Pharmacological Reports
2008, 60, 369–381
ISSN 1734-1140

Comparison of cardioprotective effects of salvianolic acid B and benazepril on large myocardial infarction in rats

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Abstract:
In the present study, we compared cardioprotective effects of salvianolic acid B (Sal B) and the angiotension-converting enzyme inhibitor, benazepril, in rats with large myocardial infarction (MI). The large MI was produced by coronary artery ligation for 4 weeks in rats. The rats were divided into the following groups: sham operation; MI; MI + Sal B (100 mg/kg by a gavage, once a day for 4 weeks) and MI + benazepril (1 mg/kg by a gavage, once a day for 4 weeks). Echocardiogram, hemodynamic and hemorheological changes, angiogenesis, infarct size and cardiac remodeling, as well as messenger ribonucleic acid (mRNA) of vascular endothelium growth factor (VEGF) were measured. The following similar effects were observed in MI rats treated with Sal B and benazepril: (1) a marked improvement of echocardiographic, hemodynamic and hemorheological parameters, (2) significant reduction of infarct size, (3) significantly attenuated heart hypertrophy, left ventricular (LV) dilatation and fibrosis. The unique effects of Sal B were: angiogenesis and augmented VEGF expression in the border and remote noninfarcted LV area. These results suggest that Sal B and benazepril exerted beneficial cardioprotective effects. However, Sal B enforced some different modality than benazepril, which might improve myocardial microcirculation by augmenting VEGF expression and promoting angiogenesis besides similar effects to benazepril.

Key words:
salvianolic acid B, benazepril, large myocardial infarction, heart function, blood viscosity, angiogenesis, infarct size, ventricular remodeling

Abbreviations: ACE – angiotensin-converting enzyme, DAB – diaminobenzidine, ECs – endothelial cells, HF – heart failure, HR – heart rate, LV – left ventricular, LV+d*p*/d*t*max – maximal rate of LV systolic pressure, LV-d*p*/d*t*min – minimum rate of LV systolic pressure, LVEDP – LV end-diastolic pressure, LVEDV – LV end-diastolic volume, LVEF – LV ejection fraction, LVFS – LV fractional shorting, LVSP – LV systolic pressure, LVESV – LV end-systolic volume, LVVCS – LV velocity of circumferential, MI – myocardial infarction, ROS – reactive oxygen species, RV – right ventricular, Sal B – salvianolic acid B, VEGF – vascular endothelium growth factor, VEGFR2 – VEGF receptor type 2, vWF – von Willebrand Factor

Introduction

Salvia miltiorrhiza (Danshen), a popular Chinese herb, has been widely and successfully used for treating angina pectoris, myocardial infarction (MI) and stroke [7]. Salvianolic acid B (Sal B), one of the major water-soluble compounds of Danshen, is the most abundant and bioactive member of the salvianolic

acids in Danshen (showing antioxidant, hepatoprotective and many other actions) [10]. It is also a major component in eight commercial FuFang danshen products [34], and was assigned as the marker species for Danshen in the 2005 edition of Chinese Pharmacopoeia. Experimental studies have shown that Sal B has possessed many biological activities of the Danshen herb. For example, it was reported to possess sedative, antioxidant, hepatoprotective and antifibrogenic effects [12], inhibit platelet aggregation, improve coronary microcirculation, enhance angiogenic processes [11], and protect against injury in the heart caused by ischemia-reperfusion [33].

Benazepril, an angiotensin-converting enzyme (ACE) inhibitor, has become an important medicine in prevention of cardiovascular diseases in developed countries [36]. ACE inhibitors have so far proved to be the most successful class of drugs in the treatment of heart failure (HF). Clinical studies have shown that ACE inhibitors reduce mortalities and improve symptoms and long-term outcome of acute myocardial infarction (AMI) [20]. Experimental studies showed that ACE inhibitors administered chronically before AMI might limit myocardial infarct size, improve cardiac function, and prevent cardiac hypertrophy [36], and experimental evidence has also indicated that harmful remodeling could be inhibited by ACE inhibitors, which has led to great interest in their therapeutic potential [24].

Compared to benazepril, there is a very limited animal experimental study information available to demonstrate the mechanisms of Sal B's cardioprotective effects on large MI. But Sal B is responsible for many of the Danshen's therapeutic actions, especially improving coronary microcirculation, antioxidant, and free radical scavenging effects [31]. Except for hemorrheology and ventricular remodeling effects, no studies using treatment with Sal B have been done to investigate its effect on angiogenesis, ventricular dilation, and collagen deposition. It is also not known whether Sal B can induce angiogenesis by the vascular endothelium growth factor (VEGF). Furthermore, there is no literature reporting comparative studies on this aspect of effects of Sal B and benazepril.

The aim of the present study was to compare the cardioprotective effects of Sal B and benazepril on cardiac dysfunction, blood viscosity, ventricular remodeling, angiogenesis and VEGF expression in the models of large MI in rats.

Materials and Methods

Animals

Male Sprague-Dawley rats, purchased from Zhejiang Experimental Centre (cleaning grade), and initially weighing 200–220 g (aged 10 weeks) were used in the study. The rights of experimental animals were ensured *quantum satis ad* during experiment. All rats were housed under constant conditions at a temperature of 23 ± 1°C, humidity of 40 ± 5%, and on a 12-h light/dark-cycle. Rats had free access to a standard diet and to drinking water. The animal experiments were performed in accordance with the standards established by the Guide for the Care and Use of Laboratory Animals of Zhejiang Province, and approved by the local Ethics Committee. The whole lab proc was carried out under the permission and surveillance of the Ethical Committee.

Drugs

Salvianolic acid B (purity > 99%) was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Benazepril was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental myocardial infarction

MI was produced in rats by ligation of the left anterior descending coronary artery for 4 weeks. The surgical procedure was performed according to a previous study [23] with minor modifications. Briefly, the rats were anesthetized with urethane (1.2 g/kg, *ip*), and then underwent a left thoracotomy. The incised area was extended using forceps and the pericardium was opened. After tracheal intubation, the rats were ventilated by a respirator (ALC-V8, Shanghai, China) with room air with a tidal volume of 25 ml/min and a respiratory rate of 70 cycles/min. The heart was exteriorized, and ligated at the proximal left anterior descending coronary artery 2–3 mm from its origin between the pulmonary artery conus and the left atrium with a 4–0 prolene suture. The heart was returned to its normal position, and the thorax was closed. Sham-operated rats underwent the identical surgical procedure as described above except that the suture was not tightened around the coronary artery. In the present study, the operation-related mortality was approximately 20% 24 h after operation.

