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E-mail: jzus@zju.edu.cn

Bone marrow mesenchymal stem cell transplantation combined with perindopril treatment attenuates infarction remodelling in a rat model of acute myocardial infarction

WANG Jian-an[†1], LUO Rong-hua[2], ZHANG Xing[2], XIE Xiao-jie[2],
HU Xin-yang[2], HE Ai-na[2], CHEN Jie[2], LI Jia-hui[2]

([1]*Department of Cardiology, the Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, China*)

([2]*Center of Lab, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, China*)

[†]E-mail: wang_jian_an@tom.com

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Abstract: Objective: This study was performed to evaluate whether implantation of mesenchymal stem cell (MSC) would reduce left ventricular remodelling from the molecular mechanisms compared with angiotensin-converting enzyme inhibitors (ACEIs) perindopril into ischemic myocardium after acute myocardial infarction. Methods: Forty rats were divided into four groups: control, MSC, ACEI, MSC+ACEI groups. Bone marrow stem cell derived rat was injected immediately into a zone made ischemic by coronary artery ligation in MSC group and MSC+ACEI group. Phosphate-buffered saline (PBS) was injected into control group. Perindopril was administered p.o. to ACEI group and MSC+ACEI group. Six weeks after implantation, the rats were killed and heart sample was collected. Fibrillar collagen was observed by meliorative Masson's trichome stain. Western Blotting was employed to evaluate the protein expression of matrix metalloproteinase (MMP)-2, matrix metalloproteinase (MMP)-9 in infarction zone. The transcriptional level of MMP2, MMP9 and tissue inhibitor of matrix metalloproteinase (TIMP)-1 in infarction area was detected by reverse transcriptase PCR (RT-PCR) analysis. Results: The fibrillar collagen area, the protein expression of MMP2, MMP9 and the transcriptional level of MMP2, MMP9 mRNA in infarction zone reduced in MSC group, ACEI group, and MSC+ACEI group. No significant difference was detected in the expression of TIMP1 mRNA among the 4 groups. Conclusion: Both MSC and ACEI could reduce infarction remodelling by altering collagen metabolism.

Key words: Remodelling, Acute myocardial infarction, Perindopril, Bone marrow mesenchymal stem cell
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INTRODUCTION

Ventricular remodelling after acute myocardial infarction not only changes the topography including ventricular enlargement and lengthening of the non-contractile region, but also changes the microstructure's myocardial apoptosis, myocardial hypertrophy, increasing content of collagen and expression of MMP (matrix metalloproteinase). This remodelling can lead to decline of ventricular function and adversely affect the prognosis for survival. Thus, reversing the procedure of ventricular remodelling would be desirable for the treatment of AMI (acute myocardial infarction).

Activation of the circulating and tissue rennin-angiotensin-aldosterone system (RAAS) could lead to ventricular remodelling. The therapeutic use of ACEI (angiotensin-converting enzyme inhibitor) is beneficial for reducing ventricular remodelling. Optimization studies demonstrated that angiotensin-converting enzyme inhibitors attenuate ventricular remodelling after acute myocardial infarction (Møller *et al*., 2004).

MSCs (mesenchymal stem cells) are pluripotent stem cells within the marrow microenvironment (Pittenger *et al*., 1999). Clinical studies showed that MSC significantly improved left ventricular function

after intracoronary injection of patient with AMI (Strauer *et al*., 2001). However, little information is available about the infarction remodelling after bone marrow mesenchymal stem cell transplantiation. The purpose of this study was to investigate: (1) whether transplantation of MSC could reduce the fibrillar collagen content, the expression of MMP as compared to treatment of ACEI; (2) whether transplantation of MSC could enhance the beneficial effect of ACEI in reducing ventricular remodelling.

MATERIALS AND METHODS

Preparation of MSC

The experimental rats were anesthetized with intraperitoneal injection of 4% chloral hydrate solution (1 ml/100 g). Tibia and thigh bones were collected and pulverized, then transferred to a sterile tube and mixed with 5 ml phosphate-buffered saline (PBS) solution containing 7500 U heparin, then added into Dulbcco's Modified Eagle Medium (DMEM). The tube was centrifuged at 900×g for 20 min. The cell suspension was loaded on Lympholyte solution. The cells were centrifuged at 900×g for 25 min. The middle one third of total volume were transferred into a tube containing DMEM, and centrifuged at 900×g for 25 min. The cell pellet was then resuspended in culture medium and seeded in culture flasks, and maintained at 37 °C in 5% CO_2 humidified air. After washing off the unattached cells during the period of 48 h, the adherent cells were cultured in DMEM containing 20% fetal bovine serum and antibiotics. The medium was changed once every 5 d.

