



Melatonin alleviates lipopolysaccharide-induced myocardial injury by inhibiting inflammation and pyroptosis in cardiomyocytes

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Background: Melatonin (MT) has been shown to protect against various cardiovascular diseases. However, the effect of MT on lipopolysaccharide (LPS)-induced myocardial injury is poorly understood. This study aims to evaluate the effects of MT on LPS-induced myocardial injury *in vitro*.

Methods: H9C2 cells were divided into a control group, MT group, LPS group, and MT + LPS group. The control group was treated with sterile saline solution, the LPS group received 8 µg/mL LPS for 24 h, MT + LPS cells were pretreated with 200 µmol/L MT for 2 h then with 8 µg/mL LPS for 24 h, and the MT group received only 200 µmol/L MT for 2 h. The CCK-8 assay and lactate dehydrogenase (LDH) activity assay were used to analyze cell viability and LDH release, respectively. Intracellular reactive oxygen species (ROS) and the rate of pyroptosis were measured using the fluorescent probe dichloro-dihydro-fluorescein diacetate (DCFH-DA) and propidium iodide (PI) staining, respectively. The cell supernatants were used to measure the levels of inflammatory cytokines, including IL-6, TNF- α , and IL-1 β by enzyme-linked immunosorbent assay (ELISA). The protein levels of iNOS, COX-2, NF- κ B, p-NF- κ B, NLRP3, caspase-1, and GSDMD were detected by western blot.

Results: MT pretreatment significantly improved LPS-induced myocardial injury by inhibiting inflammation and pyroptosis in H9C2 cells. Moreover, MT inhibited the activation of the NF- κ B pathway, and reduced the expression of inflammation-related proteins (iNOS and COX-2), and pyroptosis-related proteins (NLRP3, caspase-1, and GSDMD).

Conclusions: Our data suggests that MT can alleviate LPS-induced myocardial injury, providing novel insights into the treatment of sepsis-induced myocardial dysfunction.

Keywords: Melatonin (MT); lipopolysaccharide (LPS); myocardial injury; inflammation; pyroptosis

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Introduction

Sepsis is a complex life-threatening organ dysfunction syndrome caused by a dysregulated host response to infection that leads to an uncontrolled immune response (1). Cardiac dysfunction is prevalent in patients with sepsis (2,3), who have a poor prognosis (4,5). Therefore, the survival of septic patients may be associated with improving cardiac function.

Myocardial injury is commonly observed in patients with sepsis, which is mainly induced by lipopolysaccharide (LPS), a bacterial endotoxin found in the cell wall of Gram-negative bacteria (6-8). In cardiomyocytes, LPS activates nuclear factor- κ B (NF- κ B) which then increases the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), speeding up the synthesis and release of inflammatory cytokines (6,9). The overexpression and release of proinflammatory cytokines induced by LPS, such as interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α), have been associated with cardiac dysfunction, including myocardial depression (10). In addition, Sepsis is closely associated with pyroptosis, which is a novel type of programmed cell death (11). ROS-dependent pyroptosis has also been recognized as an important pathway mediating LPS-induced myocardial injury (12). Pathogen-associated molecular patterns (PAMPs), such as LPS, could induce reactive oxygen species (ROS) production, which can initiate NLR family pyrin domain containing 3 (NLRP3) inflammasome activation then activating caspase-1, subsequently promoting pyroptosis and the secretion of IL-1 β (13-17). ROS inhibitors have been reported to downregulate NLRP3 mRNA expression, indicating that NLRP3 gene expression is partly modulated by ROS generation (18,19). Accordingly, inhibiting the inflammation and ROS-dependent pyroptosis in cardiomyocytes may be a promising treatment for sepsis-induced myocardial injury.

Melatonin (MT) is a vital neuroendocrine hormone synthesized by the pineal gland (20). Although the primary functions of MT are associated with sleep cycle regulation (21), it recently has shown strong effects of anti-inflammation, anti-oxidation, alleviating adipose and endothelial pyroptosis (22-25). Several studies have shown that MT could protect against myocardial injury (26-30) via regulating apoptosis, autophagy, improving mitochondrial function. However, the underlying mechanisms that MT improves LPS-induced myocardial injury remain unclear. In particular, no study has found that melatonin could alleviate LPS-induced myocardial injury by inhibiting inflammation and pyroptosis, simultaneously.

In this study, we hypothesize that MT attenuates LPS-induced myocardial injury by inhibiting NF- κ B/iNOS/COX-2-induced inflammation and ROS-dependent NLRP3-mediated pyroptosis in H9C2 cardiomyocytes. In addition, we present the following article in accordance with the MDAR checklist (available at <http://dx.doi.org/10.21037/atm-20-8196>).

Methods

Cell culture

The H9C2 cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in DMEM (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified atmosphere containing 5% CO₂/95% air at 37 °C. Moreover, 0.05% trypsin was used to digest cells, and cell medium was replaced every 3 days. H9C2 cells were treated with different stimuli in 6-well plates or 96-well plates.

