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Original Article

Vitexin exerts cardioprotective effect on chronic myocardial ischemia/reperfusion injury in rats via inhibiting myocardial apoptosis and lipid peroxidation

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Abstract: Purpose: The aim of this study was to explore the cardioprotective effect of vitexin on chronic myocardial ischemia/reperfusion injury in rats and potential mechanisms. Methods: A chronic myocardial ischemia/reperfusion injury model was established by ligating left anterior descending coronary for 60 minutes, and followed by reperfusion for 14 days. After 2 weeks ischemia/reperfusion, cardiac function was measured to assess myocardial injury. The level of ST segment was recorded in different periods by electrocardiograph. The change of left ventricular function and myocardial reaction degree of fibrosis of heart was investigated by hematoxylin and eosin (HE) staining and Sirius red staining. Endothelium-dependent relaxations due to acetylcholine were observed in isolated rat thoracic aortic ring preparation. The blood samples were collected to measure the levels of MDA, the activities of SOD and NADPH in serum. Epac1, Rap1, Bax and Bcl-2 were examined by using Western Blotting. Results: Vitexin exerted significant protective effect on chronic myocardial ischemia/reperfusion injury, improved obviously left ventricular diastolic function and reduced myocardial reactive fibrosis degree in rats of myocardial ischemia. Medium and high-dose vitexin groups presented a significant decrease in Bax, Epac1 and Rap1 production and increase in Bcl-2 compared to the I/R group. It may be related to preventing myocardial cells from apoptosis, improving myocardial diastolic function and inhibiting lipid peroxidation. Conclusions: vitexin is a cardioprotective herb, which may be a promising useful complementary and alternative medicine for patients with coronary heart disease.

Keywords: Vitexin, myocardial ischemia/reperfusion injury, apoptosis, Epac-1, Rap-1, aortic rings

Introduction

In recent years, the coronary heart disease is a major factor to rates of mortality and morbidity, particularly in the aged people, which has become a serious health problem in the global scale place [1-3]. The risk and morbidity of coronary heart disease gradually increase as people grow older [4]. It can be estimated that coronary heart diseases will be likely to become the major cause of death among global diseases in 2020 [5]. However, aimed at coronary heart diseases, no effective therapy is currently available, besides supportive treatment. The ischemia and reperfusion in cardiac surgery is still the cause of myocardial injury in despite of improvements in surgical care [6, 7]. Paradoxically, reperfusion exacerbates the myocardial ischemia damage [8]. It is a phenomenon referring to myocardial ischemia/reperfusion injury (MIRI) that reestablishing of coronary-artery blood flow can be not only reduced but also exacerbated myocardial damage [9, 10]. In addition, it can result in cardiac cell death and ventricular dysfunction due to prolonged ischemia and reperfusion, and the disease mortality may be lowered, where the heart is protected by reducing ischemia/reperfusion injury [5, 11, 12]. Molecular mechanisms of MIRI injury are complex, involving many factors and links, and the oxygen free radical damage, Ca^{2+} overload and cell apoptosis are possibly important links

of the pathogenesis of myocardial ischemia/reperfusion injury [13-16].

According to many researches [17-19], the flavones, which were separated from numerous plants, have protective effects against myocardial ischemic injury. Vitexin (8-C-b-D-glucopyranosyl-apigenin), belonging to flavonoids compounds, is separated from the hawthorn leaf of China which is an effective monomer composition [20-22]. It is reported that vitexin can lower blood pressure and exert anti-inflammatory effect [23]. Vitexin also exerts effects of protecting cardiac hypertrophy [22], and inhibiting platelet aggregation [24], vascular smooth contractility [25] and apoptosis [21, 26]. Our previous study showed that vitexin had a protective effect on acute myocardial ischemia/reperfusion injury and myocardial cells stuffed hypoxia reoxygenation in vivo or in vitro [17, 27]. However, the potential mechanism of protection remains to be further study in depth. This study was to explore the protective effects of vitexin for chronic myocardial ischemia/reperfusion injury in rats and the effect of vitexin on the antioxidant defense system, cell apoptosis and signaling pathways of Epac-1/Rap-1.

