MIF Facilitates the Therapeutic Efficacy of Mesenchymal Stem Cells Derived Exosomes in Acute Myocardial Infarction Through Up-regulating miR-133a-3p

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Research
Abstract

**Background** The purpose of this study was to explore whether exosomes derived from MIF engineered umbilical cord MSCs (ucMSCs) exhibit better cardioprotective effects in a rat model of AMI and the mechanisms underlying it.

**Results** Exosomes isolated from ucMSCs (MSC-Exo), MIF engineered ucMSCs (MIF-Exo) and MIF down-regulated ucMSCs (siMIF-Exo) were used to investigate cellular protective function in human umbilical vein endothelial cells (HUVECs) and H9C2 cardiomyocytes under hypoxia and serum deprivation (H/SD) and infarcted hearts in rats. Compared with MSC-Exo and siMIF-Exo, MIF-Exo significantly enhanced proliferation, migration and angiogenesis of HUVECs and inhibited H9C2 cardiomyocytes apoptosis under H/SD *in vitro*; MIF-Exo also significantly inhibited cardiomyocytes apoptosis, reduced fibrosis area and improved cardiac function in infarcted rats *in vivo*. Exosomal miRNAs sequence and qRT-PCR confirmed miRNA-133a-3p remarkably increased in MIF-Exo. The biological effects of HUVECs and H9C2 cardiomyocytes were attenuated with incubation of MIF-Exo and miR-133a-3p inhibitors. And these effects were accentuated with incubation of siMIF-Exo and miR-133a-3p mimics, followed by p-AKT protein level up-regulated.

**Conclusion** MIF-Exo enhance the effects on promoting angiogenesis, inhibiting apoptosis, reducing fibrosis and preserving heart function, *in vitro* and *in vivo*. The miR-133a-3p and its downstream AKT signal pathway were involved in these biological activities of MIF-Exo.

Introduction

Exosomes are small extracellular vesicles with a diameter of 30–150 nm and contain multifarious proteins, mRNAs, and microRNAs et al. A growing number of evidences show the regulatory role of exosomes in biological processes, including organs crosstalk as well as intercellular signaling. They have the effect of anti-apoptosis and are used to treat animals in acute myocardial infarction (AMI). Compared with mesenchyme stem cells (MSCs), exosomes retain the function of parent cells and have many advantages such as long term stability, easy internalization into receptor cells, minimal immunological rejection and easy operation. So exosomes transplantation is a promising cell-free therapy for ischemic heart disease.

Among the cargoes of exosomes, microRNAs (miRNAs) have been demonstrated to control important processes that conduce to the pathological consequences of AMI. miRNAs are small non-coding RNAs, regulating gene expression, leading to degradation or translation inhibition of the mRNAs. More and more studies have shown that miRNAs are functional in apoptosis, angiogenesis, and fibrosis after myocardial infarction.

Exosomes can secrete from many kinds of cell types including MSCs and macrophage migration inhibitory factor (MIF) engineered MSCs. MIF is a pro-inflammatory cytokine, which is widely expressed
in many kinds of cells including MSCs. Recent studies show that MIF plays an important role in cell survival and proliferation. Our previous study showed that exosomes from MSCs with overexpression of MIF compared with MSCs had better cardiac protection in rats with AMI.

In this study, we explored the mechanism of exosomes from MIF engineered umbilical cord MSCs (ucMSCs) in cardiac protective function. In vitro, we found that exosomes from MIF engineered ucMSCs (MIF-Exo) had the better effects of cell survival, blood vessel formation and cell migration than non-engineered ucMSCs (MSC-Exo). In vivo, we found that MIF-Exo had the better cardiac protective ability to reduce fibrosis area, promoting capillaries formation, inhibiting apoptosis, and improving heart function than MSC-Exo. miRNA sequence and quantitative real-time PCR (qRT-PCR) found that miR-133a-3p expression significantly increased in MIF-Exo than MSC-Exo. The cardioprotective effects were attenuated with inhibition of exosomal miR-133a-3p. These data indicate that exosomal miR-133a-3p at least partially mediates the function of MIF-Exo in the ischemic heart.

