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ORIGINAL CONTRIBUTION

Melatonin attenuates sepsis-induced cardiac dysfunction via a PI3K/Akt-dependent mechanism

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Abstract Myocardial dysfunction is an important manifestation of sepsis. Previous studies suggest that melatonin is protective against sepsis. In addition, activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway has been reported to be beneficial in sepsis. However, the role of PI3K/Akt signaling in the protective effect of melatonin against sepsis-induced myocardial dysfunction remains unclear. Here, LY294002, a PI3K inhibitor, was used to investigate the role of PI3K/Akt signaling in mediating the effects of melatonin on sepsis-induced myocardial injury. Cecal ligation and puncture (CLP) surgery was used to establish a rat model of sepsis. Melatonin was administrated to rats intraperitoneally (30 mg/kg). The survival rate, measures of myocardial injury and cardiac performance, serum lactate dehydrogenase level, inflammatory cytokine levels, oxidative stress level, and the extent of myocardial apoptosis were assessed. The results suggest that melatonin administration after CLP surgery improved survival rates and cardiac function, attenuated myocardial injury and apoptosis, and decreased the serum lactate dehydrogenase level. Melatonin decreased the production of the inflammatory cytokines TNF-α, IL-1β, and HMGB1, increased anti-oxidant enzyme activity, and decreased the expression of markers of oxidative damage. Levels of phosphorylated Akt (p-Akt), unphosphorylated Akt (Akt), Bcl-2, and Bax were measured by Western blot. Melatonin increased p-Akt levels, which suggests Akt pathway activation. Melatonin induced higher Bcl-2 expression and lower Bax expression, suggesting inhibition of apoptosis. All protective effects of melatonin were abolished by LY294002, the PI3K inhibitor. In conclusion, our results demonstrate that melatonin mitigates myocardial injury in sepsis via PI3K/Akt signaling activation.

Keywords Sepsis · Myocardial dysfunction · Melatonin · Cecal ligation and puncture · Inflammation · Oxidative stress · PI3K/Akt signaling

Introduction

Sepsis, which is characterized by a systemic inflammatory response to severe infection and progressive organ damage, is the leading cause of death in hospitalized patients worldwide [6, 76]. Sepsis can trigger damage to various organs; for instance, it can cause brain injury, cardiac dysfunction, kidney injury, liver injury, and lung injury [7, 24, 38, 44, 63, 67, 87, 94, 106, 107, 127]. Of the complications of sepsis, cardiac dysfunction is a typical manifestation and is closely associated with increased mortality [80, 94, 105]. Sepsis patients experiencing cardiac dysfunction have a 70–90 % mortality rate, while patients without cardiac dysfunction experience only a 20 %

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mortality rate [80, 86]. Therefore, it is important to develop a novel therapeutic agent against sepsis-induced cardiac depression.

Melatonin, a hormone mainly secreted by the pineal gland, is known to exert various biological effects, such as cardioprotection [4, 82, 108], neuroprotection [3, 117], anti-tumor activity [42, 43], anti-inflammatory activity [2, 78], and anti-oxidant activity [35, 124]. Recently, it has been suggested that melatonin exhibits protective effects against cardiac dysfunction induced by ischemia/reperfusion [25, 36, 68, 119, 121]. In addition, there is some evidence that melatonin protects against sepsis-induced cardiac dysfunction, which may be related to melatonin's ability to attenuate mitochondrial dysfunction, disrupt apoptosis, decrease inflammation, and prevent oxidative damage [64, 85, 123], but the mechanisms of these actions are unclear.

The phosphatidylinositol-3-kinase (PI3K)/Akt pathway, a well-conserved family of signal transduction molecules, coordinates a variety of intracellular signals, controls cell response to extrinsic stimuli, and regulates cell proliferation and survival [16, 30, 81, 101]. The PI3Ks and the downstream serine/threonine kinase Akt (also known as protein kinase B or PKB) regulate cellular activation, inflammatory responses, chemotaxis, and apoptosis [16]. It has been demonstrated that PI3K/Akt pathway activation is protective against myocardial ischemia–reperfusion injury [31, 41, 53, 88, 99]. Furthermore, of critical significance to the present study, activation of the PI3K/Akt signaling pathway has been suggested to improve cardiac dysfunction and mortality during sepsis [18, 33, 51, 55, 61, 65, 75, 104, 120, 129]. However, the precise role of the PI3K/Akt signaling pathway in melatonin's protection against sepsis-induced myocardial injury remains unclear.

Therefore, we hypothesized that melatonin protects against sepsis-induced myocardial injury via a PI3K-dependent mechanism. In order to investigate the underlying mechanism of melatonin's cardioprotective effects, we established an animal septic model using the cecal ligation and puncture (CLP) method and then evaluated cardiac function in the presence and absence of LY294002 (LY), a PI3K inhibitor.

Materials and methods

Animals

All experiments were performed on healthy adult male Sprague–Dawley rats that weighed between 220 and 250 g. The rats were obtained from the animal center of the Fourth Military Medical University. Rats were kept under pathogen-free conditions at about 22 °C on a 12 h light–dark cycle with free access to food and water. This study was performed according to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (National Institutes of Health Publication No. 85-23, revised 1996) and was approved by the Ethics Committee of the Fourth Military Medical University.

Reagents

Melatonin (Mel), LY294002 (LY), and 4′,6-diamino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The lactate dehydrogenase (LDH) ELISA kit was purchased from Jiancheng Bioengineering Institute (Nanjing, China). Rat TNF-α and IL-1β ELISA kits were purchased from Thermo Fisher Scientific (MA, USA). The HMGB1 ELISA kit was purchased from IBL International (Germany). Superoxide dismutase (SOD) and malondialdehyde (MDA) kits were purchased from Sigma-Aldrich (St. Louis, MO, USA). The catalase (CAT) kit was purchased from Beyotime (Shanghai, China). Antibodies against Akt, phospho-Akt (Ser473), Bcl-2, Bax, and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). The rabbit anti-goat, goat anti-rabbit, and goat anti-mouse secondary antibodies were purchased from Beyotime (Shanghai, China). Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) kits were purchased from Roche (Mannheim, Germany).

Cecal ligation and puncture (CLP) model

Fasting was performed for 8 h for all rats but water was allowed ad libitum before the experiments. The CLP model was established as previously reported with some modifications [113]. In brief, after rats were anesthetized with intraperitoneal injection of chloral hydrate (350 mg/kg), they were immobilized on an aseptic operating table. In a sterile operation environment, a 2–3 cm abdominal midline incision was made to expose the cecum, which was ligated below the ileocecal valve and punctured once with an 18-gauge needle. A small amount of stool was squeezed through the puncture site. The bowel was then situated back in the abdomen and the incision was sutured with a sterile 5–0 silk. The rats in sham-operated group underwent a similar operation without cecal ligation and puncture. All animals received fluid resuscitation with 0.9 % saline solution (subcutaneously, 40 mL/kg of body weight) immediately after the surgery.

