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Original Article

Danggui Buxue decoction promotes angiogenesis by up-regulation of $VEGFR_{1/2}$ expressions and down-regulation of $sVEGFR_{1/2}$ expression in myocardial infarction rat

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Abstract

Background: The traditional herbal compound Danggui Buxue decoction (DBD), has long been used for the prevention and treatment of cardiovascular diseases, however, the underlying molecular mechanism for its effect remains still unknown. So this study would to investigate the effect of DBD on cardiac damage induced by myocardial infarction (MI) challenge.

Methods: SD Rats with ligation of left anterior descending (LAD) coronary artery were randomly divided into MI, MI plus Betaloc Zok, MI plus DBD high dose, and MI plus DBD low dose group, together with sham-operated group. After corresponding treatment for consecutive 4 weeks, cardiac function was evaluated by hemodynamics with the method of pressure-volume conduit system. Cardiac histological morphology, microvascular density and the expressions of VEGF and $VEGFR_{1/2}$ mRNA and their relative protein including VEGF, membranous $VEGFR_1$ ($VEGFR_1$), soluble $VEGFR_1$ ($sVEGFR_1$), $VEGFR_2$, and $sVEGFR_2$ were examined by hematoxylin & eosin staining, immunohistochemical staining and quantitative polymerase chain reaction and western blot assay, respectively.

Results: It showed that a significant impaired cardiac function and a remarkably inducible increase in fibrotic scar formation, microvascular density and VEGF mRNA expressions in MI rats. While DBD treatment could markedly boost cardiac angiogenesis further, hinder fibrotic scar formation, and improve declined cardiac function. Apart from the up-regulation of VEGF mRNA and VEGF and the down-regulation of $sVEGFR_{1/2}$, high dose of DBD dedicated to increasing $VEGFR_1$ mRNA and $VEGFR_1$ expression, while low dose to elevating $VEGFR_2$ mRNA and $VEGFR_2$ expression.

Conclusion: The present study demonstrated that DBD could accelerate cardiac angiogenesis, restrain fibrous scar formation and thus ameliorate cardiac function in post-MI, via the active regulation of VEGF/VEGFRs signaling pathway.

Keywords: Cardiac angiogenesis; Cardiac function; Danggui buxue decoction; Myocardium infraction; VEGF; VEGFRs

Abbreviations: BZ, Betaloc Zok; CO, cardiac output; DBD, Danggui Buxue decoction; EDP, end-diastolic pressure; EDV, end-diastolic volume; EF, ejection fraction; ESP, end-systolic pressure; ESV, end-systolic volume; H&E, Hematoxylin and eosin; LAD, left anterior descending; LV, left ventricle; MI, myocardial infarction; SV, stroke volume; SW, stroke work; VEGF, vascular endothelial growth factor; VEGFRs, VEGF receptors.

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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1. Introduction

Coronary heart disease is common one of the contemporary senile diseases and also is the leading cause of morbidity and mortality in the elderly.[1] Myocardial infarction (MI) or acute MI, known as a severe type of coronary heart disease, occurs due to the enhanced energy metabolism desire in stressed heart at the consequence of prolonged myocardial ischemia in response to occlusion of coronary artery. To date, the general strategy in clinic used to treat MI involves immediate coronary circulation recanalization for ischemic myocardial tissue reperfusion, including urgent surgeries, percutaneous coronary intervention and coronary artery bypass graft for example,[2] and/or long-term medicine assistance like vasodiators, beta-receptor blockers and calcium antagonists as anti-anginal agents, anti-platelets, and anticoagulants.[3,4] However, none of these interventions is dedicated to the cardiac structural and functional recovery during MI therapy. Angiogenesis, referring to the establishment of a mature coronary circulation network through expansion and remodeling of pre-existing vasculature,[5] is an important compensatory mechanism under various of pathological stimuli like MI stress, and in order to maintain myocardial cells viability as soon as possible. On the basis of the above, therapeutic angiogenesis is proposed and regarded as a promising approach to restore oxygen and nutrients supply to ischemic region, rescuing damaged cardiomyocyte against apoptosis and thereby ameliorating cardiac function, which has been verified successfully in multiple forms of animal models with ischemic heart disease.[6,7]

Danggui Buxue decoction (DBD), composed of the dry roots of Radix Astragali and Radix Angelicae sinensis at a weigh ration of 5:1, is prescribed as a classic and representative formula characterized by replenishing 'Qi' and nourishing blood. In China, DBD (or modified) has been traditionally applied for the prevention and treatment of coronary heart disease,[8−12] attributed to its complicated bioactive effects, such as anti-inflammation,[13] anti-oxidation,[14] anti-fibrosis,[15] cardiac protection,[16] anti-tumor,[17] immuno-regulation,[18] and like-estrogen.[19] Recent studies have shown that DBD had a notable pro-angiogenesis activity, which was associated with reinforcing vascular endothelial growth factor (VEGF) and its receptors (VEGFRs) expressions.[20−23] Accordingly, we hypothesize that DBD in improving ischemic condition may rely on the control of VEGF/VEGFRs signaling pathway. VEGF, acted as a powerfully pro-angiogenic factor, highly binds and activates upon membranous $VEGFR_1$ and $VEGFR_2$ ($VEGFR_1$ and $VEGFR_2$) expressed on vascular endothelial cells and then initiates angiogenesis process.[24] Whereas there being another two soluble receptors $sVEGFR_1$ and $sVEGFR_2$ synthesized due to the alternative splicing in the process of $VEGFR_1$ as well as $VEGFR_2$ mRNA transcription has been identified to exhibit an inhibitory effect on angiogenesis when activated by VEGF.[25,26] Considering these, that DBD is how to affect the expression of VEGF and $VEGFR_{1/2}$ mRNA and their corresponding proteins in infarction myocardium would be the crucial point.