Results

Characterization of MSCs and exosomes derived from ucMSCs

We examined the morphology and multiple differentiation potential of ucMSCs and found that osteogenesis, adipogenesis and chondrogenesis of ucMSCs were confirmed by Alcian blue staining, Oil red staining and Alizarin red staining (Fig. 1A). ucMSCs were positive for surface markers of CD44, CD73, CD105, and negative for markers of CD45, CD31 and CD34 (Fig. 1B). To verify whether lentiviral modification conducted successfully, green fluorescence was observed in both MIF-engineered ucMSCs (MIF-MSC) and MIF down-regulated ucMSCs (siMIF-MSC) under microscope (Fig. 1C). In addition, western blot showed that MIF protein level significantly decreased in siMIF-MSC compared with ucMSCs and MIF-MSC (Fig. 1D). Exosome specific markers of TSG101, CD81 and CD63 were positive in MIF-Exo, MSC-Exo and siMIF-Exo (Fig. 1E). The particle size and concentration of Exo were similar among the three groups (Fig. 1F). TEM shows that the morphology of exosomes from MIF-Exo and siMIF-Exo is a typical cup-shaped structure, with a size of around 100 nm (Fig. 1G).

MIF-Exo exhibited better protective effects on HUVECs and H9C2 cardiomyocytes than MSC-Exo under hypoxia and serum deprivation (H/SD) in vitro

In order to examine whether MIF-Exo has better cellular protective effects compared with MSC-Exo and siMIF-Exo, neovascularization, migration, proliferation and anti-apoptosis of HUVECs and H9C2 cells were evaluated. After Dil (10 μmol/L, 1μL) was labeled with exosomes for 6 hours, the labeled exosomes then co-cultured with H9C2 cells and HUVECs for 6 hours and 24 hours under H/SD. Confocal images showed that both H9C2 and HUVECs could uptake labeled exosomes. And exosomes absorption by cells was presented as a time dependent manner (Fig. 2A).

Angiogenesis of HUVECs significantly increased in the MIF-Exo group compared with PBS, MSC-Exo and siMIF-Exo groups. And there was no significant difference between siMIF-Exo group and PBS group
Migration rate of HUVECs also significantly enhanced in the MIF-Exo group compared with PBS, MSC-Exo and siMIF-Exo groups (Fig. 2D-E). TUNEL and cytometry flow all showed that the percentage of apoptosis cells remarkably reduced in the MIF-Exo group compared with PBS, MSC-Exo and siMIF-Exo groups. There was no significant difference between siMIF-Exo group and PBS group (Fig. 2F-G, J-K). EdU positive cells remarkably increased in the MIF-Exo group than PBS, MSC-Exo and siMIF-Exo groups (Fig. 2H-I). Collectively, these results suggest that MIF-Exo exhibit better cellular protective effects on HUVECs and H9C2 cardiomyocytes compare with MSC-Exo under H/SD in vitro.

**MIF-Exo effectively preserved cardiac function in rats with MI in vivo**

In order to evaluate the benefits of MIF-Exo cardiac function in vivo, PBS, MIF-Exo, MSC-Exo, and siMIF-Exo were injected at the border of infarction area in rats (Fig. 3A). Six hours after MI model establishing and exosomes injection, large areas were positive for Dil labeled exosomes in the myocardium (Fig. 3B) and near the endothelium (Fig. 3C). Two weeks after MI, LVEF significantly increased in MIF-Exo group compared with PBS group. Until 4 weeks after MI, not only LVEF remarkably improved in the MIF-Exo group compared with PBS, MSC-Exo and siMIF-Exo groups, but also LVFS significantly increased in the MIF-Exo group compared with the other three groups (Fig. 3D-E). These results suggest that MIF-Exo preserve heart function in infarcted rats.

