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ORIGINAL ARTICLE

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Melatonin attenuates lung ischaemia–reperfusion injury via inhibition of oxidative stress and inflammation

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Abstract

OBJECTIVES: Lung ischaemia–reperfusion injury is a complex pathophysiological process due to the production of reactive oxygen species and the generation of inflammatory reaction. We investigated the protective effects and the corresponding mechanism of melatonin (MT), a potent free-radical scavenger, on lung injury induced by ischaemia–reperfusion in a mouse model.

METHODS: Adult male C57BL/6J mice ($n = 30$) were randomly and equally allocated into 5 groups: sham controls, IR, IR + 10 mg/kg MT, IR + 20 mg/kg MT and IR + 30 mg/kg MT. Lung ischaemia–reperfusion injury was induced by thoracotomy followed by clamping of the left hilum for 1 h and subsequent reperfusion for 2 h.

RESULTS: Histological scoring analysis showed that lung parenchymal damage was ameliorated in the melatonin pretreatment groups when compared with the IR group, with the IR + 20 mg/kg MT group showing the strongest effect among the melatonin pretreatment groups. Wet-to-dry weight ratio, detection of malondialdehyde, protein expressions of inflammatory factors (tumour necrosis factor-α, interleukin-1β, NF-κB and IKK-γ) and apoptotic factors (cleaved caspase-3 and Bax/Bcl-2), as well as TUNEL assay showed changes similar to those of the lung injury scores in all groups. In contrast, the examination of superoxide dismutase showed a pattern contrary to that of the lung injury score in all groups. In addition, immunohistochemistry staining showed that the expressions of the antioxidants glutathione peroxidase and glutathione reductase were increased in the melatonin pretreatment groups.

CONCLUSIONS: This study demonstrated that melatonin pretreatment attenuated lung ischaemia–reperfusion injury via inhibition of oxidative stress, inflammation and apoptosis.

Keywords: Melatonin • Ischaemia–reperfusion • Lung injury • Oxidative stress • Apoptosis • Inflammation

INTRODUCTION

Lung ischaemia–reperfusion injury (LIRI) is a complicated pathological process seen in various clinical conditions such as lung transplantation, cardiopulmonary bypass cardiac surgery, resuscitation for circulatory arrest and pulmonary embolism [1, 2]. Particularly in lung transplantation, the most severe form of LIRI may lead to primary graft failure and remains a significant cause of morbidity and mortality [3]. Therefore, new and effective strategies are needed to improve the clinical outcomes for patients with LIRI.

A growing body of evidence supports that the acute severe inflammatory reaction and the generation of oxidative stress and reactive oxygen species (ROS) play essential roles in LIRI. ROS cause lung injury directly by inducing oxidative stress or indirectly by inducing apoptosis. In addition, ROS lead to the release of inflammatory cytokines, such as tumour necrosis factor (TNF)-α and interleukin (IL)-1β, through activation of inflammatory processes. These effects result in progressive injuries of the lung parenchyma, the gaseous exchange system and pulmonary circulations [4–6]. Therefore, strategies that target the generation and propagation of inflammatory response, ROS and oxidative stress may have therapeutic potential.

Melatonin (MT) or N-acetyl-5-methoxytryptamine, an indole, is synthesized and secreted nocturnally by the pineal gland [7]. Melatonin is a receptor-independent free-radical scavenger with potent antioxidant and anti-inflammatory properties [8, 9]. Melatonin not only inhibits the generation of ROS but also activates several antioxidant enzymes, such as superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx) [10, 11]. Furthermore, melatonin decreases the production of malondialdehyde (MDA), indicating that melatonin plays a protective role in lipid peroxidation and cell damage [12]. Numerous studies have shown that melatonin protects against ischaemia–reperfusion (I/R) injury in liver, kidney, heart and other organs [13–15]. There is also evidence that melatonin protects

tissue damage during I/R injury of the lung [16, 17]; however, the mechanism underlying the protection against lung I/R injury by melatonin in experimental models is poorly understood. The aim of this study was to determine the effect and mechanism of melatonin therapy on lung I/R injury in a mouse model.

