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Original article

Attenuation of Myocardial ischemia reperfusion injury by Geniposide preconditioning in diabetic rats

Jia wang[a,1], Xie De-qiong[c,1], Da-qing Hong[b], Qing-qiong Zhang[b], Jiong Zhang[b,*]

[a] General Practice Center of Electronic Science and Technology, Sichuan Academy of Sciences & Sichuan Provincial People's Hospital, Chengdu, 610072, China
[b] Department of Nephrology, Electronic Science and Technology, Sichuan Academy of Sciences & Sichuan Provincial People's Hospital, China
[c] Department of Nephrology, Yibin Second People's Hospital, China

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ABSTRACT

Studies have shown that the NRF-2 /HO-1 pathway participates in myocardial ischemic reperfusion injury (MI/R) and that Geniposide (GEN) could protect the myocardial against MI/R. This study aims to examine the protective effects of GEN on MI/R in diabetic rats and further explore the possible mechanism of action. During MI/R in rats, NRF-2 /HO-1signals changed significantly including NRF-2 and HO-1up-regulation, resulting in heart dysfuction, histological damage and increasing oxidative stress and cell apoptosis. Treatment with GEN can significantly improve the general condition and heart function in diabetic rats with decreasing the expression of cTnI, CK-MB, blood glucose, MDA, ROS, cell apoptosis and pathological damage in MI/R. In addition, GEN precondition can also significantly increase the weight of rats and the activity of SOD, CAT and GPx with up-regulating the expression of NRF-2 and HO-1 in MI/R. This study implied that Geniposide has a protective effect on myocardial ischemia reperfusion injury in diabetic rats, and its mechanism is associated with activating NRF2/HO-1 signaling pathway to suppress oxidative stress.

1. Introduction

Diabetes mellitus is an important risk factor for cardiovascular disease. The incidence rate of ischemic cardiomyopathy on diabetic patients is about four times more than that of non-diabetic patients. Moreover, the prognosis of diabetic patients is worse and the fatality rate is higher [1]. Previous studies have shown that diabetic patients suffering from myocardial ischemia reperfusion injury leads to more serious impairment of heart function, higher incidence of myocardial infarction and heart failure [1,2]. Unfortunately, the current treatment for this disease is either ineffective or has side effects. Therefore, it is an important and urgent subject to seek further more effective and minor side- effect therapy to treat this disease. It is a pity that the pathologic mechanisms of the disease is still not yet elucidated so far. Most of study displayed oxidative stress play a vital role in it. Therefore. Suppressing oxidative stress may be a promising measure to lessen cardiac ischemic reperfusion injury in diabetic patients.

Geniposide, also known as geniposide, is a cyclic iridoid glycoside. It is a monomer extracted from Gardenia jasminoides [3]. It has been demonstrated that GEN has multiple pharmacological activities including regulating immunity, reducing apoptosis, suppressing oxidative stress, etc. [3,4]. Moreover, there is a study demonstrated that GEN can attenuate organ ischemia reperfusion injury [5]. Therefore, this study is to confirm the protective effect of geniposide on myocardial ischemia reperfusion injury in STZ-induced diabetic rat and its mechanism on oxidative stress.

2. Methods

2.1. Animals

The animal experiments were performed according to the guidelines of laboratory animal care and were authorized by the Institutional Animal Care and Use Committee of the West China Hospital of Sichuan University.

Thirty male Sprague-Dawley (SD) rats, weighing 200–220 g at 6-8weeks age were purchased from the Laboratory Animal Center of Sichuan University and housed in 22 °C air-conditioned room with a 12 h light/dark cycle and free access to drinking water and having a standard diet.

2.2. Regent

Geniposide was purchased from Shanghai Chunyou Biotechnology Company in China. Streptozotocin was obtained from

* Corresponding author.
E-mail address: zhangjiong831224@163.com (J. Zhang).
[1] Jia wang and Xie De-qiong are equal contribution to the article.