Experimental protocol

Three-hundred rats were randomly assigned to five groups: the Sham group received the sham operation and no drug treatments; the CLP group received the cecal ligation and puncture (CLP) surgery; the CLP + Mel group received

the CLP surgery and melatonin; the CLP + Mel + LY group received the CLP surgery and both melatonin and LY treatments; and the CLP + LY group received the CLP surgery and LY treatment. All rats had free access to food and water. Twenty rats from each group were used to evaluate survival rates, and forty rats from each group were used for other experiments. The survival rate was evaluated 7 days after the sham or CLP operation. Melatonin dissolved in 1 % ethanol (dissolved in normal saline) was administered intraperitoneally at a dose of 30 mg/kg per injection per rat, at 3, 6, 12, 18, and 24 h after surgery. LY or the same volume of vehicle was intraperitoneally injected at a dose of 10 mg/kg per rat every 2 days for a total of four times before CLP surgery. The dose and administration routes of melatonin and LY were chosen based on previous reports [69, 123].

Evaluation of survival rate

The rats in each group had free access to food and water and were kept under pathogen-free conditions. The survival rate was evaluated within 7 days after the sham or CLP operation.

Evaluation of cardiac function by echocardiography and invasive hemodynamic assessment

Transthoracic echocardiographic examinations were established under isoflurane anesthesia (2 %) of rats in each group 48 h after CLP. Echocardiographic images were obtained using an ACUSON echocardiography instrument equipped with a 13 MHz phased-array transducer (Siemens, USA). The M-mode images of left ventricular (LV) dimensions were obtained. The left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) were recorded.

After the echocardiography, a high-fidelity pressure-transducing catheter was inserted via the right carotid artery into the left ventricle to measure the left ventricular pressure (LVP). When the rats returned to stable conditions, left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), and their first derivative with respect to time ($\pm dp/dt_{max}$) were continuously measured.

Evaluation of morphological changes of myocardial tissues

Rats were killed at 48 h after surgery, and left ventricular myocardial tissues were collected. Tissue sections of the myocardium were stained with hematoxylin–eosin (H&E) staining, and morphological changes were evaluated using light microscopy at a magnification of 400×.

TUNEL staining

Myocardial apoptosis was analyzed using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. The paraffin-embedded tissue was cut into sections 4–5 μm thick. Then, 50 μL of TUNEL reaction mixture was added to each sample, and the slides were incubated in humidified atmosphere for 60 min at 37 °C in the dark and then rinsed with PBS (pH 7.4) three times, for 5 min each time. To detect the nuclei, the slides were incubated with DAPI for 5 min at room temperature in the dark, rinsed with PBS three times, for 5 min each time, and observed using fluorescence microscopy. The TUNEL-positive cells produced green fluorescence and the nuclei produced with blue fluorescence. The apoptotic index was calculated as the ratio of the number of TUNEL-positive neurons to the total number of nuclei.

Measurement of LDH release

The activity of lactate dehydrogenase in the serum was detected using a commercially available ELISA kit, according to the manufacturer's instructions. The LDH activity was expressed as U/L.

Evaluation of inflammatory cytokines

Inflammatory cytokines in the serum and myocardial tissue were measured 48 h after surgery by using commercially available TNF-α, IL-1β, and HMGB1 ELISA kits, according to the manufacturer's instructions. Data were analyzed using a microplate reader (Multiskan Spectrum, Thermo Scientific, USA).

Measurement of CAT, SOD, and MDA

The serum and myocardial tissue were collected 48 h after surgery to measure CAT and SOD activities and MDA content using commercially available kits, according to the manufacturer's instructions. Data were analyzed using a microplate reader (Multiskan Spectrum, Thermo Scientific, USA).

Western blot

Left ventricular myocardial tissues were collected and lysed with lysis buffer. After sonication, the lysates were centrifuged, and the proteins were separated using SDS–PAGE and then transferred to Immobilon-NC membranes (Millipore, Boston, MA, USA). After being blocked with 5 % skim milk in Tris-buffered saline at room temperature for 2 h, the membrane was incubated with primary antibodies against p-Akt, Akt, Bcl-2, Bax, and β-actin (1:1000)

overnight at 4 °C, washed three times with TBST, and then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at 37 °C. The blots were imaged using a Bio-Rad imaging system (Bio-Rad, Hercules, CA, USA) and quantified using the Quantity One software package (West Berkeley, CA, USA). The value for the sham group was defined as 100 %.

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). SPSS 18.0 was used to analyze data in this study. Survival rates were calculated using Fisher's exact test. Comparisons among multiple groups were assessed by one-way analysis of variance. The LSD *t* test was used to make intergroup comparisons. Probabilities of 0.05 or less were considered statistically significant.

Results

Melatonin improved survival rate in septic rats

The survival rate is shown in Fig. 1. The 7-day survival rate in the sham group was almost 100 %. However, 7 days after CLP surgery, there was a dramatic decrease in the survival rate for the CLP group (17.8 %) (versus sham group, $P < 0.05$). With the administration of melatonin, the survival rate in the CLP + Mel group increased significantly to nearly 50 % (versus CLP group, $P < 0.05$). However, LY294002 treatment abolished the protective effect of melatonin; the survival in the CLP + Mel + LY group (28.6 %) was much lower than that in the CLP + Mel group (versus CLP + Mel group, $P < 0.05$).

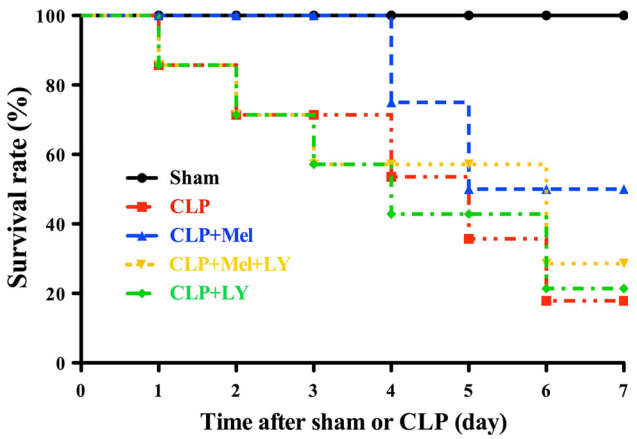

Fig. 1 Effect of melatonin on the 7-day survival rate after CLP surgery. Rats were treated with melatonin after CLP, and melatonin was given at 3, 6, 12, 18, and 24 h after CLP surgery. LY294002 was intraperitoneally injected at a dose of 10 mg/kg or the same volume of vehicle every 2 days for a total of four times before CLP surgery. Values are expressed as survival percentage ($n = 20$ for each group). $^{**}P < 0.05$ in comparison to the sham group, $^{\#\#}P < 0.05$ in comparison to the CLP group, and $^{\$\$}P < 0.05$ in comparison to the CLP + Mel group. *CLP* cecal ligation and puncture, *Mel* melatonin, *LY* LY294002

Melatonin improved cardiac function in septic rats

We assessed cardiac function with echocardiography. As shown in Fig. 2, melatonin significantly decreased myocardial injury induced by sepsis in the CLP + Mel group (versus CLP group, $P < 0.05$), as evidenced by improved cardiac function. To investigate whether PI3K plays a critical role in melatonin's cardioprotective effect, LY294002 was used to inhibit the PI3K/Akt pathway. As expected, the cardiac function of rats in the CLP + Mel + LY group was lower than that in the CLP + Mel group, indicating that PI3K plays a key role in the protective effect of melatonin against sepsis-induced myocardial injury.

