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Original Article

Angiogenesis function of salidroside in myocardium of rats with myocardial ischemia

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Abstract: This study is to determine the effect of salidroside (SAL) on myocardium of rats with myocardial infarction (MI) and to analyze its possible mechanism. Left coronary artery of Sprague-Dawley rats was ligated to establish MI model. The rats were randomly divided into model group, SAL groups (10, 20, and 40 mg/(kg/d) groups), and the sham-operated group, with 8 rats per group. Hemodynamic changes in rats were determined, and the segmental heart samples were used for H&E or Masson staining. Expression of vascular endothelial growth factors (VEGF) and cluster of differentiation 34 (CD34) were analyzed by semi-quantitative PCR and Western blot. Compared with the sham-operated group, myocardial tissue was damaged and the collagen volume fraction was significantly increased ($P<0.01$). Compared with the model group, the number of angiogenesis in all the SAL treatment groups was significantly increased while the collagen volume fraction was significantly decreased ($P<0.01$). In addition, mRNA and protein levels of VEGF and CD34 in the cytoplasm of SAL treated myocardial tissues were significantly increased compared with the model group ($P<0.01$). SAL can obviously promote angiogenesis in myocardial tissue of rats after MI.

Keywords: Salidroside, myocardial infarction, angiogenesis, vascular endothelial growth factors, CD34

Introduction

Myocardial infarction (MI) is categorized as a kind of ischemic heart disease with the highest morbidity and mortality among all ischemic heart diseases. The current treatment for MI is mainly Western medications and invasive surgery, which include coronary artery bypass graft and percutaneous transluminal coronary angioplasty. These treatment measures effectively reduced mortality in patients through alleviating the symptoms but not reducing the hypertrophya and death of residual myocardial, and these measures are incapable for the prevention of the transition to heart failure in patients [1-4]. In addition, some of the patients are not suitable for these treatments for a variety of reasons. Under this circumstance, proangiogenic therapy, as a noval treatment strategy, has been proposed for the treatment of patients with MI. Animal studies have proven that angiogenesis has the potential to recover ischemic myocardial perfusion, reduce myocardial fibrosis and infarct size, increase the thickness of the blood vessel wall, as well as increase left ventricular systolic pressure. Short-term clinical trial also received relatively positive results. However, the current proangiogenic therapy is still in the primary stage because of many potential risk factors [5-8].

From the traditional Chinese medicine view, MI results from blood stasis. The blood-activating and stasis-eliminating effect is the important strategy for the treatment of MI, which is consistent with the angiogenic therapy in modern medicine. The stems of Rhodiolarosea (R. rosea) has been used as a traditional Chinese medicine to treat MI by activating blood circulation, and salidroside (SAL) is the active component of R. rosea. In this study, using pathological methods and molecular biological approaches, the angiogenesis effect of SAL on myocardium of rats with myocardial infarction (MI) and its possible mechanism was investigated.

Materials and methods

Reagents

Salidroside, with purity higher than 99.3%, was purchased from the National Institute for the

Control of Pharmaceutical and Biological Products (Lot: 20130096; Beijing, China).

Animals

A total of 60 adult 8-week old male Sprague-Dawley rats weighing 200-240 g were purchased from Henan experimental animal center (License: SCXK (Henan province) 2010-0002). They were allowed standard chow pellets and drinking water ad libitum. The experimental protocol was ethically approved by the Animal Care Committee of Nanyang Institute of Technology. Rats were randomly divided into five groups (8 animals each). Rats of the sham-operated control (sham) and MI groups were administrated with saline solution by oral gavage. The other three group of rats were administrated with 10 mg/(kg/d), 20 mg/(kg/d) or 40 mg/(kg/d) SAL by oral gavage each day for four weeks.

