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Original Paper

Mesenchymal Stem Cell–Derived Exosomes Improve the Microenvironment of Infarcted Myocardium Contributing to Angiogenesis and Anti-Inflammation

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Key Words
MSC • Exosome • Microenvironment • Angiogenesis • Myocardial infarction

Abstract
Background/Aims: Bone marrow mesenchymal stem cells (MSCs) widely applied for treating myocardial infarction face survival challenges in the inflammatory and ischemia microenvironment of acute myocardial infarction. The study hypothesized that MSC-derived exosomes play a significant role in improving microenvironment after acute myocardial infarction and aimed to investigate the paracrine effects of exosomes on angiogenesis and anti-inflammatory activity. ***Methods****:* MSCs were cultured in DMEM/F12 supplemented with 10% exosome-depleted fetal bovine serum and 1% penicillin–streptomycin for 48 h. MSC-derived exosomes were isolated using ExoQuick-TC. Tube formation and T-cell proliferation assays were performed to assess the angiogenic potency of MSC-derived exosomes. Acute myocardial infarction was induced in Sprague-Dawley rats, and myocardium bordering the infarcted zone was injected at four different sites with phosphate-buffered saline (PBS, control), MSC-derived exosomes, and exosome-depleted MSC culture medium. ***Results:*** MSC-derived exosomes significantly enhanced the tube formation of human umbilical vein endothelial cells, impaired T-cell function by inhibiting cell proliferation *in vitro*, reduced infarct size, and preserved cardiac systolic and diastolic performance compared with PBS markedly enhancing the density of new functional capillary and hence blood flow recovery in rat myocardial infarction model. ***Conclusions:*** Exosomes stimulate neovascularization and restrain the inflammation response, thus improving heart function after ischemic injury.

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Introduction

At present, bone marrow mesenchymal stem cells (MSCs) are widely applied for treating myocardial infarction, which include myocardial necrosis, interstitial hyperemia, and edema of myocardium, accompanied by infiltration of inflammatory cells. MSCs protect ischemic cardiomyocytes by differentiation properties and by secreting paracrine factors [1]. However, transplanted MSCs face survival challenges in the inflammatory and ischemia microenvironment of acute myocardial infarction [2]. In recent years, researchers partly improved the survival rate of transplanted cells through the following 2 ways: (1) pretreatment of transplanted cells by ischemic preconditioning and genetic modification [3] and (2) improvement of the microenvironment of myocardial infarction [4, 5]. These studies illustrated that the interactions between microenvironment and transplanted cells is an important repair mechanism in stem cell treatment of damaged myocardium.

Multiple experiments have shown that stem cells played a repair function through the release of beneficial factors [6], which include the vascular endothelial growth factor (Ang-1/2), inflammatory factor (interleukin-10, tumor necrosis factor-α), and apoptotic factor [7, 8]. Some of these factors can promote the proliferation of the host vascular system and cardiomyocyte survival, so as to improve the microenvironment of myocardial infarction and enhance the effects of stem cells. In addition to these factors, exosomes (30–100 nm small membrane vesicles) were also derived from various stem cells and progenitors [9, 10] including MSCs [11, 12]. Exosomes mediated cell–cell microcommunication by their inherent molecules such as nucleotides, proteins, and bioactive lipids [13]. They played an important role as key transporters of paracrine factors in angiogenesis, immune regulation, and tissue regeneration [12]. However, the contribution of exosomes to cardiac microenvironment after acute myocardial infarction still needs better understanding.

This study hypothesizes that MSC-derived exosomes play a significant role in the improvement of microenvironment after acute myocardial infarction. To address this hypothesis, the paracrine effects of exosomes on angiogenesis and anti-inflammatory activity were investigated by treating the various cells with MSC-derived exosomes. It was demonstrated that exosomes reprogram the microenvironment of acute myocardial infarction, promoting the heart function after an ischemic injury.

Materials and Methods

Cell culture

All animal experimental procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (1996). The protocols in this study were approved by the Ethics Committee of the First Affiliated Hospital of Soochow University. Bone marrow was extracted from Sprague-Dawley (SD) rats. SD bone marrow–derived MSCs were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Gibco, CA, USA) [14]. MSCs were passaged and identified by flow cytometry. Passage 3 cells were used for the following experiments. Human umbilical vein endothelial cell (HUVEC) lines were obtained from American Type Culture Collection (VA, USA). The cell lines were cultured in DMEM/high glucose (Hyclone, UT, USA) containing 10% FBS and 1% penicillin–streptomycin. Single-cell suspensions of spleen lymphocytes were prepared and cultured in RPMI1640 (Hyclone) containing 10% FBS and 1% penicillin–streptomycin.