In addition, we used invasive hemodynamic evaluation methods to assess cardiac function. As shown in Fig. 3, melatonin treatment significantly caused an increase in LVSP and LV ± dP/dt_{max} and a dramatic decrease in LVEDP in the CLP + Mel group (versus CLP group, $P < 0.05$). Consistent with electrocardiography results, LY294002 markedly suppressed cardiac function in the CLP + Mel + LY group (versus CLP + Mel group, $P < 0.05$), suggesting again that the PI3K pathway plays a role in protection against sepsis-induced myocardial injury by melatonin.

Melatonin attenuated myocardial injury as indicated by HE staining

As shown in Fig. 4, the myocardial sections were stained with hematoxylin and eosin to evaluate damage to the myocardium. In the sham group, the cardiomyocytes were intact and there was no evidence of necrosis or inflammatory cell infiltration. The cardiac muscle cross striations were clearly visible. In the CLP group, necrosis and inflammatory cell infiltration were evident and the cardiac muscle cross striations were no longer visible. Melatonin administration attenuated the injury due to CLP surgery. However, co-treatment with melatonin and LY abolished melatonin's protection against sepsis-induced myocardial injury, indicating that PI3K activation is involved in melatonin's protective effect.

Melatonin alleviated myocardial apoptosis in septic rats

To evaluate apoptosis induced by sepsis, TUNEL was performed. As shown in Fig. 5, melatonin treatment significantly

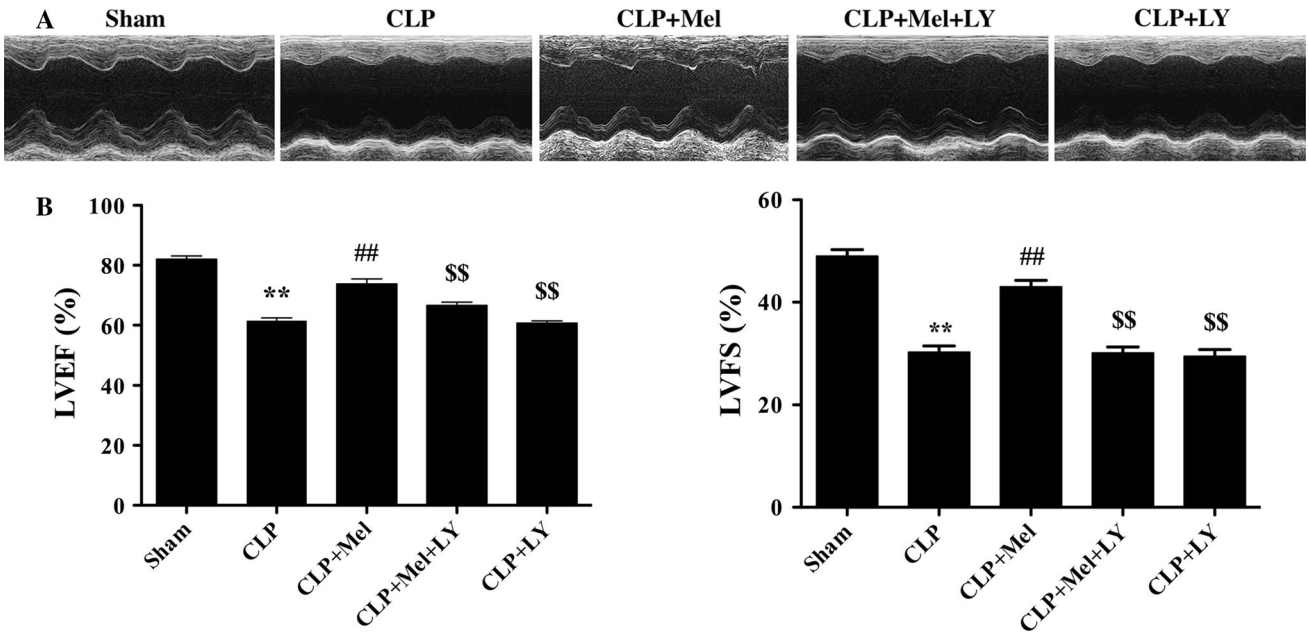

Fig. 2 Echocardiography evaluation suggests that cardiac dysfunction is attenuated by melatonin. **a** The evaluation of cardiac function by echocardiography. Representative M-mode images are shown. **b** Left ventricle ejection fraction. The results are expressed as the mean ± SEM ($n = 8$ for each group). $^{**}P < 0.05$ in comparison to the sham group, $^{\#\#}P < 0.05$ in comparison to the CLP group, and $^{\$\$}P < 0.05$ in comparison to the CLP + Mel group. *Mel* melatonin, *CLP* cecal ligation and puncture, *LVEF* left ventricle ejection fraction, *LY* LY294002

