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Effects of Betulinic Acid Derivative on Lung Inflammation in a Mouse Model of Chronic Obstructive Pulmonary Disease Induced by Particulate Matter 2.5

Authors' Contribution:

Study Design A

Data Collection B

Statistical Analysis C

Data Interpretation D

Manuscript Preparation E

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Background: Chronic obstructive pulmonary disease (COPD) is mainly induced by the increased content of particulate matter 2.5 (PM_{2.5}) in the atmosphere. This study aimed to evaluate the effects of betulinic acid derivative on lung inflammation in a mouse model of chronic obstructive pulmonary disease induced by particulate matter 2.5.

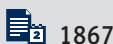
Material/Methods: The mice were given a PM_{2.5} (25 μl) suspension for 7 days by the intranasal route to establish a COPD model. The content of TNF-α and IL-6 in the BALF samples was measured by commercially available ELISA kits.

Results: The PM_{2.5}-induced higher LDH and ACP levels were significantly alleviated in mouse lung tissues by treatment with betulinic acid derivative. Treatment with betulinic acid derivative also suppressed PM_{2.5}-induced increase in AKP and ALB levels in mouse lung tissues. Betulinic acid derivative reversed PM_{2.5}-mediated suppression of SOD activity and elevation of NOS level in mouse BALF. Moreover, the PM_{2.5}-induced excessive NO and MDA levels in mouse BALF were significantly reduced ($P < 0.05$) by treatment with betulinic acid derivative. Treatment with betulinic acid derivative improved TNF-α and IL-6 levels in BALF induced by PM_{2.5} exposure. Betulinic acid derivative inhibited PM_{2.5}-induced acute inflammatory exudate, alveolar septae damage, and inflammatory cell infiltration in lungs of mice.

Conclusions: In the mouse model of PM_{2.5}-induced COPD, betulinic acid derivative reduced the degree of lung inflammation and downregulated inflammatory mediators. These findings support the need for further in vivo studies and clinical evaluation of betulinic acid derivative in patients with COPD.

Keywords: **Anti-Inflammatory Agents • Diterpenes • Particulate Matter • Pulmonary Disease, Chronic Obstructive**

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Background

Pulmonary diseases in Chinese people are mainly induced by inhaling air contaminated with particulate matter (PM), which alters lung histology. High PM levels, especially PM_{2.5} due to human intervention, has led to a rapidly increased mortality rates in patients with respiratory diseases [1]. PM_{2.5} is particulate matter in the air having particle diameter larger than 2.5 μm. The PM_{2.5} is harmful mainly because of its ability to adsorb pollutants, heavy toxic elements, viruses, and bacteria from the atmosphere [2,3]. PM_{2.5} is characterized by its potential to remain in the air for long times and to enter pulmonary tissues after inhalation, which adversely affects human health [2,3]. Mechanistic investigations have shown that elevated levels of reactive oxygen species (ROS) and mediators of inflammation induced by PM_{2.5} are responsible for pulmonary tissue injury and damage [4-6]. Examination of lung tissues revealed marked damage in patients after inhalation of PM_{2.5} [7,8].

The COPD is characterized by lung airway blocking due to inflammation and subsequent degradation of pulmonary tissues [9,10]. Generally, COPD is detected in chain cigarette smokers and people frequently exposed to PM_{2.5} [2-4, 11]. Compounds having anti-inflammatory potential are effective in treatment of respiratory diseases such as COPD [12-15]. Currently available compounds used for COPD treatment have adverse effects such as diarrhea, decrease in body weight, vomiting, and headaches [16-18]. Thus, discovery of drug candidates for treatment of respiratory diseases induced by PM_{2.5} is immediately required.

Secondary metabolites isolated from diverse plant species are useful for inhibition of many respiratory diseases, including COPD, bronchitis, chronic pneumonia, and asthma [19-21]. Amenable functionalities and several biological activities associated with triterpenes have attracted attention of clinicians globally [22]. Other properties of triterpenes have demonstrated effective anti-inflammatory [23], anti-allergic, and HIV-inhibitory effects [4]. Therefore, this study aimed to evaluate the effects of a betulinic acid derivative on lung inflammation in a mouse model of COPD induced by particulate matter 2.5.

