

Exosomes From Adipose-derived Mesenchymal Stem Cells Protect the Myocardium Against Ischemia/Reperfusion Injury Through Wnt/ β -Catenin Signaling Pathway

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Abstract: Mesenchymal stem cells (MSCs) and their secreted exosomes exert a cardioprotective role in jeopardized myocardium. However, the specific effects and underlying mechanisms of exosomes derived from adipose-derived MSCs (ADMSCs) on myocardial ischemia/reperfusion (I/R) injury remain largely unclear. In this study, ADMSC-derived exosomes (ADMSCs-ex) were administered into the rats subjected to I/R injury and H9c2 cells exposed to hypoxia/reoxygenation (H/R). Consequently, administration of ADMSCs-ex significantly reduced I/R-induced myocardial infarction, accompanied with a decrease in serum levels of creatine kinase-myocardial band, lactate dehydrogenase, and cardiac troponin I (cTnI). Simultaneously, ADMSCs-ex dramatically antagonized I/R-induced myocardial apoptosis, along with the upregulation of Bcl-2 and downregulation of Bax, and inhibition of Caspase 3 activity in rat myocardium. Similarly, ADMSCs-ex significantly reduced cell apoptosis and the expression of Bax, but markedly increased cell viability and the expression of Bcl-2 and Cyclin D1 under H/R. Furthermore, ADMSCs-ex observably induced the activation of Wnt/ β -catenin signaling by attenuating I/R- and H/R-induced inhibition of Wnt3a, p-GSK-3 β (Ser9), and β -catenin expression. Importantly, treatment with Wnt/ β -catenin inhibitor XAV939 partly neutralized ADMSC-ex-induced antiapoptotic and

prosurvival effects in H9c2 cells. In conclusion, we confirmed that ADMSCs-ex protect ischemic myocardium from I/R injury through the activation of Wnt/ β -catenin signaling pathway.

Key Words: adipose-derived mesenchymal stem cells, exosomes, ischemia/reperfusion, Wnt/ β -catenin

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INTRODUCTION

Ischemic heart disease exhibits a high morbidity and mortality worldwide because of irreversible myocardial damage and eventually heart failure. Timely restoration of coronary blood flow is the most effective therapy for reducing myocardial infarct (MI) size and improving the clinical outcome.¹ However, reperfusion can also cause fatal myocardial ischemia/reperfusion (I/R) injury in jeopardized myocardium.² Therefore, effective strategies are urgently needed to protect cardiomyocytes against I/R injury.

Mesenchymal stem cells (MSCs) have emerged as one of the most important mediators for cardioprotection.^{3,4} It has been reported that MSC transplantation can protect the myocardium against myocardial infarction and ameliorate heart failure through various mechanisms,⁵ such as proangiogenic and anti-apoptotic pathways.^{6–8} Human umbilical cord tissue-derived MSCs can preserve cardiac function by enhancing angiogenesis, inhibiting apoptosis, and augmenting proliferation.⁷ Transplantation of adipose-derived MSCs (ADMSCs) can improve functions and remodeling of infarcted myocardium.⁹ However, the clinical application of MSCs is heavily restricted because of its potential risk of malignant transformation. Recently, the biological effects of paracrine factors derived from MSCs have attracted more and more attention.

Exosomes are small membrane-bound nanovesicles with a size of 30–100 nm, which can be obtained from a variety of sources,¹⁰ including the culture medium of MSCs.¹¹ It has been reported that exosomes show important roles in cell-to-cell communication by transferring genetic materials and proteins to target cells.¹² Moreover, exosomes are able to exhibit similar functional properties with their source cells.¹³ Previous reports suggest that exosomes secreted by MSCs possess cardioprotective effects.^{14–16} MSC-derived exosomes have been proved to enhance myocardial viability and prevent adverse remodeling after myocardial I/R injury.¹⁷ However, the specific effects and mechanisms of exosomes derived

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from various MSCs for myocardial I/R injury remain largely unknown.

Among all types of MSCs which are responsible for cardiac regenerative medicine, ADMSCs are more beneficial for clinical applications not only because of their multipotent character and plasticity¹⁸ but also because they are abundant and easily harvested by minimally invasive surgical techniques.¹⁹ It has been reported that combination of ADMSC and ADMSC-derived exosomes (ADMSCs-ex) can protect kidney from acute I/R injury.¹⁹ However, whether and how ADMSCs-ex play a protective role for myocardial I/R injury remain to be elucidated. In this study, ADMSCs-ex were found to efficiently protect the myocardium from I/R-induced necrosis and apoptosis and hypoxia/reoxygenation (H/R)-induced injury. Further studies indicate that ADMSCs-ex exert a cardioprotective effect by reducing myocardial injury through activation of the Wnt/ β -catenin signaling pathway.

