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Protective effect of a polyphenols-rich extract from *Inonotus Sanghuang* on bleomycin-induced acute lung injury in mice

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ABSTRACT

Mushroom *Phellinus linteus* ("*Sanghuang*" in Chinese) is a popular medicinal polypore used to treat several disorders through its various biological functions. *Inonotus sanghuang* is claimed to produce general immune-potentiating and strengthening, anti-inflammatory, anti-tumor and anti-microbial properties, but its effect on acute lung inflammation and oxidative stress are not clearly understood. To determine the effect and mechanism of the polyphenols-rich ethyl acetate fraction from wild *I. sanghuang* extract (ISE) on acute lung injury (ALI) induced by bleomycin (BLM), female C57BL/6 mice were fed ISE (0%, 0.15% or 0.6% in diet) for 4 weeks prior to challenge with BLM. Bronchoalveolar lavage fluid (BALF) from lung, spleen and lung tissues were collected on day 3 after BLM challenge for histological, oxidative stress, molecular and biochemical analysis. ISE supplementation improved pathological features in lung injury scores and reduced lung wet-to-dry ratios. Moreover, ISE reduced inflammatory cell infiltration and the pro-inflammatory cytokines including IL-1β, IL-6 and TNF-α in BALF, decreased the MPO activity and the MDA level and increased the SOD, CAT and GSH-Px activities in lung tissue homogenates. Further mechanism analysis demonstrated that dietary ISE inhibited NF-κB signal. Finally, peripheral immune function analysis showed that ISE had less effect on immune response including splenocyte producing inflammatory cytokines and T cell proliferation except for IL-1β and IL-2. Our findings indicate the possibility that dietary ISE attenuates ALI induced by BLM through correcting the inflammation and oxidation balance at least in part via inhibiting NF-κB signal *in vivo*, suggesting that ISE might be a valuable medicinal food effective in improving lung injury.

1. Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) consist of acute hypoxic respiratory failure combined with bilateral lung infiltration, which are associated with pulmonary and non-pulmonary risk factors and are not primarily caused by left atrial hypertension [1]. Although advances in supportive treatment and mechanical ventilation strategies, there is still higher mortality of approximately 40% [2,3]. Several drugs have been developed to treat this condition, but there is < 50% for five-year survival rate and the serious adverse effects are found during long-term treatment. Therefore, for these reasons, phytochemicals from traditional Chinese medicines, fruits and vegetables have emerged as attractive alternatives for the prevention and/or treatment of ALI and ARDS.

Although the mechanisms underlying the development of ALI and ARDS is still unclear, inflammation and reactive oxygen species (ROS) have been long proposed to be mediators of ALI and ARDS [4–6]. During the first, or exudative phase of ALI, the lung's initial response to injury is characterized by the lung inflammation secreted by activated resident alveolar macrophages, permeability of the alveolar-capillary barriers mediated by innate immune-cells and accumulation of protein-rich fluids in the interstitium. Lung inflammatory cytokines continue to induce the recruitment of neutrophils and monocytes or macrophages, as well as activation of alveolar epithelial cells and T cells that further

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contribute to inflammation and tissue injury [7]. The epithelial repair processes occur during the second, or proliferative, phase of ALI when lung edema is reabsorbed and the provisional matrix restores alveolar function. Finally, the final, or fibrotic, phase of ALI does not occur in all patients but is strongly associated with the need for prolonged mechanical ventilation and increased mortality [6].

Bleomycin (BLM), a mixture of glycopeptides derived from *Strepromyces verticillus*, is a useful drug in chemotherapy and is well known to have the toxicity such as pulmonary fibrosis in humans as well as in BLM-induced animal models. Although BLM-induced models are usually considered a model of pulmonary fibrosis, its treatment is also associated with acute inflammation and oxidative stress of ALI [8,9]. Early inflammation stage of ALI has been proved using BLM-induced murine models due to its low complexicity and high reproducibility [8]. In mice, in the early inflammation stage of lung injury, infiltrating inflammatory cells including macrophages, neutrophils and lymphocytes are recruited in the alveoli by BLM, of which neutrophils and macrophages release pro-inflammatory cytokines IL-1β, TNF-α and IL-6 and ROS, and then lead to ALI within a week [5,10]. Thus, identifying and characterizing new drugs to target inflammation and ROS will be important to prevent and/or treat ALI and ARDS.

Diet or phytochemical derived from a variety of dietary components, as a modifiable environmental factor, has been known to be able to modulate the inflammation, ROS and immune response [11]. Mushroom *Phellinus linteus* ("*Sanghuang*" in Chinese) is a popular medicinal polypore used throughout China, Japan and Korea [12] and has been reported to have benefits for the improvement of blood circulation, relief of stomachaches, anti-inflammation, anti-tumors, anti-diabetes and pneumonia [13,14]. Ethanol extract of *Inonotus sanghuang* (*I. sanghuang*) mycelia produced from liquid fermentation has been proved to have antioxidant activity due to the existing phytochemicals (polyphenols) such as rutin, eriodictyol, naringenin and sakuranetin [15]. Our recent *in vitro* study have shown that the anti-oxidant, anti-proliferative, anti-inflammation and anti-microbial activities have been found in *I. sanghuang* exact from another *sanghuang* species, wild *I. sanghuang* from the Aershan Region of Inner Mongolia (Inner Mongolia, China) [16,17]. In addition, the mycelium of *sanghuangporus* ethanol extract mainly consists of polyphenolic compounds that exhibits its protective effect on LPS-induced ALI in mice via anti-inflammation and anti-oxidation [18]. However, there are no studies exploring the anti-inflammatory and immuno-modulating properties of wild *I. sanghuang* from the Aershan Region of Inner Mongolia and its use for the prevention/treatment of inflammation-related diseases.

Thus, the aim of this study was to determine whether wild *I. sanghuang* extract could attenuate lung injury induced by BLM in mice, as well as explore a possible mechanism of this action.

2. Materials and methods

2.1. Identification of I. sanghuang extract polyphenols

I. sanghuang was collected from the Aershan Region of Inner Mongolia (Inner Mongolia, China) and ethyl acetate fraction (EAF) of *I. sanghuang* powder was prepared as previously described [16]. We characterized polyphenols in this fraction and identified 6 compounds, namely rutin, quercetin, quercitrin, icarisid II, isorhamnetin and chlorogenic acid by comparing their retention times monitored at 280 nm (HPLC-DAD) and in total ion chromatogram mode (LC-MS-IT-TOF) to authenticated standards as previously described [16]. Thus, we use EAF as *I. sanghuang* extract (ISE) for this study. Concentrated ISE was stored at −20 °C until further use.