Isolation and analysis of exosomes

MSCs were cultured in DMEM/F12 supplemented with 10% exosome-depleted FBS and 1% penicillin–streptomycin for 48 h. MSCs-derived exosomes were isolated using ExoQuick-TC (System Bioscience, CA, USA) according to the manufacturer's protocol [15]. The exosome pellets were re-suspended in phosphate-buffered saline (PBS). The protein levels of CD63, a representative marker of exosomes, were detected by flow cytometry. The concentration of exosomes was assessed using the Bicinchoninic Acid Assay Kit

(Beyotime, Suzhou, China). The ultrastructure of exosomes was analyzed using Libra 120 transmission electron microscopy (Zeiss, Geman) [16].

MSC-derived exosomes uptake by HUVEC

MSCs were labeled with a fluorescent dye CM-DiI (Molecular Probes, US) by incubating them in the CM-DiI working solution (1 μM) for 15 min at 37°C, followed by washing with PBS and centrifuging at 100,000 g for 2 h at 4°C. The excess dye was removed by precipitation of exosomes. HUVECs were previously cultured to 80% confluency and incubated with DMEM containing CM-DiI-labeled exosomes for 12 h at 37°C with 5% CO_2. After incubation, cells were washed with PBS and fixed in 4% paraformaldehyde at room temperature. The nucleus of cells were stained using medium containing 4,969-diamidino-2-phenylindole (DAPI; Vector Laboratories, USA). Cellular uptake of MSC-derived exosomes by HUVECs was observed using an inverted fluorescent microscope (IX71-A12FL/PH, Olympus, Japan).

Tube formation assay

To assess the angiogenic potency of MSC-derived exosomes, HUVECs were seeded at 1.0×10^4 cells/cm^2 in Matrigel-coated 96-well plate (Matrigel, BD Biosciences, CA, USA). HUVECs were incubated with exosomes for 12 h to allow the formation of tube-like structures. DMEM/High glucose supplemented with 10% FBS (D-FBS) was used as a control. Other groups included exosome-depleted culture medium and frozen MSC–derived exosomes. The cells were viewed under a light microscope (IX53, OLYMPUS, Japan); the images of the capillary network were acquired, and total tube lengths formed were measured using the ImageJ software (National Institutes of Health, MD, USA). Tube formation assays were performed in triplicate, and 2 independent experiments were conducted [17].

T-cell proliferation assay

Spleen lymphocytes were collected from healthy SD rats using lymphocyte separation medium (MP Biomedicals, CA, USA), and then wash with RPMI. The cells were labeled with 10 μM carboxyfluorescein diacetate succinimidyl ester (CSFE, Life Technologies, CA, USA). The CSFE-labeled cells were seeded in CD3-coated 96-well plates and treated with 10 μg/ml MSC-derived exosomes, followed by culturing for 5 days. Spleen lymphocytes were harvested and were detected by fluorescence-activated cell sorting (FACSCalibur, BD Biosciences, CA, USA).

Animals

The experiments were performed as previously described [18], according to which acute myocardial infarction was induced in healthy male SD rats (250–300 g body weight), anesthetized with chloral hydrate at dosage 20 mg/kg, intubated, and ventilated. The left anterior descending coronary artery was permanently ligated with a 6-0 Prolene suture (Ethicon Inc, CA, USA). After 60 min, the viable myocardium bordering the infarcted zone was injected at 4 different sites with a total of 100 μl of the following solutions: PBS, 80 μg of MSC-derived exosomes, and exosome-depleted MSC culture medium. Transthoracic echocardiography was performed on week 4 post-myocardial infarction equipped with a 15-MHz linear transducer (LOGIQ 500, GE Yokokawa Medical Systems, Japan). The left ventricular fractional shortening (LVFS, percent) and left ventricular ejection fraction (LVEF, percent) were measured from 2D long-axis views through the infarcted area [19].