Treatment of cells

LPS (Sigma, USA) and MT (Sigma, USA) were dissolved in sterile deionized water to obtain a final concentration of 8 μ g/mL and 200 μ mol/L, respectively. The control group was treated with sterile saline solution, and the LPS group received 8 μ g/mL LPS for 24 h. Cells in the MT + LPS group were pretreated with 200 μ mol/L MT for 2 h, then 8 μ g/mL LPS for 24 h. The MT group received only 200 μ mol/L MT for 2 h. After incubation, cells were collected, and lactate dehydrogenase (LDH) levels, ROS levels, cell viability, and protein expression were analyzed. The levels of superoxide dismutase (SOD), malondialdehyde (MDA), IL-6, TNF- α , and IL-1 β in culture medium were estimated.

Cell viability

According to the manufacturer's instructions, CCK-8 assay kit (Bestbio, Shanghai, China) was used to determine the cell viability in 96-well plates. Twenty four hours after cell treatment, 10 μ L of sterile CCK-8 was added to each well and incubated in the dark for 2 h at 37 °C. The absorbance at 450 nm was determined using a microplate reader.

LDH activity assay

LDH activity was detected to estimate cell injury by a LDH

assay kit (BeastBio, Shanghai, China) according to the manufacturer's instruction.

Enzyme-linked immunosorbent assay (ELISA)

Commercial ELISA kits (Jianglai, Shanghai, China) were used to measure the concentrations of inflammatory cytokines, including TNF- α , IL-6, and IL-1 β , and oxidative stress biomarkers such as SOD and MDA.

Measurement of ROS generation

The levels of intracellular ROS were measured with the fluorescent probe dichloro-dihydro-fluorescein diacetate (DCFH-DA; Sigma, USA). After treatment with stimuli, cells were incubated with DCFH-DA (50 μ M) at 37 °C for 30 min. Then, PBS was used to wash the cells twice. The average fluorescence intensity was analyzed using an image analysis system.

Western blot

After treatment with the stimuli, radioimmunoprecipitation assay (RIPA) buffer (Beyotime, China), containing protease inhibitor cocktail (Beyotime, China) including phenylmethylsulfonyl fluoride (PMSF; Beyotime, China), was used to lyse the cells, and all homogenates were centrifuged for 15 min at 12,000 rpm at 4 °C. 10% SDS-PAGE gels were used to load equal amounts of denatured protein, which was then transferred onto polyvinylidene difluoride (PVDF) membranes. 5% nonfat milk was applied to block membranes at room temperature for 1 h. The membranes were then incubated overnight with primary antibodies against iNOS (1:1,000, NOVUS, USA), COX-2 (1:500, Abcam, USA), NF- κ B (1:1,000, Abcam, USA), p-NF- κ B (1:1,000, Abcam, USA), NLRP3 (1:1,000, NOVUS, USA), caspase-1 (1:1,000, NOVUS, USA), GSDMD (1:1000, Abcam, USA), and GAPDH (1:10,000, Abcam, USA). A fluorescent secondary antibody (1:2,500, CST, USA) was subsequently used to incubate membranes for 2 h at 4 °C. Then, TBST was used to wash the membranes 3 times, each time for 5 minutes. The quantification of protein expression was normalized to GAPDH.

Propidium iodide (PI) staining

After stimulation, the cells were collected and washed 3 times with 1 \times PBS. Then, 1 \times assay buffer was used to

wash the cells. 5 μ M PI were then mixed with 1 \times assay buffer and stained per well at 37 °C for 15 min. Finally, DAPI (Sigma, USA) were used to stain cells for 5 min. A fluorescence microscope was used to acquire the images of the cells immediately after staining. The the average fluorescence intensity and the percentage of positive cells were assessed with Image J.

Statistical analysis

All values are expressed as the mean \pm SD. The statistical analyses were done by GraphPad Prism version 8.0 (GraphPad Software, USA). One-way ANOVA or two-way ANOVA followed by Tukey's test were performed to analyze the differences among experimental groups. P values <0.05 were considered to be statistically significant.

Results

The effect of LPS on cell viability and LDH release in H9C2 cells

To confirm the effects of LPS on cellular activity and LDH release, cardiomyocytes were exposed to different concentrations of LPS. The viability of H9C2 cells was negatively correlated with LPS concentration. In response to 8 μ g/mL LPS, cell viability was significantly lower compared to the control group (*Figure 1A*). In addition, LDH release increased in a concentration-dependent manner (*Figure 1B*), and the highest LDH release was observed after treating cells with 8 μ g/mL LPS.

The effect of LPS on inflammatory cytokines in cell supernatants and oxidative stress biomarkers in H9C2 cells

After treating cells with different concentrations of LPS, the supernatants were collected to identify the levels of inflammatory cytokines. IL-6 and TNF- α levels were positively correlated with LPS concentration (*Figure 1C,D*). Furthermore, the levels of ROS increased in a dose-dependent manner (*Figure 1E*), the activity of SOD decreased (*Figure 1F*), and the levels of MDA (*Figure 1G*) and IL- β (*Figure 1H*) increased.

