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# Improvement of the survival and therapeutic effects of implanted mesenchymal stem cells in a rat model of coronary microembolization by rosuvastatin treatment

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**Abstract. – OBJECTIVE: As the impairment of myocardial micro-environments with local inflammatory reactions due to coronary micro-embolization (CME) reduces the survival of transplanted stem cells (SCs). We hypothesized that rosuvastatin treatment could improve the SC survival and enhance their therapeutic effects.**

**MATERIALS AND METHODS: Rat bone marrow-derived mesenchymal stem cells (BMSCs) were infected with lentivirus carrying the green fluorescent protein (GFP) gene. To develop a CME model, rats were injected with a suspension of microthrombotic particles (MTPs) into the left ventricle to obstruct the ascending aorta. GFP-BMSCs were injected with MTPs simultaneously. Rosuvastatin treatment was started 7 days before BMSC transplantation and ended 7 days after transplantation.**

**RESULTS: Expressions of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were examined by the molecular methods. GFP-positive BMSCs were detected by fluorescence staining. Neovascularization was determined by immunohistochemistry. Myocardial morphology was identified by H&E and Masson's trichrome staining. Cardiac function was quantified by echocardiography. Three days after CME, the multifocal myocardial necrosis with extensive infiltration of inflammatory cells was observed, accompanied by high expression of TNF-α and IL-1β. Rosuvastatin treatment reduced the infiltration of inflammatory cells and TNF-α and IL-1β expression. 28 days after transplantation, BMSC therapy with rosuvastatin promoted the survival of implanted cells by ≈45-fold while compared with BMSC therapy alone. BMSC therapy with rosuvastatin (instead of BMSC therapy alone) upregulated the VEGF and bFGF expression, increased the capillary density and improved the cardiac function.**

**CONCLUSIONS: These data suggested that rosuvastatin treatment promoted the survival of transplanted SCs and enhanced their therapeutic effects for CME.**

*Key Words:*
Coronary microembolizaiton, Mesenchymal stem cells, Rosuvastatin, Transplantation.

## Introduction

Coronary microembolization (CME) can lead to a series of adverse cardiac events: myocardial contractile dysfunction, arrhythmias, reduction in coronary reserve, myocardial macro-infarction or micro-infarction, death[1-6]. CME is encountered frequently in acute coronary syndromes and during the coronary interventions. Considerable efforts have been made to prevent and treat CME and CME-induced cardiac events, but with limited achievement[2,4,7,8].

Recently, some investigations showed that transplantation of autologous skeletal myoblasts can improve hemodynamics and left ventricular (LV) function in dogs with CME-induced chronic heart failure[9]. Stromal cell-derived factor 1 has also been shown to improve LV function and perfusion in a porcine model of myocardial infarction induced by CME[10]. However, a damaged myocardial micro-environment in the host usually leads to poor survival of transplanted cells due to ischemia, ischemia-reperfusion injury, the inflammatory reaction and apoptosis[11,12], which limit the benefits of stem-cell therapy[13-15]. Therefore, it remains unclear whether the transplanted stem cells such as bone marrow-derived mesenchymal stem cells (BMSCs) could survive well and improve cardiac performance in case of impaired myocardial environment after CME.

It has been clearly established that the inhibitors of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase (statins) can protect


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against the inflammatory reaction and the oxidant stress[16] *via* their pleiotropic effects, which are independent of the lowering of cholesterol levels. Rosuvastatin, known as a "super-statin", has been shown to possess several advantageous pharmacologic properties (e.g.: strong anti-inflammatory effects). Nevertheless, its role in reducing CME-induced impairment of the myocardial micro-environment (thus enhancing the survival and therapeutic effects of implanted BMSCs) is still unknown.

We tested the hypothesis that the rosuvastatin treatment can improve the CME-induced damaged myocardial microenvironment and, thereby, enhance the survival of the transplanted BMSCs and their therapeutic effects.

## Materials and Methods

### Ethical Approval of the Study Protocol

The study protocol was approved by the Care of Experimental Animals Committee of Fujian Medical University (Fuzhou, P. R. China). All experiments involving animals complied with the recommendations in the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health (NIH), Bethesda, MD, USA).

### Animal Care

Sprague-Dawley rats (280-320 g) were obtained from the Animal Center of Fujian Medical University and housed in the animal facilities of Union Hospital.

### Isolation, Culture, Identification and Labeling of BMSCs

The mesenchymal stem cells were prepared from the bone marrow of rats as described by Fan et al[17]. BMSCs were cultured in the low-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Hyclone, Jülich, Germany). Non-adherent cells were removed by changing the medium at 24 h, and every 72h thereafter. Passage-3 BMSCs were analyzed by using a fluorescence-activated cell sorter (Becton Dickinson Biosciences, San Jose, CA, USA) for their surface marker expressions with antibodies against CD11b.PE, CD45.PE, C90.FITC (Biolegend, San Diego, CA, USA), CD44.PE (Serotec, Kidlington, UK), and CD34.PE (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Passage-1 BMSCs were infected with pGC FU-green fluorescent protein (GFP)-Lentivirus (GeneChem, Beijing, P. R. China) at a multiplicity of infection of 8. All BMSCs were expanded to passage-3 and maintained in warm DMEM before transplantation. The efficiency of the gene transfection was evaluated by flow cytometry and fluorescence microscopy (TE2000-U flow cytometer; Nikon, Tokyo, Japan).

### Creation of a CME Model, Cell Transplantation, and Rsv Administration

The CME model was generated as described by Li et al[18]. Briefly, a sternotomy was undertaken in rats at the second and third intercostal space with ligation of mammalian arteries. The pericardium was opened and the ascending aorta was exposed fully. Allogeneic BMSCs ($2\times10^6$ cells per rat) and microembolic particles (5 mg per rat) dissolved in 0.3 mL DMEM were injected into the left ventricular (LV) chamber with a 26-G needle during 10-s occlusion of the ascending aorta. Rats in the control group received an injection of the same quantity of microembolic particles dissolved in 0.3 mL DMEM, and rats in the sham group received an injection of the same volume of DMEM only. At the end of transplantation, the chest was closed, the rat was extubated appropriately and allowed to recover. After surgery, all rats received the antimicrobial therapy (penicillin 400,000 U, i.p.) every day for 3 days. As reported previously[19], the rosuvastatin (Crestor®, AstraZeneca UK, Macclesfield, UK) treatment (3.0 mg/kg body weight per day) was started 7 days before BMSC transplantation and stopped 7 days after transplantation.