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Sigma-Aldrich (MO, USA). Kits for detecting cardiac troponin I (cTnI), creatine kinase isozyme (CK-MB), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reactive oxygen species (ROS), cell apoptosis and malondialdehyde (MDA) were purchased from Nanjing built biotech company. Primary antibodies against nuclear factor transcription factor-2 (NRF-2), heme oxidase-1 (HO-1) (CST, USA), and β-actin were all obtained from Cell Signaling Technology (Boston, MA, USA). HE staining Color reagent was provided by pathology department of West China Hospital, Sodium pentobarbital, protein extraction kit and Blood sugar test paper were all obtained from Wuhan Google Biology.

2.3. Establishment of a T2D rat model

T2D rat model was induced as previously described [6]. 30 SD rats were intraperitoneally injected with 1% streptozotocin (55 mg/kg) dissolved in citrate buffer. After 72 h, blood was collected through tail vein and blood glucose was measured by blood glucose meter. If the blood glucose persisted for more than 16.7 mmol/L for one week and accompanied by obvious excessive drinking and eating, polyuria symptoms showed that T2D rat model was successfully constructed. 30 II diabetic rats were successfully constructed.

2.4. Grouping II diabetic rats

T2D rat were randomly equally divided into the following 3 groups (n = 10). (1) sham operation group (Sham), myocardial ischemia reperfusion injury group (MI / R) and geniposide group (GEN). The rats in GEN Group were preconditioned with gardeniside by intragastric administration (100 mg/kg) before, once a day for 7 days. Sham group and MI / R group were given the same volume of saline by intragastric for 7 days, once a day. The specific dosage and mode of administration are in line with reference [7].

2.5. MI/R protocol

The MI/R injury model was constructed as previous description. T2D Rat were anesthetized with pentobarbital sodium (50 mg/kg) and fixed in supine position. ECG was recorded and monitored in each group. Tracheal intubation was used to connect the ventilator to assist the rats breathing. Moreover, the body temperature of the rats were maintained at 37 °C by use of a heated operating table. The chest of theT2D Rat were opened via a left lateral thoracotomy. And the left anterior descending branch of the coronary artery was ligated with silk thread to induce ischemia for 30 min, followed by 4 h reperfusion. Ischemia was demonstrated by observing Color changes in the ischemic area of myocardial tissue and reperfusion was achieved via loosening the ligature. The rats in Sham group were operated as the same as the above, but without ligating left anterior descending branch of the coronary artery.

2.6. Evaluating myocardial systolic and diastolic function

The left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP) and left ventricular maximal systolic/diastolic velocity ($\pm$ dp/dtmax) of the rats in all group were measured by physiological recorder following repefusion.

2.7. Assessing blood glucose, body weight, cell apoptosis and relative content of ROS in rats

Following reperfusion, blood glucose was measured by blood glucose meter and body weight was measured by body weight meter. TUNEL was used to evaluate the cell apoptosis. DCFH-DA fluorescence staining was used to determine the content of ROS in myocardium. At the end of reperfusion, the myocardial tissue was washed with 0.1 mmol/L cold phosphate buffer (PH = 7.4) and dried by filter paper. The myocardial tissue was grinded with 9 times cold saline to prepare 10% tissue homogenate. According to the instructions of ROS kit, the fluorescence intensity of ROS in myocardial tissue was measured by using 488 nm excitation wavelength and 525 nm emission wavelength on a multifunctional enzyme labeling instrument. ROS level (%) = (experimental group OD / normal group OD value) X100.

2.8. Evaluating Mocardial pathology, cell apoptosis and examining acute myocardial injury enzyme

Following reperfusion, abdominal aorta blood was taken from rats in each group. The supernatant was centrifuged at room temperature for 10 min at 3000 r /min. The contents of CK–MB and cTnI in the supernatant were detected by the Laboratory of West China Hospital. Heart was frozen in $-80\,^{\circ}C$ refrigerator. Part of myocardial muscle tissue was fixed with 10% paraformaldehyde, routinely dehydrated, paraffin embedded in paraffin, cut into 4um section, dewaxed and transparent then H&E staining and TUNEL staining in cardiology laboratory. Morphological structure was observed under light microscope and recognized by morphological scoring standard [8]. 0 points: no injury; 1 points: mild injury; interstitial edema and localized necrosis; 2 points (moderate injury): extensive cardiomyocyte swelling and necrosis; 3 points (severe): necrosis with vasoconstriction and atrophy; 4 points (very serious): diffuse vasoconstriction, hemorrhage and myocardial necrosis. TUNEL was used to evaluate the cell apoptosis. Microscopically, the nuclei of normal myocardial cells were blue, and the nuclei of apoptotic cells were brown and yellow. The apoptotic index (AI) was expressed as the percentage of apoptotic cells in the total number of cardiac myocytes, and expressed as the percentage (%).