Establishment of MI model

MI model was constructed by left anterior descending coronary artery ligation method. Briefly, rats were anesthetized by 10% chloral hydrate intraperitoneally at the time of operation, and ventilator was used after endotracheal intubation (Respiratory rate: 50-60 times/min; tidal volume: 2-3 mL/100 g). Changes in heart rate were observed by cardiac function software and ST-T segment before and after MI. The left side of the heart was exposed through a fourth intercostal thoracotomy. The heart was exposed by opening the pericardium. A ligation was made at 3 mm from the end of the left anterior descending coronary artery. Successful MI was confirmed by ST segment elevation in electrocardiographic alteration, as well as visual assessment of regional cyanosis of the ischemic region in the left ventricle (LV). For the sham group, the ligation of the left anterior descending coronary artery was not conducted, but the other procedures were the same as in the model group. Penicillin was intraperitoneally injected after surgery (800,000 units). MI model was considered successful if rats were alive 24 h after surgery.

Hematoxylin-eosin (HE) staining

Apical myocardial tissues of rats were taken and frozen with liquid nitrogen. Myocardial tissues were fixed in 10% formalin, and sections were cut in midpoint of the line along the long axis from apex to base of the left ventricular tissue at about 4 μm thick. After washing with running water and distilled water, sections were stained with hematoxylin for 3-5 min. After washing again with running water, sections were differentiated with 1% HCl in 70% alcohol. Then sections were stained with eosin for 1-4 min after washing with running water. After dehydration and differentiation in alcohol, sections were mounted and observed under Nikon Tis microscopy (Nikon, Tokyo, Japan). From each slice, six radially oriented fields (×400) were selected at random and integrated microvascular were counted.

Masson staining and determination of collagen volume fraction (CVF)

Apical myocardial tissues of rats were taken, and Masson's trichrome staining was used to evaluate collagen content. After dehydration and dewaxing, sections were differentiated with 1% HCl for 3-5 s. Morphology of myocardial collagen was assessed by picrosirius red-staining for 3 min. After washing with running water and distilled water, sections were treated with phosphomolybdic acid for 1 min. After washing again with running water, sections were stained with 2% aniline blue solution for 2 min. After dehydration and differentiation in alcohol, sections were mounted and observed under microscopy. Collagen was stained blue and myocardium was stained red. CVF was quantified in tissue sections using Nikon NIS-Elements Software BR (Nikon, Tokyo, Japan). CVF was then calculated as the area occupied by myocardial collagen divided by the total area of the tissue section. From each slice, six radially oriented fields (×400) were selected at random and the mean CVF was calculated.

RNA extraction and semi-quantitative PCR assay

A total of 100 mg myocardial tissues from infarct area were taken from rats and frozen at -80°C. Tissues were homogenized and centrifuged at 4°C and 12,000 rpm. The total RNA was extracted using TRIzol® isolation reagent (Thermo Fisher Scientific, Vilnius, Lithuania) according to the manufacture's construction. A total of 2 ug of total RNA was reverse transcribed into cDNA using the reverse transcription kit (Invitrogen, Carlsbad, CA, USA). The ex-

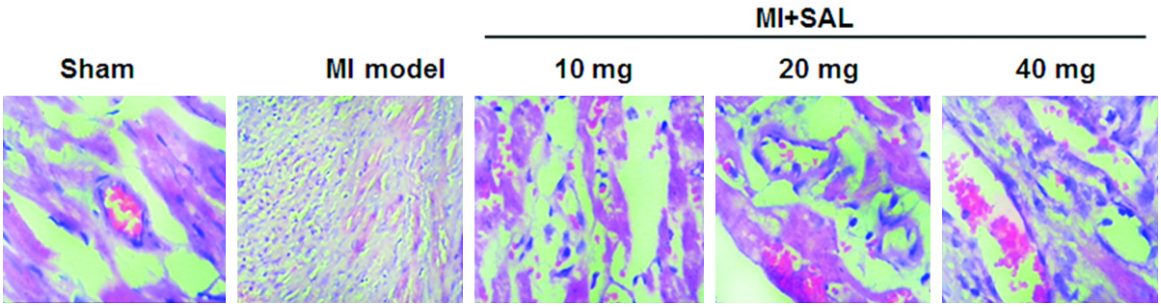

Figure 1. Pathological changes of myocardial tissue. Apical myocardial tissues of sham group, model group and SAL (10 mg/kg, 20 mg/kg and 40 mg/kg) treated rats were taken for H&E staining. Myocardial tissue stains red color and fibrosis stains pink color.

