

## Original Article

# Short-term intermittent administration of CXCR4 antagonist AMD3100 facilitates myocardial repair in experimental myocardial infarction

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**The binding of the stromal cell-derived factor-1 $\alpha$  (SDF-1 $\alpha$ ) to the cysteine (C)-X-C motif chemokine receptor 4 (CXCR4) has emerged as a key signal for stem and progenitor cells trafficking to the circulation from the bone marrow. Our aim was to investigate the role of daily intermittent administration of AMD3100 (a specific reversible CXCR4 receptor antagonist) during the healing process after myocardial infarction (MI). Wistar rats were subjected to MI and AMD3100 was injected intraperitoneally after surgery. *SDF-1 $\alpha$*  mRNA expression was measured by real-time polymerase chain reaction. Histology changes were analyzed with immunofluorescence, Masson's trichrome staining, and wheat germ agglutinin. The number of leukocytes in peripheral blood was measured by complete blood cell count analysis. The activities of matrix metalloproteinase-2/9 (MMP-2/9) were determined by gelatin zymography. The expression level of *SDF-1 $\alpha$*  mRNA in the infarcted tissue was enhanced rapidly (6 h), peaked at 24 h, and then declined to the normal level at 7 days post-MI. AMD3100 further enhanced the increase of *SDF-1 $\alpha$*  in infarct area. Increased leukocytes were observed in AMD3100-treated groups. The mobilization of c-kit<sup>+</sup> stem/progenitor cells and enhanced neovascularization were augmented by AMD3100. Additionally, AMD3100 improved ventricular remodeling, which was revealed by the decrease of infarct size, viable cardiomyocyte cross-sectional area and left ventricle (LV) expansion index, and the increase of LV free wall thickness. The activities of MMP-2/9 were up-regulated by AMD3100. In conclusion, short-term intermittent administration of AMD3100 could accelerate the wound healing process in**

**experimental MI and be a potential therapy for the treatment of MI.**

**Keywords** myocardial infarction; ventricular remodeling; AMD3100; tissue repair

Received: September 27, 2012

Accepted: February 21, 2013

## Introduction

Recent advances in coronary reperfusion strategies, i.e. thrombolysis and percutaneous coronary intervention, have tremendously reduced the mortality rate of patients suffered from myocardial infarction (MI). In the meantime, the majority of post-infarction survivors underwent an irreversible pathological evolution from compensated left ventricle (LV) function to heart failure [1]. Stem cells, which are capable of self-renewal and differentiation into specialized progeny, could theoretically replace damaged tissues after MI, which might be a promising strategy to improve post-infarction LV remodeling.

Stem/progenitor cells express a number of surface molecules that mediate the homeostasis between the retention in bone marrow (BM) and mobilization into the peripheral blood. Stromal cell-derived factor-1 $\alpha$  (SDF-1 $\alpha$ ) and its receptor the cysteine (C)-X-C motif chemokine receptor 4 (CXCR4), play an important role in this process [2]. Accumulating evidence has shown that SDF-1 $\alpha$ /CXCR4 axis is a promising molecular target for post-infarction myocardial repair [3,4].

AMD3100, a novel small molecule that acts as a specific CXCR4 antagonist, is shown with an efficient ability for BM hematopoietic stem and progenitor cells (HSPCs) mobilization with clinical therapeutic potential [5]. However, current work using continuous chronic administration of AMD3100 (by osmotic pump) after experimental MI demonstrated that this protocol could even worsen post-infarction LV remodeling [6,7]. Results from pharmacokinetic studies indicated that the tight binding of AMD3100 with CXCR4 is reversible, and with a plasma half-life of 0.9 h in rodents after intraperitoneal injection [8–10]. Therefore, it is conceivable that continuous AMD3100 administration would simultaneously contribute to a persistent occupation of CXCR4, and would thus compromise stem/progenitor cells homing to the injured myocardium, which is largely dependent on local expression of SDF-1 $\alpha$ .

Therefore, in the present study, we hypothesized that short-term daily intraperitoneal injection of AMD3100 may induce stem/progenitor cell mobilization along with enhanced homing, and accordingly, may improve post-infarction LV remodeling by facilitating SDF-1 $\alpha$ /CXCR4 interaction in injured myocardium. In addition, we also examined the timing of AMD3100 administration (1 h post-MI and 24 h post-MI), and extended the follow-up to 3 months to evaluate the long-term effect of intermittent AMD3100 treatment.

