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The role of Parkin protein in cardiac function and ventricular remodeling in myocardial infarction rats

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Abstract. – OBJECTIVE: This study aims to explore the role and the mechanism of Parkin protein in cardiac function and ventricular remodeling in myocardial infarction (MI) rats, and to provide a new sight for the treatment of myocardial infarction.

MATERIALS AND METHODS: Fifty Sprague-Dawley (SD) male rats were randomly divided into 5 groups: sham operation group (Sham group), model group (MI group), low-dose Parkin group (L-Parkin group), middle-dose Parkin group (M-Parkin group) and high-dose Parkin group (H-Parkin group). The rat model of myocardial infarction was established by ligation of the anterior descending branch. Small animal ultrasound was used to measure cardiac function. The myocardial infarct size was observed by triphenyltetrazolium chloride (TTC) staining. The pathological changes of myocardial tissues were observed by hematoxylin-eosin (HE) staining. The myocardial cell apoptosis was detected by TUNEL assay. The mRNA expression of matrix metalloproteinase 2 (MMP2), matrix metalloproteinase 9 (MMP9), tissue inhibitor of matrix metalloproteinase 1 (TIMP1), tissue inhibitor of matrix metalloproteinase 2 (TIMP2) were detected by qRT-PCR. The expression of Parkin protein in myocardial tissue of rats was detected by Western-blot.

RESULTS: Compared with MI group, left ventricular end-systolic volume (LVESV) and left ventricular end-diastolic volume (LVEDV) in Parkin overexpressing group were significantly decreased ($p<0.05$), while the value of left ventricular short axis shortening (FS) and left ventricular ejection fraction (EF %) in Parkin overexpression group were significantly increased ($p<0.05$). Overexpression of Parkin improved abnormal structure of myocardial tissue, reduced the size of myocardial infarct, made the arrangement of myocardium fibers more neatly and made the stain of myocardial cells more uniformly. Apoptosis index (AI) values were significantly decreased ($p<0.05$), and MMP2, MMP9, TIMP1 and TIMP2 mRNA levels were significantly decreased ($p<0.05$), while Parkin protein expression was significantly elevated in a dose-dependent manner ($p<0.05$).

CONCLUSIONS: After treatment with Parkin in myocardial infarction rats, the relevant mRNA levels decreased, the number of apoptotic cells decreased, the myocardial fiber morphology returned to normal, the myocardial infarct size decreased, and the cardiac function of rats improved. Therefore, Parkin therapy plays an active role in cardiac function and ventricular remodeling in myocardial infarction rats.

Key Words
Parkin, Myocardial infarction, MMP2, MMP9.

Introduction

Myocardial infarction (MI) is a cardiovascular disease that seriously harms human health and is one of the leading causes of death and disability in the world. The survey found that there are about 1.2 million new patients with myocardial infarction every year in the United States, and 32.6% of them died of myocardial infarction[1]. Although recent applications of new technologies such as coronary thrombolysis and percutaneous coronary intervention (PCI) have resulted in a reduction in acute mortality after myocardial infarction, there

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is still a significant increase in the incidence and mortality of heart failure in patients with ventricular remodeling after myocardial infarction[2]. How to inhibit the occurrence and development of ventricular remodeling after myocardial infarction and delay heart failure are increasingly concerned by the medical community.

MI can be caused by multiple factors with the coronary blockage which disturbs the blood flow as the most common reason. Coronary blockage results from atherosclerotic plaque and throbosis which will induce irreversible damage and quickly spread into other areas and induce billions of cell death[3,4]. Thus, these will result inefficient mechanical blood pumping, which causes congestive heart failure to the patient[4,5]. Up to date, the best choice for heart failure patients is full heart transplant, but this is limited by few factors, which include the quantity of ready donors and the immune response by the patient and long-term graft failure potentials[6]. Recently, stem cell injection and biomaterial injection were introduced to the patients with heart failure[7,8]. However, the survival rate of these cells is still low. Thus, new strategies are needed for the treatment of MI.

Parkin protein was originally thought to be closely related to Parkinson's disease (PD)[9]. Mutations in Parkin were reported to be one of the major causes for early onset of familial PD[10,11]. So far, most studies about Parkin protein have been limited to their role in the brain. Studies have shown that in the brain, Parkin protein is closely related to autophagy clearance of damaged mitochondria[12]. In addition, it has recently been reported that Parkin protein mediates mitochondrial clearance in mouse myocardial ischemic preconditioning experiments[13]. Although Parkin protein is highly expressed in the heart [10], the role of Parkin in myocardial infarction remains unclear. Therefore, in this research, we established the method of ligation of anterior descending coronary artery to establish a myocardial infarction model in rats to determine the role of Parkin in cardiac function and ventricular remodeling in rats with myocardial infarction.

