

Original Article

Protective effects of melatonin on lung and liver injuries in a rat model of acute sepsis

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Abstract: Melatonin exhibits remarkable potential as an anti-inflammatory, antioxidative, and anti-apoptotic agent. This study aimed to investigate the protective effects of melatonin on sepsis-induced lung and liver injuries in rats. Male Wistar rats were subjected to cecal ligation and puncture (CLP) operation to induce sepsis. Melatonin (30 mg/kg) was administered intraperitoneally at 0, 3, 6 and 12 hours after CLP treatment. Melatonin significantly improved survival and ameliorated histopathological damage of lung and liver in the CLP-challenged rats. Melatonin retarded CLP-caused deleterious hemodynamic changes of the rats, including hypotension, tachycardia, and hyporeactivity to norepinephrine. Moreover, melatonin alleviated CLP-induced pulmonary and hepatic dysfunction. Melatonin reduced CLP-increased plasma levels of tumor necrosis factor- α , interleukin-1 β (IL-1 β), IL-6, high mobility group protein box 1, and nitric oxide (NO). The myeloperoxidase activity, inducible nitric oxide synthase expression, and NO level in the lung and liver of CLP-insulted rats were markedly suppressed by melatonin. Melatonin attenuated CLP-triggered oxidative stress, as shown by the reduction of malondialdehyde, increased activities of superoxide dismutase and catalase, and elevation of glutathione content. In addition, melatonin inhibited CLP-induced pulmonary and hepatic cell apoptosis by reducing caspase-3 activity, downregulating the pro-apoptotic cleaved caspase-3 and Bax expression, and upregulating anti-apoptotic Bcl-2 and phosphorylated-Akt levels. Taken together, melatonin effectively attenuated CLP-induced septic lung and liver damages via its anti-inflammatory, antioxidative, and anti-apoptotic properties and may be a novel agent in the therapy of sepsis-caused multiple organ failure.

Keywords: Melatonin, sepsis, cecal ligation and puncture, inflammation, oxidative stress, apoptosis

Introduction

Sepsis, one of the leading causes of death in intensive care units worldwide, is a systemic inflammatory response syndrome to infection [1]. Despite recent improvements in surgical techniques and critical care medicine, the overall mortality of sepsis remains high, ranging between 30% and 50% [2]. Severe sepsis can lead to multiple organ failure (MOF) [3]. Among sepsis complications, lung and liver dysfunctions are the typical manifestations and key contributors to mortality in septic patients [4, 5]. Therefore, new therapeutic approaches against sepsis-induced lung and liver injuries should be urgently developed.

A hyperactive systemic inflammatory response with a large number of inflammatory cytokine releases and excessive generation of free radicals (reactive oxygen and nitrogen species;

ROS/RNS) is the distinct characteristic of sepsis [6]. Sepsis is a serious stage of bacterial infection. During sepsis development, bacterial components may activate the inflammatory cascades, thereby leading to the release of inflammatory mediators, including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), IL-6, and high-mobility group protein box 1 (HMGB1) [7]; consequently, neutrophil infiltrates various organs (e.g., lung, liver and heart) to induce endothelial and epithelial injuries, vascular leakage, edema, and vasodilatation, subsequently causing the development of MOF [8]. Oxidative stress, as a result of the inflammatory responses inherent with sepsis, leads to mitochondrial dysfunction, which contributes to organ damage [9]. In addition, inflammatory stress-induced apoptosis is a main cause of septic injury [10, 11]. Thus, exploring new drugs with effective anti-inflammatory, antioxidative, and anti-apoptotic profiles to reduce the inci-

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dence and mortality of this devastating condition would be valuable.

Melatonin (N-acetyl-5-methoxytryptamine), a hormone mainly secreted by the pineal gland, exerts protective effects because of its anti-inflammatory, antioxidative, and anti-apoptotic activities [12-14]. Melatonin is beneficial for reversing symptoms of septic shock [15, 16]. Melatonin alleviates sepsis-induced cardiac dysfunction and brain injury by decreasing the production of pro-inflammatory factors, such as TNF- α , IL-1 β and HMGB1 [17, 18]. Melatonin protects tissues against oxidant damage by directly scavenging the free radicals and indirectly promoting antioxidant enzyme expression [14]. Melatonin also exerts a strong anti-apoptotic effect [14, 17, 18]. However, the protective effects and underlying mechanisms of melatonin against sepsis-induced lung and liver injuries are yet to be investigated.

In this study, a cecal ligation and puncture (CLP)-induced septic rat model was used to explore the roles and potential mechanisms of melatonin in sepsis-induced lung and liver injuries. Melatonin improved the survival rate and reduced the hemodynamic changes of the rats with CLP treatment. Melatonin alleviated histopathological changes and dysfunctions of lung and liver in CLP-treated rats, and mitigated CLP-induced inflammatory response and oxidative stress. Melatonin also inhibited CLP-induced lung and liver cell apoptosis. Overall, melatonin protected lung and liver from CLP-induced septic injury via its anti-inflammatory, antioxidative, and anti-apoptotic properties, suggesting that melatonin may be used as a valuable agent for the therapy of septic lung and liver damage.

Materials and methods

Animals

Male Wistar rats (aged 10-12 weeks, weighing 280-320 g) were purchased from the Animal Experimental Center of Henan Province, China. The rats were kept in a room with constant temperature ($22 \pm 2^\circ\text{C}$) at a 12 hour light and dark cycle with free access to food and water under pathogen-free conditions. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of He-

alth (NIH Publication No. 85-23, revised 1996) and approved by the Ethics Committee of the First Affiliated Hospital of Xi'an Jiaotong University.