2.9. Examining the content of MDA and detecting the SOD, CAT and GPx activity in myocardium

At the end of reperfusion, some myocardial tissue was washed with 0.1 mmol/L cold phosphate buffer (PH = 7.4) and dried by filter paper. The myocardial tissue was grinded with 9 times cold saline to prepare 10% tissue homogenate. The activities of SOD, CAT and GPx in the myocardial were detected by Dinitrobenzoic acid method. Moreover, the content of MDA in the myocardial was detected by the method of thiobarbituric acid chromogenic.

3. Western blotting analysis

The myocardial tissue was homogenized followed by collecting supernatants to examine the protein concentrations by the method of BCA. Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. The membranes were then incubated with antibodies against NRF-2(1:500), HO-1 (1:400) and β-actin (1:3000) overnight at 4 °C, followed by incubation with an HRP-conjugated secondary antibody (Abmart, China, 1:3000) at room temperature for 2 h. The reactive bands were visualized by use of the ECL-Plus reagent (Amersham, Piscataway, NJ). The density of band was quantified by the Quantity One analytic software (UVP, USA).

3.1. Statistical analysis

Values are expressed as mean $\pm$ SEM. Group comparisons were processed by using the Student's *t*-test or the one-way analysis of variance (ANOVA) test. In all cases, $P < 0.05$ was considered as statistical significance.

4. Results

4.1. GEN alleviates MI/R-induced change of body weight and blood sugar

As expected, in comparison to the Sham group, MI/R can significantly raise the blood sugar and decrease the body weight of T2D rats ($P < 0.05$). However, GEN can alleviate MI/R-induced change of body weight and blood sugar as shown in Table 1, the GEN group has lower blood sugar and higher body weight than the MI/R group.

4.2. GEN attenuates MI/R-induced acute myocardial injury

To explore the effect of GEN on the acute myodical injury, we examine the expression of CK-MB and cTnI in the serum. Which are widley used as markers of acute myocardial injury. The result displayed that compared with the Sham group, the expression of CK-MB and cTnI in the serum were significantly increased ($P < 0.05$). In addition, we found pretreatment with GEN can remarkably decrease the serum expression of CK-MB and cTnI as demonstrated lower lever of CK-MB and cTnI in the GEN group than in the MI/R group as shown in the Table 2. This result indicated GEN can attenuate MI/R-induced acute myocardial injury.

4.3. GEN reduces MI/R-induced acute myocardial pathological injury

To further ascertain the protective effect of GEN on the MI/R, we evaluate the effect of GEN on the pathological morphology of myocardial. H&E staining showed that in comparison with the Sham group, the MI/R group has more vascular thickening, myocardial fibers swelling, myocardial atrophy and inflammatory cell infiltration which indicated more serious pathological injury and higher pathological injury score ($P < 0.05$) as shown in Fig. 1. As expected, pretreatment with GEN can obviously reduce the pathological injury of myocardial as displayed less vascular thickening, myocardial fibers swelling, myocardial atrophy, inflammatory cell infiltration and myocardial pathological injury score in the GEN group than in the MI/R group. This result indicated GEN can reduce MI/R-induced acute myocardial pathological injury.

4.4. GEN improves MI/R-induced damage of myocardial systolic and diastolic function

Compared with the Sham group, the MI/R group has lower LVEDP, LVSP and ± dp/dtmax absolute value which indicates that MI/R can severely impair the systolic and diastolic function of the heart ($P < 0.05$). But, pretreatment with GEN can obviously improve myocardial systolic and diastolic function as shown in Table 3 higher LVEDP, LVSP and ± dp/dtmax absolute value in the GEN group than in the MI/R group.

