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Original Article

Danshen improves damaged cardiac angiogenesis and cardiac function induced by myocardial infarction by modulating HIF1α/VEGFA signaling pathway

Fen Ai[1], Manhua Chen[2], Wei Li[1], Yang Yang[1], Guizhong Xu[1], Feng Gui[1], Zhenxing Liu[1], Xiangyan Bai[1], Zhen Chen[1]

Departments of [1]Emergency, [2]Cardiology, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science & Technology, Wuhan 430014, Hubei Province, China

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Abstract: Objective: The traditional Chinese medicinal Danshen (Salvia miltiorrhiza), has long been used to treat cardiovascular diseases, however, the mechanism underlying its effects remain unclear. Here, this study would to investigate the effects of Danshen injection on myocardial infarction-induced cardiac damage. Methods: Danshen was injected into mice models at low dose (3 g/kg per day) or at high dose (6 g/kg per day) after left anterior descending coronary artery (LAD) ligation. After 4 weeks, cardiac function was evaluated by echocardiography. Paraffin sections of the hearts were used for isolectin GS-IB4 staining. Protein and mRNA expression levels of HIF1α and VEGFA were evaluated by western blotting and real-time polymerase chain reaction (RT-PCR). Results: The hearts showed significantly impaired angiogenesis and slightly increase of HIF1α and VEGFA expression after LAD ligation. The angiogenesis defect and heart failure were partially rescued in Danshen treatment mice with great increase of HIF1α and VEGFA mRNA levels and protein expression. Conclusion: These results illustrated that the protective effects of Danshen injection in responsive cardiac angiogenesis were at least in part due to increased HIF1α and VEGFA expression.

Keywords: Danshen, cardiac angiogenesis, heart failure, HIF1α, VEGFA

Introduction

Coronary heart disease is expected to become the leading worldwide cause of death by 2020 [1]. Neovascularization is a critical compensatory mechanism in the myocardial stress response of myocardial infarction [2]. The compounding effect of responsive cardiac angiogenesis and the increased cardiac demand for oxygen and nutrients contributes to the transition into pathological adaption, and eventually heart failure [3, 4]. The basic idea is to reactivate the neovascularization program, which mainly consists of angiogenesis, arteriogenesis, and vasculogenesis, and then to match the increasing demands of the stressed heart so that the heart is capable of dealing with the challenges without deteriorating to heart failure. Activation of the cardiac angiogenesis program is a promising therapeutic strategy validated in multiple forms of cardiac disease animal models [5-12]. Chinese traditional drug, Danshen, belonging to the Labiatae family of flowering plants, are commonly used in the clinic for the prevention and treatment of cardiovascular diseases, including atherosclerosis, hypertension, diabetes, and chronic heart failure and so on [13-15]. However, the function and mechanism of Danshen on left anterior descending coronary artery (LAD) ligation-induced cardiac damage are still unknown. The hypoxia-inducible factor 1α-vascular endothelial growth factor A (HIF1α-VEGFA) molecular signaling pathway is the most powerful and well-studied pathway in cardiac angiogenesis. Preclinical animal studies using proangiogenic factors such as HIF1α and VEGFA have achieved remarkable success [7-10]. Therefore, identifying the function of Danshen on LAD ligation-induced cardiac damage and whether the drug could improve the levels of proangiogenic factors such as VEGFA, HIF1α, which would be the critical point in this study.

Materials and methods

Materials

Primary antibody against VEGFA (ab51745; 1:200) was purchased from Abcam (Cambridge, MA, USA). And primary antibody against HIF1α (H1alpha67, NB100-105; 1:500) was purchased from Novus Biologicals (Littleton, CO, USA). GAPDH (#MB001; 1:1,000) was purchased from Bioworld Technology (St. Louis Park, MN, USA). TRIZol (#15596018) was purchased from Life Technologies (Invitrogen, NY, USA). The bicinchoninic acid protein assay kit was purchased from Pierce Biotechnology, Inc. (Rockford, IL, USA). Isolectin GS-IB4 staining (I21411) was purchased from Life Technologies (Invitrogen, NY, USA).

