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HSP20-mediated cardiomyocyte exosomes improve cardiac function in mice with myocardial infarction by activating Akt signaling pathway

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Abstract. – OBJECTIVE: To explore the role of heat shock protein 20 (HSP20)-mediated cardiomyocyte exosomes in the cardiac function in mice with myocardial infarction via the activation of the protein kinase B (Akt) signaling pathway.

MATERIALS AND METHODS: A total of 30 mice were enrolled to establish the model of myocardial infarction. Next, these mice were divided into three groups, namely Blank group (healthy mice), Model group (mouse models of myocardial infarction), and HSP20 group (mouse models of myocardial infarction transfected with lentivirus to overexpress HSP20). After that, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining assay was performed to detect myocardial apoptosis. Reactive oxygen species (ROS) accumulation in myocardial tissues was determined via dihydroethidium (DHE) staining assay. Western blotting was employed to analyze the expression level of Akt. The expression levels of inflammatory factors tumor necrosis factor-alpha (TNF-α) and interleukin 1 beta (IL-1β) in HSP20-mediated cardiomyocyte exosomes were measured through quantitative real time polymerase chain reaction (qRT-PCR).

RESULTS: Compared with that in Blank group, the number of cardiomyocyte exosomes was increased in Model group and HSP20 group under anoxic conditions ($p<0.05$). The results of quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) proved that the HSP20 messenger ribonucleic acid (mRNA) expression in mediated cardiomyocyte exosomes was significantly lower in Model group than that in Blank group ($p<0.05$), while in HSP20 group, it was overtly higher than that in Model group but clearly lowered compared with that in Blank group ($p<0.05$). The protein expression of Akt in cardiomyocyte exosomes was evidently decreased in Model group compared with that in Blank group ($p<0.05$), while it was notably increased in HSP20 group compared with that in Model group ($p<0.05$). In comparison with Blank group, Model group had significantly elevated mRNA expression levels of TNF-α and IL-1β. The mRNA expression levels of TNF-α and IL-1β in HSP20 group were remarkably lower than those in Model group ($p<0.05$). The results of TUNEL assay revealed that the overexpression of HSP20 affected myocardial apoptosis. The myocardial apoptosis index in Model group [(38.42±2.52) %] was higher than that in Blank group [(9.74±1.21) %], HSP20 group had a significantly decreased myocardial apoptosis index [(22.36±2.13) %] in comparison with Model group ($p<0.05$). In accordance with DHE staining comparison, the accumulation of ROS in myocardial tissues in Model group was significantly higher than that in Blank group ($p<0.05$) and HSP20 group ($p<0.05$).

CONCLUSIONS: We demonstrated that HSP20-mediated cardiomyocyte exosomes activate the AKT signaling pathway, repress TNF-α and IL-1β factors, and alleviate myocardial infarction.

Key Words:
HSP20, Akt, Myocardial infarction, TNF-α, IL-1β, Exosomes.

Introduction

With the continuous development of economy, improvement in the quality of life, a faster pace of work and life and gradually-westernized dietary habits, the incidence rate of myocardial infarction

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largely shows an upward tendency, with lower age of onset. Cardiovascular diseases have gradually become the leading cause of death worldwide. The statistics have revealed that among patients with cardiovascular and cerebrovascular diseases, acute myocardial infarction is one of the most important causes of death[1]. Moreover, in China, the frequency of myocardial infarction attack is increasing in a short period of time, and the age of onset is getting lower and lower. Therefore, it is very important to determine the predisposing factors and risk factors for myocardial infarction and use these factors in preventing the disease[2,3]. Furthermore, most patients miss the best time to treat because they ignored the early symptoms and received no treatment, thereby leading to so severe myocardial infarction that results in death, so the prevention and treatment of myocardial infarction should be strictly controlled. Exosomes, intracellular multivesicular bodies, contain various bioactive constituents such as proteins and ribonucleic acids (RNAs), which are molecules for intercellular information transfer and vectors for genetic material transfer. Heat shock protein 20 (HSP20) mediates multiple signaling pathways induced by exosomes in myocardial cells, thus regulating biological functions[4]. For the past few years, a tremendous advance on HSP20 has been made in China, and the significance of HSP20 in smooth muscle and its tissues is clarified[5]. Some studies[6] have manifested that β epinephrine triggers the expression of HSP20 in the heart. Besides, overexpressed HSP20 enhances cardiac function and protects the heart. Protein kinase B (Akt), a class of autokinase, plays a vital role in various cell activities[7]. Moreover, it exerts an important cardioprotective effect[8,9]. In this work, mouse models of myocardial infarction were established to explore the influence of HSP20-mediated cardiomyocyte exosomes on cardiac function in mice with myocardial infarction by activating the Akt signaling pathway, providing a favorable basis for the subsequent therapy of myocardial infarction.