### Experimental Protocol

Rats were allocated randomly to five groups of 24 rats (Figure 1): sham (chest open only without CME); control (CME model only: no interventions); BMSCs (treated with BMSCs only after CME); Rsv (treated with rosuvastatin only after

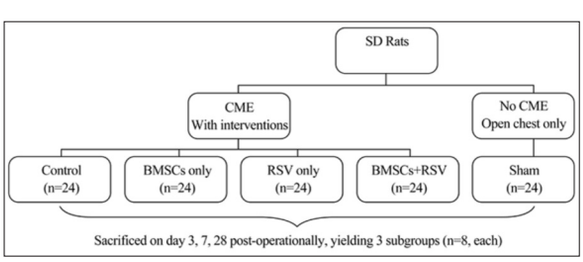


**Figure 1.** Experimental protocols and animal groups. CME, coronary microembolization; BMSCs, bone marrow-derived mesenchymal stem cells; RSV, rosuvastatin.

CME); Rsv+BMSCs (treated with a combination of rosuvastatin and BMSCs). Rats from each group were killed 3, 7, and 28 days after surgery to yield three subgroups of 8 rats.

### Echocardiographic Study

The cardiac function was assessed by transthoracic echocardiography 3 and 28 days after transplantation. Short-axis two-dimensional view of the left ventricle was taken at the level of the papillary muscles to obtain M-mode recordings by using a 10-MHz electronic-phased-array transducer (Vingmed Ultrasound VIVID7; GE Healthcare, Piscataway, NJ, USA). Left ventricular end-systolic diameter (LVESD) and left ventricular end-diastolic diameter (LVEDD) were measured in M-mode tracings on the LV short axis view at the level of the chordae tendineae. The left ventricular fractional shortening (LVFS) (%) was calculated as (LVEDD – LVESD)/LVEDD × 100) and the left ventricular ejection fraction (LVEF) was calculated by using a cubic formula[20]. All parameters were obtained by taking mean measurements from three consecutive cardiac cycles. Echocardiographic images were digitized and recorded at different experimental stages.

### Preparation of the Tissue Samples

Eight rats per group were killed under anesthesia 3 and 28 days after transplantation. Blood samples were collected and used for enzyme-linked immunoassay (ELISA). Heart tissue was harvested and cut (in transverse) into three parts. Middle-level tissue samples (thickness, 2 mm) were embedded in OCT medium for preparation of frozen sections. Six thin heart sections (thickness, 5 μm) were cut consecutively for histologic studies. The remaining heart tissue was used for the preparation of homogenates and molecular studies. All tissue samples were prepared in an identical manner.

### Immunofluorescence

In order to ensure that the transplanted BMSCs had sufficient time to differentiate into cardiomyocyte-like cells, three sections from each heart of 28-day rats were used to assess cellular differentiation. Briefly, the frozen tissue sections were co-incubated with goat anti-GFP antibody conjugated with fluorescein isothiocyanate (Abcam, Cambridge, UK) and rabbit anti-cardiac troponin I (cTnI) primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The next day, the sections were washed with phosphate-buffered saline (PBS) and incubated with mouse anti-rabbit secondary antibody conjugated with sulforhodamine 101 acid chloride (Texas Red; Santa Cruz Biotechnology) at 37°C for 30 min. Finally, the nuclei were labeled with 4',6-Diamidino-2-phenylindole dihydrochloride (Sigma-Aldrich, St. Louis, MO, USA). The sections were imaged using a laser confocal scanning microscope (LSM510; Zeiss, Jena, Germany) to identify the differentiation of BMSCs into cardiomyocyte-like cells or fusion with host cardiomyocytes. For each section, 5 high-power fields (HPF) in a cell-transplanted zone were selected randomly for analysis. Survival of the transplanted BMSCs was quantified as the number of GFP-positive cells per HPF.

### Real-time Quantitative Polymerase Chain Reaction (PCR)

The total RNA was extracted with TRIzol® reagent (Gibco, Billings, MT, USA) from the cardiac tissue of rats 7 and 28 days after transplantation. RNA (4 μg) was reverse-transcribed by using a reverse transcriptase kit (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions. Reactions involved 2 μL cDNA, 0.5 μL primers and 12.5 μL SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA), as well as water added to a final volume of 25 μL. PCR was undertaken at the following cycling conditions: initial denaturation at 95°C for 10 min followed by 45 cycles of amplification with 95°C for 15s and 60°C for 1 min. Primers were: 5'-ACCTCACCAAAGCCAGCACAT-3' and 5'-TCACAGTGAACGCTCCAGGAT-3' for the rat vascular endothelial growth factor (VEGF), 5'-TTGAACGCCTGGAGTCCAAT-3' and 5'-TGACCTCAGCATGGAGGAACT-3' for the rat basic fibroblast growth factor (bFGF), and 5'-GAGATTACTGCCCTGGCTCCTA-3' and 5'-CATCGTACTCCTGCTTGCTGAT-3' for β-actin. The dissociation curve was analyzed to confirm specific amplification. All cDNA samples were amplified in triplicate and normalized against a triplicate of β-actin in the same plate. Data[21] were expressed as $2^{-\Delta\Delta Ct}$.

### Western Blotting

Proteins were extracted from tissue samples 7 and 28 days after transplantation, and their concentrations were determined by using a bicinchoninic acid (BCA) protein assay (Beyotime

Biotechnology, Jiangsu, P.R. China). Subsequently, the protein (60 μg) was loaded onto a 12% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis and then transferred into polyvinylidene fluoride membranes (Amersham, Amersham, UK). Western blotting was carried out with a 1:500 dilution of primary antibody (anti-VEGF and anti-bFGF; Sigma-Aldrich) and a 1:10000 dilution of horseradish peroxidase-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. Bands were analyzed by using ImageJ v1.36 (NIH) to verify relative expression of VEGF or bFGF (defined as the ratio of optical density of VEGF or bFGF over GAPDH).

### Histologic Analysis

At least three short-axis ring-like sections covering all LV segments were obtained from each heart at the level of the near apex, papillary muscle, and mid-mitral valve for further histologic analyses. Sections from each heart on day 3 were stained with hematoxylin and eosin (H&E). Multifocal myocardial necrosis and infiltration of inflammatory cells were observed under Image-Pro Plus v5.0 (Media Cybernetics, Rockville, MD, USA). Sections from each heart of day-28 rats were also stained with Masson's trichrome for observation of myocardial fibrosis. By immunohistochemical staining, expression of cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1β) was detected on day 3 and small-vessel regeneration was detected on day 28. Paraffin sections (thickness, 5 μm) were incubated with primary antibodies (anti-TNF-α and anti-IL-1β; Santa Cruz Biotechnology; anti-factor VIII-related antigen; Dako, Glostrup, Denmark) and horseradish peroxidase-conjugated second antibodies. Color development during the immunoperoxidase staining was done with 3,3'-Diaminobenzidine, and sections were counterstained by using hematoxylin. Small-vessel density in the infarcted myocardium was determined. Three sections from each heart were selected for analysis and five fields from each section were counted randomly. Capillary density was quantified as the number of factor VIII-related antigen-positive vessels per HPF.

