Original Article

Protective effects of pueraria isoflavones against myocardial ischemia injuries

Weihai Liu1, Zonghui Ma1, Jiping Liu2, Bin Wang2

1Affiliated Hospital of Shaanxi University of Chinese Medicine, Xianyang, Shaanxi, China; 2Shaanxi University of Chinese Medicine, Xianyang, Shaanxi, China

Received January 19, 2019; Accepted March 11, 2019; Epub May 15, 2019; Published May 30, 2019

Abstract: Objective: The aim of the current study was to examine the protective effects of Pueraria isoflavones on cardiomyocytes. Methods: A total of 36 SD rats were randomly divided into the sham group (group A), AMI model group (group B), and Pueraria isoflavones intervention group (group C, 50 mg/kg). Each group included 12 rats. Three days after model establishment, the rats were sacrificed, myocardial cells and blood serum of the rats were collected, miR-181a expression in myocardial tissues was detected by qRT-PCR, expression of Bax, Bcl-2, and Caspase-3 proteins in myocardial tissues was detected by Western blot, concentrations of TNF-α ELISA, MDA, and SOD in the serum were detected by ELISA, and apoptosis of cardiomyocytes was recorded using the TUNEL kit. Results: Relative expression of miR-181a in group A was significantly lower than that in group B and group C (all P < 0.05). Relative expression of miR-181a in group B was significantly higher than that in group C (P=0.004). Expression of Bax and Caspase-3 proteins in group B was the highest. It was significantly different from that in group A and group C (all P < 0.05). Expression of Bax and Caspase-3 proteins in group C was significantly higher than that in group A (both P < 0.05). Expression of Bcl-2 proteins in group B was the lowest and significantly different from that in group A and group C (all P < 0.05). Expression of TNF-a and MDA in group B was the highest and significantly different from that in group A and group C (all P < 0.05). Expression of TNF-a and MDA in group C was significantly higher than that in group A (all P < 0.05). Expression of SOD in group B was the lowest and significantly different from that in group A and group C (all P < 0.05). Apoptosis of cardiomyocytes in group B was significantly higher than that in group A and group C (all P < 0.05), while apoptosis of cardiomyocytes in group C was significantly higher than that in group A (P < 0.05). Conclusion: Pueraria isoflavones play a protective role in cardiomyocytes by increasing the activity of SOD, decreasing apoptosis of cardiomyocytes and oxidation of free radicals, inhibiting expression of inflammatory factors TNF-α and miR-181a, and improving expression of cell apoptosis proteins.

Keywords: Pueraria isoflavones, cardiomyocytes, miR

Introduction

Acute myocardial infarction (AMI), the most common cardio-cerebral vascular disease in clinical practice, is mainly caused by atherosclerotic occlusion of the coronary artery. This leads to the interruption of blood flow and local necrosis of cells due to long-term ischemia and hypoxia in the cardiomyocytes. Patients will often suffer from fevers, retrosternal pain, and leukocytosis, possibly even heart failure and arrhythmia [1, 2]. Lobed Kudzuvine Root (LKR) is the dried root of the leguminous plant-kudzu. Its major chemical component is Pueraria isoflavones [3]. Previous studies shown that [4] Pueraria isoflavones promote coronary artery dilatation of animals, decrease blood pressure, slow the heartbeat, and increase blood flow of coronary arteries. Thus, they play a protective role to cardiomyocytes.

MicroRNAs are specific, non-coding, and single-stranded RNAs with a length of approximately 21nt. They regulate mRNA degradation of target genes by complementarily pairing the 3'-UTR region of target genes [5]. Studies have shown that [6] more than 30% of human genes are regulated by miR, according to bioinformatics. These include very important physiological processes, such as growth and development of human beings, proliferation and apoptosis of cells, cell differentiation, and generation and...
Protective effects of Pueraria isoflavones

development of tumors. miR-181a is one of the important members in the miR-181 family. It plays regulatory roles in multiple cancers, such as lung cancer, breast cancer, and squamous-cell carcinoma [7-9]. Previous studies have shown that miR-181a is closely associated with AMI. The study of Zhu et al. [10] demonstrated that miR-181 expression increased significantly in the plasma of patients with acute myocardial infarction, compared to healthy people. A study by Liu et al. showed that Pueraria isoflavones prevent AMI caused by severe burns [11]. However, their relationship was not confirmed by these studies.