**MIF-Exo promoted angiogenesis and cardiomyocytes survival in rats with MI in vivo**

Besides cardiac function, we also investigated the MIF-Exo effects on fibrosis angiogenesis and anti-apoptosis in vivo. Masson staining indicated that the fibrosis area significantly decreased in the MIF-Exo group compared with PBS, MSC-Ex and siMIF-Exo groups (Fig. 4A, E). To reveal the mechanism of exosomal therapy, immunofluorescence with antibodies against CD31 and α-SMA were used to stain capillary and arteriole. Four weeks after MI, the capillary density significantly increased in the MIF-Exo group compared with PBS, MSC-Exo and siMIF-Exo groups (Fig. 4B, F). The arteriolar density also raised in the MIF-Exo group compared with the other three groups (Fig. 4C, G). TUNEL showed that apoptotic cells significantly decreased in the MIF-Exo group compared with PBS, MSC-Exo and siMIF-Exo groups (Fig. 4D, H). These effects of MIF-Exo reducing fibrosis area, promoting angiogenesis, and protecting cell from apoptosis are the reason for benefit of improving cardiac repair.

**miR-133a-3p expression significantly increased in MIF-Exo**

In order to explore the molecular mechanisms of cardioprotective effects of MIF-Exo, exosomal miRNAs sequencing was performed on MIF-Exo, MSC-Exo and siMIF-Exo. There were 73 up-regulated and 102 down-regulated miRNAs in MIF-Exo compared with siMIF-Exo (Fig. 5A, C). Forty-one up-regulated and 70 down-regulated miRNAs were in MIF-Exo compared with MSC-Exo (Fig. 5D, F). Top 15 up-regulated and down-regulated miRNAs were listed (Fig. 5A-B, D-E). In these up-regulated miRNAs, there were 15 miRNAs were overlapped (Fig. 5G). Of them, qRT-PCR confirmed that miRNA-133a-3p most remarkably increased in MIF-Exo compared with MSC-Exo and siMIF-Exo (Fig. 5H). It was previously reported that miRNA-133a-
3p is associated with cardiovascular disease regulation. So in this study, we focused on the cardiac protection of exosomal miRNA-133a-3p.

**Gain and loss function of exosomal miR-133a-3p on pro-angiogenisis, proliferation, and apoptosis in HUVECs and H9C2 cells in vitro**

Gain and loss function studies were carried out to identify the effects of exosomal miR-133a-3p on pro-angiogenisis, proliferation, and apoptosis in HUVECs and H9C2 cells in vitro. HUVECs and H9C2 cells were transfected by miR-133a-3p mimics, miR-133a-3p inhibitor and their negative controls (NC) successfully and were validated by qRT-PCR (Supplementary Fig. 1). We found that incubation of MIF-Exo and miR-133a inhibitor compared with MIF-Exo and miR-133a inhibitor NC could remarkably attenuate the effects of MIF-Exo on pro-angiogenisis (Fig. 6A, C), and proliferation (Fig. 6B, D) in HUVECs. And incubation of siMIF-Exo and miR-133a mimics compared with siMIF-Exo and miR-133a mimics NC could accentuate the effects of MIF-Exo on promoting tube formation (Fig. 6E, G) and proliferation (Fig. 6F, H) in HUVECs. We also found that co-culturing of MIF-Exo and miR-133a inhibitor compared with MIF-Exo and miR-133a inhibitor NC could increase apoptotic cell number (Fig. 6I, K) in H9C2 cells. And co-culturing of siMIF-Exo and miR-133a mimics compared with siMIF-Exo and miR-133a mimics NC could suppress pro-apoptotic effects (Fig. 6J, L). Taken together, these results indicate that miR-133a-3p is a key player in MIF-Exo induced cellular protection.

**miR-133a-3p inhibited apoptosis, enhanced angiogenesis and promoted proliferation via AKT pathway in HUVECs and H9C2 cells, and promoted VEGF protein expression in HUVECs**

To further identify the potential signal pathway during miR-133a-3p playing role in biological activity, miR-133a-3p mimics and miR-133a-3p mimics NC were incubated with H9C2 cells and HUVECs. In HUVECs, AKT protein phosphorylation increased, followed by Bcl-2 protein level up-regulated and cleaved caspase-3 down-regulated; the protein expression of VEGF also increased, in miR-133a-3p mimics group. These effects could be reversed by mimics NC (Fig. 7A, C). In H9C2 cells, an enhanced AKT phosphorylation in miR-133a-3p mimics group, and adjusting its downstream targets Bcl-2 (up-regulated) and cleaved caspase-3 (down-regulated) (Fig. 7B, D). In summary, these data suggest that miR-133a-3p inhibits cardiomyocytes apoptosis, enhances angiogenesis and proliferation via AKT signal pathway, and increases VEGF levels in HUVECs.

