

## Original Article

# Protective effect of ginsenoside Rh3 on myocardial ischemia-reperfusion injury in rats by regulation of p38 MAPK/caspase-3 signaling pathway

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**Abstract:** Objective: To investigate the protective effect of ginsenoside Rh3 on myocardial ischemia-reperfusion injury (MIRI) in rats by regulation of the p38 mitogen-activated protein kinase (MAPK)/caspase-3 signaling pathway. Methods: Fifteen male SD rats were randomly and equally divided into a myocardial ischemia-reperfusion group (MY group), a sham operation group (SS group), and a ginsenoside Rh3 treated group (GR group). The myocardial infarction size, the degree of myocardial tissue lesioned, cell proliferation, cell apoptosis, the expression level of p38 MAPK, and caspase-3 mRNA were respectively analyzed. Results: The myocardial infarction size in GR group was significantly decreased when compared with that in the MY group ( $21.68 \pm 6.17\%$  vs  $53.93 \pm 4.14\%$ ,  $P < 0.05$ ). The degree of myocardial tissue lesions in the GR group was lower than that in the MY group ( $P < 0.05$ ). Compared with the MY group, the proliferation of cardiomyocytes in the GR group was quicker ( $P < 0.05$ ). In addition, the apoptotic cardiomyocytes in the GR group was significantly less than that in the MY group ( $26.97 \pm 3.75\%$  vs  $61.35 \pm 6.38\%$ ,  $P < 0.05$ ). Moreover, the expression level of p38 MAPK and caspase-3 mRNA in the GR group was lower than that in the MY group (both  $P < 0.05$ ). Conclusion: Ginsenoside Rh3 may be a protective effect on MIRI in rats by regulation of the p38 MAPK/caspase-3 signaling pathway.

**Keywords:** Ginsenoside Rh3, p38 mitogen-activated protein kinase, myocardial ischemia-reperfusion injury, caspase-3

## Introduction

In clinical practice, myocardial ischemia-reperfusion is one of the most common complications in patients with heart disease. When the blood supply of ischemic myocardial tissue in patients with myocardial ischemia-reperfusion recovers suddenly, it results in myocardial tissue injury [1, 2]. Due to a decline in physical functions and other problems, the incidence of heart disease in the middle-aged and elderly population is significantly higher than that in adolescents. Both coronary artery and coronary heart disease, which can lead to heart failure, cardiac arrest, and even sudden death, are major heart diseases [3]. Myocardial ischemia is a common complication of these different heart diseases. It has been found that the damaged myocardium can be repaired through special treatment. Myocardial ischemia-reper-

fusion, which can reduce the mortality rate caused by myocardial ischemia, is one of these predominant methods. However, it may bring about other diseases like reperfusion arrhythmia [4]. As the king of herbs in Chinese medicine, ginseng is with wide medical applications [5]. The main component of ginseng is ginsenoside, which helps to prevent cell apoptosis, cell senescence and so on. Ginsenoside plays an important role in the treatment of chronic diseases, especially myocardial ischemia-reperfusion [6]. p38 mitogen-activated protein kinase (MAPK) is a signal transduction pathway regulated by extracellular signals. It was found that the p38 MAPK/caspase-3 signaling pathway is widely present in various animal models and cell lines and plays an important role in regulating gene expression and cell cycle control [7-9]. Here, the myocardial infarction size, the degree of myocardial tissue lesions, cell

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proliferation, cell apoptosis, the expression level of p38 MAPK and caspase-3 mRNA were separately analyzed. Comparisons were conducted among the three groups to investigate the protective effect of ginsenoside Rh3 on myocardial ischemia-reperfusion injury (MIRI) in rats by regulation of p38 MAPK/caspase-3 signaling pathway.

