cardiovascular diseases, though its efficacy in treating myocardial ischemia/reperfusion injury (MI/RI) is yet to be fully elucidated. This study is an endeavor to explore the potential protective mechanism of GEN against MI/RI. To simulate the MI/RI condition, the left anterior descending artery was occluded for 30 min, followed by a reperfusion period of 120 min in a rat model. Three dosages (50, 100, or 150 mg/kg) of GEN were intraperitoneally injected to the Sprague-Dawley rats once a day, for seven days before the ligation of the artery. The rats were categorized into sham group, MI/RI group, and three different dosages GEN-treated groups. As the results showed, the pretreatment with GEN mitigated myocardial injury, reduced infarct volume, inhibited apoptosis, enhanced superoxide dismutase activity, and decreased malondialdehyde and myeloperoxidase activity, as well as serum creatine kinase-MB and lactate dehydrogenase levels. Moreover, GEN ameliorated MI/RI by downregulating protein expression of toll-like receptor 4, myeloid differentiation primary response 88, and p-nuclear factor-κB. In conclusion, the pretreatment of GEN may be considered as a potential therapeutic option for MI/RI.

Key words: Geniposide; myocardial ischemia/reperfusion injury; nuclear factor-κB; inflammation; toll-like receptor 4.

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Introduction

Acute myocardial ischemia, a type of ischemic heart disease that is of particular concern, is a common and leading cause of death worldwide.[1] Thrombolytic therapy and primary percutaneous coronary intervention and are employed to reestablish blood flow to the ischemic myocardium.[1] Nevertheless, the reperfusion technique has been demonstrated to have a dual nature: while it can alleviate myocardial ischemia symptoms, it may simultaneously worsen myocardial damage.[1] Patients may experience myocardial ischemia/reperfusion injury (MI/RI) as a result of ischemia-reperfusion injury following acute myocardial infarction during reperfusion.[2] The issue of MI/RI has garnered considerable clinical attention. Calcium overload, oxygen-derived free radicals, inflammatory response, apoptosis, and autophagy are relevant to the MI/RI mechanism.[3] Ischemia/reperfusion-induced inflammatory reactions are among the most significant aspects of MI/RI.[4] Toll-like receptors (TLRs) are vital proteins that play a crucial role in non-specific immunity by recognizing pathogen-associated molecular patterns and inducing cytokines to combat infections.[5] To date, more than 10 subtypes of TLRs have been identified in the human body, each capable of recognizing different receptors. Furthermore, all TLR ligands can serve as immune adjuvants. TLR-4 can promote the formation of active oxygen free radicals and activate multiple cytokines. The nuclear factor kappa B (NF-κB) pathway is implicated in tissue injury and stress response, and MI/RI can activate this pathway in the subsequent process of oxidative stress and calcium overload.[5] TLR4 and NF-κB activate proinflammatory cytokine expression, playing a critical role in myocardial inflammation, infarction, ischemia-reperfusion injury, and heart failure.[6]

In the quest for active agents for MI/RI treatment, natural products from Chinese herbal medicine show promising therapeutic potential. *Gardenia jasminoides* J. Ellis is a traditional Chinese herb with a wide range of pharmacological properties. It is commonly used to treat inflammation, hepatic disorders, and hypertension. Geniposide (GEN), an iridoid glycoside isolated from *Gardenia jasminoides* J. Ellis, possesses various pharmacological activities.[7] Increasing evidence suggests that GEN can reverse mitochondrial dysfunction and inhibit hypoxia/reoxygenation-induced cardiomyocyte apoptosis in H9C2 cells.[8,9] This mechanism may be relevant to the activation of Glucagon-Like Peptide-1 Receptor (GLP-1R) as well as Phosphoinositide 3-kinase/Protein Kinase B (PI3K/AKT) pathways.[10] Recent study suggests that GEN shows anti-diabetic effect on MI/RI diabetic rats *via* Nuclear Factor Erythroid 2-Related Factor 2/Heme Oxygenase-1 (NRF2/HO-1) pathway activation, suppressing oxidative stress response.[11] Moreover, several studies demonstrate that GEN effectively inhibits the inflammatory response through TLR4-mediated pathways.[7,12] However, the effects and mechanism of GEN on MI/RI inflammatory response remain poorly understood.