Materials and Methods

Research Object

Fifty healthy specific-pathogen-free (SPF) male Sprague Dawley (SD) rats, weighing (220±30) g, 3 months old, were purchased from Jinan Pengyue Experimental Animal Breeding Co., Ltd. and animal production license number: SCXK (Lu) 2014-0007. Rearing environment: maintain a temperature range of (25±5)°C, average humidity of (55±5) % under the standard conditions, 12 hours of light and dark cycle, free diet and drinking water. The experiments were approved by the Affiliated Yantai Yuhuangding Hospital of Qingdao University Animal Care and Ethics Committee. The surgery was performed according to the "Guidelines for the care and use of experimental animals".

Animal Grouping

Fifty rats were randomly divided into 5 groups (n=10), sham operation group (Sham group), model group (MI group), low-dose Parkin group (L-Parkin group), middle-dose Parkin group (M group), high-dose Parkin group (H-Parkin group).

Preparation of Parkin Overexpression Model

Forty-eight hours before the preparation of MI model, three groups of Parkin overexpressing rats were exposed to the heart by thoracotomy, and 10 μL, 20 μL, and 40 μL of Parkin over-expressed recombinant adenovirus (pAd / mCMV; Sunbio, Shanghai, China) were injected into the myocardium. The titer was 1.0×10^{10} pfu/mL and local color changes from red to white after successful injection.

Rat MI Model Construction

Rats were anesthetized by intraperitoneal injection of 1% sodium pentobarbital (40 mg/kg) and connected to a ventilator. Respiratory frequency was maintained at 50-60 beats/s. An horizontal incision was carried out between the third and fourth ribs on the left chest to open the chest and expose the heart. The left anterior descending artery (LAD) was ligated with a 6-0 absorbable suture below the root of the left atrial appendage. After ligation, the pulse weakens near the ligation site and the anterior chamber of the ventricle became shallow. In addition, electrocardiogram (ECG) leads II showed elevated ST segment elevations, initially demonstrating successful modeling. The sham group underwent sham surgery in the same manner except that the suture was placed on the LAD without ligation [14].

Cardiac Function Test in Rats

Five groups of rats were examined by Cardiac M-mode ultrasonography at 72 hours after the model was established. Visual Sonics Vevo 770 high-resolution small animal ultrasound detection system and rat RMV 707B high-frequency probe were used, with a frequency of 21 MHz, a mea-

surement depth of 3 cm, a measurement speed of 200 mm/s, and a short axial plane in the left ventricle. Above, left ventricular internal diameter at end-diastole (LVIDd) and left ventricular internal diameter at end-systole were measured (LVIDs). Through software analysis, the LVESV, LVEDV, FS and EF% were calculated.

Sample Collection

After rats were tested for cardiac function, they were anesthetized by intraperitoneally injected with 1% sodium pentobarbital (40 mg/kg). The rats were sacrificed by breaking neck and then the hearts were cut off quickly by opening the chest. The heart was washed with ice cold physiological saline and the heart general morphology was observed with naked eyes. Triphenyltetrazolium chloride (TTC) staining was performed, photographed after fixation with 4% paraformaldehyde, and paraffin embedded for HE staining. The remaining tissues were quickly frozen in liquid nitrogen and transferred at -80°C freezer for RNA extraction to check the mRNA level by qRT-PCR and for Western-blot to check the protein level.

Assessment of Myocardial Infarct Size by TTC Staining in Rats

The remaining tissues were freezed at-70°C for 15 min and quickly the long axis was taken of the vertical heart from the apex of the heart to the ligation junction. The myocardium was removed parallel to the tip of the heart, and the cardiac muscle was discarded at the tip of the heart. The rest was evenly cut into 5 pieces, about 1 mm thick, immediately clamped, flattened into a 1% TTC-PBS solution, incubated in a water bath at 37°C in the dark for 15 min, and photographed after staining. Brick red was the normal area, gray was the infarct area. Infarct size was calculated with Image Pro Plus 6.0, myocardial infarct area (%)=(the sum of unstained area/total area of myocardial sheet) ×100%.

HE Staining

Rat hearts fixed in 4% paraformaldehyde solution were dehydrated and embedded in paraffin. The section thickness was 5 μm. The slices are routinely dewaxed with xylene and hydrated with ethanol at all levels. Hematoxylin (Solarbio, Beijing, China) was stained for 5 min and rinsed with running water. Hydrochloric acid and ethanol were differentiated for 30 s, soaked in tap water for 15 min, placed in eosin dyeing solution (Solarbio, Beijing, China) for 2 min, and routinely dehydrated, transparently and mounted. The histopathological changes were observed under a 400 × light microscope (Olympus BX51, Olympus, Tokyo, Japan).