2.2. Animals

Female C57BL/6 mice (6–8 weeks of age, 18–20 g) were provided by Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). Mice were housed three to four per cage in a controlled environment with 12:12 h light and dark cycle. They were provided free access to pathogen-free water and food. All experimental procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals by the National Institute of Health and approved by the Institutional Animal Care and Use Committee of Huaihe Hospital at Henan University (Ethics code: HHYY-2017006).

2.3. Dietary ISE supplementation

Mice were randomly divided into four groups (n = 24 mice/group) and then consumed either normal diet or the mixed diet with 0.15% or 0.6% ISE (w/w) for 4 weeks before BLM challenge and continued to consume the same diet after BLM instillation until the end of this study. After 4 weeks of feeding, mice were anaesthetized with an intramuscular injection of a ketamine (60 mg/kg)/xylazine (1.25 mg/kg) solution, followed by intratracheal instillation of BLM at 2.5 mg/kg in 50 μl of saline solution. The control mice received intratracheal saline. All mice were euthanized with CO_2 on day 3 after BLM instillation, and the tissue samples were collected for further analysis.

2.4. Histopathological evaluation of lung

Mice were euthanized on day 3 after BLM instillation and perfused with cold PBS via intracardiac route. The left lung was harvested, fixed with paraformaldehyde, and cross-sections were stained with hematoxylin and eosin (H&E) for histological lung injury as previously described [19].

2.5. Lung wet/dry weight ratio

To evaluate the formation of lung edema, the wet/dry weight ratio of left lung was determined on day 3 after BLM administration as previously described [19].

2.6. Bronchoalveolar lavage fluid (BALF) collection, cytokines and cell counting

On day 3 after BLM administration, mice were euthanized to collect BALF that was used to measure total protein levels (mg/ml in BALF), IL-1β (R&D systems, Minneapolis, MN), IL-6 and TNF-α kits (both from eBioscience, San Diego, CA) concentrations as previously described [19]. After the collected BALF was centrifuged, the cell-free supernatants were used to determine these cytokine concentrations and the cell precipitation was re-suspended in PBS solution to evaluate total infiltrating cells and cell compositions as previously described [19].

2.7. Determination of MPO activity

On day 3 after BLM administration, right lung tissues were harvested to obtain the homogenates. The supernatants from lung homogenates were prepared as previously described [19] to determine the MPO activity using MPO assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), following the manufacturer's instructions.

2.8. Evaluation of SOD, CAT, GSH-PX and MDA

On day 3 after BLM administration, right lung tissues were harvested to obtain the homogenates. The supernatants from lung homogenates were prepared and used to measure the SOD, CAT, GSH-Px activity and the MDA level as previously described [19].

2.9. Western blot analysis

Lung tissues were homogenized in RIPA lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 1 × protease

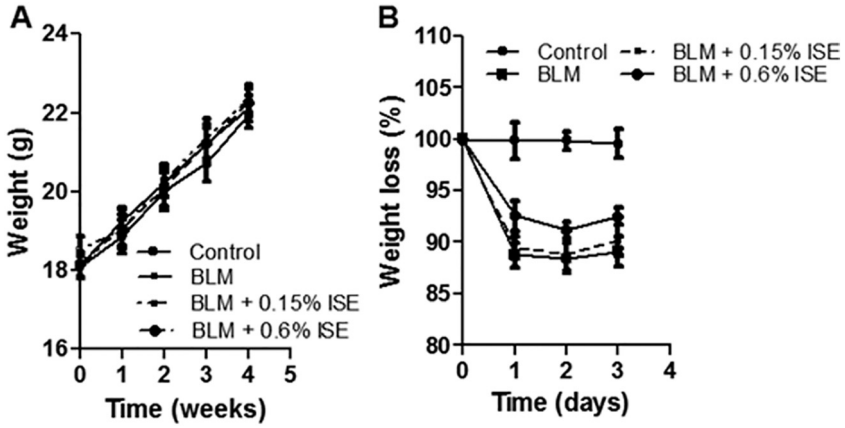

Fig. 1. Body weight change of mice fed ISE (A) before BLM instillation and the percentage of weight loss (B) after BLM instillation. Values are expressed as the mean ± SEM (n = 12 mice/group). ISE, *I. sanghuang* extract; BLM, bleomycin.

inhibitor cocktail (Roche Applied Science, Indianapolis, IN), and 1 × phosphatase inhibitor cocktail (Sigma-Aldrich). Total lung protein extract was resolved in 7.5% acrylamide gels and then transferred to nitrocellulose membranes. The membrane was blocked with 5% non-fat milk in Tris-buffered saline before being incubated, respectively with specific primary antibodies for the following proteins: phospho-IκB-α (1:1000), phospho-p44/p42 (Thr202/Tyr204) (p-Erk1/2) (1:1000), phospho-p38 (Thr202/Tyr204) (p-p38) (1:1000), and phospho-SAPK/JNK (Thr183/Tyr185) (p-Jnk) (1;1000), IκB-α (1:1000), Erk1/2 (1:1000), Jnk1/2 (1:1000), p38 (1:1000), (all from Cell Signaling Technologies, Danvers, MA), and β-actin (1:5000) (Sigma-Aldrich). The membranes were next incubated with horseradish peroxides (HRP)-conjugated secondary antibodies followed by exposure to enhanced chemiluminescent reagents (Millipore, Burlington, MA).

2.10. Splenocyte composition analysis

After mice were sacrificed on day 3 after BLM challenge, spleen was aseptically removed and single-cell suspensions were prepared to evaluate splenocyte composition using flow cytometry as described in "*Flow cytometry*" section.

2.11. Splenocyte proliferation

After single spleen cell in 24-well culture plates (2×10^6/well) was cultured in the presence of ConA (Sigma-Aldrich) at 1.5 μg/ml for 72 h, T cell proliferation was determined using flow cytometry as described in "*Flow cytometry*" section.