In this study, we initially prepared an *in vivo* model to explore whether GEN mitigates MI/RI injury, and the associated mechanisms were probed. The findings establish a basis for identifying a prospective approach to address cardiovascular conditions.

Materials and Methods

Animal raising

A cohort of 45 male Sprague-Dawley (SD) rats, with a weight range of 200 to 220 g, was acquired from the Center of Experimental Animals at the Shanghai Sippr-BK Laboratory Animal Co. Ltd. (Shanghai, China). The rats were maintained under controlled environmental conditions, with a temperature of 23±2 °C, humidity between 55-60%, and a 12-h light/dark cycle. They were given *ad libitum* access to food and water. The animal study adhered to the guidelines provided in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was conducted in compliance with laboratory animal care guidelines. The Hangzhou Eyong Biotechnological Co., Ltd. Animal Experiment Center approved and authorized the animal experiments with Certificate exhibited as No. SYXK (Zhe)2020-0024.

Animal model and GEN treatment

Following a one-week period of acclimatization, SD rats were randomized into five distinct groups: Sham, MI/RI, MI/RI+ GEN 50 mg/kg, MI/RI+ GEN 100 mg/kg, and MI/RI+ GEN 150 mg/kg. The GEN concentration was selected based on a study by Wang *et al.*[9] GEN group rats received intraperitoneal injections of different GEN doses (Sigma, USA) once a day, for seven days.[9] The sham rats were injected 0.9% saline intraperitoneally. Simply, 30 min after the last intraperitoneal injection of GEN, the MI/RI model was performed. We followed the method of MI/RI model as the previous studies mentioned.[13,14] SD rats were anesthetized after inhaling isoflurane. The rat trachea was carefully isolated and exposed using a scalpel, and a rodent ventilator was quickly connected for assisted ventilation (tidal volume: 8 mL, respiratory rate: 60 beats/min, and respiratory ratio: 5:4). A surgical procedure involving a left thoracotomy was conducted to expose the heart, followed by ligation of the left anterior descending coronary artery (a small polyethylene tube was placed 1-2 mm under the left auricle of the heart). After 30 min of compression ischemia, instant partial whitening of the left ventricular anterior wall visible to the naked eye indicated successful ischemia. The silk thread was then loosened for fluent coronary blood flow for reperfusion, and obvious hyperemia occurred in the left ventricle within a few seconds. The rats belonging to the sham group underwent identical surgical procedures, except that the suture was not fastened.

Determination of cardiac function parameters

An evaluation of cardiac function was carried out after 120 min of MI/RI. To start, a minor cut was initiated at the left atrium, followed by the insertion of a pressure-measuring sensor through the left atrium and the opening of the mitral valve, into the left ventricle. The sensor was subsequently linked to a biomedical signal acquisition and processing apparatus (PCLAB-UE; Beijing Microsignalstar, Beijing, China) to constantly track the hemodynamic parameters. Key parameters such as fractional shortening (FS), and ejection fraction (EF), along with the ascendant (+dp/dtmax) and descendant (–dp/dtmax) rates of left ventricular pressure were registered (n=6 in each group).

Sample collection

After 120 min of reperfusion, the rats were inhaled isoflurane to get euthanized. Part of the rats' blood was extracted from the carotid artery, and supernatant was collected by centrifugation for CK-MB, LDH examination (n=6 in each group). Subsequently, the hearts were excised from each group of rats. Once the myocardial infarction area was identified, the surrounding myocardial tissue was carefully dissected and trimmed for 2,3,5-triphenyltetrazolium chloride (TTC) staining (n=3 in each group). Part of the heart tissue was fixed with 4% paraformaldehyde for 15 h at 4°C and embedded in paraffin wax (n=3 in each group). Following this, 4 μm thick sections were prepared for hematoxylin and eosin (H&E) staining, TUNEL assay and immunohistochemical assay (n=3 in each group). The trimmed myocardial tissue was immediately flash-frozen in liquid nitrogen and stored at an appropriate temperature for Western blotting analysis (n=3 in each group).