Experimental protocol

The surviving rats were divided randomly into 4 groups: sham operation; MI; MI + Sal B (100 mg/kg by a gavage) and MI + benazepril (1 mg/kg by a gavage) beginning on the day following surgery, and vehicle was given to sham and MI groups once a day for 4 weeks. There were fifteen rats in each group except in gene or protein expression experiments, in which there were five, and the experiment was carried out after the 4th week.

Hemodynamic and echocardiographic changes

Two-dimensional echocardiographic studies were performed in rats under 2.0% halothane in O_2 using an echocardiographic machine equipped with a 7.5-MHz transducer (SSD-5500; Aloka, Japan) after the 4th week. Echocardiographic parameters [left ventricular end-systolic volume (LVESV), LV end-diastolic volume (LVEDV), LV ejection fraction (LVEF), LV fractional shortening (LVFS) and LV velocity of circumferential shortening (LVVCS)] were measured as described previously [15]. After finishing an echocardiographic measurement, the rat was subjected to surgical procedures to measure systemic blood pressures and hemodynamic parameters. After the instrumentation, the concentration of halothane was reduced to 0.5% to record steady-state systemic blood pressures and hemodynamic data with a pressure transducer (MP150, Biopac system, USA). Systemic blood pressures and hemodynamic parameters [heart rate (HR), LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), maximal rate of LV systolic pressure (LV + dp/d$t$max) and minimum rate of LV systolic pressure (LV – dp/d$t$max)] were recorded as described previously [6]. The study was performed in a blinded manner. After measuring echocardiographic and hemodynamic parameters, the hearts of the sacrificed rats were removed, washed with physiological saline, photographed and weighed.

Blood viscosity measurement

Every blood sample was immediately fractionated into 1 ml and 3 ml for measurements of whole blood and plasma viscosities, respectively. In order to obtain plasma for viscosity measurements, the 3 ml fractions were equilibrated at 4°C for 30 min and centrifuged at 3000 rpm at 4°C for 15 min, and then the plasma was obtained from the upper stratum. Finally, whole blood and plasma viscosities were measured by a cone-plate viscometer (PRECIL, China), at 37°C and at 3 different shear rates (10/s, 60/s, 120/s).

Cardiac morphological examination

After fixation, three cross-sections through the ventricles were cut from apex to base, and embedded. Paraffin sections (2 μm) were stained with Masson's trichrome for measurement of infarct size, hematoxylin and eosin for measurement of myocyte size, and Sirius red F3BA for determination of collagen volume fraction. The infarct size was expressed as previously described [2, 22]. To assess changes in infarct size as the infarcts evolved, histological sections of all four slices of each heart were projected onto a screen at a magnification of 10× and a planimeter was used to make the following measurements: a) areas of infarcted and noninfarcted LV myocardium, and b) lengths of the portions of the circumference of the left ventricle overlying infarcted and noninfarcted myocardium. From these measurements the following were calculated for each heart: a) percent, by area, of the left ventricle which was infarcted and b) percent of left ventricle which was infracted by circumference. These percentages represent estimates of the percent of the volume of the left ventricular myocardium which was infarcted and the percent of the surface area of the left ventricular wall which was infarcted. The mathematical justification for using two-dimensional tissue sections to make quantitative estimates of three-dimensional structures (stereology) has been described in detail elsewhere. In addition to the planimetric measurements described above, the thickness of the infarct was measured at the point of maximum thinning of the infarcted left ventricle wall.

After the cross-sections of the heart were embedded in paraffin and stained with hematoxylin-eosin, muscle fiber diameter was evaluated by direct measurements at 400× magnification only in cross sections that included a nuclear profile. A total of 40 cells per section were evaluated (I × 71 inverted microscope, Olympus, Japan). Masson's trichrome stain was used to measure interstitial fibrosis. After 3 sections per animal and 20 fields per section were scanned and computerized with a digital image analyzer (Image-pro Plus 5.0, Media Cybernetics, USA), the volume collagen fraction was calculated as the sum of all con-

nective tissue areas divided by the total area of the image [16, 17].

For the measurement of cardiomyocyte cross-sectional area and diameter in the noninfarcted LV, a total of 30 myocytes sectioned transversely for area and longitudinally for diameter at the level of the nucleus were randomly chosen from each section at 400× magnification, and traced. To measure collagen volume fraction, 16 fields in the border and remote myocardium of the noninfarcted LV and right ventricular (RV) walls per section were scanned at a magnification of 200×. The interstitial collagen volume fraction was measured while omitting fibrosis of the perivascular, epi-, and endocardial areas from the study. The collagen volume fraction was obtained by calculating the mean ratio of connective tissue to the total tissue area of all the measurements of the section. The collagen-positive areas from all sections were determined by a single investigator who was unaware of the experimental groups.

Immunohistochemistry for angiogenesis and VEGF

The immunohistochemistry procedure of von Willebrand Factor (vWF) and VEGF was performed according to a previous study [30] with a minor modification. Briefly, the hearts were fixed in 10% neutral-buffered formaldehyde for 12 h and embedded in paraffin, cut into 5 μm thick, transmural and consecutive serial sections for immunostaining of microvessels and VEGF expression.