MATERIALS AND METHODS

Animals

Male C57BL/6J wild-type mice (8–10-week old and weighing 20–30 g) were purchased from the Laboratory Animal Center of Tongji Medical College (Wuhan, China). The mice were housed and monitored in a specific pathogen-free environment. The experiment was approved by the Institutional Animal Care and Use Ethics Committee of Huazhong University of Science and Technology.

Animal grouping and melatonin preparation

Thirty C57BL/6J wild-type mice were randomly and equally allocated into 5 groups: Group 1 (the sham group); Group 2 (the IR group); Group 3 [the IR + 10 mg/kg melatonin pretreatment group (IR + 10MT)]; Group 4 [the IR + 20 mg/kg melatonin pretreatment group (IR + 20MT)] and Group 5 [the IR + 30 mg/kg melatonin pretreatment group (IR + 30MT)].

For treatment of Groups 3–5, melatonin (purchased from Sigma-Aldrich, St. Louis, MO, USA) was freshly dissolved in absolute ethanol. The resulting solution was diluted with saline until a concentration of 0.2% ethanol in saline was obtained. For treatment of Groups 1 and 2, a volume of ethanol equal to that used for the dissolution of melatonin was added to the saline solution.

Lung ischaemia–reperfusion procedure and treatment strategy

Animals were anaesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg). After ensuring an adequate depth of anaesthesia, the mice were fixed in a supine position. The sham controls underwent thoracotomy, followed by separation of the lower pulmonary ligament. Groups 2–5 underwent thoracotomies, followed by clamping of the left pulmonary hilum for 1 h and subsequent reperfusion for 2 h [18]. Groups 3–5 received specified dosages of melatonin (5, 10 and 15 mg/kg, respectively) 15 min before ischaemia and 10 min before reperfusion via intraperitoneal injection. Groups 1 and 2 were given the same volume of a solution of normal saline and ethanol at the same time points. Dosage and time points of melatonin administration were based on a previous study with minor modifications [14]. The left lung in each mouse was harvested and immediately frozen in liquid nitrogen until use.

Histological assessment of lung injury

Left lung tissues harvested from the mice were fixed in 10% formalin and embedded in paraffin. Approximately 5-mm-thick tissue sections were stained with haematoxylin and eosin to assess the injury of the lung tissues. The histological analysis followed a classic lung injury score standard [19].

Analysis of the lung wet-to-dry weight ratio

The left lower lobe of the lung was extirpated to determinate the *W/D* weight ratio, which assesses the severity of pulmonary oedema. After lung removal, the wet weight was measured; subsequently, the tissues were dried at 80°C, and the dry weight was measured after the weight became constant.

Measurement of lung superoxide dismutase activity and malondialdehyde content

It is well documented that lipid peroxidation is one of the causes for lung I/R injury. The left pulmonary tissues were homogenized on ice with 0.9% normal saline and then centrifuged for 10 min at 4000 rpm/min at 4°C. The supernatants were used for further measurements. The MDA content was determined by the thiobarbituric acid method, and the SOD activity was detected by the xanthine oxidase method using assay kits (Nanjing Jiancheng Corp., Nanjing, China) according to the manufacturer's instructions.

TUNEL assay

Paraformaldehyde-fixed, paraffin-embedded lung tissue sections were prepared. The *In Situ* Cell Apoptosis Detection kit (Chemicon, Norcross, GA, USA) was used to detect apoptotic cells according to the manufacturer's instructions, as previously described [20]. Stained cells were counted randomly.

Immunohistochemical analysis

Protein expression was determined immunohistochemically. Five-micrometre serial sections were dewaxed in xylene and rehydrated through graded alcohols. Endogenous peroxidases were blocked (3% H_2O_2, 15 min), and antigens were retrieved by microwaving the slides. Then, the slides were blocked with goat serum (1:10 dilution) for 30 min. Subsequently, sections were incubated with one of the following antibodies at 4°C overnight and diluted according to the manufacturer's protocol: GPx (1:500 dilution; Abcam, Cambridge, MA, USA) and GR (1:100 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Then, the sections were incubated with HRP-conjugated secondary antibodies followed by the Liquid DAB Substrate Chromogen System. Images were viewed and captured under an inverted microscope (Olympus, Tokyo, Japan).