Myocardial enzyme assay

After 120 min of MI/RI, following the collection of carotid artery blood from rats, the samples were allowed to rest at room temperature for 50 min. After this, the samples were centrifuged at 3000 rpm and 4°C for 10 min to obtain the plasma. Next, the upper-layer serum was collected to observe the level of serum CK-MB (7355, Meimian, Jiangsu, China) and LDH (13282, Meimian) with ELISA kits using a fully automatic biochemical analyzer (CMaxPlus, MD, China).

Malondialdehyde, superoxide dismutase, and myeloperoxidase activity assay

Myocardial tissue was homogenized to produce a 10% tissue homogenate using cold saline. The malondialdehyde (MDA) and the activity of myeloperoxidase (MPO) in the myocardium were measured using an MDA kit (Beyotime Biotechnology, Shanghai, China) and an MPO kit (Nanjing Institute of Biological Engineering, Nanjing, China), and the procedure was carried out as per the manufacturer's instructions. The dinitrobenzoic acid method was utilized to determine the activity of superoxide dismutase (SOD) in the myocardium.

Determination of myocardial infarction area

After MI/RI, the left ventricle was retained, weighed, and sliced into five sections (1-2 mm thick), followed by staining with pre-heated 1% TTC solution and incubation at 37 °C for 15 min. The sections were then fixed with 4% paraformaldehyde for 30 min. Finally, the sections were evaluated using microscopy (DM3000; Leica, Wetzlar, Germany) (n=3 in each group). The infarction area and myocardial area were measured. The area of myocardial infarction (%) was calculated as the area of myocardial infarction in each section divided by the myocardial area in the section, multiplied by 100.

Histopathological examination of myocardial tissue

Rat myocardial tissues were fixed and subsequently embedded in paraffin wax. Following this, 4 μm thick sections were prepared and stained with H&E (H3136, E4009; Sigma-Aldrich, St. Louis, MO, USA) for histological analysis. Finally, the sections were evaluated using microscopy (DM3000, Leica) at magnifications of 10×80 in 6 fields (n=3 in each group). The semi-quantitative analysis utilizing H&E staining is delineated as follows: 0 = absence of inflammatory cell infiltration and myocardial necrosis; 1 = less than 25% of the area exhibits inflammatory cell infiltration and myocardial necrosis; 2 = the area demonstrating inflammatory cell infiltration and myocardial necrosis ranges between 25% and 50%; 3 = the area showing inflammatory cell infiltration and myocardial necrosis extends from 50% to 75%; 4 = over 75% of the area is characterized by inflammatory cell infiltration and myocardial necrosis.

Assessing myocardial cell apoptosis

Myocardial tissue samples were fixed, dehydrated, embedded, and sectioned. Following TUNEL staining, with *in situ* cell death detection kit (11684795910, Roche, Basel, Switzerland), normal myocardial cell nuclei appeared blue, while the apoptotic cells were identified by brown and yellow staining of the cell nuclei. Finally, the sections were evaluated using microscopy (DM3000, Leica) at magnifications of 10×40 in 6 fields (n=3 in each group). The apoptotic index was calculated as the ratio of the number of apoptotic cells to the total number of cardiomyocytes, expressed as a percentage.

Immunohistochemical assay

Myocardial tissue sections were prepared and treated for antigen retrieval, usually citrate buffer or Tris-EDTA buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0). The heating can be performed in a water bath (90-100°C for 10-20 min), followed by incubation with H_2O_2 and bovine serum albumin. The sections were then incubated overnight with a primary antibody solution containing anti-TLR4 (ab22048, Abcam, Cambridge, MA, USA) at 4°C. As negative controls, some sections were processed in the same way as the test samples, but without the primary antibody. Afterward, the slides were incubated with a secondary antibody (HRP-conjugated Goat Anti-Mouse IgG H&L, ab205719; Abcam). The sections were treated with DAB and left for several min, then counterstained with hematoxylin. Finally, the sections were evaluated using microscopy (DM3000, Leica) at magnifications of 10×40 in 6 fields (n=3 in each group). As for the measurement of immunopositivity, a combination of manual counting and image analysis software was used. The stained slides were initially examined under a microscope, and areas of positive staining were noted. An image analysis software (Image J) was then used to quantify the intensity and extent of staining in these areas. This approach allowed us to obtain both qualitative and quantitative data on the level of immunopositivity in our samples.

