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ShexiangBaoxin pills promote angiogenesis in myocardial infarction rats via up-regulation of 20-HETE-mediated endothelial progenitor cells mobilization

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Abstract

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Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Background and aims—Therapeutic angiogenesis is a pivotal strategy for ischemic heart disease. The aim of the present study was to determine the effect and molecular mechanism of ShexiangBaoxin pills, a widely-used traditional Chinese medicine for ischemic heart disease, on angiogenesis in a rat model of myocardial infarction (MI).

Methods—We used the occlusion of left anterior descending coronary artery of Sprague-Dawley rats as a model of MI. The MI rats were treated with distilled water, ShexiangBaoxin pills, or ShexiangBaoxin pills +HET0016 (a selective blocker of the biosynthesis of 20-hydroxyeicosatetraenoic acid (20-HETE) at 10 mg/kg/day) respectively. Sham-operated rats were used as controls.

Results—Treatment with ShexiangBaoxin pills increases the level of serum 20-HETE in MI rats, which could be suppressed by HET0016 treatment. ShexiangBaoxin pills shows cardio-protective effects on MI rats, including improving cardiac function, decreasing infarction area, and promoting angiogenesis in peri-infarct area. The protective effects of ShexiangBaoxin pills are partly inhibited by HET0016. Furthermore, ShexiangBaoxin pills enhances the number of circulating endothelial progenitor cells (EPCs) and the expression of vascular endothelial growth factor (VEGF), based on immunohistochemical analysis, in peri-infarct area in MI rats, which is partly suppressed by HET0016.

Conclusions—ShexiangBaoxin pills may partially participate in angiogenesis in MI rats. The protective mechanism of ShexiangBaoxin pills may be mediated via up-regulation of 20-HETE, which promotes EPCs mobilization and VEGF expression.

Keywords

ShexiangBaoxin pills; 20-HETE; endothelial progenitor cells; vascular endothelial growth factor; angiogenesis; myocardial infarction

Introduction

Myocardial infarction (MI) has become a major cause of hospitalization and mortality in China[1,2]. The mortality of MI during the past two decades has been increasing more than 1 million deaths per year, and this trend is expected to accelerate, with the World Bank estimating that the number of individuals with MI in China will increase to 23 million by 2030[3]. Rapid and improved treatment of MI has substantially reduced the mortality that includes early myocardial reperfusion, effective antithrombotic therapy, and intensive evidence-based medication into routine clinical practice.

ShexiangBaoxin Pills is a well-known Moschus-based traditional Chinese medicines composite formula widely used in clinical practice for the treatment of cardiovascular diseases including angina pectoris and MI [4,5]. Yan and Jiang et al. reported that the main active ingredients of it are chenodeoxycholic acid, cholic acid, cinobufagin, deoxycholic acid, ginsenosideRb1, recibufogenin and ursodeoxycholic acid[6,7]. Previous studies have reported that ShexiangBaoxin pills could reduce the myocardial ischemic area, the recurrence of angina pectoris, and cardiac events in patients with angina pectoris [8]. However, the exact mechanism is not completely known.

Previous experimental and clinical studies have demonstrated that ShexiangBaoxin pills shows a pro-angiogenic effect in peri-infarct area in a rabbit model of MI [9]. Recently, myocardial angiogenesis has been recognized as one of the innovative therapeutic methods for the treatment of MI[10]. It is a process of promoting the development of the new capillary networks through migration and proliferation of previously differentiated endothelial cells [11], which can provide the supply of oxygen and nutrients to the ischemic regions of MI and improve compromised cardiac function [12,13]. The paradigm to improve therapeutic angiogenesis has focused on enhancing the formation of neovessels from preexisting, terminally differentiated endothelial cells to accelerate neovascularization. Several reports have shown that endothelial progenitor cells (EPCs), precursors derived from bone marrow, have the capacity to differentiate into endothelial cells and incorporate into areas of neovascularization via vasculogenesis [14,15]. EPCs can move to sites of ischemia and contribute to neovascularization in ischemic tissue [16]. Previous experimental and clinical studies have demonstrated that treatment of acute MI with EPCs results in a reduction in infarct size [17,18]. An *in-vitro* study by Wu et al. showed that ShexiangBaoxin pills promotes the proliferation, migration, and angiogenesis of EPCs[19].