MATERIALS AND METHODS

Ethics

All animal experimental procedures were conducted with approval from the Animal Care and Use Committee of Guangdong Medical University, following the Guide for the Care and Use of Laboratory Animals.

Isolation and Characterization of ADMSCs

ADMSCs were isolated from inguinal fat pad of Sprague–Dawley rats (Slack Experimental Animal Company, Shanghai, China) based on a published method.²⁰ Briefly, the lipoaspirate was minced and digested with 1% collagenase A (Gibco, Carlsbad, CA) and washed with phosphate-buffered saline (PBS). After filtration and centrifugation, cells were then resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% fetal calf serum (FCS; Gibco) and cultured in a humidified 5% CO₂ atmosphere at 37°C; the cells in passages 3 and 4 were identified and cultured for future experiments.

The collected ADMSCs were then observed by microscope and analyzed by flow cytometry. About 5×10^5 ADMSCs were resuspended in 1 mL of PBS and incubated at 4°C for 30 minutes with the following antirat antibodies (all from BD Biosciences, San Jose, CA): phycoerythrin-conjugated CD29, CD44, and D-related human leukocyte antigen (HLA-DR); fluorescein isothiocyanate-conjugated CD31, CD45, and CD105. Phycoerythrin-conjugated rat immunoglobulin G1 and fluorescein isothiocyanate-conjugated rat immunoglobulin G1 (BD Biosciences, San Diego, CA) were used as isotype controls. After washing, the specimens were analyzed within 1 hour using an FACSCalibur flow cytometer (BD Biosciences).

Preparation of ADMSCs-ex

ADMSCs-ex were collected and purified from ADMSC-conditioned medium by serial centrifugation and filtration steps at 4°C as previously described.²¹ The third passage of ADMSCs (10^6 cells) was seeded in 10% FCS-containing DMEM and incubated to 80%–90%

confluence for 24 hours. After washed with PBS, ADMSCs were maintained in serum-free medium for another 48 hours, and the culture supernatants of ADMSCs were then collected and sequentially centrifuged at 800 g for 10 minutes, 2000 g for 15 minutes, and 10,000 g for 30 minutes to remove dead cells and cellular debris. The supernatant was then ultrafiltrated and centrifuged with a 100,000 molecular weight cutoff devices (Millipore, Bedford, MA) at 100,000 g for 1 hour. The final exosome pellets were then obtained from 5 mL of conditioned medium by centrifugation at 100,000 g for 1 hour and resuspended in exosome-free PBS or stored at -80°C . Purified ADMSCs-ex were identified by transmission electron microscopy (JEOL JEM 2010F; Tokyo, Japan),²¹ and exosomal markers CD9, CD63, HSP70, and CD81²² were determined by western blot. The exosome fraction protein was determined using a Bicinchoninic acid (BCA) protein assay kit (Bio-Rad, Hercules, CA).

Myocardial I/R Model

Male Sprague–Dawley rats, weighing 275–300 g, were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg; Sigma, St. Louis, CA). Myocardial ischemia was produced by exteriorizing the heart with a left thoracic incision followed by making a slipknot (6–0 silk) around the left anterior descending coronary artery. After 30 minutes of ischemia, the slipknot was relaxed allowing for 3 hours of reperfusion. Forty Sprague–Dawley rats were randomly divided into 4 groups ($n = 10$ in each group)¹: Sham group, rats underwent the same surgical procedures except for coronary artery ligation²; I/R group, rats subjected to I/R injury only³; I/R+PBS group, rats subjected to I/R injury and infused with 200 μL exosome-free PBS through the tail vein within 5 minutes at the beginning of the reperfusion⁴; I/R+ADMSCs-ex group, rats subjected to I/R injury and treated with 200 μL PBS containing 400 μg of ADMSCs-ex through the tail vein within 5 minutes at the beginning of the reperfusion.

Evaluation of Serum Myocardial Enzyme and Myocardial Infarct Area

At the end of reperfusion, 5 mL blood was taken from the carotid artery. Then, serum was collected by centrifugation at 10,000 g for 10 minutes at 4°C. The levels of creatine kinase-myocardial band (CK-MB), lactate dehydrogenase (LDH), and cardiac troponin I (cTnI) were tested by enzyme-linked immunosorbent assay kits (R&D Systems, Minneapolis, MN). For the measurement of MI area, the rats were killed using cervical dislocation, and the hearts were removed and frozen at -20°C . The hearts were then cut into 2 mm slices, incubated in 1% 2,3,5-triphenyl tetrazolium chloride (TTC; Sigma) solution at 37°C for 15 minutes in the dark, and then fixed in 4% formaldehyde solution overnight. MI area was calculated as the ratio of the percentage of infarct size (TTC-negative area) to the area at risk (TTC-stained area).