Western blotting analysis

Myocardial tissue samples were homogenized with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology) and centrifuged, and protein concentration was determined using a BCA protein quantification kit (Solarbio, Beijing, China). Proteins were denatured, and the protein samples were separated on a 10% SDS-PAGE gel through electrophoresis, followed by transfer onto a PVDF membrane. The PVDF membrane was blocked with 5% milk powder, following which it was incubated with primary antibodies. The primary antibodies utilized in this study were against: β-Actin (1:1000, 4967S; Cell Signaling Technology, Danvers, MA, USA), Myeloid differentiation primary response 88 (MyD88) (D80F5) Rabbit mAb (1:1000, 4283S; Cell Signaling Technology), NF-κB p65 (D14E12) XP® Rabbit mAb (1:1000, 8242S; Cell Signaling Technology), Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb (1:1000, 3033S; Cell Signaling Technology), and TLR4 (1:300, ab217274; Abcam) followed by secondary antibodies (HRP-conjugated Goat Anti-Rabbit IgG H&L, S0001). The membranes were then placed in a chemiluminescence detector. The system captures an image of the membrane, with the bands corresponding to the target proteins appearing as dark bands against a lighter background. Finally, the bands' intensities are measured using software (Fiji processing package) that analyzes the image obtained from the imaging system. The software identifies the positions of the bands, measures intensities, and may provide data in the form of pixel intensity values or relative optical density units s (n=3 in each group).

Statistical analysis

The mean values with standard deviations were used to present the results. One-way analysis of variance or the Kruskal-Wallis H test was employed for comparing groups. A p-value less than 0.05 was deemed statistically significant.

Results

Effect of GEN on cardiac function in MI/RI-induced dysfunction rats

The chemical formula of the GEN is shown in Figure 1. The pressure sensor and the biological information collection system

were connected immediately to collect data on the cardiac function in rats. In Figure 2, the results showed that the MI/RI group rats exhibited decreased EF, FS, and ± dp/dt_{max} level in relative to the sham group rats ($p<0.05$). GEN significantly improved myocardial systolic and diastolic function, as evidenced by higher EF, FS, +dp/dt max, and -dp/dt max level in the GEN group rats contrast to the MI/RI group rats in a dose-dependent way ($p<0.05$).

Effect of GEN on myocardial infarction area in MI/RI-induced dysfunction rats

The impact of MI/RI on the myocardial infarction area in each group was assessed using TTC staining. As shown in Figure 3A, no obvious myocardial infarction was observed in sham group rats. The MI/RI group rats showed a significantly larger myocardial infarction area than the sham group rats ($p<0.01$), which significantly decreased after GEN administration in a dose-dependent way ($p<0.05$, Figure 3B).

Effects of GEN on histopathological variation in MI/RI-induced dysfunction rats

H&E staining results revealed that myocardial tissue from sham-operated rats had normal tissue morphology and structure with clear transverse striations, without any signs of inflammatory cell infiltration or histological alterations (Figure 4 A,B). In contrast, heart tissue in MI/RI rats exhibited extensive myocardial structural variations, pronounced inflammatory cell infiltration, and subendocardial necrosis. After GEN treatment, the histological features improved, showing either typical normal cardiac structure or mild architectural damage.

GEN modulated myocardial enzyme in MI/RI-induced dysfunction rats

An automatic biochemical analyzer was used to measure the levels of serum CK-MB and LDH of the myocardial tissues in rats. The MI/RI group showed significantly higher serum CK-MB and LDH levels in relative to the sham group rats ($p<0.01$, Figure 5A). Pretreatment with GEN significantly reduced these levels in MI/RI rats in a dose-dependent way ($p<0.01$, Figure 5A).

GEN inhibited oxidative stress in MI/RI-induced dysfunction rats

Assay kits were utilized to evaluate the impact of GEN on oxidative stress of the myocardial tissues in MI/RI rats. The MI/RI rats displayed increased MPO activity than sham rats ($p<0.01$, Figure 5B), while GEN significantly decreased myocardial MPO activity in a dose-dependent way ($p<0.01$, Figure 5B). The MI/RI rats exhibited higher MDA content and lower SOD activity in the myocardium than the sham rats. However, preconditioning with GEN inhibited oxidative stress activation in MI/RI rats, as evidenced by lower MDA content and higher SOD activity in the myocardium of the GEN group than the MI/RI rats ($p<0.05$ or $p<0.01$, Figure 5B).

