Melatonin protected against myocardial infarction injury in rats through a *Sirt6*-dependent antioxidant pathway

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Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online) *Adv Clin Exp Med*. 2022;31(3):277–284

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Funding sources
This work was supported by Key Fund of Department of Cardiology, Shandong University Qilu Hospital (Qingdao) (QDKY2019ZD04), People's Livelihood Science and Technology Project of Qingdao (application of DEEPVESSEL FFR in coronary artery heart disease complicated with diabetes mellitus), Qingdao Key Health Discipline Development Fund.

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Conflict of interest
None declared

Received on August 8, 2018
Reviewed on February 25, 2019
Accepted on September 1, 2019

Published online on January 25, 2022

Cite as
Wang Y, Zhang S, Ma Y, et al. Melatonin protected against myocardial infarction injury in rats through a *Sirt6*-dependent antioxidant pathway. *Adv Clin Exp Med*. 2022;31(3):277–284. doi:10.17219/acem/112060

DOI
10.17219/acem/112060

Abstract

Background. The *Sirt6*, one of the members of the sirtuin family, has been regarded as a key factor in the pathogenesis of myocardial infarction (MI) through its antioxidant defense mechanisms. A previous study reported that melatonin is an antioxidant drug that can act as an agent for cardioprotection in cardiac ischemia-reperfusion (I/R) injury. However, whether melatonin could protect against cardiac remodeling after myocardial injury via the *Sirt6*-dependent antioxidant pathway remains unknown.

Objectives. To explore the protective effects and the potential mechanisms of melatonin on MI-induced injury in rats.

Materials and methods. A cardiac remodeling model was established through left coronary artery ligation surgery. The dose of melatonin was 10 mg/kg body weight. Four weeks after the treatment for 7 successive days, the infarct size and hemodynamic parameters were evaluated. The relative mRNA level and protein level of *Sirt6* were also determined. Finally, the levels of oxidative stress, including reactive oxygen species (ROS) and superoxide dismutase (SOD), were measured, and the expression of nitric oxide (NO), inducible nitric oxide synthase (iNOS), endothelial nitric oxide synthase (eNOS) and their corresponding phosphorylation were evaluated.

Results. After the treatment with melatonin, infarct size, the left ventricular end-diastolic diameter (LVEDd), and left ventricular end-systolic diameter (LVEDd) and minimum first derivative of developed pressure (min dP/dt) decreased, while left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS) and maximum first derivative of developed pressure (max dP/dt) increased in the melatonin-MI (MM) group compared to the placebo-MI (PM) group. Furthermore, the expressions of *Sirt6*, both in mRNA and protein level, were significantly increased in the MM group treated with melatonin, as compared to the melatonin-control (MC) group treated with melatonin. In addition, melatonin enhanced SOD activity and reduced ROS levels. At the same time, we observed that the eNOS/NO signaling pathways were activated.

Conclusions. Melatonin improved cardiac function through the *Sirt6*-dependent antioxidant pathway in MI rats.

Key words: melatonin, oxidative stress, acute myocardial infarction, sirtuin

Background

Acute myocardial infarction (MI) is generally caused by coronary artery disease (CAD), which remains a leading cause of morbidity and mortality and imposes a tremendous economic burden worldwide.[1] After MI injury, the heart undergoes a series of cardiac wound-healing responses, including cardiomyocyte necrosis and apoptosis, inflammation, and cardiac hypertrophy and fibrosis. As cardiac wound healing progresses, patients suffer from post-MI left ventricular (LV) remodeling, heart failure (HF) or even death.[2,3]

Oxidative stress plays an important role in the pathophysiology of various cardiovascular disorders, including atherosclerosis, cardiac hypertrophy, cardiomyopathy, HF, ventricular remodeling, and myocardial injury after ischemia followed by reperfusion (I/R). During the development of cardiac remodeling, oxidative stress aggravates heart disease into HF.[4–6] Elevated oxidative stress may result in peroxidation of proteins, DNA and lipids, triggering mitochondria-induced cell death pathways and contributing to I/R injury.[7]

The *Sirt6* is one of the 7 mammalian sirtuin homologs and it contains a domain with NAD^+-dependent deacetylase activity.[8] Among sirtuin family members, both *Sirt1* and *Sirt6* have demonstrated cardioprotective abilities. However, no direct evidence can verify the antioxidative role of Sirt1 in protecting heart function, while *Sirt6* has been shown to play a vital protective role in cardiomyocytes in I/R through its antioxidative ability.[9]