Western blotting

Equal amounts (10–30 μg) of protein extracted from the left lung were loaded and separated by SDS–PAGE using 8–10% acrylamide gradients. Following electrophoresis, the separated proteins were electrophoretically transferred to a polyvinylidenedifluoride membrane (Sigma-Aldrich). Non-specific proteins were blocked by incubating the membrane in 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature, followed by incubating overnight with the following primary antibodies: TNF-α, IL-1β, caspase-3, Bcl-2 and Bax (Abcam) as well as p65, NF-κB and IKK-γ (Cell Signaling Technology, Danvers, MA, USA). On the following day, proteins were visualized on an ECL detection system (Beyotime Biotechnology, Shanghai, China) after incubation with the respective HRP-conjugated secondary antibodies

(1:1000 dilution). GAPDH (Sigma-Aldrich) was used as a loading control.

Statistical analysis

All results are reported as the mean ± standard deviation. The data were examined by analysis of variance (ANOVA) followed by a Bonferroni multiple comparison *post hoc* test using the SPSS software (version 16.0). Values of $P < 0.05$ were considered statistically significant between the groups.

RESULTS

Melatonin attenuates ischaemia–reperfusion-induced lung parenchymal injury

The effect of melatonin pretreatment on reducing I/R-induced lung parenchyma injury was determined by haematoxylin and eosin staining. The pathological changes in the lung tissues were observed under a light microscope. As shown in Fig. 1A, the sham group exhibited normal lung tissue structures. In contrast, severe lung damage was observed in the IR group, as indicated by widespread alveolar collapse, extensive interstitial oedema and infiltration of leucocytes. However, melatonin pretreatment ameliorated the I/R-induced lung injury, with the IR + 20MT group exhibiting the strongest improvement among the IR + MT groups. The lung injury scores paralleled the findings of the histological assessment, as the lung injury score of the sham group was low and that of the IR group was significantly higher than that of the sham group ($P < 0.01$). The lung injury scores of the IR + 10MT, IR + 20MT and IR + 30MT groups were lower than that of the IR group, with the IR + 20MT group exhibiting the lowest lung injury score among the IR + MT groups ($P < 0.05$; Fig. 1B).

Furthermore, we investigated the *W/D* ratios of the lung tissues to evaluate the extent of lung injury. Figure 1C shows that the *W/D* ratio of the lung tissues was significantly higher in the IR group than that in the sham group ($P < 0.01$). However, the *W/D* ratio in the IR + 10MT, IR + 20MT and IR + 30MT groups was significantly lower than that in the IR group, with the IR + 20MT group showing the most apparent decrease in the *W/D* ratio of the lung tissues among the IR + MT groups ($P < 0.05$).

Melatonin suppresses ischaemia–reperfusion-induced oxidative stress

The activity of SOD (a key enzyme in detoxifying intracellular O_2^- and H_2O_2) and the content of MDA (a metabolic product of lipid