### Materials and methods

#### *General information*

Fifteen male Sprague-Dawley (SD) rats weighing 250-300 g and six 1-3-day-old SD pups were purchased from Guangdong Medical Laboratory Animal Center. All rats were allowed to have one week to acclimate to the new environment. This study was approved by the Animal Ethics Committee of Dezhou People's Hospital.

#### *Modeling and grouping*

SD rats were randomly and equally divided into three groups: the first was myocardial ischemia-reperfusion group (MY group), rats in this group were modeled with myocardial ischemia-reperfusion; the second was sham-operated group (SS group), rats in this group were not treated after opening the chest; the last group was the ginsenoside Rh3 treated group (GR group), rats in this group received ginsenoside Rh3 therapy after modeling. There were 5 rats in each group.

These rats were anaesthetized by intraperitoneal injection after fasting for 12 hours. Thereafter, rats were positioned belly up. Fur on the left chest was removed and disinfection (75% alcohol) was performed on the chest. The surgery began from the space between the 4th and 5th ribs. The hearts were gently taken out after fixing the chest opener in an exposed and broadened intercostal space. Hearts in SS group were threaded without ligation. However, hearts in both MY group and GR group were ligated and monitored with an electrocardiogram. Successive increase of the ST-segment in the electrocardiogram and lifeless myocardium under the ligature indicated that the ligation was successful. After ligating for 30 min, the ligation was unfastened for myocardial reperfusion, which lasted for 120 min. The decreased ST-segment suggested that reperfusion was successful. After operation, the hearts were

carefully replaced and skin was sutured. Cardiopulmonary resuscitation was performed until rats could breathe smoothly. Rats were then provided with unlimited intake of food and fluids. Rats in the GR group were treated with intragastric administration of 0.5 mg/kg/d ginsenoside Rh3 once a day. The administration lasted for 2 weeks. At the same time, rats in the SS group received intragastric administration of normal saline. At the end of the experiment, rats in the three groups were decapitated and cardiomyocytes were collected for further study.

Hearts of rat pups were taken out under aseptic conditions. After washing with PBS, hearts were cut into pieces in serum free DMEM medium. The mixture of 1 mg/mL collagenase I and 0.25% trypsin (1:1) were used for the digestion of cardiomyocytes. In total, pieces of the hearts were digested 4 times at 37°C (15 min). At the end of each digestion, the cell suspension was harvested and provided with DMEM medium supplied with 10% serum to terminate the digestion. The cell suspension was centrifuged at 100 g for 10 min. The cell pellet was then seeded and cultured. Purified cardiomyocytes were obtained through careful collection of the non-adherent cells 2 h later. These cells were seeded at a density of  $5 \times 10^4$  cells/cm<sup>2</sup>. After culturing for 24 h, cardiomyocytes supplied in a sugar and serum free medium were incubated in an anoxic chamber for 2 h. Cardiomyocytes were then provided with only normal medium (MY group) or 0.5 mg ginsenoside Rh3 supplied normal medium (GR group), and cultured in a negative oxygen incubator for 2 h. In addition, cardiomyocytes incubated with normal oxygen conditions with normal medium was chosen as the control group (SS group). Cardiomyocytes in each group were collected for experiments concerning cell proliferation, cell apoptosis, the expression level of p38 MAPK and caspase-3 mRNA.

#### *Instruments and reagents*

2,3,5-triphenyltetrazolium chloride (TTC) dye (Sigma, USA); CCK-8 kit (Beijing Jiehui Bogao Biotechnology Co., Ltd., China); annexin-V-FITC kit (Biovision, USA); HEPES buffer solution (Procell, China); ginsenoside Rh3 (Sichuan Victory Biotechnology Co., Ltd., China); secondary anti-rabbit IgG (Beijing Bioss Biotechnology Ltd., China); GADPH (Abcam, UK); rabbit mono-

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**Table 1.** Primer sequence