Materials and methods

Ethics statement

All applicable international, national, and institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

Animals

Adult male Sprague-Dawley rats (weighing 250±20 g) were provided with the Experimental Animal Center of Anhui Medical University (License: SCXK (Wan) 2011-02). These rats were raised in white-plastic cages at a room temperature-controlled of 22±3°C and humidity of 55±15% under a 12 h light/dark cycle. Food and water were available ad libitum and permitted one week to accommodate to their environment before testing. All procedures were administrated in accordance with the guide-lines as described in the National Institutes of Health Guide for the Care and use of experimental animals and approved by the Institutional Animal Care and Use Committee at Anhui Medical University.

Drugs and reagent

Vitexin, under normal temperature preservation, was offered by Qi-xing Medicine and Technology Co., Ltd. Breviscapine injection solution was bought from Hunan Heng-sheng Pharmaceutical Co., Ltd. Hydral (10%) was purchased from Shanghai Bai-he chemical plant in China. Primary antibodies to Bax, Bcl-2 and β-actin were provided by Beijing Zhong-Shan Jin-qiao Biotechnology Co., Ltd, also having horseradish peroxidase labeling resistance against rabbit and rat IgG. Primary antibodies to Epac-1 and Rap-1 were offered by ABcam Co., Ltd. Enhanced bicinchoninic acid (BCA) protein assay kit was bought from Beyotim Institute of Biotechnology (Haiman, China). Heparin sodium injection solution was purchased from Jiangsu wan-bang biochemical pharmaceutical Co., Ltd. The total superoxide dismutase (SOD), malondialdehyde (MDA) and Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Elisa assay kits were bought from Nanjing Jian-cheng Co., Ltd. Ethanol disinfectant liquid (75%) was purchased from Hefei Tian-fan Medicine and Technology Co., Ltd. Sodium chloride injection solution (0.9%) was provided with Anhui Shuang-he Medicine and Technology Co., Ltd.

Myocardial ischemia/reperfusion injury (MIRI) model

48 male Sprague-Dawley (SD) rats were randomly divided into 6 groups: sham group, I/R group, vitexin 6, 3, 1.5 mg/kg groups and breviscapinun group. Rats were anesthetized with chloral hydrate (300 mg/kg) intraperitoneally (i.p), and then fixed in a supine position. The trachea was cannulated for artificial ventilation with room air, at a rate of 60 breaths/min. The body temperature of the rats was maintained at 37±0.5°C using an electrical heating pad (breathing ratio 1.5:1, tidal volume 30 mL/kg). Lead II of an electrocardiogram (ECG) was monitored using stainless needle electrodes, which were attached to limbs. On the left edge of thoracotomy, the chest was opened in the fourth intercostal space followed by a pericardiotomy to expose the heart. A 6-0 black silk suture was passed around the left anterior descending

(LAD) coronary artery, 2 mm from the tip of the left auricle. After 10 minutes, the end of suture was pulled through a piece of plastic tube to form a snare that occludes the artery when tightened and clamped. Successful ischemia was notarized by the appearance of regional epicardial cyanosis over the myocardial surface and myocardial infarction performance of ECG (elevation of the ST segment or height and pointed of T wave). After 60 minutes of ischemia, the coronary artery was reperfused for 60 minutes by releasing the clamp. Sham-operated animals were subjected to the same surgical procedure, but the ligation remained untied. After reperfusion, the chest of rats was closed step by step [28]. The breathing machine was removed when the recovery of spontaneous breathing for rats. These rats were fed standard at a room temperature of 25°C for 14 days. The vitexin of different doses and breviscapine were given by intravenous injection in 20 minutes before coronary artery ligation, and the same amount of saline solution was given for Sham group and I/R group.

Measurement of the elevation of the ST segment of ECG

The electrocardiogram (ECG) was continuously monitored and recorded through BL-420 biological function experiment system during the experiment. The elevations of the ST segment of baseline, ischemia for 60 minutes, reperfusion for 60 minutes, 7 days after operation and 14 days after operation were measured respectively using the image analysis software. The ST segment changes were compared between groups of each time point.