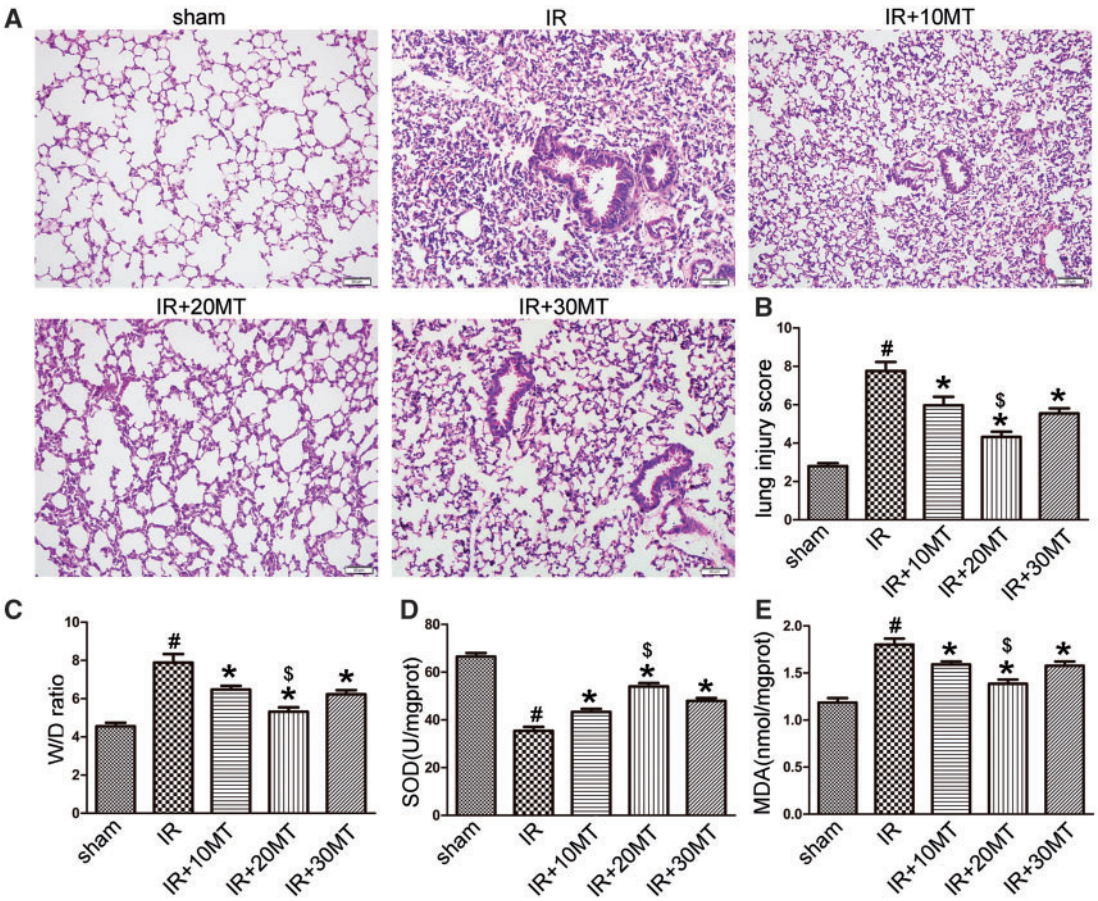

Figure 1: Melatonin attenuates lung injury and suppresses oxidative stress induced by ischaemia–reperfusion. (**A**) Haematoxylin and eosin staining analysis of lung pathological injury. Representative photomicrographs were taken at a magnification of ×200. (**B**) Lung injury score. (**C**) Lung wet/dry weight ratio. (**D**) Measurement of the lung superoxide dismutase activity. (**E**) Measurement of the lung malondialdehyde content. Data are expressed as the mean ± standard deviation and compared using 1-way analysis of variance (ANOVA) and Bonferroni *post hoc* test. $^{\#}P < 0.01$ vs the sham group, $^{*}P < 0.05$ vs the IR group and $^{\$}P < 0.05$ vs the IR + 10MT and IR + 30MT groups, $n = 6$. MDA: malondialdehyde; SOD: superoxide dismutase; W/D: wet/dry.

peroxidation) were determined to assess the effect of melatonin on I/R-induced lung oxidative stress (Fig. 1D and E).

The SOD activity was the lowest in the IR group, and melatonin pretreatment dramatically antagonized the decrease in SOD activity induced by I/R ($P < 0.05$). However, the SOD activity in the IR + 10MT and IR + 30MT groups was decreased when compared with the IR + 20MT group ($P < 0.05$). In contrast, the highest MDA level was observed in the IR group. The MDA levels were dramatically decreased in the lung tissues of the IR + MT groups when compared with the IR group ($P < 0.05$), indicating an inhibition of MDA by melatonin. The extent of inhibition in the IR + 10MT and IR + 30MT groups was reduced when compared with the IR + 20MT group.

Immunohistochemical staining showed that the expressions of GPx (Fig. 2A) and GR (Fig. 2B), 2 antioxidant indicators that are critically involved in the detoxification of ROS, were the lowest in the sham controls when compared with the other groups. Additionally, the number of positive GPx and GR cells was increased in IR mice and higher in IR + MT mice when compared with the sham controls.