### ELISA

To measure the expression of TNF-α and IL-1β in serum, blood samples from each rat on Day 3 were collected for ELISA according to the manufacturer's instructions.

### Statistical Analysis

Data were analyzed by using SPSS v13.0 (IBM, Armonk, NY, USA). Data are the mean ± SD. Differences among groups were compared by using one-way ANOVA (Analysis of Variance), followed by the Bonferroni correction. $p < 0.05$ was considered statistically significant.

## Results

### Characterization of Cultured BMSCs

Phenotypic characterization showed that BMSCs were negative for CD11b, CD34, and CD45 antigens, but positive for CD44 and CD90 antigens (Figure 2a). BMSCs with spindle morphology had a powerful ability to proliferate (Figure 2b) and were infected efficiently with GFP-lentivirus (Figure 2c, 2d).

### Changes in Myocardial Morphology

Staining with H&E (Figure 3a) and Masson's trichrome (Figure 3b) showed multiple patchy myocardial fibrosis and acute inflammation with infiltration of numerous leukocytes in micro-infarcted regions in control rats and in those treated with BMSCs only. Patchy myocardial fibrosis was seen more in sub-endocardial rather than sub-epicardial regions. In contrast, reduced fibrosis and infiltration of inflammatory cells were observed in the hearts of rats treated with rosuvastatin alone, or with rosuvastatin and BMSCs. More significant reductions were observed in the Rsv+BMSCs group than in the Rsv group.

### Expression of Inflammatory Cytokines

Expression of the inflammatory cytokines TNF-α and IL-1β was examined in rats with CME. In the myocardium, the rosuvastatin treatment reduced the expression of TNF-α and IL-1β compared with that seen in the control group ($p < 0.01$). We found that BMSC therapy with rosuvastatin further down-regulated the expression of TNF-α and IL-1β ($p < 0.01$). Transplantation of BMSCs alone did not reduce the expression of TNF-α and IL-1β (Figure 4a and 4b).

### Survival of Implanted BMSCs In Vivo

Fluorescence microscopy showed that, 28 days after BMSC transplantation, the number of GFP-labeled cells within cardiac tissues in rats treated

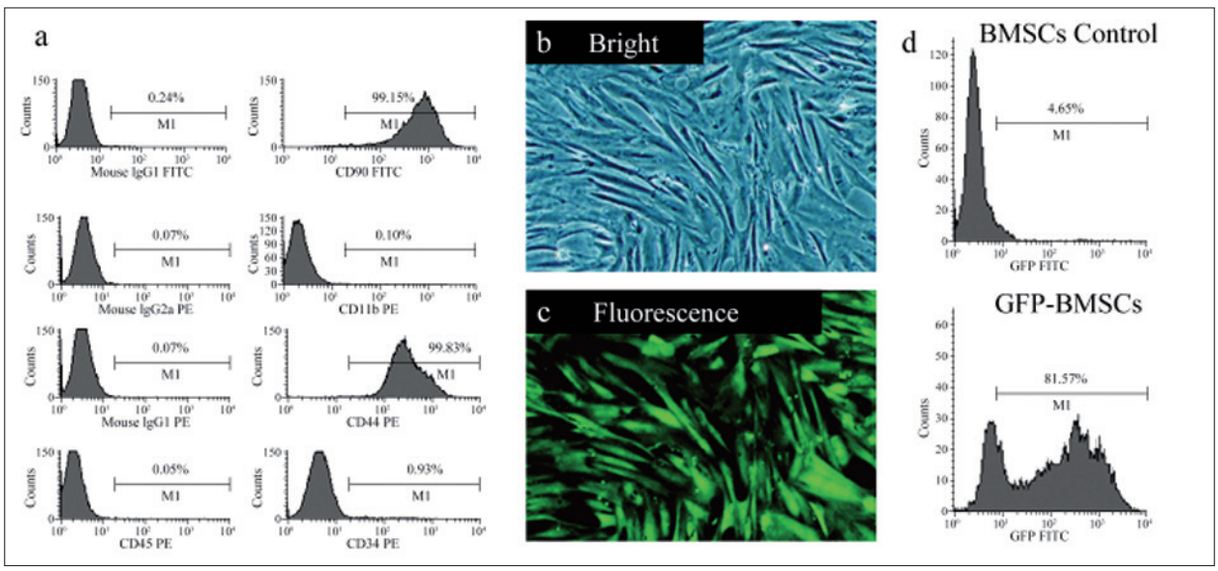


**Figure 2.** Surface markers, GFP-lentivirus infection, and multiple differentiation of rat cultured BMSCs (original amplification: b and c ×100). ***A,*** for surface markers, cultured cells had high expression of CD90 and CD44, and low expression of CD11b, CD34 and CD45, suggesting characterization of mesenchymal stem cells. ***B-D,*** after 72 h of GFP-lentivirus infection, morphologic characterization of cultured cells under bright fields ***(b)*** and fluorescence fields ***(c)*** are shown, and high efficiency of gene infection was identified by flow cytometry ***(d)***. BMSCs as defined in Figure 1. GFP, green fluorescent protein.

with rosuvastatin+BMSCs was significantly higher than that in hearts that received BMSCs only (45.5 ± 4.4 *vs.* 1.1 ± 0.2, $p < 0.01$), which suggested that rosuvastatin treatment can significantly increase the survival of implanted BMSCs in hearts after CME (Figure 5a and 5b). Surprisingly, very few transplanted BMSCs were detected in rats treated with BMSCs only; furthermore, very few transplanted BMSCs co-expressed cTnI and GFP, with cells "inlaid" into host cardiomyocytes. Conversely, most transplanted BMSCs were located around with injured cardiomyocytes and some were attached to host cardiomyocytes, with irregular spherical phases that resembled the appearance before transplantation (Figure 5b).

### Expression of Growth Factors

Expression of VEGF and bFGF in the myocardium quantified by real-time PCR showed no significant difference between the BMSCs group and the control group ($p > 0.05$). In contrast, the Rsv+BMSCs group showed higher expression of VEGF and Bfgf while compared with the control group and BMSCs group ($p < 0.01$, Figure 6a). Western blotting demonstrated consistent results with gene expression for VEGF and bFGF (Figure 6b, 6c).