Experimental groups

This study was approved by the ethics committee of the Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology (Hubei, China). In the present study, the Male BALA/c mice were divided into five groups: the Sham group without any treatment (Sham); the MI group with LAD ligation (MI); the MI+S group treated with Saline after LAD ligation (MI+S); the MI+L group treated with Danshen at low dose (3 g/kg per day) after LAD ligation (MI+L); the MI+H group treated with Danshen at high dose (6 g/kg per day) after LAD ligation (MI+H).

Left anterior descending coronary artery (LAD) ligation and Danshen treatment

Male BALA/c mice (18-25 g, 10-12 weeks) were randomized to undergo LAD ligation or Sham surgery. It indicated successful ligation when the anterior wall of the left ventricle became visible blanching and swelling of the left atrium became hypokinesis. Briefly, all mice were anaesthetized with 1% pentobarbital sodium (30 mg/kg) by intraperitoneal injection and 1 ml of 1% lidocaine by local injection. Thoracotomy was performed via the third left intercostal and then the pericardium was torn open. The LAD was ligated by 8-0 prolene. In Sham group, the suture was passed through the myocardium beneath but the LAD without ligation. For the Danshen treatment experiments, mice were intraperitoneal injected with two doses of Danshen for 4 weeks starting immediately after LAD ligation. MI+S group mice were received LAD ligation and normal saline. All of the LAD ligation-induced cardiac stress lasted for 4 weeks.

Assessments of cardiac function

After 4 weeks of LAD ligation, cardiac function was evaluated by an echocardiograph equipped with a 3-7MHz linear transducer (Acuson Sequoia™ 512 ultrasound system, Siemens Medical Solution USA, Mountain View, CA, USA). In this study, we measured LV end-systolic interior dimension (LVIDs), end diastolic interior dimension (LVIDd), ejection fraction (EF) and fractional shortening (FS).

Isolectin GS-IB4 staining

Paraffin sections of the whole heart were used for isolectin GS-IB4 staining according to the manufacturer's instructions (Invitrogen, NY, USA). Pictures were taken under the microscope objective. The isolectin GS-IB4 staining images were acquired by fluorescence microscopy. A total of 8 staining pictures from each group were quantified.

Western blotting analysis

Protein extracted from heart tissues which were lysed in radioimmunoprecipitation assay lysis buffer was used for SDS-PAGE. The proteins were subsequently transferred to nitrocellulose membranes and blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20 (TBST; Cell Signaling Technology, Inc.) for 60 min at room temperature. Membranes were probed with various primary antibodies overnight. The following day, the membranes were washed with 1× TBST, incubated for 60 min with horseradish peroxidase-labeled mouse anti-rabbit antibody and anti-avidin antibodies (Cell Signaling Technology, Inc.) in TBST fluid. Following three washes of the membrane, images were captured on film, which was placed in 10 ml LumiGLO® solution (Cell Signaling Technology, Inc.) for one minute. Following development, the images were placed into an automatic image analyzer (Bio-Rad Laboratories, Hercules, CA, USA) to determine the function of the proteins as well as the reference grayscale values. A monoclonal GAPDH antibody was used separately as a loading control.