Material and Methods

Synthesis of Drug Compound

The triterpenoid compound betulinic acid was subjected to oxidation using CrO₃/H₂SO₄/acetone to obtain compound **2** (Figure 1). Compound **2** on condensation with H₂NNH₂ in the presence of KOH delivered the desired hydrazone **3**. Reaction of compound **3** with floro-α,β-unsaturated ketone in the

presence of a base produced compound **4**, which was subsequently reacted with palladium acetate/potassium carbonate using chlorobenzene and molecular sieves to obtain the desired product **5**.

Animals

Fifty adult male mice (8 weeks old; 20-25 body weight) were provided by the Soochow University Animal Center (Suzhou, China). All mice were acclimatized under sterile conditions at 23±1°C and 60% humidity and exposed to 12-h light and dark cycles. Approval for the study was obtained from the Committee for Animal Care, the First People's Hospital of Yunnan Province, Kunming, 650032, China. The experimental procedures were conducted in accordance with the guidelines issued by the Chinese National Institute of Health.

The PM_{2.5} Collection

Airborne PM_{2.5} was collected during a 2-week period from an urban region using a Thermo Anderson sampler (Brand; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Partitioning of the membrane filter into 2×2 cm parts was followed by ultrasonic oscillation (at 110 kHz) of the pieces repeated 5 times in ultrapure water at room temperature. After filtration with steel gauze, the filtrate was subjected to centrifugation at 15 000×g for 30 min. The precipitate obtained was kept in physiological saline, subjected to sterilization in an autoclave, and then stored at 4°C.

Induction of COPD in Mice

Mice in the model and treatment groups were given suspension of PM_{2.5} (25 μl) by intranasal route daily for 7 days. The sham group was administered equal volumes of physiological saline alone. The mice administered PM_{2.5} suspension were separated into 4 groups of 10 mice each: the model group and 3 betulinic acid treatment groups (at 2-, 5-, and 8-mg/kg doses). Mice were given betulinic acid at 2-, 5-, and 8-mg/kg doses daily for 15 days through intra-gastric route in physiological saline. Mice were monitored for sensitivity, body hair pattern, and respiratory murmur in all 5 groups for 30 days. On day 31, mice were sacrificed using choral hydrate anesthesia to collect bronchoalveolar lavage fluid (BALF) and remove the lungs.

BALF Analysis

Mice were injected with 1X PBS (1 ml) into the lungs, which was then withdrawn for determination of various cytokines and oxidative markers by standard methodology [13]. The BALF samples were centrifuged at 4°C for 30 min at room temperature. Supernatant was analyzed for SOD activity and presence of malondialdehyde (MDA), lactate dehydrogenase (LDH),