Myocardial H/R Treatment

Myocardial cell H/R treatment was established in H9c2 myocardial cells (from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China) to simulate I/R injury model.

For hypoxic incubation, H9c2 cells were cultured in glucose-free and serum-free DMEM medium and exposed to a humidified incubation chamber flushed with a gas mixture of 95% N₂ and 5% CO₂ at 37°C. After 6 hours of hypoxic incubation, the cells were transferred to high glucose DMEM medium containing 10% FCS (reoxygenation medium), and reoxygenated in 95% air and 5% CO₂ at 37°C for 24 hours. H9c2 cells were randomly divided into 5 groups¹: control group, cells cultured in 10% FBS-containing DMEM medium under normoxic conditions²; H/R group, cells subjected to H/R injury only³; H/R+PBS group, cells subjected to H/R injury and 200 μL exosome-free PBS was added to the reoxygenation medium⁴; H/R+ADMSCs-ex group, cells subjected to H/R injury and 200 μL PBS containing 400 μg of ADMSCs-ex were added to the reoxygenation medium; and⁵ H/R+ADMSCs-ex+XAV939 group, cells subjected to H/R injury and 200 μL PBS containing 400 μg of ADMSCs-ex and Wnt/β-catenin inhibitors XAV939 (10 μM; Selleck, Houston, TX) were added to the reoxygenation medium.

Assessment of Myocardial Apoptosis

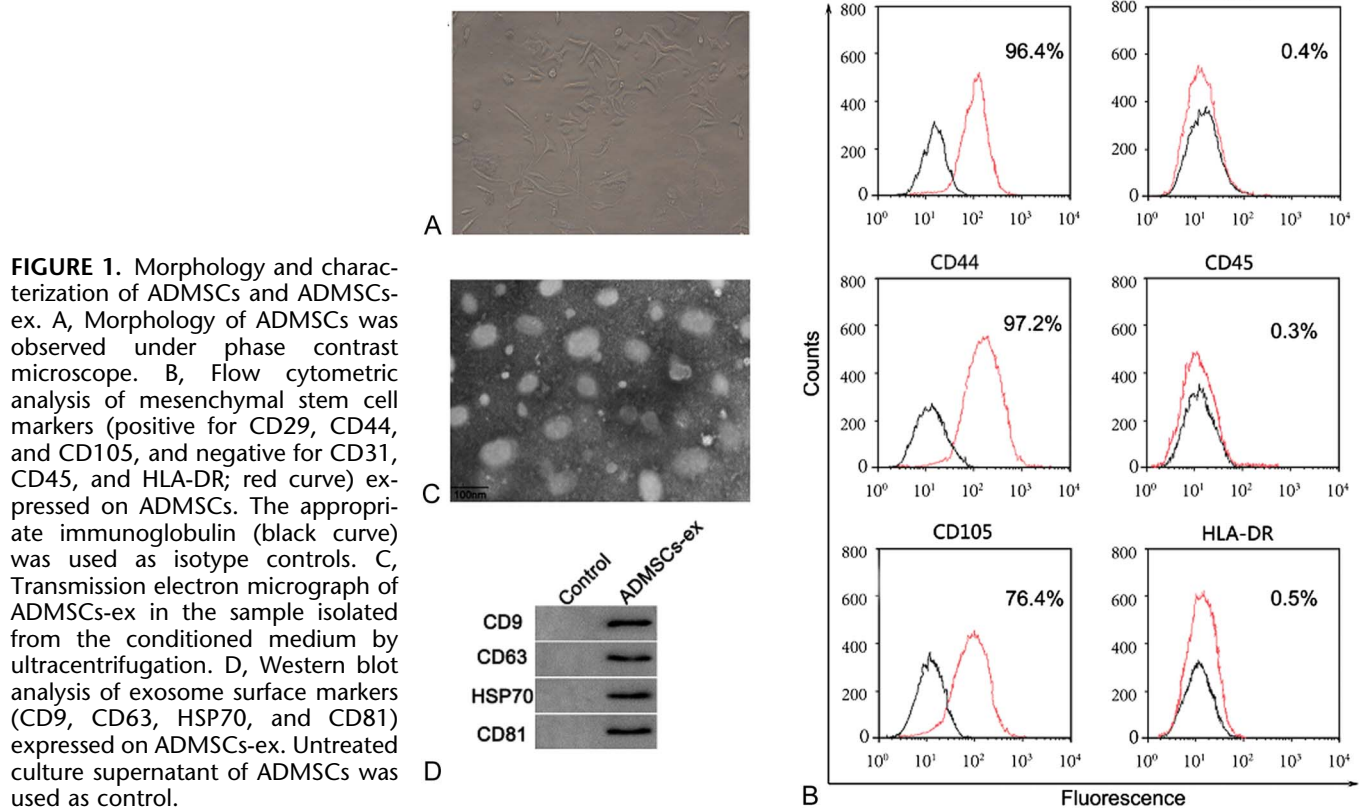
Myocardial apoptosis was detected using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay kit (R&D Systems). In addition, the expression levels of apoptosis-related Bcl-2 and Bax determined by western blot and Caspase 3 activity detected by Caspase fluorogenic protease assay kit (R&D Systems) were also used to evaluate myocardial apoptosis.