Therefore, we established rat MI model in this study, from observing the effects of DBD on cardiac systolic as well as diastolic function by evaluation of its hemodynamics, histology, and on promoting angiogenesis in damaged heart induced by ligation of left anterior descending (LAD) coronary artery, to systematically determining the expression levels of VEGF and $VEGFR_{1/2}$ mRNA and their corresponding proteins, including VEGF, $VEGFR_1$, $sVEGFR_1$, $VEGFR_2$, and $sVEGFR_2$, in hope of deeply elucidating the molecular mechanism of angiogenesis and of providing scientific theory for clinical utilization of DBD treating ischemic heart diseases.

2. Methods

2.1. Animals

The health male Sprague−Dawley rats (n = 100) weighing 250−270 g, were purchased from DaShuo Biotechnology Co., Ltd (Chengdu, Sichuan, China). Animals were raised in the cages with free access to standard rat chow and tap water and kept on a 12:12-h dark−light cycle. The whole experimental procedure was conducted with approval and under the guidelines by the Management Committee for Experimental Animals, China.

2.2. Plant materials and DBD preparation

The Chinese herbal formula DBD consists of Huangqi (Radix Astragali) and Danggui (Radix Angelicae sinensis) in a weight ratio of 5:1. Both of the dry roots were obtained from Pharmacy (Chinese herbs) affiliated in the Chengdu University of TCM (Sichuan, China), and their identity was confirmed by pharmacognostic experts. Aqueous DBD extracts were prepared with standardized procedures.[27] Briefly, Radix Astragali and Radix Angelicae sinensis were boiled together in 6-folds volume of water for 1 h. The residues from the first extraction were boiled in 8-folds volume of water for another 1.5 h. The solutions were then combined. The rotary evaporator was employed to concentrate extracted solution and make it containing 1 g of crude drug per milliliter in final. The preparation was frozen at −20 °C before usage.

2.3. Myocardial infarction model

The rats were anesthetized with chloral hydrate (30%, 0.3 g/kg, i.p.) and intubated. The heart was exposed through a limited lateral thoracotomy in the dorsal position. To induce a myocardial infarction, the pericardium was opened and the root of the left anterior descending (LAD) coronary artery was permanently ligatured with a 6-0 polypropylene suture as published previously.[28] Successful coronary obstruction was judged by immediately whitened myocardial tissue and the topical ST segment elevation for at least 30 min on the electrocardiogram.

2.4. Experimental design

Five groups were designed in this study, containing MI, MI plus Betaloc Zok (MI + BZ), MI plus DBD high (MI + DBDH) dose, MI plus DBD low (MI + DBDL) dose,

and sham group (the chest and pericardium were opened and closed without LAD ligation). In clinic, DBD and BZ were respectively recommended to orally administer 36 g crude drug (Radix Astragali, 30 g; and Radix Angelicae sinensis, 6 g) and 200 mg every day for adult (60 kg). So the dose of DBDH, DBDL and BZ was 7.56 g/kg, 3.78 g/kg, and 21 mg/kg, respectively, which was calculated according to the people and animals dose conversion table. BZ was dissolved in saline until use. Rats in sham group were garaged orally with the equal volume of saline following sham-operation, while rats of MI, MI + BZ, MI + DBDH, and MI + DBDL group represented they were challenged with saline, BZ, DBD high and low dose, respectively, after MI induced by LAD ligation.

BZ, as a positive drug in the present experiment and from AstraZeneca Pharmaceutical Co., LTD (Wuxi, Jiangsu, China), was selected for its widespreadly clinical application in cardiovascular diseases. In recent years, accumulating clinical evidence indicated that early using BZ was safe and effective for patients with MI due to its attributes of significantly dwindling MI area, blocking left ventricle remodeling, reducing heart beat rate and oxygen consume of myocardium, subsequently improving cardiac function.[29–32]

After a 4-week consecutive corresponding administration of each group, the hemodynamics of rats were detected and then the cardiac tissue was removed out for histological, immunohistochemical, qPCR and western blot analysis, respectively.

2.5. Hemodynamics

To evaluate the cardiac function, the left ventricular hemodynamics in rat was conducted with the method of pressure-volume conduit system (ADInstruments Shanghai Trading Co., Ltd, Australia). After a corresponding treatment for consecutive 4 weeks, rats were inserted pressure-volume conductance catheter from right carotid artery into left ventricle (LV) under anesthesia with 30% chloral hydrate (0.3 g/kg, i.p., Chengdu Kelong Chemical Reagent Company, Chengdu, China). The following parameters of LV hemodynamics were measured: stroke work (SW); cardiac output (CO); stroke volume (SV); end-systolic volume (ESV); end-diastolic volume (EDV); end-systolic pressure (ESP); end-diastolic pressure (EDP), ejection fraction (EF); $\pm dP/dt_{max}$; $\pm dV/dt_{max}$ and Tau.

2.6. Tissue handling

Following the surveillance of hemodynamics, the rats were sacrificed with 10% potassium chloride (Chengdu Kelong Chemical Reagent Company, Chengdu, China) through carotid artery. The heart in diastolic phase was harvested rapidly and its LV was dissected out and further cut into two parts along the lateral axis. One was fixed in 10% neutral formaldehydes (Chengdu Kelong Chemical Reagent Company, Chengdu, China), paraffin embedding, and sliced into 5 μm slices for histological and immunohistochemical staining. Another was frozen into −70 °C liquid nitrogen for Quantitative polymerase chain reaction (qPCR) and Western blotting analysis.