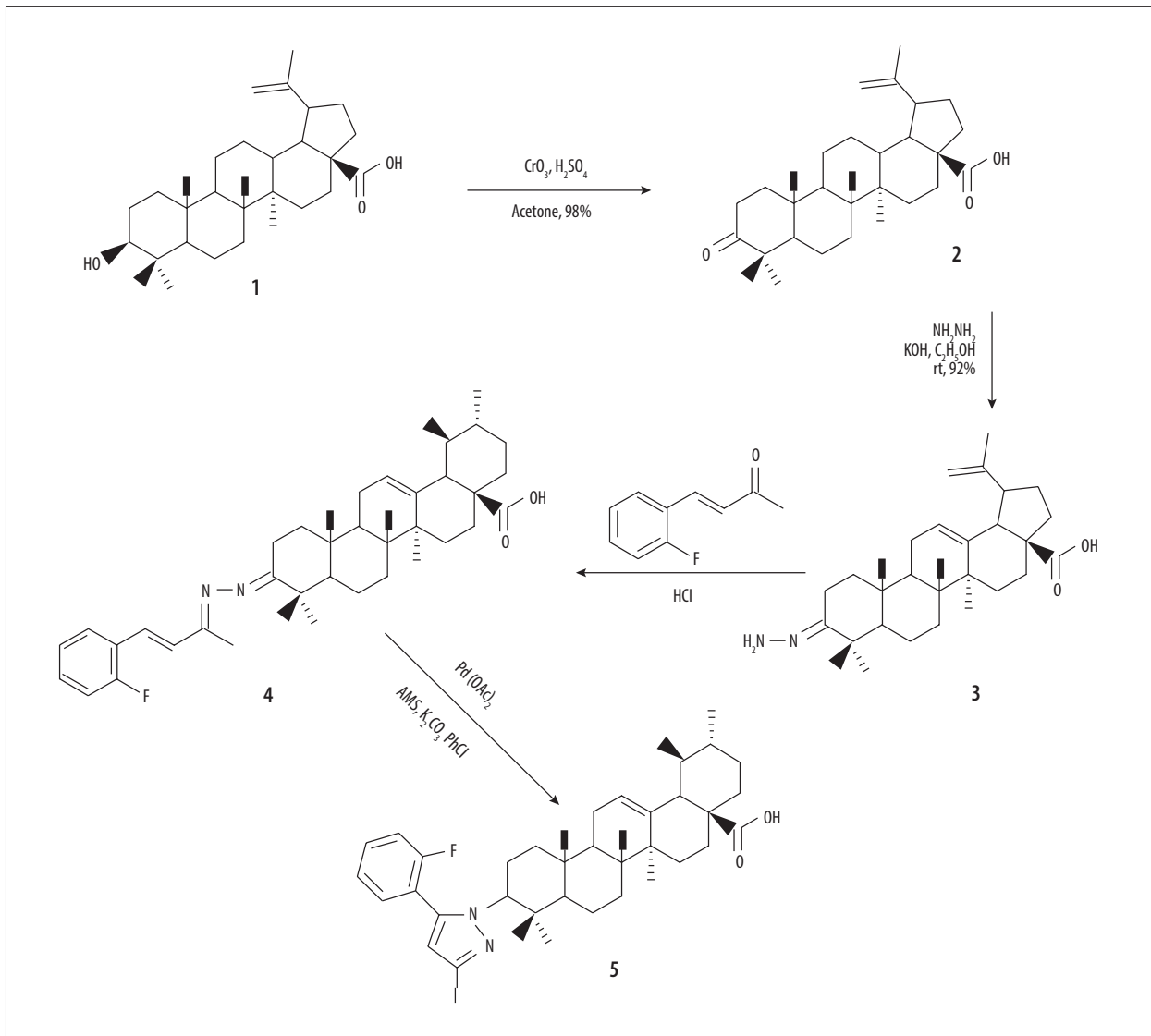


Figure 1. Synthesis of betulinic acid derivative.

alkaline phosphatase (AKP), albumin (ALB), and inducible nitric oxide synthetase (iNOS) levels using ELISA kits. The levels of $\text{TNF-}\alpha$ and IL-6 in the BALF samples were measured using commercially available ELISA kits.

Histopathological Analysis of Lungs

Histopathological alterations were examined in upper regions of the left pulmonary lobes using HE staining. Treatment of pulmonary tissues was performed with 10% buffered formalin for 24 h at 25°C to get fixed tissues. Then, dehydration was carried out with gradient ethanol followed by embedding in paraffin and subsequent slicing into thin 3- μm sections. Thin sections were dyed at room temperature after HE staining for 25 min for light microscopy examination of cell infiltration and acute inflammatory exudate.

Statistical Analysis

The data are presented as the mean \pm SD of experiments carried out independently in triplicate. Data were analyzed using one-way analysis of variance (ANOVA) followed by least significant difference post hoc testing. SPSS software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis of the data. Differences were defined as statistically significant at $P < 0.05$.