Assessment of Cell Viability

Cells were harvested after 24 hours of reoxygenation. Cell apoptosis was determined by TUNEL assay as has been noted. Cellular viability was evaluated using a Cell Counting Kit-8 (CCK-8; Sigma). Following different treatments in reoxygenation incubation for 24 hours, 10 μL CCK-8 solutions were added to the culture medium and incubated for an additional 2 hours. Subsequently, the optical density was measured at an absorbance of 450 nm, and the cell proliferation rate was calculated.

Western Blot Assays

Proteins from exosome-rich fractions, myocardial tissues, or cells were extracted with Radio-Immunoprecipitation Assay (RIPA) buffer, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblotting onto nitrocellulose membranes (Millipore). After being blocked with 5% skim milk for 1 hour, the membranes were incubated with primary antibodies against CD9, CD63, HSP70, CD81, Bcl-2, Bax, Cyclin D1, Wnt3a, Phospho-GSK-3β (Ser9) (p-GSK-3β), GSK-3β, β-catenin, and β-actin (Cell Signaling Technology, Danvers, MA) overnight at 4°C. Then the membranes were incubated with horseradish peroxidase-conjugated goat antirabbit IgG or goat antimouse IgG (Cell Signaling Technology) for 1 hour at room temperature. Proteins were visualized by the enhanced chemiluminescence detection system (Amersham, San Francisco, CA), and band intensities were quantified using ImageJ software from 3 independent results normalized by β-actin.



Statistical Analysis

The SPSS 22.0 software was used for statistical analyses. All data were expressed as mean \pm standard deviation (SD) from a minimum of 3 replicates. Differences between 2 groups were calculated using the Student's *t* test. Differences between multiple groups were calculated with one-way analysis of variance. $P < 0.05$ was considered statistically significant.

RESULTS

Morphology and Characterization of ADMSCs and ADMSCs-ex

ADMSCs showed a characteristic morphology of slender spindle-like cells (Fig. 1A). They expressed highly CD29, CD44, and CD105, but are persistently negative for CD31, CD45, and HLA-DR (Fig. 1B), as previously reported.²³ Transmission electron microscopy revealed the presence of nanovesicles with diameters ranging from about 30 to 100 nm in the extracted sample from the culture supernatants of ADMSCs (Fig. 1C). In addition, the protein markers of exosomes, such as CD9, CD63, HSP70, and CD81 were detectable in exosome-rich fractions, but absent in untreated culture supernatant of ADMSCs (Fig. 1D).

ADMSCs-ex Protect Against I/R-induced Myocardial Injury In Vivo

ADMSCs-ex implantation significantly reduced the myocardial infarction area in hearts subjected to I/R injury (Fig. 2A). The increase of serum levels of CK-MB, LDH, and cTnI induced by I/R was dramatically decreased when treated with ADMSCs-ex (Figs. 2B–D). I/R-induced apoptosis was partly attenuated after ADMSCs-ex treatment (Fig. 2E). Moreover, I/R injury led to a remarkable decrease in Bcl-2 levels and an obvious increase in Bax expression, both of which were abolished by ADMSCs-ex implantation (Fig. 2F). In addition, I/R-induced activation of Caspase 3 was dramatically decreased by ADMSCs-ex treatment (Fig. 2G).

