



Methylene Blue Protects the Isolated Rat Lungs from Ischemia–Reperfusion Injury by Attenuating Mitochondrial Oxidative Damage

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Abstract

Introduction Impaired mitochondrial function is a key factor attributing to the lung ischemia reperfusion injury (LIRI). Methylene blue (MB) has been reported to attenuate brain and renal ischemia–reperfusion injury. We hypothesized that MB also could have a protective effect against LIRI by preventing mitochondrial oxidative damage.

Methods Isolated rat lungs were assigned to the following four groups ($n = 6$): a sham group: perfusion for 105 min without ischemia; I/R group: shutoff of perfusion and ventilation for 45 min followed by reperfusion for 60 min; and I/R + MB group and I/R + glutathione (GSH) group: 2 mg/kg MB or 4 μ M glutathione were intraperitoneally administered for 2 h, and followed by 45 min of ischemia and 60 min of reperfusion.

Results MB lessened pulmonary dysfunction and severe histological injury induced by ischemia–reperfusion injury. MB reduced the production of reactive oxygen species and malondialdehyde and enhanced the activity of superoxide dismutase. MB also suppressed the opening of the mitochondrial permeability transition pore and partly preserved mitochondrial membrane potential. Moreover, MB inhibited the release of cytochrome *c* from the mitochondria into the cytosol and decreased apoptosis. Additionally, MB downregulated the mRNA expression levels of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6, and IL-18).

Conclusion MB protects the isolated rat lungs against ischemia–reperfusion injury by attenuating mitochondrial damage.

Keywords Methylene blue · Isolated lung reperfusion · Lung ischemia–reperfusion injury · Mitochondrial damage

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Introduction

Lung ischemia–reperfusion injury (LIRI) occurs in many clinical situations such as lung transplantation, pulmonary embolism, cardiopulmonary resuscitation, and pulmonary thrombosis [1, 2]. However, currently there are no effective therapies available for LIRI in clinical practice [3, 4].

Impaired mitochondrial function is strongly and directly involved in pathogenesis of LIRI [5]. As soon as reperfusion, mitochondrial respiratory chain dysfunction leads to the augmentation of reactive oxygen species (ROS). ROS directly attack the mitochondrial membrane, trigger lipid peroxidation, and activate the opening of the mitochondrial permeability transition pore (MPTP), finally resulted in mitochondrial damage [6, 7]. In the injured mitochondria, cytochrome *c* is released into the cytosol through MPTP, activating caspase-3 to induce apoptosis [8]. On the basis of the aforementioned findings, we assume that an effective pharmacological compound, which can decrease the

production of free radicals and enhance mitochondrial function, may be a promising strategy to prevent LIRI.

Methylene blue (MB) is traditionally used to treat methemoglobinemia, lithangiuria, and nitrite poisoning. Recent investigations suggest that MB has multiple biological functions [9]. Among these biological functions, the anti-oxidative properties of MB have been extensively investigated. MB easily crosses the cellular membrane, accumulates in the mitochondrial matrix, and restores mitochondrial functions such as enhancing cytochrome *c* oxidase activity, oxygen consumption, and ATP production while reducing ROS generation [10, 11]. Notably, several studies have shown that MB could attenuate ischemia–reperfusion injury in major organs (heart [12], brain [13], kidney [14], and gut [15]). Weinbroum designed a dose–response study to investigate the protective effects of MB on pancreas ischemia/reperfusion-induced lung injury [16]. Additionally, Abreu et al. [17] reported that MB attenuated pulmonary injury in lung transplantation. However, the precise role of MB on LIRI remains to be determined.

We hypothesized that MB could exert a protective effect in LIRI. Ex vivo lung perfusion has emerged as an essential tool for evaluating therapeutic interventions to reverse acute lung injury or end-stage lung diseases. In this study, we used ex vivo lung perfusion to determine the protective effects of MB on rat LIRI and found that MB protected the isolated rat lungs from LIRI by attenuating mitochondrial oxidative damage.

Materials and Methods

Drugs and Reagents

MB was obtained from Jumpcan Pharmaceutical Group (Taizhou, China). GSH (NO.70-18-8) and rhodamine 123 (NO. 62669-70-9) were purchased from Sigma-Aldrich (St. Louis, MO). The content of LDH in the perfusate (NO.A020-2, reactive oxygen species(ROS, NO.E004), malondialdehyde (MDA, NO.A003-1), adenosine triphosphate (ATP, NO.A095-1), and the activity of superoxide dismutase (SOD, NO.A001-3) assay kits were all obtained from Nanjing Jiancheng Bioengineering Institute (China). Mitochondria isolation kit NO. C1260) from the lung tissue was obtained from Beijing Applygen Technology (China). TUNEL assay was conducted with a commercial kit (NO. CW2574S, Kangwei biotechnology co. LTD, Beijing, China). Anti-cytochrome *c* antibody and anti-Cox-IV from Cell Signaling Technology (USA); anti- β -actin antibody from Abcam (Cambridge, MA, USA). All other reagents were of analytical grade and commercially available.