Immunohistochemical staining of angiogenesis was performed using UltraSensitive TM S-P kit (Maixin-Bio, China) according to the manufacturer's instruction. In brief, sections were deparaffinized and microwave-treated for 10 min twice in 10 mM sodium citrate (pH 6.0). Endogenous peroxidase in the sections was blocked by incubating them in endogenous peroxidase blocking solution for 10 min at room temperature. A rabbit polyclonal antibody against vWF protein (maixin) was used as primary antibody at a 1:70 dilution at 4°C for 18 h. After washing three times with phosphate balanced solution (PBS), sections were incubated with biotin-conjugated anti-rabbit second antibody for 10 min. They were then washed 3 times with PBS, treated with streptoavidin-peroxidase for 10 min and then washed again with PBS 3 times. Finally, specimens were incubated in diaminobenzidine (DAB) for 5 min, followed by hematoxylin counterstaining. Images from the entire sections were acquired using a digital camera system (Leica DM IL, DC 300). The slides were processed by a computerized image analyzer (Leica IM50) for quantitative assessment of vascular density in the myocardium. The number of microvessels was counted in ten random fields (magnification 200×) using computer-assisted morphometry. The number of microvessels identified from ten fields in each section were averaged and expressed as "number per square millimeter of cross-sectional area". Investigators performing immunohistochemical analysis were blinded to animal treatment [21, 30].

Immunohistochemistry of VEGF was performed as follows. The sections were deparaffinized and the endogenous peroxidase was blocked with H_2O_2 for 10 min. Then the sections were incubated with the monoclonal anti-VEGF antibody used for the study (1:100, rabbit anti-rat, Santa Cruz Biotechnology, Santa Cruz, CA). Incubation for 1 h at 37°C was followed by incubation with biotin-labeled goat anti-rabbit IgG (working solution, Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature. The sections were then incubated with SABC complex (working solution, Santa Cruz Biotechnology, Santa Cruz, CA) and developed with DAB as substrate. They were counterstained with hematoxylin, then dehydrated, cleared and covered with coverslips. The experiment was repeated on three different sections at least for each group. Ten random fields of each stained section were pictured and analyzed by a reader who was blinded to the animals' treatment status using morphometric software (Chansan, Shanghai, China). To rule out false-positive signals contributed by damaged tissue and as a control, heart sections obtained from nontreated rats were subjected to the same procedure without incubation with monoclonal antibody VEGF.

Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) reagents were from Promega Corporation, USA. Oligonucleotides for the primer were all synthesized by Invitrogen (Shanghai, China). Total RNA was extracted from 100 mg samples of frozen remote and border noninfarcted LV areas by using 1 ml of Trizol. The total RNA was reverse transcribed into cDNA by AMV reverse transcriptase (Promega,

USA). To ensure a fixed amount of initial mRNA in parallel with β-actin, amplification was performed using the following oligonucleotides: sense: 5'-GGT ATG GGT CAG AAG GAC TCC-3'; antisense: 5'-TGA TCT TCA TGG TGC TGC TAG GAG CC-3', with pre-denaturation at 94°C for 5 min, denaturation at 94°C for 40 s, annealing at 60°C for 60 s, extension at 72°C for 1 min, 30 cycles and final extension at 72°C for 10 min. The primer used for VEGF [9] was: sense: 5'-CCA TGA ACT TTC TGC TCT CTT G-3'; antisense: 5'-GGT GAG AGG TCT AGT TCC CGA-3'. PCR was performed as follows, using an authorized thermal cycler (Eppendorf, Hamburg, Germany): pre-denaturation at 94°C for 5 min; denaturation at 94°C for 1 min; annealing at 59°C for 1 min; extension at 72°C for 1 min for 35 cycles and a final extension at 72°C for 10 min.

The amplification products were separated by agarose gel electrophoresis (1.7%), stained with ethidium-bromide and the bands were analyzed by Labworks imaging acquisition and analysis software (Ultra-Violet products, Cambridge, UK).

Statistical analysis

SPSS 12.0 software package (SPSS Inc., USA) was used to analyze the results. Data are expressed as the mean ± SD. Comparisons of the time course changes between groups were performed by the use of two-way repeated-measures ANOVA. Comparisons of other data between groups were performed through the use of one-way fractional ANOVA. The Bonferroni-Holm procedure was used to correct multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

Results

Echocardiographic assessment

In the MI rats, there was a significantly compromised cardiac function. Sal B improved aggravated cardiac function, preserving LVESV and LVEDV by 52.7% ($p < 0.05$) and 78.3% ($p < 0.05$); LVEF and LVFS by 54.0% ($p < 0.05$) and 53.8% ($p < 0.05$); LVVCS by 49.3% ($p < 0.05$), respectively, compared with the MI group. Benazepril had also the similar effects (Tab. 1).

Hemodynamic Changes

In the MI group, there was a significant decrease in systolic function by 17.7% ($p < 0.01$) and 27.3% ($p < 0.01$) in LVSP and LV + d*p*/d*t*max, and a compromised diastolic function by 317.7% ($p < 0.01$) and

Tab. 1. Effects of Sal B and benazepril on hemodynamic and echocardiographic parameters in rats with large MI

	Sham	MI	MI + Sal B	MI + benazepril
Hemodynamic data				
HR (beats/min)	415 ± 5	423 ± 7	411 ± 6	416 ± 8
LVSP (mmHg)	145.4 ± 13.7	119.6 ± 8.0**	132.1 ± 7.5##	133.9 ± 8.1##
LVEDP (mmHg)	1.98 ± 0.48	8.27 ± 1.21**	4.11 ± 1.08##	4.68 ± 1.15##
LV + d*p*/d*t*max (mmHg/s)	10225 ± 1635	7432 ± 1231**	8719 ± 2522#	9813 ± 2208#
LV – d*p*/d*t*min (mmHg/s)	–6973 ± 1072	–4902 ± 408**	–5566 ± 747#	–6502 ± 700##
Echocardiographic data				
LVESV (ml)	0.118 ± 0.033	0.173 ± 0.027**	0.144 ± 0.029#	0.141 ± 0.022##
LVEDV (ml)	0.602 ± 0.063	0.722 ± 0.115**	0.628 ± 0.075#	0.624 ± 0.081#
LVEF (%)	82.3 ± 9.7	69.7 ± 7.8**	76.5 ± 6.2#	77.2 ± 5.7#
LVFS (%)	53.8 ± 10.6	41.9 ± 6.9**	48.3 ± 5.9#	49.4 ± 6.3#
LVVCS (s^{-1})	6.09 ± 0.29	5.42 ± 0.29**	5.75 ± 0.35 #	5.79 ± 0.25##

Data are shown as the mean ± SD, (n = 10). ** $p < 0.01$ *vs.* sham group; # $p < 0.05$, ## $p < 0.01$ *vs.* MI group