## Materials and Methods

### Rat model of MI

All animals were obtained from Laboratory Animal Center of the Academy of Military Medical Science (Beijing, China) and received humane cares in compliance with Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, revised 1996). MI was produced in male Wistar rats (weight, 200–250 g; age, 8–10 weeks). Ligation of the left anterior descending coronary artery (LAD) was performed as previously described [11]. Rats were anesthetized with pentobarbital sodium [40 mg/kg, intraperitoneally (ip)] and submitted to artificial ventilation (75 strokes/min, tidal volume: 8 ml/kg) using a volume-controlled rodent respirator (Harvard Apparatus, Holliston, USA). Subsequently, a thoracotomy through the left fourth intercostal space was performed, and the heart was exposed. The LAD was permanently ligated with an intramural 5-0 silk suture between the left atrial appendage and the pulmonary conus. Then chest was closed, and rats were allowed to recover. Successful operation was confirmed by electrophysiograph (Biopac Systems, Goleta, USA), with the appearance of deep S-wave, subsequent ST-segment elevation, and ventricular arrhythmia. The sham groups underwent the same surgical procedure, except for the LAD ligation. Analgesia was given after surgery (dipirone 0.1 ml, intramuscularly).

The survived rats were randomly divided into sham group ( $n = 10$ ), MI-saline group ( $n = 10$ ), and AMD3100 (Sigma Aldrich, St Louis, USA) treatment group (MI-A group,  $n = 21$ ; 1 mg/kg/day, for 6 days). The dose of AMD3100 used in the present study was adapted according to the previous study [12]. Two time points of drug administration were examined: AMD3100 was injected 1 h (immediate AMD3100, MI-IA) or 24 h post-MI (delayed-AMD3100, MI-DA). AMD3100 was suspended in phosphate-buffered saline (PBS) and injected ip. Rats were sacrificed at 7 days and 3 months post-MI for pathological evaluation, respectively. In addition, to detect SDF-1 $\alpha$  expression profile and AMD3100's mobilization effect after MI, another four groups of survived rats were used: sham-saline ( $n = 9$ ), sham-AMD3100 ( $n = 9$ ), MI-saline ( $n = 9$ ), and MI-AMD3100 ( $n = 9$ ). In each group, three rats were sacrificed at 6 h, 24 h, and 7 days post-MI for cardiac total RNA extraction and blood sampling for complete blood cell count analysis.

### Histological analysis

At the time of harvest, rats were anesthetized by intraperitoneal pentobarbital sodium (40 mg/kg), and perfused with pre-cooled PBS *via* the inferior vena cava for 5 min. The hearts were rapidly removed, prepared for paraffin embedding, and sectioned into 5- $\mu$ m slices. For immunofluorescence microscopy, serial sections were permeabilized with 0.1% Triton X-100 in PBS for 10 min, blocked for 1 h, and incubated with primary antibodies overnight at 4°C. Then the sections were washed three times with PBS, and incubated with secondary antibodies for 1 h. The c-kit<sup>+</sup> cells were labeled with anti-c-kit antibody (Santa Cruz, Santa Cruz, USA). Capillary density was evaluated after staining with isolectin B4 (endothelial cell marker; Sigma Aldrich). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma Aldrich). The mean cardiomyocyte cross-sectional area was stained with fluorescein isothiocyanate-labeled wheat germ agglutinin (WGA; Invitrogen, Carlsbad, USA) in the LV viable septal region. Histological analysis of collagen remodeling in the infarct area was determined with Masson's trichrome staining [13], which labels collagen with blue and myocardium with red. Index of expansion, collagen volume fraction (CVF), and infarct size were measured as described previously [14]. All image analyses were performed with Nikon microscope (Nikon, Tokyo, Japan) and a Microsoft Windows-based image analysis software, Image Pro Plus version 4.5 (Media Cybernetics, Silver Spring, USA).

### Gelatin zymography

The activities of gelatinase (matrix metalloproteinase-2/9, MMP-2/9) within the infarcted region at 7 days and 3 months post-MI were determined by gelatin zymography

with the following steps [15]. The LV infarcted tissues were separated and frozen in liquid nitrogen immediately. Then 100 mg frozen tissue from each heart was homogenized with lysis buffer and centrifuged at 12,000 *g* at 4°C for 10 min. About 40 µg of protein was loaded into polyacrylamide zymogram gels under non-denaturing conditions supplemented with 0.1% gelatin (Sigma Aldrich) to detect the presence of MMP-2/9. The gel was run for 1.5 h using gel electrophoresis apparatus (Bio-Rad, Hercules, USA), treated with renaturing buffer, and then incubated overnight in zymogram developing buffer at 37°C. Areas of MMP digestion were visualized by negative staining with Coomassie brilliant blue R-250 (Sigma Aldrich).