Animals and Study Groups

Male Sprague–Dawley rats weighing 280 ± 40 g were obtained from SLAC Laboratory Animal Co. Ltd. (Shanghai, China). All animal experiments were conducted in accordance with protocols approved by the Animal Care and Use Committee of Jiangnan University.

Rats were divided randomly into four groups ($n = 6$ for each group). In the sham group, the lungs were continuously perfused for 105 min. In I/R groups, the lungs were exposed to 45 min of ischemia followed by 60 min reperfusion. In I/R + MB group, rats were intraperitoneally injected with 2 mg/kg of methylene blue 2 h before surgery and then ischemia 45 min plus 60 min reperfusion [18]. In I/R + GSH group, the lungs were subjected to ischemia 45 min and perfused with the perfusate containing 4 μ M of GSH for 60 min.

Isolated Rat Lung Perfusion Model

An isolated rat lung perfusion system (interleukin [IL-2 type; Hugo Sachs Elektronik Harvard Apparatus, March Hugstetten, Germany) was used for ex vivo lung perfusion (Fig. 1). Briefly, Rats anesthetized with an intraperitoneal injection of sodium pentobarbital (80 mg/kg) were intubated after tracheotomy and ventilated during the operation. After systemic heparinization (500 IU), a cannula was inserted into the pulmonary artery and another cannula into the left atrium through a median sternotomy. Two cannulas were connected to the perfusion circuit driven by two roller pumps, and lung perfusion was started with a low flow rate (1 mL/min). Finally, the lungs were removed and suspended in a sealed chamber. All of isolated lungs were allowed to equilibrate and stabilize for 20 min before ischemia. The baseline time is defined as the end of the stabilization period. Tidal volume (V_T), airway dynamic compliance (C_{dyn}), airway resistance (R_{aw}), and pulmonary vein oxygen partial pressure (PaO_2) were measured during the perfusion.

Pulmonary Edema

At the end of experiment, the right middle lung lobe was weighed and then dried at 60 °C overnight to calculate the wet-to-dry weight ratio [19].

Histological analysis

Lung specimens were fixed in paraformaldehyde, and embedded in paraffin. Paraffin-embedded lung samples were excised into 5 μ m-thick sections and stained with hematoxylin and eosin.

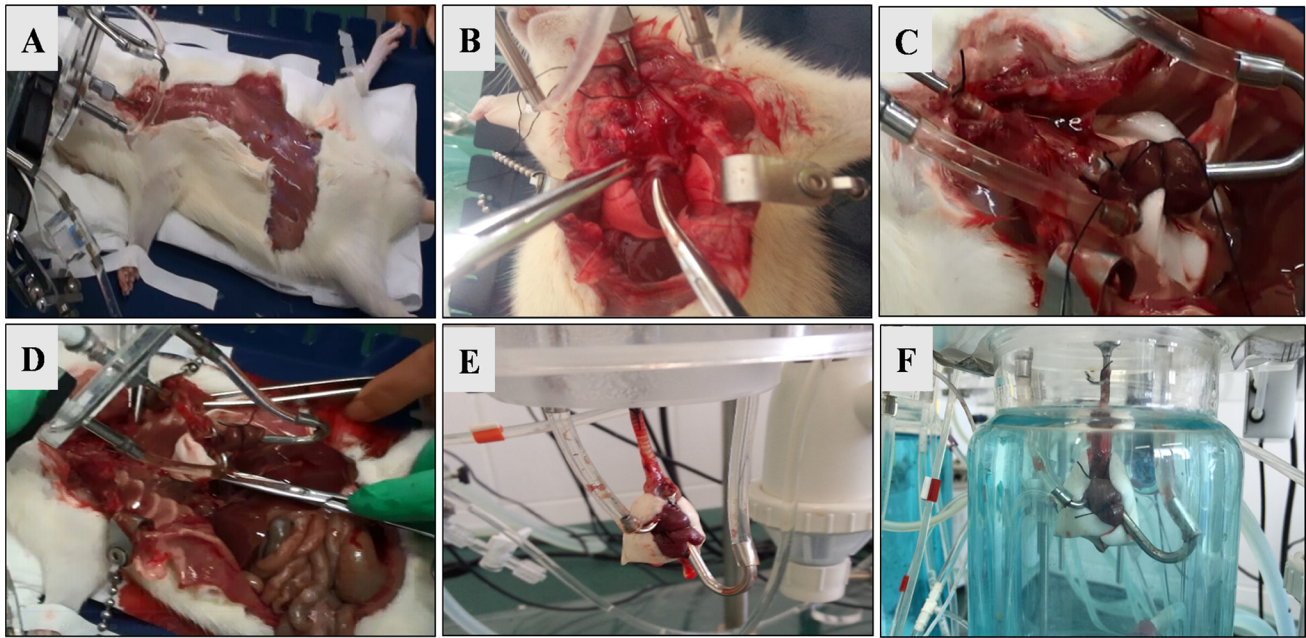


Fig. 1 **a** Expose the trachea and put the trachea cannula into the trachea. The trachea cannula is connected to the ventilation circuit (80 breaths/min; 10 mL/kg tidal volume). **b** The chest cavity is opened to expose the lung and the heart. **c** Transect the cardiac apex. Two cannulas are inserted into the main pulmonary artery from the right ventricle and the left atrium from the left ventricle, respectively. A modified Krebs–Henseleit buffer (2 mL/min) is injected into the