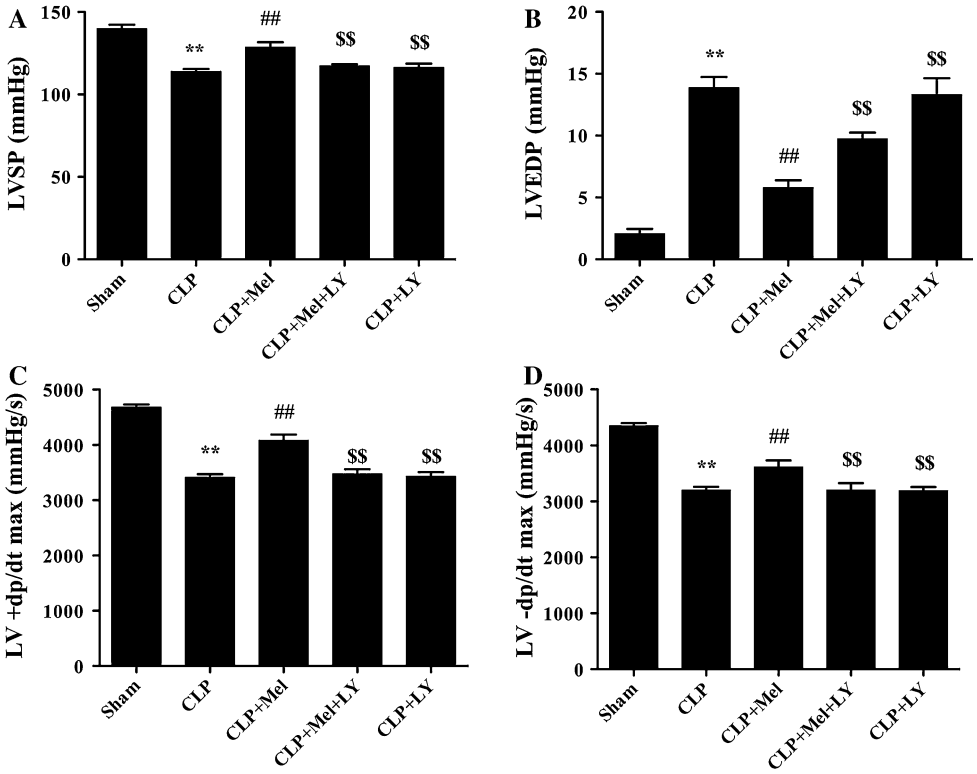

Fig. 3 Invasive hemodynamic evaluation suggests that melatonin improves cardiac function. The results are expressed as the mean ± SEM ($n = 8$ for each group). $^{**}P < 0.05$ in comparison to the sham group, $^{\#\#}P < 0.05$ in comparison to the CLP group, and $^{\$\$}P < 0.05$ in comparison to the CLP + Mel group. *Mel* melatonin, *CLP* cecal ligation and puncture, *LY* LY294002, *LVSP* left ventricular systolic pressure, *LVEDP* left ventricular end-diastolic pressure, $LV \pm dP/dt_{max}$ the instantaneous first derivation of left ventricle pressure

decreased the apoptotic index in the CLP + Mel group (versus CLP group, $P < 0.05$). However, LY abolished the protective effect of melatonin in the CLP + Mel + LY group (versus CLP + Mel group, $P < 0.05$), revealing that PI3K activation is associated with melatonin's protection against sepsis-induced myocardial apoptosis.

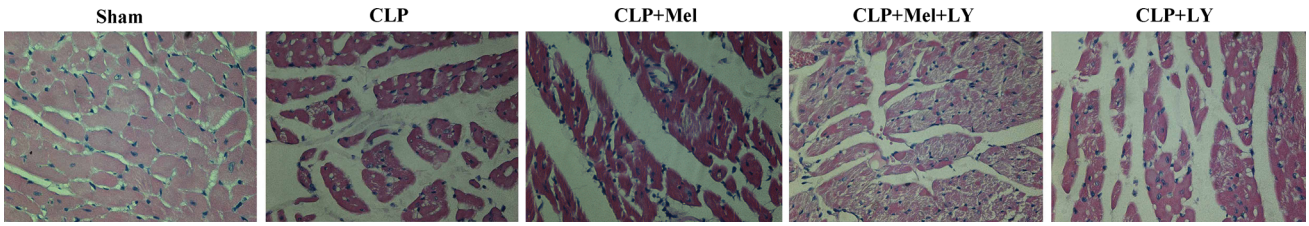

Fig. 4 Hematoxylin–eosin staining suggests that melatonin attenuates myocardial injury. Representative images of HE staining are shown (magnification ×400, $n = 8$ for each group). *Mel* melatonin, *CLP* cecal ligation and puncture, *LY* LY294002

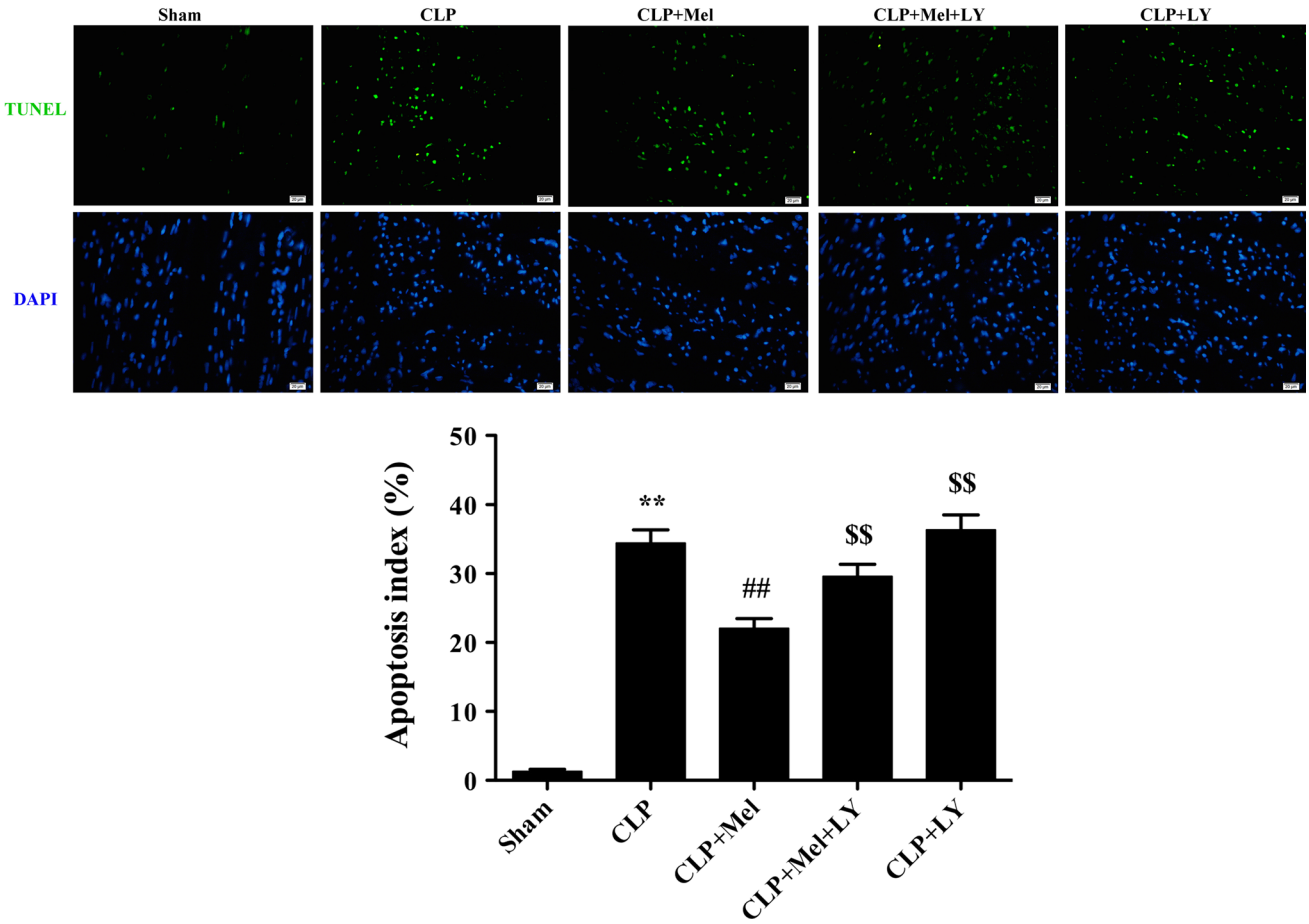

Fig. 5 Melatonin attenuation of sepsis-induced myocardial apoptosis is abolished by LY294002. Representative images of apoptosis are shown. The apoptotic cells were detected by TUNEL (*green*), and the nuclei were detected by DAPI (*blue*). The *scale bar* 20 μm. The results are expressed as the mean ± SEM ($n = 8$ for each group). $^{**}P < 0.05$ in comparison to the sham group, $^{\#\#}P < 0.05$ in comparison to the CLP group, and $^{\$\$}P < 0.05$ in comparison to the CLP + Mel group. *Mel* melatonin, *CLP* cecal ligation and puncture, *LY* LY294002