These data suggest that lung I/R increased the intrinsic antioxidant levels to protect against further I/R injury. Furthermore, melatonin played an antioxidant role during I/R-induced lung injury, and 20 mg/kg of melatonin showed the most efficient protection.

Melatonin suppresses ischaemia–reperfusion-induced inflammation

As shown in Fig. 3A and B, protein expressions of TNF-α, NF-κB, IL-1β and IKK-γ proteins, 4 indicators of inflammation, were the highest in the IR group and were significantly decreased in the IR + MT groups when compared with the IR group. Moreover, the levels of TNF-α, NF-κB, IL-1β and IKK-γ were markedly lower in the IR + 20MT group than those in the IR + 10MT and IR + 30MT groups ($P < 0.05$).

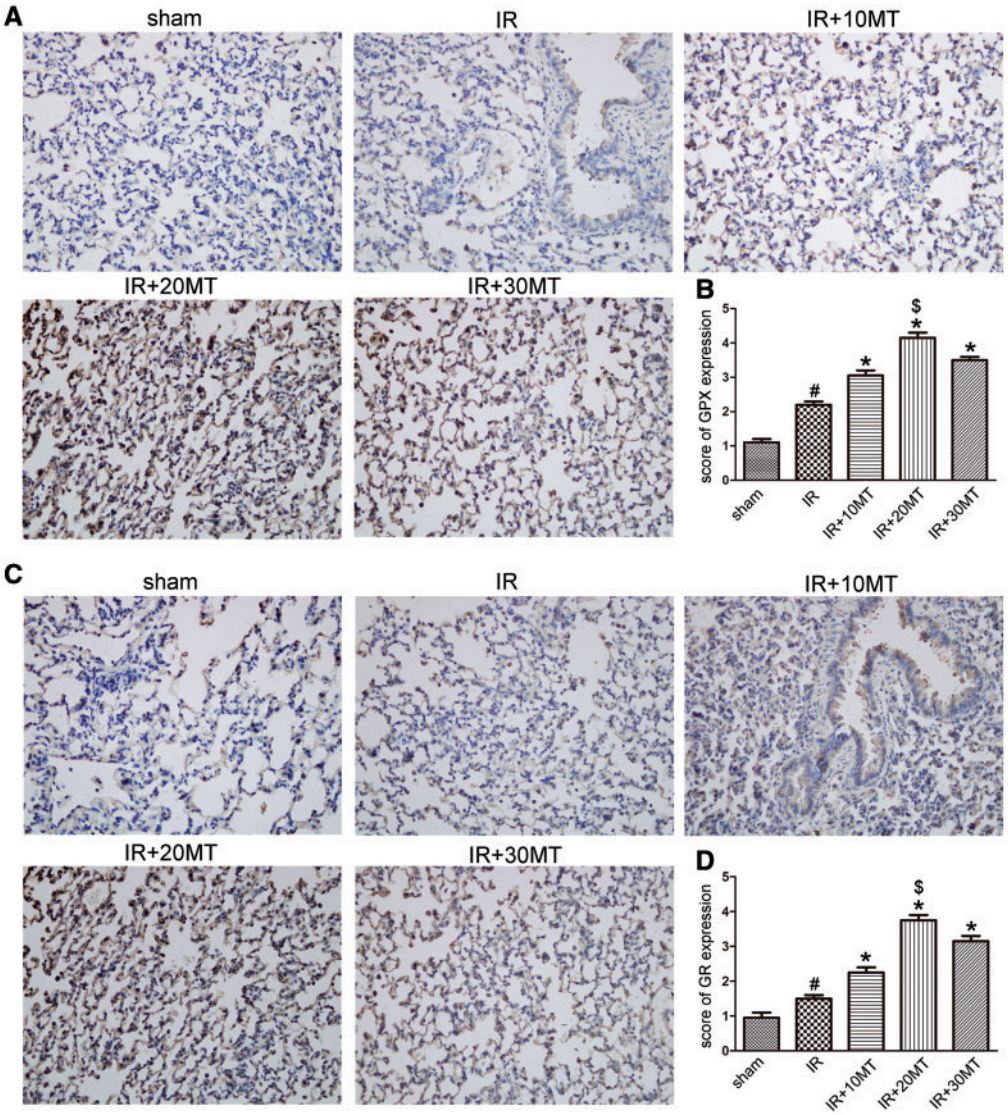

Figure 2: Immunohistochemical (IHC) analysis of glutathione peroxidase (GPx) and glutathione reductase (GR) expressions. (**A**) IHC staining of GPx + cells (brown) in lung parenchyma of the 5 groups (×400). (**B**) Comparison of the GPx expression score among the 5 groups. (**C**) IHC staining of GR + cells (brown) in lung parenchyma of the 5 groups (×400). (**D**) Comparison of the GR expression score among the 5 groups. $^{\#}P < 0.01$ vs the sham group, $^{*}P < 0.05$ vs the IR group and $^{\$}P < 0.05$ vs the IR + 10MT and IR + 30MT groups, $n = 6$.

Melatonin inhibits ischaemia–reperfusion-induced cellular apoptosis

Western blotting was performed to examine the levels of Bax, caspase-3 and Bcl-2, which are apoptosis-related proteins. Figure 3C and D reveals that the expression of Bax was the highest in the IR group, significantly lower in the IR + MT groups than in the IR group and markedly lower in the IR + 20MT than in the IR + 10MT and IR + 30MT groups. Similarly, the cleaved caspase-3 expression had a pattern parallel to that of Bax expression among the 5 groups (Fig. 3C and D). The expression of Bcl-2 showed a pattern contrary to that of the expressions of Bax and caspase-3 (Fig. 3C and D).

To further explore the effects of melatonin on cellular apoptosis, we performed the TUNEL assay to detect lung I/R-induced apoptosis. This revealed that the number of stained cells in the IR group was markedly increased when compared with the sham controls and more reduced in the IR + MT groups than in the IR group (Fig. 4). Apoptosis was more decreased in the IR + 20MT group than in the IR + 10MT and IR + 30MT groups.