20-Hydroxyeicosatetraenoic acid (20-HETE) is the ω-hydroxylation product of arachidonic acid (AA) by cytochrome P450 (CYP) 4A and 4F [20,21]. Guo *et al.* suggested that 20-HETE plays an important role in regulating the EPC functions associated with angiogenic responses [22]. Importantly, an *in-vitro* study by Chen *et al.* demonstrated that 20-HETE promotes the proliferation and migration of EPCs [23]. Therefore, we hypothesized that the cardio-protective role of ShexiangBaoxin pills in angiogenesis is mediated by promoting 20-HETE-mediated EPCs mobilization. The aim of the study was to determine the pro-angiogenic effect of ShexiangBaoxin pills and the potential underlying mechanisms in a rat model of MI.

Materials and methods

Ethics statement

The experimental protocol was approved by Animal Experimental Ethics Committee in Sun Yat-sen University. The animal study complied with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

MI model

Male Sprague-Dawley rats (250±20 g) were acclimatized in a controlled environment with 12/12-hour light/dark cycles. Rats were divided into 4 groups: sham-operated group (n=10), myocardial infarction (MI) group (n=10), MI+ ShexiangBaoxin pills and MI+ ShexiangBaoxin pills +HET0016 (n=10). MI was induced by occlusion of the left anterior descending coronary artery as described previously [24]. Briefly, anesthesia was conducted by intraperitoneal injection with ketamine (40 mg/kg) and xylazine (5 mg/kg). Then endotracheal intubation and mechanical entilation were performed. After left thoracotomy and peicardiotomy, the left anterior descending coronary artery was permanently occluded. Rats in MI+ ShexiangBaoxin pills group received a gavage of ShexiangBaoxin pills (25

mg/kg/d) [25] in distilled water for 8 weeks. HET0016 was given through intraperitoneal injection (10 mg/kg/day) [26]. ShexiangBaoxin pills was bought from Shanghai Hutchison Pharmaceuticals.

Echocardiography

Among the various cardiac contractility parameters, left ventricular (LV) ejection fraction (EF) and maximum dP/dt (dP/dt(max)) are the simplest and most used, meanwhile EF is a more accurate measure of systolic dysfunction than dP/dt(max) in a swine model of MI [27]. So, transthoracic echocardiography (IU22, Philips, Amsterdam, Netherlands) was used to assess cardiac structural and functional changes 8 weeks after MI operation. We used pentobarbital (40 mg/kg intraperitoneally) for anesthesia. Left ventricular internal diameter at end-diastole (LVIDd), left ventricular internal diameter at end-systole (LVIDs), left ventricular fraction shortening (FS) and EF were recorded.

Measurement of infarction area

Eight week after MI operation, rats were anaesthetized with pentobarbital (40 mg/kg intraperitoneally) and sacrificed after blood draw. Body weight and left ventricular weight were recorded. The left ventricular tissues were embedded in paraffin after being fixed with 4% paraformaldehyde for 24 h. Cross sections of left ventricle were at the midline of the long axis of heart. HE and Masson staining were used to assess infarction area. The extent of the infarction area was calculated as the ratio of perimeter of infarction region to total perimeter of left ventricle with Image Pro-plus 5.0 software (Media Cybernetics, Bethesda, US).

Immumohistochemical staining

Immumohistochemical staining was performed as described previously [28]. To assess the microvessel density in the peri-infarct area of MI, tissue sections were incubated with the primary antibody of CD31 (Santa Cruz, California, US) followed by HRP-conjugated secondary antibody and then staining with 3, 3-diaminobenzidine (DAB). We obtained digital photomicrographs in the peri-infarct area. The number of microvessels in 10 random fields at 400X magnification was measured and averaged. Immumohistochemical staining was also used to measure the expression of VEGF (Santa Cruz, California, US). We detected optical density of VEGF-positive cells in digital photomicrographs (400X) in peri-infarct area using image-analysis software (Image J, NIH).