Melatonin, a hormone synthesized and secreted by the pineal gland, is a well-known antioxidant.[10,11] Extensive evidence suggests that melatonin has profound cardioprotective effects.[12,13] For example, Hu et al. demonstrated that melatonin alleviated post-infarction cardiac remodeling and dysfunction by inhibiting Mst1.[14] Zhou et al. demonstrated that melatonin protected against I/R in myocardial infarction by suppressing platelet activation and function.[15] However, whether melatonin could protect against cardiac remodeling after myocardial injury via a *Sirt6*-dependent antioxidant pathway, remains unknown.

Objectives

The aim of the study was to explore the protective effects and the potential mechanisms of melatonin on MI-induced injury in rats.

Materials and methods

The animals

All the experiments were performed with the approval of the Ethics Committee at Shandong University Qilu Hospital (approval No. KYLL-KS-2021180). Three-month-old male Wistar rats weighing 250–270 g were purchased from Charles River Laboratories (Beijing, China). The rats were randomly divided into 4 groups: placebo-control (PC), melatonin-control (MC), placebo-MI (PM), and melatonin-MI (MM). Each group included 10 rats. In the PM and MM groups, the rat MI model was established through left coronary artery ligation surgery. In addition, the PC group was regarded as a negative control group, and it was designed to show whether the model was established successfully and to eliminate the influence of the operation.

Rat MI model

The rats were placed in an induction chamber and 4% isoflurane was delivered until they reached a deep plane of anesthesia. After the intubation was finished, the chest was opened at the left 4th intercostal space. The pericardial tissue was removed and the left anterior descending artery was visualized under a light microscope (model YAN-6A; Yuyan Instruments Co., Ltd., Shanghai, China) and ligated by a 6-0 silk suture (Yuyan Instruments), 1 mm below the tip of the left atrial appendage. The PC and MC groups underwent surgery without the left anterior descending artery ligation. After ligation, the left artery presented a pale appearance. Echocardiography was used to confirm the successful ligation, which would appear as ST segment and Q wave changes.

Melatonin treatment

For 7 days following the surgical procedures, melatonin (Sigma-Aldrich, St. Louis, USA) dissolved in dimethyl sulfoxide was administered to the MC and MM groups by daily intraperitoneal injection, at a dose of 10 mg/kg body weight. In the placebo groups, an equivalent volume of dimethyl sulfoxide (DMSO) was injected.

Hemodynamic measurement and determination of infarct size

A microtip catheter transducer (Millar Instruments, Houston, USA) was inserted into the right carotid artery and advanced into the left ventricle to measure the following parameters: the left ventricular end-diastolic diameter (LVEDd), left ventricular end-systolic diameter (LVESd), left ventricular ejection fraction (LVEF) and fractional shortening (FS). For the hemodynamic analysis, LV maximum first derivative of developed pressure (max dP/dt), and LV minimum first derivative of developed pressure (min dP/dt) were analyzed. Four weeks after the treatment, the animals were anesthetized again for hemodynamic measurement as described in another study.[16]

Infarct size was determined with Masson's trichrome staining as described in an earlier publication.[17] After the animals were sacrificed, the LV myocardium was dissected,

fixed in 4% paraformaldehyde, embedded in paraffin, and then cut into 7-μm sections. The sections were stained with Accustian Trichrome Stain (Masson) kit (Sigma-Aldrich). Infarct size was determined using the Microsoft Image Composite Editor v. 14.4 (Microsoft Corp., Redmond, USA).

Measurement of *Sirt6* in mRNA and protein levels

The primary rat cardiomyocytes were isolated and cultured from the animals in the 4 groups as described in a previous study.[2] When the cells were prepared, the expression of *Sirt6* in cardiomyocytes was measured using western blot and quantitative real-time polymerase chain reaction (qRT-PCR). The anti-*Sirt6* (Cell Signaling Technology, Danvers, USA) and tubulin (T5168; Sigma-Aldrich) were used. The following primers were used: *Sirt6*: forward 5′-TCTTCCAGTGTGGTGTTCCA, reverse 5′-CCTCCATGGTCCAGACTCC; *β-actin*: forward 5′- CCAACCGCGAGAAGATGA, reverse 5′- CCAGAGGCGTACAGGGATAG.