DAPI labelling

When MSCs completely occupied the flask bottom, they were incubated in medium containing DAPI (4'6-diamidino-2-phenylindole, 50 μg/ml, Sigma, USA) for 24 h. They removed from the culture bottles using 0.25% trypsin, neutralized with culture medium, and collected by centrifugation at 900×g for 5 min, and then suspended in PBS at concentration of 10^7 cells in 150 μl for transplantation.

Experimental model

Forty adult male Sprague Dawley (SD) rats (200~260 g) were enrolled in. AMI model were made by ligation of the left anterior descending coronary artery. The rats were anesthetized with 4% chloral hydrate solution (1 ml/100 g) by intraperitoneal injection, then intubated with 18G tube, and put in room with air ventilated by using animal ventilator. A thoracotomy was performed, the left anterior descending artery was ligated.

MSC implantation into ischemic myocardium

Forty rats were divided into four groups: control, MSC, ACEI, MSC+ACEI groups. PBS was injected into rat in control group and ACEI group. Perindopril was administered (p.o. 2 mg/(kg body weight)) to ACEI group, and MSC+ACEI group.

PBS medium containing MSC (total 10^7 cells, 3 sites×0.05 ml per site) or medium alone was injected into the left anterior descending coronary artery risk area (ischemic border) with a 24-gauge needle. Six weeks after MSC implantation, the rats were killed and tissue samples from ischemic area were collected.

Histology

Tissue samples from the infraction area were collected at 6 weeks after transplantation and fixed in 4% PBS formaldehyde for histological study. The samples were embedded and cut to yield 10-μm thick sections and stained with meliorative Masson's trichome stain. Interstitial fibrosis was stained blue. Some samples were frozened and cut into 4-μm thick sections, then observed under the fluorescence microscopy.

Western Blotting

Frozen hearts were crushed with a mortar and pestle, at liquid nitrogen temperature and homogenized by vibration in solution containing PMSF. The homogenate was centrifuged at 12000×g at 4 °C for 25 min, and the deposition was collected and stored at –80 °C.

Eighty micrograms of protein from rat heart homogenate was denatured in boiling water for 5 min and added to 10% SDS-PAGE and then transferred to polyvinylidene difluoride membranes. Membranes were blocked overnight with 5% (w/v) dry milk solution containing 10 mmol/L Tri-Cl, pH 8.0, 150 mmol/L NaCl and 0.05% Tween 20 (TBST) before incubation with primary antibodies diluted in TBST

(1:200 for MMP2 and MMP9; 1:1000 for β-actin). After washing, the membranes were incubated 1 h with secondary antibody diluted 1:5000 in TBST, and the labelled proteins were detected by using enhanced chemiluminescence reagents and exposed to film (Kodak, USA).

RT-PCR

The frozen hearts were crushed with a mortar and pestle at liquid nitrogen temperature. Total RNA was extracted with Trizol reagent (Gibco, USA) according to the manufacturer's instructions and quantitated by absorbance at 260 nm. The reaction volume of reverse transcription polymerase chain reaction was 25 μl. Reaction temperature was set at 42 °C for 30 min. The total reaction volume was 25 μl for PCR reaction. Primers sequences are shown in Table 1. β-actin was used as the internal standard. PCR amplification was carried out in a volume of 25 μl containing 2 μl of the template, 2.5 μl PCR buffer (10×) and 0.2 μl Taq polymerase, 2.5 mmol/L of each dNTP (TaKaRa, 2.5 mmol/L), 1.5 μl $MgCl_2$ (25 mmol/L, promega), 1 μl Primers (10 μmol/L, forward and reverse each).

Detection of PCR products

Ten microlitres of each reaction product mixed with 2 μl of 6×loading buffer was fractionated on 1.7% agarose gel. DNA Marker DL2000 (TaKaRa 300 ng/5 μl).