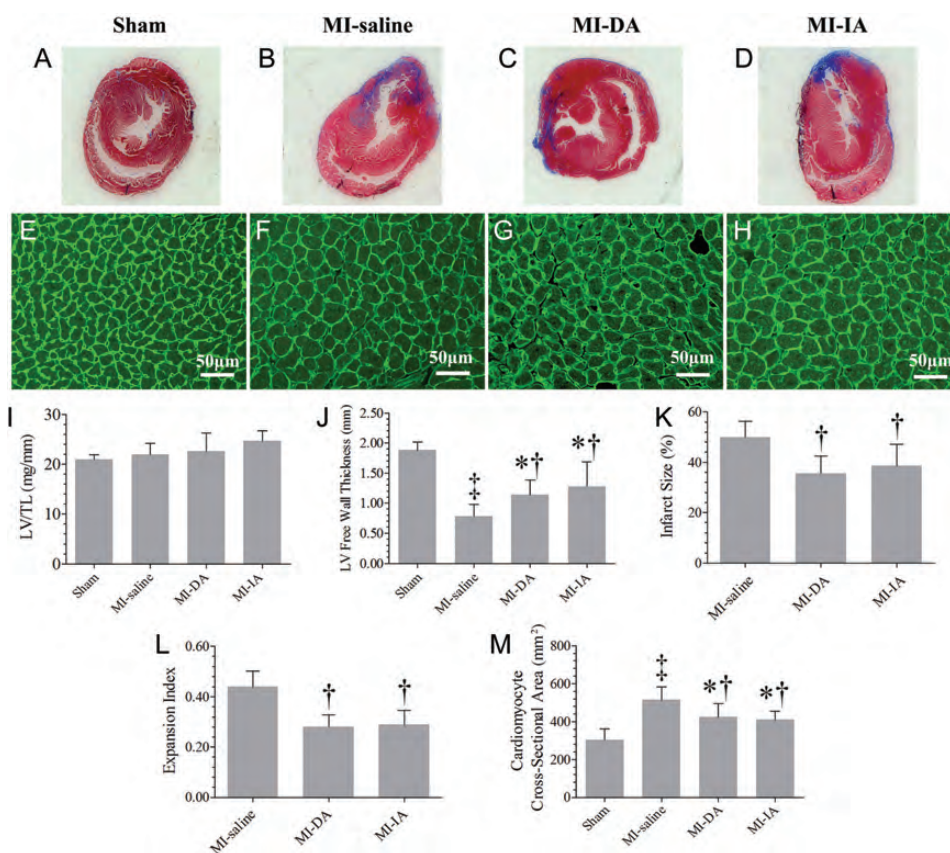
### Real-time quantitative reverse transcriptase-polymerase chain reaction

For detection the mRNA level of *SDF-1α* in the infarcted region, the real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was used. Total RNA was extracted using Trizol reagent (Invitrogen) and cDNA was synthesized from 2 µg of total RNA using Moloney

murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, USA). Real-time PCR was carried out by using an ABI Prism 7300 sequence detector (Applied Biosystems, Foster City, USA). Annealing and elongation were performed at 60°C for 1 min for all genes. Primers were as follows: *SDF-1α* forward: 5'-CCCTGCCGATTCTTTGAG-3', reverse: 5'-GCTTTTCAGCCTTGCAACA-3'; and *β-actin* forward: 5'-TCTGTGTGGATTGGTGGCTCT-3', reverse: 5'-AGAAGCATTGCGGTGCAC3'. Analysis of relative gene expression data using the  $2^{-\Delta\Delta Ct}$  method, where  $\Delta Ct = Ct_{\text{target}} - Ct_{\beta\text{-actin}}$ ,  $\Delta\Delta Ct = \Delta Ct_{\text{experimental}} - \Delta Ct_{\text{control}}$ .

### Statistical analysis

Data were presented as the mean ± standard error of the mean, and evaluated by either a Student's unpaired *t*-test, analysis of variance (ANOVA), or a two-way ANOVA. A two-tailed *P* < 0.05 was considered statistically significant. All statistical analyses were performed by using Graphpad Prism software (GraphPad Software, San Diego, USA).



**Figure 1** Amelioration of post-infarction left ventricular remodeling by AMD3100 (A–D) Masson's trichrome staining showed gross morphology of hearts from four groups at 3 months post-MI. (E–H) WGA was used to stain the myocardial membrane (green). (I–M) The left LV/TL, LV free wall thickness, infarct size, expansion index, and cardiomyocyte cross-sectional area were quantified. AMD3100 attenuated myocardial hypertrophy, improved the process of remodeling. \**P* < 0.05 (vs. sham at the same time point); †*P* < 0.05 (vs. MI-saline at the same time point); ‡*P* < 0.01 (vs. sham at the same time point).

## Results

### Improvement of post-infarction left ventricular remodeling by AMD3100

**Figure 1** shows histological examinations on heart sections from animals sacrificed 3 months post-MI. A significantly preserved LV free wall thickness, decreased infarct size, and reduced LV expansion index was observed in AMD3100-treated groups [Fig. 1(B–D,J–L)]. In addition, cardiomyocyte cross-sectional area revealed by WGA staining was also significantly attenuated by AMD3100 [Fig. 1(F–H,M)]. There was no difference of ventricle/tibial length (LV/TL) among each group. No difference was observed between MI-IA and MI-DA groups.