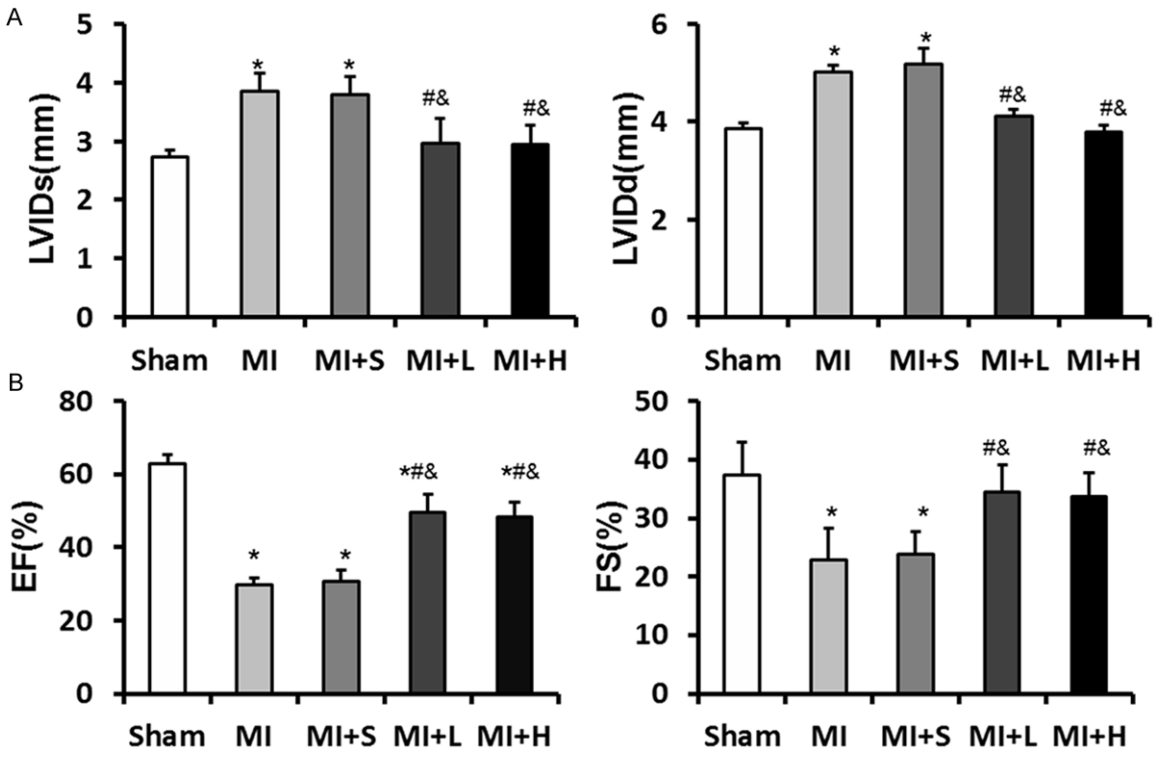

Figure 1. Danshen treatment improves cardiac function of mice after LAD ligation challenge. A. The LVIDs and LVIDd increased in MI and MI+S groups, but the MI+L and MI+H groups displayed smaller ventricular chambers than MI/MI+S group. B. The ejection fraction and the fractional shortening decreased in MI and MI+S groups 4 weeks after LAD ligation surgery. However, the MI+L and MI+H groups displayed great improvement in cardiac function when evaluated by ejection fraction and fractional shortening compared to MI and MI+S groups mice. Data are presented as the mean ± standard deviation. $^{*}P < 0.05$ vs. Sham group, $^{\#}P < 0.05$ vs. MI group, $^{\&}P < 0.05$ vs. MI+S group.