Statistical analysis

Numerical data were presented as mean±*SD*. Comparisons among groups were performed with one-way ANOVA and LSD comparison. Statistical significance was accepted while the null hypothesis was rejected at $P<0.05$.

RESULTS

Cultivated bone marrow mesenchymal stem cells were observed under phase contrast microscope. The cells displayed spindle-shape and adhered to the bottom of the flask (Fig.1).

DAPI-labeled (blue) label retaining cells were considered as the transplantation cells. Under the fluorescence microscopy, we observed that DAPI-labelled (blue) MSC was detected in the ischemic portion of the myocardium six weeks after implantation (Fig.2).

Changes of collagen fibre in the myocardium

Masson's trichrome staining revealed that collagen (blue) existed in the ischemic area (Fig.3). The area of collagen was decreased in MSC+ACEI group (Fig.3a), ACEI group (Fig.3b), MSC group (Fig.3c) compared with PBS group (Fig.3d).

MMP2 and MMP9 expression in the infraction area (Western Blotting)

MMP2 and MMP9 were highly expressed in the infraction area in the PBS group. The Western Blotting for MMP2 and MMP9 showed the band of 72 kDa and 92 kDa respectively. The band of 72 kDa was barely seen in the MSC+ACEI group. MMP2 expression was also reduced by the MSC and ACEI. MSC (Fig.4), ACEI and MSC+ACEI lowered MMP9 expression.

MMP2, MMP9 and TIMP1 expression in the infraction area (RT-PCR)

The PCR products of MMP2, MMP9 and TIMP1 were observed at the expected locations on agarose gels (Fig.5). Statistical analysis indicated that the

Table 1 Primer sequences used

Primer	Direction	Sequence (5′ to 3′)	T_m (°C)	Size (bp)
MMP2	Forward	AGGACAAGTGGTCCGCGTAAAG	59	510
	Reverse	CCACTTCCGGTCATCATCGTAGT		
MMP9	Forward	GGGAACGTATCTGGAAATTCG	57	520
	Reverse	CAGAACCGACCCTACAAAGTTG		
TIMP1	Forward	GACTAAGATGCTCAAAGGATTCG	58	360
	Reverse	ATCGCTCTGGTAGCCCTTCT		
β-actin	Forward	ATATCGCTGCGCTCGTCGTC	58	760
	Reverse	GCATCGGAACCGCTCATTGC		

Note: Cycling parameters: one cycle of 5 min at 95 °C; 32 cycles of 30 s at 95 °C, 30 s at 57~60 °C and 30 s at 72 °C; and a final extension at 72 °C for 7 min, then preserve at 4 °C

expression of MMP2, MMP9 mRNA in 3 treatment groups was significant different from that in the control group. In the MSC+ACEI, ACEI, MSC and PBS groups, the densitometry ratio of MMP2/β-actin was 0.43±0.20, 0.45±0.19, 0.46±0.14 and 0.78±0.22, respectively (P<0.05), the densitometry ratio of MMP9/β-actin was 0.40±0.13, 0.51±0.10, 0.48±0.09, and 0.70±0.20, respectively (P<0.05). However, no significant difference was detected in the expression of TIMP1 mRNA among the 4 groups (Table 2).

Both MSC and ACEI could lower MMP2 and MMP9 expression.

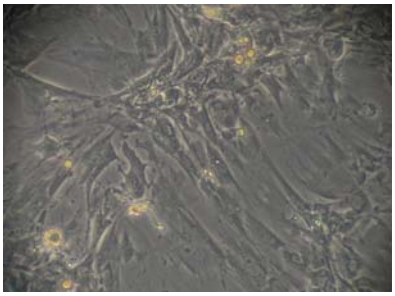


Fig.1 Biological features of cultivated bone marrow mesenchymal stem cell under inverted phase contrast microscope