### Acceleration of collagen deposition by AMD3100

As shown in **Fig. 2**, in early phase (7 days after MI), AMD3100 did not significantly alter collagen deposition in the infarct area. However, a significant increase of CVF was observed at 3 months post-MI in both AMD3100-treated groups. In non-infarct area, AMD3100 did not have an obvious impact on collagen deposition (**Fig. 2**).

### Enhanced c-kit<sup>+</sup> cell engraftment and increased angiogenesis by AMD3100

To determine the homing effect by discontinued AMD3100 treatment, we analyzed c-kit<sup>+</sup> cell engraftment in the infarct

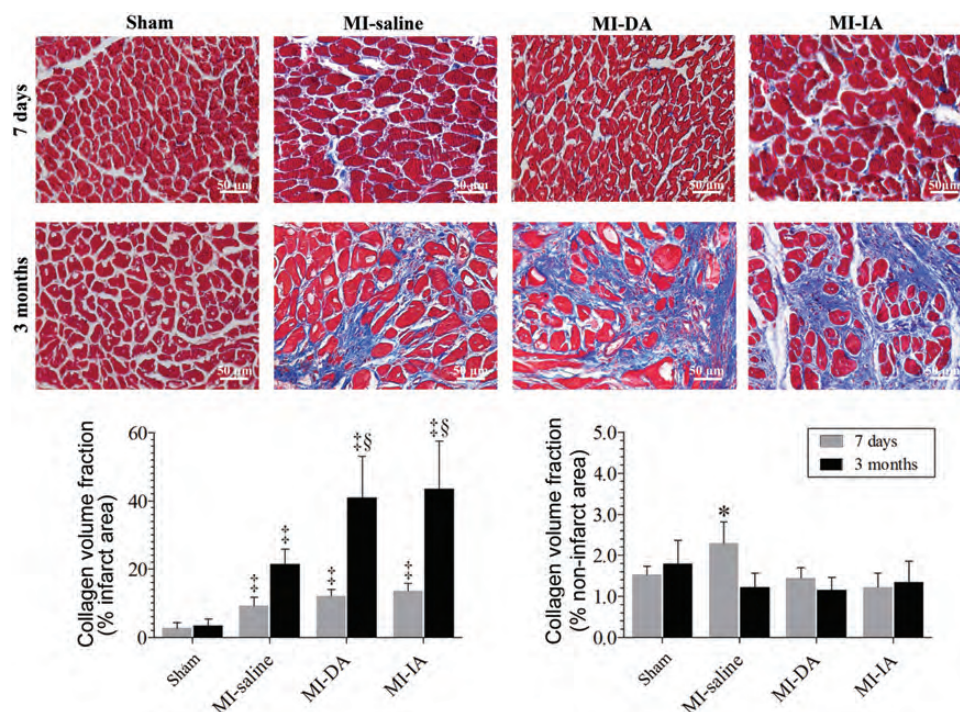
borders at 7 days. As shown in **Fig. 3**, c-kit<sup>+</sup> cells were significantly increased in AMD3100-treated groups, with an insignificant increasing trend in MI-IA group compared with MI-DA group. This effect was accompanied by a paralleled increase of isolectin B4 positive capillaries in both AMD3100-treated groups in infarct area (**Fig. 4**). There was no significant difference in capillary density among groups in non-infarct area. AMD3100 induced 1.5- and 1.7-fold increase of leukocyte counts in peripheral blood at 24 h and 7 days post-MI in MI groups, respectively (data not shown).