**Discussion**

The findings of this study are following: 1) MIF-Exo promotes migration, inhibits apoptosis, and improves tube like structure formation under H/SD in HUVECs or H9C2 cardiomyocytes. 2) MIF-Exo has better effects on preserving cardiac function and inhibiting fibrosis compared with MSC-Exo. 3) The protective effects of MIF-Exo may be at least partially mediated by miR-133a-3p, which could inhibit apoptosis and promote angiogenesis and proliferation through AKT signal pathway (Fig. 7E).
Recently, genetically modified stem cells have been used and brought good effect on repairing the injured heart. A recent study showed that MSCs with overexpressing IL-33 enhanced heart function and reduced inflammation in rats with MI.\textsuperscript{16} Another study showed that aged MSCs with overexpressing ERBB4 reduced myocardial infarct size and improved cardiac function.\textsuperscript{17} Yu et al also reported that exosomes from GATA-4 with overexpressing in MSCs could reduce cardiac injury.\textsuperscript{18} Ma et al reported that exosomes from MSCs modified with AKT could promote cardiac angiogenesis and regeneration.\textsuperscript{19} Our previous study also showed that MIF rejuvenates aged human MSCs and improves myocardial repair.\textsuperscript{12}

MSCs achieve cardiac protective function mainly through paracrine factors, such as exosome.\textsuperscript{20–23} Recent studies have shown that exosomes derived from MSCs reduce myocardial ischemic injury and preserve cardiac function after MI.\textsuperscript{24,25} Compared with stem cell transplantation therapy, exosome therapy showed improvement of no teratoma development, minimum immunogenicity, and little potential for tumorigenesis.\textsuperscript{26,27} It is a promising new cell free therapy for cardiac repair. However, more extensive testing and validation of exosomes therapeutics are needed to assure its safety and efficacy.\textsuperscript{28}

In this study, we proved that exosomes of MIF-MSCs were embellished with anti-apoptotic and angiogenesis miRNA, which inhibit apoptosis and promote angiogenesis, thus protect the cardiac function. Exosomes are extracellular vesicles containing miRNAs et al, and they are absorbed by target cells. Exosomes regulate intracellular signal pathways.\textsuperscript{29} Recent years, studies have shown that exosomal miRNAs play an important role in many biological processes.\textsuperscript{30} In current study, expression profiles of miRNAs in MIF-Exo revealed that miR-133a-3p was significantly increased in MIF-Exo and reduced in siMIF-Exo compared with MSC-Exo. It indicates that the expression of miR-133a-3p was dynamic change with overexpression or knockdown of MIF gene in ucMSCs. Exosomal miR-133a-3p was confirmed that it served as a protective factor in ischemic heart in our study.

It is reported that miR-133a-3p plays a very important role in heart and muscle development.\textsuperscript{31–33} The circulating expression of miR-133a-3p increased significantly in AMI patients compared with normal people.\textsuperscript{34} Thus, it could be considered as a diagnostic biomarker for AMI patients.\textsuperscript{35} Moreover, it was also reported to miR-133a-3p could suppress apoptosis and regulate fibrosis after MI.\textsuperscript{14} In this study, we verified that exosomes from MIF engineered ucMSCs could reduce fibrosis area and improve cardiac function in ischemic hearts. The protective effects of MIF-Exo at least partially mediated by miR-133a-3p.

We found that miR-133a-3p mimics promoted phosphorylation of AKT in HUVECs or H9C2 cells, indicating a possible molecular mechanism for the positive role of miR-133a-3p in cardioprotection. Survival signals AKT pathway has been reported as a key target in cardioprotection.\textsuperscript{36,37} miR-133a has also been well documented as a suppressor of cardiac remodeling and the mechanisms are also involving many target genes and pathways including AKT pathway.\textsuperscript{38,39} These evidences further support the cardioprotective effects of miR-133a-3p via enhancing AKT signal pathway in cardiomyocytes and endothelial cells. However, further studies are still needed to elucidate the downstream mechanisms of cardioprotective effects of exosomal miR-133a-3p in physiology and pathophysiology.
There are some limitations to this study. First, other exosomal miRNAs may also contribute to cardioprotection effects of MIF-Exo. We did not test and validate the expression and effects of those miRNAs. Second, the expression of miR-133a-3p increased significantly in MIF-Exo, but the mechanisms of miR-133a-3p were regulated by overexpression of MIF in ucMSCs have not been clarified. Third, although we confirmed the protective effects of miR-133a-3p enriched exosomes, further studies are needed to verify the safety and efficacy before used as a potential cell free therapy for cardiac repair in clinical practice.