Primer name	Primer sequence
Forward primer for caspase-3	5'-TGCGGTAGCAGCACATAATGG-3'
Reverse primer for caspase-3	5'-CCAGTGCAGGGTCCGAGGT-3'
Forward primer for GAPDH	5'-TGAACGGGAAGCTCACTGG-3'
Reverse primer for GAPDH	5'-TCCACCACCTGTTGCTGTA-3'

**Table 2.** The size of infarcted myocardialon (%)

Group	Rats (n)	Myocardial infarction size (%)	F value	P value
SS group	5	0	9.705	<0.001
GR group	5	21.68±6.17%*		
MY group	5	53.93±4.14%*#		

Note: Compared with SS group, \*P<0.05; compared with GR group, #P<0.05.

clonal antibodies against p38 MAPK (Abcam, UK); hematoxylin (Shenzhen Ziker Biotechnology Co., Ltd., China); chloroform (INEOS, USA); isopropanol (Jinan Guangyu Chemical Co., Ltd., China); ethanol (Dezhou Tongde Disinfection Products Technology Co., Ltd., China); constant temperature shaker (Chengdu Sujing Kexue Equipment Company, China); automated chemiluminescence imaging analysis system (Bio-RAD, USA).

### Myocardial infarct size

Rat hearts in the three groups were obtained and transected into 4-5 slices with a thickness of 2 mm. These slices were placed into 1% TTC buffer and incubated at room temperature for 10 min. After staining, viable and dead cardiomyocytes were displayed with different colors. Representative photos were taken when the staining was completed. Percentage of myocardial infarction size (MIS %) = (infarct size/total size) \* 100% was calculated using image analysis software (image pro plus).

### Degree of myocardial tissue lesions

Rat myocardial tissues in the three groups were embedded in paraffin after fixing in 4% formaldehyde for 15 min. Tissues embedded in paraffin were sliced at 5 µm. Thereafter, they were stained using hematoxylin and eosin staining solution.

### Cardiomyocyte proliferation

Cardiomyocytes in the three groups were harvested and resuspended in a concentration of

1×10<sup>5</sup>/mL. After culturing for 12 h, these cells were seeded into 96-well plates and cultured. At the end of cultivation, 10 µL CCK-8 solution was added into the cells. The optical density (OD) value at 450 nm was recorded with a plate reader 30 min later.

### Cardiomyocyte apoptosis

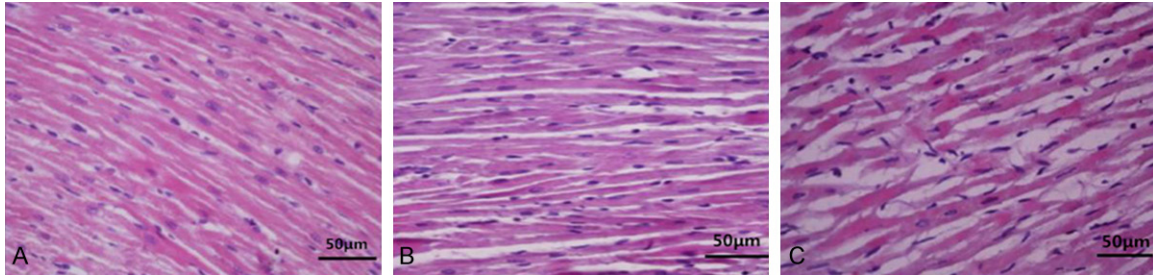
Cardiomyocyte apoptosis was detected using annexin-V-FITC kit. Annexin-V-FITC/PI staining solution was made of annexin-V-FITC, PI, and HEPES buffer solution in a ratio of 1:2:50. A volume of 100 µL cardiomyocyte suspension, which were resuspended in the staining solution at the concentration of 1×10<sup>7</sup>/mL, were incubated on a shaker at room temperature for 15 min. After that, 1 mL HEPES buffer solution was added and evenly dispersed in the suspension. The band-pass filters were excited at 488 nm to detect the presence of FITC (525 nm) and PI (620 nm) separately. Apoptotic cardiomyocytes were then identified.

