Zhike pingchuan granules improve bronchial asthma by regulating the IL-6/JAK2/STAT3 pathway

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Abstract. The present study aimed to investigate the effects of zhike pingchuan granules (ZKPC) on bronchial asthma and the underlying mechanism. A bronchial asthma mouse model was established by aerosol inhalation of ovalbumin. The changes in lung pathomorphology were observed by hematoxylin and eosin staining. The levels of IL-1β, TNF-α and IL-6 in bronchoalveolar lavage fluid (BALF) and serum were detected by corresponding ELISA kits. Levels of reactive oxygen species, malondialdehyde and superoxide dismutase in lung tissues were analyzed using corresponding kits. The expression of proteins related to apoptosis and the IL-6/janus kinase 2 (JAK2)/STAT3 pathway was detected by western blot analysis. The results showed that ZKPC significantly restored the dry/wet ratio and alleviated lung pathomorphology of bronchial asthmatic mice. In addition, ZKPC inhibits inflammation, oxidative stress levels and cell apoptosis in bronchial asthmatic mice and also suppressed the IL-6/JAK2/STAT3 pathway. Fedratinib (a JAK2 inhibitor) further strengthened the alleviative effects of ZKPC on bronchial asthma. In conclusion, ZKPC improved bronchial asthma by suppressing the IL-6/JAK2/STAT3 pathway.

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Introduction

Bronchial asthma is a heterogeneous disease characterized by chronic airway inflammation and airway responsiveness (1). The disease can occur at any age, but is more common in children and is a common cause of pediatric emergency worldwide (2). Approximately one-third of children present with wheezing before the age of two, and about one-fifths of children present with recurrent or persistent wheezing (3). The number of children with bronchial asthma in China is reported to be about 30 million (4). Childhood asthma in the young age group (aged <5 years) in developing countries is often misdiagnosed as pneumonia without effective treatment, leading to increased morbidity and mortality in this age group (5). Although the rate of bronchial asthma in China has risen from 28.7% in 2008 to 39.2% in 2016, it remains at a low level (6). With the development of traditional Chinese medicine (TCM) research, the advantages of TCM in the treatment of childhood bronchial asthma have been clinically valued (7). Therefore, it is of importance to find effective methods or drugs to treat childhood bronchial asthma from the perspective of TCM.

TCM has been widely used and demonstrated to be effective in the treatment of bronchial asthma (8-10). Zhike pingchuan granules (ZKPC) play a role in relieving coughing and reducing

sputum to relieve asthma. Their ingredients include *Gingko*, *Lumbricus*, *Perilla* leaves, rhizome *Pinelliae preparata*, *Mibaibu stemona root*, *Radix asteris*, *Folium eriobotryae*, *Platycodon grandiflorus*, *Scutellaria baicalensis*, apricot kernel, peach kernel, *Cortex mor*i and *Digupi*. Jin *et al* (11) demonstrated that *Ginkgo lactone* showed anti-inflammatory and anti-oxidant effects in a rat model of $A\beta_{1-40}$ induced Alzheimer's disease, improving nerve injury and cognitive function. Thorpe *et al* (12) indicated that the *Gingko biloba* extract EGb 761 could inhibit inflammation and thermal hyperalgesia for the treatment of inflammatory pain. Wuwei dilong decoction could inhibit the infiltration and spread of inflammatory cells in asthma (13). In addition, the active ingredients of *Aster tataricus* and *Pinellia ternata* were both demonstrated to have anti-inflammatory properties (14,15).