Materials and Methods

Main Antibodies, Drugs, and Instruments

Triphenyl tetrazolium ammonium chloride (Linuo Pharma, Jinan, China), exosome extraction kit (ExoQuick-TC kit) and exosome fluorescent labeling (Promega, Madison, WI, USA), terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) staining kit (Promega, Madison, WI, USA), RNA extraction kit (Promega, Madison, WI, USA), quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) kit (Beverly, MA, USA), RNA reverse transcription kit (Sigma-Aldrich, St. Louis, MO, USA), rabbit anti-rat HSP20 primary antibody (Upstate, Richfield Springs, NY, USA), goat anti-rabbit AF555 secondary antibody (Upstate, Richfield Springs, NY, USA), phosphor-Akt (Beverly, MA, USA), penicillin/streptomycin (Hyclone, South Logan, UT, USA) and paraffin (Nalgene). A microscope (Nikon, Tokyo, Japan), a slice embedding machine (Thermo-Fisher Scientific, Waltham, MA, USA), a refrigerated centrifuge (Thermo-Fisher Scientific, Waltham, MA, USA), an ultra-clean workbench (Antai Airtech Co., Ltd., Suzhou, China), an upright microscope (Olympus, Tokyo, Japan), a freezing microtome (Leica, Munchen, Germany), a refrigerator (Sanyo, Osaka, Japan), a paraffin slicing machine (Leica, Munchen, Germany), a dryer (Leica, Wetzlar, Germany) and a fluorescence microscope (Olympus, Tokyo, Japan).

Animal Grouping and Cell Culture

This investigation was approved by the Animal Ethics Committee of Yantaishan Hospital Animal Center. A total of 30 male mice weighing (230+30) g and aged 8 weeks old [laboratory animal production license number: SCXY (Guangdong) 2018-0011] purchased from the Laboratory Animal Center of Southern Medical University were housed in an animal room with normal illumination at a temperature of 25°C and humidity of 45%, with free access to food and water. After one week, grouping, modeling, and experiments were performed. These mice were divided into three groups: Blank group (healthy rats, n=10), Model group (mouse models of myocardial infarction, n=10), and HSP20 group (mouse models of myocardial infarction injected with 5 μmol lentivirus to overexpress HSP20 *via* tail vein once per day for 7 days). Mice in Blank group were provided with normal drinking water and illumination and subcutaneously injected with normal saline. Those in Model group and HSP20 group were subjected to ligation of left main coronary artery to replicate models of acute myocardial infarction. After successful modeling, mice in Blank group, Model group, and HSP20 group were anesthetized by injecting chloral hydrate and then sacrificed, and the heart was taken out.

Next, the heart was placed in phosphate-buffered saline (PBS) to rinse off the residual blood, cut into pieces (about 2 mm^3) and digested with 1 g/L type II collagenase for 5 min. Thereafter, 100 mL/L fetal bovine serum medium was added to terminate the digestion when the fragments were completely digested. Then, cells were collected in a centrifuge tube and centrifuged at 1000 r/min for 8 min, followed by precipitation of cells to culture suspension. After that, the suspension was collected in a 50 mL culture flask and subjected to differential attachment for 60 min. Next, non-adherent cells were aspirated to a new culture flask (plate) for continuous cultivation, i.e., culture of primary myocardial cells.