Cardiac functional assessment

Rats were fasting for 24 hours after last time to give drugs and free drinking water. After anesthetized, the rats were fixed for supine position, and a longitudinal incision was section on the neck right side skin. The arterial indwelling needle was inserted into the right carotid artery which was fully exposed and full of heparin saline (50 u/mL), and the BL-420 biological function experiment system (Chengdu Tai-meng science and technology Co., Ltd.) was used to assess the rat heart rate, left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP). The left ventricular pressure maximum rising/falling rate (+dp/dtmax, -dp/dtmax) was derived by computer algorithms.

Determination of NADPH, SOD activity and MDA content in serum

After finishing hemodynamic index detection, the blood was collected for 2-3 ml through artery indwelling needle and centrifuged for 10 minutes (3500 r/min). The supernatant of blood was aimed to measure the activities of malondialdehyde (MDA) and superoxide dismutase (SOD) and nicotinamide adenine dinucleotide phosphate (NADPH) by assay kits. Experiments were carried out with the corresponding commercial kits according to the manufacturer's instructions, and then were detected respectively at 440 nm and 340 nm by spectrophotometry. That TBA method was used to detect MDA content of the serum, and Enzyme-linked Immunosorbent method was used to detect the activity of SOD and NADPH in the serum.

Hematoxylin eosin (HE) and Sirius Red staining

The left ventricular ischemic tissue of rat had been taken and followed by 4% paraformaldehyde fixation which continued for 24 hours. Then were paraffin-embedded and sectioned at 4 μm-thick through a rotary microtome. At last, they were underwent HE [29] and Sirius Red staining [30]. The pathological change was observed under optical microscope.

Western blot

The expressions of Epac1, Rap1, Bax and Bcl-2 were detected using Western Blot [31]. The left ventricular tissues were got from the heart of operation and washed with normal saline (NS). RIPA lysis buffer containing PMSF was added into myocardial tissue and the supernatant was harvested by centrifugation (4°C, 12000 r/min, 10 minutes). The protein concentration of each supernatant sample was detected across a BCA protein assay kit. The protein lysates (50 μg) were electrophoresed and separated on 10% sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE), and transferred to PVDF membranes. The process continued for 120 minutes at 100 mA. Then was closed 1 hour at 5% (w/v) nonfat milk sealing fluid. Next, the PVDF membranes were

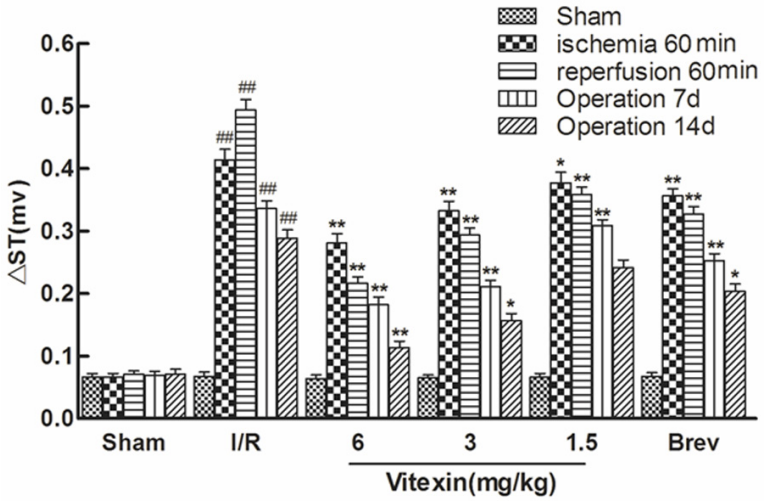

Figure 1. Vitexin inhibited chronic IRI-induced altitude of ST segment. $^{\#\#}P <$ 0.01 compared with sham group. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with I/R group. Data are expressed as mean ± SD; n=8 in each group. I/R, ischemia/reperfusion; Brev, breviscapine.