pulmonary artery. **d** Free the heart–lung block by dissecting the surrounding tissues. **e** Carefully remove the heart–lung block from the chest cavity and place it in the perfusion circuit. **f** Remove the ventilation circuit, and the lung is ventilated by negative pressure (end-inspiratory chamber pressure -8 cmH₂O; end-expiratory chamber pressure -2 cmH₂O). The trachea cannula is connected with the pressure sensor of the perfusion system to record the ventilation data

Oxidative Stress Assays

The lung tissues and perfusate were collected at the end of reperfusion. The content of LDH in the perfusate was determined according to the manufacturer's recommended protocol. The content of reactive oxygen species, malondialdehyde (MDA), adenosine triphosphate (ATP), and the activity of superoxide dismutase (SOD) in lung tissues were determined using commercially available kits (Jiancheng, Nanjing, China).

Mitochondrial Swelling Assay

Mitochondria from the lung tissue were isolated using a discontinuous percoll gradient method according to the manufacturer's instructions (Applygen, Beijing, China). Mitochondrial swelling due to MPTP opening was assessed as follows [20, 21]: the freshly prepared mitochondria (0.5 mg protein/mL) were added to the assay buffer (125 mmol/L sucrose, 50 mmol/L KCl, 2 mmol/L KH₂PO₄, and 10 mmol/L HEPES). Swelling was initiated by the addition of CaCl₂ (200 μmol/L). The decrease in OD at A520, which indicated the extent of opening of MPTP, was measured at 37 °C for 10 min.

Mitochondrial Membrane Potential Assay

Changes in mitochondrial membrane potential (MMP) were measured in the presence of 0.2 mM rhodamine 123 (Sigma-Aldrich, St. Louis, MO) as described by Emaus et al. [22]. The isolated mitochondria (0.5 mg protein/mL) were incubated in the assay buffer (225 mmol/L mannitol, 70 mmol/L sucrose, 5 mmol/L HEPES, pH 7.2). The discharge of Rh123 was induced by CaCl₂ (40 μmol/L). The fluorescence intensity was measured using a fluorospectrophotometer (Shanghai instrument analysis instrument, China) at 503 nm excitation and 525 nm emission wavelengths.

Western Blotting

For western blotting analysis, equal amount of proteins (40 μg) was loaded onto SDS–polyacrylamide gel for separation and electrotransferred onto nitrocellulose membranes. The membranes were incubated with anti-cytochrome *c* antibody, anti-Cox-IV (Cell Signaling Technology), or anti-β-actin antibody (Abcam, Cambridge, MA). After incubation with HRP-conjugated secondary antibodies, specific protein bands on the blots were visualized with a chemiluminescence detection system.

Terminal-deoxynucleotidyl Transferase-Mediated Nick End Labeling (TUNEL) Assay

TUNEL assay was conducted with a commercial kit (Kangwei biotechnology co. LTD, Beijing, China). TUNEL-positive cells were counted below five non-continuous high-power fields ($\times 400$) in each group. The percentage of TUNEL-positive cells was calculated.

Quantitative Real-time PCR (qRT-PCR)

The total RNA was isolated using Trizol (Invitrogen) and reverse transcribed into cDNA using a PrimeScript RT reagent Kit (TaKaRa). The cDNA was subjected to the Light Cycler[®] 480 (Roche) using SYBR Fast qPCR Mix (TaKaRa). Gene expression was normalized to GAPDH. All primers used in this study are listed in Table 1.

Statistical Analysis

Data were analyzed with SPSS 13.0 software and expressed as mean \pm standard deviation (SD). The nature of hypothesis testing was two tailed. For comparing the parameters (tidal volume, lung compliance, and PaO₂) between groups during 90 min of observation, a two-way analysis of variance (ANOVA) for repeated measurements was used followed by the post hoc least significant difference test or Dunnett's *t* test, treat I/R as a control, and compared all other groups against it. The difference was considered significant if $P < 0.05$ was observed.

Results

MB Improved LIRI-Induced Changes in Pulmonary Parameters

The IL-2-type lung isolation system can monitor the pulmonary physiology and mechanics in real time. We first evaluated the potential protective effects of MB on LIRI-induced pulmonary dysfunction. At baseline, the parameters had no significant differences among the groups. In this ex vivo ischemia–reperfusion rat model, lungs in the I/R group showed a significant decrease in V_T , C_{dyn} , and PaO₂

and increase in R_{aw} after 30 and 60 min of reperfusion compared to the sham group. In contrast, the lungs in the I/R with MB or GSH treated groups showed a significant increase in V_T , C_{dyn} , and PaO₂ and lower R_{aw} (Table 2).