### Alteration of Capillary Density

Capillary density in the infarct zone in the Rsv+BMSCs group was significantly higher than that in the control group and BMSCs group (29.5 ± 1.9 in the Rsv+BMSCs group *vs.* 11.5 ± 1.1 in the control group and 12.2 ± 1.9 in the BMSCs group, $p < 0.01$); however, no significant difference in capillary density was identified between the control group and BMSCs group ($p > 0.05$) (Figure 7).

### Changes in Cardiac Function

Compared with sham rats, LVEF and LVFS in the control group were significantly lower 3 and 28 days after cell transplantation ($p < 0.01$). Conversely, LVES and LVEF showed significant improvements in the Rsv group and Rsv+BMSCs group at all corresponding stages compared with the control group ($p < 0.01$). At 28 days in the Rsv group and Rsv+BMSCs group, LVFS increased significantly (33 ± 1% and 42 ± 1% *vs.* 27 ± 3%, $p < 0.01$, respectively), and LVEF increased significantly (70 ± 2% and 81 ± 2% *vs.* 62 ± 4%, $p < 0.01$ respectively); however, the BMSCs group rendered no significant improvement in LVFS (28 ± 2% *vs.* 27 ± 3%, $p = 0.38$) or LVEF (63 ± 3% *vs.* 62 ± 4%, $p = 0.34$). Furthermore, LVFS and LVEF in rats treated with rosuvastatin+BMSCs were much higher than those in rats treated with rosuvastatin only (Figure 8a-d). Compared with sham rats, LVEDD increased significantly in the control group with more prominent increments on Day 28 ($p < 0.01$). 28 days

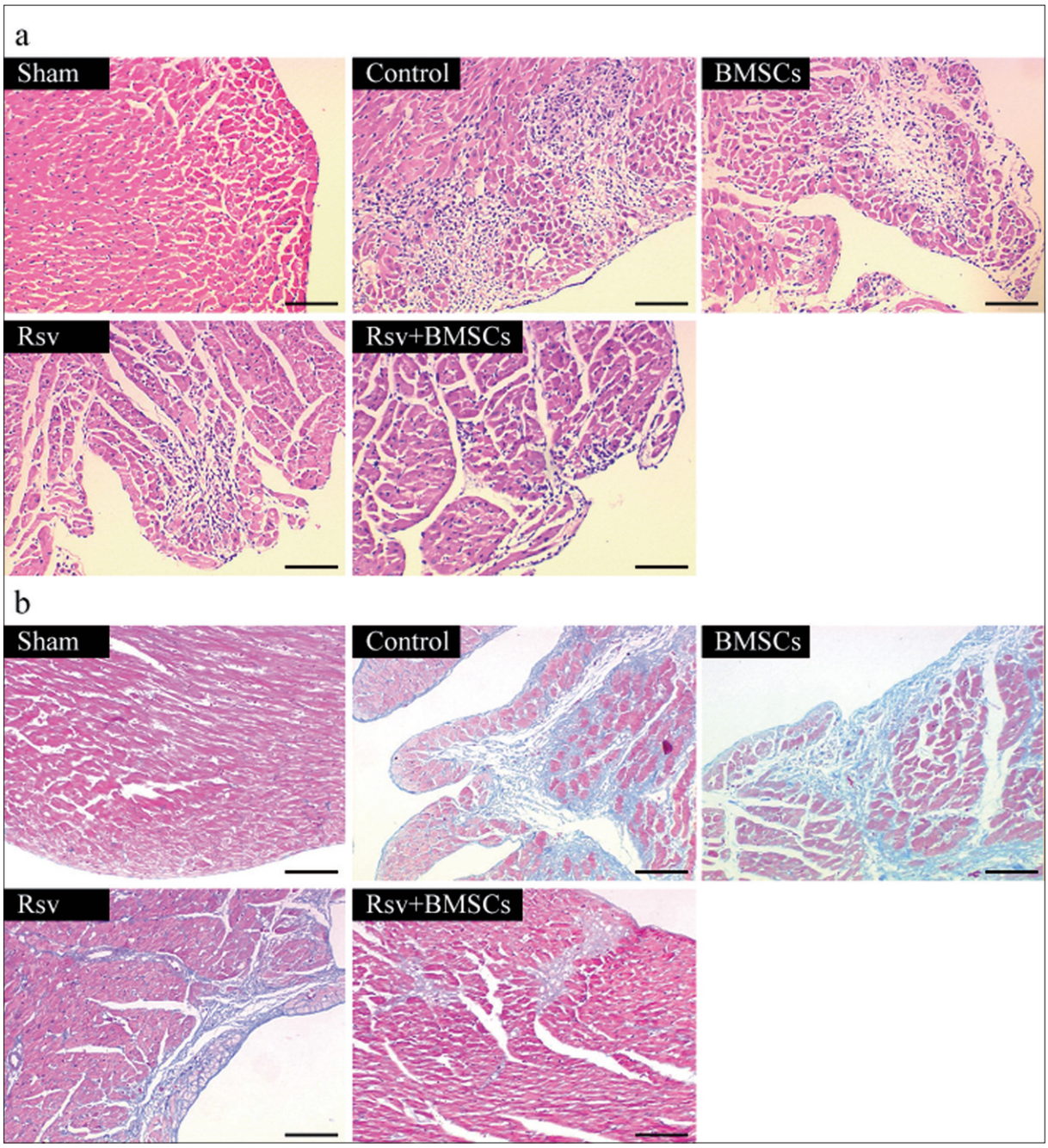


**Figure 3.** H&E ***(a)*** and Masson's trichrome staining ***(b)***. Severe infiltration of inflammatory cells and fibrosis are observed in control and BMSCs groups, and less infiltration and fibrosis are seen in Rsv and Rsv+BMSCs groups. More significant improvement was observed in the Rsv+BMSCs group than in the Rsv group. Group abbreviation is as defined in Figure 1. Scale = 100 μm.

after cell transplantation, LVEDD decreased significantly in the Rsv group (5.78 ± 0.07 mm, $p < 0.01$) and Rsv+BMSCs group (5.50 ± 0.35 mm, $p < 0.01$) compared with the control group (6.08 ± 0.12 mm), but this tendency was not observed in the BMSCs group (6.07 ± 0.26 mm *vs*. 6.08 ± 0.12 mm; $p = 0.82$); moreover, LVEDD decreased to a greater extent in the Rsv+BMSCs group while compared with the Rsv group ($p < 0.01$, Figure 8b,e). These data clearly demonstrated that treatment with Rsv and BMSCs can improve cardiac performance.