Isolation and Purification of Exosomes

Cultured primary myocardial cells were suspended *via* trypsinization and centrifuged at 4°C and 2,000 g/min for 30 min in accordance with the procedures on Total Exosome Isolation Kit (Invitrogen, Carlsbad, CA, USA), and the supernatant was taken. Next, 500 μL exosome extract was added to 1,000 μL supernatant, mixed via perversion, and placed at 4°C overnight. Thereafter, the mixture was centrifuged at 4°C and 10,000 g/min for 60 min, the supernatant was discarded, and the sediment was re-suspended by addition with 200 μL Buffer for later use. The above exosome stock was taken to extract exosomes using the Total Exosome RNA Isolation Kit (Invitrogen, Carlsbad, CA, USA) according to its instructions, and exosomes extracted were stored in an airtight container at -80°C. A transmission electron microscope was used to observe the exosome. The exosome stock was prepared for later use in experiments.

TUNEL Staining Assay

TUNEL staining of myocardial cells in each group of mice: Sections were deparaffinized, hydrated and washed, and water was removed. Then, the sections were added with TdT reaction solution and 50 mg exosomes, respectively. Next, fluorescein labeling was conducted during catalysis. Thereafter, the sections were frozen according to the steps for frozen section preparation, taken out, and rinsed for 3 times (15 min in total). Later, the sections were completely covered with 0.1% Triton and placed at room temperature for 5 min. After that, rabbit anti-rat HSP20 primary antibody was added for incubation at 4°C overnight, followed by rinsing with PBS for 3 times. Then, goat anti-rabbit AF555-labeled fluorescent secondary antibody was added for incubation at room temperature for 60 min. Apoptotic cells were yellow-brown when observed using the microscope. Apoptotic myocardial cells showed yellow-brown positive granules. Anti-quenching agent containing DAPI was added, and an inverted fluorescence microscope was utilized for observation.

Detection of Reactive Oxygen Species (ROS) in Myocardial Tissues Through Dihydroethidium (DHE) Staining

Mouse myocardial cells and 50 mg exosome were placed in an EP tube, added with buffered saline solution, probe solution and tissue perfusate, and diluted to a concentration of 20%, followed by staining. Depending on the ROS content in cells, 4 μL dihydroethidine was added for incubation in a dark room at 37°C for 20 min. After that, cells or tissues were washed with fresh solution. Thereafter, the sections were frozen in accordance with the steps for frozen tissue section preparation, and then the frozen tissue sections were taken out, washed in PBS for 3 times, and rinsed for 15 min. 10 mmol/L HSP20 primary antibody was diluted at 1:400, and 20 μmol/L goat anti-rabbit AF555-labeled secondary antibody was diluted at 1:500. Next, the tissues were covered with staining solution and incubated in a wet box at room temperature or 37°C for 60-90 min. The fluorescence intensity was observed using the inverted microscope.

Analysis of Akt Expression Via Western Blotting

Mouse cardiomyocyte exosome solution was collected into an Eppendorf (EP) tube and added with 2 μL trypsin for digestion that was terminated 3 min later, and the cells were continuously pipetted, followed by centrifugation. After that, the liquid was collected and precipitated. Next, 200 μL radioimmunoprecipitation assay (RIPA) lysis solution (100:1) (Beyotime, Shanghai, China) was added, and the supernatant was collected, centrifuged at low temperature and 12,000 r/min for 10 min, added with prepared lysis solution and stirred evenly. After sedimentation, the solution was put on ice for 10 min of lysis. The protein concentration was measured through the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Then, protein denaturation was conducted via a metal bath, followed by cryopreservation at –80°C. Thereafter, 10 μg protein sample was taken,

subjected to electrophoresis, transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), blocked with 5% skim milk powder for 1 h, and incubated with HSP20 primary antibody (1:400) at 4°C overnight and then goat anti-rabbit AF555-labeled secondary antibody (1:500) at room temperature for 60 min. After that, the membrane was washed with PBS for 3 times, followed by development with enhanced chemiluminescence (ECL) and film exposure.