MB Attenuated LIRI-induced Pulmonary Injury

To further explore the beneficial effects of MB on IR-induced pulmonary injury, we investigated the pulmonary morphology, wet/dry ratio, and the activity of LDH in the perfusate. As shown in Fig. 2b, there were severe perivascular edema and intra-alveolar hemorrhage in the I/R group, whereas MB and GSH decreased histological damages (Fig. 2c, d). Additionally, the pulmonary wet/dry ratio significantly decreased in I/R + MB group and I/R + GSH group compared to that in the I/R group, indicating that the lungs treated with MB or GSH could reduce LIRI-induced pulmonary edema (Fig. 2e). Furthermore, MB and GSH significantly diminished the IR injury induced release of LDH in lung tissues (Fig. 2f).

MB Protected Against LIRI-Induced Oxidative Stress

Considering that excess ROS has key destructive effects on LIRI, we then examined whether MB could inhibit LIRI-induced oxidative stress. As shown in Fig. 3a, c, ROS production and MDA concentration significantly increased in I/R group when compared with that in the sham group. Interestingly, MB or GSH treatment markedly reduced the production of ROS and MDA content. Moreover, MB or GSH ameliorated the decrease in SOD activity induced by LIRI (Fig. 3b). These results indicated that MB and GSH alleviated LIRI-induced oxidative stress.

MB Attenuated LIRI-induced Mitochondrial Dysfunction

To define the role of MB in improving mitochondrial functions, we isolated the mitochondria and evaluated their function. The decrease of absorbance at 540 nm indicated that mitochondrial swelling induced by I/R was inhibited by treatment of MB or GSH (Fig. 4a). Similarly, MMP in the I/R group was significantly lower than that in the sham

Table 1 Primers for real-time PCR

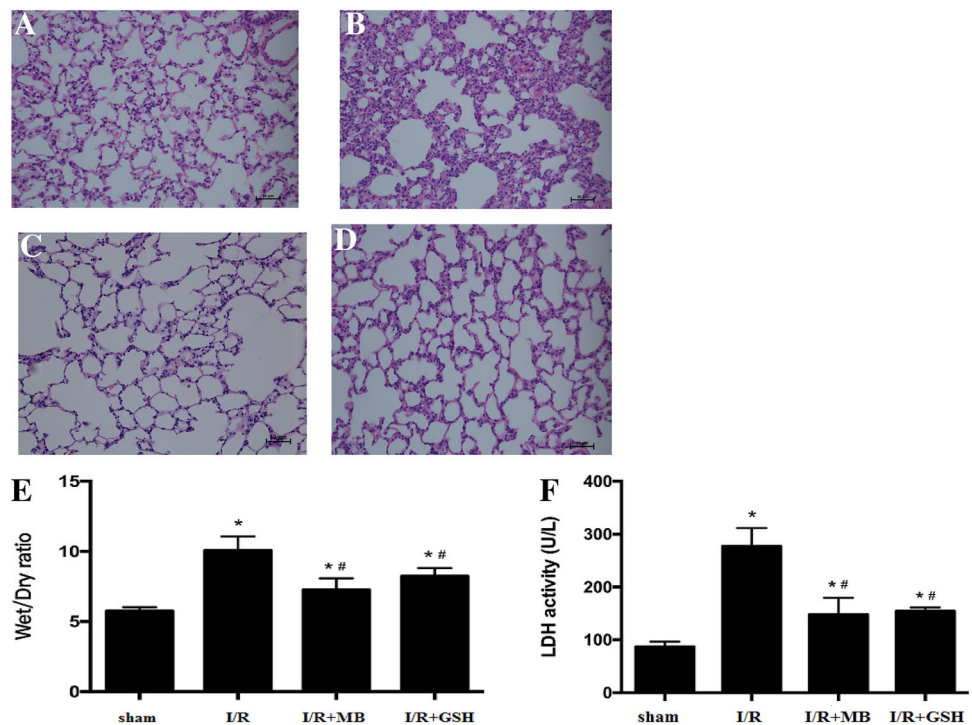
Genes	Forward	Reverse
TNF- α	5'-GGTGATCGGTCCCAACAAGGA-3'	5'-CACGCTGGCTCAGCCACTC -3'
IL-1 β	5'-CACGCTGGCTCAGCCACTC -3'	5'-GGGTTCCATGGTGAAGTCAAC-3'
IL-6	5'-GGGTTCCATGGTGAAGTCAAC-3'	5'-GGTCCTTAGCCACTCCTTCTGT-3'
IL-18	5'-ATATCGACCGAACAGCCAAC-3'	5'-ATATCGACCGAACAGCCAAC-3'
GAPDH	5'-GACATGCCGCTGGAGAAAC-3'	5'-GACATGCCGCTGGAGAAAC-3'