These data indicate that lung I/R-induced dramatic cellular apoptosis and melatonin pretreatment inhibited apoptosis after the lung I/R challenge.

DISCUSSION

A wide variety of animal models have been used to seek the mechanisms and potential treatments of LIRI, such as clamping of the pulmonary artery, clamping the hilum and transplanting the lung [2, 21]. In this study, we applied a widely used model by clamping the pulmonary hilum, which resulted in complete ischaemia and anoxia, to explore the effects of melatonin pretreatment on lung I/R injury, leading to 2 striking conclusions [2]. First, melatonin therapy substantially reduced lung injury scores, lung *W/D* ratios and lung parenchyma damage when compared with untreated LIRI at 2 h after I/R injury challenge. These findings reinforced those of previous studies that melatonin plays a protective role in lung I/R injury [22, 23]. In addition, we further investigated the mechanism of melatonin in reducing lung I/R injury, which may be involved in suppressing inflammatory response, reducing oxidative stress and inhibiting cell apoptosis. Second, 20 mg/kg was found to be a relatively more effective dose to ameliorate lung I/R injury in mice.

Previous studies have determined that I/R of the lung may not only play significant but also different roles in the development of lung injury [24, 25]. The mechanisms of lung I/R injury are associated with neutrophil activation, release of numerous inflammatory cytokines (TNF-α and IL-1β) and oxygen radicals, which lead to lung cellular damage, necrosis and apoptosis [25]. Elevation in circulating TNF-α and IL-1 contributing to organ I/R injury has been reported [26]. In addition, the expression levels of these inflammatory mediators were correlated with the degree of lung injury, including early pulmonary neutrophil infiltration, pulmonary microvascular permeability and pulmonary oedema, demonstrating the important role of these inflammatory cytokines in the modulation of the primary stages of I/R injury [27].