Measurement of HSP20, Tumor Necrosis Factor-Alpha (TNF-α) and Interleukin 1 Beta (IL-1β) Expression Levels by QRT-PCR

A total of 50 mg mouse cardiomyocyte exosome solution was taken, and the RNA kit was used for RNA extraction. The reverse transcription and fluorescence qRT-PCR were conducted according to the instructions of the RNA reverse transcription kit and qRT-PCR kit. 50 mg mouse myocardial cells were separately extracted, added with polyacrylamide gel for protein separation, transferred onto a film, blocked with 5% skim milk powder for 1 h, washed with Tris-Buffered Saline and Tween (TBST), and incubated with HSP20 primary antibody and goat anti-rabbit secondary antibody. After that, the transfer to membrane was carried out again, and relative expression levels of corresponding TNF-α, HSP20 and IL-1β were obtained. Primer sequences are shown in Table I.

Statistical Analysis

Experimental data were expressed as mean ± standard deviation. *t*-test and one-way analysis of variance (ANOVA) were employed to analyze statistical significance. Statistical Product and Service Solutions (SPSS) 21.0 software (IBM, Armonk, NY, USA) was used for statistical analysis of all data. $p<0.05$ suggested the difference was statistically significant.

Results

Structure of Cardiomyocyte Exosomes

The morphological structure of exosomes was observed using the transmission electron microscope (´100 nm and ´50 nm), and it could be seen that the exosome was about 40-100 nm. Compared with that in Blank group, the number of exosomes was increased in Model group and HSP20 group under anoxic conditions ($p<0.05$) (Figure 1).

Messenger RNA (mRNA) Expression of HSP20 in Cardiomyocyte Exosomes

The results of qRT-PCR analysis confirmed that the mRNA expression of HSP20 in cardiomyocyte exosomes was overtly lower in Model group than that in Blank group ($p<0.05$), and in HSP20 group, it was significantly higher than that in Model group ($p<0.05$) but clearly lower than that in Blank group ($p<0.05$), implying that the mRNA level of HSP20 is high in healthy mice and low in mice with myocardial infarction (Figure 2).

Overexpressed HSP20-Mediated Cardiomyocyte Exosomes Activated Akt

Western blotting analysis revealed that Akt protein expression was significantly decreased in Model group compared with that in Blank group ($p<0.05$), and in comparison, with Model group, HSP20 group exhibited a notably elevated Akt protein expression ($p<0.05$) (Figures 3 and Figure 4).

Table I. Primer sequences.

Gene		Primer sequence
HSP20	Forward	5'-CCAGAGGAAATCTCTGTCAAGG-3'
	Reverse	5'-TGGAACTCTCGAGCAATGAACC-3'
TNF-α	Forward	5'-ATCTTCTCAACCCCCGAGTGG-3'
	Reverse	5'-GGGTTTGCTACAACATGGGC-3'
IL-1β	Forward	5'-CCATCATAAAGGGCGACACAGG-3'
	Reverse	5'-TTTCGAGGACCGACCTCTACAC-3'
β-actin	Forward	5'-TTCCTTCCTGGGTATGGAATC-3'
	Reverse	5'-TGTGTTGGCATAGAGGTCTTA-3'

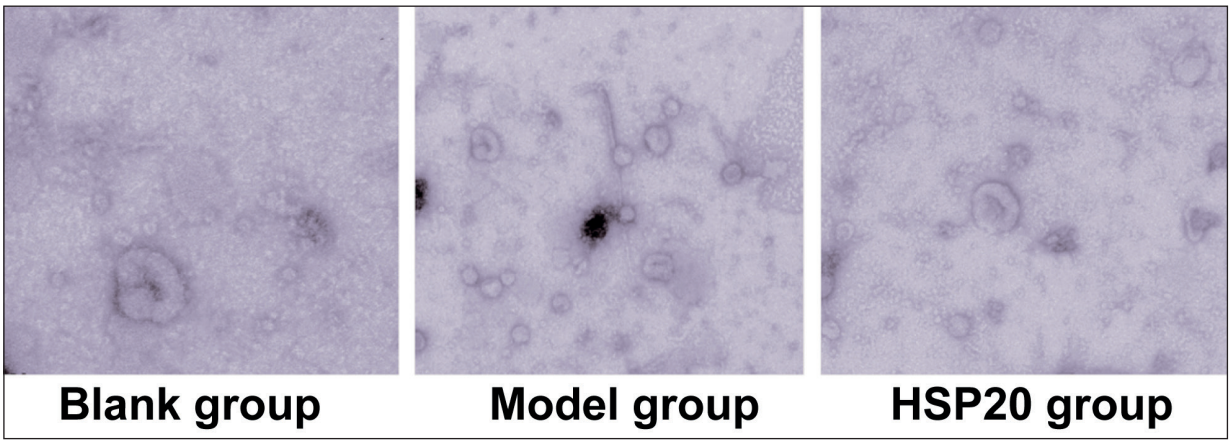

Figure 1. Structure of cardiomyocyte exosomes in each group.