Table 2 Lung parameters at different time (mean \pm SD, $n = 6$)

Items	Groups	Baseline	Reperfusion		
			0 min	30 min	60 min
C_{dyn} (mL/cmH ₂ O)	Sham	0.47 \pm 0.04	0.40 \pm 0.05	0.35 \pm 0.06	0.33 \pm 0.04
	I/R	0.47 \pm 0.05	0.28 \pm 0.09*	0.24 \pm 0.06*	0.13 \pm 0.03*
	I/R + MB	0.51 \pm 0.10	0.35 \pm 0.09	0.36 \pm 0.05 [#]	0.32 \pm 0.09 [#]
	I/R + GSH	0.50 \pm 0.05	0.33 \pm 0.12	0.37 \pm 0.06 [#]	0.33 \pm 0.05 [#]
V_T (mL)	Sham	2.05 \pm 0.28	1.83 \pm 0.21	1.62 \pm 0.25	1.47 \pm 0.23
	I/R	2.07 \pm 0.26	1.28 \pm 0.39*	1.17 \pm 0.21*	0.63 \pm 0.19*
	I/R + MB	2.20 \pm 0.30	1.47 \pm 0.25*	1.53 \pm 0.20 [#]	1.40 \pm 0.25 [#]
	I/R + GSH	2.13 \pm 0.21	1.30 \pm 0.17*	1.47 \pm 0.22 [#]	1.27 \pm 0.21 [#]
R_{aw} (cm H ₂ O/mL/s)	Sham	0.26 \pm 0.01	0.27 \pm 0.01	0.27 \pm 0.01	0.29 \pm 0.02
	I/R	0.27 \pm 0.02	0.29 \pm 0.04	0.35 \pm 0.05*	0.55 \pm 0.04*
	I/R + MB	0.25 \pm 0.01	0.26 \pm 0.01	0.26 \pm 0.01 [#]	0.28 \pm 0.03 [#]
	I/R + GSH	0.25 \pm 0.01	0.26 \pm 0.01	0.26 \pm 0.01 [#]	0.28 \pm 0.06 [#]
PaO ₂ (mm Hg)	Sham	90 \pm 10	84 \pm 14	86 \pm 10	70 \pm 10
	I/R	88 \pm 11	50 \pm 14*	61 \pm 12*	63 \pm 23*
	I/R + MB	89 \pm 10	69 \pm 14* [#]	81 \pm 14* [#]	68 \pm 14 [#]
	I/R + GSH	88 \pm 12	59 \pm 16* [#]	75 \pm 13* [#]	62 \pm 12* [#]

* $P < 0.05$ versus Sham group, [#] $P < 0.05$ versus I/R group

Fig. 2 Effects of MB on the pulmonary morphology, wet/dry ratio, and the content of LDH in the perfusates of the different groups. **a–d** Histological observation of pulmonary injury. Typical images were chosen from each experimental group (original magnification $\times 200$); **a** sham group; **b** I/R group; **c** I/R + MB group; and **d** I/R + GSH group. **e** wet/dry ratio. **f** The content of LDH in the perfusate. All values are expressed as mean \pm SD. The experiment was repeated three times ($n = 6$ for each group). * $P < 0.05$ versus the sham group, [#] $P < 0.05$ versus I/R group



group, whereas there was a significant increase in MMP in the I/R + MB group and the I/R + GSH group (Fig. 4b). To further define the role of MB in improving mitochondrial functions, we examined ATP concentrations in the pulmonary tissues. Consistent with the aforementioned data, the results showed that ATP concentration was markedly reduced in I/R group when compared with that in

the sham group. Importantly, MB or GSH treatment apparently increased ATP production (Fig. 4c).

Fig. 3 Effects of MB on the production of ROS (a), SOD activity (b), and the MDA concentration (c) in lung tissues. All values are expressed as mean \pm SD. The experiment was repeated three times; ($n = 6$ for each group). * $P < 0.05$ versus sham group, # $P < 0.05$ versus I/R group