TUNEL Detection of Myocardial Apoptosis in Myocardium

Rat hearts soaked in 4% paraformaldehyde solution were routinely paraffin-embedded and subjected to coronal section of myocardial tissue (4 μm) (paraffin-embedded tissue paraffin machine, paraffin section/Leica, Germany). Five pieces were cut in each specimen and the sections were dehydrated by conventional xylene and dehydrated by gradient ethanol. The cell apoptosis was quantified by TUNEL method using apoptotic detection kit (batch number: ZK-8005, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China). 400 fields of light microscope (BX50 type/Olympus, Japan) were used to randomly select 5 fields of vision. The cell lines were brown or brown-yellow and had the morphological features of apoptotic cells were determined as apoptotic cells. The apoptotic index (AI) was calculated which reflects the degree of apoptosis. AI=(apoptotic positive cell number/total cell number) × 100%.

qRT-PCR detection of MMP2, MMP9, TIMP1, TIMP2 mRNA Expression Levels in Myocardial Tissue

Collected tissue samples were centrifuged (10000 rpm, 10 min) after grinding at 4°C. Total RNA was extracted using TRIzol (15596018, Invitrogen, Carlsbad, CA, USA). The concentration and quality of RNA were assessed using the 260/280 nm absorbance ratio of 1.8-2.0. The total RNA was reverse transcribed into cDNA by using the reverse transcription kit (Applied Biosystems, Waltham, MA, USA). qRT-PCR was performed using Mastercycler nexus X2 (Eppendorf, Hamburg, Germa-

Table I. Primers sequence (Shanghai Shenggong Biological Engineering Technology Service Co., Ltd.).

Name	Sequences
MMP2	Forward:5′- CTATTCTGTCAGCACTTTGG-3′
	Reverse: 5′- CAGACTTTGGTTCTCCAACTT -3′
MMP9	Forward: 5′- AAATGTGGGTGTACACAGGC -3′
	Reverse: 5′- TTCACCCGGTTGTGGAAACT-3′
TIMP1	Forward: 5′- CATGGAGAGCCTCTGTGGAT -3′
	Reverse: 5′- TGTGCAAATTTCCGTTCCTT-3′
TIMP2	Forward: 5′-CGCCAAAGCAGTGAGCGAGAAG -3′
	Reverse: 5′- GCCGTGTAGATAAATTCGATGTC -3′
GAPDH	Forward: 5′- AGCCCATCACCATCTTCCAG -3′
	Reverse: 5′- CCTGCTTCACCACCTTCTTG -3′

ny). Conditions: 94°C 5 min, 94°C 45 s, 59°C 60 s, 72°C 90 s (35 cycles). Data were processed using the $2^{-\Delta\Delta Ct}$ method [15] and relative expression levels were calculated using GAPDH mRNA as an internal reference. Primers used for PCR are listed in Table I.

Western Blot Analysis Expression of Parkin Protein in Rat Myocardial Tissue

The tissue samples were mechanically dispersed, centrifuged (10000 rpm, 10 min) and supernatants were taken. The protein concentration was measured by BCA kit (Solarbio, Beijing, China), and 30 μg protein sample were mixed with 10% SDS gel buffer 1:1 and heated at 95°C for 5 min to denature the protein. Polyvinylidene difluoride (PVDF) membrane (Merck, Darmstadt, Germany) was transferred at 80 V for 30 min, blocked at 4°C for 1 h with 5% nonfat dry milk in TBST solution, and rabbit anti-Parkin (1:1000, ABIN2878901, antibodies-online, Aachen, Germany) polyclonal antibody was diluted with TBST containing 3% bovine serum albumin (BSA), reacted overnight at 4°C. After rewarming, they were incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1:1000, ABIN101988, antibodies-online, Aachen, Germany) for 1 h, washed and visualized with electrochemiluminescence (ECL) luminescence substrate for 3-5 min. Protein expression levels were normalized with GAPDH and gray-scale scans and quantification were performed using Image J (NIH) software.

Statistical Analysis

SPSS 20.0 (SPSS IBM, Armonk, NY USA) was used to analyze the monitoring data. The data analysis results were expressed as Mean±standard deviation (mean±SD). The *t*-test was