The effect of MT on cell viability and LDH release in LPS-treated H9C2 cells

When cells were treated with MT (50–200 μ mol/L) for

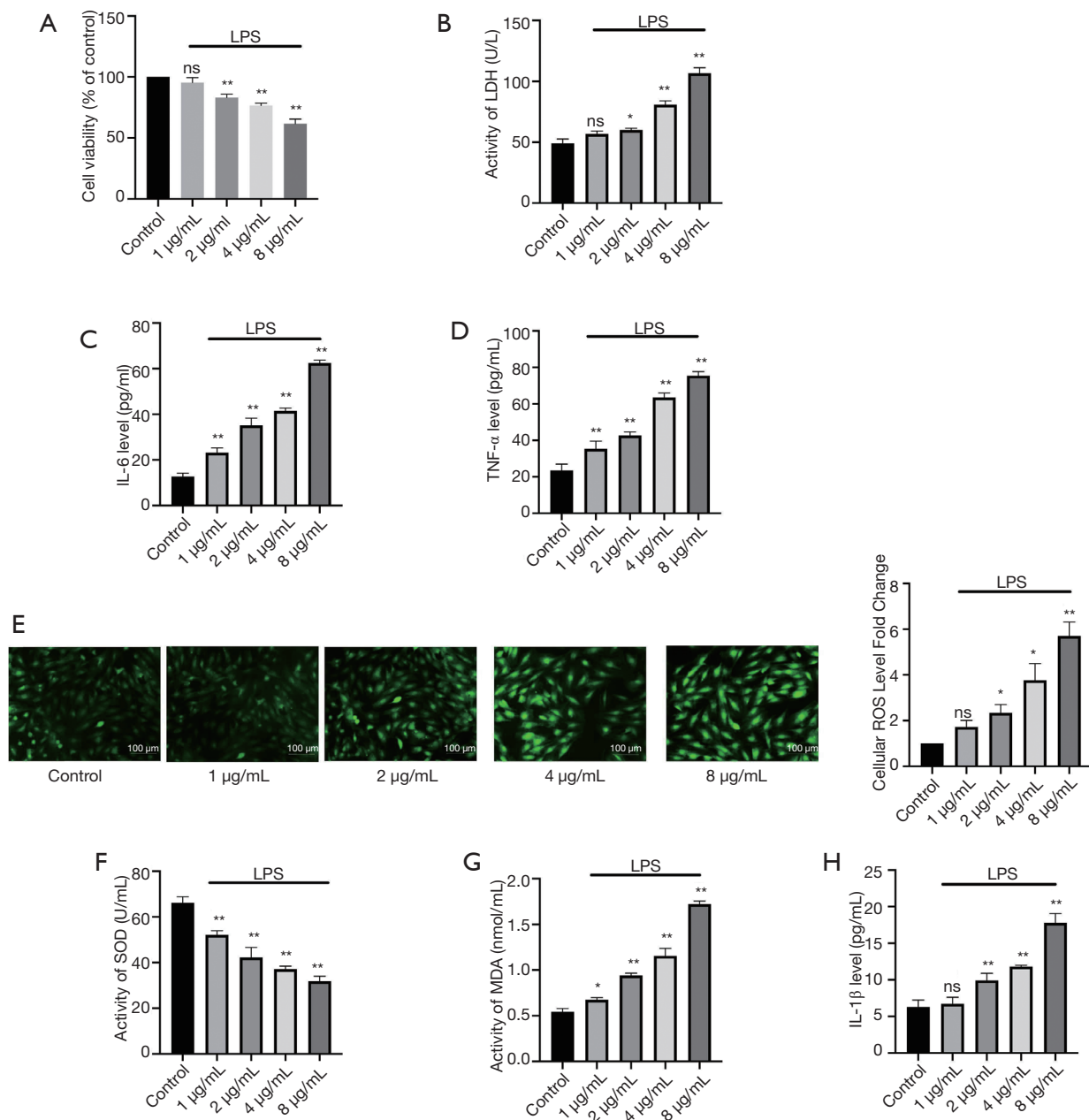


Figure 1 Dose-dependent effects of lipopolysaccharide (LPS) on H9C2 cells. (A) Cell viability was measured using the CCK-8 assay. (B) Lactate dehydrogenase (LDH) release was detected by LDH activity assays. (C,D) The levels of inflammatory cytokines, including IL-6 and TNF- α , were detected by ELISA. (E) The levels of intracellular reactive oxygen species (ROS) were detected by fluorescent probe dichloro-dihydro-fluorescein diacetate (DCFH-DA) (scale bar: 100 μ m, 100 \times magnification). (F) The activity of superoxide dismutase (SOD), (G) the content of malondialdehyde (MDA), and (H) the levels of IL-1 β were detected by ELISA. Data are shown as mean \pm standard deviation (SD) (n=6). *, P<0.05 and **, P<0.01 compared to control group; ns, P>0.05 compared to control group.