GEN reduces cell apoptosis in MI/RI-induced dysfunction rats

Subsequently, we evaluated myocardial cell apoptosis using TUNEL staining. Results demonstrated that the MI/RI rats exhibited higher cell apoptosis levels in the myocardium than the sham

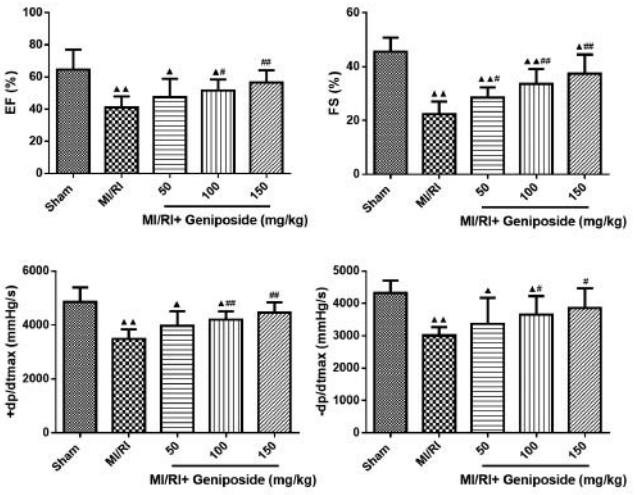

Figure 2. GEN improved cardiac function in ischemia/reperfusion injury (MI/RI)-induced dysfunction rats. After MI/RI, fractional shortening (FS), ejection fraction (EF), and left ventricular maximal systolic/diastolic velocity (+dp/dtmax and -dp/dtmax) of the myocardial tissues in rats were recorded with pressure sensor and the biological information collection system ($\bar{\chi}$±s, n=6). ▲▲ $p<0.01$, ▲$p<0.05$ *vs* sham group, #$p<0.05$, ##$p<0.01$ *vs* MI/RI group.

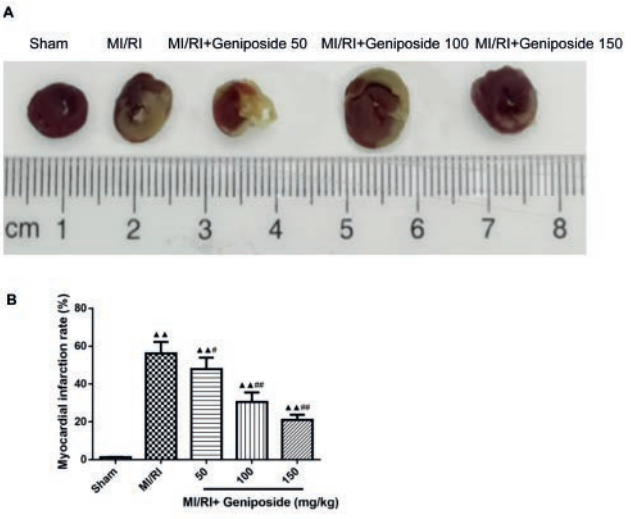

Figure 3. Effect of GEN on myocardial infarction area in MI/RI-induced dysfunction rats. **A**) Representative image of 2,3,5-triphenyltetrazolium chloride (TTC) staining of the myocardial tissues in rats ($\bar{\chi}$±s, n=3). **B**) Myocardial infarction rate of the myocardial tissues in rats ($\bar{\chi}$±s, n=3). ▲▲$p<0.01$ *vs* sham group, #$p<0.05$, ##$p<0.01$ *vs* MI/RI group.

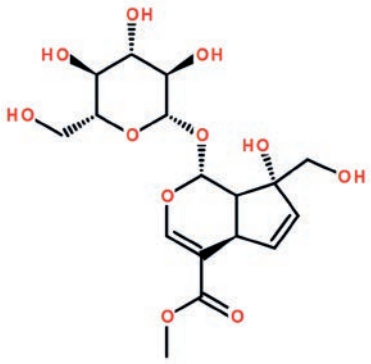

Figure 1. Chemical structure of geniposide.

rats (Figure 6 A,B). Furthermore, GEN significantly reduced MI/RI-induced cell apoptosis, as evidenced by lower cell apoptosis levels in the GEN group than MI/RI group in a dose-dependent way, but not with low-dose treatment ($p<0.01$, Figure 6 A,B).