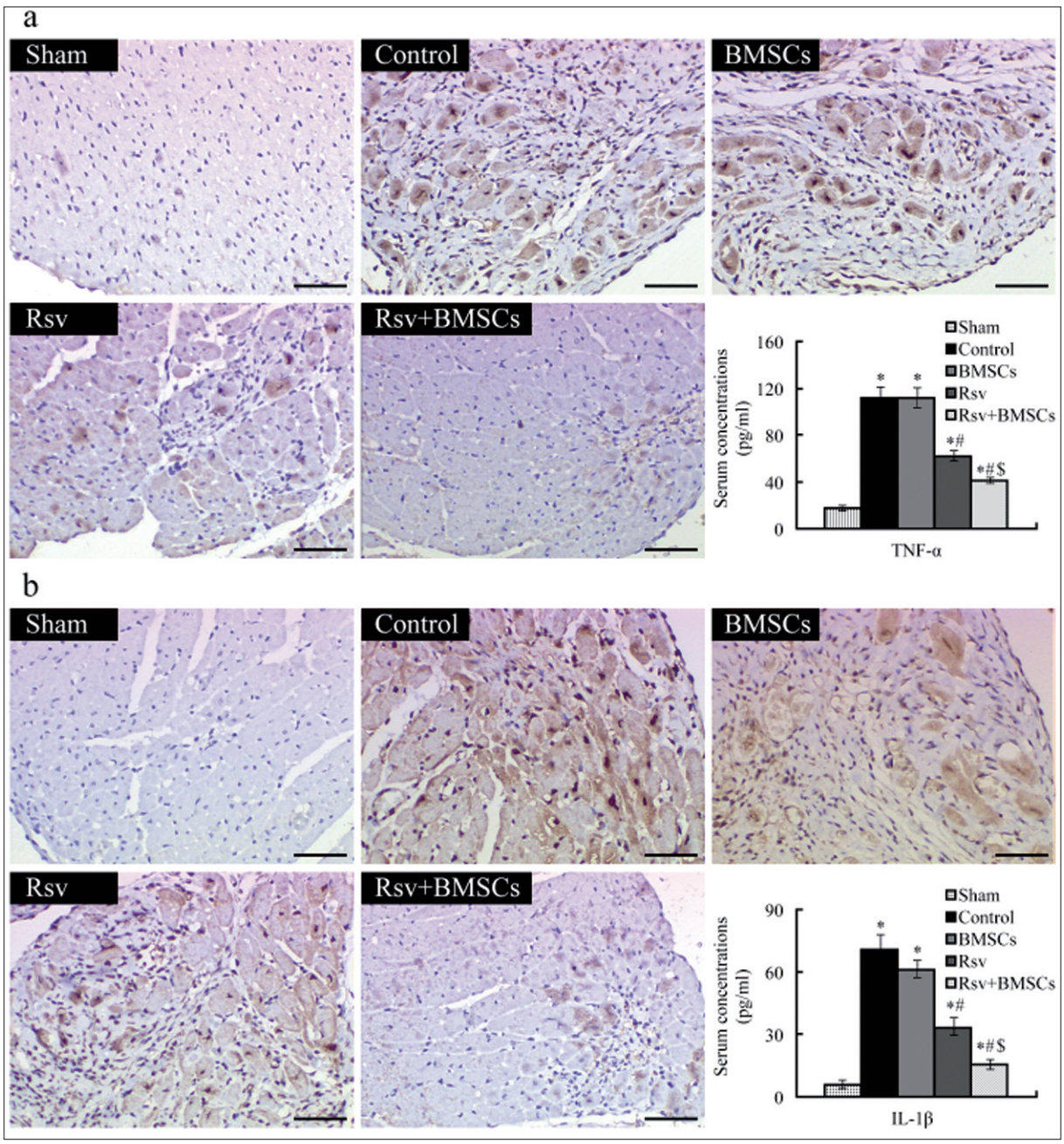


**Figure 4.** Immunohistochemical staining and ELISA for TNF-α ***(a)*** and IL-1β ***(b)*** in the myocardium 3 days after cell transplantation. Strong expression of the pro-inflammatory cytokines TNF-α and IL-1β is detected in CME and BMSCs groups. Rosuvastatin treatment and BMSC therapy with rosuvastatin, respectively, suppresses the inflammatory reaction. BMSC therapy with rosuvastatin shows the lowest expression of TNF-α and IL-1β. No protein is detected in the sham-operation group. Histogram (low right) shows ELISA results. Group abbreviation is as defined in Figure 1. ELISA, enzyme-linked immunosorbent assay. TNF, tumor necrosis factor. IL, interleukin. Scale = 100 μm. Values are the mean ± S.D (n = 8 per group). $^{*}p < 0.01$ *vs*. Sham. $^{\#}p < 0.01$ vs. CME. $^{\$}p < 0.01$ *vs*. Rsv.

## Discussion

Major findings of our study were: (1) the transplantation of BMSCs alone did not notably improve cardiac function; (2) the short-term treatment with a high dose of rosuvastatin can protect the myocardial micro-environment of the host against CME-induced acute dysfunction of the microcirculation and local inflammatory reactions, thereby improving the survival of trans-