Tab. 2. Effects of Sal B and benazepril on blood viscosity in rats with large MI

Group	PV (mPa.s)	WBV (10/smPa.s)	WBV (60/smPa.s)	WBV (120/smPa.s)
Sham	1.051 ± 0.038	12.75 ± 2.37	7.66 ± 1.24	6.48 ± 1.07
MI	1.135± 0.076*	19.96 ± 3.30**	10.57 ± 1.25**	8.50 ± 0.94**
MI + Sal B	1.035 ± 0.034$^{\#}$	11.10 ± 1.47$^{\#\#}$	7.04 ± 0.67$^{\#\#}$	6.03 ± 0.54$^{\#\#}$
MI + benazepril	1.103 ± 0.050	15.99 ± 2.08$^{\#}$	9.16 ± 0.77$^{\#}$	7.74 ± 0.57$^{\#}$

Data are shown as the mean ± SD (n = 10). * $p < 0.05$, ** $p < 0.01$ *vs.* sham group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs.* MI group. PV – plasma viscosity, WBV – whole blood viscosity

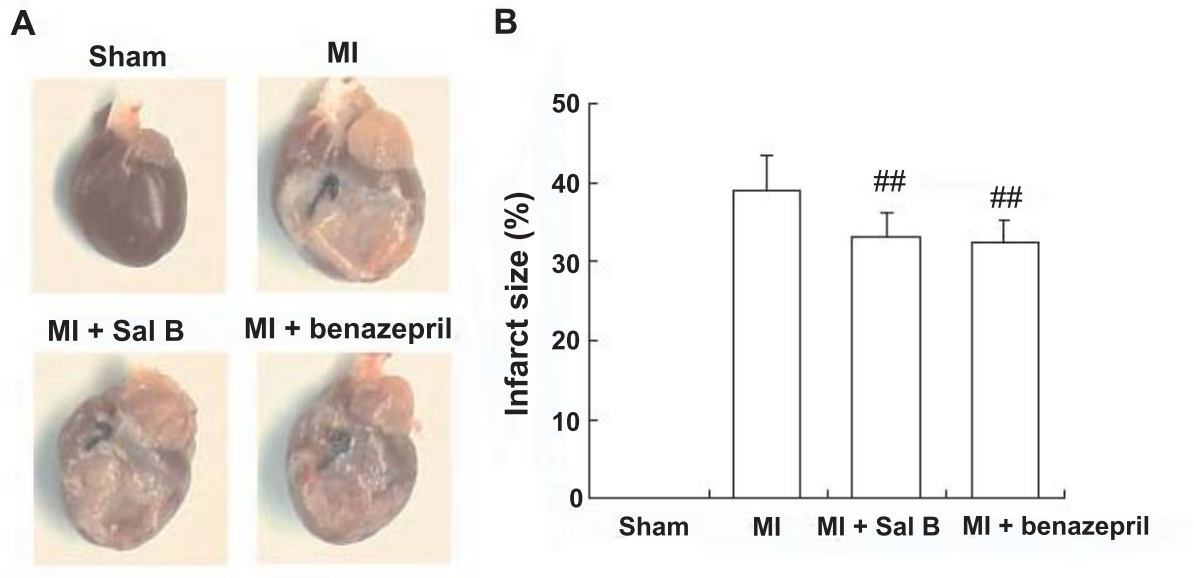

Fig. 1. Effects of Sal B and benazepril on infarct size in rats with large MI. **(A)** Representative morphological photographs of hearts. **(B)** Infarct size. Data are shown as the mean ± SD (n = 10). $^{\#\#}$ $p < 0.01$ *vs.* MI group

29.7% ($p < 0.01$) in LVEDP and LV – d*p*/d*t*min relative to the sham group, respectively. Administration of Sal B and benazepril brought about a marked amelioration in changes of systolic and diastolic functions ($p < 0.05$ and $p < 0.01$, respectively) compared with the MI group. HR did not significantly differ between all groups (Tab. 1).

Blood viscosity changes

Induction of large MI led to a significant increase in whole blood and plasma viscosities compared with the sham group ($p < 0.01$). Administration of Sal B reduced the level of blood and plasma viscosities to near normality ($p < 0.01$). Also, blood viscosity was significantly lower in benazepril-treated rats than in rats with large MI ($p < 0.05$); however, the effect in Sal B-treated rats was more significant than that in benazepril-treated rats (Tab. 2).

Infarct size

Typical morphological photographs of hearts in the 4 groups and infarct size as a proportion of LV size are shown in Figure 1. Infarct size was significantly reduced in the hearts of Sal B- and benazepril-treated groups compared with the MI group ($p < 0.01$).