ADMSCs-ex Suppress H/R-induced Cell Injury in H9c2 Cells In Vitro

To further evaluate the effect of ADMSCs-ex on myocardial I/R injury, we constructed H/R-induced H9c2 cell models in vitro to simulate myocardial I/R injury. As shown in Figure 3A, treatment with ADMSCs-ex significantly reduced H/R-induced increase in the percentage of apoptotic cells. ADMSCs-ex implantation also effectively rescued cell viability under H/R condition (Fig. 3B). Moreover, H/R-induced downregulation of Bcl-2 and Cyclin D1 and upregulation of Bax were significantly abrogated by ADMSCs-ex treatment (Figs. 3C, D).

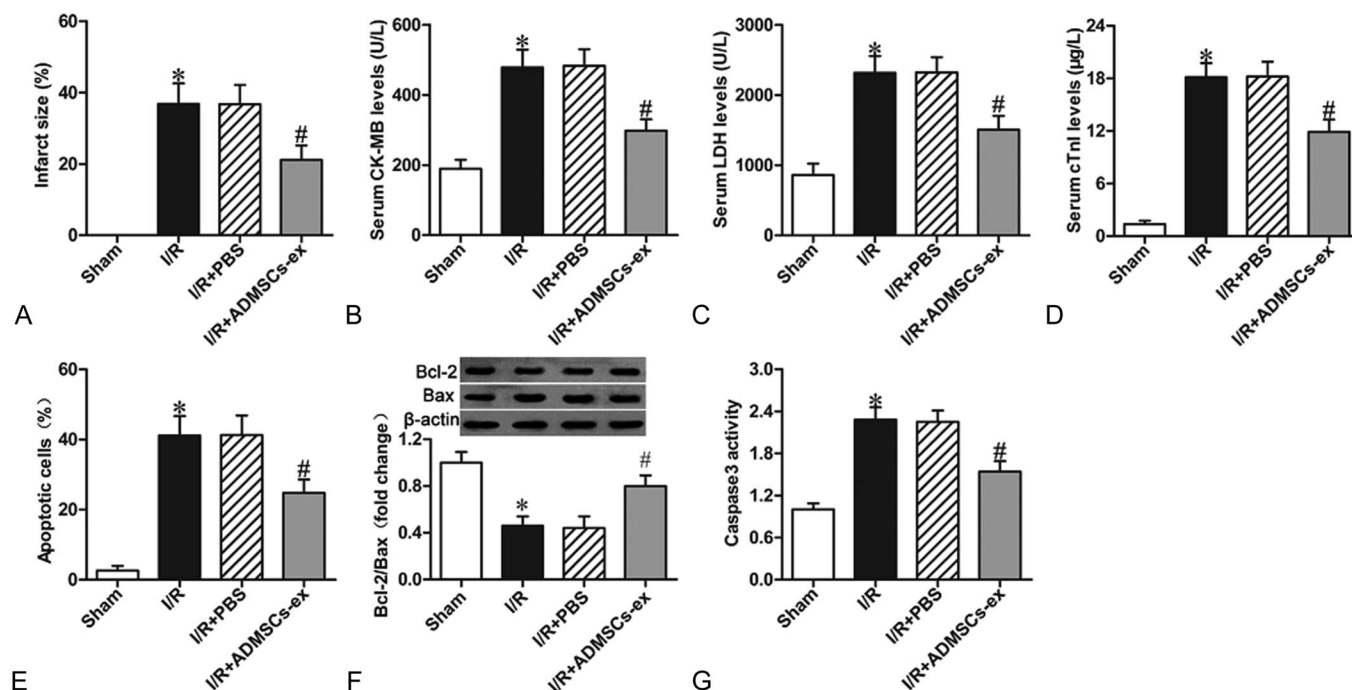


FIGURE 2. ADMSCs-ex reduce I/R-induced myocardial necrosis and apoptosis in vivo. Rats were subjected to I/R injury and treated with ADMSCs-ex, or not. A, Percentage of myocardial infarction area in different groups. B–D, The serum levels of CK-MB (B), LDH (C), and cTnI (D) in the myocardia of rats were determined by enzyme-linked immunosorbent assay. E, Percentage of TUNEL-positive apoptotic cells among total cardiomyocytes in the myocardia of rats. F, Representative western blot analysis of protein expression of Bcl-2 and Bax in myocardia of rats. β -actin was used as the loading control. The bar graph shows the ratio of Bcl-2/Bax. G, Changes of Caspase 3 activities in the myocardium of rats. Data are expressed as mean \pm SD ($n = 3$). * $P < 0.05$ versus Sham group, # $P < 0.05$ versus I/R group.