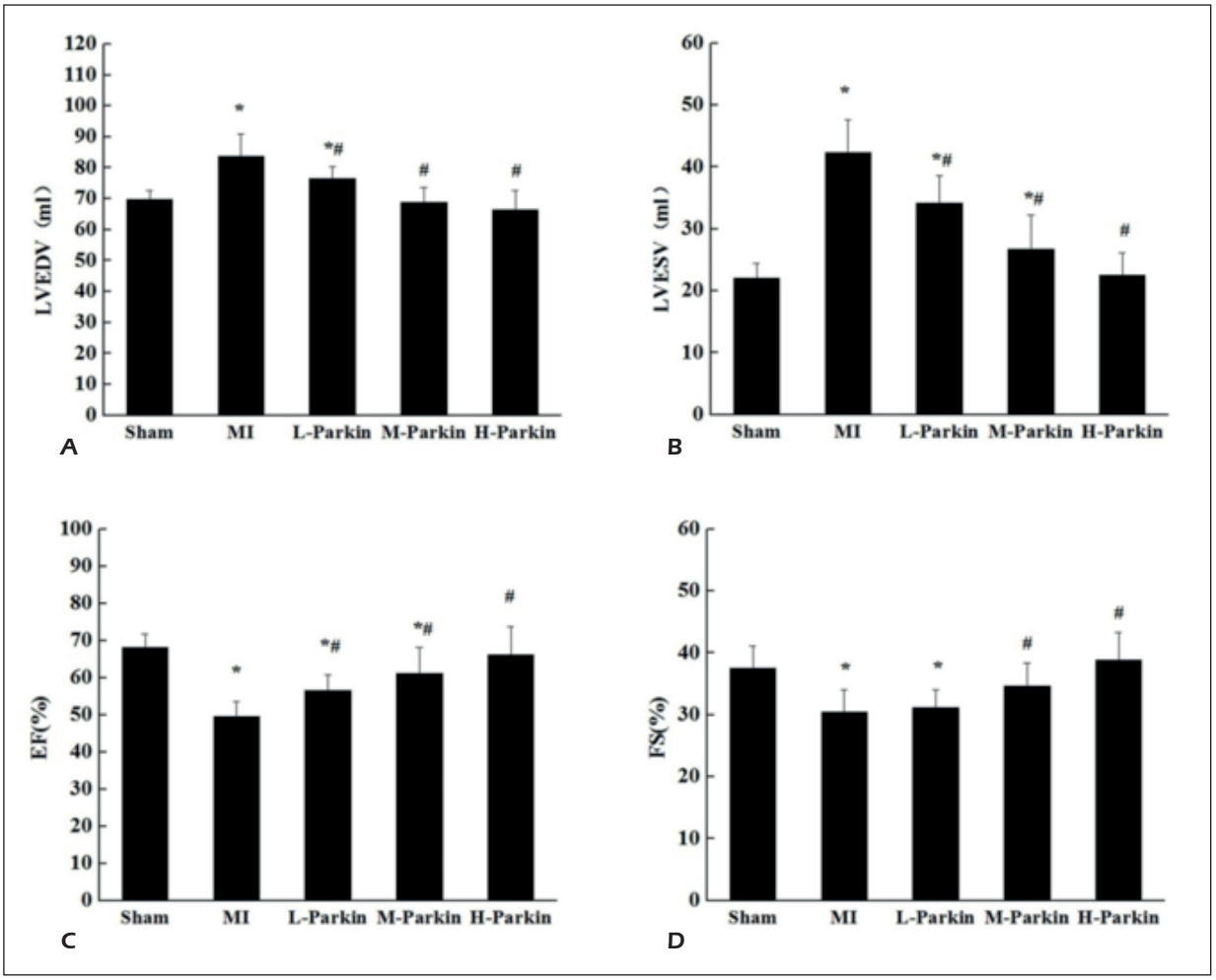

Figure 1. Effects of Parkin Overexpression on Heart Function in MI Rats. **A** and **B**. LVEDV and LVESV value in each groups. **C** and **D**. EF and FS values in each group. Compared with sham group, *p<0.05; compared with MI group, #p<0.05.

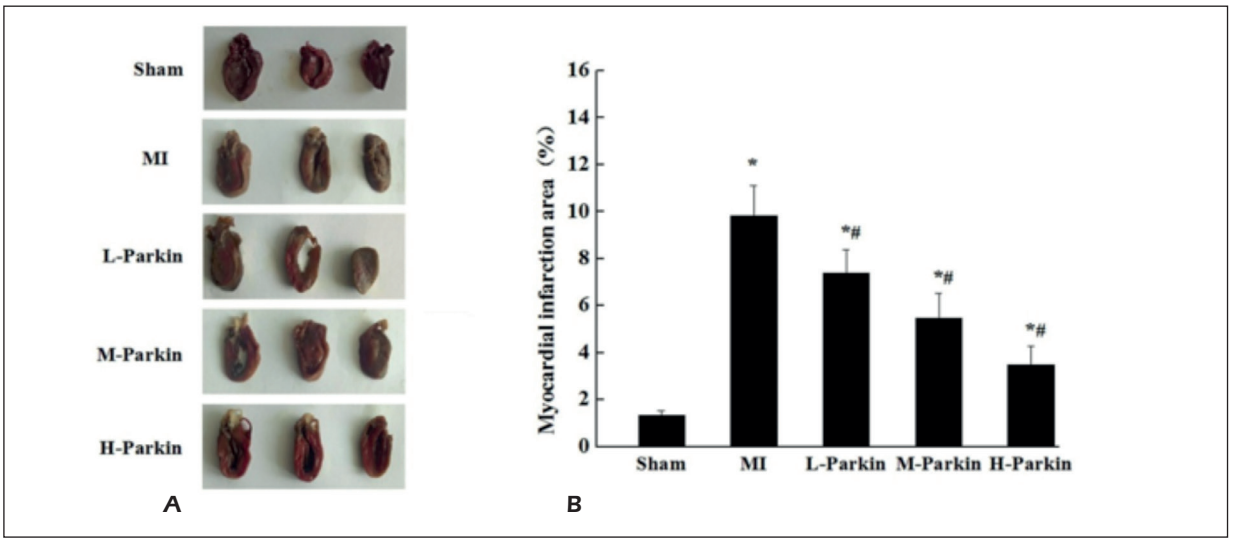

Figure 2. Effect of Parkin overexpression on myocardial infarct size. **A**, TTC staining of rat myocardial tissue in each group. **B**, Quantification of the TTC staining in each group. Compared with sham group, *$p<0.05$; compared with MI group, $^{\#}p<0.05$.