2.12. Cytokine production

After splenocytes in 24-well culture plates (2×10^6/well) was cultured in the presence of lipopolysaccharide (LPS) (1.0 μg/ml) for 24 h for production of inflammatory cytokines IL-1β, IL-6 and TNF-α or in the presence of ConA at 1.5 μg/ml for 72 h for production of T cell cytokines IL-2, IL-4 and IFN-γ. The cell-free supernatants were harvested to access IL-4, IL-6, TNF-α (all kits from eBioscience), IL-2 and IFN-γ (both kits from BD Pharmingen) using ELISA kits following the manufacturer's instructions.

2.13. Flow cytometry

To determine the composition of splenocytes, splenocytes were blocked using anti-CD16/32 (Fc block, BD Pharmingen) and then multi-stained with fluorescence-conjugated anti-Gr-1, anti-F4/80, anti-CD19, and anti-CD3 Abs (all from eBioscience). In addition, T cell proliferation was measured with fluorescence-conjugated anti-CD3 and anti-Ki-67 (eBioscience) as previously described [19] after splenocytes were stimulated with ConA for 72 h. Data were acquired by BriCyte® E6 (Mindray, Shenzhen, China) and analyzed using FlowJo 10.0.

2.14. Statistical analysis

All data were expressed as the mean ± SEM. The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA) followed by Tukey's HSD *post-hoc* test for multiple comparisons using Prism 6.0 software. Significance was set at $P < 0.05$.

3. Results

3.1. Phenolic contents of ISE

To analyze the basic chemical composition of ISE, we estimated phenolic components from EAF of *I. sanghuang*. Total phenolic content estimated in EAF was 43 μg/mg (gallic acid equivalents/mg ISE). The calculated amount of phenolic delivered per mice per day, based on the average diet consumption of 3 g/day, were 19.35 and 77.4 μg for 0.15% and 0.6% ISE, respectively.

3.2. ISE does not affect body weight change and prevent weight loss induced by BLM

Mice were fed diet with different doses of ISE for 4 weeks and then treated by BLM to induce ALI while continuing their ISE consumption. Dietary ISE did not affect body weight change compared to control diet groups (**Fig. 1**A); while weight loss was observed after BLM administration, such effects were not reversed by ISE (Fig. 1B).

3.3. ISE attenuates BLM-induced lung injury

On day 3 after BLM administration, the saline injection mice presented normal lung structure (**Fig. 2**A and E). However, the lung of BLM-treated mice displayed significant inflammatory alterations that was characterized by extensive morphological damage, such as hemorrhage, interstitial edema, thickening of the alveolar wall and infiltration of neutrophils into the parenchyma and alveolar spaces of lung (Fig. 2B and E). While ISE supplementation for 4 weeks improved BLM-induced lung histopathological damage in a dose-dependent manner (Fig. 2C, D and E).

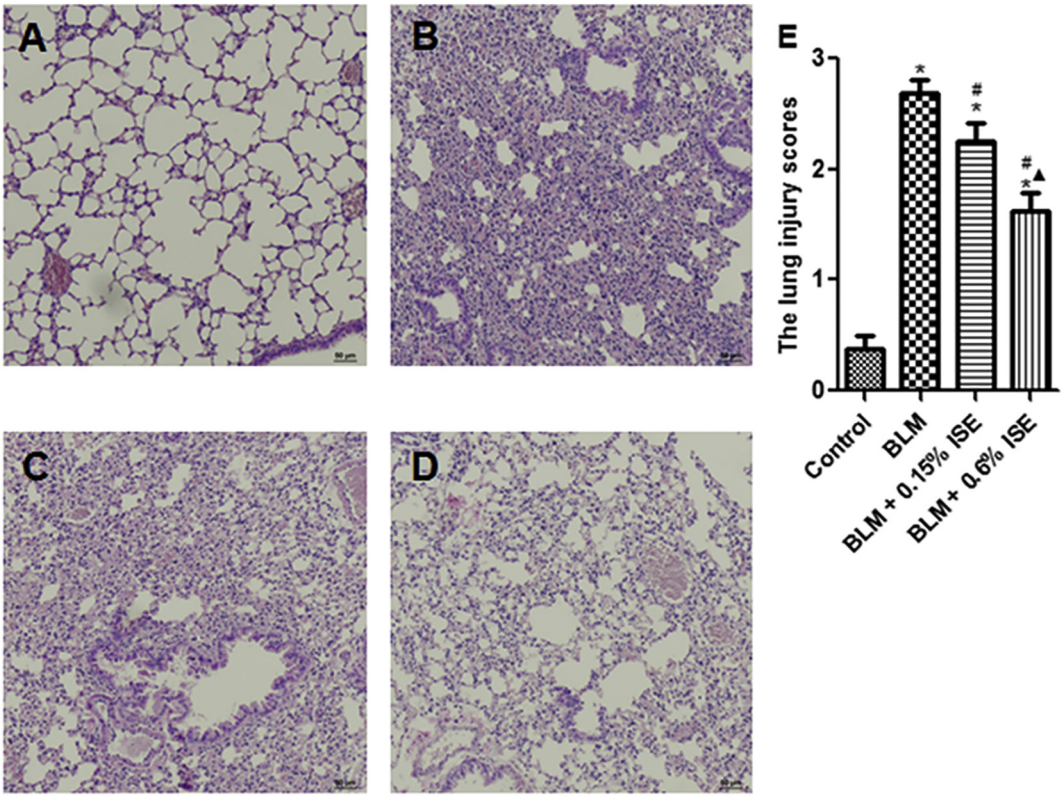

Fig. 2. Effects of ISE supplementation on BLM-induced lung injury (original magnification 100 X). Pictures are representative images of 12 mice per group (A to D) and lung mean injury scores were shown in (E). Values are expressed as the mean ± SEM (n = 12 mice/group). $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. BLM; $^{\blacktriangle}P < 0.05$ vs. BLM + 0.15% ISE. ISE, *I. sanghuang* extract; BLM, bleomycin.

3.4. ISE improves BLM-induced acute pulmonary edema and capillary permeability during inflammation

BLM administration induces an inflammatory response characterized by the accumulation of water in the lung. To assess the effect of ISE supplementation on lung edema, the lung W/D weight ratios were evaluated on day 3 after BLM administration. BLM treatment caused a notable increase of lung W/D ratios compared to control mice. Dietary ISE dose-dependently reduced the lung W/D ratios in mice treated by BLM (**Fig. 3**A). In addition, BLM administration caused a sharp rise of total proteins in BALF (Fig. 3B). While such rise was blocked by ISE treatment in a dose-dependent manner.