4.5. GEN decreases MI/R-induced up-regulation of ROS and cell apoptosis

In order to explore the mechanism involved in the protective effect of GEN in MI/R injury, we examine the content of ROS and the expression of cell apoptosis in the myocardial. The result showed the MI/R group has higher expression of ROS and cell apoptosis in the myocardial than the Sham group and GEN can significantly decrease the MI/R-induced up-regulation of ROS and cell apoptosis as manifested lower expression of ROS and cell apoptosis in the GEN group than in the MI/R group as displayed in Fig. 2.

4.6. GEN suppress the MI/R-induced activation of oxidative stress

Following above, we evaluate the effect of GEN on the oxidative stress in the MI/R. Compared with the Sham group, the MI/R group has higher content of MDA and lower activity of SOD, CAT and GPx in the myocadial than Sham group. However, precondition with GEN can significantly suppress the MI/R-induced activation of oxidative stress as displayed GEN group has lower content of MDA and higher activity of SOD, CAT and GPx in the myocadial than MI/R group in Table 4.

4.7. GEN promotes the MI/R-induced the activation of NRF-2/HO-1signalling

In order to explore the mechanism associated with GEN suppressing oxidative stress, we evaluate the NRF-2/HO-1signal pathway. As shown in Fig. 3, MI/R can induce the activation of NRF-2/HO-1signalling as displayed higher expression of NRF-2 and HO-1in the MI/R group than the Sham group. GEN can further promote the activation of NRF-2/HO-1signal pathway as demonstrated higher expression of NRF-2 and HO-1in the GEN group than in the MI/R group. Moreover, the molecular structure of GEN is as shown in Fig. 4.

5. Discussion

It is a classic way for inducing diabetes in rats by intraperitoneal injection of streptozotocin [6]. In order to better simulate and apply to clinical practice, the diabetic model of rats was established by intraperitoneal injection of streptozotocin as reported in our study. The results showed that the blood glucose of rats continued to be more than 16.7 mmol/L for 1 week after injection of streptozotocin 72 h, accompanied by significant excessive drinking, eating, urinary symptoms and weight loss, which was in line with previous studies [6]. It indicated that the diabetic rat model was successfully established. In addition, we also ligated the left anterior descending branch of the coronary artery to induce the model of MI/R in diabetic rat. The results showed that compared with Sham group, MI/R can increase the blood glucose, the expression of CK-MB and cTnI and the myocardial pathological injury of diabetic rats with decreasing the weight and the function of cardiac systole and diastole in rats. Which implied it is successful to induce the model of MI/R model in diabetic rats. Geniposide pretreatment can significantly reduce the expression of CK-MB and cTnI, the degree of myocardial pathological damage, cell apoptosis and blood sugar with improving cardiac contraction and diastolic function and increasing the weight of rats, which proves that geniposide can alleviate MI/R in diabetic rats.

Table 1
Effect of geniposide on the weight and the blood glucose in diabetic rats (n = 10, $\bar{x} \pm s$).

Group	N	weight(g)	blood sugar (mmol/l)
Sham	10	210 ± 16	18.2 ± 2.2
MI/R	10	185 ± 11**	23.4 ± 3.3**
GEN	10	196 ± 14#	19.1 ± 2.6#

Note: Compared MI/R group with Sham group, ** P<0.01; Compared GEN group with MI/R group, # $P < 0.05$;

Table 2
Effect of GEN on the expression of cTnI and CK-MB in diabetic rats (n = 10, $\bar{x} \pm s$).

Group	N	cTnL(U/L)	CK-MB(U/L)
Sham	10	122 ± 21	160 ± 30
MI/R	10	825 ± 80**	1980 ± 155**
GEN	10	675 ± 55#	920 ± 80#

Note: Compared MI/R group with Sham group,** P<0.01; Compared GEN group with MI/R group, # $P < 0.05$;