GEN downregulates TLR4 expression in myocardial tissues in MI/RI-induced dysfunction rats

Immunohistochemistry results revealed that TLR4 expression was negligible in the myocardial tissues of sham group (Figure 7 A,B). DAB staining localization proved a substantial increase in TLR4 expression in the MI/RI group than sham group. TLR4 expression was notably reduced in the ischemic area following high and moderate-dose GEN treatment, but not with low-dose treatment in comparison to the MI/RI group ($p<0.05$ or $p<0.01$).

GEN suppresses TLR4/NF-κB pathway in myocardial tissues in MI/RI-induced dysfunction rats

To further examine the impact of GEN on TLR4-mediated signaling, we assessed p65, MyD88, the downstream molecules of TLR4 (Figure 8 A,B). Western blot analysis demonstrated that TLR4, p-p65 and MyD88 expressions were upregulated in the ischemic area in the MI/RI rats relative to the sham rats ($p<0.01$).

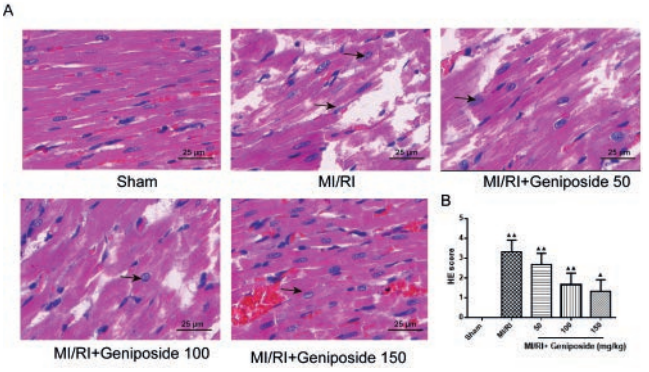

Figure 4. Effects of GEN on histopathological variation in MI/RI-induced dysfunction rats. **A**) Representative image of H&E staining of the myocardial tissues in rats, original magnification 800× scale bar: 25 μm ($\bar{\chi}$±s, n=3). **B**) Semi-quantitative assessment of the histological lesions of the myocardial tissues in rats ($\bar{\chi}$±s, n=3), ▲p<0.05, ▲▲p<0.01 *vs* sham group.

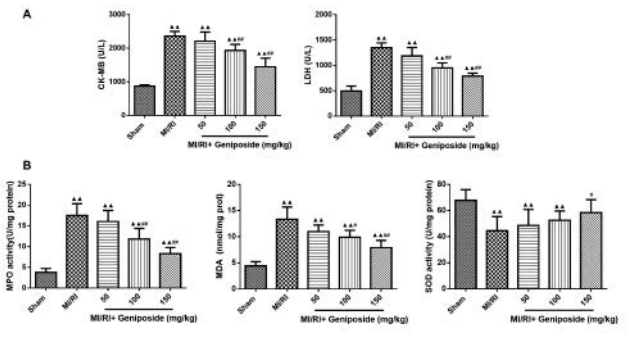

Figure 5. GEN modulated myocardial enzyme and inhibited oxidative stress in MI/RI-induced dysfunction rats. **A**) Content of serum CK-MB and LDH of the myocardial tissues in rats. ($\bar{\chi}$±s, n=6). **B**) Content of MDA and activity of MPO and SOD in the rat myocardial tissues. ▲▲p<0.01 *vs* sham group ($\bar{\chi}$±s, n=6), #p<0.05, ##p<0.01 *vs* MI/RI group.

