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Original Article

Promotive effect of salvia extract on angiogenesis of myocardium in rats with myocardial infarction

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Abstract: Objective: This study is to determine the effect and mechanism of salvia extract on angiogenesis of the myocardium in rats with myocardial infarction (MI). Methods: Left coronary artery of Sprague-Dawley rats was ligated to establish MI model. Forty rats were randomly divided into five groups: sham operation group, MI model group, and three salvia extract groups (10, 20, 40 mg/kg/day), with each group consisted of 8 rats. The salvia extract groups were orally administered with salvia extract while the MI group and sham operation group were fed with the same volume of saline. The rats were sacrificed 4 weeks later, and segmental heart samples were used for hematoxylin and eosin staining, masson staining, or electron microscopy. The expression of vascular endothelial growth factors (VEGF) and cluster of differentiation 34 (CD34) were analyzed using immunohistochemistry. Results: Compared with sham operation group, morphology of myocardium in MI group was disordered. Part of myocardial cell outline disappeared, showing obvious fibrosis in necrotic myocardial tissues, along with fuzzy or disappeared vascular ultrastructure. Compared with MI group, the number of new vessels in all salvia extract groups was increased, and the endothelial cell morphology was relatively complete according to the electron microscopy. The protein expression levels of VEGF and CD34 in the cytoplasm of myocardial tissues showed a trend that all salvia extract groups > MI group > sham operation group ($P < 0.01$). Conclusions: The present study demonstrates that salvia extract promotes angiogenesis in myocardial tissues of rats with MI.

Keywords: Salvia, myocardial infarction, angiogenesis, pathology, endothelial cells

Introduction

According to the World Health Organization, 7.25 million people die of ischemic myocardial disease in the world every year, accounting for 12.8% of all deaths [1]. Myocardial infarction (MI) belongs to the category of ischemic heart disease, and the number of deaths caused by MI is the highest among all ischemic heart diseases [1]. In the past twenty years, a variety of therapeutic measures for MI have been used to effectively reduce the mortality rate of patients. Systemic thrombolytic therapy and percutaneous coronary angioplasty are the most successful treatments, which can effectively restore the reperfusion of ischemic myocardium. However, these treatment measures are still difficult to prevent and reverse the development of heart failure in patients [2, 3]. Angiogenic therapy has been proposed as a new treatment strategy, and already achieved positive effects in short-term clinical treatments [4, 5].

It is reported that vascular endothelial growth factor (VEGF) has a prominent role in post-MI angiogenesis through direct effect on endothelial cell survival and proliferation, regulating new vessel formation and permeability as well as the recruitment of inflammatory and regenerative cells [6]. However, evidence has shown that single angiogenesis therapy such as VEGF treatment can lead to multiple potential risk factors such as drug resistance and teratogenesis [7].

Recently, therapeutic potential of natural products or traditional Chinese medicine (TCM) against MI attracts more and more concerns [8-13]. One of the important strategies for the treatment of MI with TCM is “promoting blood circulation to remove blood stasis”. Natural products of TCM also have advantages to treat MI due to the complexity, chemical diversity, and biological properties of them. Danshen (Radix Salviae miltiorrhizae et Rhizoma), the roots and rhizomes of Salvia miltiorrhiza Bunge,

is one of the well-known TCMs commonly used for activating circulation [14]. It is reported that Danshen can reduce the production of reactive oxygen species (ROS) in MI mouse model [15]. Salvianolate, a highly purified aqueous extract from Danshen, is demonstrated to have protective effects on microvascular flow that is associated with elevated superoxide dismutase activity, thioredoxin activity and glutathione concentration, and to reduce malondialdehyde concentration in porcine model of myocardial ischaemia and reperfusion [16].

CD34 is a member of a family of single-pass transmembrane sialomucin proteins that show expression in hematopoietic progenitor cells during embryonic phase and then in the vascular endothelial cells [17]. CD34 is used as a marker to select whole bone marrow-derived mononuclear cells or medullar cells, which are often used as a source of EPCs for preclinical studies of therapeutic cell therapy for angiogenesis [18]. Although previous studies have suggested that the cardioprotective effect of the root of salvia is related to multi-mechanisms and multi-targets, the angiogenesis effect related to VEGF and CD34 has not been studied before. In the present study, we aimed to determine the effect of salvia extract on angiogenesis after MI in rats, and to investigate its mechanism of action.