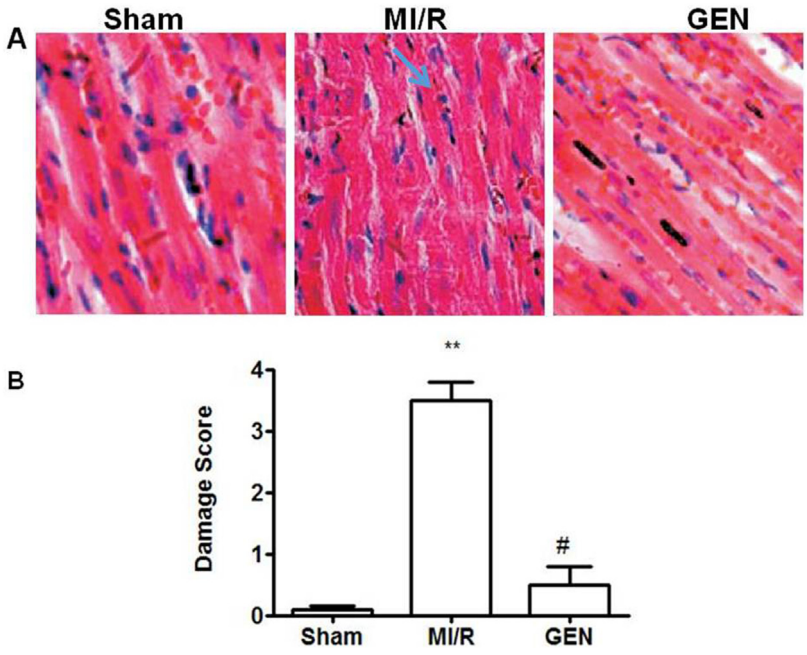

Fig. 1. Effect of GEN on pathologica injury were evaluted by HE staining(n = 10, $\bar{x} \pm s$).
Representative microphotographs of HE staining, original magnification × 40;(B) Semi-quantitative assessment of the histological lesions, $^{**}P < 0.01$ (MI/R vs. Sham); $^{\#}P < 0.05$ (GENvs. MI/R).

Table 3
Effect of GEN on the cardiac systolic and diastolic function in diabetic rats (n = 10, $\bar{x} \pm s$).

Group	N	LVSP (KPa)	LVEDP (KPa)	dp/dtmax(KPa/s	-dp/dtmax(KPa/s)
Sham	10	20.1 ± 2.6	1.5 ± 0.4	1470 ± 180	-1450 ± 450
MI/R	10	$6.2 \pm 1.1^{**}$	$5.6 \pm 0.9^{**}$	$750 \pm 110^{**}$	$-650 \pm 200^{**}$
GEN	10	$16.6 \pm 2.2^{\#}$	$3.2 \pm 0.7^{\#}$	$950 \pm 150^{\#}$	$-950 \pm 250^{\#}$

Note: Compared MI/R group with Sham group, $^{**}P<0.01$; Compared GEN group with MI/R group, $^{\#}P<0.05$;

Recent studies show that excessive oxidative stress plays an important role in the occurrence and development of diabetes mellitus [9]. Excessive ROS from overproduction and scavenging blocked in MI/R causes to reduce the activity of phospholipid bilayer peroxidation of myocardial cell membrane by peroxidation, then resulting in myocardial damage, and even heart function decline [9]; Excessive ROS can destroy the mitochondrial membrane of myocardial cells, Which can lead to myocardial damage by activation of mitochondrial apoptosis pathway. In addition, there is active oxidative stress in diabetic patients per se and MI/R may further aggravate oxidative stress in diabetic patients, which resulted in further decreasing the activities of antioxidants such as SOD, CAT and GPx and increasing the contents of oxidant-promoting substances such as MDA in the myocardium, then further aggravating myocardial damage [1,9]. As we know, the activity of SOD, CAT and GPx can also be used as an objective index to evaluate the ability of scavenging activity (ROS) and the content of MDA can indirectly reflect the degree of damage to tissue and cells. Therefore, the activity of SOD, CAT and GPx and the content of MDA and ROS in myocardium