2.7. Hematoxylin and eosin (H&E) staining

The testing specimens were fixed in 10% formalin solution for 48 h, and processed for paraffin embedding and sectioning. The paraffin slices (5 μm) were stained with H&E (Zhuhai Baso Biotechnology Co., Ltd, Zhuhai, China) for routine morphological analysis. Images were captured by microscope (Olympus, Japan) at a magnification of ×400.

2.8. Immunohistochemical staining

After a regular dewaxing, hydration and thermal remediation, the slices were added 3% hydrogen peroxide for 10 min at room temperature for blocking the activity of endogenous peroxidase. Then, the primary antibody (Bioss Biotechnology, Beijing, China) was used and it was washed with 0.1 M PBS solution for three times, with 2 min each time. After 15–20 min of adding the secondary antibody (Zhongshan Goldbridge Biotechnology, Beijing, China) at room temperature, it was also washed with 0.1 M PBS solution for three times, with 2 min each time. DBA kit (Zhongshan Goldbridge Biotechnology, Beijing, China) was applied for coloration. The primary antibody was replaced by PBS as the negative control, and the remained steps are the same as above. The microvascular density value was figured as five visions of each ischemic region selected randomly at a ×200 magnification. The discrete endothelial cells, endothelial cell clusters and cords were scored as one microvessel thereof.

2.9. Quantitative polymerase chain reaction (qPCR)

The total RNA was extracted in line with the instructions of trizol kit without RNAase. Under the manufacturer's manual of RT-PCR kit (Life, USA), 20 μl reverse transcription PCR system was prepared firstly, and incubated at 25 °C for 5 min, 50 °C for 60 min, and 70 °C for 15 min in succession, to synthesize cDNA. 2 μl cDNA was taken as a template to prepare 20 μl PCR amplification system. qPCR instrument (Life, USA) was employed to commit the amplification. The required primers were listed in Table 1. The reaction conditions included the denaturation at 94 °C for 30 s, renaturation at 60 °C for 30 s, and extension at 72 °C for 60 s, with total 40 cycle. β-actin was functioned as a reference object and the mRNA expression quantity of VEGF, $VEGFR_1$ and $VEGFR_2$ were calculated with the $2^{-\Delta\Delta Ct}$ method.

2.10. Western blot

RIPA with the certain quantity was added into testing cardiac tissues to dissociate cells for 10 min at 4 °C, and the dissociation solution was centrifuged for 10 min at 4 °C with a centrifuged speed of 12,000/min. Coomassie brilliant blue (Zhongshan Goldbridge Biotechnology, Beijing, China) assay was supplied to determine the protein concentration of the supernate. After that, 50 μg of protein sample was added in the 10% SDS-PAGE gel of every well and the protein was isolated using vertical electrophoresis (Liuyi Instrument, Beijing,

Table 1
Specific sequence of primes.

Primer name	Forward (5′-3′)	Reverse (5′-3′)
VEGF	GAGATGAGCTTCCTGCAGCATA	TCACCGCCTTGGCTTGTC
$VEGFR_1$	GGTGTCTGCTTCTCACAGGATATT	TGGCTCACTGTAGTAGGCAGAGA
$VEGFR_2$	GCTGTCGCTCTGTGGTTCTG	GTCGCCACACTCAGTCACCA
β-actin	ACACGGCATTGTCACCAACT	TGGTACGACCAGAGGCATACA

China). Half-dry transfer system (Liuyi Instrument, Beijing, China) was employed to transfer membrane for 30 min with 130 mA and about 20 V and then the film was immersed into the primary antibody against VEGF, $VEGFR_1$, $VEGFR_2$, $sVEGFR_1$ and $sVEGFR_2$ (Zhongshan Goldbridge Biotechnology, Beijing, China) for the incubation at 4 °C over night. β-actin (Zhongshan Goldbridge Biotechnology, Beijing, China) was worked as a loading control. After being washed with TBST (10 mM) for 10 min three times, it was again immersed into the secondary solution (1:3000 dilution for VEGF, $VEGFR_1$, $VEGFR_2$, $sVEGFR_1$ and $sVEGFR_2$, and 1:1000 dilution for β-actin) at the room temperature for 60-min incubation. Then, the film was washed by 10 mM of TBST solution three times with 10 min at one time. The ECL reagent (Tianluhai Photosensitive Materia, Tianjin, China) was dropped on the film for the exposure in the dark environment. Statistics were performed on the gray value of each protein band using "image J" software.

2.11. Statistical analysis

All values were expressed as *Mean* ± *SD*. Comparisons among groups were made by Analysis of Variance (*ANOVA*) followed by LSD test or rank sum test when appropriate. *P*-value of less than 0.05 was considered to be statistically significant.

3. Results

A total of 100 SD male rats (10 subjected to sham operation, and 90 subjected to LAD ligation) were performed in this study. In the process of establishing MI model, 40 rats were failed; of these, 18 deaths during the surgery, 8 lacks of spontaneous respiration, 6 failures in ligation by negative depict on electrocardiogram, and 8 deaths after surgery at 24 h. The survival 50 MI rats were randomly placed into four groups, namely, MI group (n = 12), MI + BZ group (n = 12), MI + DBDH group (n = 13), MI + DBDL group (n = 13). The 10 sham-operated controls survived until the end of the study. The marker of successful MI was the whitened ventricle and the topical ST segment elevation on electrocardiogram (Fig. 1).