Immunohistochemistry

Rats were sacrificed 4 weeks after myocardial infarction. For immunohistochemistry, hearts were embedded in paraffin and cut into 8-mm thin sections. The tissues were stained by Masson trichrome staining (HT15 Trichrome Stain Kit, Sigma). Viable tissue and scar area within the infarcted region were measured on each section by tracing the infarcted borders manually using the ImageJ software. Six sections were analyzed per heart.

SD model rats were anesthetized with chloral hydrate at a dose of 20 mg/kg, intubated, and ventilated 4 weeks after myocardial infarction. SD rats were treated with intravenous injection of 10 units heparin, followed by injection of 200 μl staining reagent Griffonia Simplicifolia lectin I (Vector Labs, CA USA). Rats were kept in a ventilator for 10 min, and the heart tissues were fixed with 10% paraformaldehyde. The new functional capillary density of hearts was measured by anti-Griffonia simplicifolia I (lectin 1:100) and anti-

Alexa Fluor 594 donkey anti-goat IgG antibodies. Alpha-smooth muscle actin (α-SMA) is commonly used as a marker of myofibroblast formation (BioLegend, CA, USA).. Newly formed blood vessels were also counted as α-SMA under the fluorescence microscope. Inflammation in myocardial infarction tissue was observed by hematoxylin–eosin staining.

Statistical analysis

Data were represented as mean ± SEM. Statistical analyses were performed by Graph Pad Prism 5 software (GraphPad Software Inc., CA, USA). The analysis of variance test followed by Newman-Keuls test was used for multi-group comparisons. Differences were considered statistically significant with a *P*-value of < 0.05.

Results

Characterization of exosomes

Exosomes derived from MSCs using ExoQuick method were observed to be 50–100 nm in diameter by transmission electron microscopy. Most of them presented membrane vesicles. Vesicles were morphologically compared in fresh and frozen (at −80°C)-thawed exosomes, and it was found that bi-layer membrane vesicles were significantly reduced in the case of frozen-thawed exosomes(Fig. 1A). CD63 as a representative marker of exosomes was detected by flow cytometry (Fig. 1B). These data demonstrated that MSC-derived exosomes were successfully purified.

Cellular uptake of MSC-derived exosomes by HUVEC and spleen lymphocytes

The fluorescence micrograph showed that DiI-labeled exosomes were localized in the cytoplasm of HUVECs (Fig. 1C). It implied that MSC-derived exosomes can be internalized by endothelial cells.