### Expression level of p38 MAPK

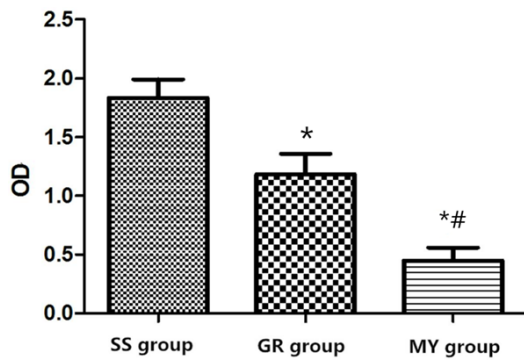
Cardiomyocytes lysates were separated by SDS/PAGE and transferred onto polyvinylidene fluoride (PVDF) membrane. Proteins were labeled with primary antibodies as follows: rabbit monoclonal antibodies against p38 MAPK (1:500), and GAPDH (1:1,000). The membrane was detected by secondary anti-rabbit IgG (1:2,000) conjugated to peroxidase using automated chemiluminescence imaging analysis system. The quantity of each band was determined using optical density analysis. The relative expression level of target protein was calculated as the ratio of optical density in the target band to the internal reference band.

### Expression level of caspase-3 mRNA

Total RNA samples of cardiomyocytes were extracted using the classical chloroform/isopropanol/ethanol method. The reverse transcription of RNA was performed according to the manufacturer's instructions. Quantitative real-time PCR was subsequently conducted in triplicate with specific primers (Table 1) and 2 \* Hot Start SYBR Green qPCR Master Mix in a PCR machine. The relative expression levels were normalized against the internal control



**Figure 1.** Myocardial tissue (200×). A. The degree of myocardial tissue lesions in the SS group; B. The degree of myocardial tissue lesions in the GR group; C. The degree of myocardial tissue lesions in the MY group.



**Figure 2.** Cardiomyocyte proliferation. Compared with SS group, \* $P < 0.05$ ; compared with GR group, # $P < 0.05$ .

(GAPDH). The real-time qPCR was set as following: 95°C, 10 min; 95°C, 15 s; 60°C, 30 s; 72°C, 30 s; 95°C, 5 min. The number of cycles was 40. Expression of caspase-3 mRNA was relatively quantified using 2- $\Delta\Delta CT$ .

#### Statistical methods

All data were analyzed using SPSS statistical software version 22.0. The myocardial infarction size, the degree of myocardial tissue lesions, cell proliferation, cell apoptosis, the expression level of p38 MAPK and caspase-3 mRNA in each group were analyzed by analysis of variance (ANOVA). The difference was statistically significant when  $P$  value was less than 0.05.

## Results

### Myocardial infarction size

As displayed in **Table 2**, there was no clear myocardial infarction in the SS group; the size of the myocardial infarction in the MY group was the largest. The myocardial infarction size

in the GR group was significantly decreased when compared with the MY group ( $21.68 \pm 6.17\%$  vs  $53.93 \pm 4.14\%$ ,  $P < 0.05$ ).

### Degree of myocardial tissue lesions

The myocardial fibers in the SS group were well arranged. The myocardial fibers in the MY group were severely broken, and a large amount of cytoplasm was swollen. The myocardial fibers in the GR group were disordered, and a small amount of cytoplasm appeared in the myocardium. It was clear that the degree of myocardial tissue lesions in the GR group was better than in MY group (**Figure 1**).

### Cardiomyocyte proliferation

As illustrated in **Figure 2**, the proliferation of cardiomyocytes in the SS group was the fastest while it was the slowest in the GR group. Moreover, cardiomyocytes in the GR group proliferated significantly faster than that in the MY group ( $P < 0.05$ ).