Results

Chemistry

Cyclized betulinic acid derivative 5 was synthesized for in vivo evaluation against PM_{2.5} induced COPD in the mouse model

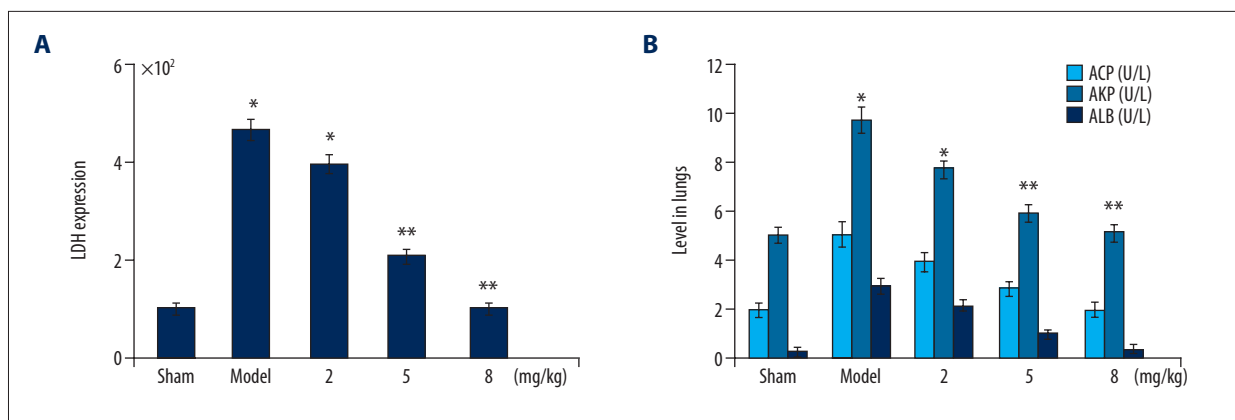


Figure 2. (A, B) Betulinic acid suppresses PM2.5-mediated inflammatory molecules. The PM2.5-exposed mice were treated with 2-, 5-, and 8-mg/kg doses of betulinic acid. The excised lung tissues were assessed using ELISA kits for LDH, AKP, ALB, and ACP levels after completion of treatment. * $P < 0.05$ and ** $P < 0.02$ vs sham group.

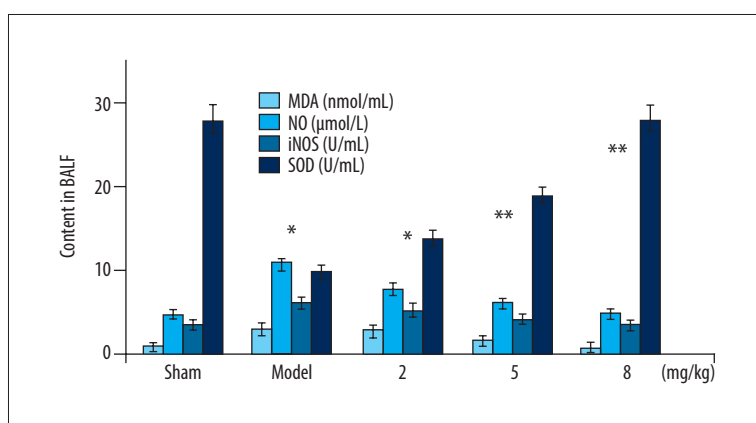


Figure 3. Betulinic acid suppresses PM2.5-mediated promotion of oxidative markers. The PM2.5-exposed mice were treated with 2-, 5-, and 8-mg/kg doses of betulinic acid. The BALF samples were assessed for MDA, NO, NOS, and SOD levels by ELISA. * $P < 0.05$ and ** $P < 0.02$ vs sham group.

taking into consideration diverse biological properties and bio-availability of triterpenoids in living systems. Cyclized betulinic acid derivative **5** was obtained from betulinic acid **1** using a sequence of reactions consisting of oxidation and condensation, followed by acid catalyzed reaction with α,β -unsaturated ketone and, finally, palladium acetate/potassium carbonate-mediated cyclization (**Figure 1**).