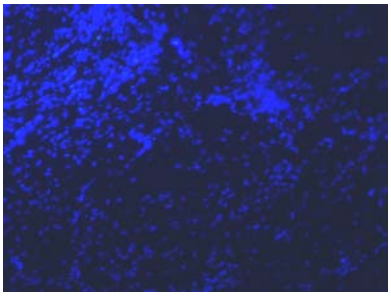
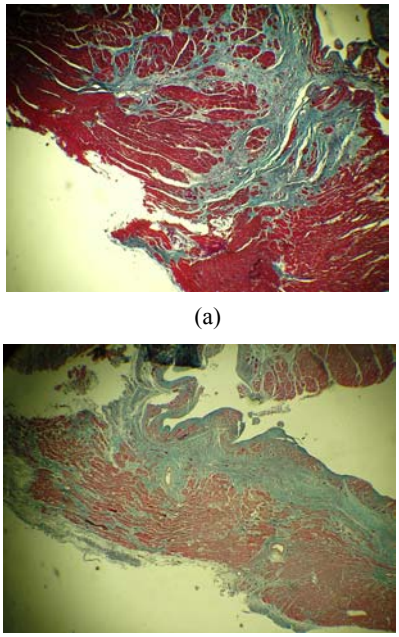
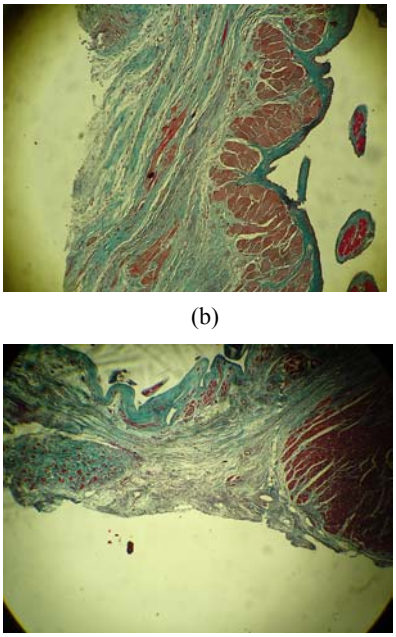


Fig.2 Six weeks after implantation, DAPI-labelled (blue) MSC was detected in the ischemic portion of the myocardium



(a)



(b)

(c)

(d)

Fig.3 Masson's trichrome staining of left ventricular tissue of rat in infraction area among 4 group (magnification, ×40). Masson's trichrome stain was used to identify alterations of collagen in infraction area. (a) MSC+ACEI group; (b) ACEI group; (c) MSC group; (d) Control group

Table 2 Comparison of MMP2, MMP9 and TIMP1 expression in the infraction area in different groups after six weeks

	MSC+ACEI	ACEI	MSC	PBS
MMP2/β-actin	0.43±0.20*	0.45±0.19*	0.46±0.14*	0.78±0.22
MMP9/β-actin	0.40±0.13#	0.51±0.10#	0.48±0.09#	0.70±0.20
TIMP1/β-actin	1.31±0.53	1.17±0.40	0.93±0.19	1.10±0.33

* MMP2/β-actin, P<0.05 vs the PBS group; # MMP9/β-actin, P<0.05 vs the PBS group

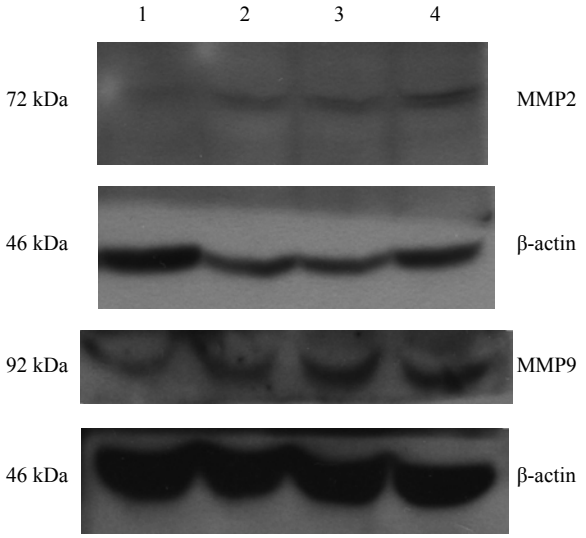


Fig.4 Expression of MMP2 and MMP9 in infraction area six weeks after implantation
Lane 1: MSC+ACEI group; Lane 2: ACEI group; Lane 3: MSC group; Lane 4: Control group