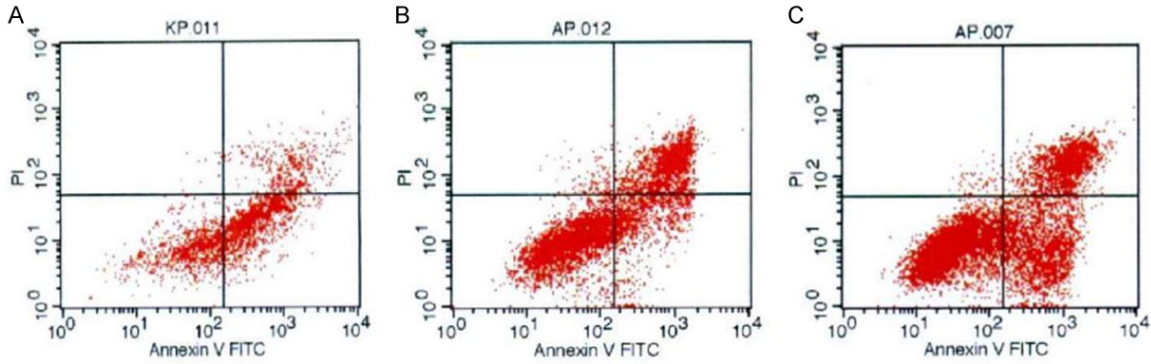
### Cardiomyocyte apoptosis

The number of apoptotic cardiomyocytes in the MY group was the most while it was the least in the SS group. Compared with the MY group, the number of apoptotic cardiomyocytes in the GR group was significantly reduced when compared with the MY group ( $26.97 \pm 3.75\%$  vs  $61.35 \pm 6.38\%$ ,  $P < 0.05$ ). Details were shown in **Figure 3**.

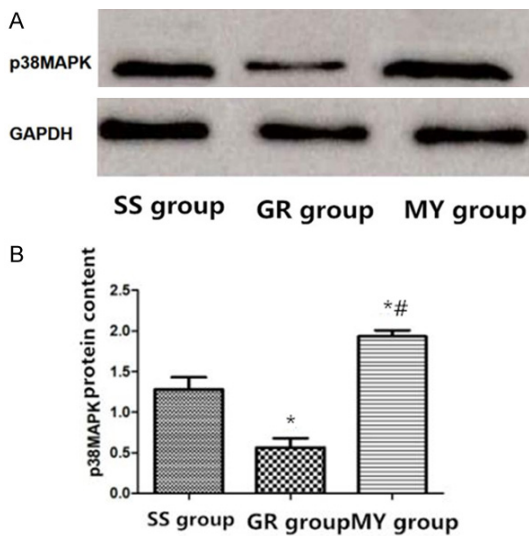
### Expression level of p38 MAPK

As shown in **Figure 4**, the expression level of p38 MAPK in the MY group was the highest while it was the lowest in the GR group. It means that the expression level of p38 MAPK in the





**Figure 3.** Cardiomyocyte apoptosis. A. Apoptotic cardiomyocyte in the SS group; B. Apoptotic cardiomyocyte in the GR group; C. Apoptotic cardiomyocyte in the MY group.



**Figure 4.** The expression level of p38 MAPK. A. The expression level of p38 MAPK; B. Relative quantity of p38 MAPK. Compared with SS group, \* $P < 0.05$ ; compared with GR group, # $P < 0.05$ .

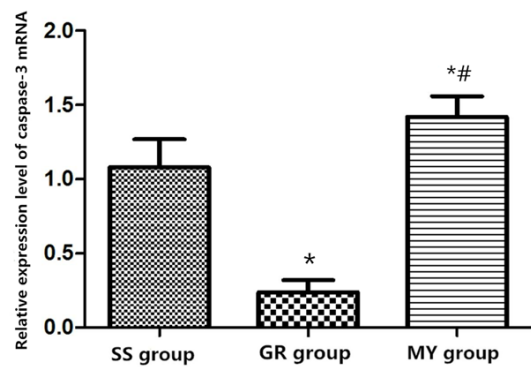
GR group was decreased when compared with either the MY group or the SS group (both  $P < 0.05$ ).