Overexpressed HSP20 Cardiomyocyte Exosomes Activated Akt Signal and Affected TNF-α and IL-1β

QRT-PCR analysis proved that compared with that in Blank group, the mRNA expression level of TNF-α in mouse cardiomyocyte exosomes was significantly increased in Model group (p<0.05), and it was evidently lowered in HSP20 group compared with that in Model group (p<0.05). The mRNA expression level of IL-1β in mouse cardiomyocyte exosomes was significantly higher in Model group than that in Blank group (p<0.05), and it was remarkably lower in HSP20 group than that in Model group (p<0.05) (Figures 5 and 6).

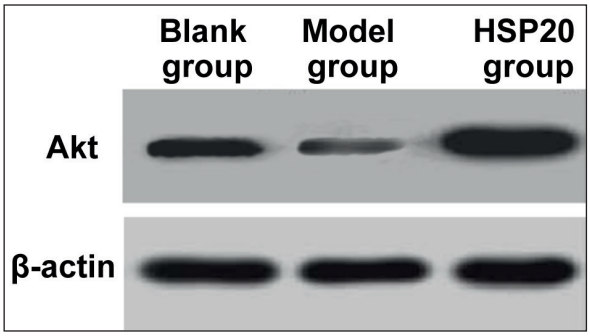

Figure 3. Akt protein expression in cardiomyocyte exosomes in each group of mice.

Effect of HSP20 Overexpression on Myocardial Apoptosis

The results of TUNEL assay showed that HSP20 overexpression had an influence on myocardial apoptosis. Compared with that in Blank group [(9.74±1.21) %], the myocardial apoptosis index in Model group [(38.42±2.52) %] was increased (p<0.05). Compared with that

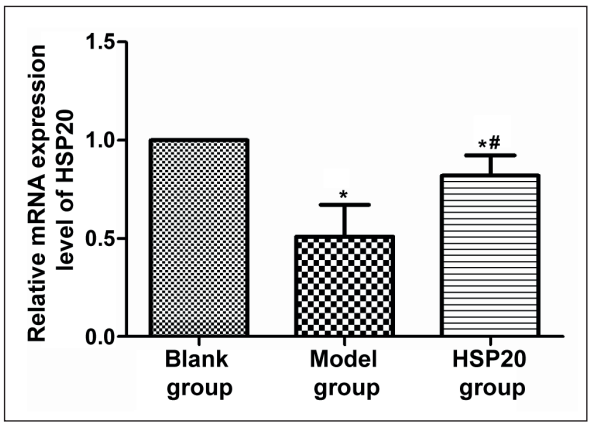

Figure 2. Relative mRNA expression level of HSP20. Note: *p<0.05 *vs.* Blank group, #p<0.05 *vs.* Model group.

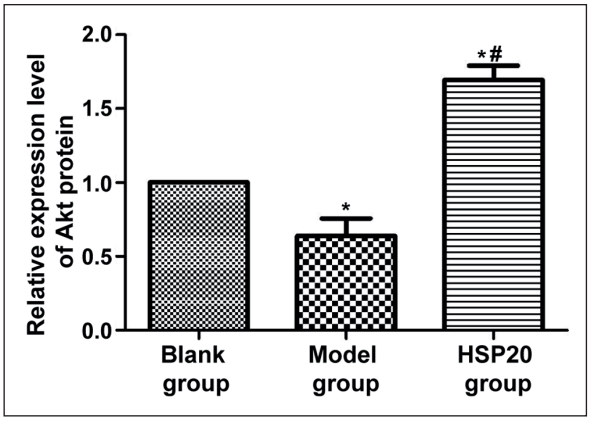

Figure 4. Relative expression level of Akt protein in cardiomyocyte exosomes in each group of mice. Note: *p<0.05 *vs.* Blank group, #p<0.05 *vs.* Model group.