MIF engineered ucMSCs derived exosomes enhance the effects on promoting angiogenesis, improving proliferation, inhibiting apoptosis, reducing fibrosis and preserving heart function, in vitro and in vivo. The miR-133a-3p and its downstream AKT signal pathway were involved in these biological activities of MIF-Exo.

Materials And Methods

Ethics statements

All animal care and surgical procedures were performed with approval from the Nanjing Medical University Animal Care and Use Committee (IACUC-1709019).

Cell culture and identification

Umbilical cord MSCs from healthy donors were purchased from the Clinical Center of Reproductive Medicine in Nanjing. ucMSCs, H9C2 cells and human umbilical vein endothelial cells (HUVECs) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, USA) supplemented with 10% foetal bovine serum (FBS, Gibco, USA), 100 U/mL penicillin, 100 µg/ML streptomycin and 110 mg/mL sodium pyruvate. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. ucMSCs used for experiments were between passages 3 and 6.

Umbilical cord MSCs were characterized by the expression of cell surface markers. They were washed with 2% FBS/phosphate-buffered saline (PBS) and incubated at 4°C for 30 minutes with 5 µl of a monoclonal antibody specific for CD31, CD34, CD45, CD73, CD44 and CD105 (BD Biosciences, San Jose, CA, USA). Unstained ucMSCs were used as controls. FACS Canto II (BD Biosciences, San Jose, CA, USA) was used for cytometry analysis.

Exosome extraction and identification

Exosomes were isolated as previously reported. ucMSCs were cultured to 80% confluence in the complete medium, washed three times with PBS, and subsequently cultured with exosomes free DMEM for 48 hours. The conditioned medium was collected and centrifuged at 1500 g for 30 min to remove apoptotic bodies and cell debris followed incubation with RiboTM Exosome Isolation Reagent (RiboBio, China) for 12 hours at 4°C. The supernatant was centrifuged at 2,000 g for 30 min. The supernatant was discarded, and the pellet was suspended in PBS and stored at -80°C. BCA kit (Thermo,
USA) was used to analyze concentration. Western blotting was used to identify surface markers of exosomes including TSG101 (14497), CD63 (25682) and CD81 (66866) (Proteintech, USA). Transmission electron microscopy (TEM, HITACHI, H-600IV, Japan) Nanoparticle Tracking Analysis (NTA, Malvern Instruments, UK) were used to determine particle morphology and the particle size distributions of isolated exosomes.

To evaluate whether exosomes could be absorbed by cells, 1 µmol Dil (a red fluorescent cell linker for general cell membrane labeling) was used for labeling exosome, and then Dil labeled exosomes were incubated with target cells for 6 hours and 24 hours. Nuclei were stained with 4’, 6-diamidino-2-phenylindole (DAPI). Confocal images were taken by Zeiss laser-scanning confocal microscope (LSM5 Live, Carl Zeiss, Germany).

**RNA extraction and quantitative real-time PCR (qRT-PCR)**

RNA was extracted from exosomes and cells by TRIzol (Invitrogen, Carlsbad, CA, USA) according to the protocols of manufacturer. Spectrophotometer (NanoDrop-2000, ThermoFisher Scientific) was to inspect the quantity and quality of RNA. Then miRNAs were reverse transcribed by miRNAs reverse transcription kit (Applied Biosystems) by using thermal circulatory apparatus (Applied Biosystems, Foster City, CA, USA). qRT-PCR was conducted using a SYBR® Green PCR Master Mix (Applied Biosystems) following the instructions. PCR cycling conditions were 95 °C for 5 minutes, 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. Gene expression data were standardized with the values for Cel-mir-39 (exosomal) and U6 (cellular). The sequences of primers used in the study were shown in Supplementary Table 1.