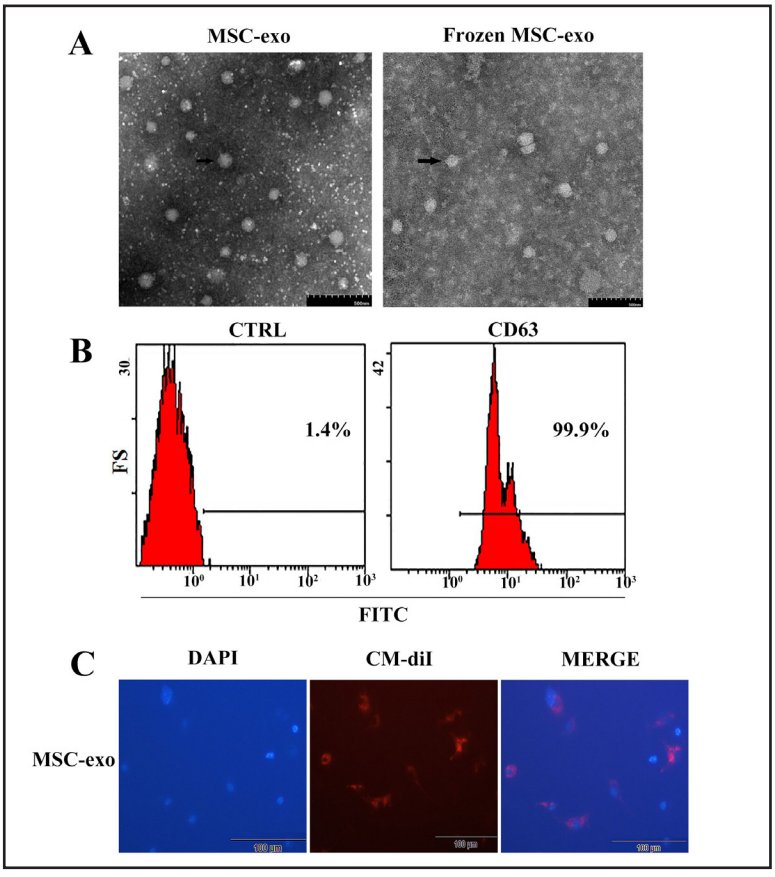

Fig. 1. Characterization and cellular uptake of exosomes. (A)The ultrastructure of exosomes was analyzed by transmission electron microscopy (reference bar, 500 nm). MSC-derived exosomes and frozen exosomes both showed membrane vesicles. (B) Flow cytometric analysis of exosomes secreted by MSCs. Left panel: control (anti-CD63 antibody; no exosomes); right panel: exosomes with FITC-conjugated anti-CD63. (C) HUVEC were incubated with MSC-derived exosomes labeled with CM-diI. Fluorescence photomicrographs showed DAPI-stained HUVEC nuclei (blue), CM-diI–labeled exosomes (red). Merge picture showed exosomes uptake into HUVEC.

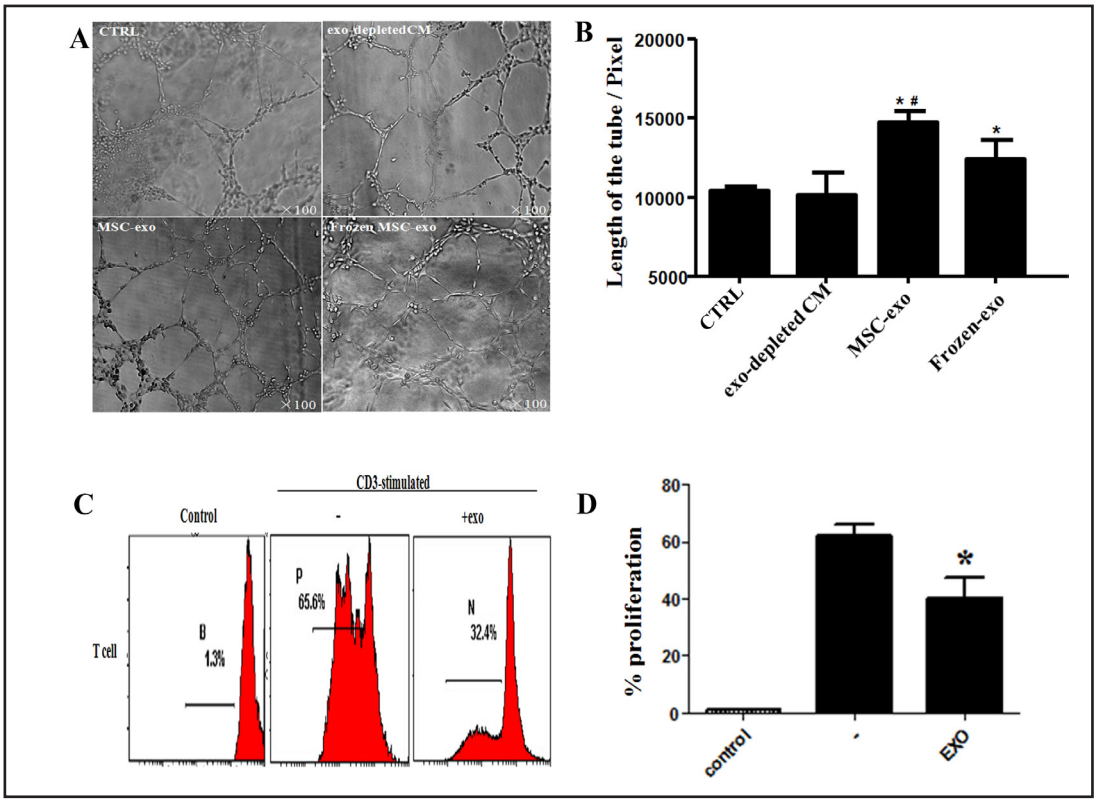

Fig. 2. MSC-derived exosomes promote angiogenesis and inhibit proliferation of lymphocytes. HUVEC cells were seeded in a Matrigel-coated well. (A) Photomicrographs showed a significant increase in tube formation in the cells incubated with the MSC-derived exosomes. (B) Statistical analysis of tube length in different conditions (* $P < 0.05$ vs. exosome-depleted CM, # $P < 0.05$ vs frozen MSC-exosome). (C) Spleen lymphocytes were stained with carboxyfluorescein diacetate succinimidyl ester, stimulated with monoclonal antibodies against CD3, and then cultured for 96 h. Proliferation was quantified by fluorescence-activated cell sorting analysis. (D) Statistical analysis of T cell proliferation in the presence or absence of exosomes. Columns, mean (n = 3); bars, * $P < 0.05$ compared with exosome-depleted CM.