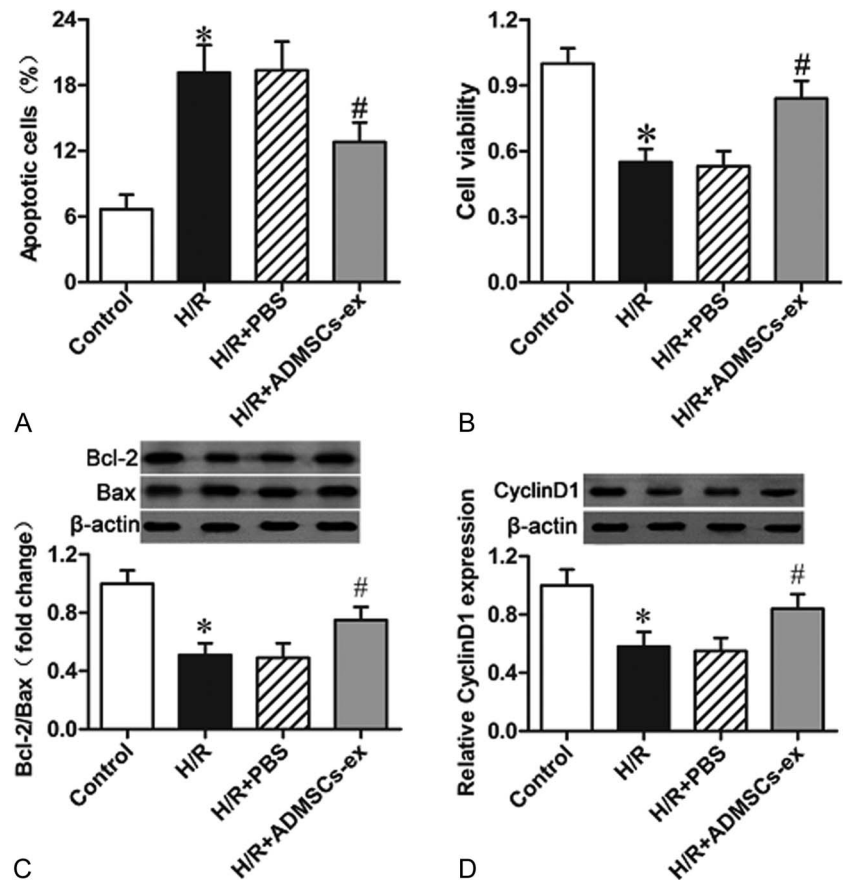


FIGURE 3. ADMSCs-ex attenuate H/R-induced apoptosis and promote cell survival in H9c2 cells. **A,** Percentage of TUNEL-positive apoptotic cells in different groups. **B,** Cell viability in different groups was determined by CCK-8 assay. **C and D,** Representative western blot analysis of Bcl-2 and Bax (**C**), as well as Cyclin D1 (**D**) levels in H9c2 cells. β -actin was used as the loading control. Bar graph shows the ratio of Bcl-2/Bax (**C**) and quantification of Cyclin D1 (**D**) expression. Data are expressed as mean \pm SD ($n = 3$). * $P < 0.05$ versus control group, # $P < 0.05$ versus H/R group.

ADMSCs-ex Activate Wnt/ β -catenin Signaling to Protect Against Myocardial I/R Injury

Western blot analysis showed that ADMSCs-ex significantly inhibited I/R or H/R-induced decrease in the expression of Wnt3a, p-GSK-3 β (Ser9), and β -catenin in rat myocardium (Fig. 4A) and in H9c2 cells (Fig. 4B), but not in the expression of GSK-3 β , indicating that ADMSCs-ex implantation induced activation of the Wnt/ β -catenin signaling pathway. Importantly, ADMSCs-ex-induced decrease of cardiac apoptosis and increase of cell viability can be partially abolished by the Wnt/ β -catenin inhibitor XAV939 (Figs. 4C, D). Furthermore, the upregulation of Bcl-2 and Cyclin D1 concomitant with the downregulation of Bax triggered by ADMSCs-ex was partly abolished in H9c2 cells after XAV939 treatment (Fig. 4E).

DISCUSSION

Regardless of great advances in the treatment of cardiovascular disease, ischemic heart disease remains one of the leading causes of death around the world. Myocardial infarction and myocardial I/R injury have become great problems in clinical treatment. Exosomes secreted by MSCs were considered a new therapeutic strategy for cardiovascular diseases because of their protective effects on myocardium after myocardial infarction or I/R injury.^{4,14,24} Here, in this study, we demonstrated that the administration of ADMSCs-ex significantly ameliorated I/R-induced myocardial necrosis and

apoptosis in a rat myocardial I/R injury model, reduced H/R-induced myocardial apoptosis, and enhanced myocardial viability in H9c2 cardiomyocytes. The possible mechanisms underlying the cardioprotective effects of ADMSCs-ex may be associated with the activation of Wnt/ β -catenin signaling pathway.