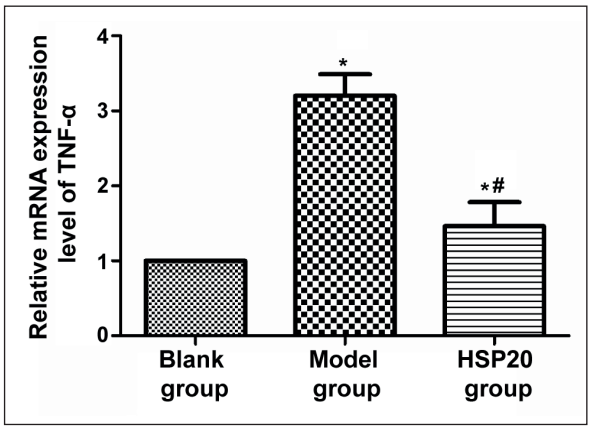

Figure 5. Relative mRNA expression level of TNF-α in cardiomyocyte exosomes in each group of mice. Note: *p<0.05 *vs.* Blank group, $^{\#}p$<0.05 *vs.* Model group.

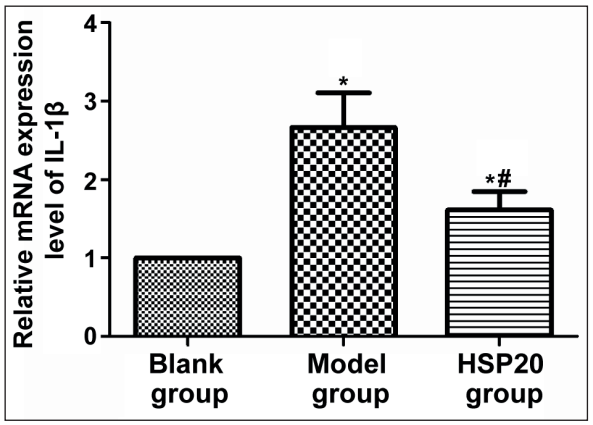

Figure 6. Relative mRNA expression level of IL-1β in cardiomyocyte exosomes in each group of mice. Note: *p<0.05 *vs.* Blank group, $^{\#}p$<0.05 *vs.* Model group.

in Model group, the apoptotic index in HSP20 group [(22.36±2.13) %] was significantly lowered (p<0.05) (Figures 7 and 8).

Impact of HSP20 Overexpression on ROS in Myocardial Tissues

Based on DHE staining results, the accumulation of ROS in myocardial tissues was overtly increased in Model group compared with that in Blank group (p<0.05), and it was significantly decreased in HSP20 group compared with that in Model group (p<0.05), suggesting that the overexpression of HSP20 is able to reduce the accumulation of ROS in myocardial tissues after myocardial infarction (Figure 9).

Discussion

Myocardial infarction ischemia leads to apoptosis of myocardial cells, and apoptotic cells release toxic substances to further aggravate apoptosis[10,11]. Besides, these direct injuries give rise to left ventricular remodeling, ultimately resulting in heart failure. Therefore, reducing the apoptosis of myocardial cells and maintaining a good microenvironment are of great importance for avoiding ventricular remodeling and maintaining cardiac function[12]. Research has manifested that HSP20-mediated cardiomyocyte exosomes can improve the cardiac function after myocardial infarction, relieve fibrosis and enhance vascular density. Overexpressed HSP20-mediated cardiomyocyte exosomes are associated with enhanced Akt