Airway inflammation is an important factor in the development of bronchial asthma. IL-6 is synthesized and secreted by lymphocytes and mononuclear macrophages after activation and is related to local inflammatory response, participates in the body's inflammatory damage process and is also the main inflammatory factor of bronchial asthma (16,17). Janus kinase 2 (JAK2)/STAT3 signaling plays an important role in various biological activities, such as tumorigenesis and inflammation (18). JAK2 can phosphorylate STAT3, which activates STAT3 and downstream target genes (19). Studies have shown that JAK2/STAT3 signaling is crucial for the pathogenesis of asthma (20,21). Furthermore, Simon *et al* (22) found that STAT3 inhibition could suppress proliferation of airway smooth muscle cells in asthma. Therefore, the JAK2/STAT3 signaling pathway plays crucial roles in the development and progression of asthma. Yan *et al* (23) demonstrated that JAK2 is associated with airway remodeling in bronchial asthma. Shi *et al* (24) indicated that IL-6 could promote the activation of the JAK2/STAT3 pathway.

Therefore, the present study aimed to investigate the therapeutic effects of ZKPC on bronchial asthma, as well as the underlying mechanism related to the IL-6/JAK2/STAT3 pathway.

Materials and methods

Mouse model of bronchial asthma. A total of 60 male C57BL/6J mice (specific-pathogen-free; age, 6-8 weeks; weight range, 16-20 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., and were maintained under a 12-h light/dark cycle at room temperature (22±2˚C) with a humidity of 45% and allowed *ad libitum* access to water and food. Each group contained 10 mice. Normal saline (5 ml) was suspended with 200 mg aluminum hydroxide to prepare a 4% aluminum hydroxide gel. A total of 1.5 mg ovalbumin (OVA; Thermo Scientific Fisher, Inc.) was fully dissolved in 2.5 ml normal saline, which was then suspended in 2.5 ml 4% aluminum hydroxide gel to prepare the sensitizing solution. Mice was intraperitoneally injected with 0.2 ml/day of newly prepared sensitizing solution for 15 days in total. ZKPC granules (manufactured by Henan Shizhen Pharmaceutical Co., Ltd.) were purchased from the Second Affiliated Hospital of Henan University of Chinese Medicine. ZKPC solution was prepared by mixing ZKPC granules with distilled water to obtain a final ZKPC solution concentration of 0.1 g/ml.

In the first set of experiments, from days 15-30, the mice in the Model group were placed in a transparent airtight chamber and inhaled aerosolized 1% OVA solution naturally for 30 min/day (n=10). Mice in the control group were treated with normal saline in the same manner (n=10).

In the ZKPC-Low (L) group, mice were administered with equivalent doses of ZKPC (0.525 g/kg) by gavage 30 min prior to aerosol inhalation (n=10). In the ZKPC-High (H) group, mice were administered with ZKPC at four times the equivalent dosage (2.1 g/kg) by gavage 30 min prior to aerosol inhalation (n=10). At 24 h after the last aerosol inhalation, mice were anesthetized via intraperitoneal injection of 1% pentobarbital sodium (40 mg/kg), and blood from the eyeballs (0.2-0.3 ml) and bronchoalveolar lavage fluid (BALF) were obtained. Subsequently, anaesthetized mice were killed by cervical dislocation and lung tissues were extracted from mice after confirmation of cardiac arrest. The control group and model group were given equal amounts of distilled water.

In the second set of experiments, mice in the fedratinib (Fedr) group were administered with Fedr (60 mg/kg) by gavage 30 min prior to aerosol inhalation (n=10). In the ZKPC + Fedr group, mice were administered with Fedr (60 mg/kg) and ZKPC at four times the equivalent dosage (2.1 g/kg) by gavage 30 min prior to aerosol inhalation (n=10). The dose of Fedr was determined and modified based on a previous study (25). Mice health and behavior were monitored every day. All animal experiments were approved by the Animal Experimental Ethics Committee of Henan University of Traditional Chinese Medicine (approval no. 20190412WZ).

Dry/wet (D/W) ratio of the lungs. The lower lobe of the right lung was taken, of which the wet weight was recorded. The lung dry weight was recorded after baking in an oven for 72 h. The D/W ratio of lung was then calculated.

