Melatonin reduces myocardial cell damage in myocardial ischemia/reperfusion rats by inhibiting NLRP3 activation

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ABSTRACT

Melatonin (Mel) is an endogenous hormone with many pharmacological effects, such as sedation, hypnosis, antidepressant, blood pressure regulation, anti-inflammatory and anti-tumor. It is mainly synthesized by pineal gland in vivo. Mel can regulate the function of cardiovascular system and effectively reduce myocardial cell injury, thus playing a role in myocardial protection, but its specific mechanism is unclear. Ischemia-reperfusion injury (IRI) often leads to poor prognosis and complications in patients with cardiovascular diseases, and inflammatory reaction mediated by nod-like receptor thermoprotein domain-related protein 3 (NLRP3) is an important reason for the further aggravation of subsequent injury after IRI. Rats were intervened with Mel or NLRP3 inhibitor MCC950 for 10 days, and then the IRI rat model was established. After that, rats were anesthetized and killed, and myocardial tissues were collected for experiments. The experimental results showed that Mel reduced the myocardial infarction area, decreased aspartate aminotransferase (AST), creatine kinase myocardial band (CK-MB), lactate dehydrogenase (LDH) and malondialdehyde (MDA), and significantly inhibited the expression of reactive oxygen species (ROS), NLRP3, caspase-1 and apoptosis-associated speck-like protein containing a CARD (ASC), but its effect on superoxide dismutase (SOD) was opposite. Therefore, Mel may improve autophagy and inflammation during myocardial ischemia/reperfusion and may decrease myocardial cell damage by inhibiting NLRP3.

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Introduction

According to the China Cardiovascular Disease Report 2018, myocardial disease has come to represent a major health concern in recent years (1). When a heart organ or tissue is short of blood, it will inevitably be damaged, and reinjection in time can limit the degree of damage (2). However, after reinjection, additional damage will occur, which is similar to a chaotic biological restart, which limits the number of salvageable tissues, this compound injury is called myocardial ischemia/reperfusion injury (IRI) (3). Acute heart failure and myocardial infarction may be the main clinical signs and symptoms of IRI, and pathological factors such as inflammation and myocardial apoptosis are involved (4). Therefore, suppression of the inflammatory response and the production of ROS may serve a key role in rescuing IRI

Melatonin (Mel) is a multifunctional molecule that is naturally produced in the body, and its diversity and versatility reflect its range of effects, such as scavenging free radicals and hypnosis (5,6). Previous studies on Mel mainly focused on its regulation of circadian rhythm and paid more attention to its application in improving sleep (7). However, Mel also plays a certain role in anti-apoptosis, anti-inflammatory and anti-oxidative stress in addition to the above effects (6,8,9). A previous study identified the presence of Mel receptors in human blood vessels, which provides novel research directions and indicates that Mel serves a key role in cardiovascular disease and may thus directly control the occurrence and development of cardiovascular disease (10). Many acute cardiovascular events often occur in the early morning, which may be related to the circadian rhythm of the heart. It can be inferred that the regulatory mechanism of melatonin may be involved in the aggravation or remission of cardiovascular diseases (11,12). In addition, some studies have found that the level of Mel in patients with myocardial infarction is low, so it can be inferred that increasing the level of Mel can effectively reduce the risk of acute cardiovascular events (13).

The NOD-like receptor thermal protein domain associated protein 3 (NLRP3) is a member of the LR inflammatory corpuscle family (14). According to Lamkanfi et al. (15), NLRP3 inflammatory corpuscle is a part of the innate immune system, which can be overactivated by IRI, and then produce IL-1β and IL-18 to cope with cell stress, and then NLRP3 can integrate ASC to activate caspase-1, which can obviously aggravate the inflammatory reaction of the body and amplify the resulting inflammatory damage (16). In addition, in animal experiments, mice lacking ASC and Caspase-1 have significantly reduced their inflammatory reaction and subsequent injuries (including infarction development, myocardial fibrosis and dysfunction), and the use of Caspase-1 inhibitor can significantly protect the myocardial cells of mice with acute myocardial infarction model (17,18). A recent report indicated that some molecules and drugs (e.g., via dopamine and Mel) can regulate the activation of NLRP3 (19). Based on the above, we established an IRI rat model to observe the ef-

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fects of Mel on the levels of NLRP3 and myocardial tissue in rats and to clarify the mechanism of Mel on NLRP3 in IRI.