3.1. Effect of DBD on hemodynamics

As shown in Table 2, related hemodynamic parameters were monitored to assess cardiac function among groups. Compared with sham group, LAD ligation challenge resulted in a enlarged left ventricular chamber and impaired cardiac function with the declined SW, CO, SV, ESP, EF, dP/dt_{max}, $-dP/dt_{max}$, dV/dt_{max}, and $-dV/dt_{max}$ ($P < 0.01$ in all), and the elevated ESV, Tau, EDV, and EDP ($P < 0.05$ or $P < 0.01$). Betaloc Zok, DBD high or low dose treatment for consecutive 4 weeks could significantly went up SW, CO, SV, ESP, EF, $-dP/dt_{max}$, dV/dt_{max}, and $-dV/dt_{max}$ ($P < 0.05$ or $P < 0.01$), and brought down ESV as well as EDV ($P < 0.05$ or $P < 0.01$). In addition, dP/dt_{max} level was higher in the DBD high dose treated group than that in the MI model group ($P < 0.01$).

3.2. Effect of DBD on histopathology of myocardium

In terms of H&E staining, cardiomyocytes in cardiac tissue were arranged orderly, nuclei and cytoplasms were respectively stained blue and red color, and collagen fibers were presented with a varying red color. A comparison of cardiac tissue in MI group with that in sham group showed a severe injures that myocardial cells appeared a large amounts of death, losting their normal ordered structure and fibrous scar tissues replacing original myocardial tissues appeared. After administration of Betaloc Zok, DBD high and low doses, the formation of fibrous scar tissues were less markedly as compared to that in MI group (Fig. 2A).

3.3. Effect of DBD on microvascular density in myocardium

The influence of DBD on stimulating angiogenesis reaction was also accomplished. Microvascular density in the peri-infarct border was measured by using Immunohistochemical analysis and the results displayed evident induction in cardiac capillaries as a result of LAD ligation stress because of compensatory mechanism evoked for the salvage of damaged myocardial cells when compared to that in sham-operation group ($P < 0.05$). Compared with MI group, the amounts of blood vessels were evidently boosted among MI + BZ, MI + DBDH and MI + DBDL group ($P < 0.01$ in all) (Fig. 2B and C).

3.4. Effect of DBD on related mRNA expressions levels of VEGF, $VEGFR_1$ and $VEGFR_2$

Since VEGF and its specifical receptors (VEGFRs) are crucial for pro-angiogenesis in response to ischemic stress, mRNA levels of VEGF, $VEGFR_1$ and $VEGFR_2$ were

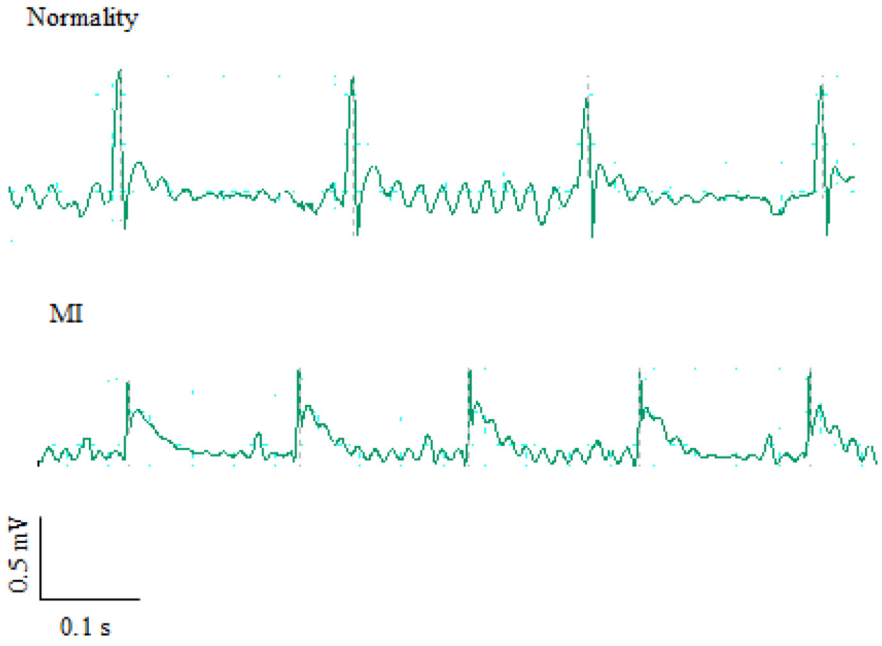

Fig. 1. The representative ECG before and after MI.

examined, respectively (Fig. 3). Similar to angiogenesis, mRNA expression of VEGF instead of both $VEGFR_1$ and $VEGFR_2$ was remarkably raised in heart tissue with MI, as compared to that in sham group ($P < 0.05$). On the basis of MI model, VEGF mRNA expression were further reinforced after administration of BZ ($P < 0.05$), DBD high ($P < 0.05$) and low dose ($P < 0.05$), respectively. As for mRNA levels of $VEGFR_1$ and $VEGFR_2$, there was no statistic difference among sham group, MI group, and MI + BZ group ($P > 0.05$). However, remarkable elevation in mRNA levels of $VEGFR_1$ was showed in DBDH tested group ($P < 0.05$) with respect to DBDL tested group incrementing $VEGFR_2$ mRNA expression increase ($P < 0.05$).