Hematoxylin and eosin (H&E) staining. Lung tissue was collected, fixed with 10% formalin solution at 4˚C for >24 h and dehydrated in different concentrations (low to high) of ethanol. Samples were then embedded in paraffin, which were cut into 5 μm sections. Following hematoxylin staining for 3 min and eosin staining for 3 min at room temperature, the slices were sealed. Finally, the pathological morphology of mouse lung tissue was observed under a full-field pathological slice scanner.

ELISA. BALF and serum (stored at -80˚C) were thawed. The levels of IL-1β, TNF-α and IL-6 in BALF and serum were detected by IL-1β ELISA kit (cat. no. PI301), TNF-α ELISA kit (cat. no. PT512) and IL-6 ELISA kit (cat. no. PI326), respectively, according to the manufacturer's protocol (Beyotime Institute of Biotechnology).

Detection of oxidative stress factors. Part of the lung tissue was prepared into 10% tissue homogenate, which was centrifuged at 10,000 x g for 20 min at 4˚C. The supernatant was collected and tested with reactive oxygen species (ROS; cat. no. E-BC-K138-F; Elabscience Biotechnology Inc.), malondialdehyde (MDA; cat. no. S0131S; Beyotime Institute of Biotechnology) and superoxide dismutase (SOD; cat. no. S0109; Beyotime Institute of Biotechnology) kits.

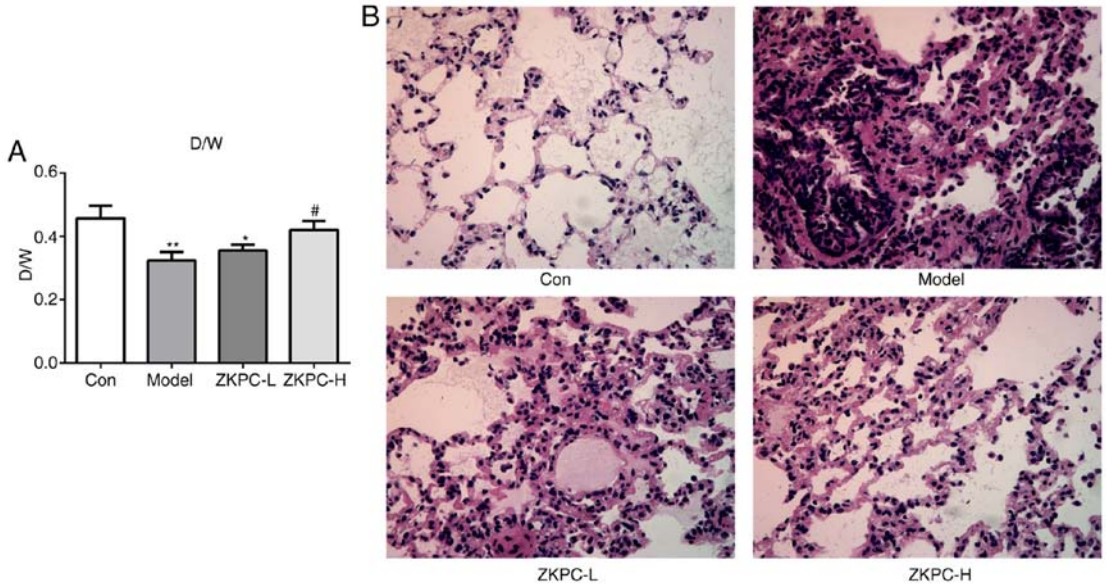

Figure 1. ZKPC improves bronchial asthma-induced lung injury. (A) D/W ratio of lung tissues was calculated after weighing. *P<0.05 and **P<0.01 vs. Con group. $^{\#}$P<0.05 vs. Model group. (B) Lung pathomorphology was observed by hematoxylin and eosin staining (magnification, x400). n=10. D/W, dry/weigh; Con, control; ZKPC, zhike pingchuan granules; L, low; H, high.