DISCUSSION

Structural cardiac remodelling involves hypertrophy of the non-infarction area and lengthening of the infarction region after acute myocardial infarction. It has been suggested that alternation of fibrillar collagen content and activation of MMPs induce lengthening of the infarction region. In acute myocardial infarction patients, myocardial fibrillar collagen was degraded by matrix metalloproteinases. The level of MMP transcription may affect infracted area healing and LV (left ventricular) remodelling. If the fibrillar collagen increased, activation of MMP was increased, then lead to pathological left ventricular remodelling. Decrease of MMP activity may facilitate collagen accumulation. Myocardial MMP activation shows a time-dependent spectrum with the development of pressure-overload (Mujumdar and Tyagi, 1999). Garcia *et al*.(2005) studied the relation between abnormal cell wall motion and stimulation of MMP in left ventricle by electric stimulates atrial and ventricular sites of five open-chest anesthetized dogs, and found that dyskinesia induced by epicardial activation stimulates MMP activity in the heart.

MMPs play a pivotal role in tissue remodelling processes. MMP activity was upregulated during MI

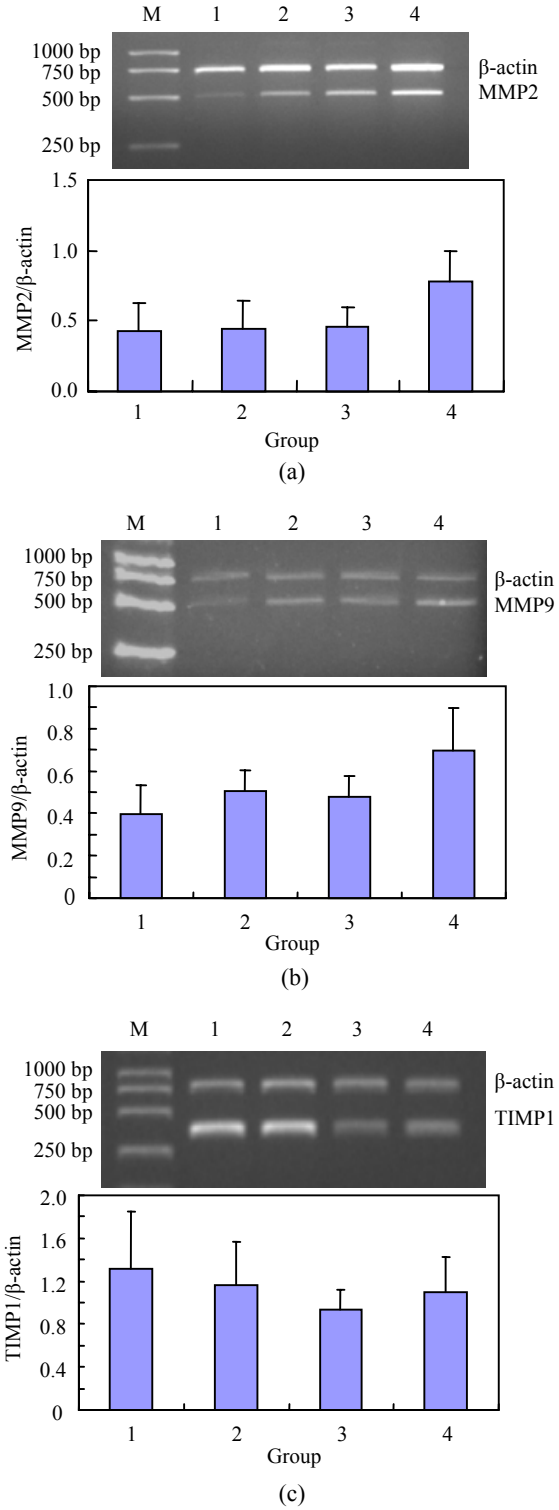


Fig.5 PCR reaction product revealed by agarose gel electrophoresis (upper) and relative contents of (a) MMP2, (b) MMP9 and (c) TIMP1 mRNA to β-actin values are expressed as mean±*SD* (lower).
M: DNA marker; Lane 1: MSC+ACEI group; Lane 2: ACEI group; Lane 3: MSC group; Lane 4: Control group

(myocardial infarction). This process was regulated by several cytokines. Cytokine tumor necrosis factor (TNF) alpha may relate to left ventricular remodelling. Yang and co-workers studied the changes of plasma levels of TNF alpha and matrix metalloproteinase-2,3,9 (MMP-2,3,9) expressions in myocardium of congestive heart failure (CHF) patients and found plasma levels of TNF alpha increased in patients with CHF. The expressions of MMP-2,3,9 were significantly increased in patients with CHF compare to controls. This effect support that TNF alpha might mediate the upregulation of MMP (Yang *et al*., 2004). TNF alpha increased human atrial myofibroblast proliferation and MMP9 secretion predominantly via the TNF-R1 receptor (Porter *et al*., 2004). Xie *et al*.(2004)'s experimental research on regulation of MMP2 and MMP9 expression and activity in adult rat cardiac fibroblasts in response to interleukin-1 beta and demonstrated that activation of ERK1/2 and JNKs play a pivotal role in the regulation of MMP9 expression and activity; activation of NF-kappaB stimulates expression of MMP2 and MMP9.