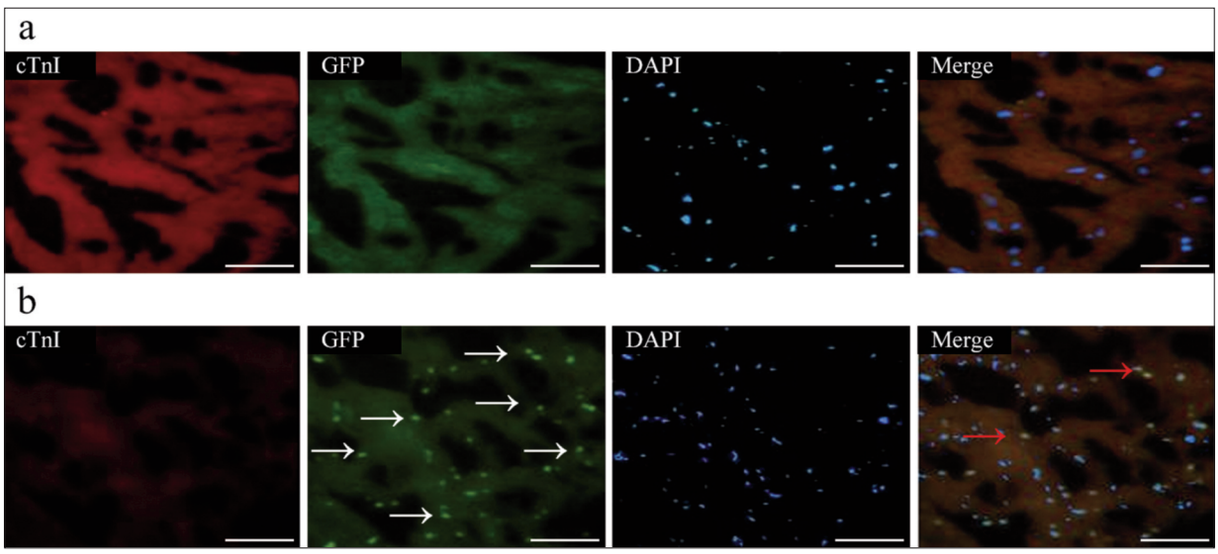


***Figure 5.*** Representative confocal laser scanning micrographs of transplanted BMSCs in the myocardium 28 days after cell transplantation. Nuclear staining by DAPI is shown in blue, GFP expression in green, and the cardiac-muscle marker cTnI in red. GFP-positive cells (*white arrows*) are deemed to be surviving transplanted BMSCs. Very few transplanted BMSCs are observed in the BMSCs group ***(a)***. The number of surviving stem cells in BMSCs+Rsv group is significantly higher than that in the BMSCs group ***(b)***. In the BMSCs+Rsv group, few cells co-express GFP and cTnI (*red arrows*) ***(b)***. BMSCs and group abbreviation are as defined in Figure 1. GFP is as defined in Figure 2. Scale = 50 μm

planted BMSCs; (3) a combination of rosuvastatin and BMSC transplantation has significantly improved the cardiac performance. The present work is the first to demonstrate that rosuvastatin pretreatment can enhance the survival and therapeutic effects of the implanted BMSCs in the setting of CME.

According to the "seed and soil" hypotheses, the fate of transplanted cells *in vivo* is determined primarily by the donor cells and the myocardial micro-environment of the host. It has been demonstrated that the transplanted cells (regardless of their origin) did not survive well in the host myocardium, with cell death of 70-93% in the first 3 days and further cell loss over time, which thus limited the therapeutic effects of transplanted cells[22-25]. To enhance the survival of grafted cells, the pretreatment of donor cells with a hypoxic stimulus or growth factors, or with anti-apoptotic and pro-survival genetic modification, carry unknown risks (including a tumorigenic risk)[26]. To avoid an unfavorable micro-environment in the acute phase of myocardial ischemia, delayed application of cell transplantation could bypass the best "time window" for inhibition of the ventricular remodeling[27], and also is impractical because the cell transplantation is usually undertaken immediately after the acute coronary intervention. Therefore, efficacious measures to improve the survival of donor cells are lacked, and modification of the impaired myocardial micro-environment seems to be theoretically possible.

In a model of myocardial infarction, Yang et al[28] demonstrated that the pretreatment with atorvastatin improved the survival of transplanted cells. Our data showed that in a rat model of CME, short-term treatment with a high dose of rosuvastatin significantly promoted the survival of transplanted BMSCs and improved the cardiac performance, suggesting that modification of the unfavorable myocardial microenvironment may be crucial.

The main factor influencing the survival of stem cells is that transplanted cells are highly sensitive to ischemia/hypoxia, ischemia-reperfusion injury, and subsequent inflammation and apoptosis – the unfavorable micro-environment that accompanies acute myocardial infarction (AMI) and which cannot be avoided completely during the reperfusion therapies[29]. In addition to possessing the same pathophysiology as AMI, CME is characterized by acute dysfunction of the microcirculation and impaired myocardial perfusion as evidenced by "no-flow" or "low-flow"; moreover, unlike infracted conduct coronary arteries (which can be opened readily by reperfusion methods) CME, once oc-