3.5. ISE decreases inflammatory cell infiltration and neutrophil activation in BLM-treated mice

As shown in Fig. 4, BLM administration increased the total infiltrating cells (Fig. 4A) and cell compositions including macrophages (Fig. 4B), neutrophils (Fig. 4C), and T cells (Fig. 4D) and such increase was attenuated by ISE supplementation. In addition, we explored the effect of ISE on the MPO activity (a marker of neutrophilic inflammation) in lung tissues. We found that the MPO activity in lung tissues was increased by BLM and this increase was suppressed by dietary ISE (Fig. 4E).

3.6. ISE decreases BALF inflammatory cytokine concentration in BLM-treated mice

As shown in Fig. 5, BLM increased the levels of IL-1β, IL-6 and TNF-α in BALF when compared with control group. While such increase was markedly reduced by ISE supplementation in a dose-dependent manner.

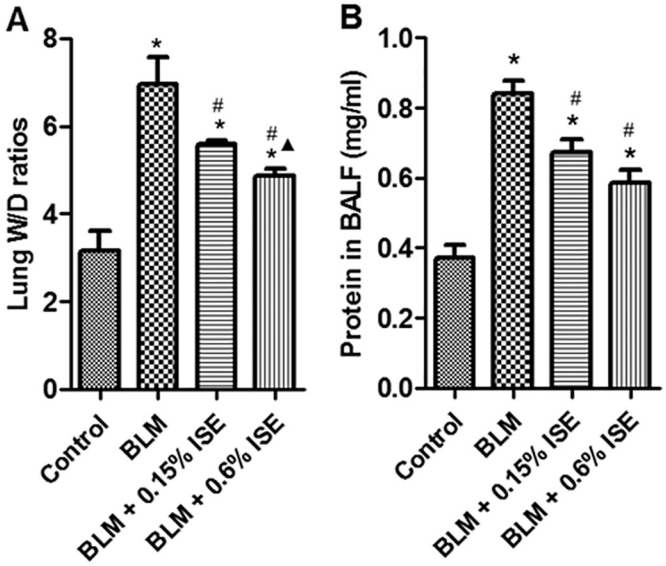

Fig. 3. Effects of ISE supplementation on the lung Wet/dry ratios (A) and the BALF protein levels (B) of BLM-treated mice. Values are expressed as the mean ± SEM (n = 12 mice/group). $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. BLM; $^{\blacktriangle}P < 0.05$ vs. BLM + 0.15% ISE. ISE, *I. sanghuang* extract; BLM, bleomycin.

3.7. ISE changes the oxidative stress balance in the lung of BLM-treated mice

The oxidative stress analysis showed that BLM treatment resulted in a significant decrease in antioxidant enzyme activity such as SOD (Fig. 6A), CAT (Fig. 6B) and GSH-Px (Fig. 6C) when compared with

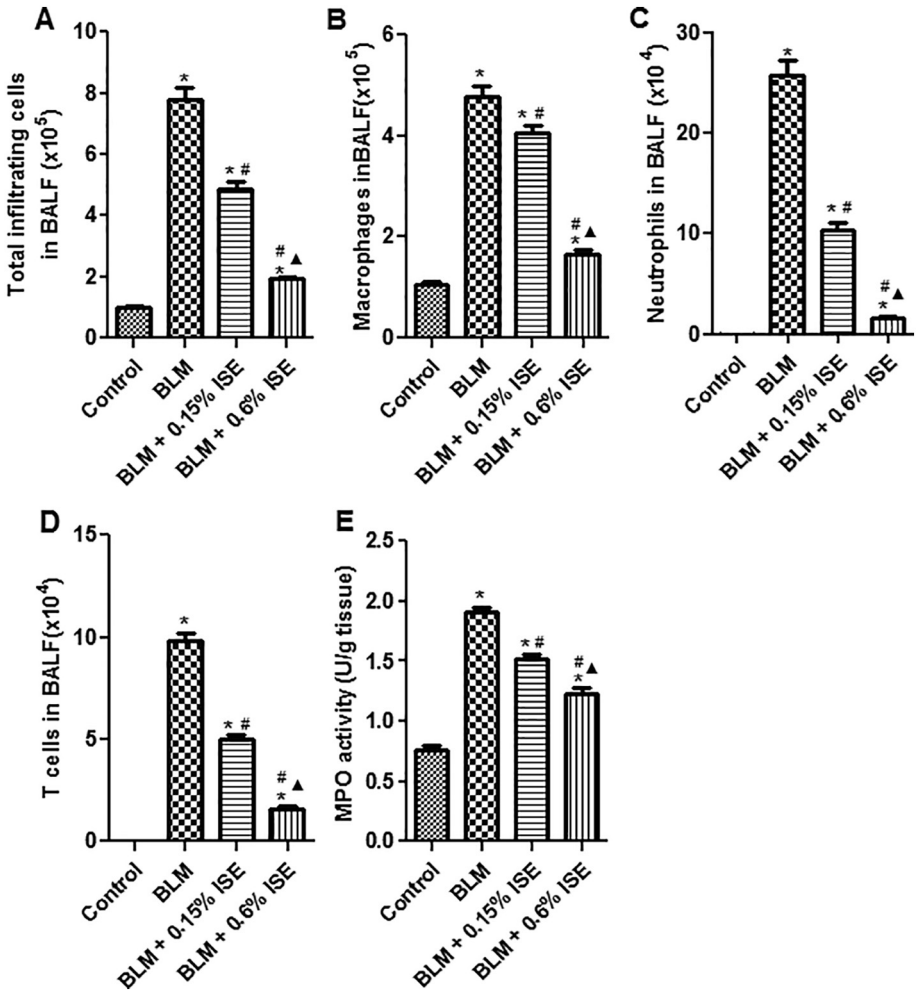

Fig. 4. Effects of ISE supplementation on inflammatory cell infiltration and neutrophilic inflammation in the BALF and lung tissues of BLM-treated mice, respectively. The total infiltrating cells (A) in the BALF were measured by trypan blue assay and the cell compositions including macrophages (F4/80, B), neutrophils (Gr-1, C) and T cells (CD3, D) in the BALF were determined using flow method. MPO activity (E) in lung homogenate was measured using MPO assay kit described in "*Materials and methods*" section. Values are expressed as the mean ± SEM ($n = 12$ mice/group). $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. BLM; $^{\blacktriangle}P < 0.05$ vs. BLM + 0.15% ISE. ISE, *I. sanghuang* extract; BLM, bleomycin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

control group. While treatment with ISE dose-dependently increased these antioxidant enzyme activities. Furthermore, BLM dramatically increased the MDA levels (Fig. 6D) and such rise was markedly blocked by ISE supplementation.