Analysis of oxidative stress (ROS and SOD)

Intracellular reactive oxygen species (ROS) production in cardiomyocytes was evaluated with a fluorometric assay using 2',7'-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, USA), according to the manufacturer's protocols. The cells were incubated with DCFH-DA for 1 h, after which the fluorescence intensity was measured at 488 nm and 525 nm emission wavelengths. Lysate and cardiomyocytes from fresh rat hearts were collected before total superoxide dismutase (SOD) activity was determined, using a commercial SOD Kit (Beyotime Biotech Inc., Jiangsu, China). The 50% inhibition activity of SOD (IC50) values was determined under an optic density (OD) of 560 nm, using a Model 550 Microplate Reader (Bio-Rad Laboratories Inc., Hercules, USA).

Nitric oxide measurement

The level of nitric oxide (NO) in MI cardiac tissue was detected using a diagnostic assay kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) as described in a previous study.[18]

Measurement of iNOS and eNOS with western blot

Total proteins were extracted from LV tissues. Protein concentrations were detected using a Pierce BCA Assay Kit (Thermo Fisher Scientific Inc., Waltham, USA). Then, samples containing 20 μg protein were resolved with electrophoresis on 10% polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. Rabbit anti-endothelial nitric oxide synthase (eNOS), anti-inducible nitric oxide synthase (iNOS), polyclonal antibodies, rabbit anti-P1177 eNOS, and rabbit anti-GAPDH monoclonal antibody (all from Abcam, Cambridge, USA) were used to detect the levels of iNOS, eNOS and phosphorylated eNOS (p-eNOS) in this assay.

Statistical analyses

Data were shown as median and interquartile range (IQR) due to a small sample size. Statistically significant differences among the groups were analyzed using the Kruskal–Wallis test. Pairwise multiple comparisons post hoc tests were carried out using Dunn's test with a Bonferroni correction. Two-tailed, unpaired Mann–Whitney tests were used to analyze the comparisons between 2 groups. The value of $p < 0.05$ was taken to indicate statistical significance.

Results

Melatonin decreased the myocardial infarct size in the rat MI model

As shown in Fig. 1A, in which the fibrotic scar appears blue and the viable myocardium is red, melatonin did not affect the cardiac structure in the MC group, while in the MM group, melatonin treatment decreased the myocardial infarct size ($U = 0$; $p < 0.0001$). The quantitative analysis confirmed this finding (Fig. 1B).

Melatonin improved hemodynamic parameters

Hemodynamic analyses were performed in all 4 groups. In the MM group, LVEDd, LVESd and min dP/dt decreased, while LVEF, LVFS and max dP/dt increased, but there was no significant difference after Dunn's Bonferroni adjustment (Table 1). No differences in these parameters were observed in the MC group, as shown in Fig. 2.

Melatonin increased *Sirt6* expression both in mRNA and protein levels

After treatment with melatonin, the expressions of *Sirt6* both in mRNA ($p < 0.0001$ after adjustment) and protein levels ($p < 0.0001$ after adjustment) were increased in the MM group compared to the PM group, as shown in Fig. 3 (Table 1).

Melatonin ameliorated MI-induced oxidative stress

Because of the fact that increased oxidative stress deteriorated cardiac remodeling, 2 oxidative stress-related