used for the data analysis between the two groups. Multiple comparisons were evaluated by repeated measures analysis of variance (ANOVA). One-way ANOVA was used to compare the mean of multiple samples. The comparison between any two means was performed by LSD method. The LSD method was applied in the comparison between two groups. $p<0.05$ was considered statistically significant.

Results

Rat Cardiac Function

LVESV and LVEDV in the hearts of the MI rats increased by 91.4% and 20.2%, respectively ($p<0.05$) (Figure 1A, B), and the EF and FS values decreased by 27.2 % and 18.7 %, respectively ($p<0.05$) (Figure 1C, D), when compared with the sham group. Parkin treatment group had significantly decreased LVESV and LVEDV values in the heart, when compared with MI group, while EF and FS values were significantly increased. There was a statistically significant difference between the MI group and Parkin treatment group ($p<0.05$) in a dose-dependent manner. These results showed that Parkin overexpression can improve cardiac and vasomotor function.

Assessment of Myocardial Infarct Size in Rats

Myocardial infarct size in the MI group accounted for 9.83% of the left ventricle, while it only accounted for 1.32% in the Sham group. Thus, there was a significant difference ($p<0.05$) between these two groups. The Parkin overexpression significantly improved myocardial structural abnormalities after myocardial infarction in rats, and the myocardial infarct size was reduced (Figure 2). Parkin's overexpression in the low, middle and high treatment groups had a significant decrease in the infarct size range ($p<0.05$), when compared with MI group.

Myocardial Pathological Changes in Rats

As shown in Figure 3, the myocardial cell nuclei appeared as blue and the cytoplasm and collagen fibers appeared as pink. In the Sham group, the myocardial fibers were arranged neatly, and the nuclei and cytoplasm were evenly stained. In the MI group, the myocardial cells were significantly sparse, and the myocardial fibers were broken and disordered. The Parkin overexpression in low, medium, and high treatment groups had more regular myocardial fibers and more uniform myocardial cells when compared with MI group. These results suggested that Parkin overexpression can improve myocardial fiber alignment.

Myocardial Apoptosis in Myocardial Tissue

As shown in Figure 4, the number of myocardial apoptotic cells in MI rats increased significantly when compared with Sham group