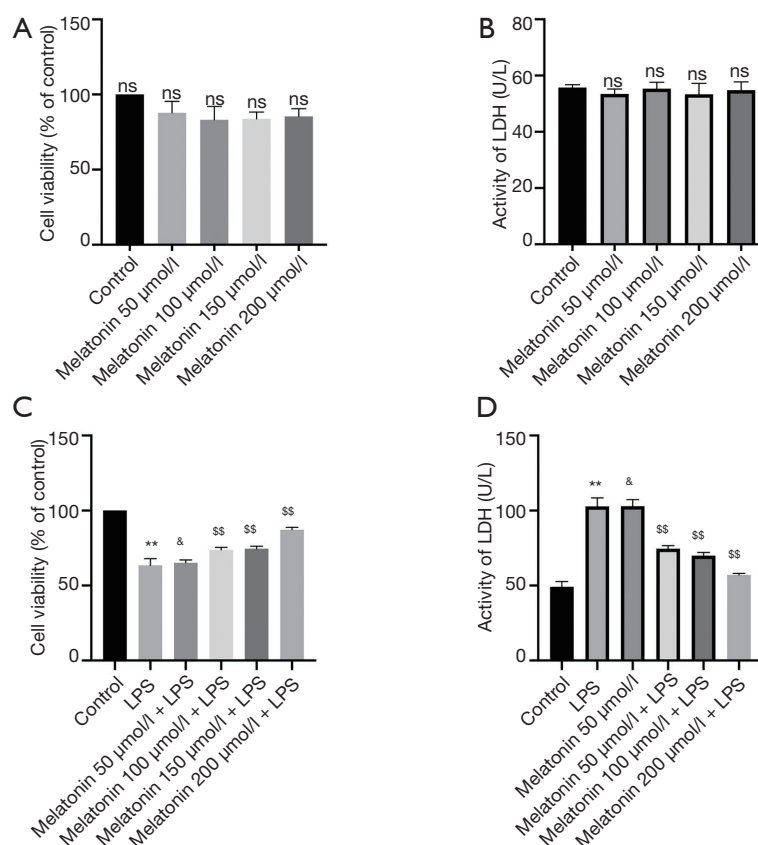


Figure 2 The effect of melatonin (MT) on cell viability and lactate dehydrogenase (LDH) release in lipopolysaccharide (LPS)-treated H9C2 cells. (A) The toxicity of MT on cells was measured using the CCK-8 assay and (B) the activity of LDH. (C) The effects of MT on LPS-induced H9C2 cell injury were detected using the CCK-8 assay and (D) LDH release. Data are shown as mean \pm standard deviation (SD) (n=6). **, P<0.01 compared to control group; ^{ss}, P<0.01 compared to LPS group; ns, P>0.05 compared to control group; [&], P>0.05 compared to LPS group.

24 h, cell viability and LDH release did not significantly change (Figure 2A,B). Subsequently, we evaluated the effect of MT on LPS-induced cardiomyocyte injury. Pretreatment with MT for 2 h significantly protected cells from LPS-induced injury in a concentration-dependent manner. Cell viability gradually recovered, and LDH release decreased, compared to the LPS group. Statistically, a significant inhibitory effect of MT on cytotoxicity commenced at 200 μ mol/L (Figure 2C,D).

The effect of MT on inflammatory cytokines in cell supernatants

Inflammatory responses and cytokine release play a crucial role in LPS-induced cell injury. After LPS treatment, the expression of inflammatory cytokines (IL-6, TNF- α) in

cell supernatants dramatically increased. In contrast, MT (200 μ mol/L) pretreatment down-regulated IL-6 and TNF- α levels (Figure 3A,B).

The effect of MT on the expression of iNOS, COX-2, and the activation of the NF- κ B pathway in LPS-treated H9C2 cells

To investigate the possible anti-inflammatory mechanisms of MT on LPS-induced myocardial injury, we explored the protein expression of iNOS and COX-2, and the activation of NF- κ B in cardiomyocytes. After exposed to LPS, the levels of iNOS, COX-2, and p-NF- κ B were upregulated. This effect was reversed after adding MT (Figure 4A,B,C,D,E).

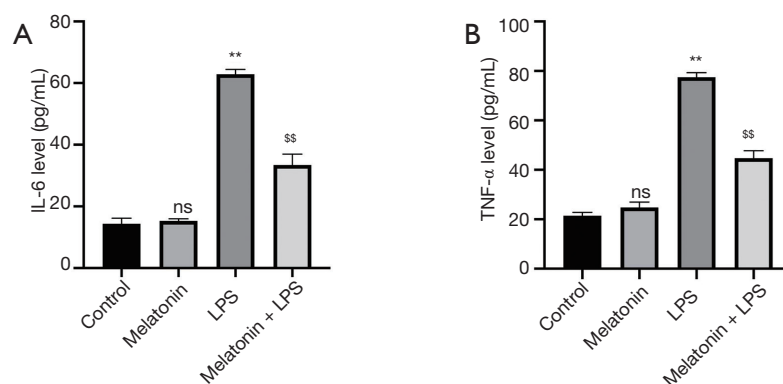


Figure 3 The effect of melatonin on interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) in lipopolysaccharide (LPS)-treated H9C2 cells. The levels of (A) IL-6 and (B) TNF- α in cell supernatants were determined by ELISA. Values were shown as mean \pm standard deviation (SD) (n=6). **, P<0.01 compared to control group; ^{ss}, P<0.01 compared to LPS group; ns, P>0.05 compared to control group.

