EXAMINATION OF THE PROTECTIVE EFFECT OF 6-SHOGAOL AGAINST LPS-INDUCED ACUTE LUNG INJURY IN MICE VIA NF-ĸB ATTENUATION

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Abstract: Acute lung injury (ALI), the causative factor for acute respiratory distress syndrome, results in significant morbidity and mortality. Due to a lack of effective therapeutic options for ALI, the development of novel therapies is urgently needed. NF-ĸB, an inflammatory mediator necessary for the evolution of ALI, could serve as an important target for novel agents to prevent disease progression. The present study was designed to evaluate the protective effect of 6-shogaol against lipopolysaccharide (LPS)-induced ALI in mice, and its mechanism of action. Our results suggest that 6-shogaol significantly attenuates elevated levels of various proinflammatory cytokines, such as tumor necrosis factor alpha (TNFα), IL-1β and IL-6. Moreover, the influx of neutrophils, increased protein concentration and edema were also suppressed in mice pretreated with 6-shogaol. These observations were also confirmed by histopathological examination of lung tissues, which suggests that 6-shogaol significantly improves the pathological condition to normal in a dose-dependent manner. A docking study of 6-shogaol was also performed, with the NF-ĸB p50 homodimer bound to a ĸB site, to determine its possible inhibitory effects. Our results show that 6-shogaol was efficiently accommodated in the deep cleft of the active site, lined with residues Tyr57, Val58 and Cys59, Tyr143, Lys145 and Lys146 of Chain B of p50 NF-ĸB.

Key words: acute lung injury; NF-ĸB; 6-shogaol; cytokines; LPS

INTRODUCTION

Acute lung injury (ALI), the causative factor of acute respiratory distress syndrome, is often characterized by numerous events, such as severe hypoxemia, alveolar-capillary barrier damage, pulmonary inflammation, noncardiogenic lung edema and multiple organ failure. Direct and indirect injuries of the lungs are considered the main etiological factors in the onset of this disease. Pneumococcal infection, release of gastric substances, sepsis, major trauma and acute pancreatitis are inducing factors for the development of ALI [1,2]. Studies have indicated that around 190000 cases of ALI were reported in the USA, and it results in the death of 74500 patients each year [3]. The nuclear factor-ĸB (NF-ĸB) pathway plays a pivotal role in the pathogenesis of ALI [4-8]. NF-ĸB is found in the cytoplasm of unstimulated cells in a dormant form

bound to its inhibitor, IĸBα [9]. The activation of NF-ĸB and its translocation from the nucleus, for binding to its associated DNA attachment site from its bound form, is triggered by external stimuli. Thus, after its release in free form, NF-ĸB regulates the expression of numerous genes, and results in inflammation in ALI. Despite the high morbidity and mortality associated with ALI, there are no effective therapeutic options for its treatment. Therefore, the development of novel therapies, particularly those that target NF-ĸB, is urgently needed. Plant or plant-based products have received considerable attention for the treatment of ALI due to significant therapeutic effects and relatively low toxicity. These therapeutic products act as antioxidant and anti-inflammatory agents to arrest the key pathways responsible for progression of ALI.

Ginger (*Zingiber officinale* Roscoe) has been widely used throughout the world because of its aromatic spicy nature. In Chinese and Indian traditional medicine in particular it has also been used as a herbal therapy, with benefits for treating a wide range of ailments, including tenderness, heartburn, queasiness, vomiting, aches/pain, the common cold and diarrhea [10]. Compounds derived from phenylpropanoids act as a basic skeleton for the active-constituents of ginger, including gingerols and shogaols [11]. Both of these products are present in fresh and dried ginger. Gingerol (sometimes referred to as [6]-gingerol) is the active constituent of fresh ginger. In contrast, shogaol (or 6-shogaol), a dehydrated product of gingerols, is found in larger amounts in dried as opposed to fresh ginger [12]. The results of previous studies suggest that shogaol has prominent antiproliferative and anti-inflammatory effects [13]. With respect to its anti-inflammatory action, 6-shogaol suppressed the generation of metabolites of both cyclooxygenase and lipoxygenase, and arachidonic acid. Moreover, the organic extracts from dried ginger rhizomes inhibited lipopolysaccharide (LPS)-induced prostaglandin E2 (PGE2) production. However, there have been no or very limited studies to elucidate the effect of 6-shogaol on LPS-stimulated ALI. Based on these considerations, the present study evaluated the pharmacological effects of 6-shogaol (6-SH) in an LPS-induced mouse model of ALI, and its mechanism of action.