### Increased expression of *SDF-1 $\alpha$* in the infarcted region of post-MI rats

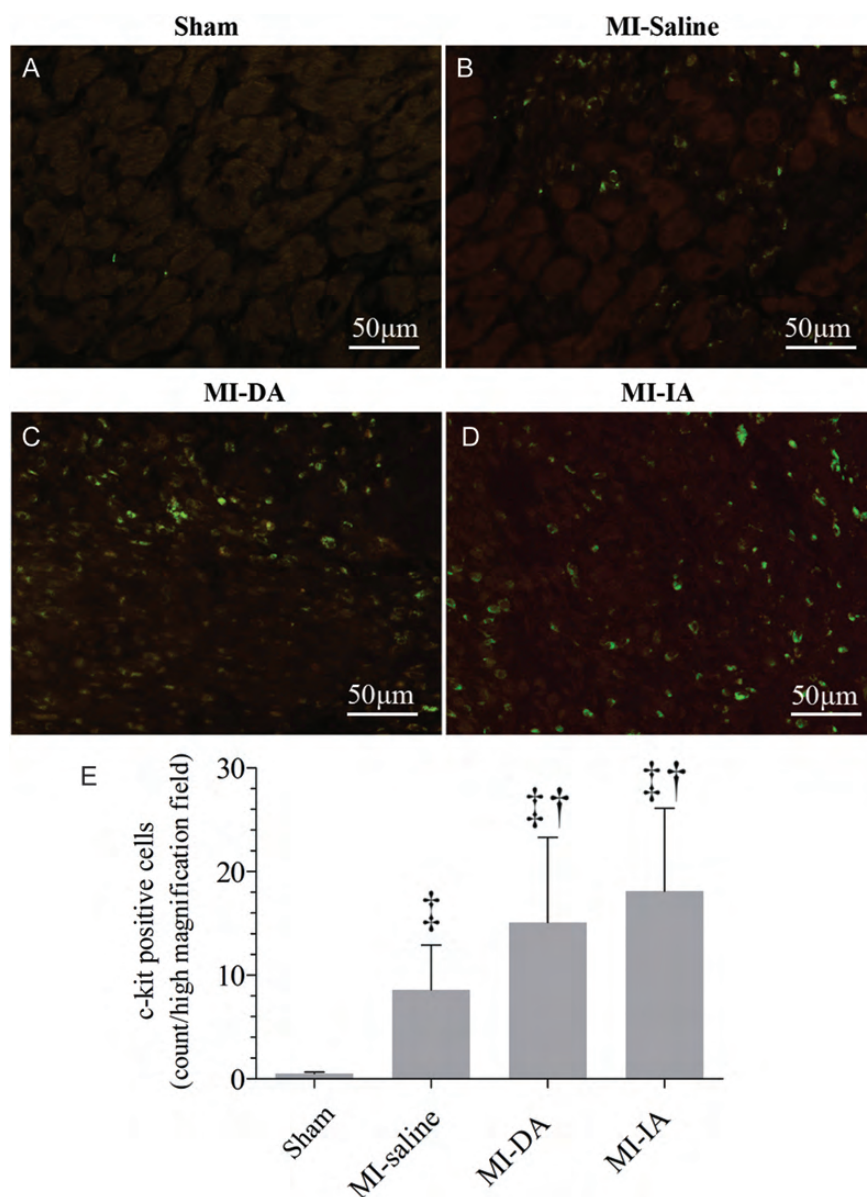
To determine the role of AMD3100 in *SDF-1 $\alpha$*  mRNA expression after MI, we serially analyzed the *SDF-1 $\alpha$*  mRNA expression at different time points by quantitative RT-PCR. The expression level of *SDF-1 $\alpha$*  mRNA in MI-saline group was significantly and rapidly (6 h) increased, and then reached the plateau 24 h post-infarction. This effect was significantly amplified by AMD3100 treatment (**Fig. 5**).

### AMD3100 regulates the activity of MMP-2/9

MMP-2/9 enzymatic activities in LV sample were analyzed by gelatin zymography [Fig. 6(A,B)]. At 7 days post-MI, there was no statistical difference of MMP-2 activity between untreated and treated rats in MI groups. At 3



**Figure 2** AMD3100 improves collagen remodeling Representative Masson's trichrome staining of heart samples from four groups: sham, MI-saline, MI-DA group, and MI-IA group at two different time points. The graph reveals a significant elevation of CVF in the infarct area of AMD3100-treated rats. The data of CVF in the non-infarct area stayed the same in all groups except increased in saline group 7 days post-MI. \* $P < 0.05$  (vs. sham at the same time point);  $^{\dagger}P < 0.01$  (vs. sham at the same time point);  $^{\S}P < 0.01$  (vs. MI-saline at the same time point).



**Figure 3 Representative immunofluorescence images of c-kit<sup>+</sup> positive cells in infarcted area** Assessment of recruitment of c-kit<sup>+</sup> positive cells into infarcted area was conducted with immunostaining for c-kit<sup>+</sup> cells (green) and quantified in lower panel. AMD3100 enhanced the recruitment of c-kit<sup>+</sup> cells into ischemic region. †*P* < 0.05 (vs. MI-saline at the same time point); ‡*P* < 0.01 (vs. sham at the same time point).

months post-MI, AMD3100 elevated the activity of MMP-2 compared with saline group [*P* < 0.05; **Fig. 6(C)**].