Therefore, the current study aimed to explore the protective effects of Pueraria isoflavones on cardiomyocytes, examining its relationship with miR-181a.

Materials and methods

Animal sourcing

A total of 36 Sprague-Dawley rats were sourced from the Animal Laboratory Center. All rats were males with a body weight of 160 g ± 10 g. They were fed in the Animal Laboratory Center by a designated person. The temperature of the laboratory was 20~25°C. Humidity was maintained at a range of 45~60% and noise was kept <50 db. The rats were permitted to drink and eat freely. Lamination was given every 12 hours.

Principle reagents and instrument

Rabbit-anti-rat Bax, Bcl-2, Caspase-3 monoclonal antibodies, β-actin antibody (from R&D, USA, AF820, AF810, AF835, MAB8929); Trans Script Green miRNA Two-Step qRT-PCR Super Mix kit and Easy Pure miRNA Kit (TransGen Biotech, Beijing, China, AQ202-01 and ER601-01); Primers designed and synthesized by Shanghai Sangon Biotech (see Table 1 for details); TNF-α ELISA kit, MDA kit, SOD kit, and TUNEL apoptosis detection kit (Shanghai Beyotime Biotechnology, PT512, S0131, S0060 and C1091); RIPA lysate and BCA protein assay kit (Sigma Aldrich, USA, R0278, BCA1); Sodium pentobarbital (Sigma, USA); PCR instrument (ABI, USA, 7500); Pueraria isoflavones (Hangzhou East Biopharm Co., LTD). All procedures were approved by the Animal Care and Use Committee of the Affiliated Hospital of Shaanxi University of Chinese Medicine and in accordance with guidelines of the National Institute of Health.

Model establishment

A total of 36 rats were randomly divided into the sham group (group A), AMI model group (group B), and intervention group (group C). Rats in the three groups were given sodium pentobarbital (3%, 1.5 mL/kg) by intraperitoneal injection for anesthesia. After anesthesia, the rats were kept in the supine position with their limbs fixed. Next, 75% ethanol was applied for routine disinfection and tracheas were used to connect the animals to the ventilator. The rats were cut through along the parallel ribs on the left (2~3 cm). Subcutaneous tissue was isolated, the thoracic cavity of the rats was opened, and surgical ligation of the left anterior descending coronary artery was performed. When the arched ST segment was elevated, the AMI model was established successfully. Afterward, the thoracic cavity was closed and tissues were gradually sutured. The ventilator was removed when the rats recovered spontaneous respiration. They were then given anti-infective treatment (penicillin). Rats in group A only received threading without ligation. They were observed for 24 hours after successful model establishment. Twenty-four hours after modelling, rats in group C were given Pueraria isoflavones at 50 mg/kg, 1 day/time, by intragastric administration. Rats in groups A and B were given the same volume of normal saline for 3 days by intragastric administration. After the operation, they were fitted with collars to prevent self-harm. They were fed in a single cage and allowed to eat and drink freely.

Sample collection

After 3 days, the rats were euthanized with chloral hydrate (10%, 0.4 mL/100 g). Myocardial tissues were removed from the infarct area. Blood was drawn from the abdominal aorta. It was centrifuged at 3,000 rpm for 10 minutes, collecting serum for subsequent tests.

Expression of Bax, Bcl-2, and Caspase-3 proteins in myocardial tissues of rats

Total protein was extracted from collected myocardial tissues using RIPA lysis. Concentrations of proteins were detected using the BCA meth-
Protective effects of Pueraria isoflavones

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Upstream primer</th>
<th>Downstream primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-181a</td>
<td>5'-AACATTCAACGCTGCTGGTGAATG-3'</td>
<td>5'-CTCCTTGGAATACGTTTGGGCTCCTGTA-3'</td>
</tr>
<tr>
<td>U6</td>
<td>5'-CTCGCTTCGGCACAGAATTTGCGT-3'</td>
<td>5'-AACGCTTCACGAATTTGCGT-3'</td>
</tr>
</tbody>
</table>

od and adjusted to 4 μg/μL. SDS-PAGE electrophoresis was applied for separation. After electrophoresis, the proteins were transferred to PVDF membranes. Ponceau S was used for staining, PBST was applied for immersion for 5 minutes, 5% skim milk powder was adopted for sealing for 2 hours, and the primary antibody (1:1000) was added to seal overnight at 4°C. The primary antibody was removed by flushing the membranes. Horseradish peroxidase-labeled goat anti-mice secondary antibody (1: 5000) was then added. Incubation was performed at 37°C for 1 hour. TBST was used for rinsing 3 times, for 5 minutes each time. Images were developed in darkness. Excessive liquid on the membranes was removed with filter paper and ECL lamination was applied. Thus, images were developed. Protein bands were scanned and gray values were analyzed with Quantity One software. Relative expression level of protein=gray value of the target protein band/gray value of the β-actin protein band.