3.5. Effect of DBD on expressions of VEGF, $VEGFR_{1/2}$ and $sVEGFR_{1/2}$ proteins

After a measurement of VEGF and $VEGFR_{1/2}$ mRNA expression, its corresponding proteins which included VEGF, membranous $VEGFR_{1/2}$ ($VEGFR_{1/2}$) and soluble $VEGFR_{1/2}$ ($sVEGFR_{1/2}$) were analyzed. Both immunoblot images and semi-quantitative determination of each group were exhibited in Fig. 4 and Table 3 The data uncovered that there was no statistic significance in above proteins between MI group and sham group ($P > 0.05$ in all). Compared to MI group, BZ treatment only markedly increased VEGF contents ($P < 0.05$) but had no obvious effect on the other four proteins ($P > 0.05$

Table 2
Hemodynamic results of each group (means ± SD).

Group	n	Dose (mg/kg)	SW (mmHg·μL)	CO (μL/min)	SV (μL)	ESV (μL)	ESP (mmHg)
Sham	10	–	3373.5 ± 635.2	18763.8 ± 4564.7	48.9 ± 11.4	20.1 ± 3.9	110.9 ± 18.5
MI	12	–	834.5 ± 96.2$^{\#\#}$	5817.2 ± 654.9$^{\#\#}$	15.7 ± 8.3$^{\#\#}$	67.8 ± 8.8$^{\#\#}$	62.3 ± 18.8$^{\#\#}$
MI + BZ	12	21	2512.9 ± 171.8**	17994.4 ± 6561.6**	42 ± 16**	30.6 ± 2.6**	89.6 ± 10.9*
MI + DBDH	13	7.56	2345.6 ± 288.3**	16962.2 ± 5593.5**	36.6 ± 13.4**	39.7 ± 3.2**	94.3 ± 15.9*
MI + DBDL	13	3.78	2209.1 ± 214.7**	13452.3 ± 5144.6**	37.2 ± 11.8**	35.8 ± 5.2**	82.6 ± 15.6*

EF (%)	Tau (ms)	EDV (μL)	EDP (mmHg)	$-dP/dt_{max}$ (mmHg/s)	$-dV/dt_{max}$ (μL/s)	dP/dt_{max} (mmHg/s)	dV/dtmax (μL/s)
73.7 ± 16.4	13.6 ± 3.5	66 ± 4.6	13.4 ± 6.9	6340.6 ± 412	2666.1 ± 882.2	5000.1 ± 177.2	3015.9 ± 951.3
38.2 ± 11.9$^{\#\#}$	20.1 ± 8$^{\#}$	93.2 ± 8.2$^{\#\#}$	25.3 ± 6.4$^{\#\#}$	3366.7 ± 650.9$^{\#\#}$	958 ± 68.3$^{\#\#}$	3124 ± 102$^{\#\#}$	1145.9 ± 159.2$^{\#\#}$
69.1 ± 18.8**	15.3 ± 2.1	77.7 ± 2.8*	17.3 ± 4.8	4504.9 ± 467.3*	2403.9 ± 248.3**	3943 ± 217.1	2912 ± 247.8**
50 ± 20.8$^{\#**}$	18.2 ± 8.1	75.8 ± 3.6*	18.1 ± 7.9	5562.2 ± 288.1**	2320 ± 195.8**	4845.1 ± 255.9**	2643 ± 242.9**
58 ± 22.9$^{\#**}$	18.2 ± 4.1	73.4 ± 6.2*	16.9 ± 4.9	4999 ± 304.1*	2248.2 ± 167.2**	3666.8 ± 162.5	2281.5 ± 204.4**

The Sham denotes the rats without myocardial infarction (MI) and treated with saline, and the MI, MI + BZ, MI + DBDH, and MI + DBDL denote the rats with MI and treated with saline, Betaloc Zok, and DBD high and low doses, respectively. SW = stroke work; CO = cardiac output; SV = stroke volume; ESV = end-systolic volume; ESP = end-systolic pressure; EF = ejection fraction; EDV = end-diastolic volume; EDP = end-diastolic pressure. 100 mm Hg = 13.33 k P. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with sham group; $^{*}P < 0.05$, $^{**}P < 0.01$, compared with model group.