Promotion of angiogenesis and inhibition of in vitro lymphocyte proliferation by MSC-derived exosomes

The results showed that both fresh and frozen exosomes enhanced the tube formation of HUVECs. There was no difference in the same experiment between exosome-depleted culture supernatant and control groups. Further, MSC-derived exosomes significantly promoted tube formation of HUVECs compared with frozen MSC-derived exosomes (14576.15 ± 767.61 vs. 12328.63 ± 1109.99, respectively, $P < 0.05$) (Fig. 2A–B). The results indicated that fresh MSC-derived exosomes greatly promoted angiogenesis *in vitro*. Furthermore, the effect of MSC-released exosomes on the anti-inflammation response was examined by analyzing the proliferation of T-cells when treated with exosomes *in vitro*. The results showed that the proliferation of CD3-stimulated T-cells significantly decreased when treated with exosomes (Fig. 2C–D).

Improvement in cardiac function and amelioration of fibrosis after myocardial infarction by MSC-derived exosomes

Infarcted hearts injected with exosomes showed a higher LVEF compared with the PBS-injected control group (Fig. 3A–B). When myocardial infarct size and percentage myocardium infarcted were analyzed after Masson trichrome staining, histological graphs showed significantly reduced cardiac fibrosis in the MSC-derived exosome group than in PBS

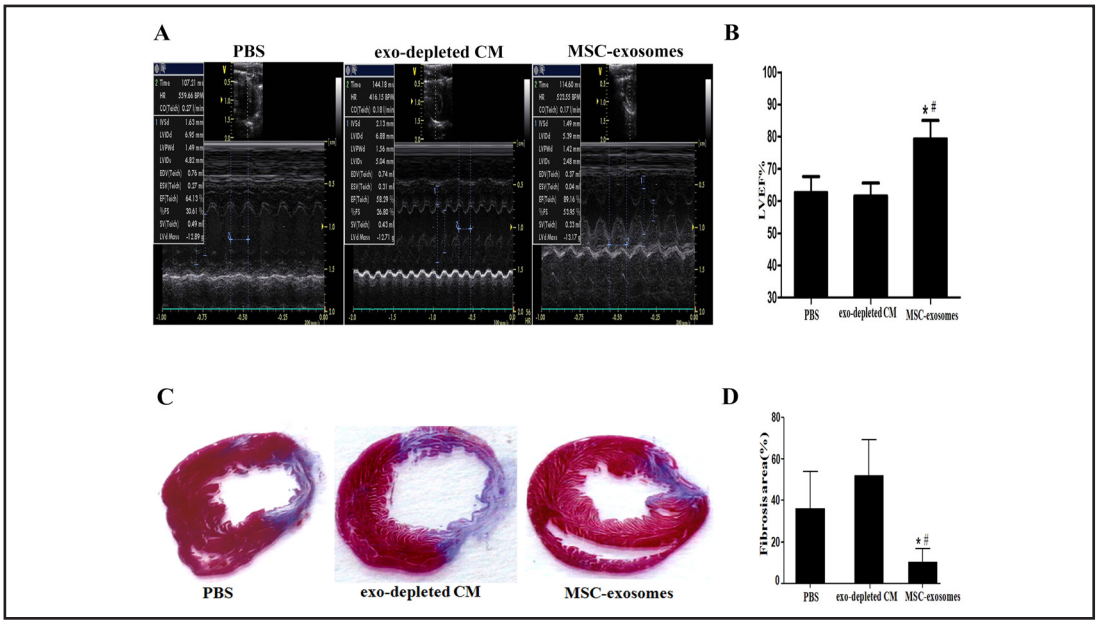

Fig. 3. Functional effects of exosomes *in vivo*. (A) Exosomes improve echocardiographic performance of the infracted heart at 4 weeks after myocardial infarction. Representative echocardiographic images from at least six cardiac contractile cycles of the hearts from control and experimental rats are provided. (B) The mean percentages of the left ventricular ejection fraction were calculated. * P < 0.05 vs. exosome-depleted CM, # P < 0.05 vs phosphate-buffered saline (PBS) (C) Micrography showed the infract size using Masson trichrome staining in various groups 4 weeks post-infarction. Blue represents the infarction part of myocardial fibrosis tissue. Red represents the normal myocardium tissue. (D) Quantification of infarct size in various groups. Data represent the means (* P < 0.05 vs. exo-depleted CM, # P < 0.05 vs. PBS).

and exosome-depleted CM groups (Fig. 3C–D). Taken together, these data showed that MSC-derived exosomes play an important role in cardiac remodeling post-myocardial infarction.