Apoptosis of cardiomyocytes in rats

Myocardial tissues of the rats were obtained, fixed with 4% paraformaldehyde, dehydrated with gradient ethanol, embedded with paraffin, and cut into slices. TUNEL apoptosis detection was used to detect the apoptosis of cardiomyocytes, strictly following manufacturer instructions. After staining, an optical microscope found that normal cardiomyocytes displayed blue nuclear, while apoptotic cardiomyocytes showed yellow or brownish yellow nuclear. Five fields were randomly observed for each slice. Apoptotic cardiomyocytes in the fields were then observed and counted (apoptosis rate= total quantity of apoptotic cells/total number of cells *100%).

Expression of miR-181a in myocardial tissues of rats

Total RNA was extracted from collected myocardial tissues with the EasyPuremiRNA kit. Extracted total RNA was examined for purity, concentrations, and integrity using an ultravio-

Detection of TNF-α, MDA, and SOD in serum of rats

Collected serum was added to 50 μl of standard solution with different concentrations. The resulting solution was placed in the blank micro-well. Next, 50 μL of distilled water and 50 ul of antibody were added to the blank control well. Afterward, 40 ul of the sample was added to the remaining micro-wells first, then further adding 10 ul of antibody labeled by biotin. The plate was then sealed and incubated at 37°C for 30 minutes. The plate was then washed and incubated at 37°C for 30 minutes. When the plate was flushed, the flush liquid fully filled each well without overflowing. It was discarded after 30 seconds of standing and patted dry 5 times. Moreover, 50 ul of enzyme labeled solution was added to each well. The plate was sealed again and incubated at 37°C for 60 minutes. The plate was then flushed again 5 times. The last time, absorbent paper was used to dry the plate thoroughly. Horseradish peroxidase labeled solution, at 100 μl/well, was added to seal the plate and incubation was performed at 37°C for 15 minutes, avoiding light. Afterward, the color substrate TMB, at 100 μl/well, was added.
Incubation was conducted at room temperature for 20 minutes, void of light. Finally, the stop solution, at 50 μl/well, was added. A microplate reader was used within 15 minutes to determine the maximum absorption wavelength at 450 nm. Three groups of duplicate wells were set. The experiment was repeated 3 times. MDA and SOD were determined following kit instructions.

Statistical methods

Collected data was statistically analyzed with SPSS 20.0 software package (Guangzhou Bomai). The graph was plotted using GraphPad Prism 7 (Shanghai Beka). Measurement data are expressed as mean ± standard deviation (mean ± SD). Variance analysis was used for comparisons among groups and LSD t-test was applied for comparisons between groups after variance analysis. P < 0.05 indicates statistical differences between the two groups.

Results

Relative expression of miR-181a in myocardial tissues of rats

The current study detected relative expression levels of miR-181a in myocardial tissues of 3 groups of rats 3 days after model establishment. Expression of miR-181a in group A, group B, and group C was significantly different (F=22.139, P < 0.001). Relative expression of miR-181a in myocardial tissues of group A (0.842 ± 0.252) was significantly lower than that of group B (1.625 ± 0.322) and group C (1.258 ± 0.287) (P < 0.001, P=0.001). Relative expression of miR-181a in myocardial tissues of group B was significantly higher than that of group C (P=0.004) (Figure 1).

Expression of Bax, Bcl-2, and Caspase-3 proteins in myocardial tissues of rats

Expression of Bax, Bcl-2, and Caspase-3 was detected in myocardial tissues of rats. Expression of Bax in each group was different (P < 0.05). Expression of Bax in group B was the highest and different from that in group A and group C (P < 0.001, P < 0.001). Expression of Bax in group C was significantly higher than that in group A (P < 0.001). Expression of Bcl-2 in each group was different (P < 0.05). Expression of Bcl-2 in group B was the lowest and different from that in group A and group C (P < 0.001, P < 0.001). Expression of Bcl-2 in group C was significantly different from that in group A (P=0.033). Expression of Caspase-3 in each group was different (P < 0.05). Expression of Caspase-3 in group B was the highest and different from that in group A and group C (P < 0.001, P < 0.001). Expression of Caspase-3 in group C was significantly higher than that in group A (P < 0.001) (Figure 2).