Melatonin mitigated LDH leakage in septic rats

LDH release is an indicator of myocardial injury, so we measured LDH levels in the serum. As shown in Fig. 6, CLP surgery triggered a dramatic increase in serum LDH release. Melatonin administration significantly decreased the LDH release after surgery in the CLP + Mel group (versus CLP group, $P < 0.05$). As expected, LDH release was markedly higher for the CLP + Mel + LY group (versus CLP + Mel group, $P < 0.05$), indicating that PI3K participates in melatonin's protection against sepsis-induced myocardial injury.

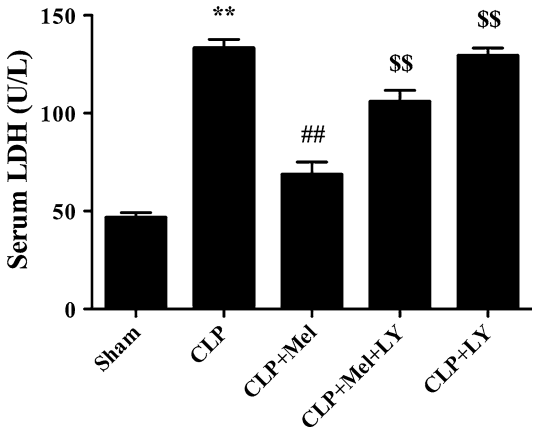

Fig. 6 Effects of melatonin and LY294002 on sepsis-induced LDH release. The LDH release was determined using an ELISA kit according to the manufacturer's instructions. The results are expressed as the mean ± SEM ($n = 8$ for each group). $^{**}P < 0.05$ in comparison to the sham group, $^{\#\#}P < 0.05$ in comparison to the CLP group, and $^{\$\$}P < 0.05$ in comparison to the CLP + Mel group. *Mel* melatonin, *CLP* cecal ligation and puncture, *LY* LY294002

Melatonin lowered inflammatory cytokines production in serum and myocardial tissue of septic rats

Subsequently, the effects of melatonin on inflammatory cytokines in serum and myocardial tissue of septic rats were assessed as another measure of myocardial injury. As shown in Fig. 7, the levels of the inflammatory cytokines TNF-α, IL-1β and HMGB1 in the serum and myocardial tissue were markedly lower in the CLP + Mel group than those in the CLP group ($P < 0.05$); LY abolished the effect of melatonin.

Melatonin decreased oxidative stress in serum and myocardial tissue of septic rats

As shown in Fig. 8, the effects of melatonin on sepsis-induced oxidative stress in the serum and myocardial tissues were evaluated. In the CLP + Mel group, the levels of SOD and CAT in both the serum and myocardial tissues were markedly higher than those in the CLP group ($P < 0.05$); LY abolished the effect of melatonin. MDA level, which is a marker of oxidative damage, was lower in the CLP + Mel group than that in the CLP group; LY abolished the effect of melatonin.

The role of Akt, Bcl-2, and Bax in the protective effects of melatonin

To further investigate the molecular mechanism underlying melatonin-mediated cardioprotection against sepsis, we detected p-Akt/Akt, Bcl-2, and Bax protein levels by Western blot. As shown in Fig. 9, CLP surgery significantly increased Akt phosphorylation relative to the sham group. Akt phosphorylation was further enhanced with melatonin administration. However, LY abolished the increase in Akt phosphorylation when co-administered with melatonin. As shown in Fig. 9, CLP surgery induced a dramatic increase in Bax expression relative to the sham group. Melatonin administration decreased the expression of Bax, but this decrease was significantly abolished by co-administration with LY. In contrast, CLP dramatically decreased Bcl-2 expression. Melatonin administration increased the expression of Bcl-2, but this increase was significantly abolished by co-administration with LY.

Discussion

In this study, we found that melatonin attenuated myocardial dysfunction induced by sepsis. Melatonin improved cardiac function, mitigated myocardial apoptosis, and decreased inflammation and oxidative stress associated with sepsis. The protective effect of melatonin was closely associated with the activation of the PI3K/Akt signaling pathway. Melatonin administration dramatically increased the 7-day survival rate of rats that underwent CLP surgery and attenuated cardiac dysfunction and myocardial apoptosis observed 48 h after CLP surgery. Moreover, melatonin lowered the release of inflammatory cytokines such as TNF-α, IL-1β, and HMGB1 as well as the production of MDA, which is an oxidative lipid product. It also increased the activity of anti-oxidant enzymes, such as SOD and CAT. However, these protective effects of melatonin were abolished by treatment with LY294002, a PI3K signaling inhibitor, indicating that melatonin acts via the activation of PI3K/Akt signaling.

Sepsis, the systemic inflammatory response to infection, causes high mortality among the critically ill [6, 45], primarily as a result of multiple organ damage. Myocardial dysfunction is regarded as a critical manifestation of this syndrome [21, 32, 58, 71, 89, 95, 110]. The underlying mechanisms of myocardial dysfunction during sepsis include circulatory changes [46, 93, 94], autonomic dysregulation [96, 97], metabolic changes [102], mitochondrial dysfunction [15, 22], cell death (necrosis and apoptosis) [47, 84], inflammation, and oxidative stress [8, 23, 59]. However, the pathogenesis of sepsis-induced myocardial dysfunction is complex and involves a multitude of molecular players. Studies have suggested that there is a reduction in the levels of cardiac dihydropyridine receptors, such as L-type calcium channels, during sepsis [60, 128]. A recent study suggested that mitochondrial nitric oxide (NO) could be involved in myocardial depression [115]. As for the inflammatory cytokines, TNF-α and IL-1 might be involved [8].