MATERIALS AND METHODS

Materials

6-shogaol was procured from the Sigma Aldrich (St. Louis, MO, USA). Dexamethasone (DEX) was supplied by Changle Pharmaceutical Co. (China). The TNF-α, IL-6 and IL-1β ELISA kits were procured from Biolegend (USA). The assay kit obtained from Nanjing Jiancheng Bioengineering Institute of Nanjing (China) was used for the estimation of myeloperoxidase (MPO).

Animals

Male BALB/c mice, weighing approximately 18-20 g, were used for performing biological assays. All animals were housed in appropriate laboratory cages and fed with standard laboratory diet and water *ad libitum*. Before starting any experiment, all mice were acclimatized to the standard laboratory environment for a minimum of 3 days. Animal experiments were performed in accordance with the Institutional Animal Ethical Committee guidelines.

Induction of ALI in mice via administration of LPS

Six groups of mice were used, each was comprised of 12 healthy male BALB/c mice. The classification of the groups was as follows: one group served as a control group, the second group was treated with LPS, while the third, fourth and fifth groups received LPS + 6-SH at a concentration of 10, 20 and 40 mg/kg, respectively. The last group received LPS and DEX. The 6-SH and DEX were administered intraperitoneally and the mice in the control and LPS groups received an equal volume of PBS instead of a drug. After 1 h, the mice were anesthetized with diethyl ether. Lung injury was induced via intranasal instillation of 10 μg of LPS in 50 μl. The control mice were given 50 μl PBS without inducing agent. The bronchoalveolar lavage fluid (BALF) was collected three times through a tracheal cannula with sterile PBS to a total volume of 1.3 mL.

Lung wet to dry weight (W/D) ratio measurement and protein analysis

The lungs were removed after the mice were euthanized, and the corresponding wet weight was quantified. To obtain the dry weight, the lungs were placed in a drying oven at 60°C for 24 h. The relative difference of the wet lung against the dry lung was easily interpreted as the severity of edema in the tissues. The BALF was collected and protein content was determined by BCA method. The protein level was expressed in milligram protein per milliliter in BALF.

Inflammatory cell counts of BALF

To quantify the inflammatory cell count, pellets of the cells were obtained by centrifugation of the fluid collected from each sample. The centrifugation was carried out at 4°C, 3000 rpm (800×g) for 10 min. The resulting pellets were resuspended in PBS for total cell counts using a hemocytometer.

Cytokine assays

The concentrations of various pro-inflammatory markers, such as, TNF-α, IL-6, and IL-1β, in the BALF were measured by ELISA kits according to the standard protocol provided by the manufacturer (BioLegend, Inc., USA). In brief, the standards along with samples were added to a 96-well plate after treating the well plate with specific monoclonal antibody. A biotinylated detection antibody was added to the above, followed by the addition of avidin-horseradish peroxidase. 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution was subsequently added, that gave a blue color to the resulting solution. Finally, when the reaction was stopped, the color changed from blue to yellow. Then, the optical density (OD) of the microplate was read at 450 nm.

Pulmonary MPO activity in acute lung injury mice

The mice were killed 24 h after the LPS challenge. Lung tissues were frozen in liquid nitrogen and then homogenized in PBS. The MPO activities in the lung homogenates were examined using an MPO determination kit (Nanjing JianCheng Bioengineering Institute, China). The rest of the homogenate was centrifuged at 2000×*g* for 10 min at 4°C. The supernatant was used to detect the levels of MDA according to the manufacturer's instructions (Nanjing JianCheng Bioengineering Institute, China).

Histological analysis of lung tissue

Dissected lungs of mice were fixed with 10% buffered formalin, embedded in paraffin. After staining with hematoxylin and eosin (H&E), alterations in lung tissues were observed under a normal light microscope at 200× magnification.

Docking analysis

Protein preparation

Docking was carried out against the structure of the NF-kappa B p50 homodimer bound to a kappa B site for a possible inhibitory effect of 6-SH. The co-crystallized ligands, bound water molecules and cofactors were separated from the proteins before starting any docking experiments. The CHARMM force field was used for the minimization of the target protein using steepest descent (gradient <0.1) and conjugate gradient algorithms (gradient <0.01). A suitable active-site was identified in the target protein via a defined active site module within the Discovery Studio 2.5.