CLP-induced septic rat model

Sepsis was induced by CLP as described previously [19]. After the rats were anesthetized with intraperitoneal injection of 50 mg/kg sodium pentobarbital, a small mid-abdominal incision was made, and the cecum was exposed. The cecum was isolated and ligated below the ileocecal valve with a 3-0 silk suture, punctured twice at opposite ends with an 18 gauge needle, and returned into the abdominal cavity. Afterward, the abdominal incision was closed in two layers, and the animals received normal saline solution (50 ml/kg body weight) subcutaneously to prevent dehydration. The sham-operated rats underwent the same surgical procedure, except that the cecum was neither ligated nor punctured.

Experimental protocols

Sixty rats were randomly assigned to three groups ($n = 20$ for each group) as follows: (1) sham group: rats received the sham operation with neither ligation nor puncturing; (2) CLP group: rats underwent CLP surgery; and (3) CLP + melatonin group: rats underwent CLP surgery and melatonin treatment. Melatonin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 1% ethanol (dissolved in normal saline) was administered intraperitoneally at 30 mg/kg per injection per rat at 0, 3, 6 and 12 hours after CLP surgery. The sham and CLP groups were given equal amount of normal saline (with 1% ethanol) at the same durations after surgery via the same routes noted above. Animals were sacrificed 24 hours after the sham or CLP surgery, except for the survival studies. Survival rate was evaluated within 7 days after the sham or CLP operation.

Measurement of hemodynamic parameters

Changes in hemodynamics, including mean arterial blood pressure (MAP), heart rate (HR), and pressor responses to norepinephrine (NE), were measured every 4 hours after sham or CLP surgery. Briefly, after anesthetization, the left carotid arteries of the rats were cannulated with a polyethylene-50 catheter, exteriorized to

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the back of the neck, and connected to a pressure transducer (Statham, Oxnard, CA, USA) for the measurement of MAP and HR, which were displayed on a Gould model TA5000 polygraph recorder (Gould Inc., Valley View, OH, USA). After recording the baseline hemodynamic parameters, animals were given intravenous injection of 1 µg/kg NE to examine their vascular reactivity. The value of pressor responses to NE at time 0 h of each group was calculated as 100% to normalize the baseline value of pressor responses to NE of all groups.

Sample collection

Blood samples were collected at 0, 12 and 24 hours after the surgeries and immediately centrifuged at 3,000 g for 10 minutes. The plasma was decanted and separated into two parts; one part of the plasma was stored at 4°C within 1 hour for biochemical analysis. Another part was stored at -80°C for later measurements of inflammatory factors. Each volume of blood removed was immediately replaced with the injection of an equal volume of sterile saline. Animals were sacrificed 24 hours after sham or CLP surgery, and the lung and liver were harvested. One half of the tissue samples were fixed with 10% formalin for histological examination and the remaining samples were stored at -80°C until use.

Histopathological examination

Fixed lung and liver samples were successively dehydrated and paraffin embedded. Tissue sections (4 µm) were deparaffinized, rehydrated gradually, stained with hematoxylin and eosin (HE), and examined under a light microscope (Olympus, Tokyo, Japan). The slides were evaluated by two experienced pathologists blinded to the treatment.

Assessment of lung wet/dry (W/D) weight ratio

Rat lungs were excised, and the wet weight was immediately recorded. Subsequently, the lungs were placed in an incubator at 70°C for 48 hours until the weight was unchanged, and the dry weight was recorded. The wet-to-dry weight ratio was calculated as follows: W/D ratio = (wet weight-dry weight)/dry weight.

Evaluation of lung and liver functions

Lung function was evaluated by analyzing the levels of pH, PaO₂, PaCO₂, bicarbonate (HCO₃⁻),

and base excess in the plasma using an arterial blood gas analyzer (AVL Scientific Corp., Roswell, GA, USA). Liver function was determined on the basis of the enzymatic analysis of glutamate pyruvate transaminase (GPT) and glutamate oxaloacetate transaminase (GOT) in the plasma. GPT and GOT activities were assayed using a biochemical blood analyzer (Fuji Photo Film Co., Ltd., Tokyo, Japan).

Measurement of LDH release

The activity of plasma LDH was detected using a commercially available ELISA kit (Jiancheng Bioengineering Institute Nanjing, Jiangsu, China) according to the manufacturer's instructions. The LDH activity was expressed as U/L.

Measurement of inflammatory cytokines

Inflammatory cytokines in the plasma were measured at 0, 4, 12 and 24 hours after surgery by using commercially available TNF-α, IL-1β, IL-6 and HMGB1 ELISA kits (BD Biosciences, San Diego, CA, USA), in accordance with the manufacturer's instructions. Data were analyzed using a microplate reader at 490 nm (Thermo Scientific, MA, USA).

Measurement of MPO activity

MPO activity was measured using commercial kit (Jiancheng Bioengineering Institute Nanjing, Jiangsu, China) in accordance with the protocol of the manufacturer. The fresh lung and liver tissues were homogenized for preparation of the supernatants to detect MPO activity. MPO activity was measured by spectrophotometer (Beckman Inc., Palo Alto, CA, USA) at 460 nm and expressed in U/g tissue.