Expression of TNF-α, MDA, and SOD in serum of rats

Concentrations of TNF-α, MDA, and SOD in serum of the rats were detected. Expression of TNF-α was significantly different among the different groups (P < 0.05). Expression of TNF-α in group B was the highest and different from that in group A and group C (P < 0.001, P < 0.001). Expression of TNF-α in group C was significantly higher than that of group A (P=0.001). Expression of MDA was significantly different among the different groups (P < 0.05). Expression of MDA in group B was the highest and different from that in group A and group C (P < 0.001, P=0.002). Expression of MDA in group C was significantly higher than that of group A (P=0.037). Expression of SOD was significantly different among the different groups (P < 0.05). Expression of SOD in group B was the lowest and different from that in group A and group C (P < 0.001, P < 0.001). Expression of SOD in
Protective effects of Pueraria isoflavones

Apoptosis of cardiomyocytes in rats

Apoptosis of cardiomyocytes in each group was significantly different (F=46.212, P < 0.001). Apoptosis of cardiomyocytes in group B was significantly higher than that in group A and group C. Expression of Bcl-2 protein in group C was significantly lower than that in group A. Expression of Bcl-2 protein in group C was significantly higher than that in group A and group C. Expression of Caspase-3 protein in group C was significantly higher than that in group A; *implies differences between the two groups (**P < 0.001). D Representative Western blot images.

Discussion

Previous studies have shown that miRNA may regulate target genes in cardiovascular diseases, playing regulatory roles in the apoptosis and necrosis of cardiomyocytes. Studies have suggested that miR-181a, as an important miRNA, was highly expressed in the serum of AMI patients, showing promise as a prognostic factor of AMI. The current study detected relative expression levels of miR-181a in cardiomyocytes of rats in each group, after model establishment. Result showed that expression of miR-181a in myocardial tissues of group B was significantly higher than that of group A. Expression of miR-181a in group C (Pueraria isoflavone intervention group) was significantly decreased, suggesting that Pueraria isoflavones inhibit relative expression of miR-181a. Previous studies have shown that miR-181a may negatively regulate expression of TNF-α, according to dual-luciferase reporter assay. Therefore, this study detected expression of TNF-α in the serum of rats. TNF-α, an important inflammation promoting cytokine in the human body, is mainly generated by monocytes and macrophages. Studies have shown that TNF-α is not expressed in normal cardiomyocytes. However, in the case of myocardial infarction, ischemic and hypoxic myocardial tissues will activate cardiomyocytes and mononuclear macrophages in the regional myocardium. This will result in the generation of massive TNF-α in infarcted areas and infarcted border areas. The current study detected expression of TNF-α in the serum of rats, finding that expression of TNF-α in group B (without intervention) was significantly increased and different from that in group C (Pueraria isoflavone intervention group). Results suggest that Pueraria isoflavones effectively inhibited inflammation, after model establishment. However, present results also demonstrated that expression of TNF-α increased with an increase of miR-181a expression. No negative regulation was observed. It was speculated that maybe TNF-α was regulated by multiple targets instead of only one target gene. In addition, detection of TNF-α expression was based on concentra-
Protective effects of Pueraria isoflavones

Figure 3. Expression of TNF-α, MDA, and SOD in the serum of rats. A. Expression of TNF-α in serum of group B was the highest, compared with that in group A and group C. Expression of TNF-α in group C was significantly higher than that in group A. B. Expression of MDA in group B was the highest, compared with that in group A and group C. Expression of MDA in group C was significantly higher than that of group A. C. Expression of SOD in group B was the lowest, compared with that in group A and group C. Expression of SOD in group C was significantly lower than that of group A. *implies differences between the two groups (**P < 0.001).

Figure 4. Apoptosis of cardiomyocytes in each group. Apoptosis of cardiomyocytes in group B was significantly higher than that in group A and group C. Apoptosis of cardiomyocytes in group C was significantly higher than that in group A and differences were statistical. *implies differences between the two groups (**P < 0.001).