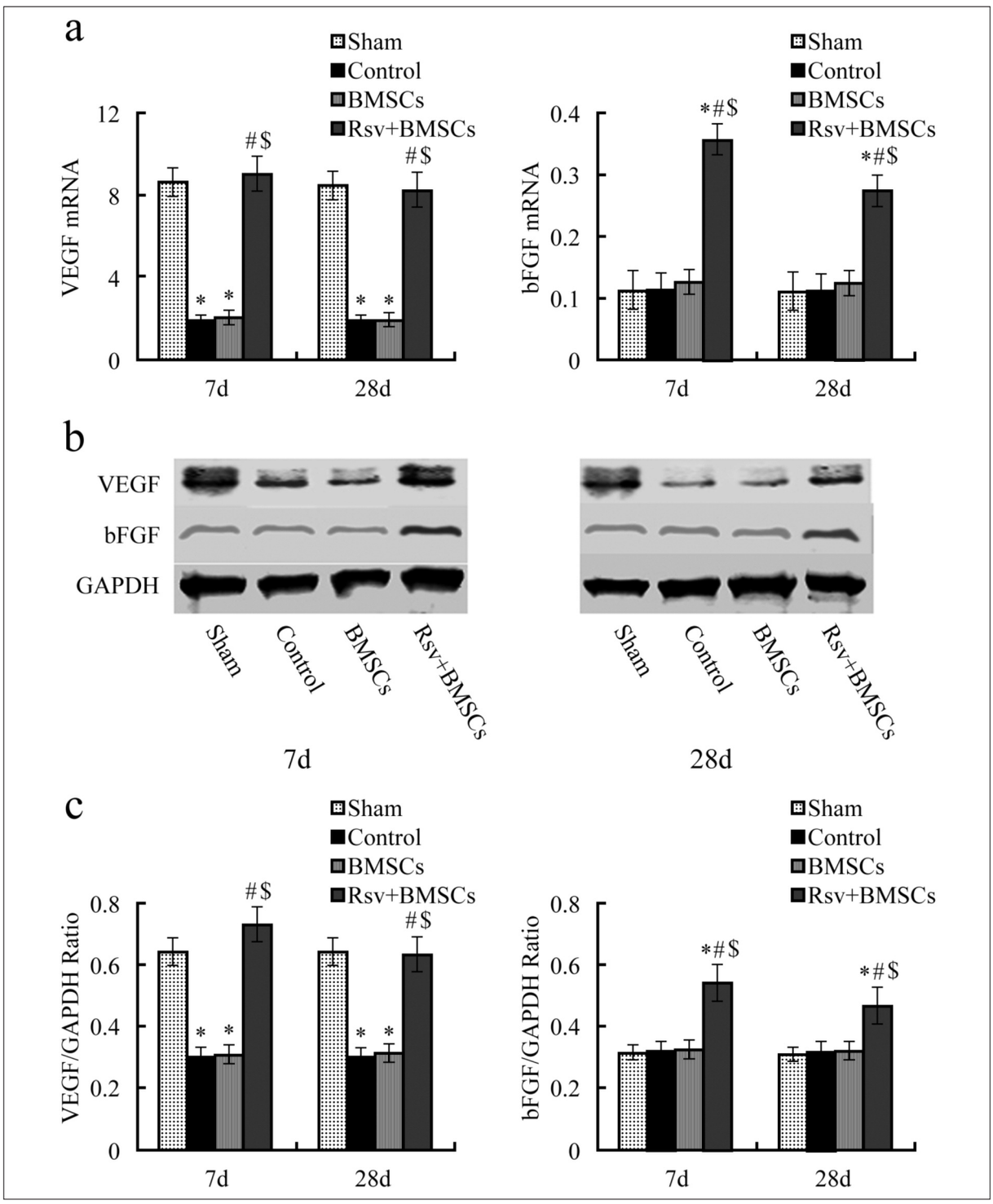


**Figure 6.** Expression of genes and proteins for paracrine factors in the myocardium 7 and 28 days after cell transplantation. ***a,*** Quantitative real-time PCR reveals that the gene expression of VEGF and bFGF is enhanced by BMSC therapy with rosuvastatin but not by BMSC therapy alone. ***b,*** Representative graphs for expression of VEGF and bFGF protein by western blotting. ***c,*** Quantified western blotting shows that the protein expression of VEGF and bFGF is upregulated by BMSC therapy with rosuvastatin, but not by BMSC therapy alone. Group abbreviation is as defined in Figure 1. VEGF, vascular endothelial growth factor. bFGF, basic fibroblast growth factor. GAPDH, glycelaldehyde-3-phosphate dehydrogenase. Data are the mean ± S.D (n = 8 per group). $^{*}p < 0.01$ vs. sham. $^{\#}p < 0.01$ vs.CME. $^{\$}p < 0.01$ *vs*. BMSCs.

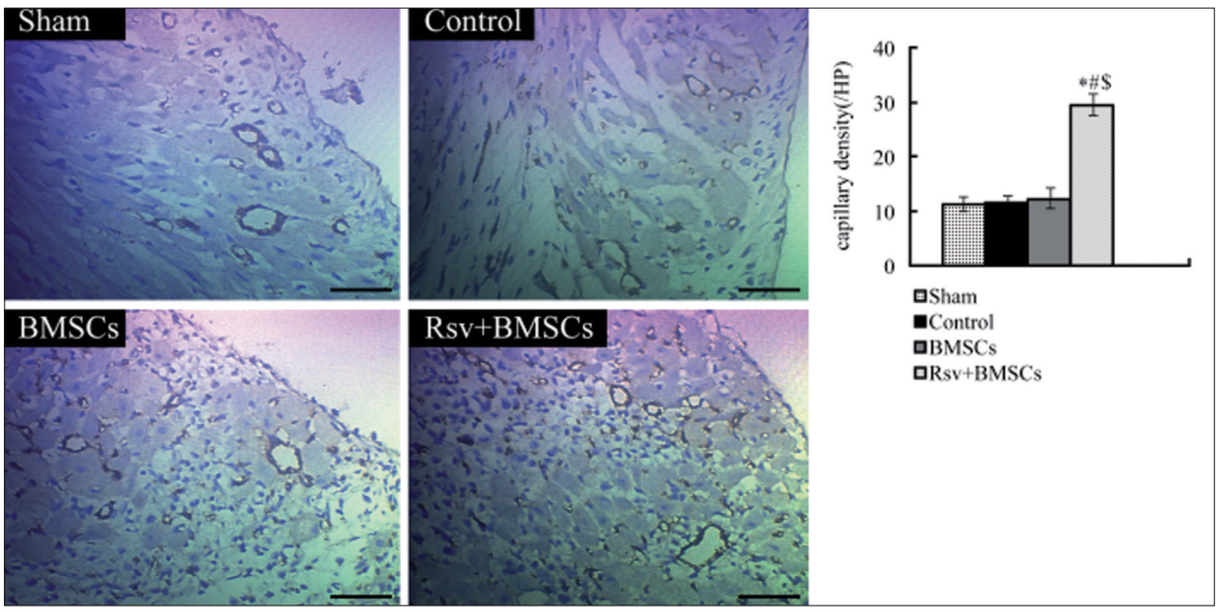


**Figure 7.** Neovascularization and quantitative analyses of capillary density in the infarcted region 28 days after cell transplantation. CME and BMSC therapy leads to poor capillary regeneration, respectively. Stem-cell therapy with rosuvastatin promotes neovascularization significantly. Quantitative data on capillary density are shown in a histogram (*low right*). Group abbreviation is as defined in Figure 1. Scale = 100 μm. Data are the mean ± S.D (n=8 per group). $^{*}p < 0.01$ *vs*. sham. $^{\#}p < 0.01$ *vs*. CME. $^{\$}p < 0.01$ *vs*. BMSCs.

curred, it cannot be treated effectively. Accordingly, the myocardial microenvironment in this situation is even more unfavorable for the survival of transplanted cells.

To create a favorable microenvironment before the cell transplantation is essential in the case of CME. It has been clearly established that the statins can protect against inflammatory reactions and oxidant stress[16] *via* their pleiotropic effects, which are independent of lowering of cholesterol levels. Atorvastatin for Reduction of Myocardial Damage During Angioplasty-Acute Coronary Syndromes (ARMYDA-ACS) and ARMYDA-RECAPTURE trials have suggested that the pretreatment with a high dose of atorvastatin before coronary interventions can reduce the post-procedural myocardial necrosis. Underlying mechanisms for these beneficial effects may be explained mainly by the potent anti-inflammatory functions of atorvastatin, which markedly inhibit the expression of multiple inflammatory factors, improve the endothelial function in the microcirculation and decrease the peri-procedural CME and myocardial injury[2,30-35]. Rosuvastatin has been shown to possess several advantageous pharmacologic properties (e.g., strong anti-inflammatory effects). We found that high-dose and short-term administration of rosuvastatin significantly inhibited release of TNF-α and IL-1β in the myocardium after CME, and promoted the survival of implanted BMSCs by ≈45-fold.