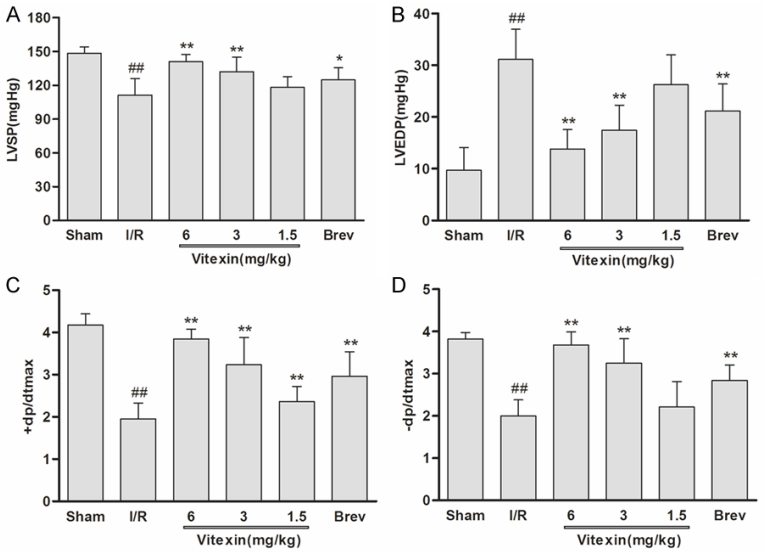

Figure 2. Vitexin improved myocardial diastolic function of left ventricular. (A) LVSP (mmHg); (B) LVEDP (mmHg); (C) +dp/dtmax; (D) +dp/dtmax. $^{\#\#}P < 0.01$ compared with sham group. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with I/R group. Data are expressed as mean ± SD; n=8 in each group. I/R, ischemia/reperfusion; Brev, breviscapine.

soaked on a suitable box joining respectively Epac-1 (1:1000 dilutions), Rap-1 (1:3000 dilutions), Bax (1:250 dilutions) and Bcl-2 (1:250 dilutions) and hatched for one night at 4°C. The PVDF membranes were washed and incubated to horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse, goat anti-rabbit) for 1 hour. At last, the gray value of each belt, detected by an enhanced chemiluminescence ECL Western Blotting detection reagent and visualized by a Bioshine ChemiQ4600 imaging system (Shanghai Bioshine Scientific instrument Co., Ltd), was calculated by Image J Image analysis software. β-actin protein served as an internal calibration.

Detection of contractive-relaxative aortic ring [32]

When finishing collecting blood, the 1 cm telecentric thoracic aorta was obtained after the thoracotomy, and cut lightly into 3 mm in length. The BL-420 biological function experiment system was recorded the changes of the contractive-relaxative aortic ring in the process of experiment simultaneously. The Kreb's fluid (containing Nacl, Kcl, $Cacl_2$, KH_2PO_4, $MgSO_4$, $NaHCO_3$, $C_6H_{12}O_6 \cdot H_2O$) was incubated every 15 minutes and continued 45 minutes on a vascular tension system device. The vascular ring were contracted and reached its maximum by high potassium solution (60 mmol/L). The vascular ring was soaked into phenylephrine solution, and was transfused the ladder concentration of acetylcholine (Ach, 0.01, 0.1, 1, 10, 100 mmol/L).

Statistical analysis

The results were averaged and expressed as mean ± SD and data were evaluated using the statistical analysis program (SPSS 17.0). Statistical analysis was performed with one-way analysis of variance (ANOVA). A *P* value of less than 0.05 was taken as statistically significant.

Results

Vitexin inhibited chronic IRI-induced altitude of ST segment

Compared with Sham group, I/R group was significantly elevated (P < 0.01) on the ST segment

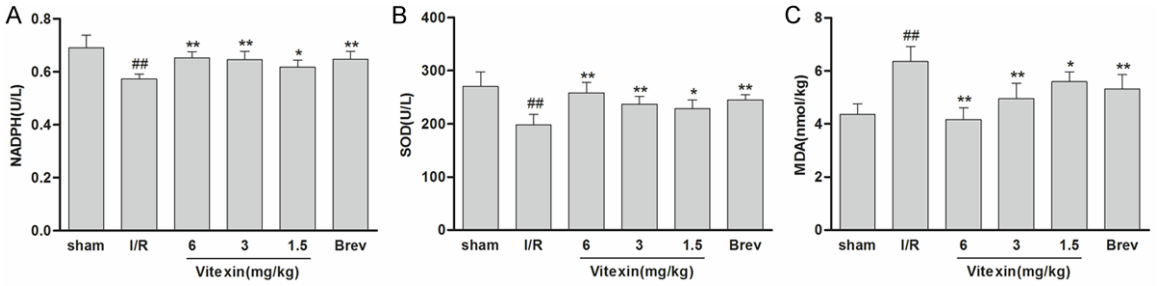

Figure 3. Vitexin alleviated myocardial injury through inhibiting lipid peroxidation. (A) NADPH (U/L); (B) SOD (U/L); (C) MDA (mmol/kg). $^{\#\#}P < 0.01$ compared with sham group. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with I/R group. Data are expressed as mean ± SD; n=8 in each group. I/R, ischemia/reperfusion; Brev, breviscapine.