Exosomes are small membrane-bound nanovesicles containing many functional biomolecules, such as nucleic acids and proteins.²⁵ They play important roles in cell-to-cell communication by transferring these biomolecules from a donor cell to a recipient cell.^{25,26} In this study, we successfully isolated and characterized exosomes from ADMSCs, which are positive for CD29, CD44, and CD105, but negative for CD31, CD45, and HLA-DR.²³ Our observations demonstrated that the protein-containing nanovesicles with diameters from 30 to 100 nm in the extracted samples were positive for CD9, CD63, HSP70, and CD81, the specific markers of exosomes,²⁷ confirming that the nanovesicles are exosomes.

Myocardial infarction size is an important index for assessing the consequences of myocardial I/R injury. The myocardial enzyme spectrum indexes CK-MB, LDH, and cTnI serve as sensitive indicators to assess the severity of myocardial infarction.²⁸ A prominent infarct size and increased activities of CK-MB, LDH, and cTnI observed in this research are indicative of myocardial damage and cardiac insufficiency in rat after I/R injury. Previous studies indicating that exosomes derived from cardiac progenitor and

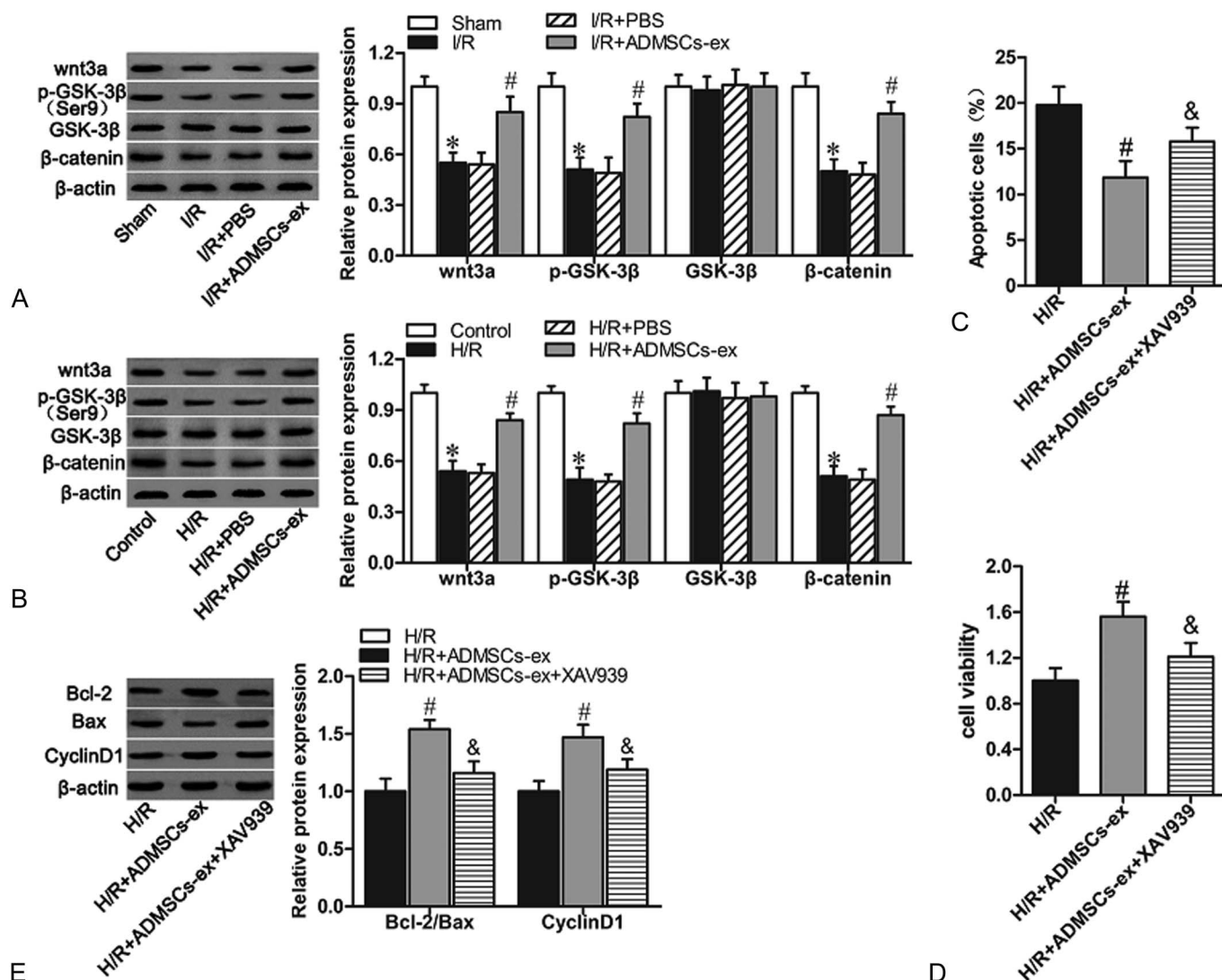


FIGURE 4. Wnt/ β -catenin signaling involves in ADMSC-ex–induced cardioprotective effects. A and B, Representative western blot analysis for Wnt3a, p-GSK-3 β (Ser9), GSK-3 β , and β -catenin in rat myocardium (A) and in H9c2 cells (B). β -actin was used as the loading control. Bar graph shows the quantification of Wnt3a, p-GSK-3 β (Ser9), GSK-3 β , and β -catenin expression. C, Percentage of TUNEL-positive apoptotic cells in different groups. D, Cell viability in different groups was determined by CCK-8 assay. E, Representative western blot analysis of Bcl-2, Bax, and Cyclin D1 levels in H9c2 cells. β -actin was used as the loading control. Bar graph shows the quantification of Bcl-2/Bax and Cyclin D1 expression. Data are expressed as mean \pm SD (n = 3). * P < 0.05 versus Sham or control group, # P < 0.05 versus I/R or H/R group. & P < 0.05 versus H/R+ADMSCs-ex group.

plasma can protect myocardium from I/R injury.^{29,30} In this study, ADMSCs-ex infusion not only diminished the infarct size but also attenuated the serum levels of CK-MB, LDH, and cTnI, suggesting that ADMSCs-ex exerted cardioprotective effects after I/R injury.