TIMP is an inhibitor of MMP. Four different TIMP species have been identified. TIMP binds to activated MMPs in 1:1 ratio (Spinale, 2002). TIMP1 knock-out mice developed more large left ventricular end-diastolic volume and mass compare to wild-type mice. Whereas left ventricular systolic pressure and cardiac output were similar in both group. Myocardial fibrillar collagen content was reduced in TIMP1 knock-out mice (Roten *et al*., 2000). In TIMP1-deficient mice, left ventricular remodelling process accelerated after myocardial infarction, which demonstrated that myocardial TIMP1 plays a regulatory role in post-MI remodelling (Ikonomidis *et al*., 2005; Roten *et al*., 2000). If MMP1 increased but TIMP1 did not, left ventricular dysfunction and dilatation would occur (Papadopoulos *et al*., 2005). In spontaneously hypertensive rat model, it could be found that myocardial TIMP levels decreased while MMP activity increased in the process of decompensation to LV failure.

Bone marrow contains hematopoietic stem cell and mesenchymal stem cell, with the latter having the capacity of self-renewal and differentiation into multiple lineages, whether MSC could differentiate into cardiac cell is still in controversy. Many experts consider that MSC would infuse into cardiac cell. Earlier studies showed that transplantation of MSC improves heart function (Chen *et al*., 2004). However, little information is available on the effect of MSC on ventricular remodelling after acute myocardial infarction.

Does MSC attenuate the degree of LV remodelling? If so, what is the underlying molecular mechanism of that process? Recent clinical and experimental studies imply MSC transplantation significantly improved left ventricular function after AMI. Our study tested the hypothesis that MSC attenuates the degree of LV remodelling by altering the expression of MMPs. Xu *et al*.(2005) reported that MSC transplantation in rats after myocardial infarction attenuates extracellular matrix (ECM) remodelling. In that study, they found that collagen type I, collagen type III, MMP1, TIMP1 mRNA expression were decreased by MSC transplantation, whereas no significant difference was observed for MMP1 between the MI group and the MI+MSC group. In our study, we focused on the change of infarction area and found that MMP2 and MMP9 upregulated in infarction area, probably through a paracrine-mediated mechanism (Fedak *et al*., 2005). Fedak transplanted smooth muscle cells to the dilating left ventricle of cardiomyopathic hamsters, and found that left ventricular dilatation was attenuated and TIMP:MMP ratio was increased in the transplantion group, as compared to the control group. This phenomenon indicates that cell transplantion might reduce the capacity for matrix degradation.

The therapeutic use of ACEI is beneficial for reducing ventricular remodelling. Early initiation of ACEIs attenuates collagenolytic activity (Papadopoulos *et al*., 2004). ACEI also reduced cardiac remodelling in end stage heart failure, the ACE inhibitors captopril and ramipril inhibited MMP2 and MMP9 activities (Reinhardt *et al*., 2002). Our data are consistent with previous viewpoint. The main evidence of this study is that the mRNA and protein expression of the MMP2 and MMP9 is significantly decreased in group MSC or ACEI. But there was no difference in TIMP1 among the 4 groups. One possible interpretation of this finding was that MMPs are mainly regulated by cytokine.

Our present study demonstrated the following effects of MSC transplantation in a rat model of AMI: (1) decrease in collagen in the infarction area of the

myocardium; (2) decreased MMP2 and MMP9 level in the myocardium. The beneficial effect of MSC transplantation on reducing ventricular remodelling is mediated by a decrease in MMPs expression.

In summary, we have demonstrated that mesenchymal stem cell transplantation could reduce the collagen fibre in the myocardium and MMP2, MMP9 in infarction zone.

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