Western blot analysis

Heart extracts were lysed in RIPA buffer (50 mM Tris-HCL pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with complete protease inhibitor cocktail tablets (Roche). Cell debris and tissue sediment were removed by centrifugation at 12,000 rpm for 10 min. Lysates were separated by 10–12% SDS-PAGE gel, transferred to PVDF membranes (Millipore). CD31 (Santa Cruz, California, US), VEGF (Santa Cruz, California, US) proteins were detected by use of antibodies, respectively, and then by an HRP-conjugated secondary antibody. The level of GAPDH (Cell Signaling Technology; 1:1000) protein was also measured as a loading control. The protein bands were

visualized by the ECL detection system (Thermo, Boston, MA) and the densities of the bands were quantified and normalized against GAPDH by image software (Thermo).

Detection of serum 20-HETE

Serum 20-HETE level was detected with an ELISA kit (R&D, Detroit, Michigan, USA). The process was performed according to manufacturer's instructions.

Flow cytometry analysis of circulating EPCs

We used flow cytometry to detect circulating EPCs in rats 7 days after MI. Briefly, blood samples were drawn from the eye socket vein. Blood samples (100 μl) were incubated at 4°C for 30 min with fluorescent conjugated antibodies, including anti-CD34-PE (Santa Cruz, California, US), anti-CD133-FITC (Biorbyt, Cambridge, UK) and anti-VEGFR2-PE-Cy5 (Biorbyt, Cambridge, UK). Fluorescent conjugated isotype IgGs were used as controls. Then erythrocytes were eliminated via incubation with lysis buffer and washing with PBS. Cells-labeled with fluorescence were counted with FACS flow cytometer (Becton Dickinson) and analyzed with Cell Quest Software. The percentage of EPCs defined as double-positive cells of CD34+CD133+,CD34+VEGFR2+ and CD133+VEGFR2+ in monocytes (10,000 events) was calculated.

Statistical analysis

Normal distribution data was described as mean ± standard deviation (SD). Student's *t* test was used to compare the differences between two groups. The comparison between multiple groups was performed using one-way ANOVA followed by a Bonferroni test. The SPSS version 13.0 (SPSS Inc., Chicago, IL, US) was used for statistical analyses. A two-tailed *p*-value <0.05 indicates significant difference.

Results

ShexiangBaoxin pills increases circulating 20-HETE in MI rats

The comparison of left ventricular weight and body weight among groups is shown in Fig. 1. The rats in MI group had lower body weight as compared to rats in sham-operated group. Treatment with ShexiangBaoxin pills or HET0016 (a blocker of 20-HETE synthesis) did not affect body weight. There was no significant difference in left ventricular weight in rats given different treatments. Rats treated with ShexiangBaoxin pills under MI for 8 weeks increased serum 20-HETE levels, which were determined by ELISA assay. Notably, treatment with HET0016, a selective 20-HETE blocker, significantly reduced 20-HETE levels (Fig. 2).

ShexiangBaoxin pills improve cardiac function and reduced infarction area in MI rats through up-regulating 20-HETE

Fig. 3 shows the representative M-mode echocardiograms of rats given different treatments. Echocardiography showed that MI rats had increased LVIDd and LVIDs as compared to sham-operated rats, and ShexiangBaoxin pills ameliorated the changes in LVIDs in MI rats (Fig. 4). Decreased EF adn FS, which indicate reduced cardiac function, were observed in

MI rats. Rats treated with ShexiangBaoxin pills under MI showed significant improvement in cardiac function, whereas 20-HETE blockade by HET0016 partly suppressed the protective effect of ShexiangBaoxin pills on cardiac function. Fig. 5A shows the representative images of HE staining and Masson staining of cardiac slices of rats given different treatment. Administration of ShexiangBaoxin pills attenuates infarction area in MI rats, whereas the effect of ShexiangBaoxin pills on infarction area were suppressed by HET0016 (Fig. 5B).

ShexiangBaoxin pills promote angiogenesis inperi-infarct area in MI rats through up-regulating 20-HETE

Immumohistochemical staining with CD31 showed that ShexiangBaoxin pills promotes angiogenesis in peri-infarct area of rats under MI, while HET0016 suppressed the pro-angiogenic effect of ShexiangBaoxin pills (Fig. 6). We also detected the expression of CD31 in protein (Fig. 7). We found that ShexiangBaoxin pills could increase the level of CD31 in rats under MI, while HET0016 suppressed the effect of ShexiangBaoxin pills on CD31. These results suggest that ShexiangBaoxin pills promotes angiogenesis partially through up-regulating 20-HETE levels.