Table 1. Effect of SAL on the number of microvascular and collagen content in rat myocardial tissue

Groups	Dose/mg/(kg/d)	No. microvascular	CVF/%
Sham	-	5.2±0.4*	9.5±2.2**
MI	-	3.4±0.2	44.7±8.3
SAL	10	12.6±2.3**	36.3±5.4**
	20	18.4±3.2**	22.4±6.0**
	40	24.7±2.6**	17.2±2.8**

CVF, collagen volume fraction. *P<0.05, **P<0.01, compared with MI group.

pression of β-actin was used as internal control. Semi-quantitative PCR was performed with 2 ug of cDNA. For mRNA quantification, the reaction mixture was incubated for 1 cycle at 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, 58°C for 1 min, 72°C for 1 min, and 72°C for 5 min. Primer used for amplification was as follows, VEGF forward: 5'-ATGAACTTTCTGCTCTCTTGGG-3' and reverse: 3'-CTCTCCTATGTGCTGGCTTTG-5'; CD34 forward: 5'-CCTGCCGTCTGTCAATGTTTC-3' and reverse: 3'-GCACTCCTCGGATTCCTGAAC-5'; β-actin forward: 5'-CGTTCACATCCGTAAAGACCTC-3' and reverse: 3'-TAGGAGCCAGGGCAGTAATCT-5'. The amplification products (10 ug) were separated by 1.5% agarose gel electrophoresis, and the acquired images were analyzed by AlphaView SA and the relative mRNA expression was expressed as the densitometric value ratio of VEGF or CD34 band to β-actin band.

Western blot analysis

Myocardial tissue homogenates were obtained as described above and total protein was extracted. A total of 20 ug Protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane. After blocking with 5% skimmed milk for 2 h, the membranes were probed with the following antibodies: rabbit anti-CD34 (1:1000; bs-2038R; Beijing Biosynthesis Biotechnology co., Ltd), rabbit anti-VEGF (1:1000; bs-1313R; Beijing Biosynthesis Biotechnology co., Ltd) and mouse anti-β-actin (1:5000; BM-0627; Wuhan BosterBiotechnology co., Ltd, China). Goat anti-rabbit IgG antibodies (1:2000) conjugated to HRP (Boster Biotechnology Co., Ltd., Wuhan, China) were used as secondary antibodies. Signal detection was performed using chemiluminescence reaction (ECL) (Boster Biotechnology Co., Ltd., Wuhan, China). The acquired images were analyzed by Image lab 3.0 (Bio-Rad Laboratories) and the relative protein expression was expressed as the densitometric value ratio of CD34 or VEGF band to β-actin band.

Statistical analysis

Data were expressed as mean ± S.D. Statistical significance was determined with paired *t*-tests using SPSS 16.0 (SPSS Statistics/IBM Corp, Chicago, IL, USA). *P*-values <0.05 were considered statistically significant.