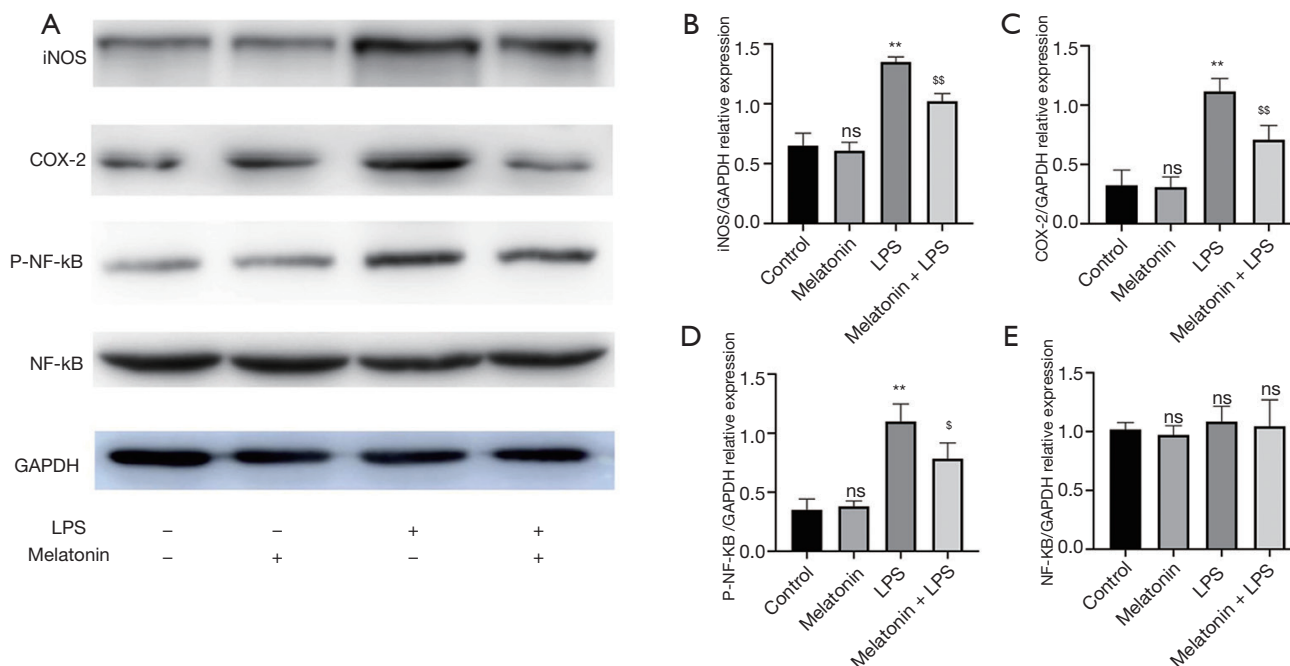


Figure 4 The effect of melatonin on the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), nuclear factor- κ B (NF- κ B) and p-NF- κ B in lipopolysaccharide (LPS)-treated H9C2 cells. A “+” symbol indicates presence and a “-” symbol indicates the absence of the relevant treatment. (A) iNOS, COX-2, NF- κ B and p-NF- κ B protein expression in H9C2 was analyzed by western blot. The relative protein expression levels of (B) iNOS/GAPDH, (C) COX-2/GAPDH, (D) p-p65/GAPDH, and (E) p65/GAPDH in H9C2 cells were analyzed. Data are shown as mean \pm standard deviation (SD) (n=3). **, P<0.01 compared to control group; ^s, P<0.05 compared to LPS group; ^{ss}, P<0.01 compared to LPS group; ns, P>0.05 compared to control group.