Betulinic Acid Inhibits Inflammation in Mice with PM-induced COPD

Exposure to PM2.5 significantly increased LDH and ACP levels in the mouse lung tissues compared to the sham group (**Figure 2**). The AKP and ALB levels were also much higher in PM2.5-exposed mice compared to the sham group. The PM2.5-induced LDH and ACP levels were significantly alleviated in mouse lung tissues after treatment with 2-, 5-, and 8-mg/kg doses of betulinic acid. Treatment with 2-, 5-, and 8-mg/kg doses of betulinic acid also suppressed the PM2.5-induced increase in AKP and ALB levels in mouse lung tissues.

Betulinic Acid Inhibits PM2.5-induced Oxidative Stress Factors in Mice

The activity of SOD in PM2.5-exposed mice was suppressed and that of NOS was promoted compared to sham mice (**Figure 3**). Betulinic acid reversed the PM2.5-mediated suppression of SOD activity and elevation of NOS level in mouse BALF at 2-, 5-, and 8-mg/kg doses. The NO and MDA levels in BALF of PM2.5-exposed mice were significantly ($P < 0.05$) elevated compared to the sham group. The PM2.5-induced excessive NO and MDA levels in mouse BALF were significantly ($P < 0.05$) reduced by treatment with 2-, 5-, and 8-mg/kg doses of betulinic acid.

Betulinic Acid Alleviates PM2.5-induced Excessive Cytokine Release in Mice

Continued exposure to PM2.5 leads to decreased levels of anti-oxidants in lung tissues, especially in chain smokers [24,25]. The effect of betulinic acid on TNF- α and IL-6 levels in PM2.5-exposed mice were measured at 2-, 5-, and 8-mg/kg doses by ELISA (**Figure 4**). The BALF of PM2.5-exposed mice showed

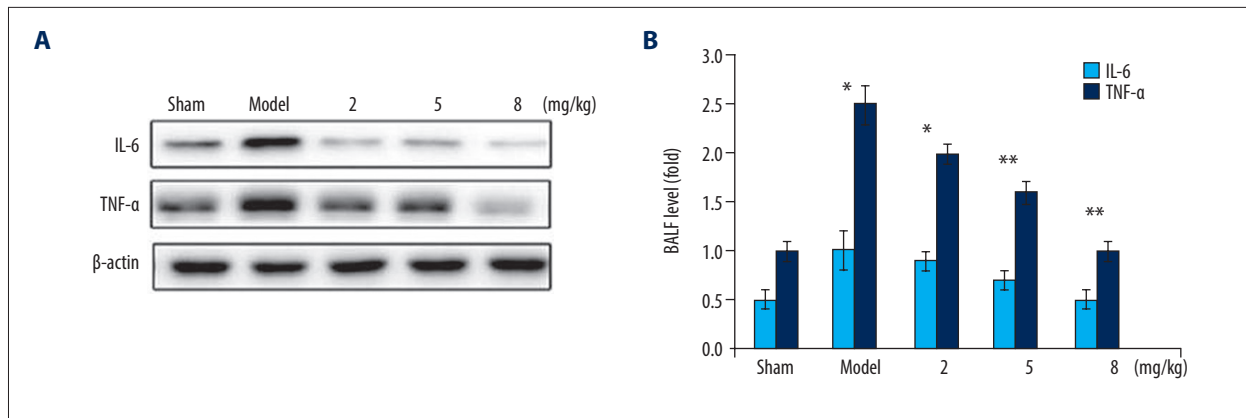


Figure 4. Betulinic acid inhibits PM2.5-mediated production of TNF- α and IL-6 levels in BALF of mice. The PM2.5-exposed mice were treated with 2-, 5-, and 8-mg/kg doses of betulinic acid. The BALF samples were analyzed by (A) western blotting and (B) ELISA assays for TNF- α and IL-6 content. * $P < 0.05$ and ** $P < 0.02$ vs sham group.