Determination of nitrite

The amounts of nitrite in lung and liver tissues and blood were measured using a colorimetric reaction with the Griess reagent (Promega, Madison, Wisconsin, USA) [20]. Lung and liver tissues were cooled in ice-cold distilled water and homogenized (0.1 g/ml). The crude homogenate was centrifuged at 20,000 g for 20 minutes at 4°C. Approximately 100 µl of samples were incubated with 100 µl of Griess reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride; 1% sulfanilamide in 5% phosphoric acid; 1:1) at room temperature for 20 minutes. The optical density (OD) was read at 550 nm on

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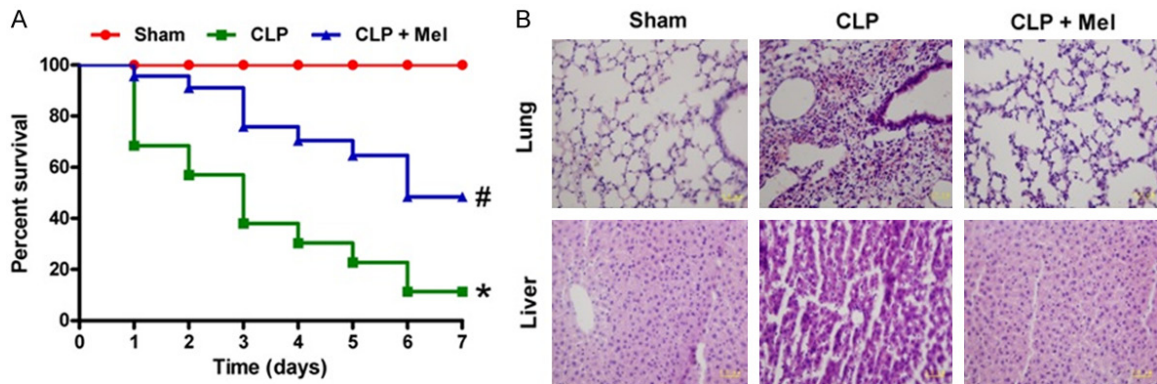


Figure 1. Melatonin reduced the mortality and histopathological changes of lung and liver in CLP-insulted rats. Rats intraperitoneally received melatonin at 30 mg/kg per injection per rat at 0, 3, 6 and 12 hours after CLP surgery. A. Seven-day survival rate was plotted with Kaplan-Meier method. B. At 24 hours after CLP surgery, rats were sacrificed, and the histopathological changes of lung and liver were evaluated using hematoxylin and eosin staining. Scale bar: 10 μ m. Data are expressed as mean \pm SD (n = 10 per group). **P < 0.01 vs. sham group; #P < 0.05 vs. CLP group.

a microplate reader (Thermo Scientific). Nitrite concentration was calculated via comparison with the OD₅₅₀ of a standard solution of known sodium nitrite concentrations.

Measurement of MDA, SOD, CAT and GSH

The lung and liver tissues were homogenized and centrifuged at 3,000 g for 20 minutes at 4°C. MDA and GSH contents and CAT and SOD activities in the supernatants were measured using commercially available assay kits (Jiancheng Bioengineering Institute Nanjing, Jiangsu, China) according to the instructions of the manufacturers. The ODs were measured at 530 (MDA), 450 (SOD), 240 (CAT) and 405 nm (GSH) with a microplate reader (Thermo Scientific). MDA concentration was expressed as nmol/g tissue. SOD and CAT activities were expressed as U/mg protein. GSH concentration was expressed as μ mol/g tissue.

Western blot analysis

Lung and liver specimens were lysed using RIPA lysis buffer (Beyotime). The homogenates were centrifuged at 4,000 g for 10 minutes at 4°C, and the supernatants were collected to detect the protein expression. Equal amounts of protein from lung and liver tissues were subjected to separation on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes (Millipore, Boston, MA, USA). After blockage with 5% skim milk in Tris-buffered saline

with Tween-20 (TBST) and shaking at room temperature for 1 hour, the following procedures were performed: the membranes were incubated overnight with the primary antibodies against iNOS (Abcam, Cambridge, UK), caspase-3 (Cell Signaling Technology, Beverly, MA, USA), cl-caspase-3 (Cell Signaling Technology, Beverly, MA, USA), Bcl-2 (Abcam, Cambridge, UK), Bax (Cell Signaling Technology, Beverly, MA, USA), Akt (Cell Signaling Technology, Beverly, MA, USA), p-Akt (Cell Signaling Technology, Beverly, MA, USA), and β -actin (Cell Signaling Technology, Beverly, MA, USA); membranes were washed with TBST three times and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Beyotime, Shanghai, China) at room temperature for 1 hour followed by washing with TBST three times. The protein bands were detected with the enhanced chemiluminescence detection reagent (Pierce, Rockford, IL, USA) using a Bio-Rad imaging system (Bio-Rad, Hercules, CA, USA). The band densities were quantified by scanning densitometry using the Quantity One software package (West Berkeley, CA, USA).

Measurement of caspase-3 activity

Caspase-3 activity was determined by using the colorimetric assay kit (Assay Designs, Ann Arbor, Mich, USA), in accordance with the manufacturer's instruction, to evaluate the apoptotic cells of lung and liver. Results were expressed as U/ μ g protein.

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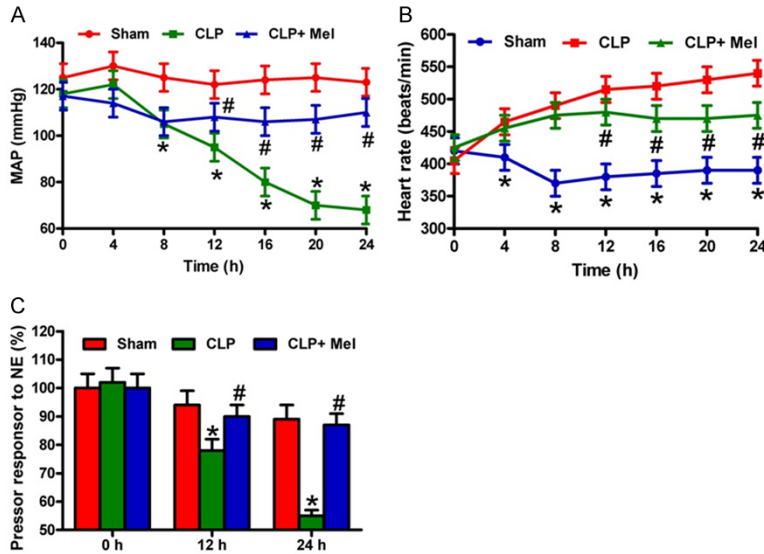