Materials and methods

Animals

Male Sprague-Dawley rats (8 weeks, 200-240 g) were purchased from Experimental Animal Center of Henan Province (license No. SCXK (Yu) 2010-0002; Certificate of Conformity No. 1000142). Rat MI model was constructed by the ligation of left anterior descending branch of coronary artery. For MI model group, the rats were anaesthetized by intraperitoneal injection of 10% chloral hydrate. Then, the thoracic cavity of the rat was opened and the heart was exposed, before the coronary artery was ligated at 3 mm from its root part. Subsequently, the heart was returned to the thoracic cavity, and the cut was sutured. Intraperitoneal injection of penicillin was performed to prevent infections. For the sham operation group, a piece of wire was only threaded at the same surgical site in the MI model group without ligation. At 48 h after the surgery, a total of 40 survived rats were evenly and randomly divided into sham operation group, MI model group, low-dose group (10 mg/kg/day salvia extract), medium-dose group (20 mg/kg/day salvia extract) and high-dose group (40 mg/kg/day salvia extract). Rats in sham operation group and MI model group were lavaged using saline at 9 o'clock every day, while rats in other groups were lavaged using respective doses of salvia extract according to body weights. After surgery, the rats were raised for a total of consecutive four weeks. All animal experiments were conducted according to the ethical guidelines of Nanyang Institute of Technology.

Hematoxylin and eosin (HE) staining

Apical myocardial tissues were frozen in liquid nitrogen, and fixed using 10% formaldehyde before being cut into sections with a thickness of 4 μm at the midpoint along the major axis of the left ventricle. Then, the specimens were washed with deionized water and stained using hematoxylin for 3-5 min. After washing with water again, the specimens were fixed in anhydrous alcohol containing 1% HCl, and stained with eosin for 1-4 min, followed by extensive washing with water. After another fixing with anhydrous alcohol, the specimens were paraffin-embedded and observed under a light microscope (Nikon Tis; Nikon, Tokyo, Japan) with a magnification of 400×.

Masson staining

Apical myocardial tissues were frozen in liquid nitrogen after excision. The specimens were dewaxed and dehydrated before washing with 1% HCl solution for 3-5 s. After staining with mild alkaline fuchsin for 3 min, the specimens were washed with deionized water. After staining with 1% phosphomolybdic acid for 1 min, the specimes were washed again with water. Subsequently, the specimens were stained with 2% aniline blue solution for 2 min and dehydrated by washing with 95% alcohol before embedding. Under the light microscope (Nikon Tis; Nikon, Tokyo, Japan), myocardial collagen fibers showed blue-green color, while myocardial tissues exhibited red color. Changes of collagen fiber composition were analyzed using NIS-Elements Software Basic Research (Nikon, Tokyo, Japan).

Transmission electron microscopy

Apical myocardial tissues were washed with saline, and soaked in 2.5% glutaraldehyde, before being cut into pieces of 0.5 mm^3. Then, the

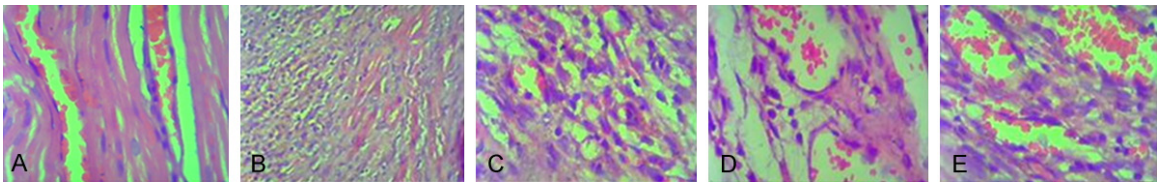

Figure 1. Effect of salvia extract on myocardial pathomorphology. Hematoxylin and eosin staining was used to visualize myocardial tissues (magnification, ×400). A: Sham operation group; B: MI model group; C: Low-dose salvia extract group (10 mg/kg/day); D: Medium-dose salvia extract group (20 mg/kg/day); E: High-dose salvia extract group (40 mg/kg/day).