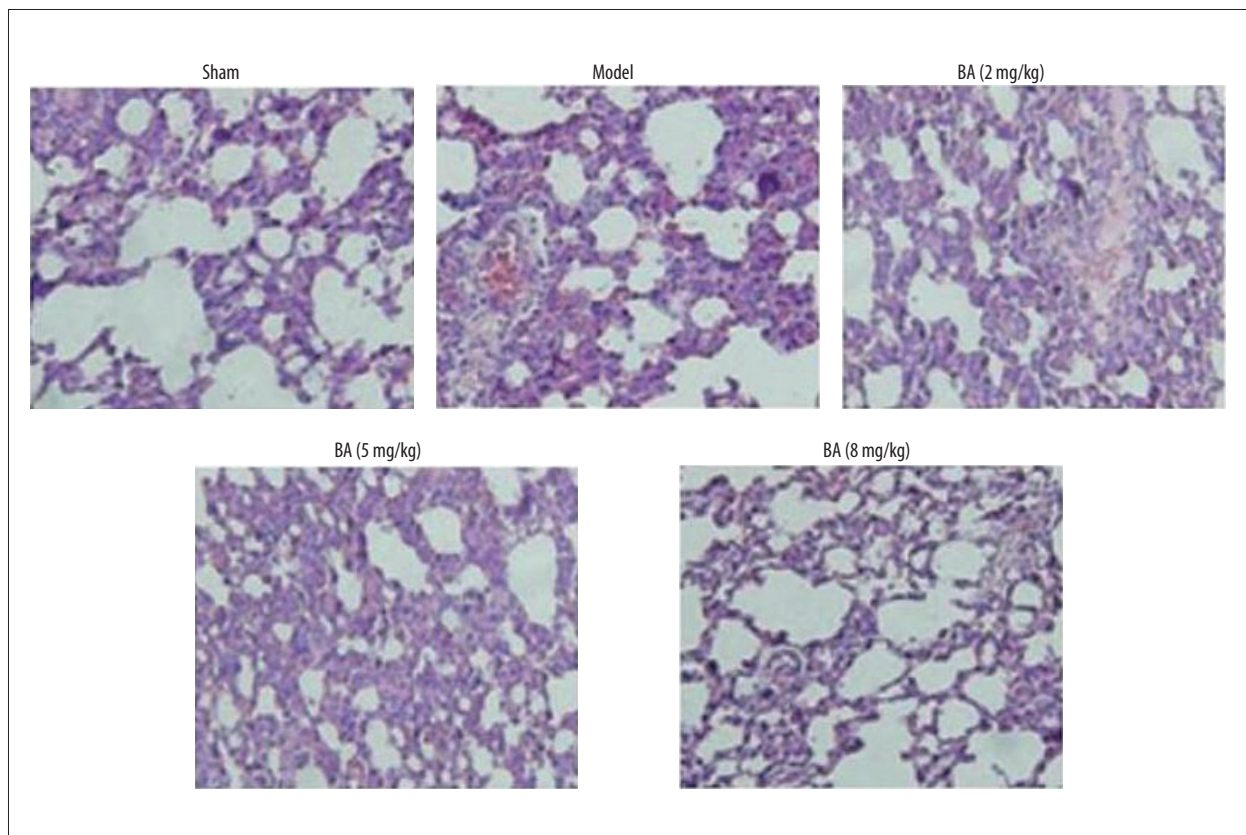


Figure 5. Betulinic acid inhibits PM2.5-induced histological alterations in mouse lungs. The PM2.5-exposed mice were treated with 2-, 5-, and 8-mg/kg doses of betulinic acid and pathological alterations were assessed by hematoxylin and eosin staining. Magnification, $\times 220$.

higher TNF- α and IL-6 levels compared to the sham group. However, betulinic acid treatment of PM2.5-exposed mice at 2-, 5-, and 8-mg/kg doses alleviated TNF- α and IL-6 levels in BALF. The decrease in PM2.5-induced TNF- α and IL-6 levels in mouse BALF was significant at 2 and 5 mg/kg and the effect was the strongest at the 8-mg/kg concentration.