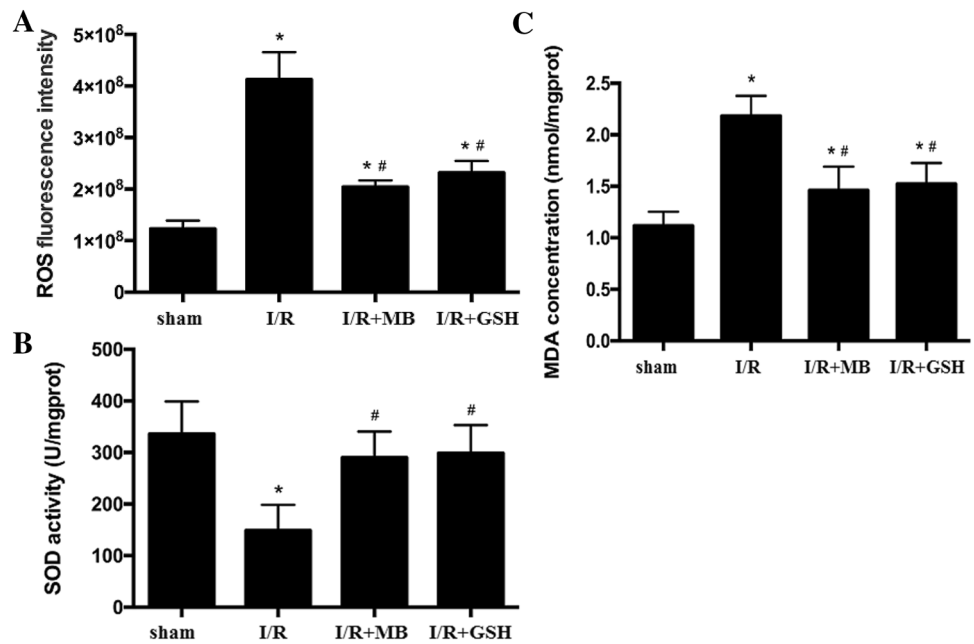
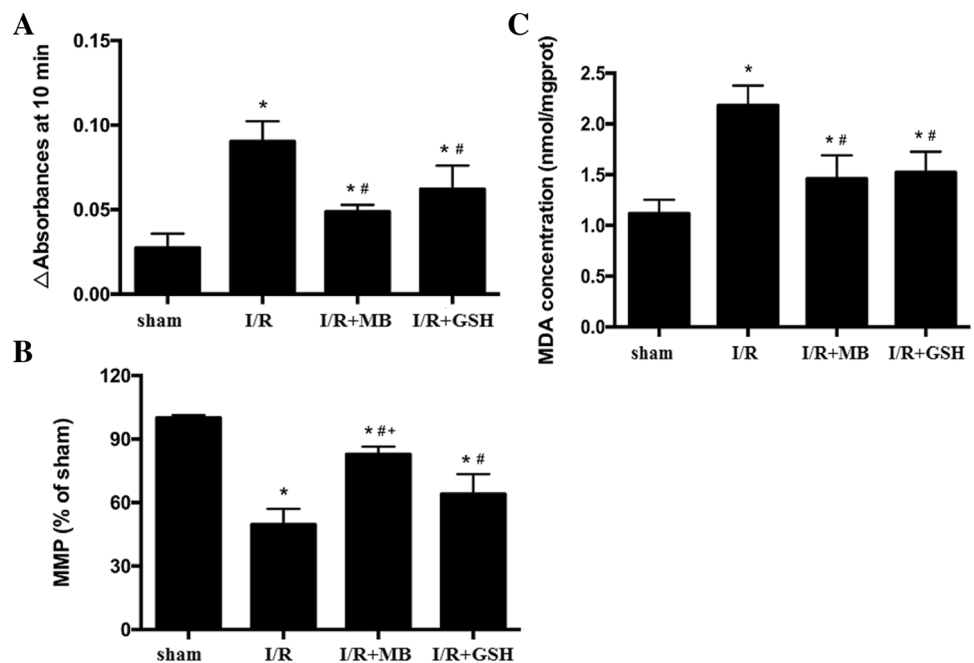


Fig. 4 Effects of MB on the opening of MPTP (a); mitochondrial membrane potential (b); and ATP production (c). All values are expressed as mean \pm SD. The experiment was repeated three times ($n = 6$ for each group). * $P < 0.05$ versus sham group, # $P < 0.05$ versus I/R group, + $P < 0.05$ versus I/R + GSH group



MB Blocked Cytochrome *c* Release From the Mitochondria into the Cytosol and Alleviated Apoptosis

It is well accepted that apoptosis plays a causal role in LIRI. Therefore, we evaluated whether the protective properties of MB against LIRI involved inhibition of apoptosis. To analyze LIRI-induced apoptotic cells, pulmonary sections were subjected to TUNEL staining, and the apoptosis indices were calculated. As shown in Fig. 5a–d, most of the pulmonary epithelial cells were positive for

TUNEL staining in the I/R group. However, the numbers of TUNEL-positive cells were significantly decreased in the I/R + MB group and the I/R + GSH group (Fig. 5e). The results of western blotting showed that the release of cytochrome *c* from the mitochondria into the cytosol was increased in the I/R group, whereas MB or GSH treatment inhibited this release (Fig. 5f). These data showed that MB could inhibit LIRI-induced apoptosis.