Results

Effect of SAL on histopathology

H&E staining was used to analyze the histopathology of myocardial tissues. In sham group, the structure of myocardial was normal, myocardial fibers were integral without significant changes and vascular morphology was com-

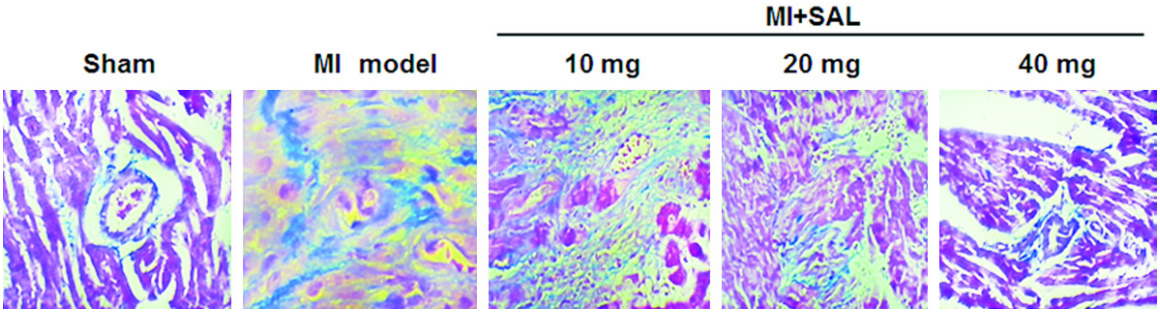

Figure 2. Myocardial Masson staining. Normal myocardium stains red-brown and fibrotic tissue stains blue.

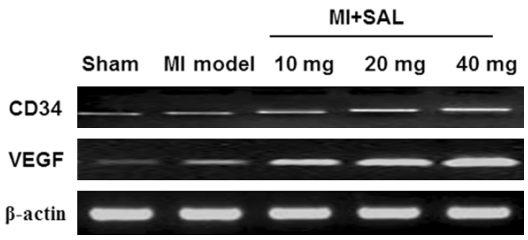

Figure 3. Semi-quantitative PCR. Myocardial tissues from infarct area were taken for RNA extraction. After reverse transcription, CD34 and VEGF mRNA expression was analyzed by semi-quantitative PCR. Expression of β-actin mRNA was used as internal control.

plete. Untreated model group showed obviously swelling of myocardial cells, disappeared nuclei of the myocardial cells, loss of transverse striations, and large numbers of invasive inflammatory cells. Compared with the model group, treatment with 10 mg/kg, 20 mg/kg and 40 mg/kg SAL resulted in relative normal myocardial arrangement, clear transverse striations, fewer fibroblasts and slight inflammatory cell infiltration (**Figure 1**). In addition, the number of microvascular was significantly increased in SAL treated groups compared with model group (**Table 1**). These results indicate that SAL could reduce the injury of myocardial cells.

Effects of SAL on myocardial fibrosis

Masson's trichrome staining was performed to evaluate fibrosis. The myocardial cells were stained red and collagenous fibers were blue. We found that the sham group exhibited tight myocardial fibers with a small portion of blue collagen. The model group showed more severe myocardial fibrosis compared to sham group, indicated by increased blue collagenous fibers and higher CVF values ($P<0.01$, **Figure 2** and **Table 1**). SAL treatment partly ameliorated cardiac hypertrophy and markedly reversed myocardial fibrosis in MI rats, and the effect of SAL was better with increased concentration (**Figure 2** and **Table 1**).

Effects of SAL on VEGF and CD34 expression

Next we analyzed the expression of VEGF and CD34. MI induced slightly higher expression of VEGF and CD34 mRNA, and SAL treatment significantly increased expression of these mRNAs compared with model group ($P<0.01$, **Figure 3** and **Table 2**). Similarly, although model group had slightly increased VEGF and CD34 protein expression, SAL treatment significantly increased the expression of VEGF and CD34 ($P< 0.01$, **Figure 4** and **Table 2**). Moreover, expression of VEGF and CD34 was increased with higher concentration of SAL (**Table 2**). Collectively, these results indicate the cardioprotective effect of SAL in acute myocardial injury.

Discussion

At the early stage after MI, ischemia of myocardial tissues leads to the body's self-protective response, stimulating the formation of granulation tissue. However, due to non-renewability of cardiomyocytes, myofibroblasts transiently appear during granulation tissue formation and become apoptotic when the scar matures. We found that SAL treatment obviously increased new blood vessels in myocardium, and histopathological examination showed more integrate vessels in high dose SAL group compared with that treated with medium or low dose of SAL.