Materials and Methods

Animals

90 Specific Pathogen Free (SPF) SD rats (250-300 g; 8 weeks) were obtained [license no. SCXK (Beijing) 20160006]. Rats followed National Institutes of Health guidelines.

Myocardial IRI model

According to the reference method (20), the formation of the rat myocardial IRI model was induced by coronary artery ligation. Rats were anesthetized with 1% pentobarbital sodium. Cut a skin incision along the middle of the suprasternal fossa. After separating the inferior mandibular gland, the anterior tracheal muscles were retracted to expose the trachea. A transverse tracheal incision was performed between the 2nd and 3rd tracheal rings. The incision length did not exceed 1/3 of the peritracheal diameter. After cleaning the tracheal contents, a tube was quickly inserted 0.5-1.0 cm into the trachea and connected to a small animal ventilator. Subsequently, the left chest hair was shaved, the area was disinfected with 75% alcohol and skin along the left edge of the sternum was incised (2 cm). The subcutaneous tissue was separated from the muscle, then the chest was opened between the 4-5 ribs to expose the heart. After ligation of the left anterior descending coronary artery for 30 minutes, the ligation was released to restore myocardial reperfusion. Subsequently, the heart was put back into the chest, the gas was squeezed out and the chest wall was sutured. After 6 h reperfusion, rats were killed by vertebral dislocation after anesthesia, and myocardial tissue was collected for subsequent experiments.

Grouping

Rats were divided into 6 groups (15 in each group): i) Sham: Only threading was performed in the LAD (without ligation); ii) Myocardial IRI: Rats were induced into IRI; iii) IRI + low dose Mel (IRI + 10 Mel): Before surgery, rats were intraperitoneally injected with Mel (10 mg/kg/d, 10 d); then, rats were induced into IRI; iv) IRI + high dose Mel (IRI + 20 Mel): Before surgery, rats were intraperitoneally injected with Mel (20 mg/kg/d, 10 d); then, rats were induced into IRI; v) IRI + blank solvent negative control (IRI + NC): Before surgery, rats were intraperitoneally injected with DMSO (10 mg/kg/d, 10 d); then, rats were induced into IRI; vi) IRI + NLRP3 inhibitor MCC950 (IRI + MCC950): Rats were intraperitoneally injected with MCC950 (3 mg/kg/d, 10 d), as previously described (21); then, rats induced into IRI.

Triphenyl tetrazolium chloride (TTC) staining

The specimens were frozen (-20°C, 30 min) and cut into 2 mm sections. Each section was placed in a 6-well plate, and then 2% TTC was added and covered with a round coverslip to stain (37°C, 30 min). After staining, the section was removed and then imaged with Canon (200DII; Canon, Inc., Tokyo, Japan). The infarct size was assessed using ImageJ 1.43 software and calculated as a percentage of infarct area (INF) over the total area at risk (AAR).

H&E staining

The specimens were treated in paraformaldehyde (4%, 4°C, 24 h), and then cut into sections (5 μm), which were dewaxed and hydrated. Next, the sections were stained with hematoxylin (0.3%, 37°C, 5 min). Following differentiation with hydrochloric acid and ethanol (30 s), placed in 0.5% eosin staining solution (0.5%, 37°C, 2 min) conventionally dehydrated, transparentized and mounted. The histopathological changes of the myocardium were observed under an optical light microscope.