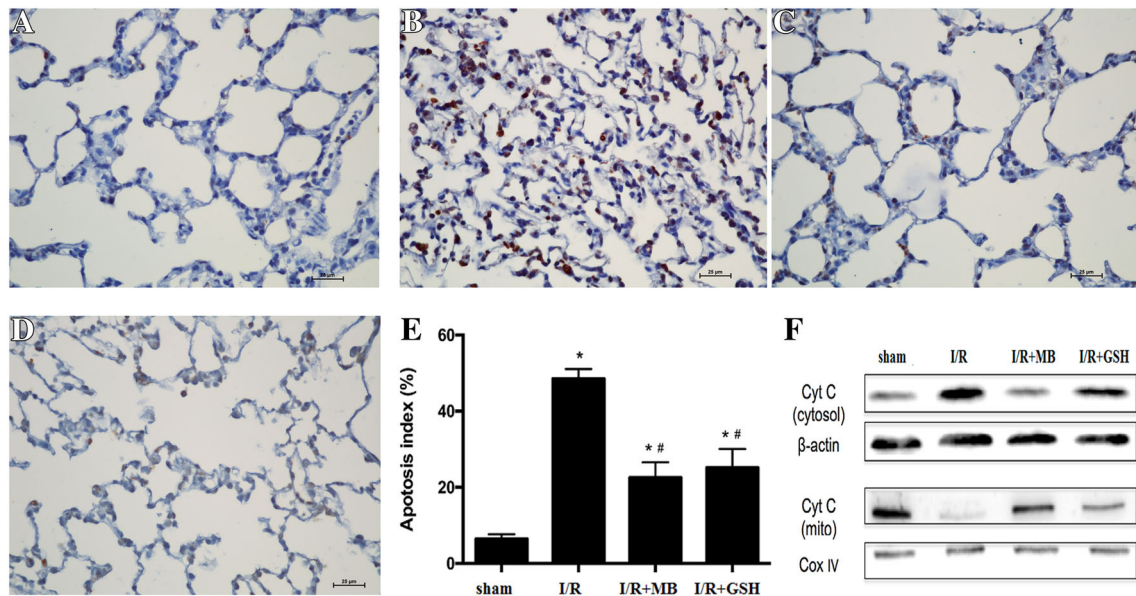


Fig. 5 Effects of MB on apoptosis in lung tissues. (a–d) Histological images of TUNEL-stained cells. e The apoptosis index. All values are expressed as mean ± SD (*n* = 6 for each group). **P* < 0.05 versus sham group, #*P* < 0.05 versus I/R group. f The content of cytochrome

c in the mitochondria and cytosol was assessed by western blotting. β-actin and Cox-IV were used as a protein loading control. The experiment was repeated three times; representative results of three independent experiments were shown

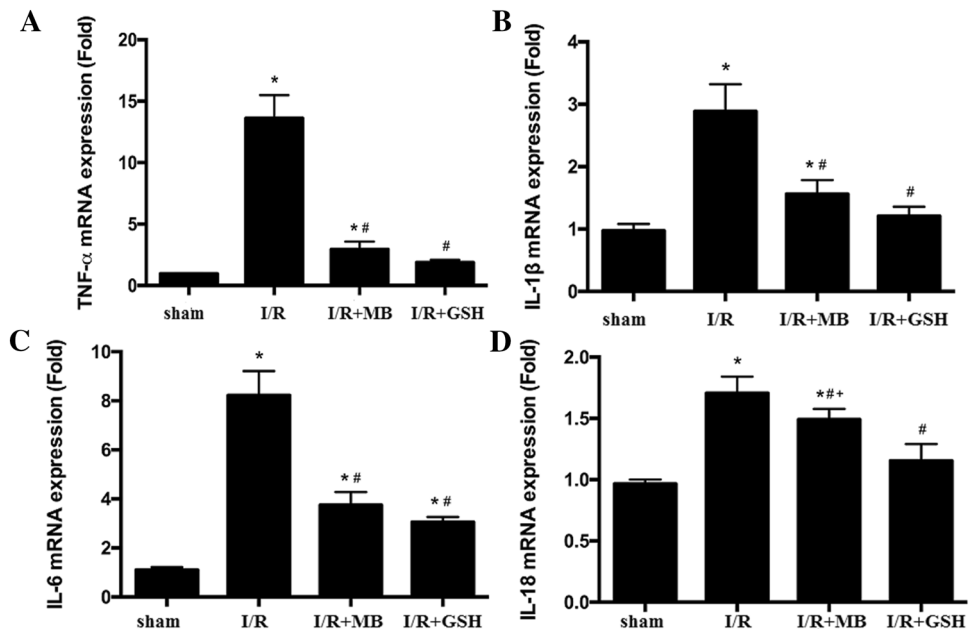
MB Downregulated the mRNA Expression Levels of Pro-inflammatory Cytokines Induced by LIRI

qRT-PCR results indicated that the mRNA expression levels of TNF-α, IL-1β, IL-6, and IL-18 were significantly elevated in the I/R group compared to those in the sham group. MB treatment downregulated the mRNA expression levels of TNF-α, IL-1β, IL-6, and IL-18 (Fig. 6).