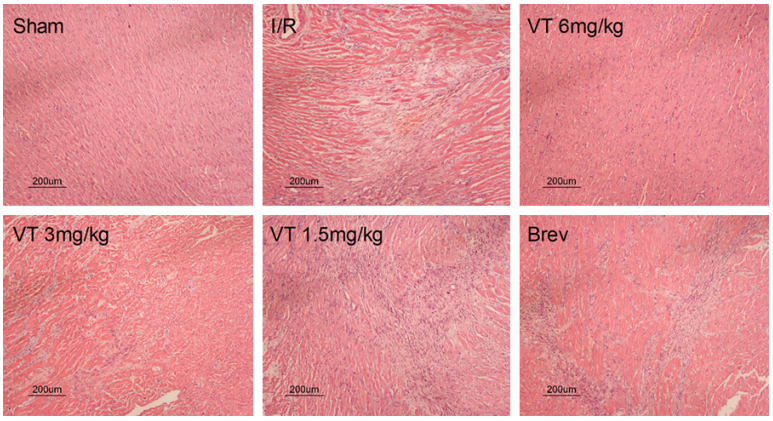

Figure 4. Vitexin protected chronic I/R-induced histopathological damage. Hematoxylin and eosin (HE) staining of the heart. Scale bar =200 μm. I/R, ischemia/reperfusion; Brev, breviscapine.

of ECG. Vitexin (6 mg/kg, 3 mg/kg) and breviscapine group inhibited significantly height of the ST segment elevation ($P < 0.01$ or $P < 0.05$) at every period on chronic myocardial ischemia/reperfusion injury in rats. The results are shown in **Figure 1**.

Vitexin improved myocardial diastolic function of left ventricular

We investigated the impacts of vitexin on cardiac function. There were significant differences in LVSP, LVEDP, +dp/dtmax and -dp/dtmax between the Sham group and the I/R group, I/R injury greatly decreased LVSP, +dp/dtmax and -dp/dtmax, and increased LVEDP in the I/R group ($P < 0.01$). However, Vitexin (6 mg/kg, 3 mg/kg) significantly elevated LVSP [from (111.2±14.7) mmHg to (139.1±7.7) mmHg, $P < 0.01$; from (111.2±14.7) mmHg to (131.0±12.2) mmHg, $P < 0.01$], +dp/dtmax [from (1.95±0.35)% to (3.8±0.2)%, $P < 0.01$; from (1.95±0.35)% to (3.2±0.6)%, $P < 0.01$] and -dp/dtmax [from (2.0±0.4)% to (3.7±0.3)%, $P < 0.01$; from (2.0±0.4)% to (3.2±0.6)%, $P < 0.01$] when compared with the I/R group, and down-regulated LVEDP [from (31.8±5.8) mmHg to (13.8±3.8) mmHg, $P < 0.01$; from (31.8±5.8) mmHg to (17.4±4.9) mmHg, $P < 0.01$]. The results showed vitexin could obviously improve the function of left ventricular on chronic myocardial ischemia/reperfusion injury in rats (**Figure 2**).

Vitexin alleviated myocardial injury through inhibiting lipid peroxidation

To detect the effects of vitexin on chronic MIRI rats, we measured several markers in serum (NADPH, SOD and MDA). Compared with the Sham group, MDA levels were significantly increased in the I/R group, and I/R group significantly reduced NADPH and SOD activities ($P < 0.01$). Treatment with vitexin (6 mg/kg, 3 mg/kg and 1.5 mg/kg) and breviscapine (3 mg/kg) group reduced MDA release and increased NADPH and SOD activities in comparison with the I/R group (**Figure 3**). The results showed that vitexin reduced myocardial injury through inhibiting lipid peroxidation.