Evaluation of ROS

The heart tissue was collected and cut into 1 mm pieces, after digesting with 0.1% collagenase I and hyaluronidase-containing digestive solution (45 min, 4°C) (22). Following incubation in 0.1% 2’,7’-dichlorofluorescein diacetate (10 μM, 37°C, 30 min). ROS was detected by flow cytometry, and the results were analyzed by CytExpert software.

Immunofluorescence

The paraffin sections of myocardial tissue were dewaxed and repaired with citrate buffer for 5 min, then rinsed with phosphate-buffered saline (PBS), blocked with 10% bovine serum albumin (BSA) and incubated with primary anti-rabbit anti-raft NLRP3, rabbit anti-rat caspase-1 and ASC polyclonal antibodies overnight at 4°C. Then, sections were washed with PBS, followed by the addition of FITC-labeled goat anti-rabbit IgG. And the tissue was stained with 10 μg/ml 4’,6-diamidino-2-phenylindole (DAPI). Finally, after washing with PBS, the tissue was sealed with a fluorescence quencher. Then the slices were observed under the microscope at a magnification of ×400.

Western blotting

The heart tissue specimens were washed with PBS, added to 150 μl pre-chilled lysis for ultrasonic lysis and centrifuged at 850 × g. After the total protein was extracted by radioimmunoprecipitation assay (RIPA) buffer, the total protein concentration was determined by a bicinechonic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Protein (30 μg) was collected to perform 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with Tris-buffered solution and incubated with anti-rabbit anti-raft NLRP3, caspase-1, ASC and β-actin polyclonal antibodies at 4°C overnight. After rewarmed, the specimens were incubated with secondary goat anti-rabbit horseradish peroxidase-conjugated IgG and washed with emission chromogenic substrate (ECL; Thermo Fisher Scientific, Inc.) for 3-5 min. β-actin was used to normalize the protein levels. ImageJ software 5.0 was used for semi-quantification.
Results

Influence of Mel intervention on myocardial infarction in myocardial tissue

TTC (Figure 1A) and H&E staining (Figure 1B) were used to assess the area of myocardial IRI and tissue structure, respectively. The infarction of IRI in the IRI group was increased (P<0.01; Figure 1A). Following high- and low-dose Mel intervention, the area of myocardial infarction decreased, which was significantly different from the IRI and the IRI + NC groups (all P<0.01). Following intervention with the NLRP3 inhibitor MCC950, the myocardial infarct size was significantly decreased (P<0.01).

In the Sham group, myocardial tissue structure was normal and cardiomyocyte arrangement was orderly (Figure 1B). On the contrary, the cardiomyocyte arrangement in the IRI and IRI + NC groups was disordered, with a number of broken lines observed. Following Mel intervention, myocardial cell arrangement was restored and broken lines disappeared in the IRI + 20 Mel, IRI +10 Mel and IRI + MCC950 groups.

Influence of Mel intervention on the expression of the oxidative stress factors AST, CK-MB and LDH of IRI rats

The rats in the IRI group exhibited higher AST, CK-MB and LDH than the Sham group (P<0.01; Figure 2). Following treatment with Mel, the expression of these factors was decreased in a dose-dependent manner. Furthermore, treatment with the NLRP3 inhibitor also significantly decreased the levels of these factors in myocardial tissue (P<0.01).

Influence of Mel intervention on MDA, SOD and ROS

In order to determine the effects of Mel intervention, the levels of MDA, SOA and ROS were also examined. Compared with the Sham group, MDA was increased in the IRI group (P<0.01; Figure 3A), whereas SOD was decreased in the IRI group (P<0.01; Figure 3B). Following intervention of Mel, MDA was significantly decreased, whereas SOD was increased (all P<0.05); these effects were more notable in the high-dose group. Treatment with NLRP3 inhibitor had similar effects to those induced by Mel (all P<0.05). Flow cytometry showed that ROS in the IRI group increased compared with those in the Sham group (P<0.01; Figure 3C), and Mel administration inhibited ROS generation in a dose-dependent manner. The addition of NLRP3 inhibitor decreased the production of ROS (P<0.01).