3.8. Anti-inflammatory effect of ISE is caused by the inhibition of NF-κB signal in BLM-treated mice

NF-κB and MAPK signals are the main pathways involved in inflammatory response [20]. To determine the possible molecular mechanism of anti-inflammatory effect of ISE, we assessed NF-κB and MAPK signaling pathways in the lung from BLM-treated mice.

NF-κB is mediating in the regulation of production of inflammatory mediators such as TNF-α and IL-6 that in turn activate MAPK- and NF-κB- dependent inflammatory pathways. As shown Fig. 7A, BLM challenge promoted the phosphorylation of IκBα and induced a reduction of IκBα level in lung tissues and ISE supplementation prevented IkBα's phosphorylation and degradation. However, BLM administration with

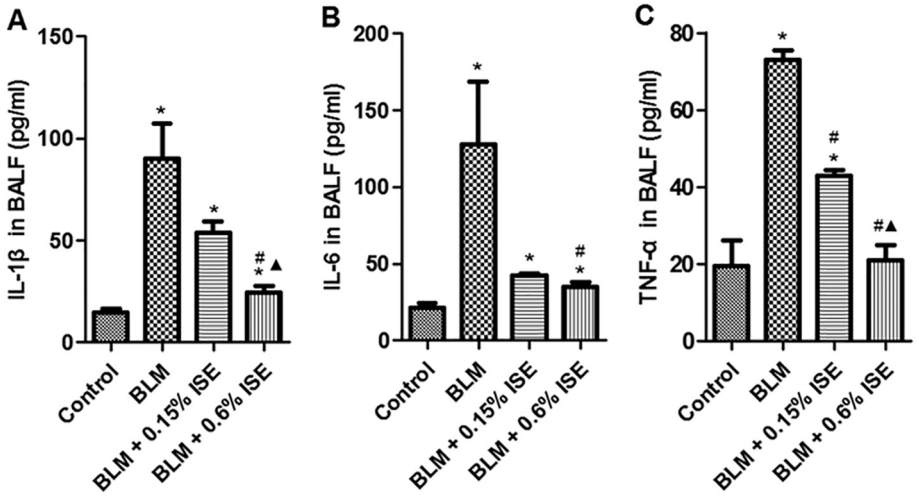

Fig. 5. Effects of ISE supplementation on inflammatory cytokine release in the BALF of BLM-treated mice. The levels of IL-1β (A), IL-6 (B) and TNF-α (C) in the BALF were measured using ELISA assay. Values are expressed as the mean ± SEM (n = 12 mice/group). $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. BLM; $^{\blacktriangle}P < 0.05$ vs. BLM + 0.15% ISE. ISE, *I. sanghuang* extract; BLM, bleomycin.

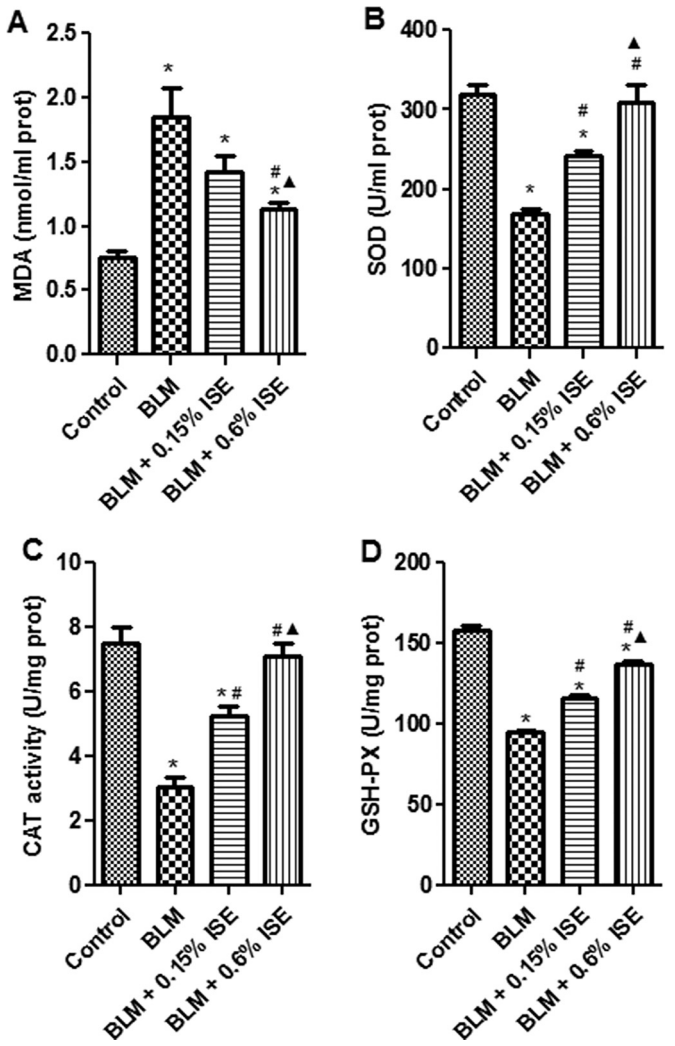

Fig. 6. Effects of ISE supplementation on the SOD, CAT and GSH-Px activities and the MDA level in the lung of BLM-treated mice. On day 3 after BLM treatment, right lung was collected and then homogenized to evaluate the SOD (A), CAT (B) and GSH-Px (C) activities and the MDA level (D). Values are expressed as the mean ± SEM (n = 12/group). $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. BLM; $^{\blacktriangle}P < 0.05$ vs. BLM + 0.15% ISE. ISE, *I. sanghuang* extract; BLM, bleomycin.

or without ISE did not change the Erk1/2, Jnk1/2, and p38 phosphorylation in lung tissues when compared with control group (Fig. 7B to D).

3.9. Effect of ISE on pro-inflammatory cytokines produced by splenocytes

Although dietary ISE dose-dependently attenuated BLM-induced ALI, it remains unclear whether ISE affects immune response from peripheral immune organs which may be involved in the pathogenesis of BLM-induced ALI. To address this, we first evaluated spleen cell compositions. No difference for total splenocytes (data not shown), neutrophils (Fig. 8A), macrophages (Fig. 8B), and B cells (Fig. 8C) was found across all groups.