ShexiangBaoxin pills increase the number of circulating EPCs and the expression of VEGF in peri-infarct area in MI rats

At day 7 after MI, ShexiangBaoxin pills significantly increased the number of circulating EPCs in rats under MI (Table 1). The effect is suppressed by HET0016. Fig. 8 shows the representative images of immumohistochemical staining with VEGF in rats given different treatment. ShexiangBaoxin pills increased the expression of VEGF in peri-infarct area of rats8 weeks after MI, whereas treatment with HET0016 attenuated the effect of ShexiangBaoxin pills on VEGF expression. And we also detected the expression of VEGF in protein (Fig. 9). In accordance with expectation, we reached the same results.

Discussion

In a meta-analysis of randomized controlled trials, Chung et al. reported that Chinese herbal medicine is found to be efficacious in lowering the risk of fatal and nonfatal cardiogenic shock, cardiac arrhythmia, myocardial reinfarction, heart failure, angina, and occurrence of total heart events, especially combined biomedicine[29]. Similar to the previous study, the present study demonstrate that ShexiangBaoxin pills improves cardiac function, and we also found that it can promote angiogenesis in peri-infarct area of rats under MI. Although it has been recognized that treatment with ShexiangBaoxin pills has beneficial effects in ischemic heart disease, the exact mechanism for these effects is still not clear. Here, for the first time, we show that the related mechanism of ShexiangBaoxin pills on angiogenesis in MI is mediated via 20-HETE-mediated EPCs mobilization.

Previous studies have demonstrated that ShexiangBaoxin pills has protective effects on cardiovascular diseases. ShexiangBaoxin pills was found to inhibit cardiac fibrosis in spontaneous hypertensive rats[30]. In clinical study, treatment with ShexiangBaoxin pills attenuates the occurrence of angina pectoris and reduces the use of nitroglycerin in patients

with angina pectoris[31]. The protective effects of ShexiangBaoxin pills on cardiac vasculature include dilating coronary arteries, improving endothelial cell function, and reducing the area of atherosclerotic plaque[32,33]. Our results are consistent with a previous study[4] that ShexiangBaoxin pills could increases cardiac microvessel density in peri-infarct area, reduces the infarction area, and improves cardiac function in MI rats.

It is well known that ShexiangBaoxin pills is composite of seven medicinal materials or extracts. Some investigation has begun to evaluate the therapeutic effects of the bioactive components and the synergistic efficacy of ShexiangBaoxin pills on MI in rats. Li *et al.* reported that ShexiangBaoxin pills displayed better regulation efficacy than those of seven combined version of these bioactive components in rat model of MI [34]. However, the specific mechanism of the pro-angiogenic effect of ShexiangBaoxin pills is still not clear and needs further investigation.

EPCs are generated in bone marrow, and these cells have the capacity to proliferate and migrate to sites of neovascularization, where they are differentiated into mature ECs [14]. The number of circulating EPCs and their angiogenic capacity are closely associated with cardiovascular events and mortality [35]. We found that ShexiangBaoxin pills could increased circulating numbers of EPCs as well as microvessel density in peri-infarct area in MI, suggesting that increasing EPCs may be an important factor to repair the cardiac damage under MI. Interestingly, an *in-vitro* study by Wu et al. showed that ShexiangBaoxin pills promoted the proliferation, migration, and angiogenesis of EPCs[19]. These findings support the notion that ShexiangBaoxin pills could mobilize bone marrow-derived EPCs to peripheral tissues and to participate in angiogenesis.