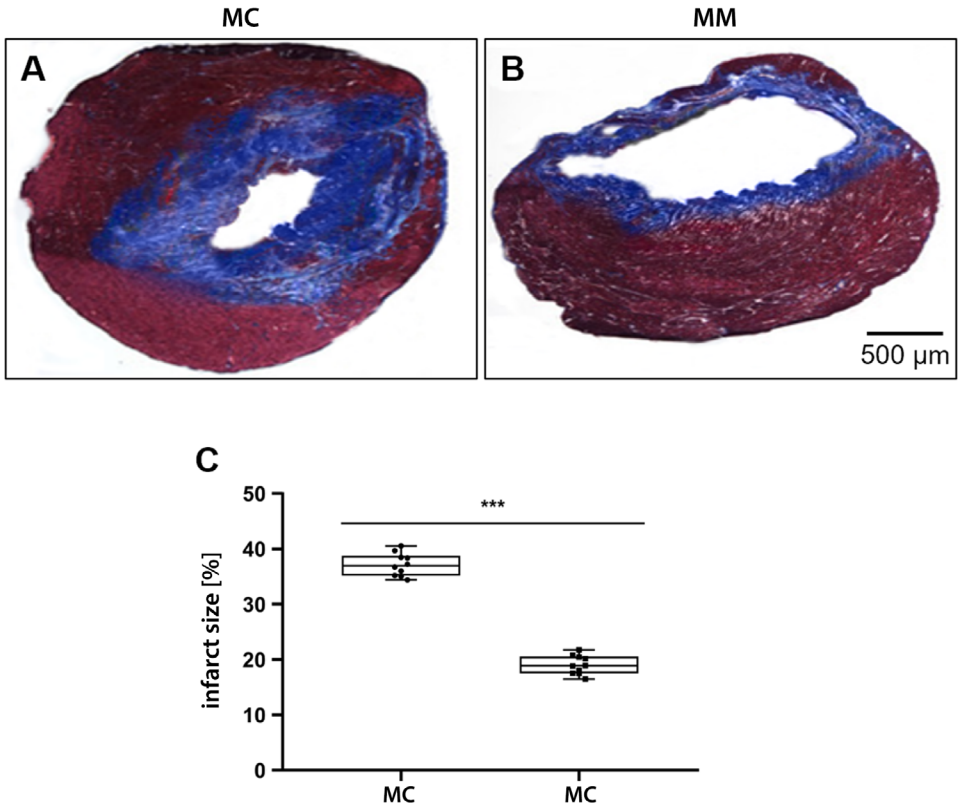

Fig. 1. Melatonin decreased myocardial infarct size in the MM. A,B. Masson's trichrome staining. The fibrotic scar is blue and the viable myocardium is red; C. The statistical analysis of infarct size

MC – the melatonin-treated sham-operated group; MM – the melatonin-treated MI group; *** $p < 0.001$.

Table 1. The comparisons between multiple groups after Kruskal–Wallis test and the comparisons between the melatonin-treated myocardial infarction group (MM) and the placebo-treated myocardial infarction group (PM) after Bonferroni adjustment

Outcomes	Kruskal–Wallis χ^2 test	df	p-value among groups	Comparisons	Bonferroni-adjusted p-value
LVEDd [%]	35.08	3	<0.0001	MM vs. PM	0.3339
LVEF [%]	32.95	3	<0.0001	MM vs. PM	0.3345
Max dP/dt [mm Hg/s]	32.93	3	<0.0001	MM vs. PM	0.3347
LVESd [%]	33.02	3	<0.0001	MM vs. PM	0.3345
LVFS [%]	34.23	3	<0.0001	MM vs. PM	0.3341
Min dP/dt [mm Hg/s]	32.98	3	<0.0001	MM vs. PM	0.3347
Sirt6 mRNA expression	32.94	3	<0.0001	MM vs. PM	<0.0001
Sirt6 protein expression	30.29	3	<0.0001	MM vs. PM	<0.0001
ROS (fold)	33.01	3	<0.0001	MM vs. PM	0.3343
SOD (fold)	33.01	3	<0.0001	MM vs. PM	0.3336
NO relative level	32.99	3	<0.0001	MM vs. PM	0.3346
eNOS protein/actin	32.97	3	<0.0001	MM vs. PM	<0.0001
p-eNOS protein/actin	33.17	3	<0.0001	MM vs. PM	0.3343
iNOS protein/actin	21.98	3	<0.0001	MM vs. PM	0.0008
p-iNOS protein/actin	24.24	3	<0.0001	MM vs. PM	0.0136

LVEDd – left ventricular end-diastolic diameter; LVEF – left ventricle ejection fraction; max dP/dt – maximum first derivative of developed pressure; LVESd – left ventricular end-systolic diameter; LVFS – left ventricular fractional shortening; Min dP/dt – minimum first derivative of developed pressure; ROS – reactive oxygen species; SOD – superoxide dismutase; NO – nitric oxide; eNOS – endothelial nitric oxide synthase; p-eNOS – phosphorylated eNOS; iNOS – inducible nitric oxide synthase; p-iNOS – phosphorylated iNOS; df – degrees of freedom.

molecules were detected. The results indicated that when treated with melatonin, the ROS level from primary cardiomyocytes was lowered in the MM group compared to the PM group, yet it was nonsignificant after Dunn's Bonferroni adjustment ($p = 0.3343$; Table 1). There was no significant difference among the PC and MC groups, as shown in Fig. 4A. The SOD activity was enhanced in the MM group compared to the PM group, but it was nonsignificant after Dunn's Bonferroni adjustment ($p = 0.3336$; Table 1), as shown in Fig. 4B.