*Expression level of caspase-3 mRNA*

As displayed in **Figure 5**, the expression level of caspase-3 mRNA in the GR group was the lowest while the highest in the MY group. It indicates that the expression level of caspase-3 mRNA in the GR group was lower than that in either the MY group or the SS group (both  $P < 0.05$ ).

**Discussion**

MIRI refers to sudden reperfusion of blood supply of ischemic myocardial tissue, resulting



**Figure 5.** Relative expression level of caspase-3 mRNA. Compared with SS group, \* $P < 0.05$ ; compared with GR group, # $P < 0.05$ .

in a larger size of myocardial infarction [10]. This complication was commonly observed in patients with myocardial ischemia, which was caused by various diseases, like myocardial infarction, and coronary arteriosclerosis, etc. [11]. In recent years, an increased incidence of ischemic heart diseases was observed among the middle-aged and elderly population. There are many treatments for ischemic cardiomyopathy, such as drugs and surgery. Among them, surgeries are one of the most effective therapies [12]. However, the therapeutic effects in some patients are not satisfying. The reason is that MIRI is prone to reoccur after treatment, causing another injury to the heart. Therefore, it is urgent to find an effective method to reduce MIRI during treatment [13].

It was reported that the p38 MAPK signaling pathway plays an important role in heart diseases, like myocardial ischemia-reperfusion, coronary heart disease, and myocardial infarction.

tion. What's more, hypertension is also associated with the activation the p38 MAPK signaling pathway [14, 15]. Wang et al. reported that the p38 MAPK signaling pathway was activated in rats with cerebral infarction. The myocardial infarction size was increased, and was accompanied by a large number of apoptotic neurons. These results indicate that active p38 MAPK signaling pathway contributes to the incidence of cerebral infarction [16]. There were reports on the relationship between the expression level of caspase-3 mRNA and ischemic lesions in patients treated with drugs for ischemic limbs. The expression level of caspase-3 mRNA corresponded positively to the amount of apoptosis in neurons. In addition, it was found that ginsenoside Rh3 could reduce neuronal apoptosis by reducing the expression level of caspase-3 mRNA [17, 18]. Zhang et al. confirmed that ginsenoside Rh3 could inhibit the p38 MAPK signaling pathway, resulting in the improvement of the occurrence, development, and relapse of the disease [19]. Ginsenoside Rh3, which displays therapeutic effects on myocardial ischemia-reperfusion in rats, could significantly inhibit the formation of thrombus and reduce the apoptosis of cardiomyocytes [20-22]. In our study, the myocardial infarction size in the GR group was significantly decreased when compared with the MY group. The degree of myocardial tissue lesions in the GR group was lower than that in the MY group ( $P < 0.05$ ). Compared with the MY group, the proliferation of cardiomyocytes in the GR group was quicker ( $P < 0.05$ ). The apoptotic cardiomyocytes in the GR group was significantly less than that in the MY group ( $P < 0.05$ ). Moreover, the expression level of p38 MAPK and caspase-3 mRNA in the GR group was lower than that in the MY group ( $P < 0.05$ ).

As a result of insufficient time and other problems, the protective effect of ginsenoside Rh3 on MIRI in rats was not studied thoroughly. In order to provide a more thorough reference for the therapeutic effect of ginsenoside Rh3 on MIRI, subsequent studies will concentrate on the comprehensive assessment of ginsenoside Rh3 on MIRI and the mechanism study of ginsenoside Rh3 on the expression level of p38 MAPK and caspase-3 mRNA.

In summary, ginsenoside Rh3 may have a protective effect on MIRI in rats by regulation of p38 MAPK/caspase-3 signaling pathway.

### Disclosure of conflict of interest

None.

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