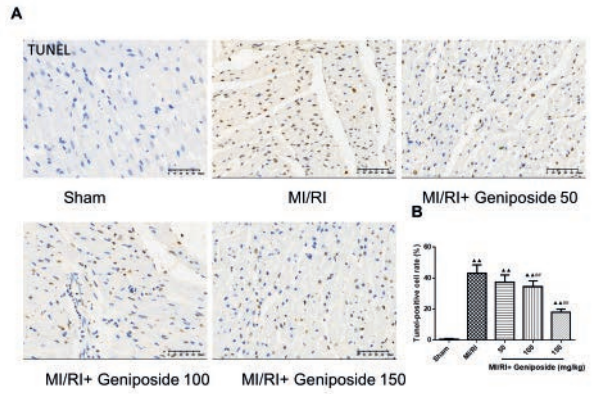

Figure 6. GEN reduces cell apoptosis in MI/RI-induced dysfunction rats. **A**) The TUNEL assay was employed to estimate cell apoptosis of the myocardial tissues in rats, original magnification 400×; scale bar: 50 μm. **B**) Semi-quantitative assessment of the cell apoptosis of the myocardial tissues in rats, ($\bar{\chi}$±s, n=3), ▲▲p<0.01 *vs* sham group, ##p<0.01 *vs* MI/RI group.

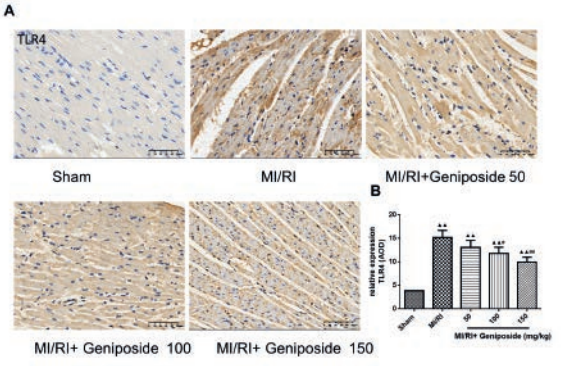

Figure 7. GEN downregulates TLR4 expression in myocardial tissues in MI/RI-induced dysfunction rats. **A**) Representative micrographs of the immunohistochemical staining for TLR4 in the myocardial tissues in rats, original magnification 400×: scale bar: 50 μm. **B**) Quantitative analysis of TLR4 staining of the myocardial tissues in rats ($\bar{\chi}$±s, n=3), ▲▲p<0.01 vs sham group, #p<0.05, ##p<0.01 *vs* MI/RI group.

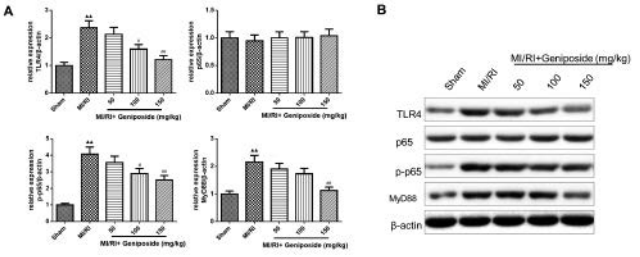

Figure 8. Protein expression of toll-like receptor 4 (TLR4)/nuclear factor-κB (NF-κB) (p65) signaling pathway in myocardial tissues in MI/RI-induced dysfunction rats. **A**) Representative immunoblots of samples from rat ventricles subjected to different treatment groups of the myocardial tissues in rats. **B** Quantitative densitometric analysis of TLR4, p65, p-p65 and MyD88 protein of the myocardial tissues in rats with β-actin as an internal standard ($\bar{\chi}$±s, n=3). β-actin was used as a control, ▲▲p<0.01 *vs* sham group, #p<0.05, ##p<0.01 *vs* MI/RI group.

Administration of GEN significantly decreased TLR4, p-p65 and MyD88 expression compared with MI/RI group at high and moderate dose group ($p<0.05$ or $p<0.01$), and no significant changes in low dose group.