Statistical analysis

Statistic Package for Social Science (SPSS) 19.0 (IBM, Armonk, NY, USA) was used to perform data analysis. One-way ANOVA was performed for multiple comparisons, followed by Tukey’s post hoc test. In total, three experimental repeats were performed and data are presented as the mean ± SD (standard deviation). P<0.05 was considered to indicate a statistically significant difference.
the Sham group (all \( P<0.01 \); Figure 4). IL-1\( \beta \) and IL-18 decreased in a dose-dependent manner following administration of Mel. Following treatment with NLRP3 inhibitor, IL-1\( \beta \) and IL-18 were also downregulated (all \( P<0.01 \)).

**Influence of Mel intervention on NLRP3, caspase-1 and ASC**

The NLRP3 (Figure 5), caspase-1 (Figure 6) and ASC (Figure 7) were analyzed by immunofluorescence and Western blotting (Figure 8). Compared with the Sham group, the NLRP3, caspase-1 and ASC were increased in the IRI and IRI + NC groups (all \( P<0.01 \)). Compared with the IRI and IRI + NC groups, these inflammasome proteins were decreased in the IRI + Mel groups (all \( P<0.05 \)). Following treatment with NLRP3 inhibitor, NLRP3, caspase-1 and ASC were decreased compared with that in IRI and IRI + NC groups (all \( P<0.01 \)).

**Discussion**

After IRI rats were induced to have a myocardial injury, the main pathological manifestations of rats were the increase of myocardial infarction area, the disorder of myocardial tissue, and the abnormal increase of serum LDH, CK-MB and AST levels, which indicated that the establishment of IRI model rats in this experiment was successful. When Mel pretreatment was given to rats for 10 days, it could obviously alleviate their oxidative stress level and inflammatory reaction. Further research showed

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**Figure 4.** Effects of Mel intervention on (A) IL-1\( \beta \) and (B) IL-18 (n=10). \(^*\)P<0.01 vs. Sham; \(^{##}\)P<0.01 vs. IRI; \(^{##}\)P<0.01 vs. IRI + NC.

**Figure 5.** Effects of Mel intervention on NLRP3 (n=5). \(^{*}\)P<0.05, \(^{##}\)P<0.01 vs. Sham; \(^{*}\)P<0.05, \(^{##}\)P<0.01 vs. IRI; \(^{##}\)P<0.01 vs. IRI + NC.

**Figure 6.** Effects of Mel intervention on caspase-1 (n=5). \(^{*}\)P<0.05, \(^{**}\)P<0.01 vs. Sham; \(^{*}\)P<0.05, \(^{**}\)P<0.01 vs. IRI; \(^{**}\)P<0.01 vs. IRI + NC.

**Figure 7.** Effects of Mel intervention on ASC (n=5). \(^{*}\)P<0.05, \(^{##}\)P<0.01 vs. Sham; \(^{*}\)P<0.05, \(^{##}\)P<0.01 vs. IRI; \(^{##}\)P<0.01 vs. IRI + NC.

**Figure 8.** Western blot analysis of the effects of Mel intervention on NLRP3, caspase-1 and ASC proteins (n=5). \(^{*}\)P<0.05, \(^{##}\)P<0.01 vs. Sham; \(^{*}\)P<0.05, \(^{##}\)P<0.01 vs. IRI; \(^{##}\)P<0.01 vs. IRI + NC.
that Mel could inhibit the activation of NLRP3 inflammatory corpuscles after IRI, and then interfere with the abnormal activation of downstream targets, indicating that Mel pretreatment could effectively prevent a series of injuries caused by IRI and improve the prognosis of patients. Its cardioprotective effect may be mediated by the abnormal inflammatory reaction triggered by NLRP3 inflammatory corpuscles.