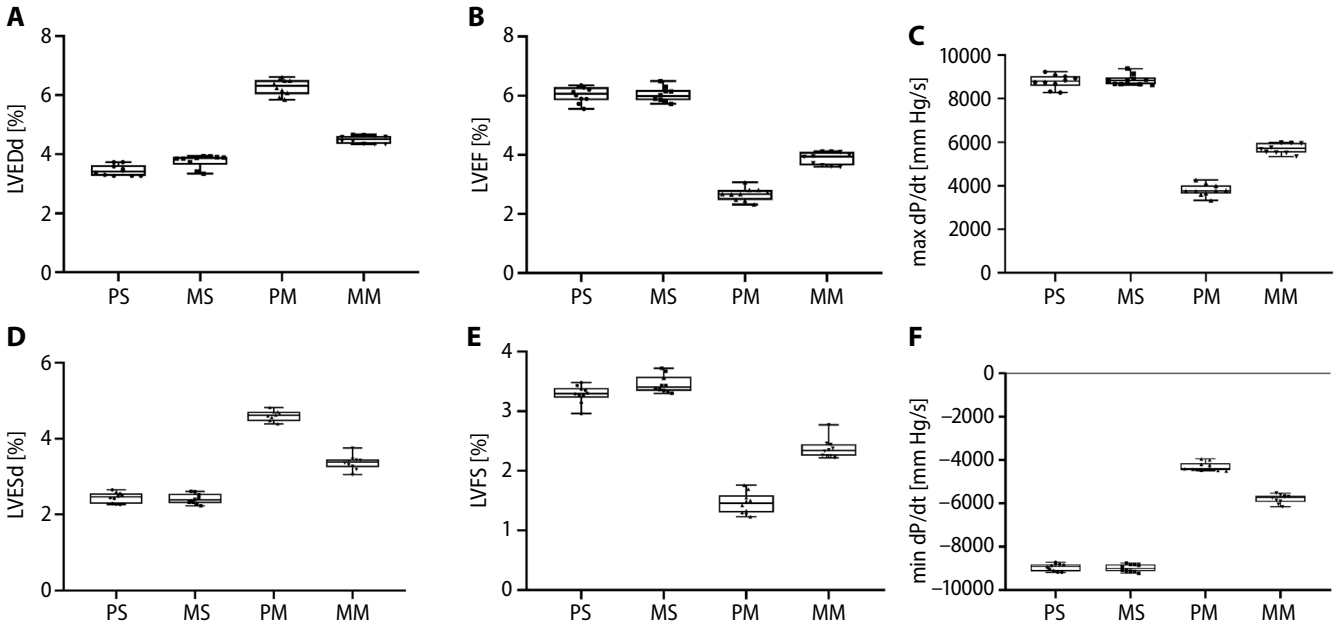

Fig. 2. Melatonin improved hemodynamic parameters. (A) LVEDd, (D) LVESd and (F) min dP/dt decreased significantly, while (B) LVEF, (E) LVFS and (C) max dP/dt increased significantly in myocardial infarction (MI) rats after treatment with melatonin. However, data became nonsignificant after adjustment

LVEDd – the lleft ventricular end-diastolic diameter; LVESd – left ventricular end-systolic diameter; LVEF – left ventricle ejection fraction; LVFS – left ventricular fractional shortening; PS – placebo-treated sham-operated group; MS – melatonin-treated sham-operated group; PM – placebo-treated myocardial infarction group; MM – melatonin-treated myocardial infarction group.