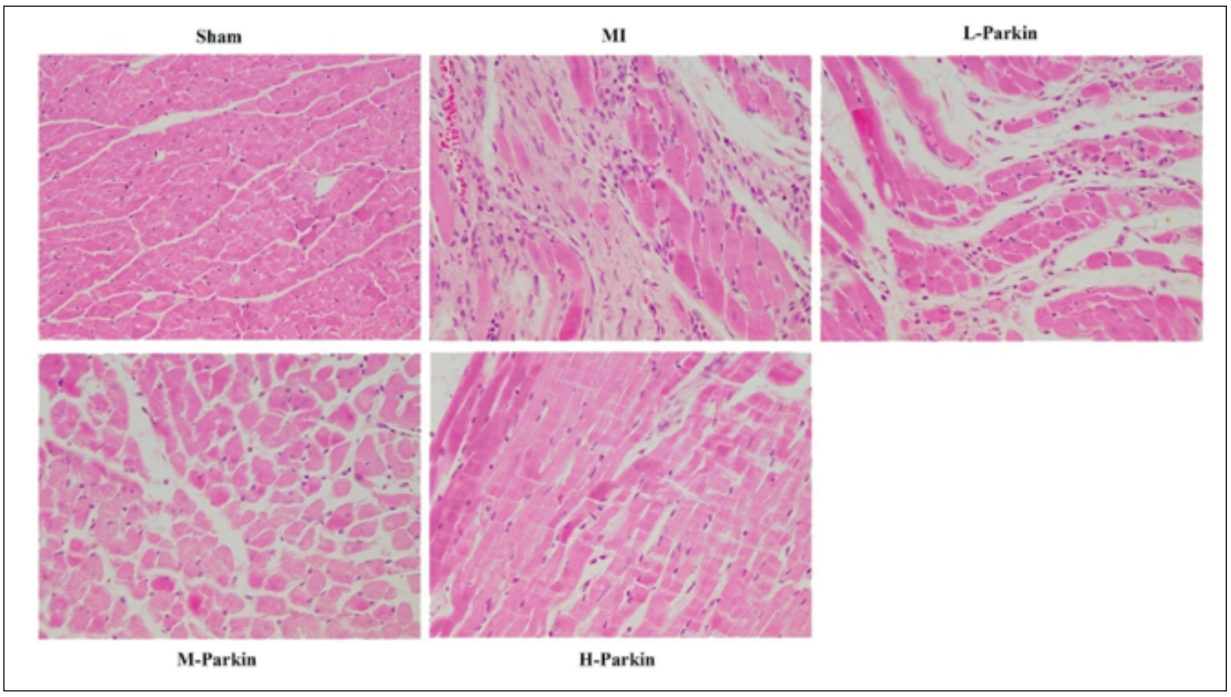

Figure 3. Rat myocardial HE staining. Rat hearts fixed in 4% paraformaldehyde solution were dehydrated and embedded in paraffin. The sections are routinely stained with HE staining kit and the histopathological changes were observed under a 400X light microscope (Olympus BX51, Olympus Japan).

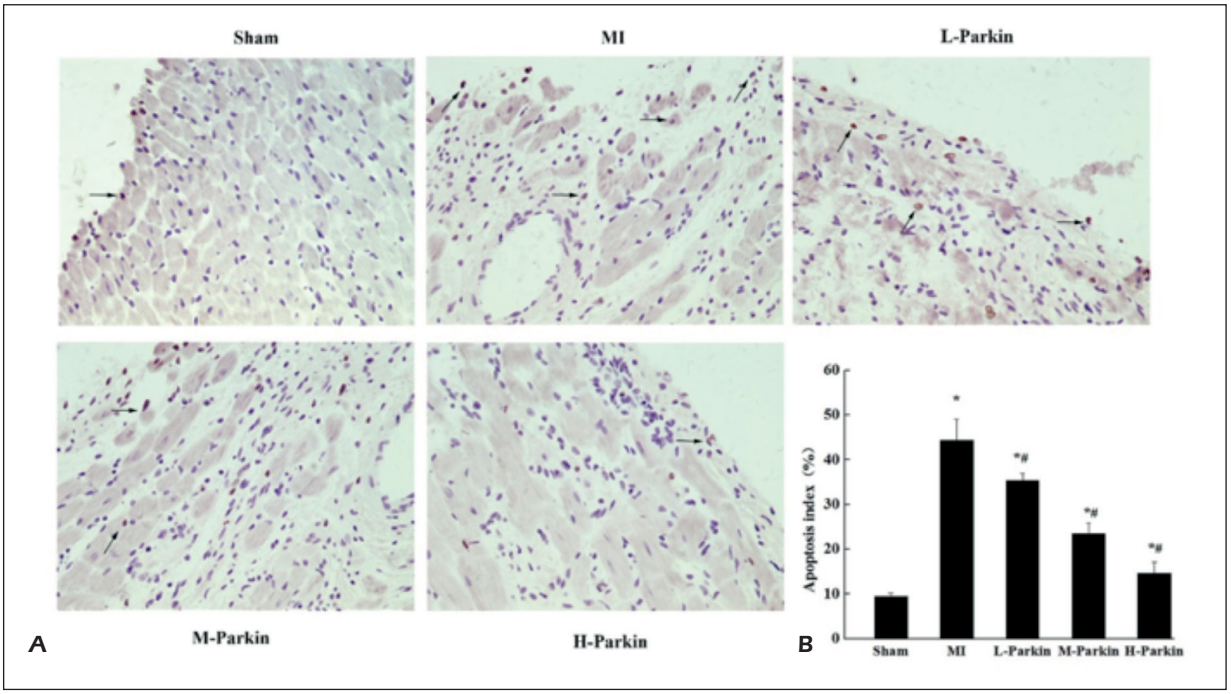

Figure 4. The effect of parkin overexpression on apoptosis of rat cardiomyocytes. **A**, Rat myocardial tissue section was stained with TUNEL. **B**, Data statistics. Compared with sham group, $*p<0.05$; compared with MI group, $^{\#}p<0.05$.