Ligand Fit docking

To exemplify the molecular interactions of 6-SH necessary for the inhibition of key enzymes, LigandFit, a shape-based method inbuilt in Discovery Studio 2.5, was utilized for docking analysis. It works on a cavity detection algorithm where a shape comparison filter was combined with the Monte Carlo technique to produce various ligand conformations. The most stable conformation of ligand was then allowed to dock into the active site of a protein using the grid resolution of 0.5 Å (default). During docking, the 5 Å from the core of the binding site was kept flexible during the minimization. This was carried out to calculate the non-bonded interactions between the ligand and catalytic residues of the target protein. The resultant docked pose was selected for further analysis and image generation.

Statistical analysis

The experimental data are presented as means±SEM. Analysis of variance (ANOVA), followed by Dunnett's test, was used to compare the data between experimental groups. P values of <0.05 were considered statistically significant. The analysis was performed using

GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com

RESULTS AND DISCUSSION

Effect of 6-SH on LPS-induced lung W/D ratio and protein concentration in the BALF

In acute lung injury, the lungs are associated with pulmonary edema. Therefore, the effect of 6-SH was determined on the W/D ratio of the lungs. The ratio was determined after 7 h of instillation of LPS via the intranasal route. After that, the lung W/D ratio (Fig. 1)
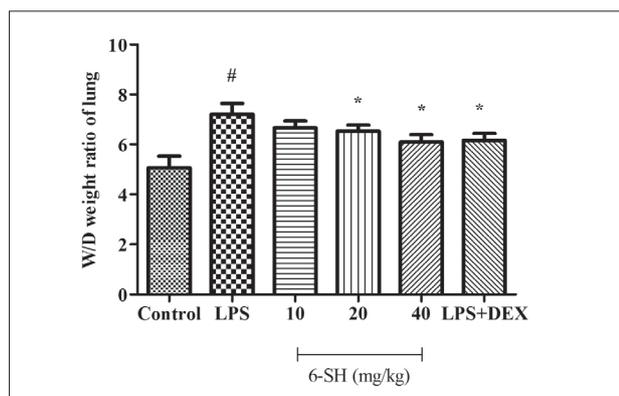

Fig. 1. Effects of 6-SH on the lung W/D ratio. 6-SH was i.p. administered at doses of 10, 20 and 40 mg/kg 1 h prior to instillation of LPS. The data are expressed as means±SEM. $P^{\#}<0.01$ *vs.* Control group, $P^{**}<0.05$, $P^{**}<0.01$ *vs.* LPS-treated group.
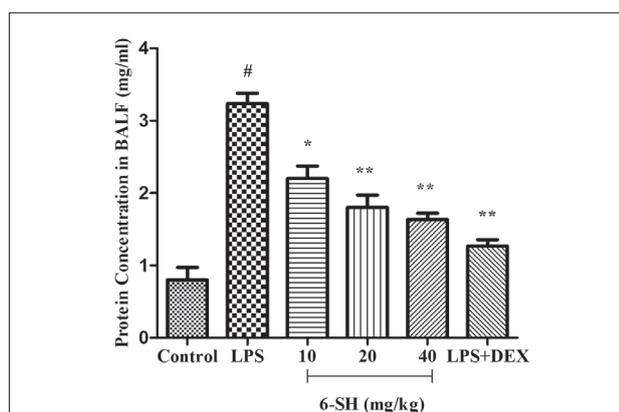

Fig. 2. Effects of 6-SH on the total protein concentration in BALF. 6-SH was i.p. administered at doses of 10, 20 and 40 mg/kg 1 prior to instillation of LPS. The values presented are the means±SEM. $P^{\#}<0.01$ *vs.* control, $P^{*}<0.05$, $P^{**}<0.01$ *vs.* LPS.

and total protein concentration (Fig. 2) were calculated. The results showed that the lung W/D ratio was augmented after LPS administration in comparison of the control group (P<0.01). However, pretreatment with 6-SH at different concentrations, i.e. 10, 20 and 40 mg/kg, or DEX drastically reduced the lung W/D ratio compared with the LPS group (P<0.05). The total protein concentration of BALF was significantly elevated in the case of the LPS group. However, in comparison with the LPS group, pretreatment with 6-SH (10, 20 and 40 mg/kg) or DEX diminished the total protein concentration significantly (P<0.05 or P<0.01).