Figure 2. Melatonin inhibited hemodynamics changes in CLP-challenged rats. Rats intraperitoneally received melatonin at 30 mg/kg per injection per rat at 0, 3, 6 and 12 hours after CLP treatment. The changes in mean arterial blood pressure (A), heart rate (B), and pressor response to norepinephrine (C) were measured at indicated durations. Data are expressed as mean \pm SD (n = 10 per group). *P < 0.05 vs. sham group; #P < 0.05 vs. CLP group.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay

The apoptotic cells of the lung and liver were measured using a TUNEL staining kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's protocol. Tissue sections were dewaxed, rehydrated, and equilibrated in TBS. The sections were then digested with 20 μ g/ml proteinase K for 20 minutes at room temperature, incubated with a mixture containing terminal deoxynucleotidyl transferase and fluorescence-labeled nucleotides, and examined under a fluorescence microscope (Olympus, Tokyo, Japan). The negative control was prepared via incubating slides with the mixture containing only deoxynucleotidyl transferase.

Statistical analysis

Data are presented as the mean \pm standard deviation (SD). Groups of data were compared with the one-way ANOVA and subsequent Tukey post hoc test for multiple comparisons. Kaplan-Meier plots were used to illustrate survival between treatment groups, and Log-rank test was used for comparison of the survival distributions among groups of rats. GraphPad Prism version 5.02 (GraphPad Prism Software Inc, San Diego, CA) was used to analyze data in this

study. Values of P < 0.05 indicated significance.

Results

Melatonin improved survival rate and alleviated lung and liver injuries in CLP-induced septic rats

We first evaluated the effect of melatonin on the survival rate of CLP-induced septic rats. As shown in **Figure 1A**, the seven-day survival rate in sham group was almost 100%. After 7 day of CLP surgery, the survival rate remarkably decreased. However, the survival rate in the CLP + melatonin group significantly increased. HE staining results revealed normal cell structure in the lung and liver of sham-operated rats (**Figure**

1B). CLP markedly induced histopathological injuries of rat lung and liver; these damages were alleviated by melatonin treatment. These data indicated that melatonin prevented CLP-induced rat lethality and histopathological changes of lung and liver from the septic rats.

Melatonin reduced the hemodynamics changes in CLP-induced septic rats

The baseline values of hemodynamic parameters, including MAP (**Figure 2A**), HR (**Figure 2B**), and pressor responses to NE (**Figure 2C**) in all groups of animals, were not different among groups. As shown in **Figure 2A**, the MAP showed no significant change during the experimental period in the sham group. A progressive decrease in the MAP of the rats in the CLP group was observed from 8 h to 24 hours. Melatonin markedly prevented the delayed decrease in MAP. CLP caused a significant increase in HR during the experimental period (**Figure 2B**). Nevertheless, melatonin attenuated the late tachycardia induced by CLP. The rats in the CLP group showed a substantial time-dependent attenuation of the pressor responses to NE (**Figure 2C**), which was nearly restored to the normal level by melatonin at 24 h after CLP. These results suggested that mela-

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Table 1. Effects of melatonin on acid-base balance and blood gases in rats with CLP-induced sepsis

		Sham (n = 10)	CLP (n = 10)	CLP + melatonin (n = 10)
pH	0 h	7.44 ± 0.01	7.43 ± 0.03	7.43 ± 0.01
	12 h	7.55 ± 0.02	7.53 ± 0.01	7.54 ± 0.02
	24 h	7.57 ± 0.02	7.50 ± 0.02	7.56 ± 0.01
PaO ₂ (mmHg)	0 h	92.3 ± 1.8	104 ± 1.3	101 ± 1.7
	12 h	90.5 ± 1.3	95.8 ± 2.1	96.2 ± 1.5
	24 h	96.2 ± 1.9	94.9 ± 2.2	89.8 ± 2.6
PaCO ₂ (mmHg)	0 h	45.5 ± 1.5	42.8 ± 2.2	44.2 ± 1.2
	12 h	34.5 ± 1.2	29.3 ± 0.9	30.8 ± 1.2
	24 h	29.2 ± 1.7	26.8 ± 2.7	27.9 ± 1.6
HCO ₃ ⁻ (mM)	0 h	30.5 ± 0.6	29.1 ± 0.7	28.4 ± 0.8
	12 h	28.2 ± 0.7	24.7 ± 0.9	27.5 ± 0.5
	24 h	27.1 ± 0.6	20.4 ± 0.9*	26.3 ± 0.6#
Base excess (mM)	0 h	6.5 ± 0.9	5.0 ± 0.7	5.6 ± 0.5
	12 h	6.4 ± 0.8	3.8 ± 0.5	4.4 ± 0.8
	24 h	6.3 ± 0.7	-1.9 ± 1.2*	5.8 ± 0.5#

Note: *P < 0.05; #P < 0.01.

tonin inhibited CLP-induced hemodynamic changes in septic rats.