Next, we cultured spleen cells with LPS to determine the production of pro-inflammatory cytokines. Splenocytes from BLM treated mice did produce less IL-1β compared to control mice (Fig. 8D) and this effect was further potentiated by ISE at 0.15%. However, no difference for IL-6 (Fig. 8E) and TNF-α (Fig. 8F) was found across all groups.

3.10. Effect of ISE on T cell proliferation and ex vivo production of T cell cytokines by splenocytes

BLM with or without ISE did not impact T cell population from splenocytes compared to control mice (Fig. 9A). However, whether T cell functions including T cell proliferation and T-cell secreting cytokine production could be affected is still unknown. Thus, in the ex vivo cell proliferation and cytokine production assays, splenocytes were stimulated with the optimal concentration of T cell mitogen ConA [21] to induce T cell proliferation and assess T cell producing cytokines. BLM treatment did not affect T cell proliferation compared to control mice and was not further changed by dietary ISE (Fig. 9B). Furthermore, no difference for T-cell secreting cytokines IL-4 (Fig. 9D) and IFN-γ (Fig. 9E) was found across all groups. However, ConA-stimulated splenocytes from BLM produced less IL-2 compared to control mice and this effect was further lessened by the concomitant 0.6% ISE (Fig. 9C).

4. Discussion

The present study is to demonstrate that dietary ISE dose-dependently ameliorates BLM-induced ALI by inhibiting inflammatory and oxidative stress responses, tissue edema, lung hemorrhage and in turn improving the pathological symptoms in the lung and its underlying mechanisms might be mediated by the inhibition of NF-κB signal. In addition, in BLM-treated mice, ISE did not change the subpopulations of splenocytes and substantially affect LPS-stimulated pro-inflammatory cytokine production and ConA-induced T cell proliferation and secreting cytokines except for inhibiting IL-1β and IL-2, respectively.

Early inflammatory response has been typically characterized by the rapidly elevated infiltrating cell recruitment, macrophages and neutrophils activation, inflammatory mediators including IL-1β, IL-6 and TNF-α within the first 3 days in BALF from BLM-induced mouse model of lung injury [9,22,23]. TNF-α, an “early-wave” cytokine, plays crucial roles in the pathogenesis of ALI and ARDS via inducing apoptosis of respiratory epithelium which contributes to the alveolar damage in IPF [24–26]. In addition, TNF-α can up-regulate the expression of pro-fibrotic cytokine TGF-β [27,28]. Evidence shows that TNF-α blockade with either anti-TNF-α Abs or TNF-α antagonist can suppress BLM-induced lung fibrosis [29]. However, TNF-α has also been demonstrated to be essential for suppressing pulmonary inflammation in BLM-induced pneumopathy [30]. IL-1β and IL-6 are another pro-inflammatory cytokines involved in lung injury and fibrotic process, and are known to act synergistically with TNF-α [31,32]. Thus, inhibition of IL-1β, IL-6, and TNF-α contributes to attenuating the inflammatory and fibrotic responses induced by bleomycin in mice. In this study, it is conceivable that dietary ISE results in a reduced BLM-induced ALI, which was confirmed by the histological findings, lung injury scores, and lung edema, following bleomycin administration and in an indirect effect on the reduction of infiltrating cells, pro-inflammatory cytokines and neutrophil activation in this model.

Furthermore, BLM administration could induce the production of ROS [33,34], which in turn activates a variety of signal transducing pathways including MAPK and NF-κB signals [35]. Previous studies have demonstrated that the intratracheal inoculation of BLM induced the phosphorylation and degradation of IκB-α and the translocation of the active dimer of NF-κB into the nucleus in the whole lung of mice [36,37]. Furthermore, exogenous or endogenously produced ROS could activate the MAP kinases which has been associated with proliferation or apoptosis [35]. Thus, we speculate that the inhibition of inflammatory mediators by ISE supplementation is likely to be mediated by regulating MAPK, NF-κB and other signals through interference with the activity of MAPK (Erk1/2, Jnk1/2, p38) and NF-κB. Indeed, we found that BLM treatment up-regulated the IκB-α phosphorylation and down-regulated an NK-κB inhibitor, IκB-α level, which is associated with an increase of MDA level and a reduction of the SOD, CAT and GSH-Px activities. However, dietary ISE did not inhibit the activation of

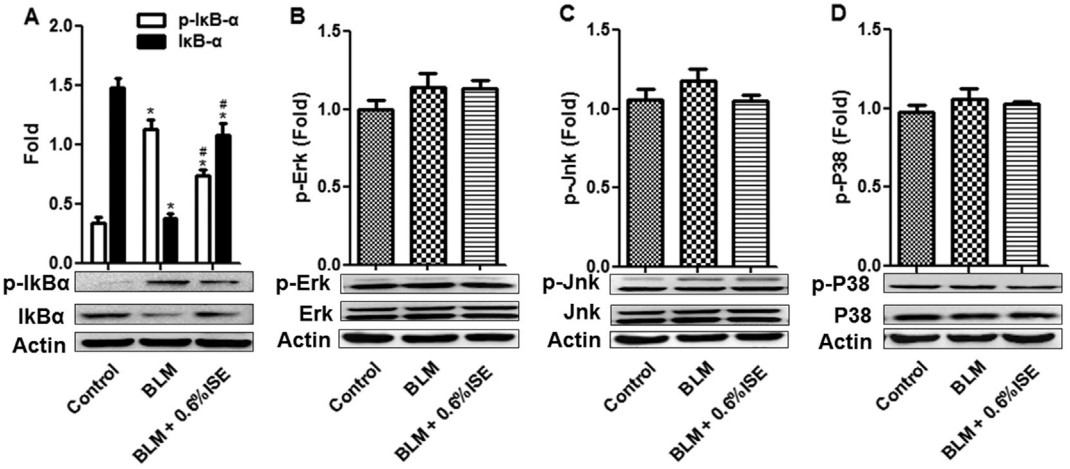

Fig. 7. Effects of ISE supplementation on NF-κB and MAPK signaling pathways in the lung of BLM-treated mice. Expression of p-IκB-α and IκB-α (A), p-Erk1/2 (B), p-Jnk1/2 (C) and p-p38 (D) in the lysates of lung tissues from each group mice were analyzed by Western-blotting assay. The value of the control was set at 1, and the relative value was presented as fold induction to that of the control. Values are expressed as the mean ± SEM of 12 samples. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. BLM. ISE, *I. sanghuang* extract; BLM, bleomycin; p-Erk, p-Erk1/2; p-Jnk, p-Jnk1/2; Erk, Erk1/2; Jnk, Jnk1/2.