**Western blotting**

Cells were lysed in a lysis buffer (Cell Signaling Technology, USA) supplemented with protease inhibitors (Calbiochem, USA) at 4 °C for 30 min, while the exosomes were lysed in 20 µL lysis buffer at 4 °C for 10 min. Total protein concentration was quantified using the BCA protein assay kit (Pierce, USA). Western blotting was performed according to the standard protocol as previously described. Antibodies used were as follows: phosphorylated-AKT (p-AKT) (4060, Cell Signaling Technology), AKT (4691, Cell Signaling Technology); Cleaved caspase-3 (29034, Signalway Antibody), Bcl-2 (ab196495, Abcam), vascular endothelial growth factor (VEGF, ab52917, Abcam), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5174, Cell Signaling Technology) and horseradish peroxidase-conjugated secondary antibody (BioSharp, China). The bands were visualized by using enhanced chemiluminescence reagents and analyzed with a gel documentation system (iBrightCL1000, Invitrogen and Image Lab Software version 3.0).

**Exosomal miRNA sequencing**

The miRNAs sequencing was carried out in MIF-Exo, MSC-Exo and simIF-Exo. Exosomal miRNA-seq analysis was performed by RiboBio (Guangzhou, China) using the Illumina HiSeq 2500 instrument. Differentially expressed miRNAs were identified through \(|\log_2(\text{fold change})| \geq 1\) and P-value < 0.05 with the threshold set for up and down regulated genes. Bioinformatics analyses including differentially
expressed miRNA analysis, prediction of target genes of miRNA, gene ontology (GO) analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were also performed by RiboBio.

**miRNAs transfection**

Transfection of miR-133-3p mimics (50 nmol/L), miR-133-3p inhibitors (100 nmol/L) and their negative controls (50–100 nmol/L) were carried out using Lipofectamine 2000 (Invitrogen, USA) according to the instructions of manufacturer. Briefly, cardiac cells were cultured to 70% confluence. miR-133a-3p mimics, miR-133a-3p inhibitor and their negative controls were mixed with a transfection reagent, then added to the cell culture at a final concentration of 50–100 nmol/L. Transfection efficiency was determined by performing qRT-PCR. The sequences of miRNAs transfected were shown in Supplementary Table 2.

**Lentiviral constructions and infection**

The lentivirus construction in this study was obtained from GENECHEM (Shanghai, China). Two lentiviruses recombinant vectors were constructed. One is pLenti-EF1a-P2A-Puro-NRCMV-MIF-3Flag, used as MIF (overexpression of MIF) virus; and the other, hU6-MCS-Ubiquitin-EGFP-IRES-puromycin, served as siMIF (knocking down of MIF) virus. ucMSCs were seeded in 24 wells plates, when reached 50% confluence, they infected with MIF overexpression lentivirus or MIF knockdown lentivirus. Pools of stable transductions were generated by selection using puromycin (0.75 µg/ml) for three days. Fluorescent signals were viewed under the microscope. The MIF expression was evaluated by western blotting.

**Co-culture experiment**

HUVECs or H9C2 cardiomyocytes were seeded in 6 wells or 24 wells plates, once reached 70% – 80% confluence, cells were incubated with PBS, MIF-Exo, MSC-Exo, and siMIF-Exo (100 µg/mL) for 24 hours. After incubation, the cells were subjected to hypoxia and serum deprivation (H/SD) for another 12 hours, and they were evaluated by further experiments.

**Tube formation of HUVECs assay**

Angiogenesis of HUVECs was assessed by using the capillary tube forming assay. Then HUVECs (30,000 cells/well) were seeded in 96 wells plates covered with growth factor reduced Matrigel (356230; BD Biosciences, San Jose, CA, USA). After 6 hours, capillary like tube formation was photographed. Tube length was quantified by Image J software (National Institutes of Health, NIH).