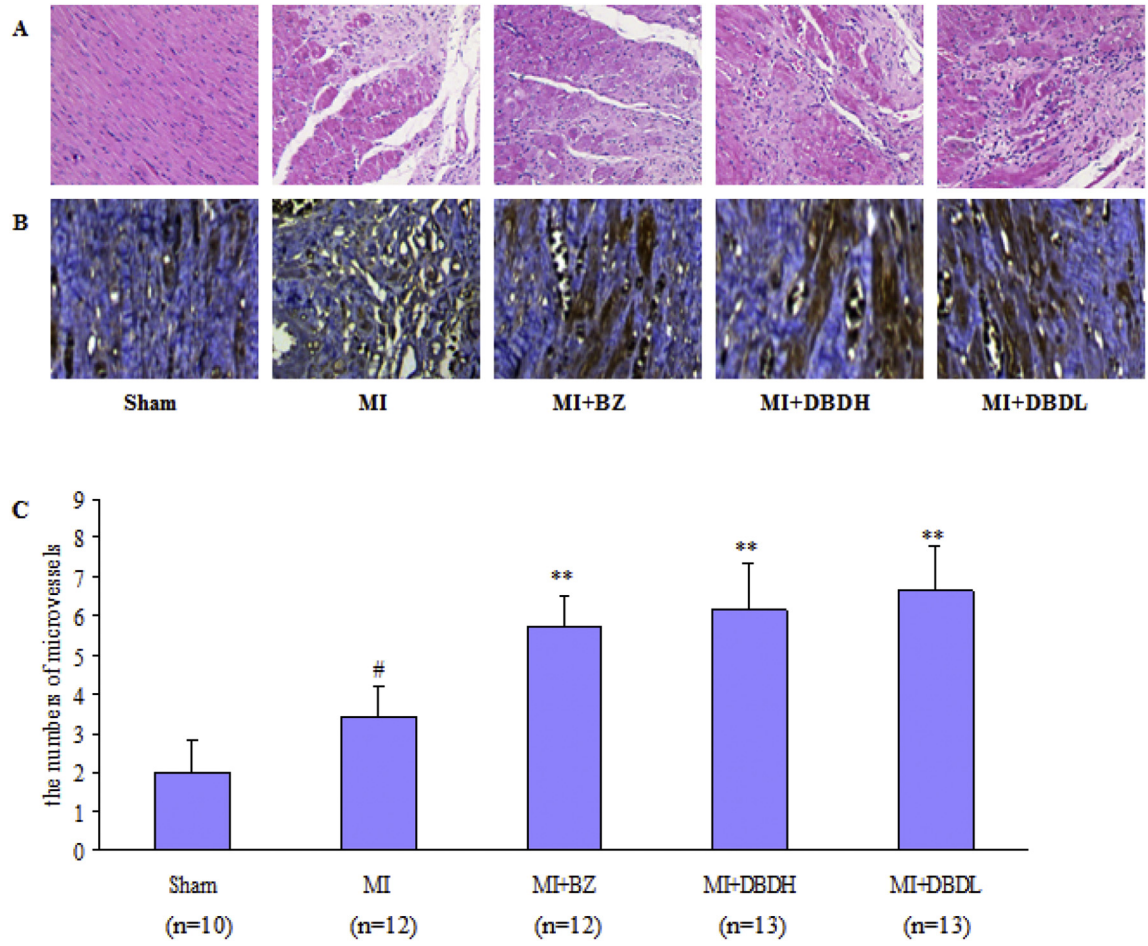

Fig. 2. A) Histopathological analysis of rat myocardial tissue by H&E staining (original magnification ×400); B) Microvascular density analysis of rat myocardial tissue by immunohistochemical staining (original magnification ×200); C) the numbers of microvessels in rat myocardial tissue of each group. The Sham denotes the rats without myocardial infarction (MI) and treated with saline, and the MI, MI + BZ, MI + DBDH, and MI + DBDL denote the rats with MI and treated with saline, Betaloc Zok, and DBD high and low doses, respectively. $^{\#}P < 0.05$, compared with sham group; $^{**}P < 0.01$, compared with model group. (means ± SD).

in all). Beside shared up-regulation of VEGF levels ($P < 0.05$ in both) and down-regulation of $sVEGFR_{1/2}$ levels ($P < 0.01$ in all), DBD high and low dose also respectively increased $VEGFR_1$ ($P < 0.05$) and $VEGFR_2$ ($P < 0.05$) levels in MI heart tissue, which was in accordance with the action mode to $VEGFR_1$ and $VEGFR_2$ mRNA expressions.

4. Discussion

Angiogenesis, a process of sprouting new capillaries, is a critical compensatory mechanism in the cardiac damage response to ischemia, but simultaneously the insufficiency of adaptive angiogenesis as to enlarged expenditure of energy in stressed myocardial cells contributes to the transition of heart into heart failure.[33,34] In clinic, there was a close relationship between reduced cardiac perfusion flow and deficient cardiac angiogenesis in the myocardium of patients suffering from ischemic heart disease.[35–38] It can be seen that accelerating cardiac angiogenesis and rebuilding coronary microcirculation might be the important therapeutic target for the treatment of ischemic heart disease, even heart failure. In consistence with the study by Lei et al.,[23] the present study revealed the crucial role of DBD in inducing responsive angiogenesis boost in the heart with MI and alleviating its deterioration into heart failure, supporting that DBD have a broadly clinical application prospect as a pro-angiogenesis agent for the management of ischemic heart disease.

It was reported that the ability of membranous $VEGFR_1$ ($VEGFR_1$) binding to VEGF which acted as pro-angiogenesis mediator was 10 times stronger than that of membranous $VEGFR_2$ ($VEGFR_2$),[39] and $VEGFR_2$ was greater than $VEGFR_1$ in regard to regulating activity of endothelial cells.[40] Moreover, normality of endothelial cells proliferation but abnormality of vascular morphology were observed in mice lack of $VEGFR_1$ gene,[41,42] whereas both endotheliocyte and some sub-typical hemopoietic cells without function were generated when mice were knocked-out $VEGFR_2$ gene.[43,44] These findings reminded us of that VEGF activating or exciting upon special $VEGFR_1$ and $VEGFR_2$ expressed on vascular endothelial cells membrane played an essential role in mediating