Apoptosis is recognized as a major mechanism of cell death and cardiac damage during myocardial I/R injury.²⁸ Various studies have demonstrated that I/R injury induced massive apoptosis in cardiomyocytes, and diminution of cellular apoptosis can ameliorate myocardial damage.^{28,29} Some earlier studies showed that exosomes derived from induced pluripotent stem cells and human cardiac progenitor cells can prevent cardiomyocytes apoptosis in ischemic myocardium

after myocardial infarction.^{31,32} Our research showed that ADMSCs-ex implantation significantly decreased I/R-induced myocardial apoptosis, rescued I/R-induced downregulation of Bcl-2, and repressed I/R-induced upregulation of Bax and Caspase 3 activity in rat myocardium. These results demonstrated that ADMSCs-ex induced antiapoptotic effects in rat cardiomyocytes after I/R injury.

To further understand the protective mechanism of ADMSCs-ex against I/R injury in cardiomyocytes, H/R injury was induced in H9c2 cardiomyocytes to simulate myocardial I/R injury in vitro. Consequently, ADMSCs-ex implantation markedly reduced H/R-induced cell apoptosis, suppressed H/R-induced downregulation of Bcl-2 and upregulation of Bax,

indicating that ADMSCs-ex attenuated H/R-induced apoptosis. Moreover, ADMSCs-ex implantation significantly reduced H/R-induced loss of cell viability, and markedly aggravated the expression of Cyclin D1, suggesting that ADMSCs-ex enhanced cell viability after H/R injury. Taken together, these results indicated that ADMSCs-ex exert antiapoptotic and pro-survival effects in H9c2 cells under H/R condition.

The Wnt/ β -catenin signaling pathway is known to be a critical regulator of survival and apoptosis of cardiomyocytes.³³ ADMSCs-ex implantation significantly abolished I/R-induced inhibition of Wnt/ β -catenin signaling. Furthermore, pretreatment with Wnt inhibitor XAV939 partly neutralized ADMSC-ex-induced antiapoptotic and pro-survival effects in H9c2 cells, suggesting that ADMSCs-ex exert cardioprotective effects in cardiomyocytes through the Wnt/ β -catenin signaling pathway after myocardial I/R. However, this study did not clarify how ADMSCs-ex affect Wnt/ β -catenin signaling and whether some molecules secreted by ADMSCs-ex are involved in this process, such as microRNAs and proteins. This question could be our next research.

CONCLUSION

In summary, we conclude that ADMSCs-ex administration of ADMSCs-ex can protect ischemic myocardium from I/R injury through activating the Wnt/ β -catenin signaling pathway by exerting the antiapoptotic and pro-survival effects on cardiomyocytes. This report may provide a novel therapeutic strategy for reducing myocardial damage in ischemic heart disease.

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