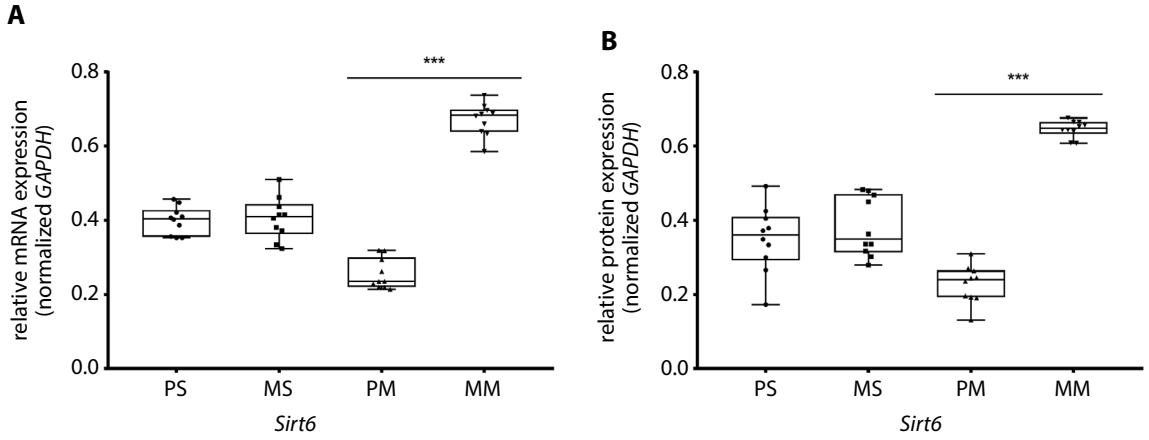

Fig. 3. Melatonin increased *Sirt6* expression both in mRNA and protein level. A. The mRNA level in cardiomyocytes in different groups; B. The protein level in cardiomyocytes in different groups

PS – placebo-treated sham-operated group; MS – melatonin-treated sham-operated group; PM – placebo-treated myocardial infarction group; MM – melatonin-treated myocardial infarction group. PM compared to MM: * $p < 0.05$; ** $p < 0.01$; ***$p < 0.001$.

Melatonin increased eNOS and NO production and inhibited iNOS production

In order to demonstrate whether the important role of melatonin as an antioxidant was involved in NO signaling, we performed assays to measure the production of NO-related molecules. The results showed that the levels of NO ($p = 0.3346$ after adjustment when compared to the PM group), eNOS ($p < 0.0001$ after adjustment) and p-eNOS ($p = 0.3343$ after adjustment when compared to the PM group) were increased in the MM group, but not in the PC and MC group, as shown in Fig. 5. This indicated that melatonin might promote cardioprotection by activating the eNOS/NO signaling pathway. At the same time, we found that the levels of iNOS ($p = 0.0008$ after adjustment when compared to the PM group) and phosphorylated iNOS (p-iNOS) ($p = 0.0136$ after adjustment when compared to the PM group) were significantly decreased when treated with melatonin in the hearts of the MM rats but not in the PC and MC groups, as shown in Fig. 5C. This indicated that melatonin might promote cardioprotection by inhibiting iNOS (Table 1).

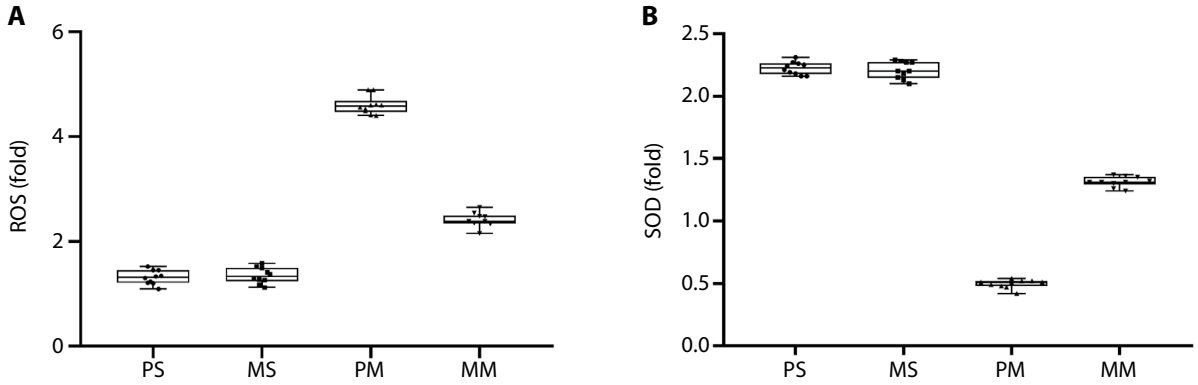

Fig. 4. Melatonin ameliorates myocardial infarction (MI)-induced oxidative stress. A. The determination of reactive oxygen species (ROS) in the 4 groups; B. The determination of superoxide dismutase (SOD) in the 4 groups. Data became non-significant after the adjustment

PS – placebo-treated sham-operated group; MS – melatonin-treated sham-operated group; PM – placebo-treated myocardial infarction (MI) group; MM – melatonin-treated MI group.