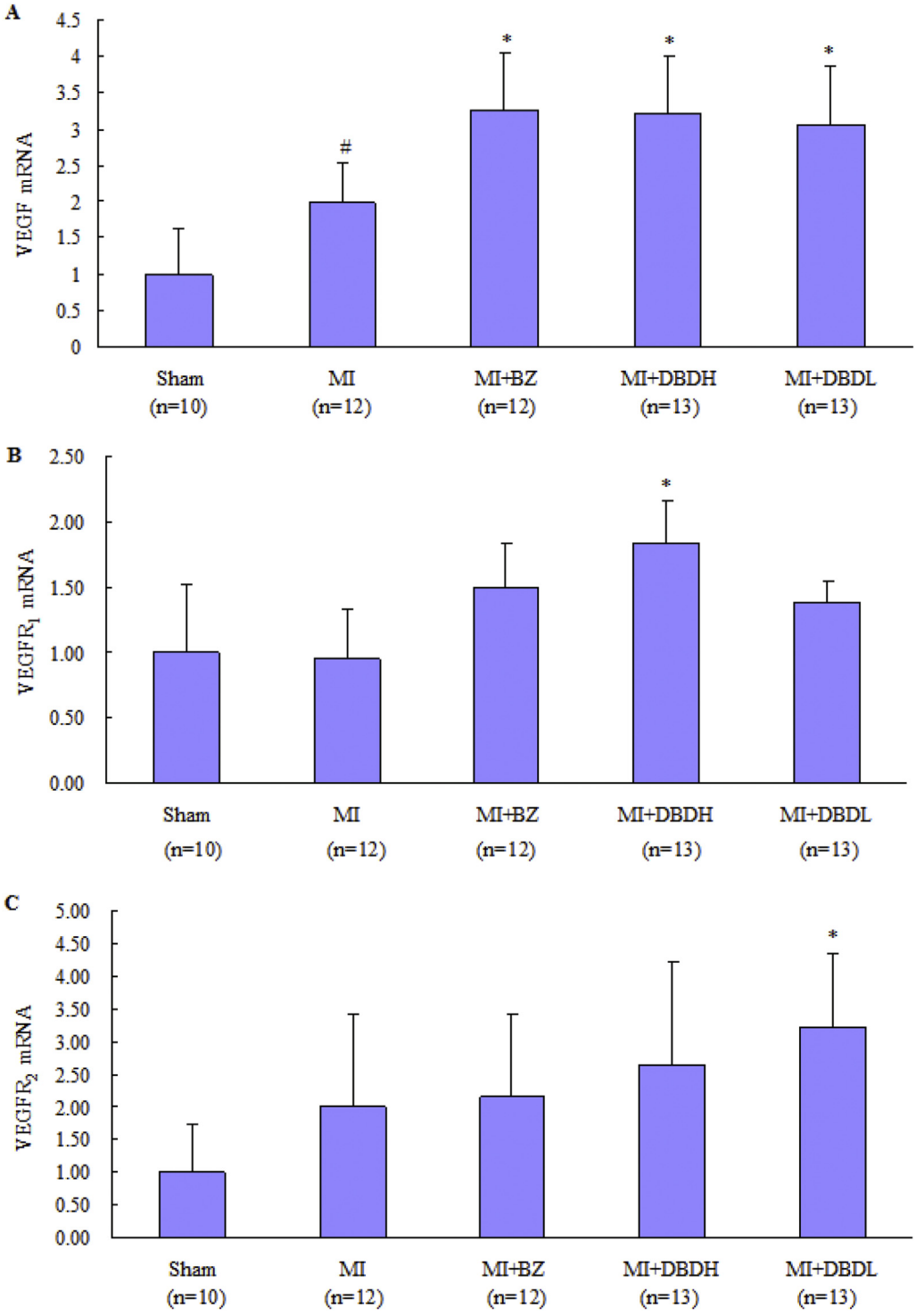

Fig. 3. mRNA expression of VEGF (A), $VEGFR_1$ (B) and $VEGFR_2$ (C) in rats myocardial infraction of each group. The Sham denotes the rats without myocardial infarction (MI) and treated with saline, and the MI, MI + BZ, MI + DBDH, and MI + DBDL denote the rats with MI and treated with saline, Betaloc Zok, and DBD high and low doses, respectively. $^{\#}P < 0.05$, compared with sham group; $^{*}P < 0.05$, compared with model group. (means ± SD, fold change relative to sham group).

diverse aspects of angiogenesis process. Our results demonstrated that administration of DBD could significantly raise VEGF mRNA and its protein expressions levels in cardiac tissue of MI, which was in agreement with the early report.[23] In addition, DBD high dose focused on promoting $VEGFR_1$ mRNA and $VEGFR_1$ expression, while low dose on stimulating $VEGFR_2$ mRNA and $VEGFR_2$ expression. This seems to be that action pattern of DBD was distinct in direct pro-angiogenesis ability depending on which of target genes and proteins invoked at different dose. Concerning another shorter

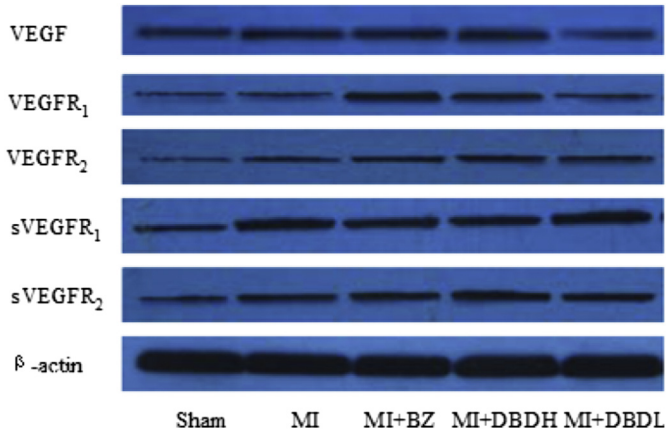

Fig. 4. Immunoblot analysis of VEGF, $VEGFR_{1/2}$ and $sVEGFR_{1/2}$ proteins in rat myocardial tissue. The Sham denotes the rats without myocardial infarction (MI) and treated with saline, and the MI, MI + BZ, MI + DBDH, and MI + DBDL denote the rats with MI and treated with saline, Betaloc Zok, and DBD high and low doses, respectively.