Angiogenesis is affected by a variety of biochemical factors. Before the formation of new blood vessels, proangiogenesis factors penetrate into endothelial cells by degrading the

Table 2. Effect of SAL on the expression of CD34 and VEGF in rat myocardial tissue

Group	Dose/mg/(kg/d)	VEGF mRNA/β-actin mRNA	Cd34 mRNA/β-actin mRNA	VEGF protein/β-actin protein	CD34 protein/β-actin protein
Sham	-	0.241±0.023	0.181±0.02	0.366±0.024	0.321±0.028
MI	-	0.321±0.027	0.231±0.025	0.421±0.031	0.267±0.022
SAL	10	0.723±0.069**	0.642±0.077**	1.076±0.094**	0.714±0.085**
	20	1.124±0.078**	0.824±0.079**	2.234±0.156**	1.497±0.123**
	40	1.579±0.08**	0.926±0.097**	2.925±0.17**	1.578±0.197**

**$P<0.01$, compared with MI group.

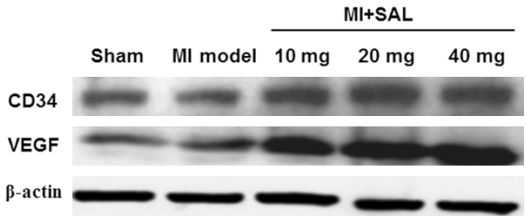

Figure 4. Western blot. Myocardial tissue homogenates were obtained and total protein was extracted. CD34 and VEGF expression was detected and expression of β-actin was used as internal control.

extracellular matrix and increasing the permeability of blood vessel wall, thus promoting the proliferation of endothelial cells [9]. Proliferation of endothelial cell is induced by soluble VEGF, and VEGF plays an important role in the formation of new blood vessels [10, 11]. Binding of VEGF to its receptor promotes the multiplication and migration of endothelial cells, mediates differentiation and migration of mesoderm precursor cells that related to angiogenesis and vascular network building, and induces mesoderm precursor cells to position in the site angiogenesis. Therefore, VEGF is one of the most important signaling proteins associated with angiogenesis and vascularization [4, 9-11]. Meanwhile, VEGF promotes the expression of integrin αvβ3, leading to vasodilatation, increased permeability of the wall, macrophage and neutrophil chemotaxis [12]. In addition, VEGF also induces the expression of extracellular matrix degrading enzymes that plays important roles in the formation of the lumen of the blood vessel [11-15]. In this study, we found upregulated expression of VEGF in MI model rats, which should be the normal pathological reaction after tissue damage. Results of semi-quantitative PCR and Western blot showed that expression of CD34, a protein mainly expressed in normal tissues and vessel, was relatively low in the myocardial tissues of rats in model group, further illustrating the reason for less integrate blood vessels in rats of model group. However, SAL treatment significantly increased VEGF and CD34 expression, and elevated VEGF would further promote the formation and maturation of new blood vessels. Furthermore, elevated CD34 is the indicator for increased number of new blood vessels.

Rhodiolarosea is a widely used traditional Tibetan medicine herb that has been shown to improve blood circulation [16]. Since it is known that the etiology and pathogenesis of MI is blood stasis due to Qi deficiency. Rhodiola has significant effects of removing blood stasis for promoting tissue regeneration as an important adjuvant for ischemic heart disease treatment [17]. MI is a kind of ischemic heart disease, therefore, Rhodiola could improve blood retention by removing blood stasis, resulting in the reconstruction of vessels and the establishment of collateral circulation.

In conclusion, our present study demonstrates that SAL could effectively promote the formation and development of new blood vessels, which further improve the hypoperfusion state at early stages of MI, control the decline in myocardial hypertrophy and contractile force, and effectively prevent heart failure.

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Disclosure of conflict of interest

None.

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