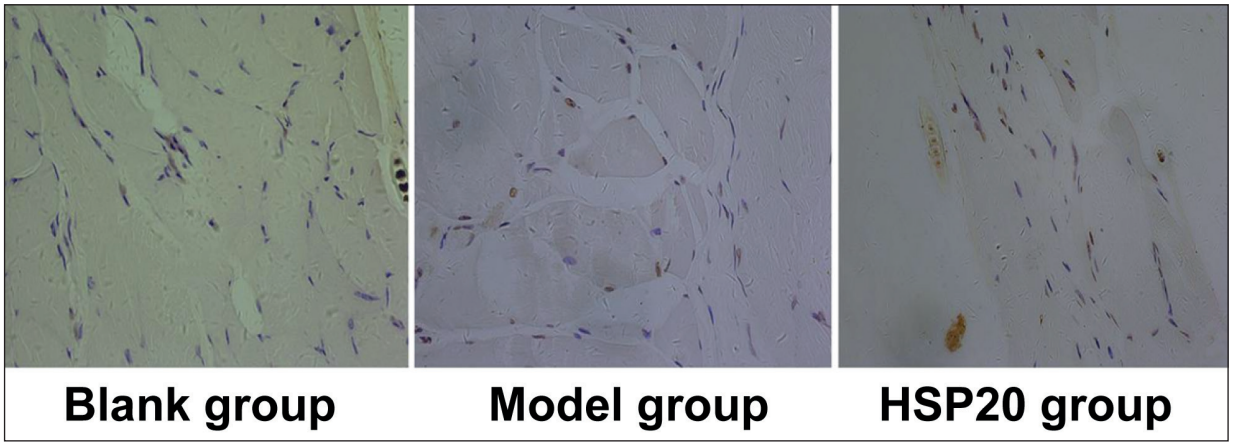

Figure 7. Comparisons of myocardial apoptosis detection results (magnification ×200).

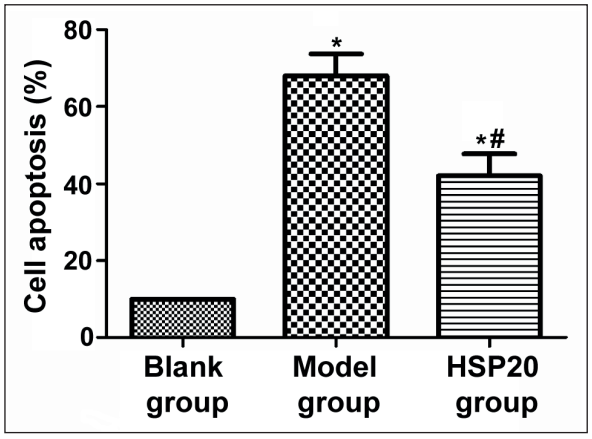

Figure 8. Comparison of myocardial apoptosis in mice. Note: *p<0.05 *vs.* Blank group, #p<0.05 *vs.* Model group.

activation and increased growth factor secretion. The ability of cells to secrete growth factors and exosomes has a close correlation with the therapeutic effects on mesenchymal stem cells. Cell exosomes are vesicle-like bodies released by cells, which have a lipid bimolecular membrane structure with a diameter of about 40-100 nm. Studies have proved that exosomes contain various cell membrane components and regulatory substances including miRNAs. One of the most attractive features of exosomes is that they own the characteristics and phenotypes of their progenitor cells[13].

The morphological structure observed under the transmission electron microscope (´100 nm and ´50 nm) showed that the size of exosomes was about 40-100 nm. Compared with that in Blank group, the number of exosomes in Model group and HSP20 group was increased in the absence of oxygen (p<0.05). Exosomes are membranous microvesicles secreted by active cells, which can be produced and secreted by almost all cells in the human body. In addition, they contain biological macromolecules such as proteins, enzymes and nucleic acids, and membrane-bound proteins and marker proteins on the surface of the membrane bilayer bind to corresponding specific proteins on cell surface so as to transfer substances between cells[14]. In this report, models of myocardial infarction were established to further study the factors affecting myocardial infarction. It was found that the mRNA expression of HSP20 in cardiomyocyte exosomes was overtly lower in Model group than that in Blank group (p<0.05), and the mRNA expression of HSP20 in cardiomyocyte exosomes in HSP20 group was clearly higher than that in Model group (p<0.05) and evidently lower than that in Blank group (p<0.05), indicating that healthy mice have a high level of HSP20, while mice with myocardial infarction have a low level of HSP20. Lee et al[15] demonstrated that the overexpression of HSP20 alleviates the fibrosis in myocardial infarction. Hua et al[16] observed that overexpressed HSP20 in the heart provides pressure protection for the heart, and its anti-apoptotic property protects the heart from damage.