Discussion

In the present study, we demonstrated for the first time that MB could protect the isolated rat lungs from LIRI, as confirmed by pulmonary function parameters, pulmonary morphology, wet/dry ratio, and LDH activity. Considering that the mitochondrion is an important target of MB, to identify the potential mechanism by which MB inhibited LIRI, we focused on the effect of MB on mitochondrial

Fig. 6 Effects of MB on the gene expression levels of pro-inflammatory cytokines including TNF-α (a), IL-1β (b), IL-6 (c), and IL-18 (d) in lung tissues. All values are expressed as mean ± SD. The experiment was repeated three times (*n* = 6 for each group). **P* < 0.05 versus sham group, #*P* < 0.05 versus I/R group, +*P* < 0.05 versus I/R + GSH group



functions. We found that MB treatment significantly attenuated LIRI-induced decrease in SOD activity and ATP generation and increase in ROS production and MDA concentration. We also found that MB preserved MMP and inhibited mitochondrial swelling. Concomitantly, lungs from MB-treated rats showed less cytochrome *c* release into the cytosol and decreased cell apoptosis rate. In addition, treatment with MB significantly down regulated the expression levels of the pro-inflammatory cytokines. Taken together, these findings strongly support the protective effects of MB on the isolated rat lungs affected by LIRI through its anti-oxidative, anti-apoptotic, and anti-inflammatory effects.

Isolated lung perfusion is a powerful experimental tool to research the treatment of acute lung injury (ALI). Many kinds of ALI animal models can be easily and rapidly developed using this approach [23]. Moreover, these models can be used to assess the histological characterization and inflammatory cytokine content in pulmonary tissues [24]. Most importantly, the model of isolated lung perfusion is a convenient tool to monitor the physiological indices such as V_T , C_{dyn} , and PaO_2 and R_{aw} in real time. V_T is the volume of gas inspired or expired during each respiratory cycle; C_{dyn} is an index of functional stiffness of the lung and reflects changes to small airways; R_{aw} is defined as the pressure difference required for a unit flow change. In this study, we successfully established the isolated LIRI model on the IL-2-type perfused lung isolation system, as evidenced by abnormal lung function, pulmonary morphology, increased wet/dry ratio, and LDH content in the perfusion. Interestingly, MB could attenuate these abnormal conditions.

In this study, GSH was selected to serve as a positive control drug. Although the protective effect of GSH on LIRI was comparable to that of MB, the very short half-life of GSH [25] and its insufficiency to rapidly cross the lipid-rich cell membrane [26] hinder its application to treat oxidative stress-associated lung diseases. MB has some unique advantages over GSH to treat LIRI. First, MB is water soluble and can rapidly cross the cell membrane [27, 28]. Second, MB has positive features such as universal availability, cost-effectiveness, and safety. Finally, MB as an FDA-approved agent has been used clinically for over 130 years. We found that MB treatment markedly improved lung function, lessened pulmonary damage, enhanced mitochondrial function, and inhibited apoptosis. These results implied that MB may be a potential agent to treat LIRI.

Mitochondrial damage is a central event in the development of IR injury [1, 5]. Recent investigations found that MB enhanced mitochondrial respiration at low

concentrations (0.5–2 mM) by shuttling electrons to oxygen in the electron transport chain [9, 15, 28]. Furthermore, MB treatment could attenuate IR injury such as stroke [12] and other brain injuries [13], and those to the kidney [14] and the gut [15]. In this study, we further found that MB treatment reduced the production of ROS and the content of MDA and enhanced the activity of SOD. We also found that MB preserved MMP and blocked the opening of MPTP. These results demonstrated the beneficial effect of MB on mitochondrial function.

Excess apoptosis always contributes to IR injury [29]. The generation of ROS combined with calcium overload lead to the opening of MPTP [30]. The opening of MPTP and the rupture of the outer membrane cause cytochrome *c* release from mitochondria to cytosol. We found that MB blocked cytochrome *c* release into the cytosol and alleviated apoptosis. This result showed the anti-apoptotic effect of MB on LIRI.

It is well known that LIRI triggers an intense inflammatory response accompanied with increased levels of pro-inflammatory cytokines [31]. Pro-inflammatory cytokines cause long-term damage in the infarcted tissue [32]. Therefore, suppression of inflammatory response might be a promising therapy for controlling organ injury [33, 34]. Consistent with Fenn's finding that MB attenuated inflammation in traumatic brain [35], we observed down-regulation of the mRNA levels of pro-inflammatory cytokines after MB treatment.

Our study has some limitations. First, although the *ex vivo* lung perfusion has many advantages in lung research, we did not use the *in vivo* IR injury model to test the protective role of MB on LIRI. Second, we only investigated the protective effect of MB before LIRI. Future studies should investigate the beneficial effect of MB after LIRI.

In conclusion, the present results indicate that MB protects the isolated rat lungs from IR injury and that the potential mechanisms of this protective effect may be associated with the restoration of mitochondrial function and the suppression of inflammatory response and apoptosis. Therefore, our findings suggest that application of MB may have a curative effect on LIRI.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval This research involved rats. The study was approved by the Animal Care and Use Committee of Jiangnan University.

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