It has been questioned whether the stem-cell transplantation benefits the functional recovery of cardiac cells because of regeneration of functional cardiomyocytes. One hypothesis suggests that functional benefit conferred to cardiomyocytes is not related to the addition of new donor-derived contractile elements but instead is due to indirect effects such as the paracrine effect of grafted cells on host tissue[36-38]. We found no evidence of the cellular differentiation and only the GFP-positive cells co-expressed the cardiac-muscle marker cTnI. Also, their morphology was not identical to the new cardiomyocyte-like cells depicted previously[39,40] but was instead like BMSCs themselves before transplantation, suggesting that cell fusion (but not cell differentiation) was dominant. It does not seem possible that the cell fusion contributed to the significant improvement of cardiac function noted in the present study. Several studies have shown that cytokines such as VEGF and bFGF are strongly associated with the paracrine effect of BMSCs[41,42]. Up-regulation of expression of VEGF and bFGF in donor cells also promoted angiogenesis and improved

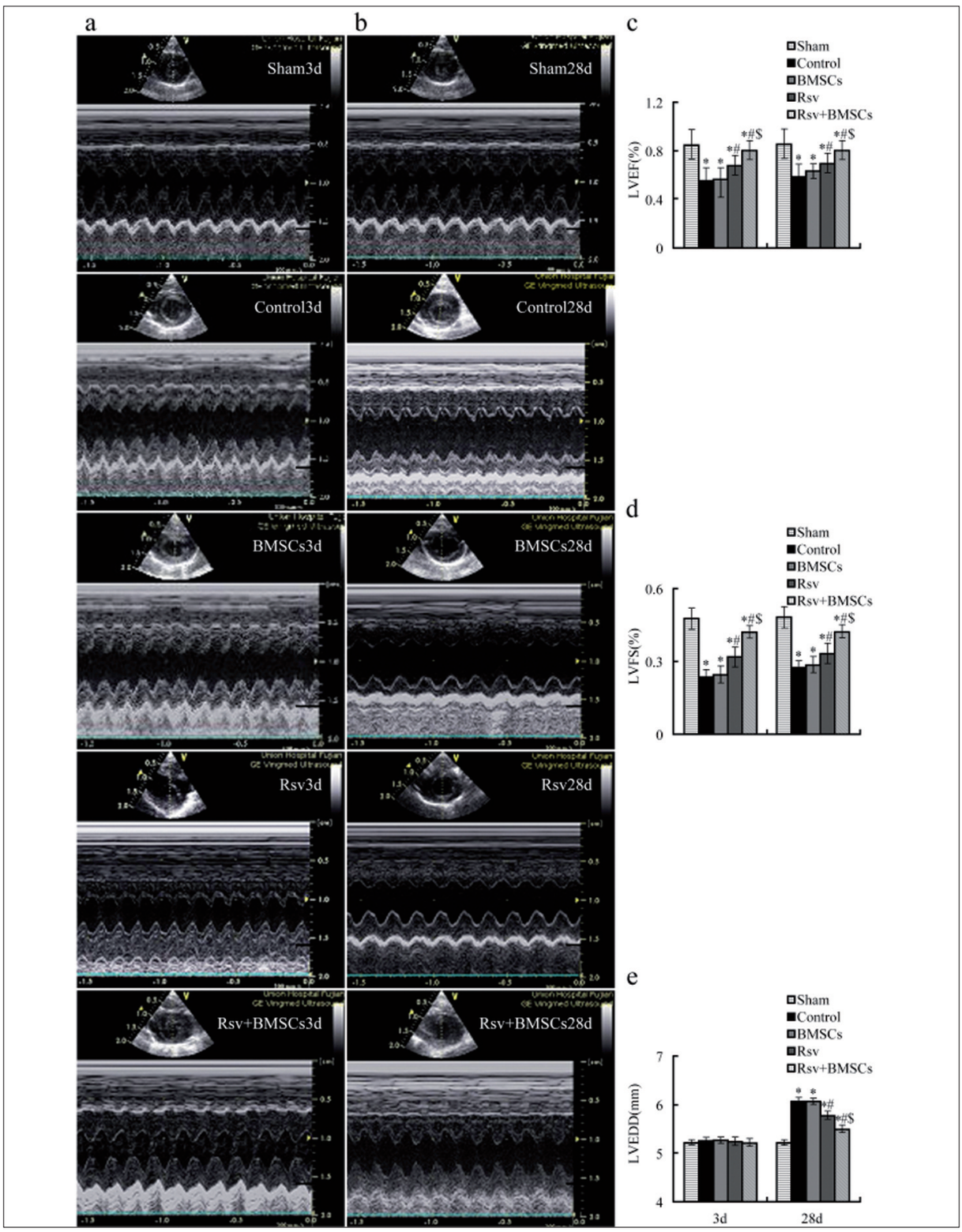


**Figure 8.** Echocardiograms and cardiac performance from various treatments. ***a*** and ***b,*** Representative M-mode echocardiograms from five groups 3 and 28 days after cell transplantation, respectively. ***c,*** Left ventricular ejection fraction. ***d,*** Left ventricular fractional shortening. ***e,*** Left ventricular end-diastolic diameter. Group abbreviation is as defined in Figure 1. LVEF, left ventricular ejection fraction. LVFS, left ventricular fractional shortening. LVEDD, left ventricular end-diastolic diameter. Data are the mean ± S.D (n=8 per group). $^{*}p < 0.01$ *vs*. sham. $^{\#}p < 0.01$ *vs*. CME. $^{\$}p < 0.01$ *vs.* BMSCs.

the regional blood flow surrounding the ischemic myocardium of the host[43,44]. Our work demonstrated that, compared with BMSC transplantation alone, a combination of rosuvastatin and BMSC transplantation further increased the expression of VEGF and bFGF as well as the density of capillaries and small arteries in post-CME myocardium 7-28 days after cell transplantation, and was accompanied by further reduced cardiac remodeling and better cardiac performance. Therefore, we've provided supportive evidence for the hypothesis that the subsequently enhanced paracrine effects mediated by surviving implanted BMSCs may be the dominant mechanism for these beneficial changes.

Taken together, we postulate that rosuvastatin treatment can improve the survival of transplanted BMSCs by reducing CME-induced inflammatory reactions in the acute phase. Greater numbers of surviving transplanted BMSCs secrete more VEGF and bFGF in subacute and chronic phases, which in turn enhance the survival of transplanted BMSCs *via* angiogenesis and tissue perfusion and, thus, lead to structural and functional benefits.

## Conclusions

Rosuvastatin administration promotes the survival and the therapeutic effects of transplanted BMSCs by improving the impaired myocardial microenvironment. This phenomenon is achieved by inhibition of acute inflammation and enhancement of the subsequent paracrine effect of surviving implanted BMSCs. These findings may provide new options for the treatment of patients with CME and novel strategies for stem-cell transplantation.

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### Conflict of Interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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