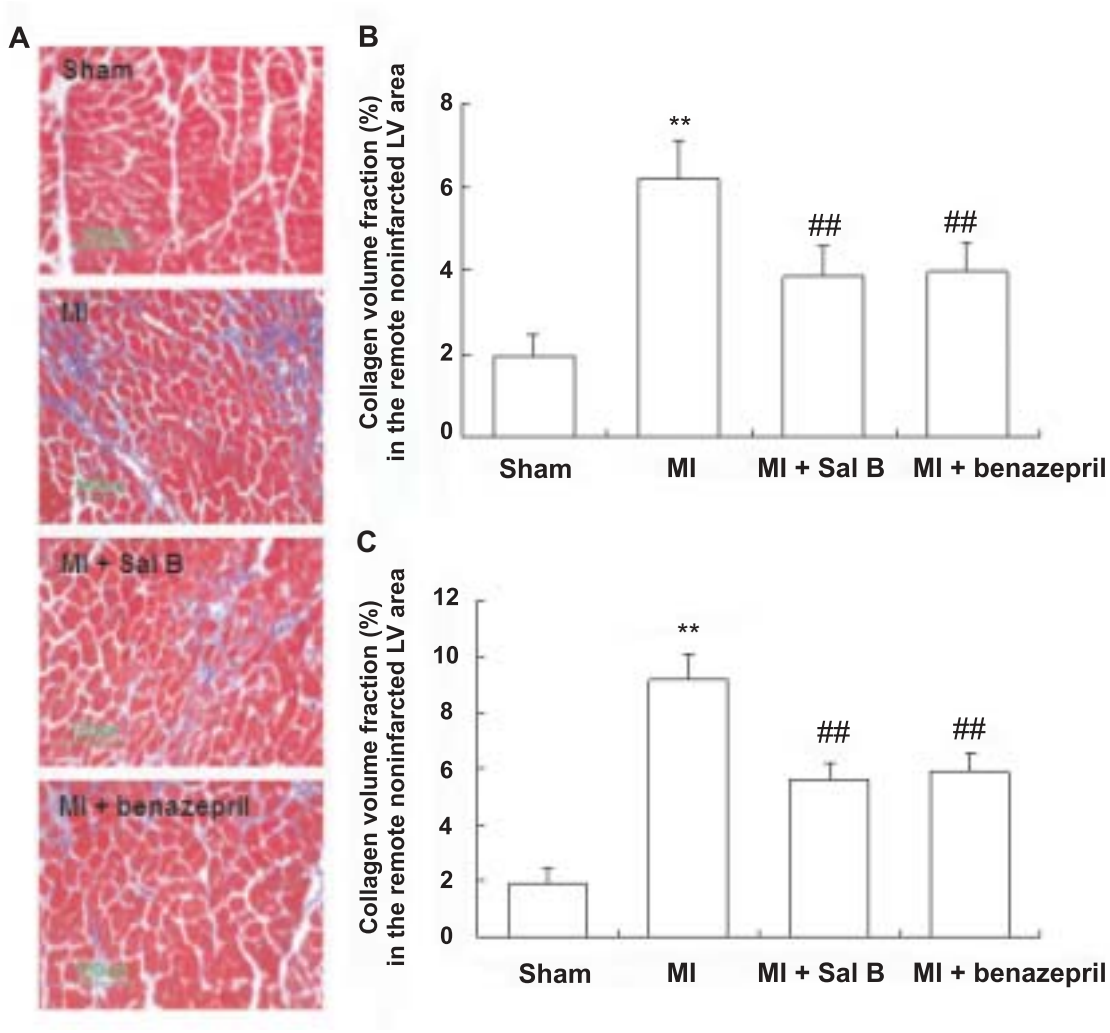

Fig. 2. Effects of Sal B and benazepril on collagen volume fraction in rats with large MI. **(A)** Representative photomicrographs of collagen volume stained with Sirius red in the remote noninfarcted LV area (200× magnification). **(B)** Morphometric analysis of collagen volume fraction in the remote noninfarcted LV area; **(C)** Morphometric analysis of collagen volume fraction in the border noninfarcted LV area. Data are shown as the mean ± SD (n = 10). ** $p < 0.01$ *vs.* sham group; ## $p < 0.01$ *vs.* MI group

Cardiac collagen volume and hypertrophy

In our studies, we examined the effects of Sal B and benazepril on collagen volume of heart in rats with large MI. In the MI group, the collagen volume fraction in the remote and border noninfarcted LV area was significantly increased relative to the sham group ($p < 0.01$). Sal B and benazepril decreased the collagen volume fraction by 49.0% ($p < 0.01$) and 45.3% ($p < 0.01$) in the remote noninfarcted LV area, and by 55.1% ($p < 0.01$) and 52.4% ($p < 0.01$) in the border noninfarcted LV area compared with the MI group, respectively, and their influences were more remarkable in the border region of MI. The study results indicated that fibrosis in the border region adjacent to infarct zone was more prominent compared with the remote zone (Fig. 2).

In the MI group, the cross-sectional area and diameter of myocytes in the noninfarcted left ventricle significantly increased compared with sham group ($p < 0.01$); Sal B and benazepril reduced it by 59.7% ($p < 0.01$) and 78.1% ($p < 0.01$) in the cardiomyocyte area, and by 62.0% ($p < 0.01$) and 74.0% ($p < 0.01$) in the cardiomyocyte diameter compared with the MI group, respectively (Fig. 3). In agreement with the above results, the heart-weight-to-body-weight ratio, which was increased in the MI group compared with sham group ($p < 0.01$), were significantly ($p < 0.01$)