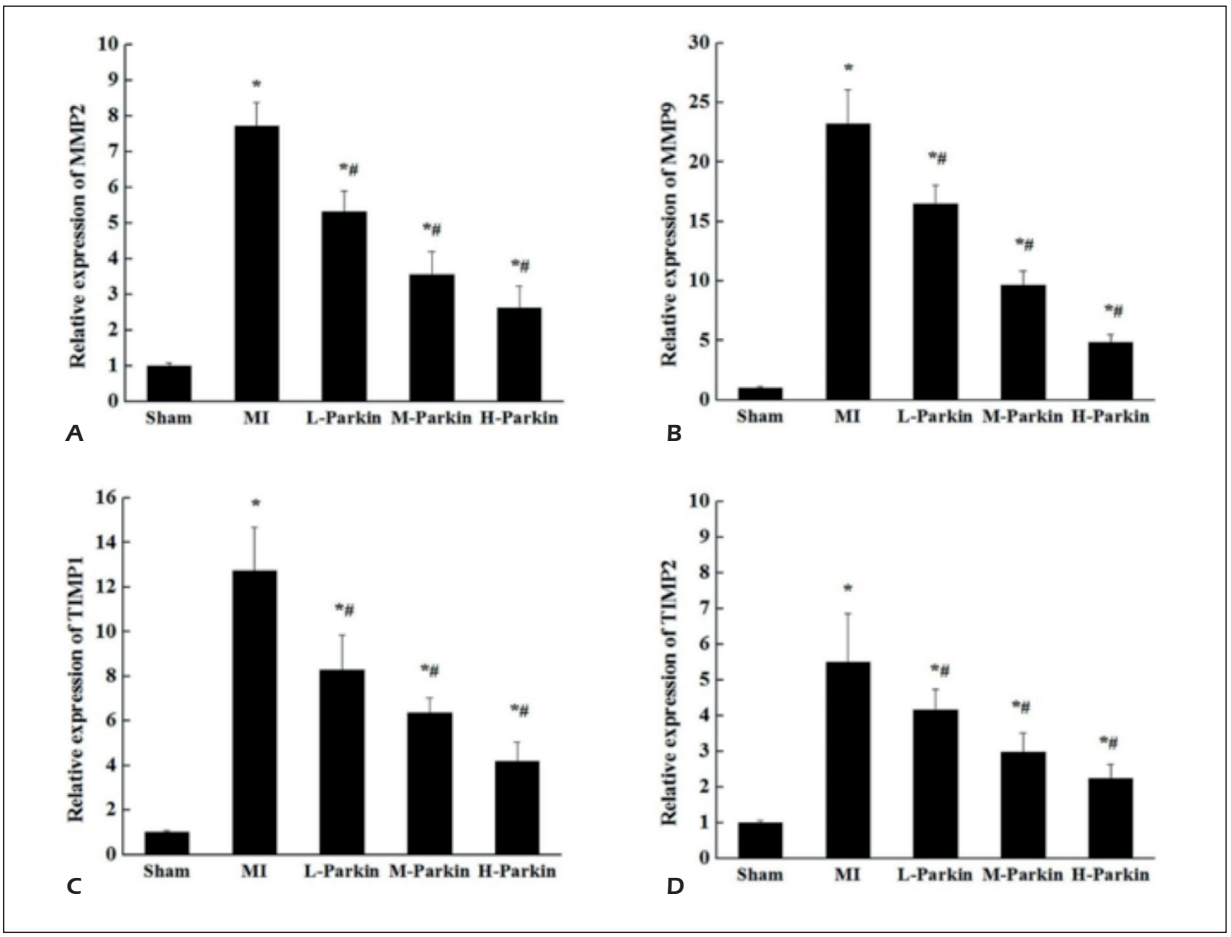

Figure 5. MI-related gene mRNA expression levels in myocardial tissue. **A**, MMP2 mRNA; **B**, MMP9 mRNA; **C**, TIMP1 mRNA; **D**, TIMP2 mRNA. Compared with sham group, *$p<0.05$; compared with MI group, $^{\#}p<0.05$.

(9.40%, 44.41%) ($p<0.05$). After Parkin treatment, the Parkin overexpression in each group was significantly lower than that in the MI group. The difference was statistically significant ($p<0.05$) and was dose-dependent.

Expression of MMP2, MMP9, TIMP1 and TIMP2 mRNA in Myocardium

As shown in Figure 5, the relative mRNA content of myocardial tissue showed that MMP2, MMP9, TIMP1 and TIMP2 mRNA levels in the MI group were significantly higher than those in the Sham group ($p<0.05$). When compared with MI group, the expression of MMP2, MMP9, TIMP1 and TIMP2 mRNA in Parkin overexpression group was significantly lower than that in MI group ($p<0.05$).

Western Blot Detection of Parkin Protein Expression

As shown in Figure 6, the Parkin protein level in the MI group was significantly decreased (0.89, 0.58) when compared with the Sham group ($p<0.05$), while when compared with the MI group, the Parkin protein expression levels in the Parkin over-expression low-, medium- and high-treatment groups increased by 0.39, 0.49, and 0.63, respectively ($p<0.05$). L-Parkin group had no significant difference compared with Sham group ($p>0.05$).