Several pro-angiogenic factors, including VEGF and 20-HETE, have been implicated to regulate the mobilization of EPCs. Previous studies showed that 20-HETE has the capability to enhance the proliferation of endothelial cells (ECs) by activating VEGF pathway [20,36]. In addition, 20-HETE blockade suppressed the pro-angiogenic effects of VEGF, epidermal growth factor and basic fibroblast growth factor [36]. Therefore, the pro-angiogenic effect of 20-HETE is mediated by mobilizing EPCs and up-regulating VEGF. In the present study, treatment with ShexiangBaoxin pills augment both 20-HETE level and the numbers of EPCs along with increasing VEGF expression in MI rats. In contrast, 20-HETE blockade by HET0016 partially attenuates the effect of ShexiangBaoxin pills on EPCs, VEGF, and angiogenesis. These results strongly suggest that the effects of ShexiangBaoxin pills on angiogenesis in MI are mediated viapromoting 20-HETE-mediated EPCs mobilization and VEGF expression.

Although 20-HETE shows the capacity of increasing proliferation of EPCs and promoting angiogenesis, the exact role of 20-HETE in cardiovascular system is complicated [37]. For example, a previous study found that 20-HETE regulates blood pressure in a tissue-specific way and could be both pro-hypertensive and anti-hypertensive feature [38]. Other study found that 20-HETE has detrimental effects on cardiomyocytes and ECs[38]. The mechanisms of some TCMs, such as ShexiangBaoxin pills, are usually through a multi-target and multi-pathway mode due to different active ingredients [39]. Hence, the roles of

20-HETE on cardiac effect might be related to the dual-directional regulation effects of ShexiangBaoxin pills.

Besides, 20-HETE, epoxyeicosatrienoic acids (EETs) are other metabolites of AA metabolized by CYP epoxygenases, and these lipid mediators can increase coronary blood flow and protect the myocardium from ischemia-reperfusion injury[40]. However, these active lipid mediators are degraded to the corresponding dihydroxyeicosatrienoicacids(DHETs) by soluble epoxide hydrolase (sEH)[41]. Zhu et al. found that sEH blockade could prevent and reverse ischemic heart disease[42]. Thus, whether the effect of ShexiangBaoxin pills on angiogenesis related to the sEH/EETs pathway requires further investigation.

There are some limitations in this study: 1) we did not compare the effect of ShexiangBaoxin pills with its main active ingredients and tested separately and 2) we did not determine the effect of ShexiangBaoxin pills on the expression and activity of CYP4A or CYP4F also 3) we did not explore whether the effect of ShexiangBaoxin pills on angiogenesis is related to angiopoietin. We will explore the effect of the main active ingredients of ShexiangBaoxin pills on angiogenesis in future studies.

Conclusions

In the present study, we demonstrate that ShexiangBaoxin pills reduces myocardial infarction area, and preserves cardiac function in a rat model of MI. The related mechanism for these biological activity may partially be through up-regulating 20-HETE, which could increase the expression of VEGF and mobilize EPCs to participate in angiogenesis. Because ShexiangBaoxin pills is a compound consisting of different ingredients, the whole effect of ShexiangBaoxin pills in rats with MI may also be involved in other mechanisms. This calls for further studies to compare the effect of ShexiangBaoxin pills with its main active ingredients and explore related mechanisms.

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Highlights

- ShexiangBaoxin pills display a remarkable pro-angiogenic role in MI rats.
- ShexiangBaoxin pills increase circulating 20-HETE in MI rats.
- ShexiangBaoxin pills increase the number of circulating EPCs and the expression of VEGF in peri-infarct area in MI rats.
- The protective mechanism of ShexiangBaoxin pills may be mediated via up-regulation of 20-HETE, which promotes EPCs mobilization and VEGF expression.

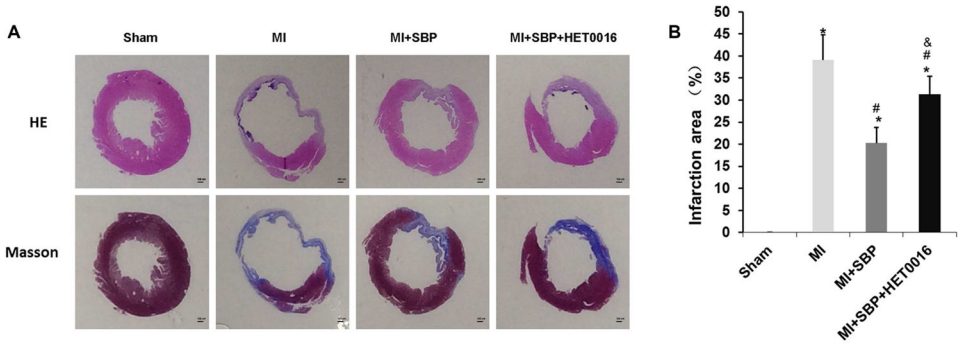

Fig. 1.