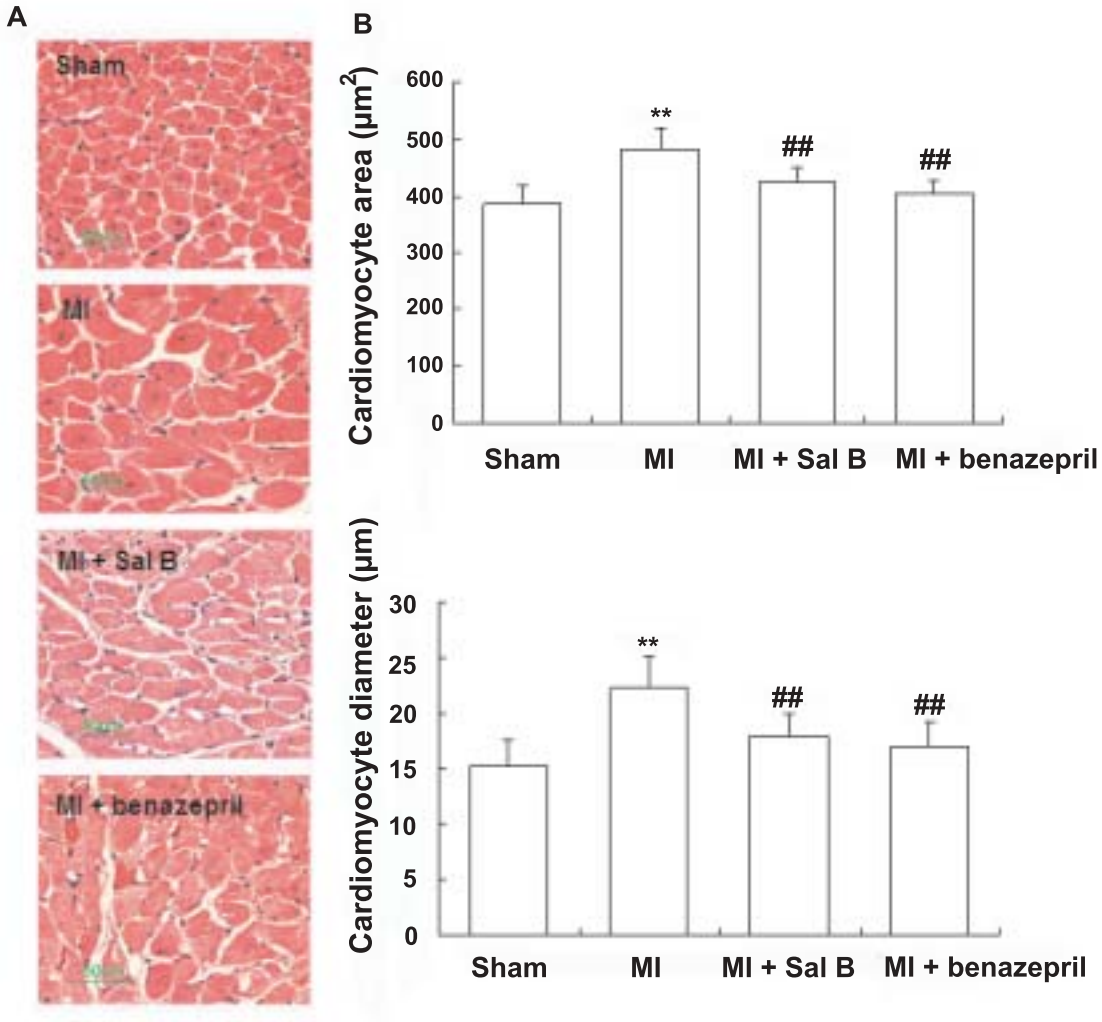

Fig. 3. Effects of Sal B and benazepril on cardiac hypertrophy in rats with large MI. **(A)** Representative photomicrographs of cardiomyocyte size stained with hematoxylin and eosin (magnification 400×). **(B)** Morphometric analysis of cardiomyocyte area. **(C)** Morphometric analysis of cardiomyocyte diameter. Data are shown as the mean ± SD (n = 10). ** $p < 0.01$ *vs.* sham group; $^{\#\#}$ $p < 0.01$ *vs.* MI group

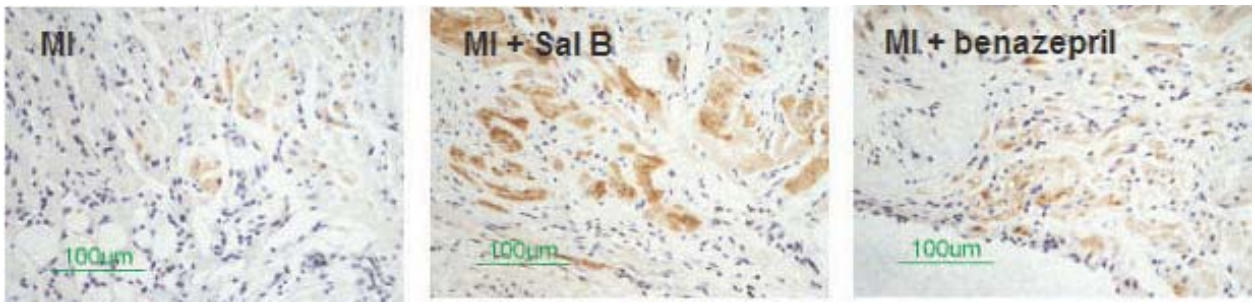

Fig. 4. Effects of Sal B and benazepril on VEGF protein expression examined by immunohistochemistry in rats with large MI. The brown part indicated the newly formed VEGF

lowered by Sal B and benazepril treatment (sham: 2.30 ± 0.12 mg/g; MI: 3.36 ± 0.15 mg/g; MI + Sal B: 2.60 ± 0.10 mg/g; MI + benazepril: 2.68 ± 0.11 mg/g). Body weight did not significantly differ between all groups (sham: 338 ± 24 g; MI: 331 ± 16 g; MI + Sal B: 342 ± 22 g; MI + benazepril: 335 ± 17 g).

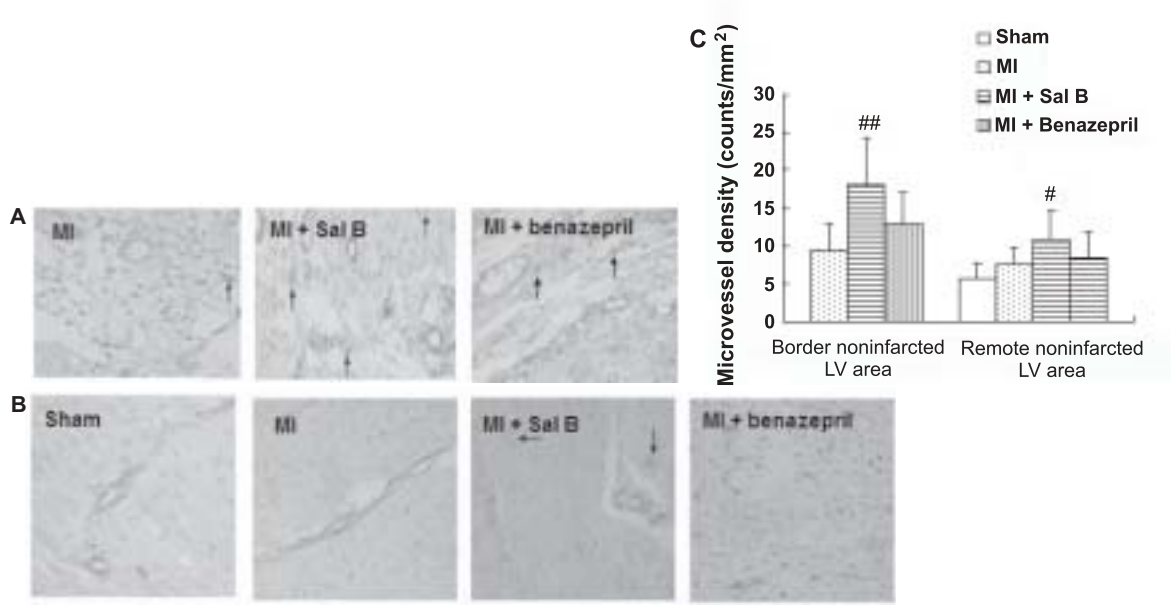

Fig. 5. Effects of Sal B and benazepril on myocardial microvessel density in rats with large MI. The arrows indicated the endothelial cells that were stained positively with vWF. **(A)** Immunohistochemical staining of vWF in the border noninfarcted LV area. **(B)** Immunohistochemical staining of vWF in the remote noninfarcted LV area. **(C)** Myocardial microvessel density in the border and remote noninfarcted LV area. Data are shown as the mean ± SD (n = 10). $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs.* MI group