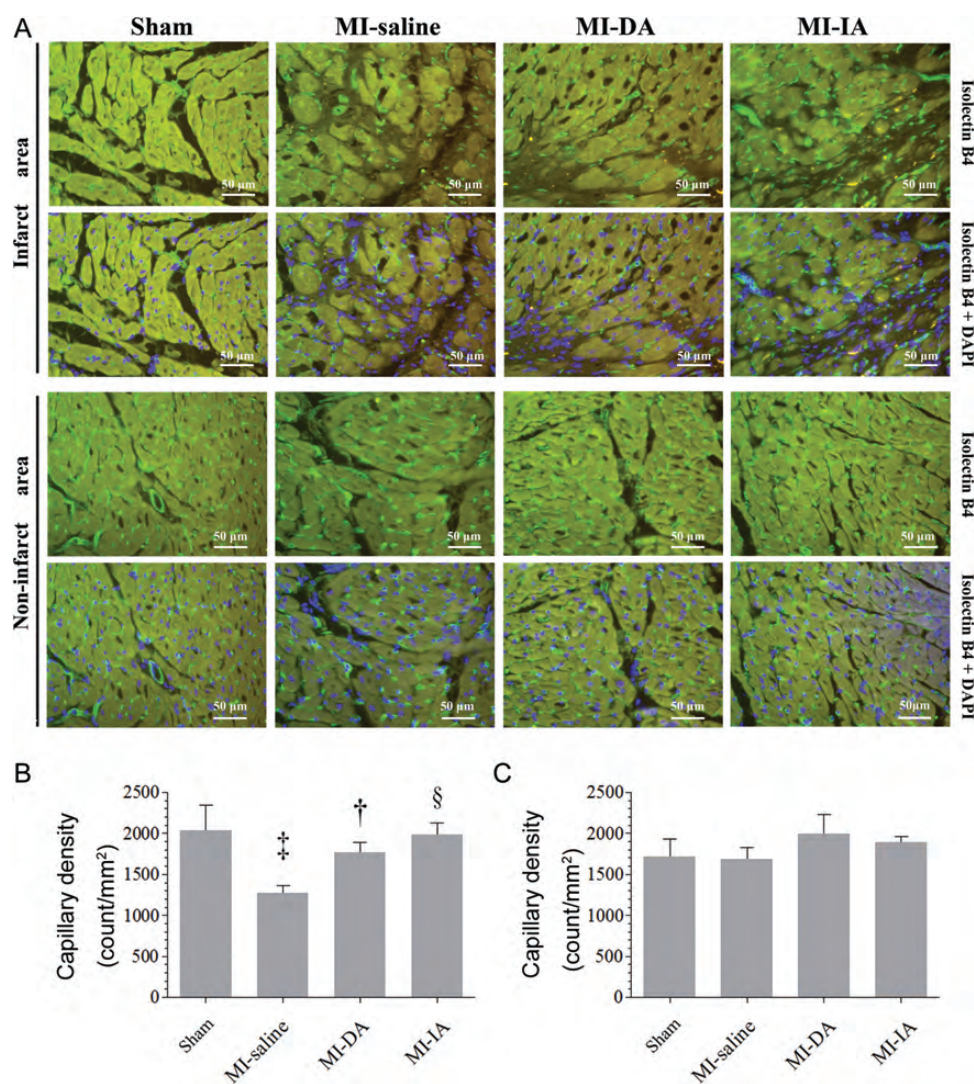
Higher MMP-9 activity was detected in MI-DA and MI-IA groups at 7 days post-MI compared with MI-saline group (*P* < 0.01). In addition, MMP-9 activity in MI-IA group was significantly higher than that in MI-DA group at 7 days post-MI (*P* < 0.05). There was no significant change of MMP-9 activity in all groups at 3 months post-MI [**Fig. 6(D)**].

## Discussion

The results presented here showed that short-term (7 days) intermittent AMD3100 administration after MI could

enhance c-kit<sup>+</sup> cells engraftment to the ischemic region, along with increased angiogenesis. These effects may contribute to accelerated wound healing, increased LV free wall, decreased infarct size and decreased compensatory cardiomyocyte hypertrophy in viable region, and ultimately may lead to an improved post-infarction LV remodeling. These data support the interpretation that short-term administration in acute phase of MI, could mobilize BM c-kit<sup>+</sup> stem/progenitor cells, and could facilitate SDF-1α/CXCR4 mediating BM c-kit<sup>+</sup> stem/progenitor cells recruitment in the local ischemic area.

AMD3100 was initially used as an anti-human immunodeficiency virus agent. In 2008, it was approved as a



**Figure 4** Representative immunofluorescence images of islectin B4 positive area in infarcted tissue. Isolectin B4 (green) was used to detect capillary vessels density in infarct and non-infarct area. Nuclei were counterstained with DAPI (blue). AMD3100 increased the capillary vessels density in ischemic area. <sup>‡</sup> $P < 0.01$  (vs. sham at the same time point); <sup>†</sup> $P < 0.05$  (vs. MI-saline at the same time point); <sup>§</sup> $P < 0.01$  (vs. MI-saline at the same time point).

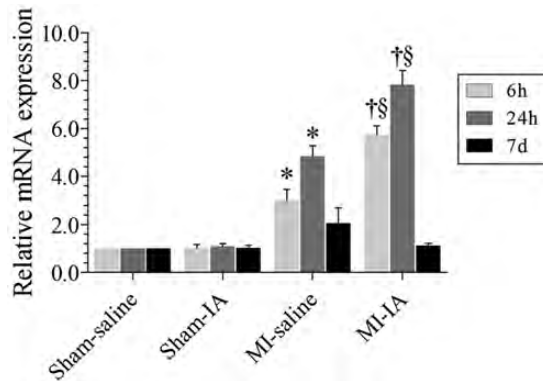
hematopoietic stem cells (HSCs) mobilizing drug by US Food and Drug Administration for autologous stem cell transplantation in patients with multiple myeloma or non-Hodgkin's lymphoma [16]. Stem cells were mobilized to replace damaged tissues *via* their paracrine factors after they arrive at the injured region [17]. Granulocyte colony-stimulating factor (G-CSF) combined with AMD3100 promoted angiogenesis in a hindlimb ischemic model [18]. These suggested that AMD3100 would be a potential therapy of ischemic heart disease *via* mobilization of stem cells.