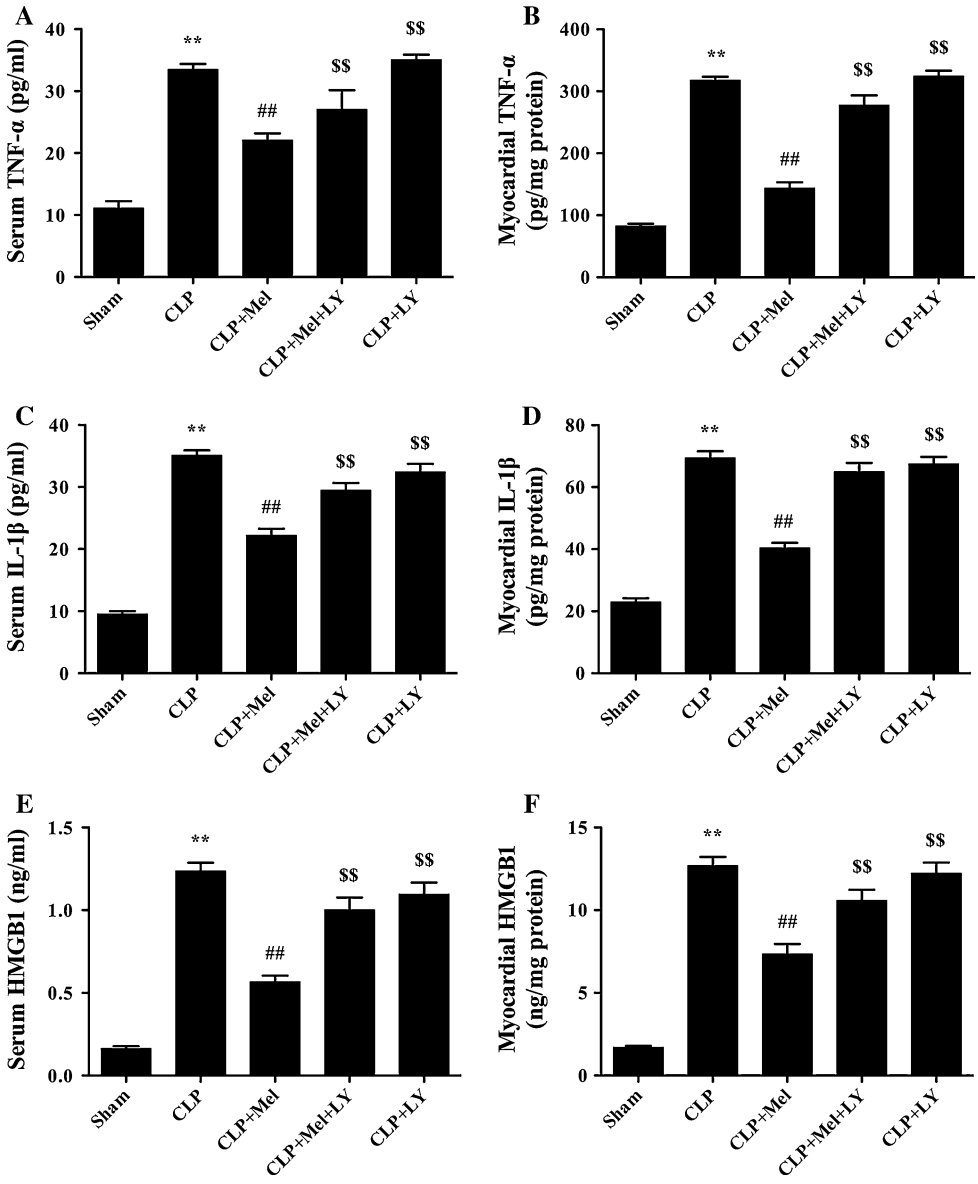

Fig. 7 Melatonin decreased the levels of inflammatory cytokines in the serum and myocardial tissues. **a** Serum TNF-α level. **b** Myocardial TNF-α level. **c** Serum IL-1β level. **d** Myocardial IL-1β level. **e** Serum HMGB1 level. **f** Myocardial HMGB1 level. The values are expressed as the mean ± SEM ($n = 8$ for each group). $^{**}P < 0.05$ in comparison to the sham group, $^{\#\#}P < 0.05$ in comparison to the CLP group, and $^{\$\$}P < 0.05$ in comparison to the CLP + Mel group. *Mel* melatonin, *CLP* cecal ligation and puncture, *LY* LY294002, *HMGB1* high-mobility group box 1

Previous studies have demonstrated the protective role of melatonin against sepsis. It has been reported that 5-hydroxy-2′-isobutyl-streptochlorin (HIS), a novel derivative of melatonin, inhibits inflammation via regulation of TRIF-dependent signaling and inflammasome activation [98]. It has been reported that melatonin is protective against sepsis-induced kidney injury [19]. In addition, with respect to sepsis-induced myocardial injury, melatonin attenuated mitochondrial impairment and improved survival rates [85, 123]. Consistent with previous results, we found that melatonin improved the 7-day survival rate after CLP surgery. The cardiac dysfunction induced by sepsis is mitigated by melatonin as evidenced by the echocardiography, hemodynamic evaluation, and morphology. Garcia et al. demonstrated the NF-κB/NLRP3 inflammasome connection during sepsis, leading to a disproportionate inflammatory response to sepsis [34]. Melatonin administration blunts NF-κB transcriptional activity via a sirtuin1-dependent NF-κB deacetylation in septic mice. In addition, melatonin decreased NF-κB-dependent proinflammatory response and restored redox balance and mitochondrial homeostasis, thus inhibiting the NLRP3 inflammasome. The study heralds a promising therapeutic application for melatonin in the treatment of sepsis. In our present study, we found that melatonin protects the heart from sepsis via a PI3K/Akt-dependent mechanism and the protective effects of melatonin can be abolished by LY294002, a specific PI3K antagonist. Therefore, melatonin, as a promising therapeutic application in the treatment of sepsis, may exert its protection via PI3K, which