Comparisons of left ventricular weight and body weight in rats among groups.

MI, myocardial infarction; SBP, ShexiangBaoxin pills. * $p<0.05$ *vs.* Sham group.

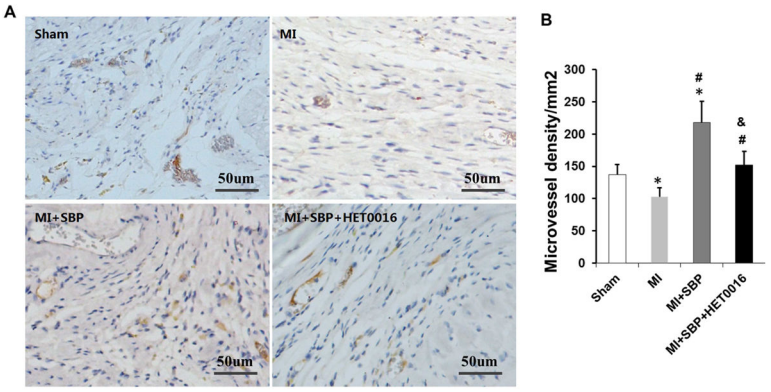

Fig. 2.

Comparisons of circulating 20-HETE in rats among groups.

MI, myocardial infarction; SBP, ShexiangBaoxin pills. * $p<0.05$ *vs.* Sham group; # $p<0.05$ *vs.* MI group; & $p<0.05$ *vs.* MI+SBP group.

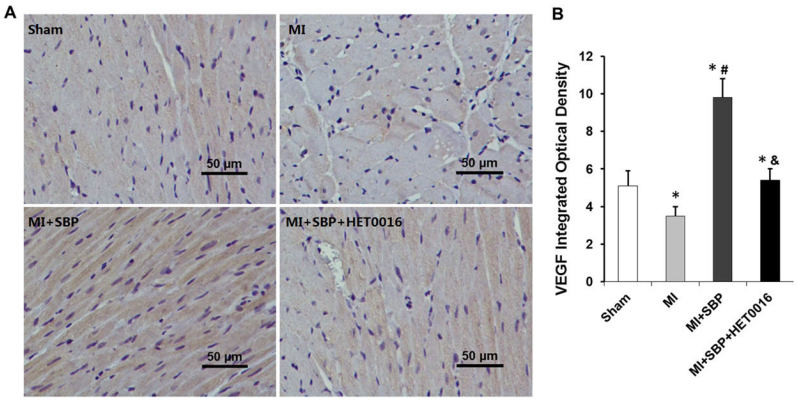

Fig. 3.

Representative M-mode echocardiograms of rats among groups.

MI, myocardial infarction; SBP, ShexiangBaoxin pills; AW, anterior wall; ID, internal diameter; PW, posterior wall.

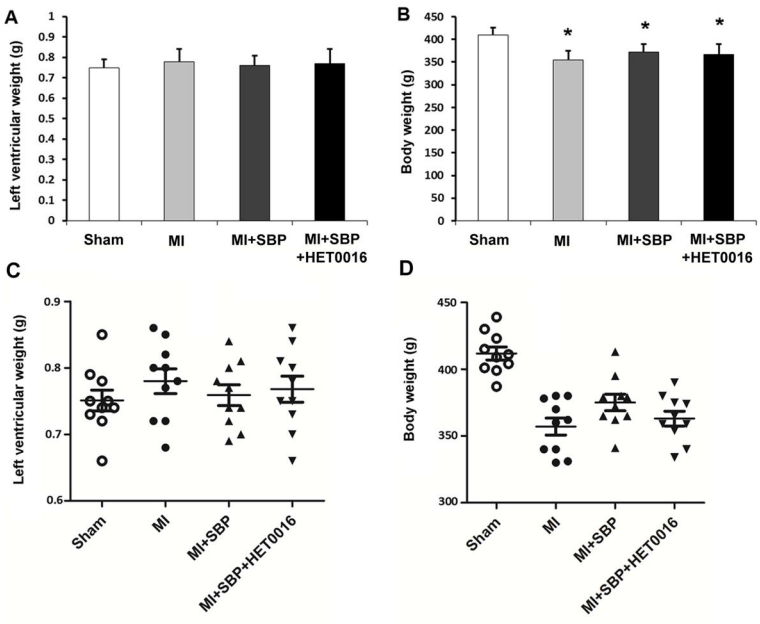

Fig. 4.