According to Western blotting analysis, the protein expression of Akt in cardiomyocyte exosomes was notably lower in Model group than that in Blank group (p<0.05), and it was clearly increased in HSP20 group compared with that in Model group (p<0.05). The mRNA expression levels of TNF-α and IL-1β in cardiomyocyte exosomes were significantly elevated in Model group compared with those in Blank group and

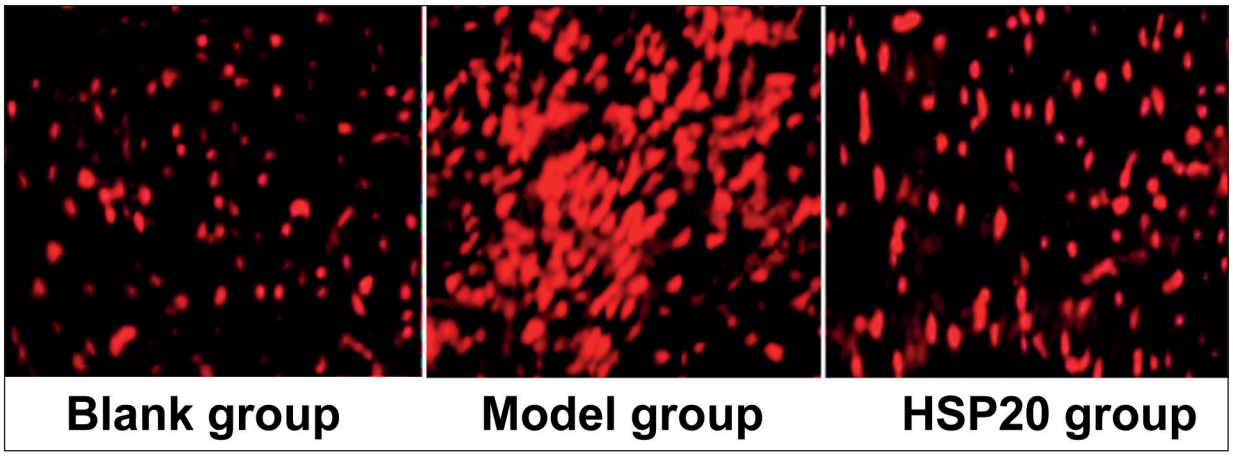

Figure 9. Comparison of ROS accumulation in mouse myocardial tissues. Note: the accumulation of ROS in Model group is notably higher than that in Blank group and HSP20 group.

HSP20 group ($p<0.05$). Experiments showed that expressed HSP20 activates Akt, inhibits TNF-α and IL-1β and protects the cardiac function from damage. Carnero et al[17] revealed that overexpressed Hspa12b activates the PI3K/Akt signal and suppresses the expressions of JNK and p38. Lei et al[18] discovered that HSP20 has an important anti-interference effect and can improve the cardiac function of infarcted myocardium, alleviate fibrosis and increase the density of HSP20 in blood vessels at the same time. HSP20 plays a vital role in protecting the heart under stress. Activated Akt is able to repress inflammatory factors in myocardial cells and improve the function of myocardial cells.

The results of TUNEL assay showed that overexpressed HSP20 exerted a certain effect on the apoptosis of cardiomyocytes. The myocardial apoptosis index in Model group [(38.42±2.52) %] was clearly higher than that in Blank group [(9.74±1.21) %] ($p<0.05$) and HSP20 group [(22.36±2.13) %] ($p<0.05$). It was found through DHE staining assay that the accumulation of ROS in myocardial tissues in Model group clearly higher than that in Blank group ($p<0.05$) and HSP20 group ($p<0.05$), implying that overexpressed HSP20 lowers the accumulation of ROS in myocardial tissues after myocardial infarction. Kajiya et al[19] pointed out that the overexpression of HSP20 plays a protective role in cell death caused by oxidative stress *in vitro*. Kadivar et al[20] manifested that overexpressed HSP20 activates Akt, increases Akt expression, enhances the secretion of VEGF, IGF-1, and FGF-2, and plays a key role in anti-cardiomyocyte apoptosis.

Conclusions

We indicated that HSP20-mediated cardiomyocyte exosomes are able to activate the Akt signaling pathway, inhibit TNF-α and IL-1β and relieve myocardial infarction.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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