tissues were fixed in 2.5% glutaraldehyde fixing solution for 3 h. Afterwards, the samples were fixed again with 1% osmium tetroxide for an hour, followed by washing with phosphate-buffered saline for 3 times of 15 min. The samples were then dehydrated using acetone (50%, 70%, 80%, 90% and 100%) for 3 times of 1015 min. Subsequently, the samples were soaked with EPON812 and acetone (1:1) for 1 h, EPON812 and acetone (3:1) for 3 h, and EPON812 for 12 h. The samples were embedded and labeled before aggregation at 37°C for 12 h, 45°C for 12 h, and 60°C for 24 h. After slicing into 50 nm thickness, the slices were stained with uranyl acetate and lead citrate, before observation under transmission electron microscope (HT7700; Hitachi, Tokyo, Japan).

Immunohistochemical staining

Apical myocardial tissues were frozen in liquid nitrogen after excision. The specimens were dewaxed and dehydrated before incubation with 3% H_2O_2 solution at room temperature for 15 min to eliminate ndogenous peroxidase activity. After washing with deionized water, antigen retrieval was achieved by heating the specimens for 2 min at 100°C in citric acid buffer (0.01 mol/L, pH 6.0). After washing with phosphate-buffered saline, the specimens were blocked using 5% bovine serum albumin for 20 min at room temperature. Then, the specimens were incubated with monoclonal rabbit anti-mouse VEGF (1:100; bs-0565R; Bioss, Beijing, China) or cluster of differentiation 34 (CD34) (1:100; bs-2038R; Bioss, Beijing, China) primary antibodies in dark at 4°C for 12 h. Afterwards, biotin-labeled goat anti-rabbit IgG secondary antibody (TC1378; Boster, Wuhan, China) was added for incubation at room temperature for 20 min, before treatment with streptavidin-biotin-peroxidase complex and 3,3'-diamino-benzidine staining (DD-1660; Boster, Wuhan, China). Following hematoxylin staining, the specimens were subjected to dehydration, transparency and mounting, before being observed under a microscope (Nikon Tis; Nikon, Tokyo, Japan) with a magnification of 400×. Positive expression of VEGF was indicated by light brown granules in cytoplasm, while positive CD34 expression was shown by dark brown granules in cytoplasm. Ten fields were analyzed for each slice, and the average absorbance of VEGF or CD34 in each field was calculated using NIS-Elements Software Basic Research (Nikon, Tokyo, Japan).

Statistical analysis

The results were analyzed using SPSS 16.0 statistical software (IBM, Armonk, NY, USA). All data were expressed as means ± standard deviations. Differences between groups were analyzed using analysis of variance. $P < 0.05$ indicated statistical significance.

Results

Structure and morphology of myocardial tissues, blood vessels and smooth muscles in all salvia extract groups are improved compared with those in MI model group

To visualize myocardial tissues, HE staining was performed. In sham operation group, the structures of myocardial cells were clear and intact, myocardial tissues were arranged in order, and red cells existed in the lumen (**Figure 1A**). In MI model group, the color of myocardial cells was lighter compared with sham operation group, the arrangement of myocardial tissues was disordered, the outline of myocardial cells was blurry, karyolysis and neutrophil infiltration were present, and hyperplasia was observed in aged granulation tissues