Promotion of angiogenesis and inhibition of T lymphocyte proliferation by MSC-derived exosomes

The fluorescence micrograph showed a large number of functional tube formation in the surrounding region of infarction areas. Representative images are shown in Fig. 4A significant increase in new capillaries was observed in the MSC-derived exosome group than in PBS (1152.17 ± 91.40 vs.704.50 ± 134.64, $P < 0.05$) and exosome-depleted CM groups (1152.17 ± 91.40 vs.704.50 ± 138.85, $P < 0.05$) (Fig. 4A–B). Meanwhile, blood vessel density increased in heart injected with exosomes than with PBS (112.67 ± 12.31 vs. 36.17 ± 9.79, $P < 0.05$, Fig. 4C–D). The micrograph after hematoxylin staining showed that myocardial cells arranged in disorder and inflammatory cells infiltrated in infarcted ventricular tissue. There were decreased inflammatory cells in the exosome group than in PBS and exosome-depleted CM groups (Fig. 4E). These results demonstrated that MSC-derived exosomes improved the microenvironment of myocardia infarction through angiogenesis and anti-inflammation.

Discussion

This study demonstrated that MSC-derived exosomes could be internalized by endothelial cells. These exosomes significantly promoted tube formation of HUVECs and also decreased the proliferation of spleen lymphocytes. Thus, MSC-derived exosomes accounted for the cardioprotection of MSCs through angiogenesis and anti-inflammation in an AMI rat model.