Melatonin alleviated the dysfunctions of lung and liver in CLP-induced septic rats

The arterial blood gas parameters, including pH, PaCO₂, PaO₂, HCO₃⁻, and base excess, were examined using an arterial blood gas analyzer to elucidate the protective effects of melatonin on CLP-induced lung dysfunction. As shown in **Table 1**, no significant difference was observed in the levels of pH, PaCO₂, and PaO₂ among the three groups. However, CLP group presented a significant decrease in the values of HCO₃⁻ and base excess. By contrast, melatonin significantly attenuated these decreases. The lung W/D weight ratio, an indicator of lung edema, was notably increased in CLP-insulted rats compared with that in the sham group. In contrast, this ratio was remarkably reduced by melatonin treatment (**Figure 3A**). The plasma levels of GPT and GOT were measured to evaluate liver dysfunction. CLP caused significant increases in plasma levels of GPT (**Figure 3B**) and GOT (**Figure 3C**). Nonetheless, the increases were terminated by melatonin treatment (**Figure 3B, 3C**). In addition, CLP increased the LDH in the plasma, but decreased by melatonin (**Figure 3D**). These data demonstrated that melatonin improved CLP-induced dysfunctions of lung and liver in septic rats.

Melatonin inhibited CLP-induced inflammatory cytokine release in the plasma of septic rats

We measured the plasma levels of TNF-α, IL-1β, IL-6 and HMGB1 by using ELISA at different time points to analyze the effects of melatonin on the CLP-stimulated release of inflammatory cytokines. The plasma levels of TNF-α (**Figure 4A**), IL-1β (**Figure 4B**), IL-6 (**Figure 4C**) and HMGB1 (**Figure 4D**) showed no significant difference in the three groups

at 0 hours after CLP (**Figure 4A-D**). CLP caused significant increase in the plasma levels of TNF-α (**Figure 4A**), IL-1β (**Figure 4B**), IL-6 (**Figure 4C**) at 4 and 12 hours, and HMGB1 (**Figure 4D**) at 12 and 24 hours after CLP. Moreover, the plasma levels of TNF-α, IL-1β and IL-6 at 4 hours were higher than those at 12 hours after CLP, whereas the plasma level of HMGB1 was lower at 12 hours than that at 24 hours after CLP. By contrast, melatonin significantly inhibited the release of the inflammatory cytokines mentioned above (**Figure 4A-D**). These results indicated that melatonin suppressed the inflammatory cytokine release in the plasma of CLP-induced septic rats.

Melatonin reduced neutrophil infiltration and iNOS/NO biosynthesis in CLP-induced septic rats

MPO activity is an indicator of tissue neutrophil infiltration [21]. The MPO activity was significantly higher in the lung and liver tissues of the CLP group than that in the control group (**Figure 5A**). However, CLP-induced neutrophil infiltration was decreased by melatonin treatment. The effects of melatonin on NO levels in the septic rats were subsequently investigated. **Figure 5B** shows that CLP increased the lung and liver NO levels, which were reduced by melatonin. Melatonin also attenuated CLP-induced NO production in the plasma (**Figure**

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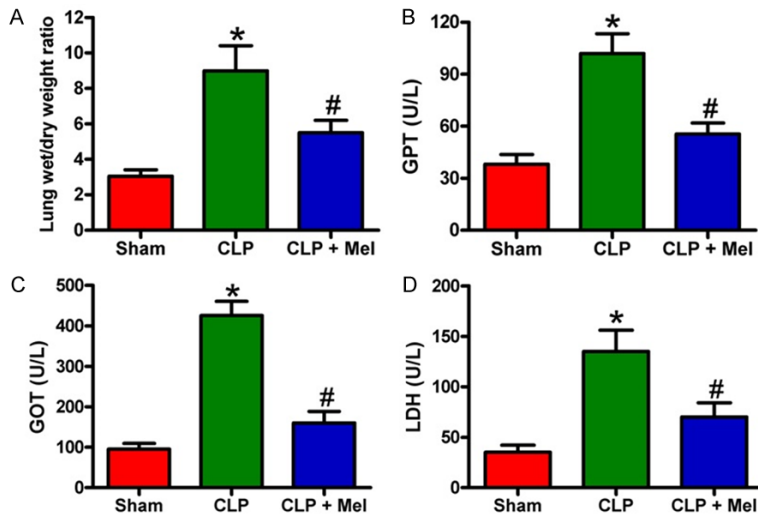


Figure 3. Melatonin mitigated the dysfunctions of lung and liver in CLP-treated rats. Rats intraperitoneally received melatonin at 30 mg/kg per injection per rat at 0, 3, 6 and 12 hours after CLP surgery. (A) Lung wet/dry weight ratio. (B-D) The plasma levels of glutamate pyruvate transaminase (B), glutamate oxaloacetate transaminase (C), and lactate dehydrogenase (D) were measured at 24 hours after CLP. Data are expressed as mean \pm SD (n = 10 per group). *P < 0.05 vs. sham group; #P < 0.05 vs. CLP group.

Melatonin attenuated CLP-induced oxidative stress in septic rats

MDA, an indicator of lipid peroxidation levels, increased in the lung and liver of the CLP group, but the increase was significantly attenuated by melatonin (Figure 6A). The activities of SOD and CAT in the lung and liver were significantly inhibited by CLP, and melatonin restored the inhibition (Figure 6B, 6C). CLP-decreased GSH level was also elevated by melatonin treatment in the lung and liver (Figure 6D). These data suggest that melatonin could suppress CLP-induced oxidative stress in rats.