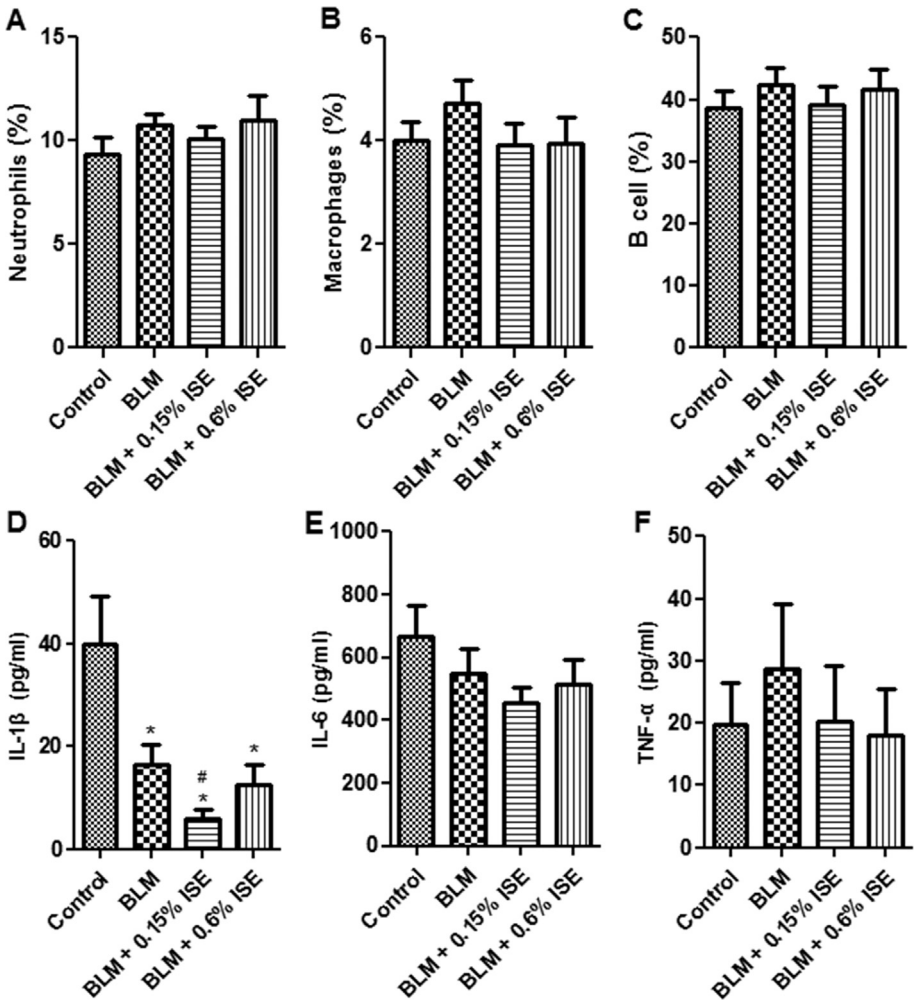

Fig. 8. Effects of ISE on cell populations and inflammatory cytokine production by splenocytes. Spleen cells were stained with fluorescence-conjugated anti-Gr-1, anti-F4/80 and anti-CD19 Abs to measure the macrophages (A), neutrophils (B), and B cells (C) using flow cytometry, respectively, or stimulated with LPS for 24 h and supernatant was used to determine production of IL-1β (D), IL-6 (E) and TNF-α (F) using ELISA assay. Values are expressed as the mean ± SEM (n = 12 mice/group). $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. BLM. ISE, *I. sanghuang* extract; BLM, bleomycin.