Effects of 6-SH on MPO activity

Myeloperoxidase (MPO) activity serves as an important marker of neutrophil influx into tissues. By measuring this activity, we could easily translate the accrual of neutrophils in the pulmonary tissues. Thus, the LPS-induced model of ALI showed the infiltration of neutrophils in the lungs, resulting in higher MPO activity. This MPO excreted by neutrophils results in the generation of an MPO-derived oxidant, which ultimately leads to the tissue damage. Thus, inhibition of MPO would prevent the associated degradation of pulmonary tissue. As shown in Fig. 3, the activity of MPO in tissues was significantly increased in comparison to the control group (P<0.01). However, administration of 6-SH showed a reduction in MPO
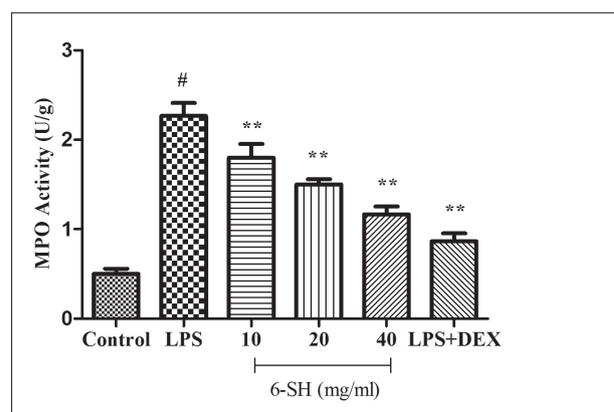

Fig. 3. Effects of 6-SH on MPO activity. 6-SH was i.p. administered at doses of 10, 20 and 40 mg/kg 1 h prior to instillation of LPS. The values expressed as the means±SEM. $P^{\#}<0.01$ *vs.* control, $P^{*}<0.05$, $P^{**}<0.01$ *vs.* LPS.
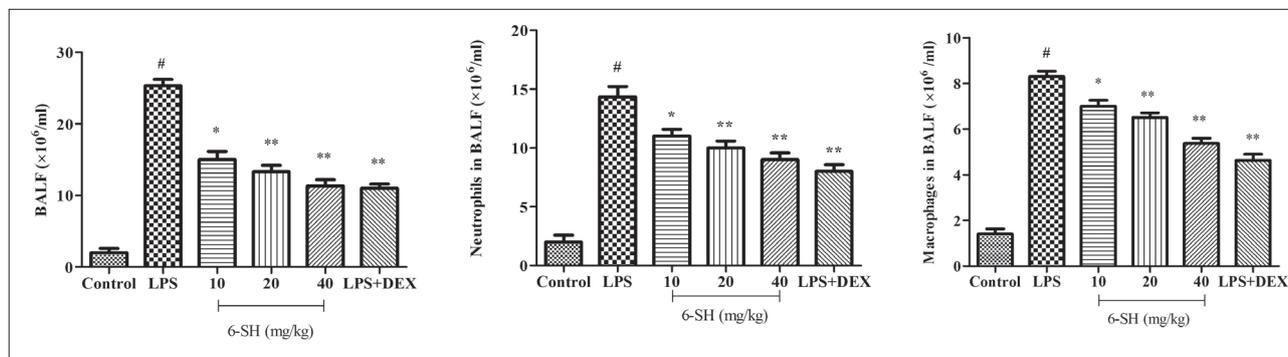
Fig. 4. Effects of 6-SH on the number of total cells, neutrophils, and macrophages in the BALF. **A** –total cells; **B** – neutrophils; **C** – macrophages. The 6-SH was i.p. administered at doses of 10, 20 and 40 mg/kg. The values presented are the means±SEM, where P#<0.01 *vs.* control, P*<0.05, P**<0.01 *vs.* LPS.

activity in a dose-dependent manner (10, 20 and 40 mg/kg) (P<0.01). This result indicates the protective effect of 6-SH on the MPO-induced tissue damage.