Betulinic Acid Inhibits Tissue Damage and Inflammation Induced by PM2.5 in Mice

The preventive effect of betulinic acid on lung damage induced by PM2.5 in mice was determined by standard methodology [23,24]. Acute inflammatory exudate, increased inflammatory

cell infiltration, and damaged alveolar septae were observed in mice exposed to PM2.5 (Figure 5). In the sham mouse group, acute inflammatory exudate, increased inflammatory cell infiltration, and damaged alveolar septae were not observed. Betulinic acid treatment inhibited PM2.5-induced acute inflammatory exudate, alveolar septae damage, and inflammatory cell infiltration in mouse lungs at 2-, 5-, and 8-mg/kg doses. The preventive effect of betulinic acid on PM2.5-induced acute inflammatory exudate, alveolar septae damage, and inflammatory cell infiltration in mouse lungs was strongest at 8-mg/kg doses.

Discussion

Reactive oxygen species are excessively produced in animal lungs by exposure to particulate matter, which is the major factor leading to bronchial alveolar wall thickening [26]. Many respiratory diseases in humans are associated with particulate matter exposure, especially to PM2.5. Pulmonary chronic inflammation induced by PM2.5 is the main cause of several diseases, including pulmonary hypertension and COPD [27]. In developing countries, sharp increases in the incidence of respiratory diseases, including asthma, pneumonia, and COPD, are being caused by increased PM2.5 content in the atmosphere.

Respiratory diseases induced by PM2.5 are a serious health issue globally; therefore, studies to develop treatments, particularly using traditional Chinese medicine, are increasingly being performed [28-30]. Lung inflammation in smokers is inhibited through reduction of chemokine expression by treatment with tuberostemonine [31]. Inflammation of lung airways in house dust mite-mediated asthma patients is inhibited by resveratrol treatment [14]. Improvement in pulmonary function has been reported in a PM2.5-induced mouse model of lung damage by treatment with GubenZhike via inflammatory cell infiltration suppression [32]. The present study showed that PM2.5 promoted aggregation of inflammatory

cells, enhanced acute inflammatory exudate, and damaged lung tissues in mice. PM2.5 exposure activates innate immune cells as well as epithelial cells, which subsequently promotes excessive inflammatory cytokine production and elevated antibacterial protein levels [33,34]. The present study found elevated levels of LDH and ACP in the mouse lung tissues exposed to PM2.5. Moreover, AKP and ALB levels in lung tissues of mice were also higher following PM2.5 exposure relative to the sham group. Level of oxidants like MDA, iNOS, TNF- α , and IL-6 also showed significant increases in mouse lung tissues after exposure to PM2.5. Additionally, SOD activity was downregulated in PM2.5-exposed mouse BALF compared to sham mice. However, betulinic acid treatment of PM2.5-exposed mice caused a significant suppression in levels of inflammatory cytokines and inhibited ROS generation in the BALF. Betulinic acid treatment effectively reversed PM2.5-mediated suppression of SOD activity in mouse BALF. In the present study, acute inflammatory exudate, increased inflammatory cell infiltration, and damaged alveolar septae were observed in mice exposed to PM2.5. However, betulinic acid treatment inhibited PM2.5-induced acute inflammatory exudate, alveolar septae damage, and inflammatory cell infiltration in mouse lungs at 2-, 5-, and 8-mg/kg doses.

Conclusions

Betulinic acid inhibits PM2.5-mediated inflammation and damage of lungs through downregulation of inflammatory molecules and increase in anti-oxidative factors. Moreover, betulinic acid treatment inhibited PM2.5-induced acute inflammatory exudate, alveolar septae damage, and inflammatory cell infiltration in mouse lungs. The findings from this study in a mouse model of particulate matter 2.5-induced COPD and lung inflammation support the need for further in vivo studies and clinical evaluation of the effects of betulinic acid derivative in patients with COPD.

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