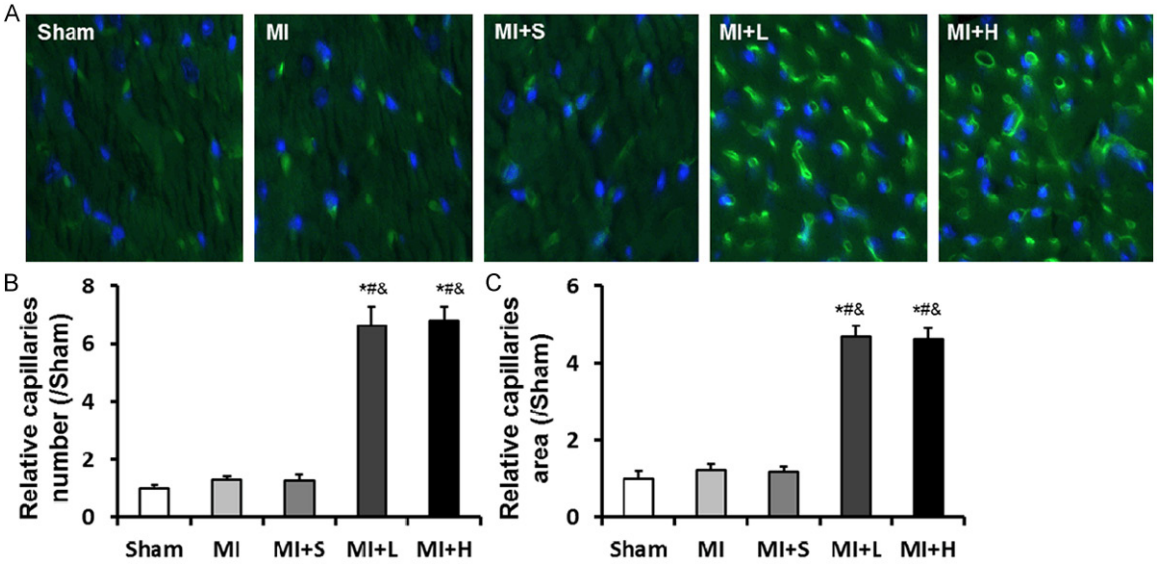

Figure 2. Danshen improves angiogenesis defect in response to LAD ligation. A. Fewer capillaries were observed in the post-MI heart by isolectin staining shown in green. Nuclear DAPI staining was shown in blue. However, the MI+L and MI+H groups displayed great improvement in capillary number compared to MI or MI+S group mice. B-C. Cardiac capillaries were quantified. Smaller capillary areas and fewer capillary numbers were observed in the MI and MI+S mice hearts compared to the Danshen treatment groups after LAD ligation. Data are presented as the mean ± standard deviation. $^{*}P < 0.05$ vs. Sham group, $^{\#}P < 0.05$ vs. MI group, $^{\&}P < 0.05$ vs. MI+S group.