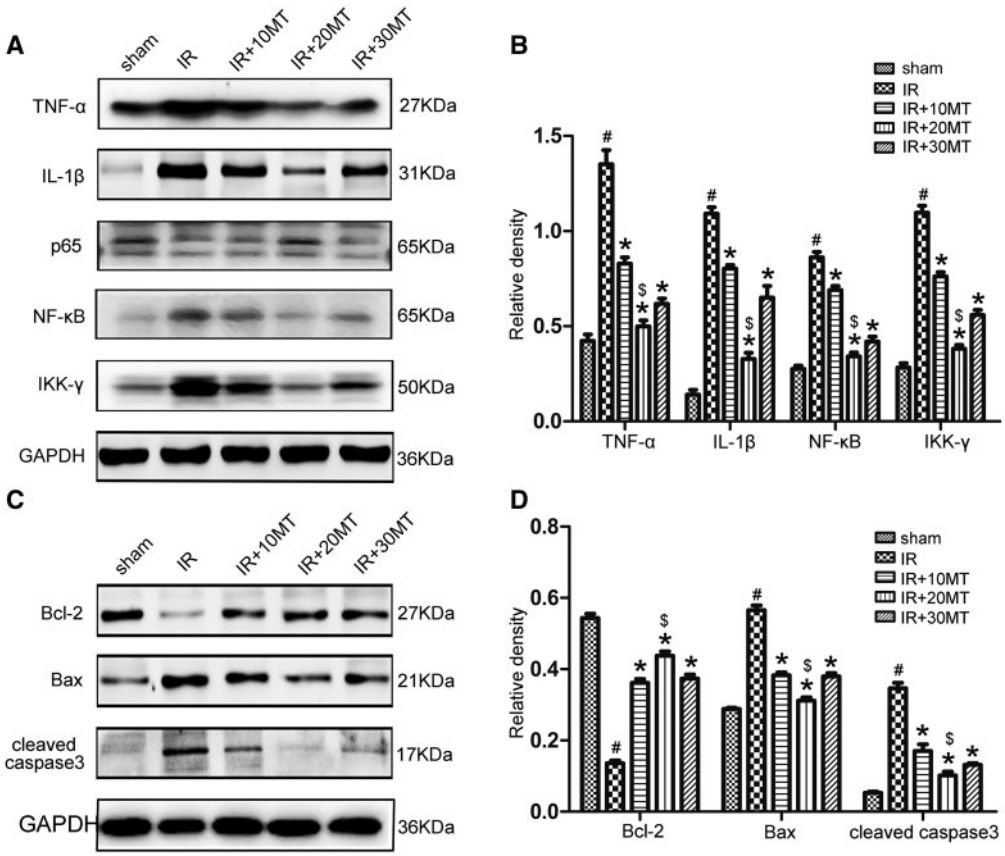

Figure 3: Western blotting analysis of the expressions of inflammatory and apoptosis-related proteins. (**A**) Protein expressions of TNF-α, NF-κB, IL-1β and IKK-γ. (**B**) Protein expressions were quantified relative to GAPDH as an internal control. (**C**) Protein expressions of Bcl-2, Bax and cleaved caspase-3. (**D**) Protein expressions were quantified relative to GAPDH as an internal control. $^{\#}P < 0.01$ vs the sham group, $^{*}P < 0.05$ vs the IR group and $^{\$}P < 0.05$ vs the IR + 10MT and IR + 30MT groups, $n = 6$. IL-1β: interleukin-1β; TNF-α: tumour necrosis factor-α.