The effect of MT on ROS-dependent pyroptosis in LPS-treated H9C2 cells

We then investigated the role of ROS in LPS-induced myocardial injury. LPS treatment increased intracellular

ROS and MDA levels, and reduced SOD activity in H9C2 cells. Nevertheless, MT pretreatment effectively reduced the production of MDA and ROS, and also improved the activity of SOD (Figure 5A,B,C). Furthermore, the number

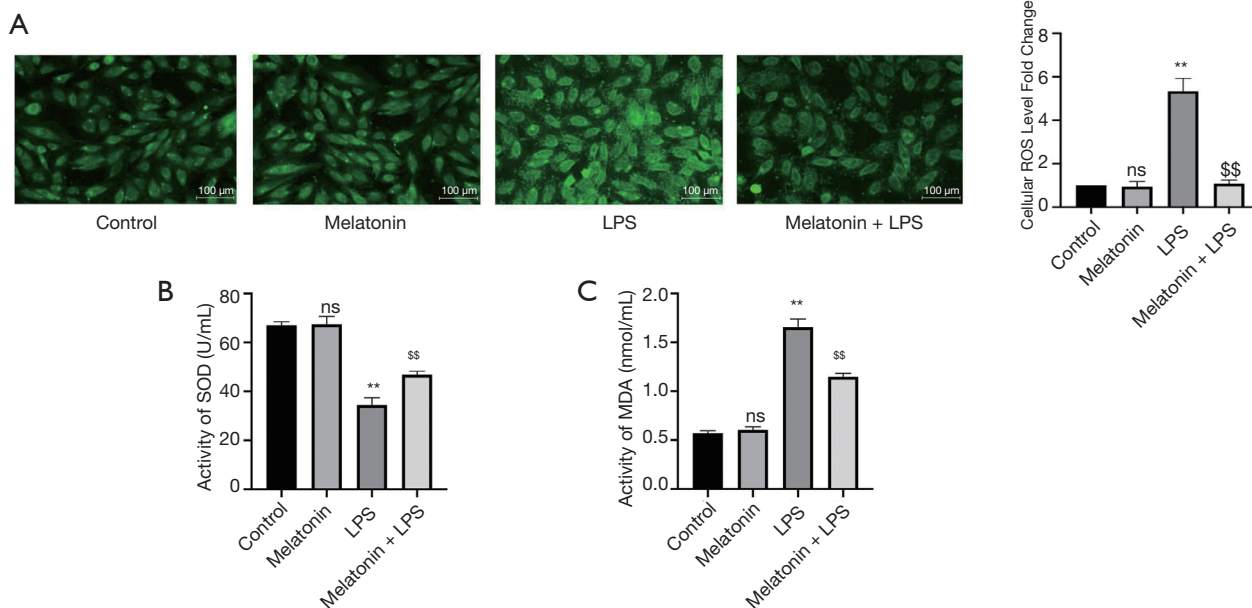


Figure 5 The effect of melatonin on the levels of intracellular reactive oxygen species (ROS), malondialdehyde (MDA), and the activity of superoxide dismutase (SOD) in lipopolysaccharide (LPS)-treated H9C2 cells. (A) ROS levels were measured by DCFH-DA (scale bar: 100 μ m. 200 \times magnification). (B) SOD activity and (C) MDA levels were measured using ELISA. Data are shown as mean \pm standard deviation (SD) (n=6). **, P<0.01 compared to control group; \$\$, P<0.01 compared to LPS group; ns, P>0.05 compared to control group.

of pyroptotic cardiomyocytes dramatically increased with the production of increased ROS stimulated by LPS. By contrast, MT alleviated cardiomyocyte pyroptosis by reducing ROS (Figures 5A and 6). The results indicated the MT improved cellular activity by inhibiting ROS-dependent pyroptosis.

The effect of MT on the expression of NLRP3, caspase-1, and GSDMD in LPS-treated H9C2 cells

The above results demonstrated that MT alleviated H9C2 cell pyroptosis by reducing the production of intracellular ROS. Thus, in the next experiment, pyroptosis proteins including NLRP3, caspase-1, and GSDMD were measured to evaluate the protective mechanism of MT on ROS-dependent pyroptosis in LPS-treated H9C2 cells. LPS treatment increased the expression of NLRP3, caspase-1, and GSDMD compared with the control group, and this effect was reversed by MT pretreatment (Figure 7A,B,C,D).

Discussion

In this study, MT pretreatment had shown a beneficial effect on LPS-induced myocardial injury. MT significantly

improved cell viability by inhibiting inflammation and reducing the levels of IL-6, IL-1 β , TNF- α , and ROS-dependent cell pyroptosis. Moreover, MT inhibited the activation of the NF- κ B pathway and reduced the expression of inflammatory proteins, such as iNOS and COX-2, and downregulated the expression of pyroptosis proteins including NLRP3, caspase-1, and GSDMD in myocardiocytes. These findings suggest that the protective effects of MT might be related to the inhibition of NF- κ B/iNOS/COX-2-dependent inflammatory responses and ROS-dependent pyroptosis.