Effect of ShexiangBaoxin pills on cardiac structure and function in MI rats.

MI, myocardial infarction, SBP, ShexiangBaoxin pills; EF, ejection fraction, FS, fraction shortening; LVIDd, left ventricular internal diameter at end-diastole; LVIDs, left ventricular internal diameter at end-systole. * $p<0.05$ *vs.* Sham group; # $p<0.05$ *vs.* MI group; & $p<0.05$ *vs.* MI+SBP group.

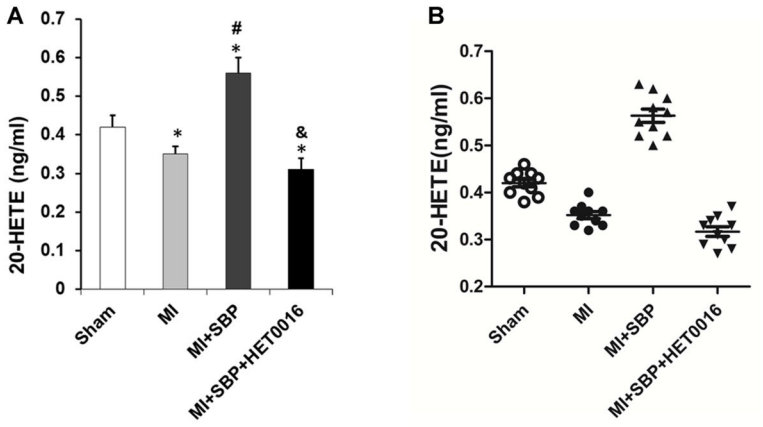

Fig. 5.

(A) Representative images of HE staining and Masson staining of cardiac slices in rats. MI, myocardial infarction; SBP, ShexiangBaoxin pills. (B) Effect of ShexiangBaoxin pills on infarction area in MI rats. MI, myocardial infarction; SBP, ShexiangBaoxin pills. * $p<0.05$ *vs.* Sham group; # $p<0.05$ *vs.* MI group; & $p<0.05$ *vs.* MI+SBP group.

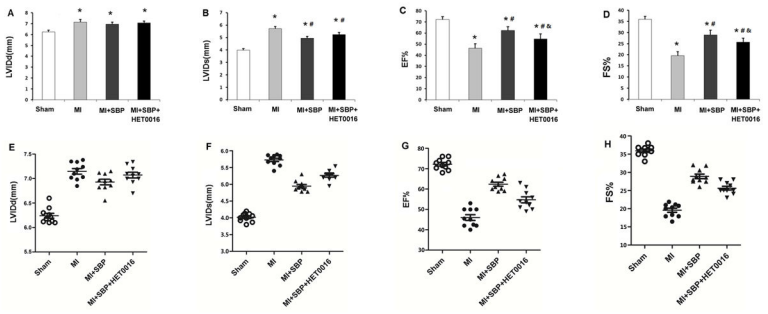

Fig. 6.

Effect of ShexiangBaoxin pills on angiogenesis in peri-infarct area in rats with MI.

MI, myocardial infarction; SBP, ShexiangBaoxin pills. * $p<0.05$ *vs.* Sham group; # $p<0.05$ *vs.* MI group; & $p<0.05$ *vs.* MI+SBP group.