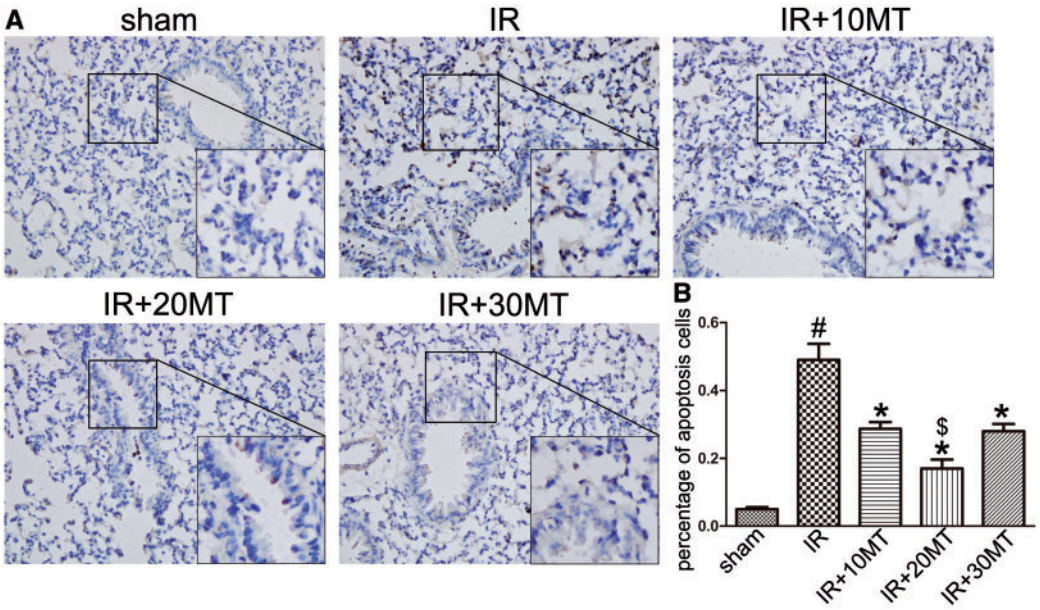

Figure 4: TUNEL assay for measuring cell apoptosis. (**A**) Representative photomicrographs show TUNEL-positive staining cells (brown) of the nucleus at magnifications of ×400. (**B**) Percentage of TUNEL-positive staining cells. $^{\#}P < 0.01$ vs the sham group, $^{*}P < 0.05$ vs the IR group and $^{\$}P < 0.05$ vs the IR + 10MT and IR + 30MT groups, $n = 6$.

Our study demonstrated increased levels of TNF-α, NF-κB, IL-1β and IKK-γ during LIRI.

To further explore the protective mechanisms of melatonin, we evaluated changes of oxidative stress in the lung. The modification of cell components induced by ROS, such as changes in MDA and SOD levels, showed an increase in the MDA level and a reduction in the SOD activity in LIRI. However, melatonin pretreatment attenuated oxidative stress induced by LIRI, suggesting that melatonin possibly exerts protective effects via downregulating oxidative stress.

Furthermore, immunohistochemical staining showed that cells with positive GPx and GR staining were increased in IR mice and higher in IR + MT mice when compared with the sham controls. These data support that melatonin, acting as a free-radical scavenger and a potent antioxidant, plays an antioxidant role during IR-induced lung injury [8, 9].

The relationships among increased inflammatory responses, oxidative stress and cell apoptosis in acute ischaemia-induced organ injury have been investigated in depth [28, 29]. This study found that the expressions of Bax and cleaved caspase-3, 2 indicators of apoptosis, were significantly higher in lung I/R injury mice when compared with the sham controls. On the contrary, the expression of Bcl-2, an antiapoptosis-related protein, showed a pattern contrary to that of Bax and caspase-3 in these groups of mice. Notably, apoptosis-related proteins were significantly decreased after melatonin administration, whereas antiapoptosis-related protein was increased among these groups 2 h after LIRI. Taken together, our findings indicate that melatonin reduces lung I/R injury and protects the lung function, possibly via downregulation of lung cellular apoptosis.

Limitations

This study has several limitations. First, all parameters in this study were examined 2 h after reperfusion, without evaluating the time-related effect of melatonin in the lung I/R mouse model. Second, our results suggest that 20 mg/kg is a relatively more effective dose to ameliorate lung I/R injury in mice, but the appropriate dose for humans or other animal models needs further study. Third, the application of the experimental model of hilar clamping to induce I/R injury does not reflect the physiological nature of I/R injury. Instead, the model of orthotopic mouse lung transplantation would provide a physiological experimental setting in which the human lung transplantation setting would be ideally reflected [30]. The results obtained in this study should be tested in the latter model.

CONCLUSION

In conclusion, this study demonstrated that melatonin pretreatment attenuated lung I/R injury by inhibiting acute inflammatory reactions, oxidative stress and cellular apoptosis, suggesting that melatonin might be a potential therapeutic approach to LIRI.

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Conflict of interest: none declared.

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