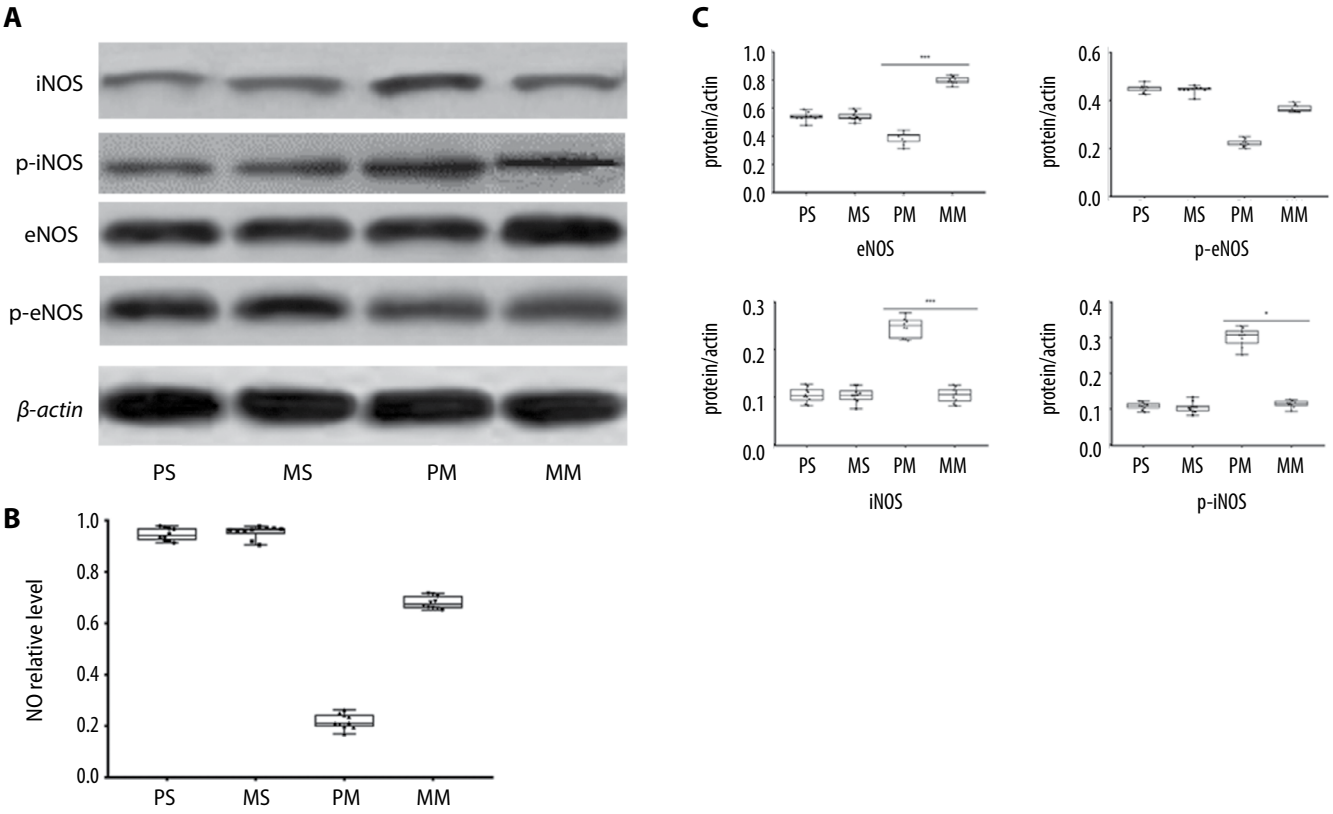

Fig. 5. Melatonin activated the endothelial nitric oxide synthase (eNOS)/nitric oxide (NO) signaling pathway and inhibited the inducible nitric oxide synthase (iNOS) signaling pathway. A. Western blot of iNOS/phosphorylated iNOS (p-iNOS)/eNOS/phosphorylated eNOS (p-eNOS) in the 4 groups; B. Relative NO levels compared in the 4 groups; C. The analysis of the proteins in the 4 groups