Melatonin alleviated lung and liver cell apoptosis in CLP-induced septic rats

TUNEL assay was performed to explore the role of melatonin in lung and liver cell apoptosis in CLP-induced septic rats. As shown in Figure 7A, CLP significantly caused cell apoptosis in the lung and liver. However, a notable decrease in the TUNEL-positive cells was observed in the CLP + melatonin group. Moreover, melatonin inhibited CLP-increased caspase-3 activity in the lung and liver (Figure 7B). The apoptosis-related molecules, including caspase-3, cl-caspase-3, Bcl-2, Bax, Akt and p-Akt, were also measured via Western blot analysis in the lung and liver tissues of CLP-induced septic

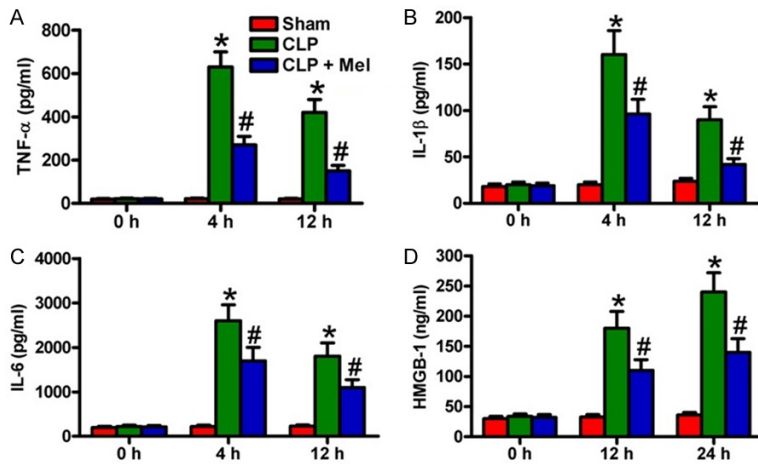


Figure 4. Melatonin suppressed the production of inflammatory cytokines in the plasma of CLP-induced septic rats. Rats intraperitoneally received melatonin at 30 mg/kg per injection per rat at 0, 3, 6 and 12 hours after CLP surgery. The releases of tumor necrosis factor- α (A), interleukin (IL)-1 β (B), IL-6 (C), and high-mobility group protein box 1 (D) in the plasma were measured by ELISAs at 0, 4, 12 and 24 hours after CLP. Data are expressed as mean \pm SD (n = 10 per group). *P < 0.05, **P < 0.01 vs. sham group; #P < 0.05 vs. CLP group.

5C). CLP-enhanced iNOS expression in lung and liver was significantly attenuated by melatonin (Figure 5D, 5E). These results suggest that CLP-induced increase in neutrophil infiltration and iNOS/NO biosynthesis was mitigated by melatonin in septic rats.

rats to investigate the potential mechanisms for melatonin effects on sepsis-induced apoptosis. Results shown in Figure 7C-G depicted that CLP significantly increased the pro-apoptotic molecules (cl-caspase-3 and Bax) and decreased the anti-apoptotic proteins (Bcl-2

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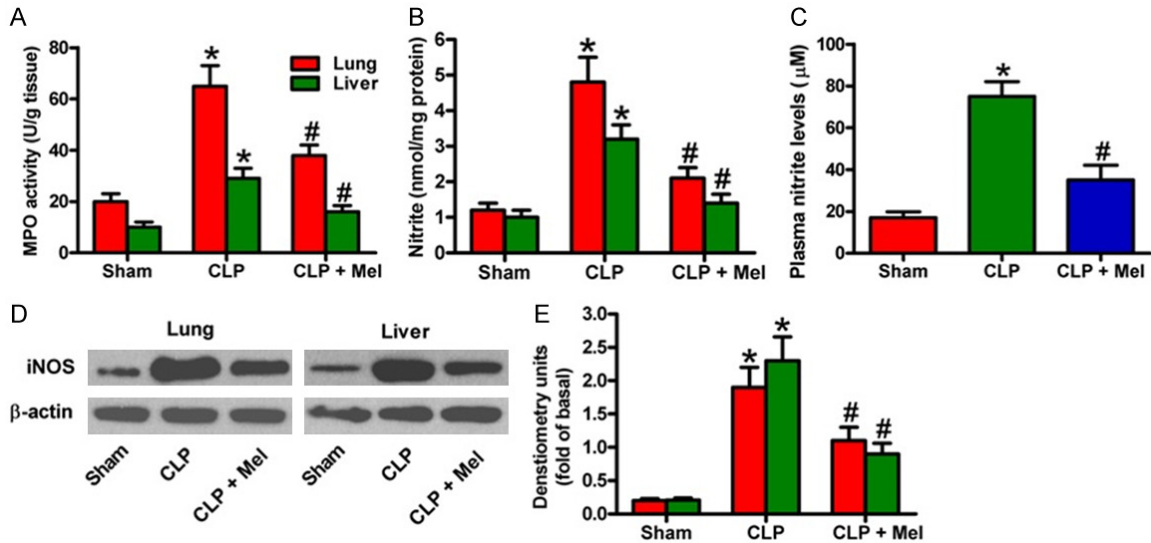


Figure 5. Melatonin reduced CLP-induced increase in myeloperoxidase (MPO) activity and iNOS/NO biosynthesis in septic rats. Rats intraperitoneally received melatonin at 30 mg/kg per injection per rat at 0, 3, 6 and 12 hours after CLP surgery. MPO activity (A) and NO production (B) in the lung and liver were measured at 24 hours after CLP. (C) The concentration of NO in the plasma was determined. (D) Representative Western blot results of iNOS expression in the lung and liver. (E). Relative protein band densities of iNOS normalized against β -actin. Data are expressed as mean \pm SD (n = 10 per group). *P < 0.05 vs. sham group; #P < 0.05 vs. CLP group.