Furthermore, the current study detected expression of MDA and SOD in the serum of rats. MDA could be regarded as the final product of oxidation reaction in the strong oxidation. Its variation could directly reflect the intensity of lipid-peroxidation. SOD, an important antioxidant enzyme, is an oxygen free radical scavenger that maintains the dynamic balance of oxygen free radicals in the body [16, 17]. After detection, expression of MDA in group B was significantly higher than that in group C, while expression of SOD was significantly lower than that in group C. This suggests that Pueraria isoflavones effectively increase the activity of SOD, remove free radicals, decrease the oxidative stress response after model establishment, and protect the rats. At the end of the study, apoptosis of cardiomyocytes of rats was detected using the TUNEL method. The apoptosis rate of cardiomyocytes in group B was significantly higher than that in group C and group A, indicating that Pueraria isoflavones could inhibit the apoptosis of cardiomyocytes. However, the specific mechanisms of inhibition and the reaction to apoptotic factors remained unclear. Therefore, expression of Bax, Bcl-2, and Caspase-3 proteins was detected in the myocardial tissues of rats. Bax and Bcl-2 are the same kind of proteins related to water-solubility. Bax is an apoptosis-promoting gene and Bcl-2, as the antagonist gene of Bax, inhibits the apoptosis of cells [18]. Caspase-3 is an important member of cysteine protease and directly participates in the apoptotic process of cells [19]. After detection, expression of Bax and Caspase-3 proteins in myocardial tissues of group C was significantly inhibited. Expression was lower than that in group B. Expression of Bcl-2 in group C was significantly higher than that in group B, suggesting that Pueraria isoflavones effectively inhibit expression of Caspase-3 and Bax proteins in cardiomyocytes and increase expression of Bcl-2, inhibiting the necroptosis and apoptosis of cardiomyocytes.
Studies have shown that Pueraria isoflavones have improved treatment of various diseases. In a study by Jung et al. [20], it was shown that the combination of metformin and Pueraria lobata extract could prevent weight gain in rats with a high-fat diet, without significant adverse effects. In the study of Tiyasatkulkovit et al. [21], it was shown that Pueraria lobata extract could effectively promote the proliferation of primary osteoblasts and inhibit the increase of osteoclasts. It is expected to become a therapeutic agent for bone loss in postmenopausal women. The abovementioned studies indicate that Pueraria isoflavones produce no negative effects on normal animals or cells. Thus, it is a potential therapeutic drug for treatment of various diseases.

Previous studies have also shown that [22] Pueraria may improve the progression of ischemia/reperfusion injuries by increasing the activity of SOD, upregulating Bcl-2, decreasing expression of Bax, and inhibiting the inflammatory reaction. Pueraria isoflavones were proven, in the current study, to play roles effectively increasing SOD activity, decreasing the apoptosis of cardiomyocytes and oxidation of free radicals, inhibiting expression of inflammatory factor TNF-α and miR-181a, and improving expression of apoptotic proteins, thus protect cardiomyocytes. However, the current study had some limitations. This study did not detect expression of TNF-α in myocardial tissues of rats. The effects of Pueraria isoflavones on expression of TNF-α in myocardial tissues required further investigation. Moreover, this study was an animal experiment. Clinical trials in clinical practice have not yet been conducted. Therefore, its effects in clinical practice also require further verification. Moreover, it is unclear how Pueraria isoflavones regulate expression of miR-181a. Studies have shown that Pueraria can improve antioxidant stress and inhibit apoptosis by acting on biologically active molecules or regulating some signaling pathways [23]. These may be the underlying mechanisms with isoflavones as well.

Results of the current study should be confirmed by examining relevant signaling pathways and molecules, investigating how Pueraria isoflavones affect expression of miR-181a.

In summary, Pueraria isoflavones protect cardiomyocytes by increasing SOD activity, decreasing apoptosis of cardiomyocytes and oxidation of free radicals, inhibiting expression of inflammatory factors TNF-α and miR-181a, and improving expression of cell apoptosis proteins.

Disclosure of conflict of interest

None.

Address correspondence to: Weihai Liu, Affiliated Hospital of Shaanxi University of Chinese Medicine, No. 2, Weiyang West Road, Xianyang 712000, Shaanxi, China. Tel: +86-13992097389; E-mail: weihailiuu@163.com

References

Protective effects of Pueraria isoflavones