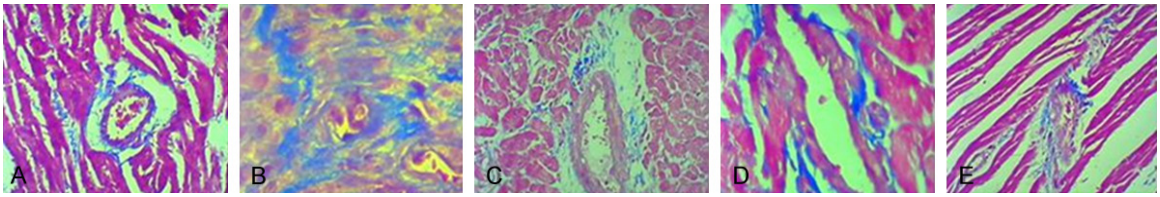

Figure 2. Effect of salvia extract on fibrosis in myocardial tissues. Masson staining was used to observe fibers in myocardial tissues (magnification, ×400). A: Sham operation group; B: MI model group; C: Low-dose salvia extract group (10 mg/kg/day); D: Medium-dose salvia extract group (20 mg/kg/day); E: High-dose salvia extract group (40 mg/kg/day).

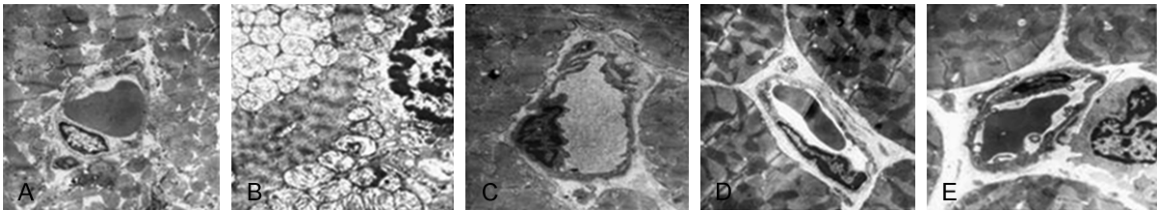

Figure 3. Effect of salvia extract on myocardial ultrastructure. Transmission electron microscopy was used to show myocardial ultrastructure (magnification, ×5000). A: Sham operation group; B: MI model group; C: Low-dose salvia extract group (10 mg/kg/day); D: Medium-dose salvia extract group (20 mg/kg/day); E: High-dose salvia extract group (40 mg/kg/day).

(**Figure 1B**). By contrast, salvia extract groups showed ordered myocardial tissues, clearer outline, darker tissue color, reduced number of fibroblasts, and milder inflammatory infiltration than MI model group. In addition, abundant new granulation tissues and blood vessels appeared in the myocardial tissues in low-dose salvia extract group (**Figure 1C**). In medium-dose salvia extract group, there were many newborn blood vessels with clear structures and intact vascular wall (**Figure 1D**). In high-dose salvia extract group, the amount of luminal smooth muscles was increased, and vascular wall was thickened (**Figure 1E**). These results suggest that the structure and morphology of myocardial tissues, blood vessels and smooth muscles in all salvia extract groups are improved compared with those in MI model group.

Treatment with salvia extract reduces the degree of fibrosis in myocardial tissues in a dose-dependent manner

To observe fibers in myocardial tissues, Masson staining was employed. In sham operation group, myocardial tissues had regular shapes and clear structures, few blue collagen fibers were observed among abundant red myocardial tissues, and blood vessels had intact shapes, distinct layers, clear red muscle layers, and few blue collagen fibers around the vessels (**Figure 2A**). In MI model group, the red myocardial tissues had disordered arrangements, obvious tissue fractures, many blue collagen fibers, disordered granulation tissues, less intact vascular shapes, and lumen closure (**Figure 2B**). By contrast, salvia extract groups showed ordered arrangements of myocardial tissues, and increased percentages of red myocardial tissues than MI model group. In low-dose group, the number of new vascular vessels in myocardial tissues was increased, but the arrangements of red myocardial tissues were not ordered, and the red color was a little light (**Figure 2C**). In medium-dose group, newborn intact vascular vessels and granulation tissues were cross-linked, vessel lumen was clear and intact, and the vascular wall was thick (**Figure 2D**). In high-dose group, red cardiac muscles accounted for most part of the myocardial tissues, with rare blue fiber tissues and abundant newborn mature vascular vessels (**Figure 2E**). These results indicate that treatment with salvia extract reduces the degree of fibrosis in myocardial tissues in a dose-dependent manner.