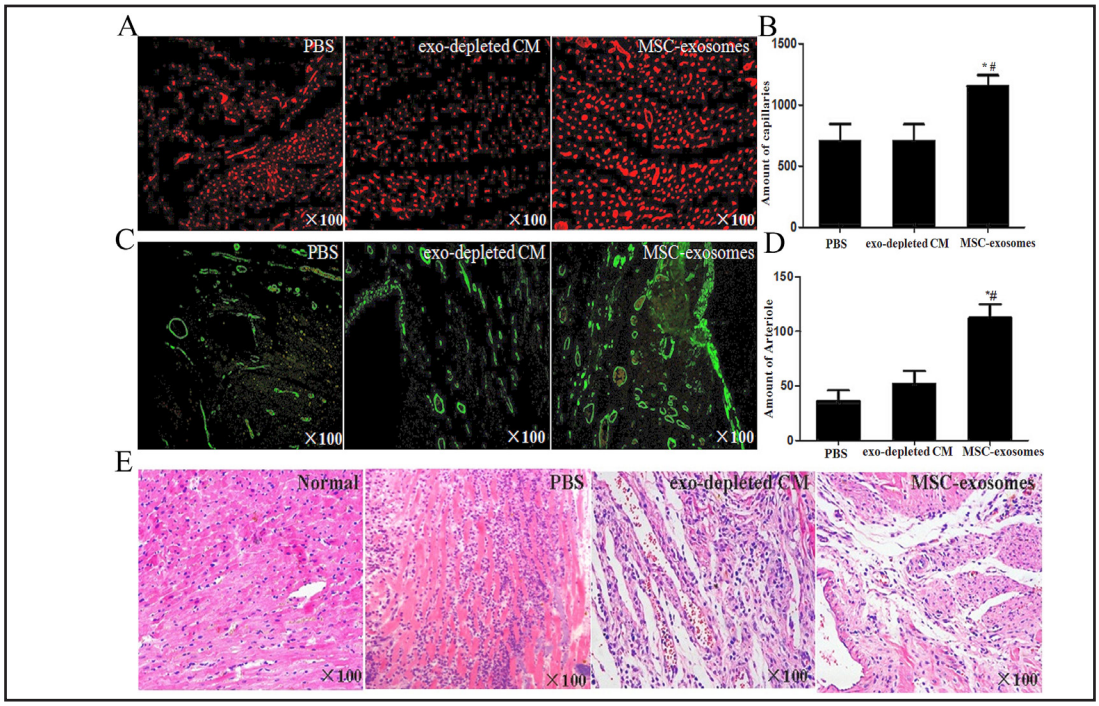

Fig. 4. Exosomes promote neoangiogenesis and decrease the inflammation in the infracted zone. The heart tissue sections were stained by Griffonia Simplicifolia lectin I (red) and alpha-smooth muscle actin (green). Blood vessel density in the infarcted zone was counted and compared among three groups. The new functional capillary density was measured by Griffonia Simplicifolia lectin I (A-B) (*P < 0.05 vs. exo-depleted CM, # P < 0.05 vs. PBS). Newly formed blood vessels were counted as α-SMA under the fluorescence microscope (C-D) (*P < 0.05 vs. exo-depleted CM, # P < 0.05 vs. PBS). Inflammation infiltration in myocardial infarction tissue was observed by HE staining (E).

Many studies have proved that a paracrine mechanism triggered by transplanted cells contributes significantly to myocardial repair [20]. Various cytokines secreted by MSCs played an important role in the improvement of myocardial function. This study demonstrated no difference in angiogenesis and improvement of heart function between the exosome-depleted conditional medium and the PBS control group. These results implied negligible roles for soluble factors secreted by MSCs. Cytokines could be easily degraded by nucleases because they lack a protective membrane film structure. Exosomes avoid the disruption of the enzymes under the protection of the mobile membrane. Exosomes may well be a key ingredient of paracrine mechanism.

It is well accepted that the protection of MSCs on the infracted hearts is mainly dependent on their angiogenesis-promoting activity[21]. Furthermore, Exosomes derived from a series of cells have shown to reduce the myocardial infarction area and improved heart function by angiogenesis in mice [22]. Recent studies have demonstrated the importance of exosomes and its wider involvement in the stimulation of angiogenesis and cytoprotection and modulation of inflammation and apoptosis[23]. Consistent with the previous observations,our results showed that exosomes accounted for the cardioprotection of MSCs through the formation of new blood vessels and reduced inflammation. Interestingly, we found the drop in the concentration of frozen exosomes and the decreased function of angiogenesis. It implyed that fresh exosomes could achieve a better therapeutic effect. Scientists also had different views about the use of fresh and frozen exosomes [11, 24]. Some research showed that intact but not lysed exosomes enhanced cardiac function after myocardial I/R injury [25], which is consistent with the findings of this study.

Instead of cell therapy, the use of non-cell therapy methods have shown several promising prospects in lesser long-term side effects such as: arrhythmia, calcification, and

multi-directional differentiation. As cell-free products, exosomes may have a potential for circumventing many of the limitations of using stem cells as therapeutic agents.

Exosomes are demonstrated to be important extracellular communicators of ischemic signaling and myocardial repair. Studies have shown their role in cell-to-cell communication by shuttling functional materials such as mRNAs, micro-RNAs (miRNAs) and protein. For instance,the key features of myocardial ischemia/reperfusion (I/R) injury involved in loss of ATP/NADH, increased oxidative stress and cell death. Proteins in exosomes exactly complemented or compensated proteomic deficiencies in ischemic/reperfused myocardium [25]. Several reports have suggested that exosomes and macrovesicles were secreted by myocardial tissue bordering the infarcted zone [26]. MiRNAs are delivered with intact functionality and have been repeatedly shown to regulate protein expression in recipient cells in a paracrine fashion [27]. Exosomes may modulate the microenvironment by heterocellular communication to initiate the repair process. The study of exosomes may reveal important signaling mechanisms for microenvironment improvement after myocardial infarction.

Conclusions

In conclusion, this study demonstrated that exosomes are the active ingredient of paracrine secretion by MSCs. MSC-derived exosomes stimulate neovascularization and restrain the inflammation response. They improve cardiac function after ischemic injury. Thus, MSC-derived exosomes play an important role in cardiac remodeling post-myocardial infarction. Further investigations are needed to illuminate long-term effects of exosome therapy for damaged hearts and analyze the composition of exosomes to find out detailed principles for traumatic myocardium.

Abbreviations

FBS (fetal bovine serum); MSCs (mesenchymal stem cells); PBS (phosphate-buffered saline); SD (Sprague-Dawley).

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Disclosure Statement

The authors declare they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

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