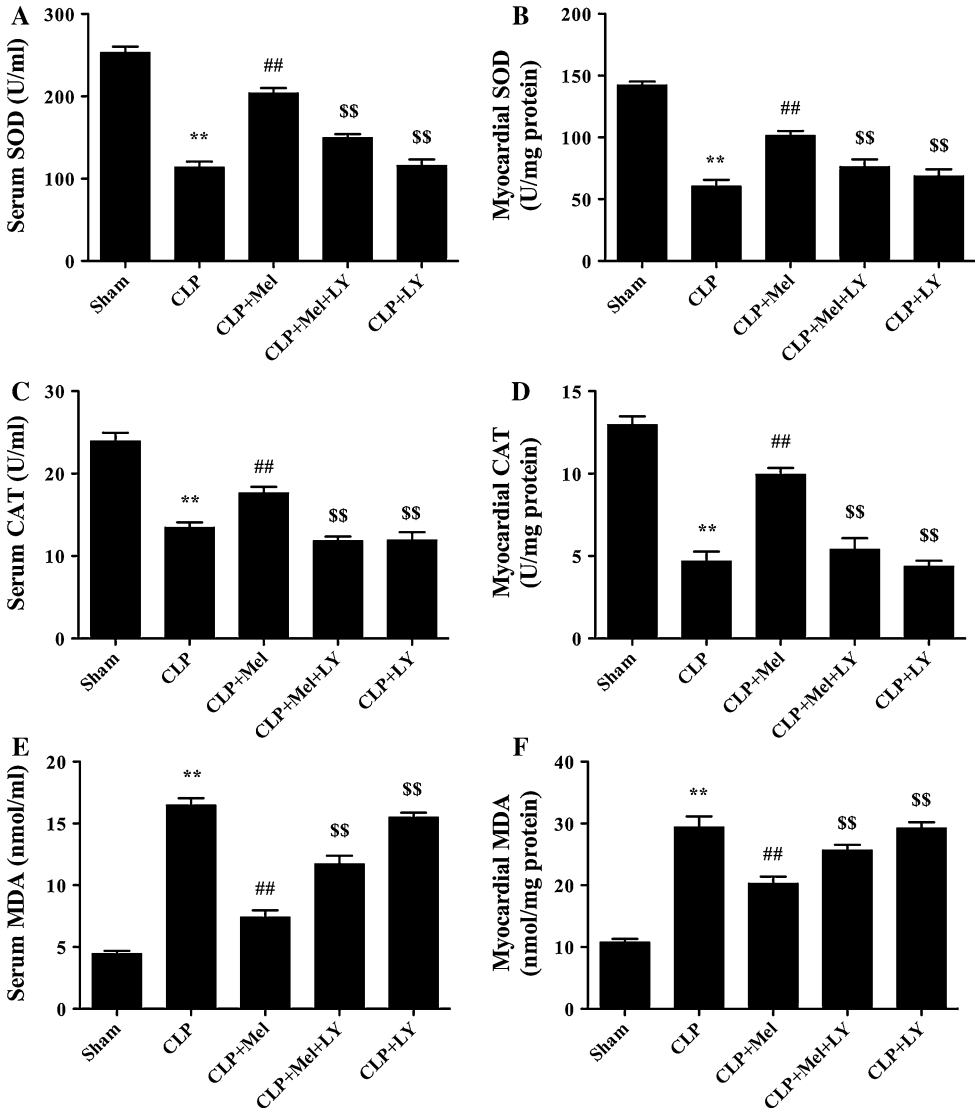

Fig. 8 Melatonin increased the levels of SOD and CAT and decreased MDA content in the serum and myocardial tissues. **a** Serum SOD level. **b** Myocardial SOD level. **c** Serum CAT level. **d** Myocardial CAT level. **e** Serum MDA level. **f** Myocardial MDA level. The values are expressed as the mean ± SEM ($n = 8$ for each group). $^{**}P < 0.05$ in comparison to the sham group, $^{\#\#}P < 0.05$ in comparison to the CLP group, and $^{\$\$}P < 0.05$ in comparison to the CLP + Mel group. *Mel* melatonin, *CLP* cecal ligation and puncture, *LY* LY294002, *SOD* superoxide dismutase, *CAT* catalase, *MDA* malondialdehyde

provides another therapeutic target. Recently, Lorente et al. described higher mortality rates for sepsis patients with high melatonin levels [70], which appears inconsistent with our results. However, some problems should be considered: (1) differences may exist between the treatment of sepsis in a CLP-induced rat model treatment and in human beings. Patients with sepsis are treated with certain kinds of antibodies and medications, which are not used in a rat model. (2) Melatonin in human originates endogenously mainly from the pineal gland in humans. In this study, increased melatonin levels in septic patients might have resulted from sepsis-induced pineal damage. However, we considered melatonin as a treatment reagent that was obtained from an exogenous source. Furthermore, melatonin has a protective effect against sepsis-induced myocardial injury. (3) Melatonin, as an endogenous hormone, is expressed at different levels at different times. Moreover, melatonin levels are associated with the circadian rhythm and may differ among individuals.

Sepsis induces the release of enormous endotoxins, such as lipopolysaccharide (LPS), triggering a cascade of proinflammatory cytokines (TNF-α, IL-1, etc.) [122]. Among the cytokines, TNF-α induces apoptosis in rat heart [83, 125]. In addition, high-mobility group box 1 protein (HMGB1) has been identified as an important late-acting mediator of inflammation in sepsis [5, 109]. HMGB1 is a nuclear non-histone DNA-binding protein that is produced extracellularly during inflammation [62]. Previous studies have demonstrated that HMGB1 can activate the immune system and induce cell proliferation, adhesion, migration, and cytokine release [40, 52, 56, 72]. In the present study, melatonin treatment significantly attenuated inflammation by suppressing the levels of TNF-α, IL-1β, and HMGB1 in the serum and myocardial tissue. Additionally, melatonin

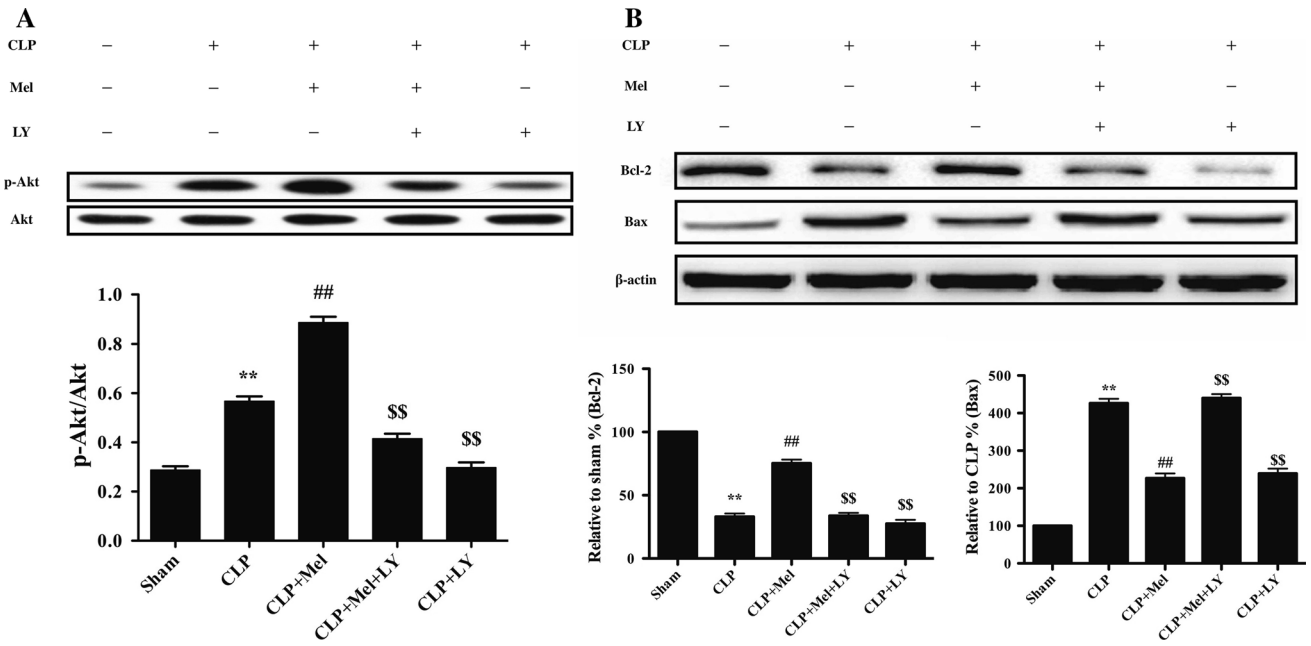

Fig. 9 Effect of melatonin on the expression of phosphorylation-Akt, Bcl-2, and Bax following sepsis. Representative images of the Western blot results are shown. Melatonin increased the ratio of p-Akt/Akt, which was significantly abolished by Akt-inhibitor LY294002. Melatonin increased Bcl-2 expression and decreases Bax expression, an effect that is reversed by the by Akt-inhibitor LY294002. The values are expressed as the mean ± SEM ($n = 8$ for each group). $^{**}P < 0.05$ in comparison to the sham group, $^{\#\#}P < 0.05$ in comparison to the CLP group, and $^{\$\$}P < 0.05$ in comparison to the CLP + Mel group. *Mel* melatonin, *CLP* cecal ligation and puncture, *LY* LY294002