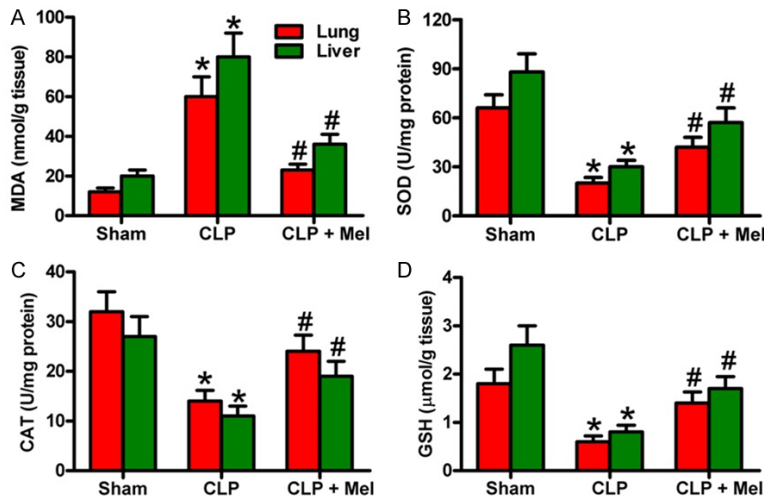


Figure 6. Melatonin reduced malondialdehyde (MDA) content and increased superoxide dismutase (SOD) and catalase (CAT) activities and glutathione (GSH) level in the lung and liver of CLP-induced septic rats. Rats intraperitoneally received melatonin at 30 mg/kg per injection per rat at 0, 3, 6 and 12 hours after CLP. The MDA content (A), SOD activity (B), CAT activity (C), and GSH level (D) in the lung and liver were measured by commercial kits at 24 hours after CLP. Data are expressed as mean \pm SD (n = 10 per group). *P < 0.05 vs. sham group; #P < 0.05 vs. CLP group.

and p-Akt). Nevertheless, melatonin reversed these changes. These results indicated that melatonin reduced the lung and liver cell apoptosis in CLP-treated rats.

els of inflammatory cytokines, such as TNF- α , IL-1 β and HMGB1, in the plasma of CLP-induced septic rats. (5) Melatonin reduced CLP-induced neutrophil infiltration into the lung and liver,

Discussion

The CLP-induced sepsis model is a widely used method for investigating the complicated mechanisms of sepsis because of its similar features to those of septic patients [22]. In this study, the CLP-induced septic rat model was used to investigate protective effects of melatonin against septic lung and liver injuries. The major findings are as follows. (1) Melatonin improved the survival rate and histopathological injuries of lung and liver of CLP-insulted rats. (2) Melatonin inhibited the hemodynamic changes of CLP-induced septic rats. (3) Melatonin ameliorated lung and liver dysfunctions in rats subjected to CLP. (4) Melatonin reduced the levels of inflammatory cytokines, such as TNF- α ,

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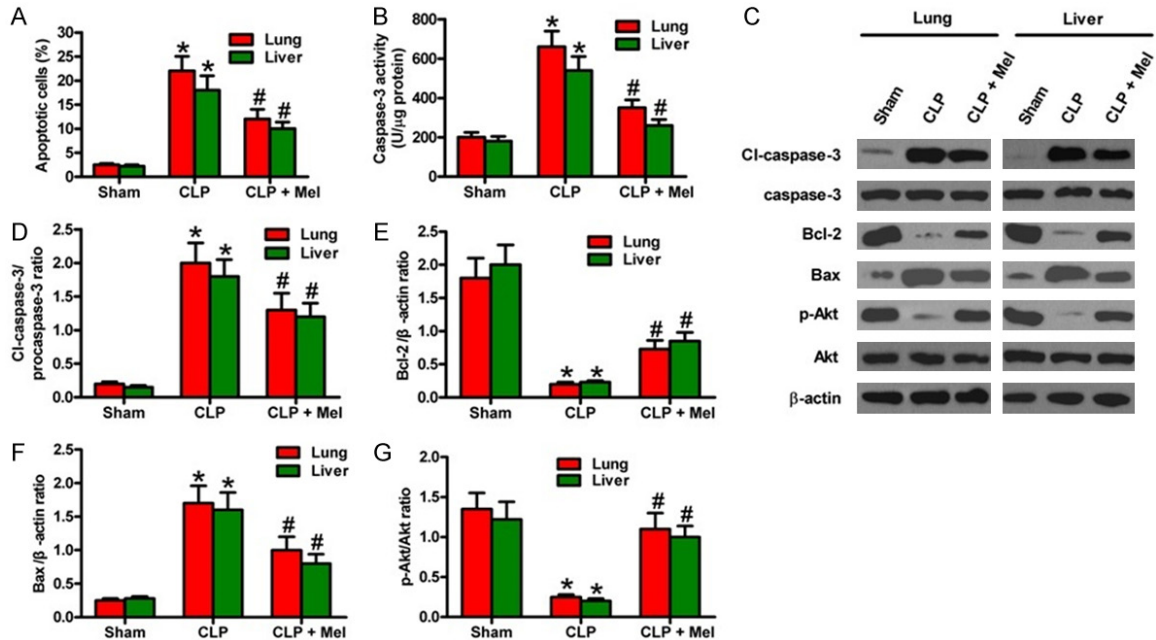


Figure 7. Melatonin inhibited CLP-induced lung and liver cell apoptosis of the septic rats. Rats intraperitoneally received melatonin at 30 mg/kg per injection per rat at 0, 3, 6 and 12 hours after CLP. (A) The percentage of apoptotic lung and liver cells were measured by TUNEL assay. (B) Caspase-3 activity was assessed to evaluate the apoptosis of lung and liver cells. (C) The expression of caspase-3, cl-caspase-3, Bcl-2, Bax, Akt and p-Akt was measured by Western blot analysis. β -actin was used as the endogenous control. Ratios of cl-caspase-3/caspase-3 (D), Bcl-2/ β -actin (E), Bax/ β -actin (F), and p-Akt/Akt (G) were calculated. Data are expressed as mean \pm SD (n = 10 per group). *P < 0.05 vs. sham group; #P < 0.05 vs. CLP group.

and decreased the pulmonary, hepatic, and plasma NO levels, as well as the expression of iNOS in the lung and liver. (6) Melatonin decreased the MDA content but enhanced the SOD and CAT activities and GSH level in the lung and liver. (7) Melatonin inhibited CLP-induced lung and liver cell apoptosis, as shown by the decrease in caspase-3 activity, down-regulation of cl-caspase-3 and Bax, and up-regulation of Bcl-2 and p-Akt. Collectively, these results demonstrate that melatonin attenuates CLP-induced inflammation, oxidative stress, and apoptosis in rats, suggesting melatonin as a useful agent for therapy of septic lung and liver injuries.