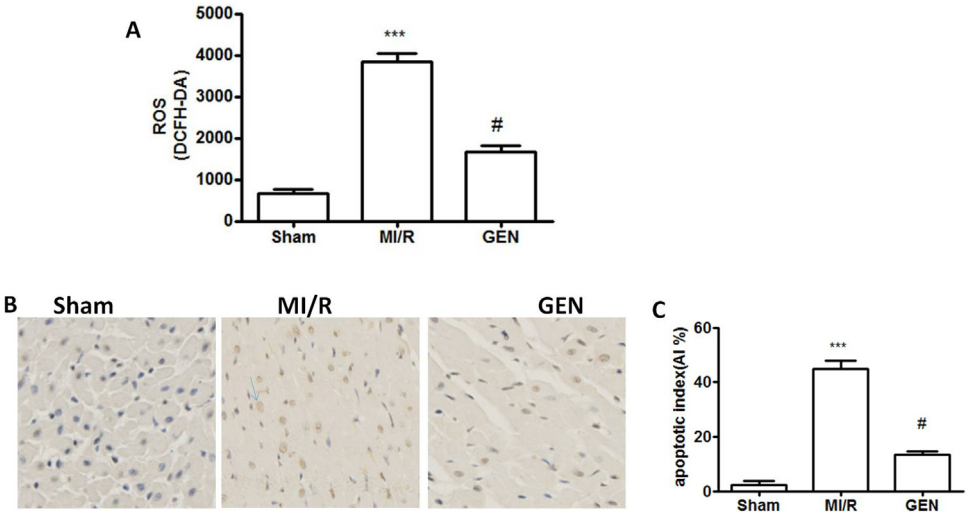

Fig. 2. Effects of GEN pretreatment on the expression of ROS and cell apoptosis(n = 10, $\bar{x} \pm s$).
(A)DCFH was employed to assess the expression of ROS in the myocardial; (B) TUNEL was employed to the expression of cell apoptosis, original magnification × 400; (C) Semi-quantitative assessment of the cell apoptosis $^{***}P < 0.001$ (MI/R vs. Sham); $^{\#}P < 0.05$ (GENvs. MI/R).

Table 4
Effect of GEN on the expression of MDA and the activity of SOD, CAT and GPx in diabetic rats ($\bar{x} \pm s, n = 10$).

Group	N	SOD(U/ml)	CAT(U/ml)	GP_X(U/mg)	MDA(μmol/l)
Sham	10	142.3 ± 16.6	112.1 ± 16.2	10.2 ± 1.7	15.2 ± 2.2
MI/R	10	91.5 ± 11.3**	76.2 ± 9.5*	4.6 ± 0.5*	32.2 ± 4.6**
GEN	10	132.2 ± 13.1#	101.2 ± 12.1#	7.8 ± 1.3#	21.5 ± 2.8#

Note: Compared MI/R group with Sham group, ** $P<0.01$; Compared GEN group with MI/R group, # $P<0.05$;

can be used to evaluate the level of oxidative stress. The results showed that compared with Sham, the contents of MDA and ROS in MI/R group were significantly increased and the activities of SOD, CAT and GPx in myocardium were significantly decreased, which further confirmed that ROS-mediated oxidative stress played an important role in MI/R in diabetic rats. Gen can significantly decrease the contents of MDA and ROS with obviously increasing the activities of SOD, CAT and GPx. Which implied that geniposide has a protective effect on MI / R in diabetic rats and its mechanism is related to inhibiting ROS-mediated oxidative damage, enhancing the antioxidant capacity of the body, and maintaining the antioxidant and oxidative balance of the body.

NRF-2 /HO -1 signaling pathway is the central antioxidant stress response pathway [10]. Oxidative stress can not only promote the transcription of NRF-2 mRNA and the synthesis of NRF-2 protein, but also facilitate the dissociation of the complex Keap-1-NRF-2, resulting in the significant increase of free NRF-2, which in turn leads to upregulate the expression of NRF-2 in the nucleus and augment the expression of downstream antioxidant proteins of NRF-2 such as HO-1. The ability of scavenging free radicals was enhanced, and the activities of SOD, CAT and GPx were increased, thus reducing oxidative stress injury and protecting cardiomyocytes [10,11]. The results showed that MI/R could increase the expression of NRF-2 and HO-1 in cardiomyocytes and geniposide could further increase the