Discussion

An increasing body of research highlights that ischemia-reperfusion is a critical contributor to the suboptimal therapeutic outcomes and prognosis in patients with cerebral ischemia. Key factors such as stress response, Ca^{2+} metabolic imbalance, acid-base disturbances, and inflammatory response during reperfusion lead to structural alterations in heart tissue and functional and metabolic abnormalities, ultimately causing myocardial apoptosis.[15-17]

It is widely accepted that reperfusion injury is more severe than ischemic injury due to the substantial production of reactive oxygen species (ROS) during the reperfusion process.[18] In this study, we assessed MDA which is an end product of lipid peroxidation generated by ROS levels to estimate the level of ROS.[19] Physiologically, low levels of ROS are produced, and the molecule plays an essential role in numerous signaling pathways. However, MI/RI results in ROS overproduction, leading to oxidative stress in the pathologic situation.[20] GEN has been associated with antioxidative properties and cardioprotective actions, with its role in inhibiting oxidative stress and inflammation being well-established.[21] Our results showed that GEN downregulates the MDA level which is highly expressed in the MI/RI rats, suggesting that GEN's cardioprotective effect might be due to the attenuation of lipid peroxidation in MI/RI rats. SOD is considered a major enzyme that acts as a free radical scavenger, preventing ROS generation. The findings revealed that SOD activities in the myocardium were significantly increased in treated with GEN compared to the MI/RI group. This further confirms that GEN protects against MI/RI *via* the mitigation of oxidative stress.

Inflammation plays a crucial role in MI/RI. Zhao *et al.*[22] observed that serum inflammatory factor levels in patients with myocardial infarction are significantly higher than those in healthy individuals, greatly affecting disease progression. Pro-inflammatory regulators contribute to MI/RI-induced inflammation, and ATPase levels are closely related to intracellular mitochondrial redox processes. Aggregation and infiltration of inflammatory cells as well as the releasement of the inflammatory cytokine are essential steps in inflammation.[23] MPO activity in the myocardium can serve as an indicator of inflammation concerning neutrophil infiltration.[24] Shen *et al.* [25] demonstrated that GEN enhances oxidative stress response while downregulating inflammation in mice. Our study supports this conclusion, showing that GEN pretreatment significantly reduces MPO activity, thus alleviating MI/RI in rats. The anti-inflammatory effect of GEN is validated by the results of H&E staining.

TLR4, a key inflammatory cytokine, plays a crucial role in initiating MI/RI-induced inflammation. Study has illustrated GEN's anti-inflammatory effects on antidiabetic actions,[26] pancreatitis,[27] and traumatic brain injury,[28] among others. However, the mechanism of action in MI/RI remains unclear. we analyzed that TLR4 expression was significantly decreased in the ischemic area after treatment with high and moderate doses of GEN compared to the MI/IR group, suggesting that GEN can reduce TLR4 expression in myocardial infarction.

Furthermore, we investigated the role of TLR4-mediated NF-κB signaling in GEN group of myocardial tissues. TLR4 is a receptor that binds to endotoxins and activates downstream pathways, including MyD88, NF-κB, and MAPK,[29] which stimulate the production of pro-inflammatory cytokines and chemokines. Previous study has shown that GEN can inhibit inflammatory cell infiltration, reduce IL-1β and TNF-α release, and suppress TLR4 expression and NF-κB activation[30]. These results suggest that GEN can protect hepatocytes from acetaminophen hepatotoxicity by inhibiting the TLR4/NF-κB signaling pathway. Similarly, the TLR4/NF-κB signaling pathway has been implicated in attenuating inflammation caused by I/R injury,[4] with p65 and p-p65 involved in MI/RI. MyD88 is an anchor protein that connects TLR with downstream signaling pathways.[31] In our study, we found that TLR4, MyD88, and p-p65 expression were enhanced in the MI/RI rats than sham rats, but treatment with moderate and high doses of GEN led to significant declines in these protein levels, indicating that GEN can reduce inflammation by inhibiting the expression of TLR4, MyD88, and p-p65 in an MI/RI model.

To summarize our findings, we have demonstrated that GEN has the potential to protect the heart from MI/RI by curbing cardiomyocyte apoptosis, diminishing neutrophil infiltration, enhancing SOD activity, and reducing MDA content and MPO activity. Further, our research identified that GEN can mitigate inflammation by restraining the expression of TLR4, MyD88, and p-p65 in the MI/RI model. This investigation accentuates the promise that traditional Chinese herbs, like GEN, hold as a resource for innovative pharmaceuticals for cardiovascular conditions. However, the pre-treatment approach to a clinically viable therapy requires further investigation. In future research, it would be insightful to examine the efficacy of GEN when administered after the onset of MI/RI, which could better emulate a real-world clinical scenario.

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