Discussion

Myocardial infarction is a cardiovascular disease featured with high mortality and morbidity and the incidence of this disease is still rising be-

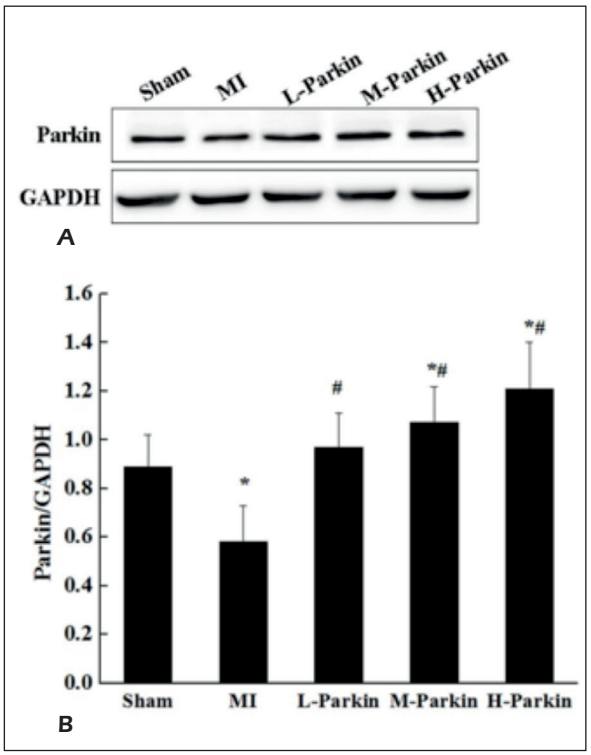

Figure 6. Parkin expression in each group. **A**, Cardiomyocytes tissue samples were mechanically dispersed and Western-blot was performed to determine the Parkin protein level in each group. **B**, Quantification of the Parkin protein expression. Compared with sham group, $*p<0.05$; compared with MI group, $^{\#}p<0.05$.

cause of the environment, heredity, lifestyle and so on [16]. MI is considered as one of the leading cause for death and disability. New technologies developed fast in the recent years, including coronary thrombolysis and percutaneous coronary intervention (PCI) that significantly reduced the occurrence of acute mortality after myocardial infarction [2,17,18]. In addition, stem cell and biomaterial based treatment were also explored as the treatment for the heart failure[7,8]. However, there is still an increase in the incidence and mortality of heart failure in patients with ventricular remodeling after myocardial infarction. Moreover, all these treatments have its own limit; thus, exploring new strategies to inhibit the occurrence and development of ventricular remodeling after myocardial infarction and delay heart failure are still urgently needed by the medical community. Parkin gene has long been recognized and reported to be the major cause for the early onset of the Parkinson disease[9-11]. Majority of the studies[12,13,19,20] about Parkin are limited in the brain and some of the study focus on mitochondrial autophagy and tumor. Intense systemic and local inflammation will occur after myocardial infarction and MI will induce autophagy when cardiomyocytes exposed to hypoxia [21]. Parkin was reported to recruited to dysfunctional mitochondria and promotes the autophagy in mitochondria and thus eliminate the dysfunction of mitochondria in the pathogenesis of Parkinson's disease[12]. Parkin also plays an important role in the autophagy of mitochondria in cardiomyocytes[22]. Moreover, it has been shown that Parkin could mediate selective clearance of mitochondria by autophagy in cells treated with mitochondrial uncoupler [23,24]. Further study revealed that Parkin was important in the mitochondrial dysfunction and disruption of autophagosomal clearance, associated with autophagy-related marker LC3 I to LC3 II in cardiomyocytes [13]. Hence, Parkin might plays a similar role during MI which will also induce the autophagy during the early onsite of MI. In this study, we applied Parkin protein treatment in the rat MI model and analyzed the output of this treatment. Surprisingly, our results indicated that Parkin overexpression significantly decrease the value of LVESV and LVEDV, while increase the value of EF and FS. Further studies indicated that overexpression of Parkin improved abnormal structure of myocardial tissue, reduced the size of myocardial infarct, made the arrangement of myocardium fibers more neatly, and made the stain of myocardial cells more uniformly. All these data proved that Parkin overexpression treatment plays a positive role on MI rat. Thus, Parkin has the potential to be explored as a treatment target clinically in the future.

Matrix metalloproteinases (MMPs) act as physiological regulators of the extracellular matrix and activity change in MMPs, especially MMP9 and MMP2 was reported to be related with the onset of acute MI and the expression of MMPs was related with LV function in patients who were diagnosed as acute MI [25-27]. MMP9 and MMP2 expression were significantly increased with the onset of acute MI [27]. In our study, we proved that overexpression of Parkin can profoundly decreased the expression of MMP9 and MMP2, which might be explained the mechanism of how this treatment plays a positive role in the rat MI model. TIMPs are reported to regulate the MMPs activation by binding to these MMPs and prevent the degradation of these MMPs[28]. In our study, TIMP1/2 expres-

sion significantly decreased in the Parkin treatment group, which suggested that the MMPs expression might be regulated by TIMP protein decrease. Thus, future work will be performed to investigate how Parkin affects the expression of MMPs and TIMPs.

Conclusions

We showed that after treatment with Parkin protein in myocardial infarction rats, the relevant genes mRNA levels decreased, the number of myocardial cell apoptotic cells decreased, the myocardial fiber morphology returned to normal, the myocardial infarct size decreased, and the heart function index of rats became better. Therefore, Parkin protein therapy plays an active role in heart function changes and ventricular remodeling in myocardial infarction rats.

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Conflict of Interests

The Authors declare that they have no conflict of interests.

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