Effect of 6-SH on the inflammatory cell count in the BALF of LPS-induced ALI mice

Lung injury becomes worse after the generation of pro-inflammatory cytokines that are released as a result of early inflammatory response. These cytokines, including TNF-α, IL-1β and IL-6 and other inflammatory mediators, play a critical role in the progression of ALI in its severe form [14,15]. It has been found that patients affected by acute respiratory distress syndrome (ARDS) showed elevated levels of TNF-α, IL-1β and IL-6 in the BALF [16]. Apart from its activity in intensifying the inflammatory process to cause associated injury, these cytokines also recruit neutrophils into the lung, which results in higher MPO activity. This higher activity leads to the release of granular enzymes, which could be associated with lung injury and pulmonary edema. Therefore, the number of inflammatory cells, such as neutrophils and macrophages, in the BALF were analyzed at 7 h after LPS challenge. As shown in Fig. 4, the number of total cells, neutrophils and macrophages compared with that of the control group (P<0.01) was found to be elevated after administration of LPS. Nonetheless, pretreatment with 6-SH (10, 20 and 40 mg/kg) or DEX appreciably lessened the number of total cells

(P<0.01), neutrophils (P<0.01) and macrophages (P<0.01). These results indicated that 6-SH exerts protective effects in ALI of mice.

Effect of 6-SH on the generation of cytokines in the BALF of LPS-treated ALI mice

As discussed above, for the progression of ALI, the release of various pro-inflammatory cytokines has played a major role. Thus, the inhibition of the generation of these cytokines results in the loss of the inflammatory response associated with it. Consequently, 6-SH was analyzed after 7 h of administration of LPS by ELISA for its activity against TNF-α, IL-1β and IL-6. As shown in Fig. 5, the levels of TNF-α, IL-6, and IL-1β in BALF were appreciably augmented in comparison to the control. However, these levels were reduced drastically after the introduction of 6-SH in different concentrations. It was surprising to note that these effects were dependent upon the concentration of 6-SH.

Effect of 6-SH on the histopathology of lungs of LPS-treated mice

The lungs of mice were collected 7 h after challenging with LPS and subjected to H&E staining. As shown in Fig. 6, lung tissues from the control group showed a normal structure and no major histopathological changes were observed (Fig. 6A). In the LPS-chal-
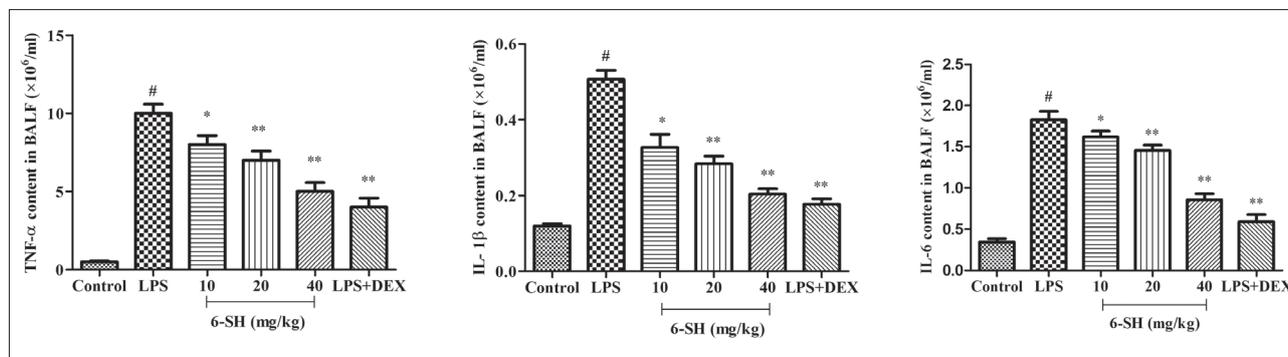
Fig. 5. Production of inflammatory cytokines TNF-α, IL-1ß, and IL-6 in different experimental groups. 6-SH was i.p. administered at doses of 10, 20 and 40 mg/kg. The values presented are means±SEM. P#<0.01 vs. control, P*<0.05, P**<0.01 vs. LPS.
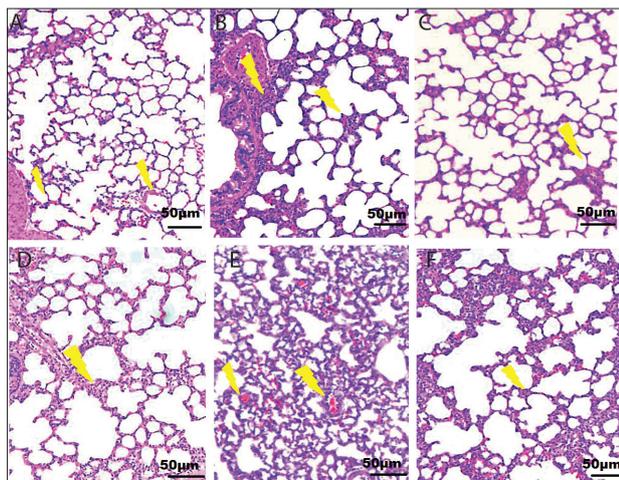