Salvia extract alleviates the injuries of endothelial cells in vascular vessels of myocardial tissues

To investigate the status of endothelial cells in vascular vessels, transmission electron micros-

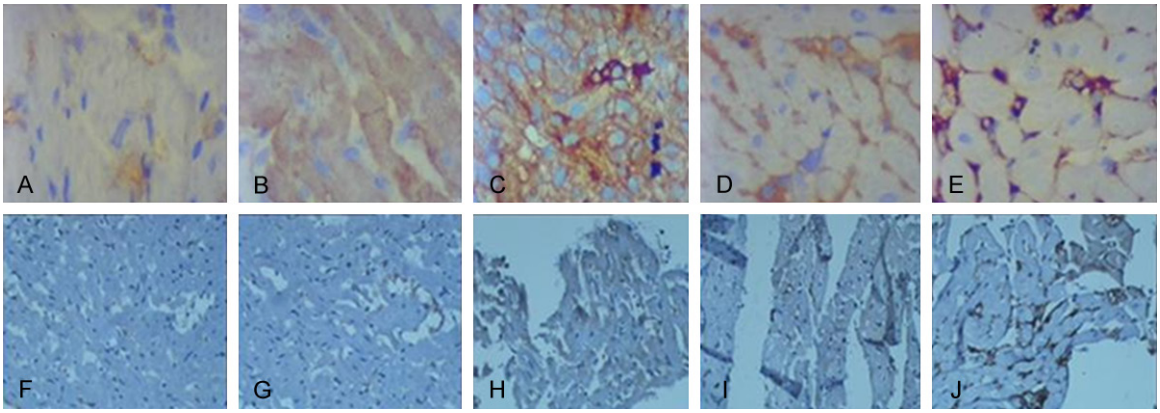

Figure 4. Effect of salvia extract on the expression levels of (A-E) VEGF and (F-J) CD34 proteins in myocardial cells of rats with myocardial infarction (magnification, ×400). (A and F) Sham operation group; (B and G) MI model group; (C and H) Low-dose salvia extract group (10 mg/kg/day); (D and I) Medium-dose salvia extract group (20 mg/kg/day); (E and J) High-dose salvia extract group (40 mg/kg/day).

Table 1. Effect of salvia extract on the expression levels of VEGF and CD34 proteins in cardiac myocytes of rats with myocardial infarction (means ± standard deviations)

Groups	No. of cases	Salvia doses (mg/kg/day)	VEGF level	CD34 level
Control	8	-	41.16 ± 2.79	12.69 ± 2.88
Model	8	-	58.24 ± 8.76	20.54 ± 3.92
Low-dose salvia	8	10	159.68 ± 17.44*	102.11 ± 10.50*
Medium-dose salvia	8	20	185.25 ± 18.52*	153.24 ± 16.66*
High-dose salvia	8	40	202.28 ± 20.72*	207.32 ± 15.85*

Note: VEGF, vascular endothelial growth factor; CD34, cluster of differentiation 34. *P < 0.01 compared with model group.

copy was carried out. In sham operation group, myocardial tissues had ordered arrangements, clear intercalated discs and clear vascular shapes; the vascular vessels had intact wall, red blood cell filling in the lumen, intact endothelial cells, apparent nucleus, smooth basal membrane, and visible pericytes around the vessels (**Figure 3A**). In MI model group, myocardial tissues had disordered arrangements, blurry intercalated discs, damaged vascular wall, injured endothelial cells, karyolysis, shrunk basal membrane, and few pericytes (**Figure 3B**). By contrast, salvia extract groups showed ordered myocardial tissues, clearer intercalated discs, and more intact vessel shapes. In low-dose group, the vascular wall in myocardial tissues was basically intact, endothelial cells were only partially damaged, and the shrinkage degree of basal membrane was alleviated but still severe (**Figure 3C**). In medium-dose group, clear vessels were observed in myocardial tissues, with intact vascular wall, clear nucleus in endothelial cells, smooth basal membrane, existence of pericytes (**Figure 3D**). In high-dose group, the condition was similar to that in medium-dose group, with clearer vessel wall, more intact endothelial cells, and visible pericytes (**Figure 3E**). These results suggest that salvia extract alleviates the injuries of endothelial cells in vascular vessels of myocardial tissues.