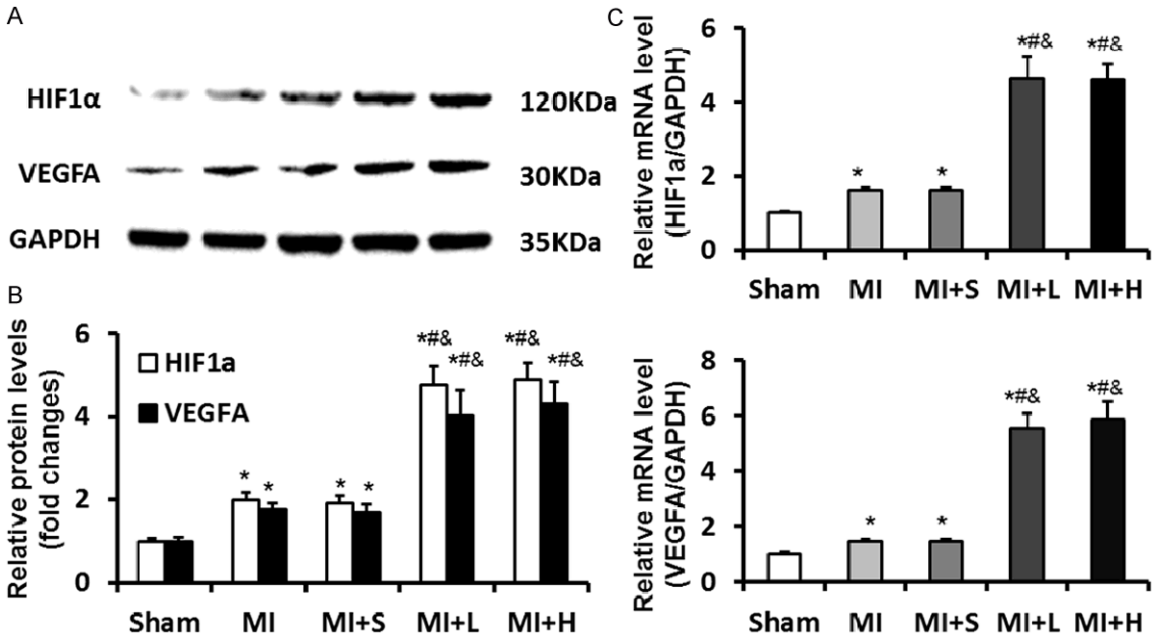

Figure 3. Danshen upregulates key proangiogenic factors HIF1α and VEGFA in response to LAD ligation stress. A. Slightly increase of HIF1α and VEGFA protein in the MI and MI+S group hearts in response to MI was shown by western blotting analysis. However, the MI+L and MI+H groups displayed great increase in protein expression of HIF1α and VEGFA. B. Quantitative results for western blotting of HIF1α and VEGFA. C. The mRNA levels of HIF1α and VEGFA in the hearts of mice following Sham or MI surgery with or without Danshen treatment. The MI+L and MI+H groups displayed great increase in mRNA levels of HIF1α and VEGFA compared to MI or MI+S group mice. Data are presented as the mean ± standard deviation. *P<0.05 vs. Sham group, #P < 0.05 vs. MI group, &P < 0.05 vs. MI+S group.

Real-time polymerase chain reaction (PCR) analysis

Total mRNA was extracted from mice myocardium tissue using TRIZol according to the manufacturer's instructions and synthesized cDNA using oligo (dT) primers with the Transcriptor First Strand cDNA Synthesis Kit. Selected gene differences were confirmed by quantitative real-time PCR using SYBR green and normalized results against GAPDH gene expression. The sequences of primers used in this study were displayed as follows: HIF1α: 5'-TTACTGAGTTGATGGGTTA-3' and 5'-TGTTTGTTGAAGGGAGAA-3'; VEGFA: 5'-CTGTGCAGGCTGCTGTAACG-3' and 5'-GTTCCCGAAACCCTGAGGAG-3'; GAPDH: 5'-ACTCCACTCACGGCAAATTC-3' and 5'-TCTCCATGGTGGTGAAGACA-3'.

Statistical analysis

Values are expressed as the mean ± standard error of the mean. Comparisons between four groups were performed using one-way analysis of variance using SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA). P < 0.05 was considered to indicate a statistically significant difference between values.

Results

Danshen improves cardiac function of mice after LAD ligation challenge

The end-point MI group mouse displayed dilated left ventricular chambers as shown in the echocardiographic analyses (**Figure 1A**). The LVIDs increased from 2.73 mm to 3.85 mm, the LVIDd increased from 3.79 mm to 4.89 mm, indicating that MI mice developed heart failure. However, the MI+L and MI+H groups displayed smaller ventricular chambers than MI group (**Figure 1A**). The ejection fraction decreased from 62.87% to 29.78% and the fractional shortening decreased from 37.39% to 22.82% in MI group 4 weeks after LAD ligation surgery, indicating the development of heart failure (**Figure 1B**). However, the MI+L and MI+H groups displayed great improvement in cardiac function when evaluated by ejection fraction and fractional shortening compared to MI group mice (**Figure 1B**). These results indicated that

Danshan treatment could improve cardiac function of mice after LAD ligation challenge.

Danshen improves angiogenesis in response to LAD ligation

In order to observe whether Danshen had an effect on angiogenesis in the animal hearts, we conducted an experiment of isolectin GS-IB4 staining in this study. Significantly smaller capillary areas and fewer capillary numbers were observed in the MI group hearts (**Figure 2A-C**). Compensatory increases in the capillary area and capillary number were found in MI+L and MI+H group mice in response to LAD ligation stress (**Figure 2A-C**). These data strongly suggested that Danshen treatment could improve severely impaired coronary angiogenesis in response to myocardial infarction challenge.