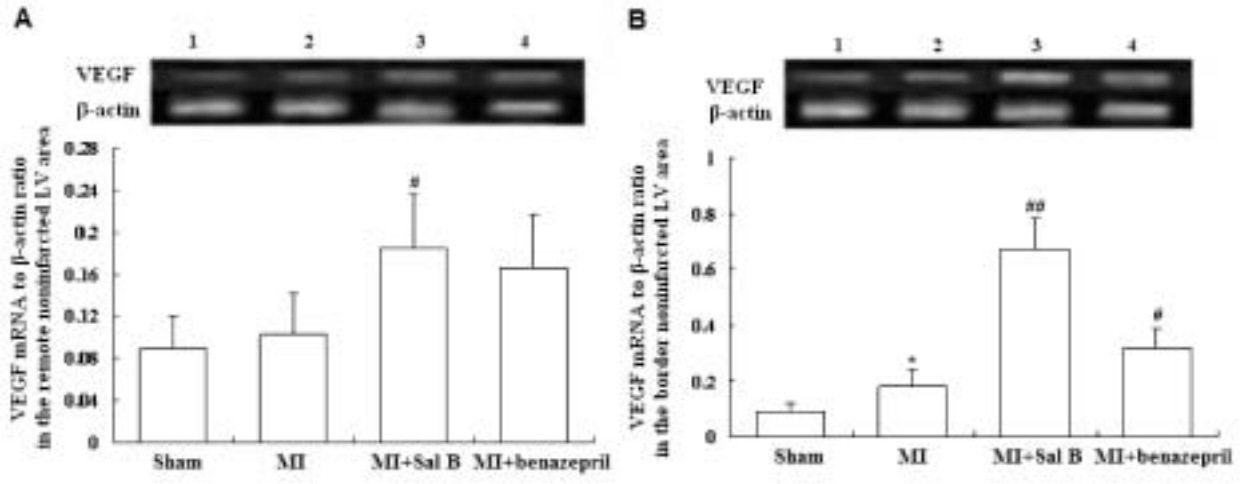

Fig. 6. The effect of Sal B and benazepril on VEGF mRNA expression in rats with large MI. **(A)** VEGF mRNA in the remote noninfarcted LV area. **(B)** VEGF mRNA in the border noninfarcted LV area. Lane 1, sham group; Lane 2, MI group; Lane 3, MI + Sal B group; Lane 4, MI + benazepril group. Data are shown as the mean ± SD (n = 5). * $p < 0.05$ *vs.* sham group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs.* MI group

Angiogenesis and VEGF expression

In MI rats, there was an increase in the density of microvessels per square millimeter. In the border noninfarcted LV area, there were an average of 9.4 ± 3.60 microvessels stained positively with vWF per square millimeter in MI group. After treatment with Sal B and benazepril, there were increases by 18.1 ± 6.33 ($p < 0.01$) and 12.9 ± 4.18 ($p > 0.05$) *vs.* the MI group, respectively (Fig. 5A, C). In the remote noninfarcted LV area, there were an average of 7.4 ± 2.37 microvessels in MI group *vs.* 5.6 ± 2.01 microvessels in the sham group ($p > 0.05$); after administration with Sal B and benazepril, they were increased by 10.8 ± 3.79 ($p < 0.05$) and 8.5 ± 3.50 ($p > 0.05$) *vs.* the MI group, respectively (Fig. 5B, C). In the border and remote noninfarcted LV area, no significant differences were observed in benazepril-treated rats compared with the MI group.

The mRNA and protein expression change in VEGF was in agreement with the above results among the 4 groups. From Figure 4 and Figure 6, we found that the VEGF expression level between sham and MI groups was not conspicuous. After administration with Sal B and benazepril, there was an increase in expression level compared with the MI group ($p < 0.05$ and $p < 0.01$); moreover, Sal B promoted the VEGF expression more effectively than benazepril, and its influence was more remarkable in the border region of MI, in which angiogenesis was more prominent compared with the remote zone.

Discussion

In the present study, we demonstrated that Sal B elicited a similar cardioprotective effect in improvement of heart function and blood viscosity, reduction of infarct size, inhibition of ventricular remodeling as benazepril, but Sal B also showed a unique effect inducing angiogenesis and augmenting VEGF expression in the border and remote noninfarcted LV area.

HF resulting from MI is a complex clinical syndrome with poor prognosis, its development is a long-term and complex process involving many factors, such as the sympathetic nervous system, renin-angiotensin system, reactive oxygen species (ROS), apoptosis, and so on. Despite considerable scientific data on the biochemical and molecular characteristics of HF, the precise molecular mechanisms responsible for HF still remain disputed. At this stage, diminution of infarct size, improvement of heart function and microcirculation, and prevention of ventricular remodeling are important end-points in the treatment of cardiovascular disorders post-infarction [1, 35].

Danshen, the rhizome of *Salvia miltiorrhiza* Bunge, is one of the most important ancient Chinese herbs, and is ranked as "supergrade" medicine in Shen-Nung's Pen-Ts'ao [27, 28]. Sal B is the most abundant and bioactive member of the salvianolic acids in Danshen [10], and studies find that Sal B has been shown to possess many of actions of the Danshen herb (such as antioxidant, hepatoprotective and so forth) [11, 12]. Benazepril has become an important medicine in treatment of cardiovascular diseases in developed countries [35]. In the present study, we compared cardioprotective effects of Sal B and benazepril in rats with large MI. In contrast to previous studies which mainly examined the acute effects of Danshen on MI [9, 25], our results demonstrated that chronic treatment with Sal B could improve systemic blood pressure, cardiac function and reverse remodeling in rats with large MI, and also showed that Sal B caused a significant prevention of compensatory hypertrophy as evidenced by decrease in heart weight indexes and cardiomyocyte hypertrophy, inhibition of cardiac dilation, as well as reduction of collagen deposition in the remote and border noninfarcted LV areas, especially in the latter. Benazepril had similar effects on cardiac function, infarct size, cardiac collagen volume and hypertrophy.