Salvia extract enhances the positive expression of VEGF and CD34 in myocardial tissues

To determine the expression of VEGF and CD34 in myocardial tissues, immunohistochemical staining was used. In sham operation group, positive expression of VEGF or CD34 was low in cytoplasm of myocardial cells (**Figure 4A** and **4F**). In MI model group, positive expression of VEGF and CD34 was increased compared with that in sham operation group, but without statistical significance (P > 0.05) (**Figure 4B** and **4G**; **Table 1**). Compared with MI model group, salvia extract treatments significantly enhanced the positive expression of VEGF and CD34 in the cytoplasm of myocardial cells (P < 0.01) (**Table 1**). Of note, the vascular vessels in low- and medium-dose groups were mainly newborn vessels, while those in high-dose group were

mainly vessels with intact shapes (**Figure 4C-E** and **4H-J**). These results indicate that salvia extract enhances the positive expression of VEGF and CD34 in myocardial tissues.

Discussion

After MI, myocardial ischemia can induce self-protective response of the body, and stimulate the formation of granulation tissues. However, aged granulation tissues will be transformed into fibrous tissues due to the poor myocardial cell regeneration ability. In the present study, HE staining, masson staining, and VEGF expression have demonstrated the formation of granulation tissues in myocardial tissues. However, electron microscopy has shown that the basal membrane of vascular vessels in myocardial tissues from MI model group is severely injured, and endothelial cells are not intact, preventing the transformation of newborn granulation tissues into functional vascular vessels. In the meantime, CD34 expression level is low in myocardial tissues from MI model group, suggesting little formation of intact vascular vessels in these rats. After treatments by salvia extract, the number of newborn vascular vessels in myocardial tissues is increased. In low-dose group, granulation tissues are mainly observed. In medium- or high-dose groups, the number of newborn vascular vessels is dramatically increased, vessel structures are intact, and endothelial cells show good integrity under electron microscope. These results suggest that salvia extract exerts its effect in a dose-dependent manner. In addition, immunohistochemical staining has shown consistent results.

The formation of new blood vessels is achieved in the form of budding. At initial stages, degradation of extracellular matrix and increase of vessel permeability induce the activation of angiogenic factors, as well as the migration and proliferation of endothelial cells. As the increase in the number of endothelial cells, newborn buds form the lumen [2-4]. In this process, VEGF and its receptor are highly expressed in vascular endothelial cells, being among the most important signal-associated proteins for angiogenesis and vascularization [4, 16-21]. Endothelial cells play important roles in the regulation of contraction and relaxation of vascular vessels, platelet aggregation, thrombosis and inflammatory responses. Injuries in endothelial cells can trigger the death of more endothelial cells, leading to reduced synthesis of vascular protective factors such as nitric oxide, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 [19]. In ischemic myocardium, VEGF not only promotes the migration and proliferation of endothelial cells, but also reduces their apoptosis [1, 16-19]. In the meantime, VEGF up-regulates the expression of endothelial nitric oxide synthase, leading to the production of more nitric oxide [20, 21]. VEGF also up-regulates the expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 via Notch/VEGF signaling pathway [19]. Therefore, enhanced VEGF expression, endothelial cell proliferation and endothelial cell integrity are required for angiogenesis. Our results also show that VEGF expression has been slightly elevated in MI model group, but this is only a normal pathological response after lesions. In addition, the expression of CD34, a characteristic marker of new vessels, is not high in MI model group, suggesting that new buds may not form real lumen. After treatment with salvia extract, the shapes of endothelial cells become intact, the number of endothelial cells is increased, and VEGF expression is elevated. These results suggest that salvia extract promotes angiogenesis in myocardial tissues of rats with MI, which effectively alleviates myocardial perfusion deficiency at the early stage of MI, decreases the degree of hypertrophy of myocardial cells, and enhances myocardial contractility, finally preventing the development of MI into heart failure.

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Disclosure of conflict of interest

None.

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