Vitexin protected chronic I/R-induced histopathological damage

The myocardium in the sham group exhibited normal myocardial architecture (**Figure 4**) and myocardial fibers (**Figure 5**). However, focal myocardial damage with uneven staining was observed. Morphological changes in affected cardiomyocytes primarily comprised different degrees of swelling, necrosis, myocytolysis and

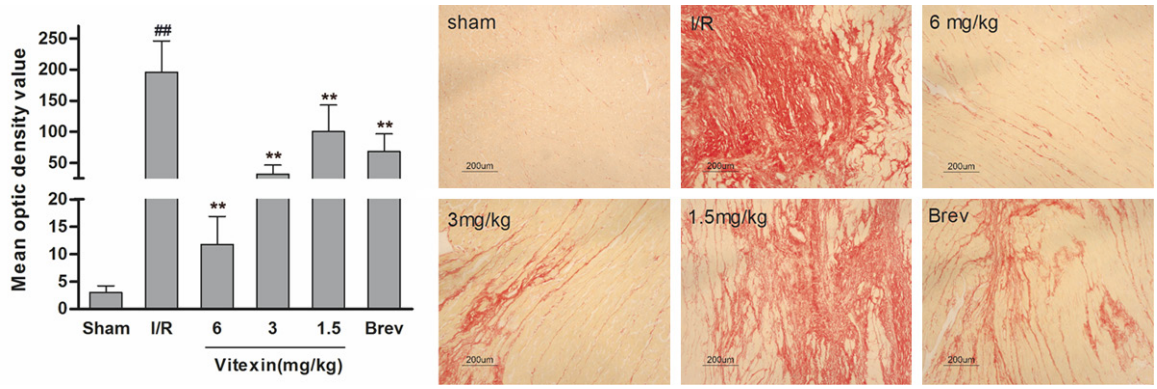

Figure 5. Vitexin protected chronic I/R-induced histopathological damage. Sirius Red staining of the heart. Scale bar =200 μm. I/R, ischemia/reperfusion; Brev, breviscapine.

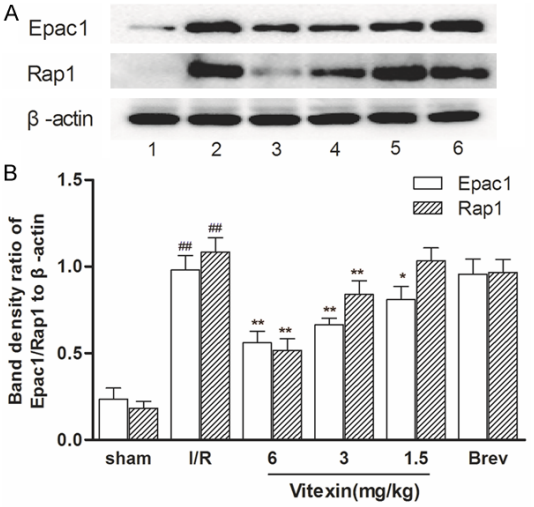

Figure 6. Effects of Vitexin on Epac1 and Rap1 expression. A: Western blotting analysis for Epac1 and Rap1 expression in heart. B: Representative images of Western blot with primary antibodies against Epac1 and Rap1, respectively. Gray values of the Western blot were normalized to the loading control β-actin. ##P < 0.01 compared with sham group. **P < 0.01 and *P < 0.05 compared with I/R group. 1: sham group; 2: IRI group; 3: vitexin 6 mg/kg group; 4: vitexin 3 mg/kg group; 5: vitexin 1.5 mg/kg group; 6: breviscapine 3 mg/kg group.