IRI is the main cause of poor clinical treatment effects of ischemic heart disease, which can lead to death, poor prognosis and serious complications. Pathologically, excessive inflammatory reaction and oxidative stress accompanying IRI can lead to irreversible changes in heart function and structure, and lead to the death of myocardial cells (23). Previous studies have shown that over-activated inflammatory reaction can significantly increase the area of myocardial infarction, and biochemical markers in the blood (such as LDH, CK-MB and AST) can be sensitive to the degree of cardiac tissue damage (24,25). As expected, in this study, compared with the IRI group, the myocardial infarction area of the high-dose and low-dose Mel groups was significantly reduced, in addition, the LDH, CK-MB and AST in the IRI group increased. On the contrary, pretreatment with Mel significantly reduced the level of these markers in serum, which further confirmed the beneficial effect of Mel on the myocardial tissue of IRI rats combined with the pathological results of heart-stained with HE.

The main mechanism of IRI-induced high-level oxidative stress on myocardial damage is to induce myocardial cell death, and the most important feature of myocardial cell scorch is the dependent reaction between NLRP3 and caspase-1 during IRI (26). A large number of ROS will be produced in cells shortly after reperfusion, and excessive ROS can activate NLRP3 and stimulate it to combine with ASC to form NLRP3 complex, which can lead to the cleavage of cell death regulatory proteins, and then induce the occurrence of myocardial cell scorch. Mel has a strong ability to scavenge ROS, which can reduce MDA and increase SOD (27,28). In this regard, we further evaluated the biochemical markers MDA and SOD in serum representing oxidative damage. In this study, IRI leads to the increase of oxidative stress, compared with the IRI model, the MDA level of mel-treated rats decreased significantly, while the SOD activity increased significantly. It shows that Mel can significantly reduce the increase of ROS level caused by IRI.

The pathophysiological mechanism of IIRI-induced heart injury involves myocardial apoptosis and inflammatory reaction. It has been previously reported that NLRP3 inflammatory corpuscles are a key component of innate immunity and an important heart injury-related disease (29). At present, the cell death mediated by NLRP3 is becoming a new target to intervene in myocardial IRI. NLRP3 inflammatory corpuscles can be assembled and activated by various dangerous signals (such as IRI, coronary heart disease and atherosclerosis), and induce inflammatory reactions similar to those caused by pathogens (30,31). When NLRP3 is activated, it can promote the secretion of IL-1β and IL-18, not only that, NLRP3 will in turn aggravate the development of coronary heart disease and IRI through the above mechanism, leading to a vicious circle. Mel is an effective antioxidant, which can stimulate the production of other antioxidants, inhibit myocardial cell apoptosis and autophagy, resist lipid peroxidation and DNA damage, alleviate inflammatory reactions, and play an effective role in heart protection by regulating various channels (32-40). In this study, the NLRP3, caspase-1 and ASC in IRI rats were significantly inhibited after Mel intervention. These findings suggest the abnormal activation of the NLRP3/ASC/caspase-1 pathway and subsequent production of inflammatory factors may be the main pathway of myocardial cell injury. In addition, Mel is a potential myocardial IRI protective agent, and its protective mechanism may be achieved through the regulation of NLRP3 inflammatory corpuscles.

To sum up, the changes in cardiac structure and the pro-inflammatory factors can be observed in myocardial IRI rats, which are related to the activation of the NLRP3/ASC/caspase-1 pathway. Besides, the protective activity of Mel on the heart may be achieved by regulating the NLRP3 inflammatory corpuscles, thus alleviating the myocardial injury caused by IRI.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors’ contributions
SL and XL designed and performed the experiments, collected, analyzed and interpreted data and wrote the manuscript. FX participated in the design and coordination of experiments and collected and analyzed data. YB collected and analyzed data. JM analyzed and interpreted data and drafted the manuscript. All authors read and approved the final manuscript. SL, XL and FX confirm the authenticity of all the raw data.

Ethics approval and consent to participate
The study was approved by the Ethics Committee of Beijing Anzhen Hospital, Capital Medical University (approval no. 2020107X).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