PS – placebo-treated sham-operated group; MS – melatonin-treated sham-operated group; PM – placebo-treated myocardial infarction (MI) group; MM – melatonin-treated MI group. PM compared to MM: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Discussion

Melatonin can counteract many pathological conditions, such as cardiovascular disorders, carcinogenesis, neurological diseases, and aging.[19] Recently, its cardioprotective function has gained a great deal of attention.[20–23] More and more evidence suggests that the cardioprotective functions of melatonin are due to its direct free radical scavenger properties, antioxidant activities and anti-inflammatory effects. In this study, we confirmed the cardioprotective role of melatonin in an animal model of MI, and for the first time we found that melatonin improved cardiac remodeling through a *Sirt6*-dependent antioxidant pathway.

Pharmacological data have clearly demonstrated the powerful antioxidant properties of melatonin.[24–26] For example, melatonin can protect the brain as a potent antioxidant, anti-inflammatory and antiapoptotic agent.[27] Melatonin has been shown to prevent granulosa cells from oxidative damage by targeting JNK-mediated autophagy, which may hold promise for patients with ovulation failure-related disorders.[28]

In order to investigate the cardioprotective role of melatonin on MI, we established a MI rat model by ligating the left anterior descending coronary artery and verifying ST elevation using eletrocardiography (ECG). Four weeks after the treatment with melatonin, infarct size and hemodynamic parameters were measured. Infarct size was reduced significantly after the treatment with melatonin, which indicated that melatonin could protect the infarcted myocardium. Similarly, the results of hemodynamic studies indicated that post-MI cardiac remodeling had improved significantly following the treatment with melatonin.

Accumulated evidence indicates that oxidative stress is responsible for the progression of cardiac remodeling.[29,30] In order to investigate whether the cardioprotective effect of melatonin functions through its antioxidant role, ROS and SOD levels were measured as the indicators of oxidative stress. Excessive ROS accumulation leads to cellular oxidative stress and elicits an increased proinflammatory response, which is associated with cardiac remodeling.[7,31] Not surprisingly, after the treatment with melatonin, we found that ROS activity reduced significantly and SOD level increased significantly in the MM group. The evidence suggested that melatonin could prevent cardiac remodeling by decreasing oxidative stress.

The NO is a widely known ventricular dilator produced from L-arginine by a family of NOS, including eNOS and iNOS.[32] It has been shown that NO plays a role in reducing oxidative stress[33] and in blocking the progression of MI.[34] In our study, the expressions of NO, iNOS, eNOS, and their corresponding phosphorylations were determined. The results indicated that the expressions of NO and eNOS were upregulated and the expression of iNOS was lowered in the MM group compared to the PM group. This evidence suggests that melatonin might suppress the processes of oxidative stress by activating the eNOS/NO signaling pathway and inhibiting iNOS signaling pathway.

Interestingly, we found that the expressions of *Sirt6*, both in mRNA and protein levels, were significantly increased in the MM group compared to the PM group. This indicates that *Sirt6* might be the target through which melatonin improves oxidative stress by activating the eNOS/NO signaling pathway in the heart.

In spite of the fact that a dose of 10 mg/kg of melatonin administered via intraperitoneal injection works cardioprotectively in the MM group, there is still no evidence to demonstrate whether this dose works for humans. Actually, it was reported that a total dose of 50 mg has been used during a major vascular surgery.[35] Regretfully, those researchers concluded that intracoronary and intravenous administration of melatonin did not improve the myocardial salvage index in patients with ST-elevation myocardial infarction (STEMI) undergoing primary percutaneous coronary intervention. Hopefully, if the dose of melatonin was increased to 10 mg/kg, it would demonstrate some beneficial effects for the heart, considering its obvious *Sirt6*-dependent antioxidative ability in I/R. However, this speculation still needs to be verified.

Limitations

Firstly, this study proved the effect of melatonin on mouse MI model, but there is still no evidence to demonstrate whether this works for humans. Secondly, the study had no designed not designed dose gradient, so the optimal dose of melatonin for MI could not be obtained. Thirdly, we only analyzed the changes of *Sirt6* expression and did not verify whether the knockout of *Sirt6* would eliminate the cardioprotective effect of melatonin.

Conclusions

The current study demonstrated for the first time that intraperitoneal injection of melatonin could improve the cardiac function via a *Sirt6*-dependent antioxidant pathway in MI rats.

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