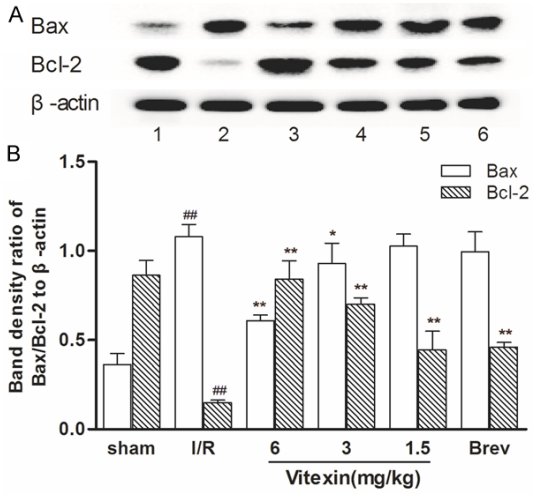

Figure 7. Effects of Vitexin on Bax and Bcl-2 expression. A: Western blotting analysis for Bax and Bcl-2 expression in heart. B: Representative images of Western blot with primary antibodies against Bax and Bcl-2, respectively. Gray values of the Western blot were normalized to the loading control β-actin. ##P < 0.01 compared with sham group. **P < 0.01 and *P < 0.05 compared with I/R group. 1: sham group; 2: IRI group; 3: vitexin 6 mg/kg group; 4: vitexin 3 mg/kg group; 5: vitexin 1.5 mg/kg group; 6: breviscapine 3 mg/kg group.

myofibrillar loss. Myocardial fibers were disrupted and arranged irregularly. Infarction foci were infiltrated with numerous neutrophils. The large numbers of myocardial fibers showed disordered structures and accumulated. Myocardial matrix exhibited infiltration of inflammatory cells, where a large number of scar tissue was exhibited. Vitexin-treated (6 mg/kg, 3 mg/kg) group markedly attenuated the myocardial damages in comparison with the I/R group.

Effects of vitexin on Epac1, Rap1, Bax and Bcl-2 expression

We analyzed the effects of vitexin on Epac1, Rap1, Bax and Bcl-2 protein expression by western blot. The results showed that the levels of Bax, Epac1 and Rap1 in the I/R group were significantly higher and Bcl-2 expression level was significantly lower than the Sham group [**Figures 6**, **7** ($P < 0.01$)]. Medium and high-dose vitexin groups presented a significant

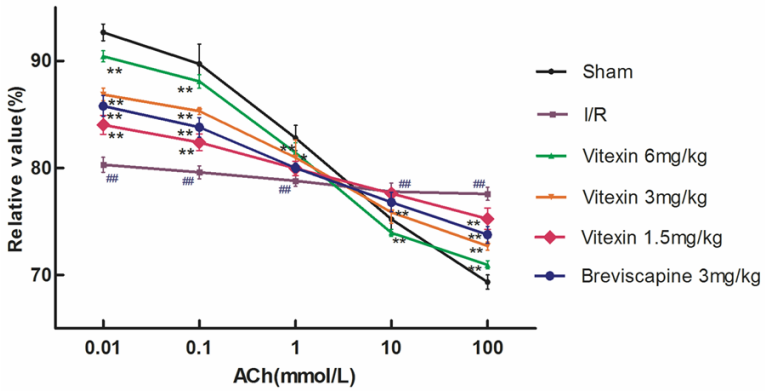

Figure 8. Effects of vitexin on tension-systaltic aorta. ##P < 0.01 compared with sham group. **P < 0.01 and *P < 0.05 compared with I/R group. I/R, ischemia/reperfusion; Brev, breviscapine.

decrease in Bax, Epac1 and Rap1 production and increase in Bcl-2 compared to the I/R group [**Figures 6**, **7** (P < 0.01)]. However, low-dose vitexin and breviscapine (3 mg/kg) showed no significant differences on the expression of Bax or Rap1 compared to the I/R group.

Effects of vitexin on tension-systaltic aorta

As shown in **Figure 8**, we used BL-420 biological experiment system to investigate the effects of vitexin on systaltic tension of aorta on chronic MIRI rats. The tension-systaltic aorta in the I/R group was remarkably declined compared with the sham group (P < 0.01). Vitexin exhibited marked improvement on tension-systaltic aorta in comparison with the I/R group. The results revealed vitexin enhanced the ability on tension-systaltic aorta, and also had a certain effect to dilate blood vessels.

Discussion

Our study demonstrated obviously a great cardioprotective of vitexin against chronic myocardial ischemia/reperfusion injury in rats, and the underlying mechanisms might be associated with the enhancement of antioxidant defense system and inhibition of apoptosis.