Fig. 6. Effect of 6-SH on histopathological changes on lung tissue of ALI mice after tissue staining. 6-SH was i.p. administered at doses of 10, 20 and 40 mg/kg. **A** – Control group; **B** – LPS group; **C** – LPS+DEX group; **D** – LPS+6-SH (10 mg/kg); **E** – LPS+6-SH (25 mg/kg); **F** – LPS+6-SH (50 mg/kg).
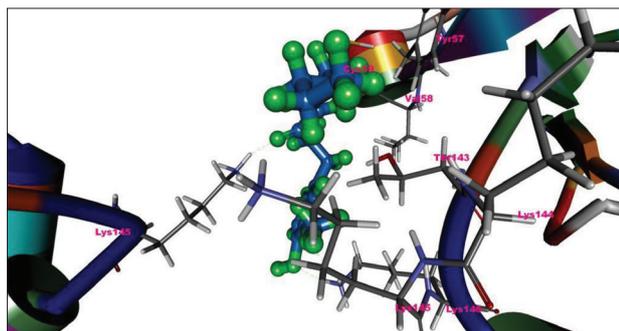

Fig. 7. Docked orientation of 6-SH in the active site of *p*50 NF-κB.

lenged group, tissues showed marked inflammation due to infiltration of neutrophils along with local fibrosis. Additionally, the thickening of the alveolar wall and congestion of the lungs were observed (Fig. 6B). These pathological alterations were extensively reversed to normal upon treatment with 6-SH (10, 20 and 40 mg/kg) (Fig. 6D-F) and DEX (5 mg/kg) (Fig. 6C).

Effect of 6-SH on the NF-κB

The diverse cellular process could be easily controlled by transcription factor NF-κB. [17]. In a dormant condition, NF-κB will exist in bound form with its inhibitor (IκBs) in cytoplasm. Once it is triggered to its active form, the p65 unit of NF-κB dissociates from its inhibitory protein IκB-α and enters the nucleus. After arriving in the nucleus, it attaches to κB binding sites and activates the transcription of specific target genes such as TNF-α, IL-1β and IL-6. Since we have explored the initial effects of 6-SH on the level of these cytokines, it is imperative to examine the effect 6-SH on the inhibition of NF-κB. For this, a molecular docking analysis of 6-SH was undertaken with the NF-κB protein model. LigandFit within Discovery Studio 2.5 was used to carry out this experiment as per the standard protocol mentioned in the experimental section. Results showed that 6-SH creates excellent H-bonds with residues like Lys145 and Lys146 (Fig. 7). They also showed the formation of the one cation-pi interaction with Lys145. It is surprising that 6-SH was efficiently buried in the active site of the NF-κB situ-

ated between Chain A and Chain B. Docking analysis showed that 6-SH was located deep in the cleft lined with Tyr57, Val58 and Cys59, Tyr143, Lys145 and Lys146 of the Chain B of p50 NF-κB. In terms of scoring profile, 6-SH was again revealed as an efficient ligand to control the activity of NF-κB with a dock score of 41.27 and ligand internal energy of -3.52.

To conclude, 6-Shogaol significantly inhibits the elevated level of various pro-inflammatory cytokines, e.g. TNF-α, IL-1β and IL-6. Moreover, the influx of neutrophils and edema were also suppressed after pretreatment with 6-Shogaol. These observations were also confirmed by a histopathological examination of lung tissues, which suggest that it significantly improve the pathological condition to normal in dose-dependent manner.

Authors' contributions: Jing-Chao Wang performed the induction of ALI in mice via administration of LPS, Li-Hua Zhou performed the histopathological analysis of the lung tissue, Hai-Jin Zhao and Shao-Xi Cai perform docking study and cytokine assays.

Conflicts of interest disclosure: The authors declare no conflict of interest.

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