CXCR4 is the cell surface receptor for SDF-1 $\alpha$ , expressed in HSCs, endothelial progenitor cells (EPCs), and mesenchymal stem cells (MSCs). It is believed that disruption of the interaction between CXCR4 and SDF-1 $\alpha$  is sufficient for anchored progenitors to break away from BM niches, resulting in rapid mobilization of these cells into the

peripheral blood [19]. SDF-1 $\alpha$  in infarcted tissue plays a pivotal role in trafficking of stem cells to the ischemic region. Studies have shown that SDF-1 $\alpha$ /CXCR4 axis plays important roles in the survival and protection of the myocardium against acute ischemia injury [20], stem and progenitor cell homing and survival [21], but it is not sufficient to induce homing in the absence of MI [22].

Low oxygen concentration results in high-level expression of SDF-1 $\alpha$  in hypoxic tissue [22] and CXCR4 in HSPCs [23]. Several reports showed a transient up-regulation of SDF-1 $\alpha$  expression in the heart during the first few days post-MI [22,24,25]. In the present study, in MI-saline group, *SDF-1 $\alpha$*  mRNA was detected in the infarcted region, reached its peak at 1 day, and declined to the normal level at 7 days post-MI, which is consistent with a previous study [24]. Moreover, higher expression level of *SDF-1 $\alpha$*  was observed in MI-IA group compared with MI-saline group,

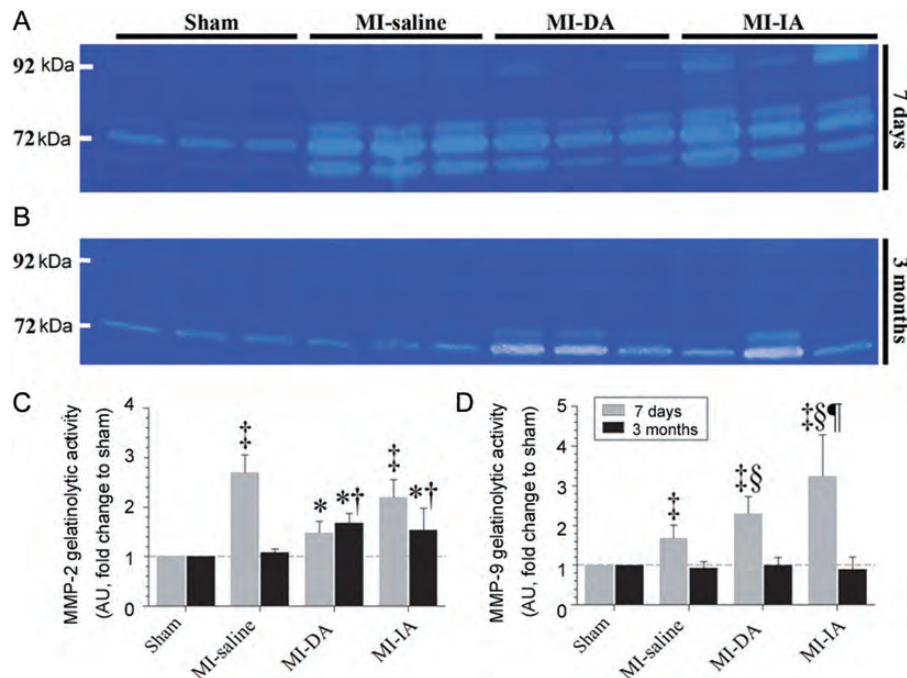
which would further enhance c-kit<sup>+</sup> stem/progenitor cells engraftment to the ischemic region. Macrophages, HSPCs, and MSCs are known to express SDF-1 $\alpha$  [26–28]. The increased expression of SDF-1 $\alpha$  in MI-IA group may be due to the AMD3100 mobilized monocyte/macrophage, MSC, and HSPC. Future work is needed to confirm this hypothesis.



**Figure 5 Dynamic changes of SDF-1 $\alpha$  mRNA expression in infarcted region** The expression levels of SDF-1 $\alpha$  at 6 h, 24 h, or 7 days post-MI were determined by real-time PCR. After correction to  $\beta$ -actin mRNA levels, SDF-1 $\alpha$ / $\beta$ -actin mRNA ratios were normalized to sham group as 1.0. The expression of SDF-1 $\alpha$  was up-regulated at 6 h post-MI, reached its peak at 24 h, declined to the normal level. AMD3100 magnified the increment. \* $P < 0.05$  (vs. sham-saline at the same time point); <sup>†</sup> $P < 0.05$  (vs. sham-IA at the same time point); <sup>§</sup> $P < 0.05$  (vs. MI-saline at the same time point).