treatment also significantly decreased myocardial apoptosis, as evidenced by the lowered apoptotic index, the increased level of Bcl-2, and decreased level of Bax. However, Gerd et al. demonstrated that the release of endotoxins is beneficial under certain conditions [9]. Preconditioned hearts (Ischemic/LPS pretreated) demonstrated increased tolerance against myocardial ischemia that was associated with a reduced TNF-α concentration and an increased TNF-α inhibitory plasma activity. Their study illustrated the important role for TNF-α as well as the TNF-α inhibitory serum activity in the progress of myocardial ischemia with respect to mortality, hemodynamics and regional myocardial blood flow, and infarct size. Consequently, TNF-α inhibitory serum activity is expected to provide new insights into cardioprotection against ischemia–reperfusion afforded by ischemic preconditioning and early exposure to LPS. Dipeptidyl peptidase (DPP)-4, which is responsible for a degradation of the hormone glucagon-like peptide-1 (GLP-1), plays a key role in glucose metabolism and in the control of glycemic status. Sebastian et al. demonstrated the DPP-4 inhibitor linagliptin improves survival and suppresses LPS-induced inflammatory pathways, improving vascular dysfunction and reducing oxidative stress in endotoxemic rats. These protective effects are associated with GLP-1-mediated decrease of iNOS expression as well as activation of the AMPK signaling pathway. And this study heralds a promising therapeutic application for linagliptin in the treatment of sepsis [103]. Another study investigated whether phosphorylation of RISK (reperfusion injury salvage kinases, proposed to be protective by previous reports in mostly rodent models) is causal for the protection of ischemia postconditioning (IPoC) [100]. In pig model of IPoC, pharmacological RISK inhibitors were used to block increases in RISK phosphorylation during reperfusion. However, differences in the infarct size were not significant, which may be attributed to species differences between rodents and larger mammals. Additionally, maintenance of acidosis and subsequent inhibition of mitochondrial permeability rather than phosphorylation of RISK mediates postconditioning in pigs [20]. To further elucidate the underlying mechanisms, Langendorff apparatus can be used to establish the global ischemia model to diminish the latent errors in sampling tissues. For now, few studies have analyzed the RISK in different species and further research is warrant.

Oxidative stress during sepsis is also another critical factor contributing to the myocardial dysfunction [57, 66]. During sepsis, excessive reactive oxygen species (ROS) production activates lipid peroxidation, leading to cell and mitochondrial membrane damage, which triggers cell apoptosis and necrosis [17, 49, 50]. In addition, ROS can modify the inner mitochondrial membrane potential and induce the release of cytochrome c into the cytosol,

eventually leading to cell apoptosis [37, 111, 118]. SOD and CAT are anti-oxidant enzymes that serve as ROS scavengers [130], and MDA is an indicator of lipid peroxidation [13, 116]. In our study, the results suggest that melatonin treatment alleviates oxidative stress via increasing the activity of CAT and SOD and decreasing the production of MDA.

PI3K/Akt signaling has been reported to play a protective role in sepsis [12, 51, 65, 112, 126]. Moreover, transgenic overexpression of Akt protects against sepsis in the mice infected with the Gram-negative bacteria [90]. Once activated, PI3K leads to the phosphorylation of Akt, leading to the phosphorylation of diverse target molecules (such as Bcl-2 family) that act to preserve mitochondrial integrity and promote cell survival [69]. Melatonin has been reported to be protective against hemorrhagic shock-induced liver injury in rats through an Akt-dependent HO-1 pathway [48]. Our results suggested that melatonin treatment significantly increased Akt phosphorylation. Taken together, our results suggest that PI3K/Akt signaling pathway is involved in the protective effect of melatonin. We hypothesize that melatonin protects against sepsis-induced myocardial dysfunction via the PI3K/Akt pathway.

MT1 and MT2, both G-protein coupled receptors, are involved in the transduction of melatonin signaling [92]. These receptors are expressed in several different organs and tissues; therefore melatonin modulates multiple aspects of human physiology, and melatonin dysfunction and its receptors are associated with sleep and circadian dysfunction [39], diabetes [14, 73], and Alzheimer's and Parkinson's diseases [1, 114]. Melatonin as a free-radical scavenger is receptor-independent; however, its indirect anti-oxidative action may be mediated by receptors [54, 91]. In addition, melatonin interacts with intracellular proteins such as calmodulin [11], calreticulin [74], or tubulin [79], and antagonizes the binding of Ca^{2+} to calmodulin [10]. Furthermore, melatonin receptors have been identified in the cardiovascular system [26, 121]. Animal studies have indicated that melatonin has dual effects on the vasculature with vasoconstriction being observed through MT1 receptor activation, and vasodilatation through MT2 receptor activation [27, 77]. Additionally, melatonin has been shown to activate PI3K/Akt pathway via its receptors [28]. As for sepsis, melatonin receptors have been suggested to mediate improvements with respect to survival in a septic model [29]. The results showed that MT1 and MT2 mediate at least a part of the effects induced by melatonin to improve survival. Therefore, we hypothesize that the activation of PI3K/Akt signaling by melatonin in septic rats is likely mediated by melatonin receptors. This, however, requires further validation using melatonin receptor antagonists.

Melatonin has not only been widely used in experimental animal research but also in clinic for human beings. It shows a relatively high adaptation for most people with different pathologies, and can be safely used in numerous disorders. The toxic dose, however, remains unknown owing to a lack of research. Our results (150 mg/kg for sepsis rats) suggest that the possible melatonin dose that can be administered for myocardial dysfunction in sepsis patients was 3 mg/kg according to dosage conversion ratio between species (human:rat is nearly 1:25–50). However, further research is needed in clinical scenarios.

In summary, our study suggests that melatonin treatment confers a significant protective effect against myocardial dysfunction induced by sepsis in a PI3K/Akt-dependent manner. PI3K/Akt activation augments anti-oxidant activity, inhibits inflammation, and suppresses apoptosis, contributing to the attenuation of myocardial depression. Our work warrants a more thorough examination of the clinical use of melatonin in the treatment of sepsis-induced myocardial injury.

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Compliance with ethical standards

Conflict of interest None declared.

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