Sepsis is a systemic inflammatory response syndrome to infection. This systemic inflammatory cascade results in neutrophil sequestration in various systemic organs, including the lung and liver. Subsequent neutrophil extravasation can lead to vascular and parenchymal cell dysfunctions [23]. Pro-inflammatory cytokines, such as TNF- α , IL-1 β and IL-6, are the most strongly associated cytokines with sepsis [15]. TNF- α is an important initiator in sepsis.

When the host is infected with bacteria, TNF- α appears early in the circulation and quickly reaches peak levels, thereby inducing microcirculation and a series of inflammatory changes in vascular endothelial cells [24]. IL-1 β and IL-6 are considered closely related to septic severity and mortality [17, 25]. HMGB1, as a late inflammatory mediator, is a key factor in the lethal effect of sepsis, and its level will directly affect the severity of the body's response and prognosis of sepsis [26]. Melatonin inhibits production of TNF- α , IL-1 β and HMGB1 in sepsis-induced cardiac dysfunction and brain injury [17, 18]. In the present study, the release of TNF- α , IL-1 β , IL-6 and HMGB1 in the plasma and the activity of MPO in the lung and liver tissue were markedly reduced by melatonin in CLP-challenged rats. TNF- α and IL-1 β can induce iNOS expression and NO production [27, 28]. NO produced by iNOS probably plays a central role in mitochondrial damage during sepsis [29]. Melatonin inhibits iNOS expression and activity in the liver and lung, and prevents endotoxemia in lipopolysaccharide-induced multiple organ dysfunction syndrome in rats [30]. In this study, melatonin decreased

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the production of NO in lung, liver, and plasma, and reduced the expression of iNOS in lung and liver of CLP-induced septic rats.

Oxidative stress is one of the most significant factors in the pathogenesis of sepsis [9]. Sepsis is associated with enhanced generation of ROS and RNS, which react with biological macromolecules, thereby producing lipid peroxides, inactivating proteins, and mutating DNA [9, 31, 32]. Lipid peroxidation can cause changes in membrane fluidity and permeability, increase the rate of protein degradation, and gradually lead to cell lysis [33]. MDA is an end product of the lipid peroxidation and reflects the damage caused by ROS [34]. In the antioxidant system, SOD and CAT are key ROS scavengers, which can specifically eliminate superoxide radicals and prevent ROS attack [35]. GSH is one of the major components of the non-enzymatic antioxidant system [36]. Melatonin exhibits both free radical scavenging and antioxidant properties. Melatonin protects against oxidative organ injury by reversing the changes of MDA and GSH content in multi-organs of a septic rat model [37]. Melatonin also ameliorates septic cardiac and brain injury by elevating SOD and CAT activities and decreasing MDA content [17, 18]. Consistent with these findings, we found that melatonin inhibited CLP-induced oxidative damage, as shown by the reduction of MDA content and enhancement of SOD and CAT activities, as well as GSH level in the lung and liver of septic rats.

Apoptosis is another key factor in the evolution of organ damage during sepsis. Blocking apoptosis improves the outcome in animals with severe sepsis [38]. During sepsis, oxidative stress is recognized as a strong mediator of apoptosis via the formation of lipid hydroperoxides [39]. ROS overproduction may induce a depletion of intracellular GSH that acts as a free-radical scavenger and a regulator of the intracellular redox state, which results in mitochondrial damage, cytochrome c release, caspase activation, and cell apoptosis [40]. NO also plays an important role in cell apoptosis. NO can react with superoxide, thereby forming the peroxynitrite, which causes lipid peroxidation, cellular damage, and apoptosis [41]. PI3K/Akt signaling plays a protective role in several septic models [17, 42-44]. When activated, PI3K causes Akt phosphorylation and subsequent phosphorylation of diverse target mole-

cules (such as Bcl-2 family) that preserve mitochondrial integrity and promote cell survival [45]. The Bcl-2 family, including anti-apoptotic (such as Bcl-2) and pro-apoptotic (such as Bax) members, acts as a crucial checkpoint upstream of the mitochondrial apoptosis pathway [46]. The caspase family is an executioner of apoptosis, in which caspase-3 is a crucial apoptotic protease in the final common pathway of the apoptotic cell death [47]. Melatonin inhibits sepsis-induced cardiac and brain apoptosis [17, 18]. In this study, phosphorylation of Akt and expression of Bcl-2 were upregulated, whereas Bax and cl-caspase-3 expression, and caspase-3 activity were reduced by melatonin treatment. These results imply that melatonin may prevent apoptosis of septic lung and liver through inhibiting the mitochondrial-initiated caspase pathway.

Conclusion

In summary, treating CLP-induced sepsis with melatonin attenuates lung and liver injuries via inhibiting inflammation, oxidative stress, and apoptosis. Our findings provide a biochemical basis for the use of melatonin as a potential agent for sepsis therapy.

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Disclosure of conflict of interest

None.

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