The c-kit is a specific marker of stem/progenitor cells [29,30]. The recruitment of CXCR4<sup>+</sup> progenitor cells to ischemic tissue is mediated in hypoxic gradients *via* hypoxia inducible factor-1-induced expression of SDF-1 $\alpha$  [23]. A previous study demonstrated that CXCR4<sup>+</sup> cells could not recruit to the SDF-1 $\alpha$  expressing site in animals with an osmotic pump releasing AMD3100, because of the persistent disruption of SDF-1 $\alpha$ /CXCR4 axis [31]. Short plasma half-life time of AMD3100 allows for enough time of SDF-1 $\alpha$ /CXCR4 axis to recover from the disruption. The rapidly increased SDF-1 $\alpha$  in the ischemic region could attract mobilized cells to injured tissue. The highest level of SDF-1 $\alpha$  was observed at 24 h post-MI, which may cause different regulations between MI-DA and MI-IA groups. In our study, more c-kit<sup>+</sup> cells were observed in MI-IA group than MI-DA group, although there is no statistical significance. The expression of SDF-1 $\alpha$  in infarct region was enhanced in MI-IA group; hence, short-term administration of AMD3100 may augment more recruited stem cells to sites of injury than single injection. The c-kit<sup>+</sup> cells in the ischemic region are not likely all from BM. Recent evidence indicated that cardiac resident progenitor cells would also express this marker [32,33]. The relative contribution of increased c-kit<sup>+</sup> cell pool in this setting remains unknown.

The mobilized c-kit<sup>+</sup> cells could be MSCs, EPCs, or HSCs. A previous study showed that HSCs could not trans-differentiate into cardiac myocytes in myocardial infarcts



**Figure 6 Analysis of MMP-2/9 enzymatic activities in LV sample by gelatin zymography** (A) 7 days post-MI. (B) 3 months post-MI. (C, D) Quantification of band intensity normalized to loading control. MMP-2 was 72 kDa, MMP-9 was 92 kDa. \* $P < 0.05$  (vs. sham at the same time point); <sup>†</sup> $P < 0.05$  (vs. MI-saline at the same time point); <sup>‡</sup> $P < 0.01$  (vs. sham at the same time point); <sup>§</sup> $P < 0.01$  (vs. MI-saline at the same time point); <sup>¶</sup> $P < 0.05$  (vs. MI-DA at the same time point).

[34]. It is commonly believed that HSCs [34], MSCs [35], and EPCs [36] can differentiate into endothelial cells. The enhanced recruitment observed in AMD3100-treated rats may contribute to elevated capillary vessels in the infarcted region. Indeed, we observed more condensed capillary vessels in the AMD3100-treated groups compared with the saline group. Additionally, MI-IA led to a stronger neovascularization than MI-DA group. The neovascularization could result in improved remodeling post-MI. The picrosirius staining showed that AMD3100 enhanced the collagen deposition and collagen maturation in infarcted region, but did not affect the fibrosis in non-infarct area. We also observed thicker LV free wall, less expansion index, and smaller infarct size in AMD3100-treated groups than the saline group. Cardiomyocyte cross-sectional area was limited in AMD3100-treated groups from WGA staining. All these data suggest the ameliorative effect of AMD3100 in the regulation of remodeling post-MI.

MMPs are a family of zinc-containing enzymes that play an important role in the remodeling process of MI [37,38]. Gelatin zymography elicited the enhanced enzymatic activities of MMP-2/9 at 7 days and declined at 3 months post-MI. The data were consistent with previous reports [39,40]. The recruited leukocytes are a major source for MMP-9 in the ischemic myocardium. In the mean time, SDF-1 $\alpha$  reduces the release of nitric oxide in endothelial cells, resulting in the up-regulation of MMP-9 [41]. The activity of MMPs in MI-DA and MI-IA groups were different at each time point, which may be the result of time of the administration.

Previous studies reported that MMPs play a role *via* proteolysis of excessive collagen, because the excessive extent of non-contractile or non-relaxable fibrosis would aggravate cardiac remodeling and reduce cardiac function. Overexpressed MMP-9 in G-CSF-treated MI mice was associated with enhanced atherogenesis and improved cardiac remodeling and function [42,43]. Moreover, MMP-9 promotes the release of vascular endothelial growth factor [44], which contributes to the cardioprotective effects. Enhanced activities of MMPs resulted from AMD3100 administration may lead to the same function to the infarcted heart.

Our results emphasized the importance of SDF-1 $\alpha$ /CXCR4 axis during remodeling process post-MI and suggested that consecutive administration of AMD3100 could be a potentially therapeutic way in MI.

## Funding

This work was supported by the grants from the National Natural Science Foundation of China (81070121 and 81170238) and Tianjin Municipal Science and Technology Committee (09ZCZDSF04200 and 11JCYBJC12000).

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