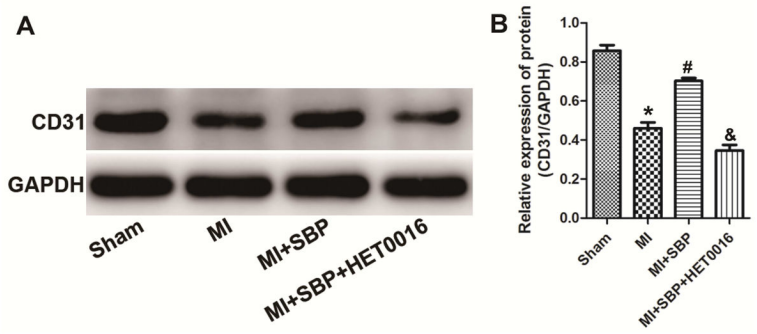

Fig. 7.

The protein expression of CD31 in MI rats given different treatments. MI, myocardial infarction; SBP, ShexiangBaoxin pills. * $p<0.05$ *vs.* Sham group; # $p<0.05$ *vs.* MI group; & $p<0.05$ *vs.* MI+SBP group.

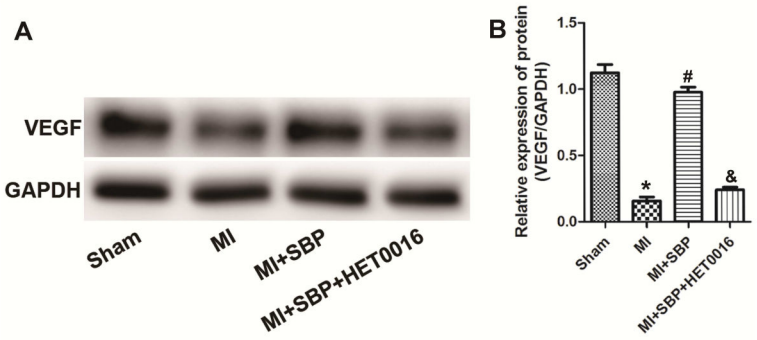

Fig. 8.

Effect of ShexiangBaoxin pills on the expression of VEGF in peri-infarct area in rats with MI.

* $p<0.05$ *vs.* Sham group; # $p<0.05$ *vs.* MI group; & $p<0.05$ *vs.* MI+SBP group.

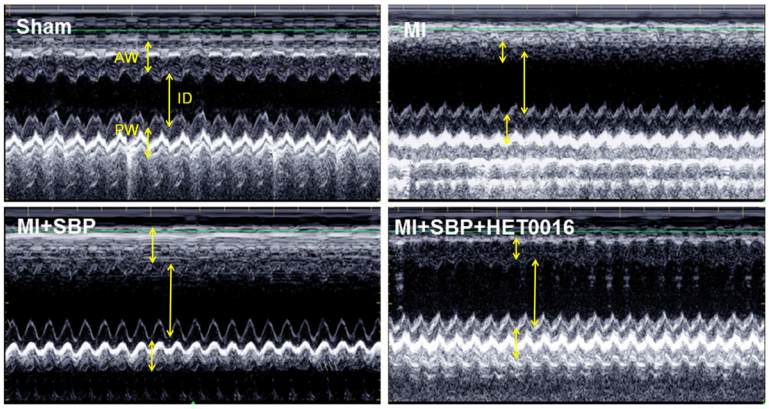

Fig. 9.

The protein expression of VEGF in MI rats given different treatments.

MI, myocardial infarction; SBP, ShexiangBaoxin pills. * $p<0.05$ *vs.* Sham group; # $p<0.05$ *vs.* MI group; & $p<0.05$ *vs.* MI+SBP group.

Table 1

The effect of SBP on the number of circulating EPCs in MI rats.

	Sham	MI	MI+SBP	MI+SBP+HET0016
CD34/CD133(%)	0.11±0.04	0.2±0.02 *	0.39±0.08 *#	0.21±0.05 *&
VEGFR-2/CD133(%)	0.2±0.03	0.28±0.08	0.5±0.07 *#	0.32±0.03 *&
VEGFR-2/CD34(%)	0.1±0.01	0.16±0.06	0.38±0.09 *#	0.18±0.05 *&

EPCs, endothelial progenitor cells; HET0016, a selective blocker of the biosynthesis of 20-HETE; MI, myocardial infarction; SBP, ShexiangBaoxin pills.

* $p<0.05$ *vs.* Sham;

# $p<0.05$ *vs.* MI;

& *vs.* MI+SBP.