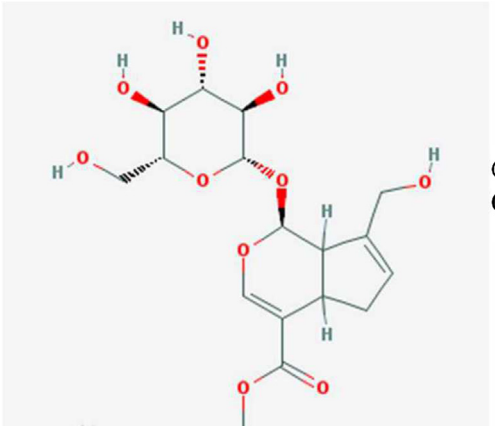

Fig. 4. The stucture of GEN.

expression of NRF-2 and HO-1, which suggested that geniposide could further promote the activation of NRF-2/ HO-1 signaling pathway to alleviate oxidative stress-mediated injury. The difference between this study and Cai Zhihui's research is that for the first time in diabetic rats, geniposide has been proved to have protective effect on myocardial ischemia reperfusion injury in diabetic rats, and its mechanism is also related to inhibiting oxidative stress. The mechanism of Geniposide inhibiting oxidative stress and alleviating myocardial ischemia-reperfusion injury in diabetic rats was preliminarily proved to be associated with activation of NRF-2/ HO-1 signaling pathway.

To sum up, gardenoside preconditioning can significantly alleviate STZ-induced myocardial ischemia/reperfusion injury in diabetic rats, and its mechanism may be associated with activating NRF-2/HO-1 signaling pathway to inhibit oxidative stress-mediated

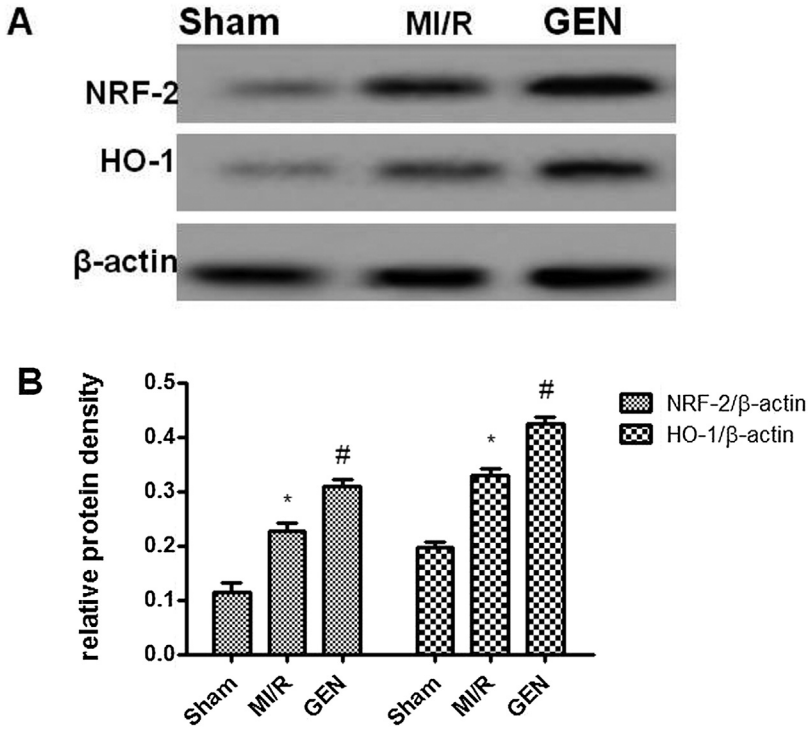

Fig. 3. Effects of GEN pretreatment on the expression of NRF-2and HO-1($n = 10, \bar{x} \pm s$).
Western blot analysis were employed to the expression of NRF-2and HO-1. (A) A representative result for Western blot analysis NRF-2and HO-1. B. Semi-quantitative analysis the relative amounts of NRF-2and HO-1 in each group of rats; Bars represent the means ± SE (n = 10). * $P < 0.05$ (MI/R vs. Sham); # $P < 0.05$ (GENvs. MI/R).

injury, which provides a new choice for clinical treatment of diabetic complications.

Disclosure of conflicts of interest

The authors declare that they have no competing interests.

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