The inflammatory response plays a vital role in myocardial injury (31). Cardiac dysfunction is closely related to inflammatory cytokines including IL-6 and TNF- α in sepsis (32,33). Clinical and experimental studies have indicated that inflammatory cytokines trigger sepsis-induced cardiac dysfunction (34,35), and inhibition of inflammatory cytokines (IL-6, TNF- α) can improve cardiac dysfunction in the septic animal model and in patients with sepsis (36,37). LPS-induced inflammatory responses could also be alleviated by MT via decreasing the release of inflammatory cytokines (38). In the present study, the experimental data indicated that MT reduced the production of the inflammatory cytokines (TNF- α ,

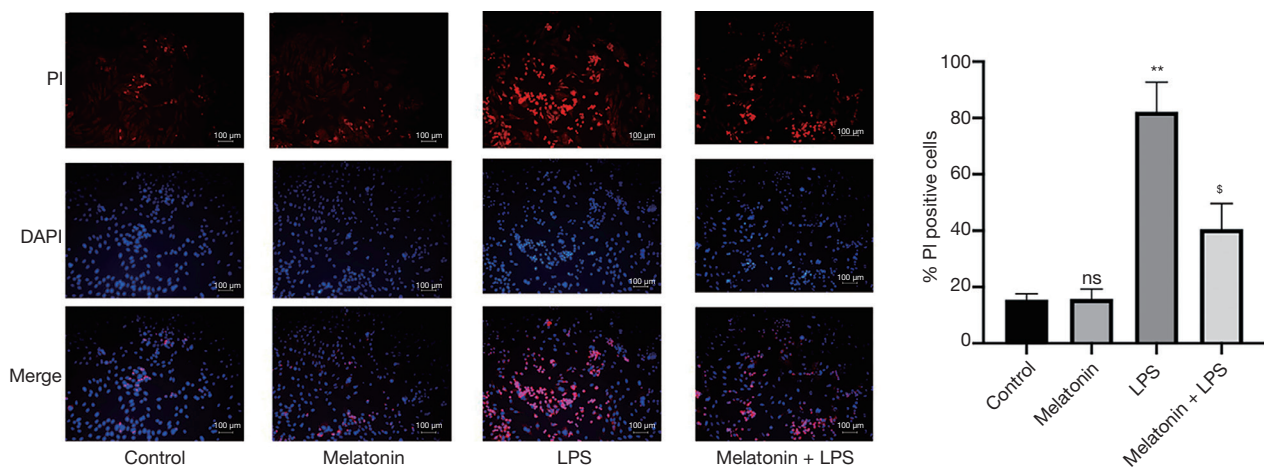


Figure 6 The effect of melatonin on pyroptotic cell death in H9C2 cells after lipopolysaccharide (LPS) treatment. Pyroptotic cells were detected using propidium iodide (PI) staining (scale bar: 100 μ m, 100 \times magnification). Data are shown as mean \pm standard deviation (SD) (n=6). **, P<0.01 compared to control group; ^s, P<0.05 compared to LPS group; ns, P>0.05 compared to control group.