Danshen upregulates key proangiogenic factors, HIF1α and VEGFA, in response to LAD ligation stress

HIF1α and its downstream target VEGFA are critical for responsive angiogenesis in the heart after stress. These factors were evaluated in order to better understand the effect of Danshen in cardiac angiogenesis. Consistent with the observation of impaired angiogenesis, the mRNA and protein levels of HIF1α and VEGFA were lightly increased in MI and MI+S myocardia compared to the Sham myocardia (**Figure 3A-C**). However, significantly increase of HIF1α and VEGFA were observed in MI+L and MI+H group mice in response to LAD ligation stress (**Figure 3A-C**). These data indicated that Danshen treatment could upregulate key proangiogenic factors, HIF1α and VEGFA, in response to LAD ligation stress.

Discussion

Danshen (Salvia miltiorrhiza) is the traditional Chinese medicine, which is used for activating blood circulation and promoting blood circulation to remove blood stasis, nourishing blood sedative and so on [16-18]. Modern pharmacological researches show that the two effective components in Danshen, lipid-soluble diterpenoid compounds which could improve blood circulation, function as antibacterial and anti-inflammatory, and water-soluble phenolic acid compounds which could function as antioxidant, anticoagulation, anti-thrombosis, blood lipid regulation and cell protection [19-24]. It is an ideal treatment drug for treating myocardial ischemia diseases, which has a broad clinic application in future. However, the mechanism of Danshen in treating cardiovascular diseases is still not very clear. In this study, it showed that Danshen could improve cardiac function of mice with myocardial infarction, and Danshen injection had the protective effects in responsive cardiac angiogenesis. HIF1α-VEGFA signaling pathway is the best studied molecular mechanism in the regulation of cardiac angiogenesis [25]. In this study, we demonstrated that Danshen functions as a new mediator in the angiogenesis process by upregulating HIF1α-VEGFA during cardiac stress. In clinics, early studies have demonstrated that the functional disruption of the coronary microvasculature and the impairments in total myocardial perfusion occurred in human failing hearts with ischemic heart disease [26, 27]. The cardiac perfusion was severely reduced below normal levels in patients with ischemic disease, suggesting that abnormal myocardial perfusion plays a vital role in the development and/or transition to heart failure [28, 29]. It was proved that there was a close relationship between the depressed cardiac perfusion and the reduced capillary density in the myocardium of patients with idiopathic dilated cardiomyopathy [30, 31]. Meanwhile, cardiac perfusion abnormalities in patients were partially improved after beta-adrenergic blocker treatment, suggesting that the coronary microvasculature was dynamic and might be an important therapeutic target for the treatment of heart failure induced by multiple etiologies [32]. Danshen is broadly used in treating coronary artery diseases. However, development of a successful therapeutic strategy is very important and requires a detailed understanding of the molecular mechanisms involved in coronary angiogenesis, which must rely on extensive bench studies. Angiogenesis, a process of sprouting new capillaries, is an adaptive response to multiple forms of stress including myocardial infarction [33, 34]. Pathological stimuli such as ischemia are also known to promote capillary growth within the myocardium. However, the compensatory enhancement of angiogenesis in response to these pathological stimuli appears to be insufficient, and this insufficiency is one of the critical factors responsible for the transition of the heart to heart failure [35]. In this

study, we revealed a critical role of Danshen in the responsive angiogenesis process in the heart with myocardial infarction, which could inhibit the transition process of the heart to heart failure. These findings in this study will have basic and clinical significance in treating myocardial infarction.

However, there is still some inadequacy in this study. In future, it could be studied whether and how Danshen could regulate cardiac apoptosis, inhibit inflammation response, mediate cardiac remodeling and improve cardiac function and so on. It will clearly illustrate the function and mechanism of Danshen in myocardial infarction treatment through these extensive bench investigations.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhen Chen, Department of Emergency, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science & Technology, No. 26 Shengli Street, Jiang An District, Wuhan 430014, Hubei Province, PR China. Tel: 027-82211481; Fax: 027-82211481; E-mail: c_z_s_s_@163.com

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