It is reported the ventricular systolic and diastolic functions were indicators which reflected cardiac function [33]. Additional studies implicated that the process of myocardial I/R resulted in the changes of cardiac hemodynamic parameters including LVSP, LVEDP, +dp/dtmax and -dp/dt max [34], which was a sensitive indicator of cardiac function [35]. In previous studies, vitexin was used for various medicinal properties, including lower blood pressure, increasing the blood flow of coronary artery and vascular smooth contractility [21, 23]. The data from this study showed that, LVSP, +dp/dtmax and -dp/dtmax were decreased significantly during reperfusion period, while LVEDP was increased in ischemia-induced rats. However, the lower of LVEDP and enhancement of LVSP, +dp/dtmax and -dp/dtmax were obviously considered as a cardioprotection by vitexin treatment, which was particularly high and medium dosage. It is reminded that vitexin could ameliorate cardiac function and protect myocardial damage against chronic myocardial ischemia/reperfusion injury.

It has been shown that the pathological process which I/R was an important contributor to the generation of reactive oxygen species (ROS) was a key, and indirectly resulted in lipid peroxidation [36]. The superoxide radical was considered a primary ROS, and was in the early stages of ischemia, and the main ROS was deemed to superoxide radical which was generated from the xanthine oxidase enzyme [37]. The highly active hydroxyl radical was inhibited by reducing the superoxide radical, which the H_2O_2 of generation through SOD catalyst transfers H_2O, O_2, catalase and oxidizing enzyme [38, 39]. MDA, which was an enzyme substrate of lipid peroxidation, was considered as an effective index of the rate of lipid peroxidation [37] and a marker for oxidative damage [38]. The NADPH oxidase also existed in outside the organization of phagocytes, including heart, liver, kidney and blood vessels, and probably also was a factor in the generation of ROS [40, 41]. MDA, SOD and NADPH, as indicators of oxidative status, reflected the degree of myocardial I/R injury. In the present research, vitexin treatment reduced myocardial I/R-induced MDA formation. Meanwhile, vitexin treatment enhanced myocardial antioxidant capacity as evidenced by increased antioxidant enzyme SOD and NADPH activity in

I/R rats. These observations indicated that vitexin protected myocardial damage against chronic myocardial ischemia/reperfusion injury in rats.

Apoptosis was a cell death mechanism which was important in development, normal tissue and disease. A previous study suggested that apoptosis played a crucial role in the development of chronic myocardial ischemic/reperfusion injury [42]. The MIRI could relieve through inhibiting cardiomyocyte apoptosis [43, 44]. In the previous report, programmed cell death was regulated by numerous genes, particularly Bcl-2 and Bax genes [45]. Oltvai [46], at 1993, reported for the first time, whether the cell was death or not, it depended on Bcl-2/Bax. Also, Ca^{2+} overload could promote cell apoptosis [47]. It was demonstrated that Epac-Rap1, which was a key way of the new cAMP signaling pathways in the myocardial tissue, had an important and extensive effect, including regulation of the myocardial cell metabolism and the endothelial barrier function [48]. The previous study indicated that Epac1 increased intracellular Ca^{2+} concentration through PLCε inositol 1, 4, 5-triphosphate receptor signaling. Rap1, an immediate effector of Epac, had been shown to contribute to Epac-mediated cells [49]. In the present study, the expression of Bax, Epac1 and Rap1 after vitexin treatment was decreased in myocardial ischemia-induced injury, while the expression of Bcl-2 was increased.

As a whole, the effects of vasodilation depended mainly on increasing vascular active substances and releasing prostaglandin by endothelial dependency [50, 51]. In the present study, we used mainly the high concentration of K^+ solution and PE for pro-shrinking effect of blood vessels. High concentration of K^+ solution led to internal flow of the extracellular Ca^{2+} by opening voltage-gated Ca^{2+} channels, while cell membrane depolarized and the extracellular Ca^{2+} increased. The results of this study suggested vitexin exhibited improvement on tension-systaltic aorta. The data demonstrated a cardioprotective effect of vitexin against chronic myocardial ischemia/reperfusion injury in rats.

Conclusion

In conclusion, we demonstrated that vitexin treatment effectively improved myocardial function and attenuated myocardial I/R injury by means of enhancement of antioxidant defense system, inhibition of Ca^{2+} overload and suppression of myocardial apoptosis. Consequently, these results suggest that vitexin is a cardioprotective herb, which may be a promising useful complementary and alternative medicine for patients with coronary heart disease.

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Disclosure of conflict of interest

None.

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