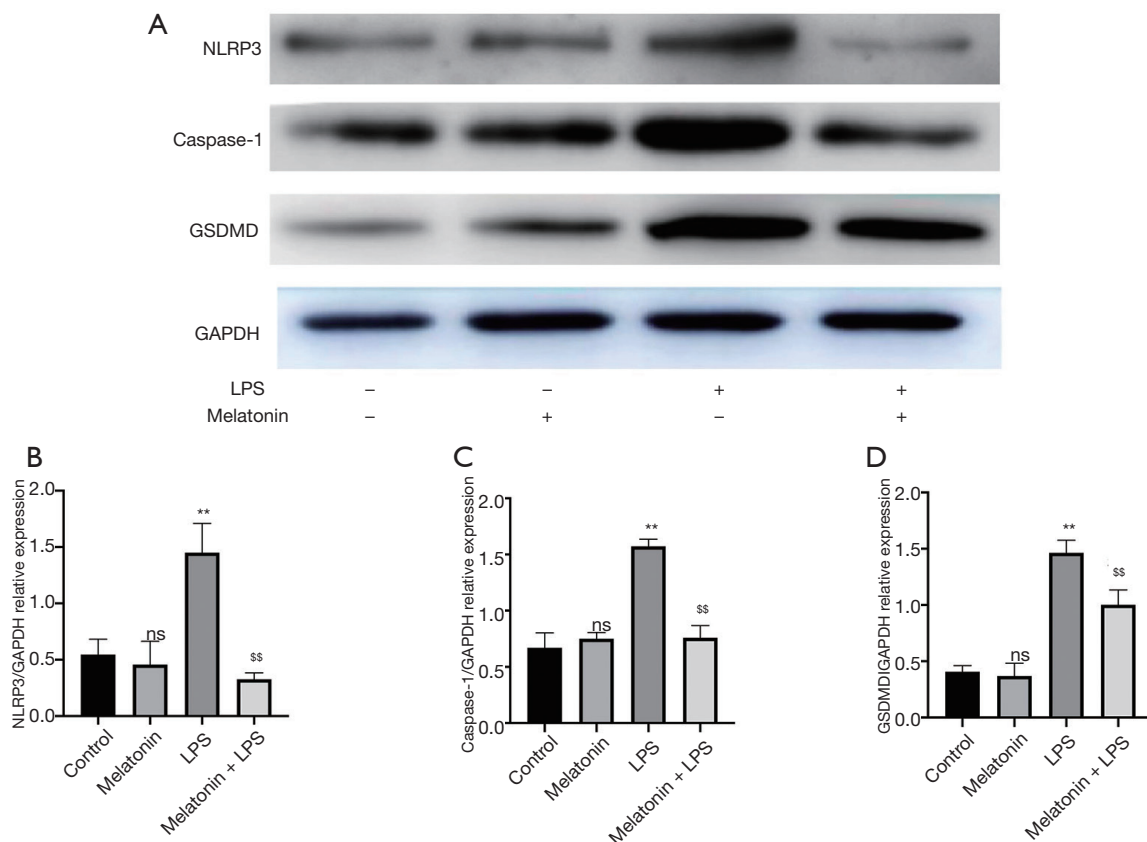


Figure 7 The effect of melatonin on the expression of NLR family pyrin domain containing 3 (NLRP3), caspase-1, and Gasdermin-D (GSDMD) in lipopolysaccharide (LPS)-treated H9C2 cells. (A) NLRP3, Caspase-1 and GSDMD protein expression in H9C2 was analyzed by western blot. The relative protein expression levels of (B) NLRP3/GAPDH, (C) caspase-1/GAPDH and (D) GSDMD/GAPDH in H9C2 cells were analyzed. Data are shown as mean \pm standard deviation (SD) (n=3). **, P<0.01 compared to control group; ^{ss}, P<0.01 compared to LPS group; ns, P>0.05 compared to control group.

IL-6), and improved the viability of H9C2 cells by decreasing LDH.

MT showed a strong anti-inflammatory effect, thus, its effect on inflammation in H9C2 cells after LPS stimulation was further investigated. Inflammation and inflammatory cytokine release is closely related to COX-2 and iNOS (39). LPS can activate the NF- κ B pathway, resulting in inflammation (40). Furthermore, phosphorylation of NF- κ B leads to the release of NF- κ B bound to the inhibitor I κ B (41) and the translocation of dimerized NF- κ B to the nucleus, thus promoting the expression of inflammatory genes including inflammatory cytokines (42). This is an important process which modulates inflammatory responses and drives the expression of iNOS and COX-2 (43). Previous studies have suggested that the expression of iNOS and COX-2 was mainly regulated by NF- κ B pathway, which was closely associated with myocardial dysfunction (44). In addition, MT could inhibit the expression of COX-2 and iNOS (45,46), which also alleviates tissue injury by inhibiting the NF- κ B pathway (24) with significantly decreased inflammation responses. However, the anti-inflammation mechanism of MT in LPS-induced myocardial injury is unclear. Our experimental results revealed that the upregulation of iNOS, COX-2, and p-NF- κ B could be effectively inhibited by MT in LPS-induced myocardial injury. Therefore, the protective effect of MT might be associated with its anti-inflammatory effects.

Oxidative stress is another important mechanism that mediates cardiovascular diseases, including myocardial injury (47). Recent studies have suggested that excessive ROS generation leads to LPS-induced cardiomyocyte injury (48,49). Excessive generation of ROS and MDA and decreased activity of antioxidant enzymes such as SOD have been observed during oxidative stress (50). This can cause damage to cellular components and macromolecules, including proteins and DNA, and can induce mitochondrial dysfunction, ultimately leading to tissue damage (51). Our results suggested that MT could improve LPS-induced cell injury by decreasing the generation of ROS and MDA, and increasing the activity of the antioxidant enzyme SOD.

The NLRP3 inflammasome could be activated by ROS-dependent signaling pathways after stimulation by PAMPs such as LPS (19). ROS are regarded as vital messengers that promote inflammasome activation. They have also been recognized to play an essential role in NLRP3 inflammasome activation (52) and drive NLRP3/caspase-1 complex activation, leading to pyroptosis (53). Under

physiological conditions, ROS are eliminated by antioxidant enzymes during cell metabolism, maintaining the balance between ROS generation and elimination (54). When ROS are continuously generated, the intracellular antioxidant defense systems cannot maintain redox homeostasis, which eventually leads to oxidative stress-induced injury and cellular damage, including cell apoptosis (55) and vascular endothelial cell pyroptosis (21,56). Our results suggested that the high-level production of ROS triggered cardiomyocyte pyroptosis, along with increased expression of NLRP3, caspase-1, and GSDMD. On the other hand, the data indicated that MT pretreatment reduced the generation of ROS, thus leading to the decreased expression of NLRP3, caspase-1, and GSDMD proteins, and reduced the release of IL-1 β . Therefore, the protective effect of MT might be associated with its antioxidant properties, which reduces the generation of ROS and, in turn, alleviates NLRP3-mediated pyroptosis in H9C2 cardiomyocytes. However, the protective mechanisms of MT in LPS-induced myocardial injury should be further elucidated in septic animal model, which is a limitation inherent to the research.

Conclusions

The present study demonstrated that MT pretreatment alleviated LPS-induced myocardial injury. Its protective effect might be related to its strong ability to inhibit NF- κ B/iNOS/COX-2-induced inflammation and ROS-dependent pyroptosis in H9C2 cardiomyocytes.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/atm-20-8196>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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