Previous studies have indicated a significant relationship between blood viscosity and the severity of coronary heart disease [13, 29]. The increased blood viscosity in rats with large MI would diminish the coronary flow, exacerbate the microcirculation disorder of the ischemic myocardium, and enlarge the ischemic and hypoxic area. In the present study, a significant improvement of blood viscosity was found in Sal B-treated rats ($p < 0.05$ and $p < 0.01$), and the effect on blood viscosity was more remarkable than that of benazepril.

Angiogenesis, which is outgrowth of new vessels from existing vessels, is a key process involved in normal development and wound repair tissue, as well as ischemic heart and limb diseases, and atherosclerosis [2, 3, 31]. During angiogenesis, endothelial cells detach from the pre-existing destabilized vessel, mi-

grate into the perivascular space and proliferate to finally mature and form new vascular structures. A number of growth factors, proteases, adhesion molecules and other angiogenic mediators that enable endothelial cell migration or proliferation regulate this process. VEGF is considered one of the most important potent growth factors in angiogenesis. VEGF activates endothelial nitric oxide synthase (eNOS) by the induction of calcium flux, the recruitment of heat-shock protein 90 (Hsp90) and the phosphorylation of nitric oxide synthase (NOS) *via* the phosphatidylinositol-3-OH-kinase [PtdIns(3)K]-Akt pathway. Upon activation, eNOS catalyzes L-arginine transformation to L-citrulline and nitric oxide (NO). NO contributes to a variety of endothelial cellular events including proliferation, migration and anti-apoptosis and so on, the events are essential early steps required for neovascularization. In addition, NO induces the mobilization and expansion of endothelial progenitor cells (EPC) in the bone marrow, EPC can differentiate into mature endothelial cell. Previous studies have shown that puerarin could promote endothelial cell function and prevent endothelial dysfunction, apoptosis and viability loss [4].

Angiogenesis can be quantified by different methods based on the microscopic evaluation of tissue vascularization using antibodies with affinity for specific epitopes on the endothelial cell, such as VEGF, CD31, CD34, and vWF or factor VIII [32]. In this study, we, by assessing vWF, found that Sal B might significantly increase the density of microvessels in the remote and border noninfarcted LV area in the rats with large MI; the results of morphological observation also proved the point. There was no evidence to show that benazepril had any favorable effects on the angiogenesis. The results suggested that Sal B enforced cardioprotective effect by inducing neovascularization, improving myocardial microcirculation.

VEGF is considered a key angiogenic growth factor and stimulates proliferation, migration, and tube formation of endothelial cells (ECs) primarily through the VEGF receptor type2 (VEGFR2, KDR/Flkl) [14]. ECs generate ROS such as $O_2^{\bullet-}$ and H_2O_2 which play a role in physiological and pathophysiological responses. Signal transduction by ROS, so-called "redox signaling" has been an emerging area of investigation. The major source of ROS in ECs is an NADPH oxidase, which is activated by numerous stimuli including VEGF, cytokines, shear stress, hypoxia and G-protein coupled receptor agonists including angiotensin II (Ang II) in ECs [5]. Role of ROS derived from NADPH oxidase in VEGF signaling is linked to angiogenesis. VEGF binding to VEGFR2 leads to the activation and translocation of the small GTPase Racl into the plasma membrane, which stimulates the NADPH oxidase in ECs. ROS derived from this oxidase may oxidize and inactivate protein tyrosine phosphatases (PTPs) which negatively regulates VEGFR2, thereby enhancing VEGFR2 autophosphorylation, and subsequent redox signaling linked to angiogenic responses such as EC proliferation and migration [8, 26]. In ECs, NADPH oxidase is one of the major sources of ROS and consists of catalytic subunits. VEGF stimulates ROS production *via* activation of NADPH oxidase, and ROS are involved in VEGFR2-mediated signaling linked to EC migration and proliferation. Moreover, ROS derived from NADPH oxidase are involved in postnatal angiogenesis. Localizing NADPH oxidase and its regulators in the specific subcellular compartment is an important mechanism for activating specific redox signaling events [27]. Previous studies have demonstrated that the endothelial cell mitogen VEGF promotes neovascularization *in vitro* [18] and *in vivo* [19], and is considered one of the most important growth factors in angiogenesis, which stimulates proliferation, migration, and tube formation of ECs, primarily through VEGFR2. We compared Sal B and benazepril with VEGF mRNA expression in rats with large MI. In this study, we found that VEGF expression in the remote and border of noninfarcted left ventricular area was significantly up-regulated in Sal B-treated group compared with MI group ($p < 0.05$ and $p < 0.01$). Perhaps, Sal B promoted angiogenesis by up-regulating VEGF expression. The detailed mechanism will need to be further examined.

In conclusion, these results suggest that Sal B and benazepril exerted beneficial cardioprotective effects such as improving significantly post-MI cardiac function and reducing blood viscosity, infarct size, cardiac collagen volume and hypertrophy. However, Sal B enforced some modality different from benazepril, which might improve myocardial microcirculation by augmenting VEGF expression and promoting angiogenesis besides similar effects to benazepril. Notwithstanding that benazepril failed to exert the significantly beneficial effects on angiogenesis and VEGF expression, its cardioprotective effect was the same. We are now investigating the possible reason of ho-

moplastic myocardial preservation between Sal B and benazepril.

Acknowledgments:
This study was supported by a project from National Postdoctor Science Foundation of China (No. 20070411201), a common project from National Natural Science Foundation of China (No. 30500661) and a grant of National Important Basic Development Plan of China (also called 973 project, No. 2005CB523405). Professor Dr Yu-ling Ma (Research Fellow, School of Pharmacy, University of Reading, GB) provided assistance with the composition and English styling of the paper.

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Received:
October 10, 2007; in revised form: January 28, 2008.