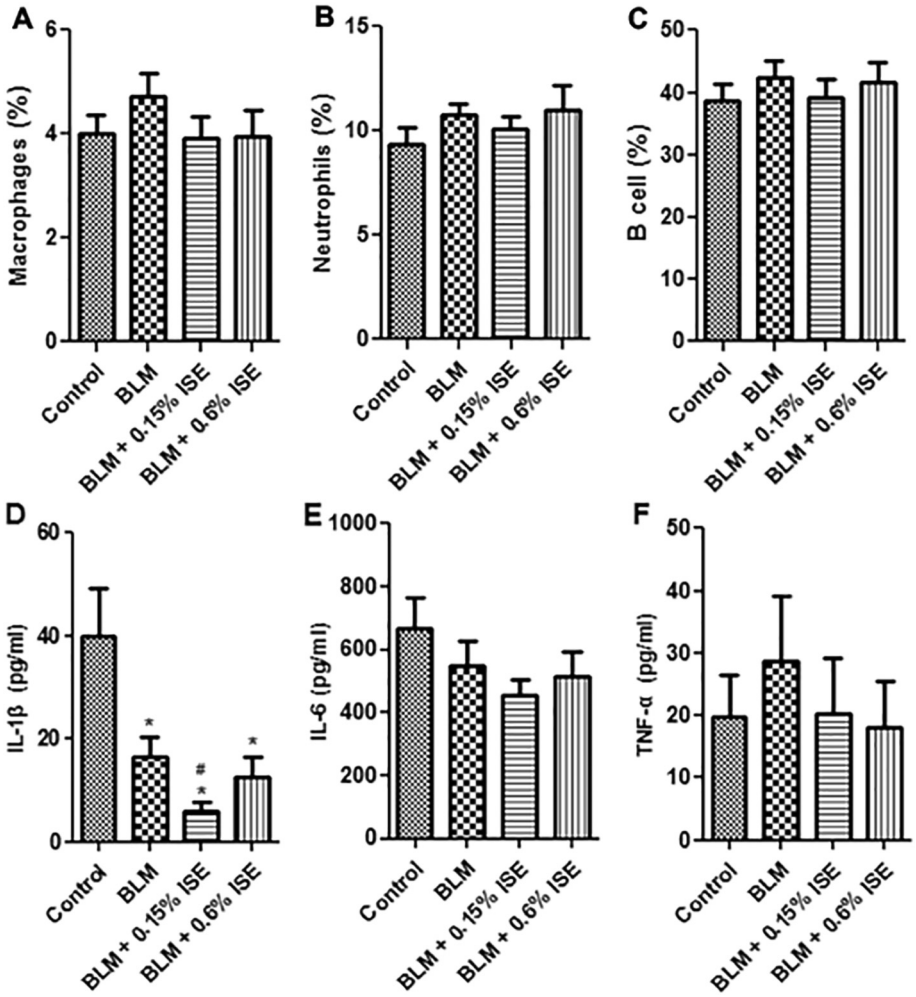

Fig. 9. Effect of ISE supplementation on T cell population and functions in splenocytes. Spleen cells were stained with fluorescence-conjugated anti-CD3 Ab to measure the T cell population (A), or stimulated with ConA for 72 h to determine T cell proliferation (B) using flow cytometry or T cell secreting cytokines including IL-2 (C), IL-4 (D) and IFN-γ (E) using ELISA assay. Values are expressed as the mean ± SEM (n = 12 mice/group). $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. BLM. ISE, *I. sanghuang* extract; BLM, bleomycin.

Erk1/2, Jnk1/2 and p38, but prevented the phosphorylation and degradation of IκB-α. Furthermore, ISE dose-dependently changed oxidative/anti-oxidative disbalance, such as up-regulation of the SOD, CAT and GSH-Px activities and down-regulation of the MDA level. These data indicate that the attenuation of BLM-induced ALI by ISE may be mediated via inhibiting NF-κB signal, oxidative stress and inflammation in vivo. However, whether ISE supplementation could directly affect oxidative stress, which in turn changes NF-κB signaling pathway and induces the production of inflammatory mediators needs to be further investigated. In addition, these data suggest that ISE might be able to prevent/treatment lung fibrosis via inhibiting early inflammation and oxidative stress. Further studies to elucidate these issues need to be investigated.

The accumulation of lung infiltrating cells in BLM-induced ALI suggests that the pathogenesis of this disease may be mediated by immune cells such as macrophages and T cells. *In vivo* depletion of T cells could attenuate lung injury and fibrosis [29,38]. Thus, alteration of systemic immunity may affect BLM-induced lung lesion. Since dietary ISE had the ability to reduce inflammatory cell infiltration in the lung from BLM-treated mice, it was of interest to assess this protective effect of ISE on systemic immunity. Zhu and coworkers have shown that BLM could decrease the number of splenocytes but un-change the number of T cells, B cells, and macrophages 2 weeks later after intratracheal incubation of BLM [39]. In addition, Sharma and coworkers have found that BLM treatment could not affect splenic $CD3^+$ lymphocyte counts, but decrease the percentage of splenic $CD3^+$ T cells and increase the percentage of splenic $CD3^+$ T cells in the hilar lymph nodes on day 3 after BLM treatment [38]. However, in this study, we did not observe any difference for splenocyte counts and percentage of lymphocyte populations including T cells, B cells, macrophages, and neutrophils on day 3 after BLM administration. Therefore, we speculate that this difference might be due to a different BLM-treatment.

Pro-inflammatory cytokines are able to cause tissue damage. Dietary ISE could decrease lung pro-inflammatory cytokine level in BALF from BLM-induced ALI mice, but whether it can affect systemic immunity such as lymphocyte proliferation and T cell-secreting cytokines is not still known. Furthermore, studies have reported the effects of BLM on T cell functions [38,39], but no report is found for splenocyte producing pro-inflammatory cytokines in BLM-treated mice. To the best of our knowledge, this is the first report indicating that BLM administration decreases IL-1β, not IL-6 and TNF-α from LPS-stimulated splenocytes; while this inhibitory effect on IL-1β was further potential by ISE sat 0.15%. However, what mechanism is involved in this effect needs to be explored.

Finally, we also evaluated T cell proliferation and T-cell secreting cytokines across all groups. In accordance with a previous study [38], there is little change in the splenic T cell proliferation and T-cell producing IL-4 and IFN-γ between BLM-treated and control group and this

effect was not affected by ISE. Although Sharma and coworkers did not find any effect of BLM treatment on IL-2 production at day 3–10 after BLM administration [38], decreased IL-2 secretion by BLM-treated splenocytes has been found on day 14 after BLM administration [39]. In the current study, we found that splenocyte secreting IL-2 was inhibited by BLM and was further lessened by ISE at 0.6%. Considering these reports and the results of the present study, it is likely to speculate that this effect of ISE would be too weak to change BLM-induced systemic immunity.

Polyphenols are the secondary metabolites of mushrooms in the genus *Inonotus*. In this study, *I. sanghuang* extract contains 6 polyphenols such as quercetin, rutin, quercitrin, icarisid II, isorhamnetin and chlorogenic acid, which have been shown to exert anti-oxidant, anti-proliferative, anti-inflammatory and anti-microbial activities in vitro [16,17]. These compounds such as quercetin [40,41], rutin [42], isorhamnetin [43] and chlorogenic acid [44] have been reported to have the protective effects on lung injury induced by LPS or BLM via either anti-oxidative or anti-inflammatory activities, which may be mediated via inhibiting NF-κB or MAPK signaling pathway [5,42–44], which suggests that one phenolic compound of ISE might have the ability to be against ALI induced by LPS or BLM. However, whether the phenolic compounds combined have the synergetic or additive effect against ALI is still unknown. Current data demonstrate that the attenuation of dietary ISE on BLM-induced ALI might be mediated at least in part by *I. sanghuang* polyphenols, suggesting the synergetic or additive effect of the components of ISE.

In conclusion, the results of this study indicate the potent anti-inflammatory and anti-oxidative effects of an ethyl acetate fraction of mushroom *I. sanghuang* in BLM-induced ALI in mice. Our results suggest that ISE attenuates experimental ALI through suppressing inflammatory mediators and correcting the oxidation/anti-oxidation disbalance mediated in part via the inhibition of NF-κB signal in lung tissues. Attenuation of inflammatory mediators may offer an effective therapeutic approach against ALI. Together, our findings indicate that a protective effect and putative mechanism of ISE's action involves inhibition of inflammation and oxidative stress, and that it has importance to be considered as anti-inflammatory agent against lung inflammation. Finally, the exact mechanism deciphering the interaction of *I. sanghuang* and its compounds to inflammatory cytokines and oxidative stress in lung injury and fibrosis warrants further investigation at cellular and molecular levels.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

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