**EdU and migration assay**

For proliferation evaluation, HUVECs were labeled with EdU for detection as described previously. HUVECs (2 × 10^4 cells/well) were seeded in 24 wells plates and they were incubated with EdU labeling reagent (Invitrogen, in 1:1,000 dilution) for 24 hours. Then cells were fixed with 4% paraformaldehyde for 30 min at room temperature, and treated with Triton X-100 for another 20 min, and then washed three times with PBS. Click-iT EdU Alexa Fluor 555 Imaging Kit (Invitrogen) was used according to
manufacturer's instructions. Finally, cells were stained with 4′, 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO, USA). Images were analyzed by Image J software.

For migration evaluation, treated HUVECs were cultured in 6 wells plates at the density of $2 \times 10^5$ cells/well with 1 mL test medium. After 24 hours, washed with PBS three times, and then scratched using a P200 pipette tip. Twelve hours later, cell migration was observed by microscopy and analyzed by using Image J software.

**Flow cytometry analysis of apoptosis**

Apoptosis was assayed by flow cytometry as described previously. For apoptosis analyzes, cells were harvested with trypsin, fixed with ice cold 70% ethanol. Apoptosis was assayed with Annexin V-fluorescein isothiocyanate and propidium iodide staining (3801660; Sony Biotechnology, San Jose, CA, USA). Data were analyzed with a CELL Quest kit (BD Biosciences).

**TUNEL analysis of apoptosis**

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were used for cell and tissue apoptosis. A TUNEL apoptosis detection kit (Roche, USA) was used to assay apoptosis. All cell nuclei were stained with DAPI. Apoptotic cells were dyed with TUNEL positive nuclei. Samples were examined with a microscope (Zeiss LSM510 META, German). The percentage of apoptotic nuclei was calculated for further analysis.

**MI model establishing and exosomes injection**

Our animal study protocol conforms to the Guide for the Care and Use of Laboratory Animals [National Institutes of Health, (NIH) Bethesda, MD, USA] and is approved by the Institutional Animal Care and Use Committee of the Nanjing Medical University for Laboratory Animal Medicine. Sprague Dawley rats (male, 6–8 weeks) were provided from Animal Core Facility of Nanjing Medical University (Nanjing, China). MI models were performed as previously described. Rats were anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg), then the left anterior descending coronary artery was ligated at 1.5 mm below the level of the inferior margin of the left auricle. After MI models successful establishing, exosomes (50ug) were equally divided into 4 portions for injection at the border of the infarction area.

**Assessment of cardiac function**

Cardiac function was evaluated with transthoracic echocardiography (Vevo 2000 high-resolution micro-imaging system) 14 days and 28 days after exosomes therapy using isoflurane inhalation (1.5%-2%). Left ventricular ejection fraction (LVEF) and left ventricular fraction shortening (LVFS) were analyzed using the Vevo 2000 workstation software.

**Masson trichrome staining**

Slides from paraffin embedded heart tissues were stained by Masson's trichrome to detect fibrosis. Infarct size was evaluated as the average ratio of fibrosis area to the total ventricular area. Images were
captured by scanning electron microscope (SU8010, Japan) and analyzed with Image J software.

**Immunofluorescence**

Immunofluorescence was performed as previously described. In brief, heart tissues were collected, fixed with 4% PFA, embedded in paraffin, and sectioned. For Immunofluorescence analyses, heart sections were stained with primary antibodies against CD31 (ab7388, Abcam, Cambridge, United Kingdom) and anti-Actin (A2066, Sigma-Aldrich). DAPI was used for nuclear counterstaining.

**Statistical analysis**

Continuous variables and categorical variables were described by means ± SEM and percentages. Independent Sample t-test was used to compare continuous variables between the two groups. Statistical differences among more than two groups were assessed by One-way ANOVA with the Bonferroni test. Chi-squared test was used for comparison between dichotomous variables. A value of P < 0.05 was considered statistically significant.

**Declarations**

**AUTHOR CONTRIBUTIONS**

WWZ, LS, FXZ contributed to the design of the study. WWZ, LS, PCZ, YWL, YQZ, JZ and WZ performed the experiments. WWZ, LS, YL, YYH, YYZ, GXC and FXZ contributed to the writing the manuscript. WWZ and LS contributed to the material support of the study. All authors read and approved the final manuscript.

**COMPETING INTERESTS**

The authors have declared that no competing interest exists.

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