soluble VEGFRs ($sVEGFR_1$ and $sVEGFR_2$), it were still translated and synthesized due to the alternative splicing in the process of respect $VEGFR_1$ and $VEGFR_2$ gene transcription,[24] which could competitively unite VEGF, interrupt the transmittance of signaling pathway,[45] and exert a negative effect on angiogenesis.[46–48] After treatment with DBD, the levels of both $sVEGFR_1$ and $sVEGFR_2$ were markedly depressed in cardiac tissue of MI rats, suggesting that DBD is likely to suppress both $sVEGFR_1$ and $sVEGFR_2$ expressions to indirectly promote angiogenesis. Taken together, DBD-produced angiogenesis directly and indirectly maybe was associated with up-regulating VEGF mRNA, $VEGFR_{1/2}$ mRNA, VEGF and membranous $VEGFR_{1/2}$ expression, and with down-regulating $sVEGFR_{1/2}$ levels in infracted cardiac tissue, but further understanding on DBD participating in the regulation of VEGF/VEGFR signaling pathway are required.

Following acute MI, a large number of myocardial cells appear death in the form of predominant apoptosis,[49] and fiber repair replacing active cardiomyocyte is initiated,[50] accompanying with cardiac function dramatic lesion which results from cardiac structural abnormality in a process known as ventricle remodeling.[51] Although the effect of DBD on cardiomyocyte apoptosis after LAD ligation did not explored in this communication, it achieved remarkable success in other study that DBD treatment could produce a protective effect on *in vitro* damaged H9c2 myocardial cells against apoptosis induced by ischemia and potentially through up-regulating p53 mRNA expression and down-regulating HIF-1α mRNA expression.[52] Our data showed that a less marked fibrosis scar in infract region was presented by H&E staining analysis when MI rat was treated with continuous 4-week DBD when compared to MI group treated with saline, which was similar with studies by Zeng et al.[53] who found administration of DBD could remarkably relieve isoprenaline-caused rat myocardial fibrosis response by blocking the synthesis of collagen protein secreted by myocardial interstitial cells, suggesting that longer treatment with DBD maybe render greater beneficial effect on protecting damaged cardiomyocyte and interfering with collagen protein synthesis as the result of continuous and persistent amelioration of the blood and nutrients supply for the infarction area.

Researcher witnessed DBD could effectively improve left ventricular systolic and diastolic function of MI rat by strength of EF and the ratio of ventricular late and early peak velocity of blood flow.[9] Furthermore, Xu et al.[54] found that DBD treatment could ameliorate cardiac function of heart failure rat. In here, it was the first time that the influence of DBD on rat cardiac function was committed by its hemodynamic assessment using pressure-volume conduit system. After MI stress, cardiac systolic function was impaired with declined SW, CO, SV as well as ESP and with heightened ESV, and diastolic function was broken as well with the height of Tau, EDV and EDP, in addition to lowered dP/dt_{max}, $-dP/dt_{max}$, dV/dt_{max}, and $-dV/dt_{max}$ for index of heart failure. And DBD treatment could remarkably reverse these disadvantageous conditions. These results suggest that DBD is capable of bettering cardiac systolic and diastolic function of MI even heart failure.

In conclusion, we uncovered the core role of DBD in the responsive cardiac angiogenesis after MI, together with apparently restraint of fibrous scar formation and improvement of cardiac function, which effectively suppressed the transition of heart into heart failure to some extent, and its underlying molecular mechanism might be involved in the positive regulation of VEGF/VEGFRs signaling pathway, including up-regulating VEGF mRNA, VEGF, $VEGFR_{1/2}$ mRNA, and $VEGFR_{1/2}$ expressions, and down-regulating $sVEGFR_1$ as well as $sVEGFR_2$ expressions.

Table 3
Semi-quantitative analysis of VEGF, $VEGFR_1$, $VEGFR_2$, $sVEGFR_1$, and $sVEGFR_2$ in myocardial tissue of each group. (means ± SD).

	n	Dose (mg/Kg)	Concentrations (mg/ml)				
			VEGF	$VEGFR_1$	$VEGFR_2$	$sVEGFR_1$	$sVEGFR_2$
Sham	10	–	0.433 ± 0.049	0.426 ± 0.086	0.345 ± 0.073	0.379 ± 0.024	0.411 ± 0.041
MI	12	–	0.474 ± 0.035	0.449 ± 0.096	0.418 ± 0.064	0.459 ± 0.065	0.401 ± 0.058
MI + BZ	12	21 mg/kg	0.564 ± 0.048*	0.495 ± 0.093	0.431 ± 0.054	0.465 ± 0.076	0.455 ± 0.018
MI + DBDH	13	7.56 g/kg	0.546 ± 0.027*	0.585 ± 0.086*	0.454 ± 0.068	0.254 ± 0.038**	0.227 ± 0.016**
MI + DBDL	13	3.78 g/kg	0.561 ± 0.061*	0.507 ± 0.021	0.590 ± 0.019*	0.248 ± 0.024**	0.216 ± 0.024**

The Sham denotes the rats without myocardial infarction (MI) and treated with saline, and the MI, MI + BZ, MI + DBDH, and MI + DBDL denote the rats with MI and treated with saline, Betaloc Zok, and DBD high and low doses, respectively. *$P < 0.05$, **$P < 0.01$, compared with model group.

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