Western blot analysis. Lung tissue samples were homogenized using RIPA buffer and centrifuged at 10,000 x g for 10 min at 4˚C. Protein concentration was determined using the BCA method. Total protein (25 μg) were loaded per lane and separated via 10% SDS-PAGE, followed transfer to PVDF membranes. Subsequently, membranes were blocked in 5% non-fat milk in TBS-Tween-20 (0.05%) buffer for 1 h at 25˚C and incubated with primary antibodies overnight at 4˚C. Membranes were then incubated with rabbit IgG horseradish peroxidase-conjugated secondary antibody (cat. no. 7074; dilution, 1:1,000; Cell Signaling Technologies, Inc.) for 1 h at 25˚C. The primary antibodies (all from Cell Signaling Technology, Inc.) used were as follows: Anti-IL-6 (cat. no. 12912; dilution, 1:1,000), anti-Bcl2 (cat. no. 3498; dilution, 1:1,000), anti-Bax (cat. no. 2772; dilution, 1:1,000), anti-cleaved (c) caspase-3 (cat. no. 9661; dilution, 1:1,000), anti-caspase-3 (cat. no. 9662; dilution, 1:1,000), anti-JAK2 (cat. no. 3230; dilution, 1:1,000), anti-phosphorylated (p)-JAK2 (cat. no. 3771; dilution, 1:1,000), anti-STAT3 (cat. no. 12640; dilution, 1:1,000), anti-p-STAT3 (cat. no. 9145; dilution, 1:2,000) and anti-GAPDH (cat. no. 5174; dilution, 1:1,000). Bands were visualized using an enhanced chemiluminescence system (Amersham Biosciences; Cytiva) and band gray values were semi-quantified using ImageJ software (version 1.0; National Institutes of Health).

Statistical analysis. Data were represented by the mean ± SD. SPSS 20.0 (IBM Corp.) was used to conduct one-way ANOVA and Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference. All experiments were replicated three times.

Results

ZKPC improves lung injury induced by bronchial asthma. The D/W ratio of model group was significantly lower compared with the control group. In the ZKPC-H group, the D/W ratio was significantly increased compared with the model group (Fig. 1A). In the model group, alveolar spaces presented with obvious hyperemia, bleeding and cell infiltration, and alveolar walls were not clearly observed. Upon increasing concentrations of ZKPC, hyperemia, bleeding and cell infiltration in tissue interspace and alveolar cavity were markedly decreased, and the structure of most alveolar walls were complete in the ZKPC-H group (Fig. 1B).

ZKPC improves inflammation and oxidative stress in bronchial asthma mice. As shown in Fig. 2A, the levels of IL-1β, TNF-α and IL-6 were significantly upregulated in BALF, which was gradually suppressed by increased concentrations of ZKPC. The levels of IL-1β, TNF-α and IL-6 in serum showed similar changes to that in BALF (Fig. 2B). As shown in Fig. 2C-E, the levels of ROS and MDA increased while SOD levels decreased in lung tissues of the model group, and ZKPC effectively reversed the levels of ROS, MDA and SOD.

ZKPC inhibits cell apoptosis and the IL-6/JAK2/STAT3 pathway in lung tissue. The expression levels of IL-6, Bax and c-caspase-3 increased while Bcl-2 expression levels declined in lung tissues of model mice. Upon increasing concentrations of ZKPC, the expression levels of IL-6, Bax and c-caspase-3 were gradually downregulated while Bcl-2 expression levels were upregulated in lung tissues (Fig. 3A). p-JAK2 and p-STAT3 levels in lung tissues of model mice was upregulated, which was partially reversed by increasing concentrations of ZKPC (Fig. 3B).

Fedratinib enhances the improvement effects of ZKPC on bronchial asthma-induced lung injury. Fedratinib treatment upregulated the D/W ratio compared with the model group, and the D/W ratio was further increased with co-treatment of ZKPC and fedratinib (Fig. 4A). Lung injury in Fedr group was alleviated while the degree of improvement was lower

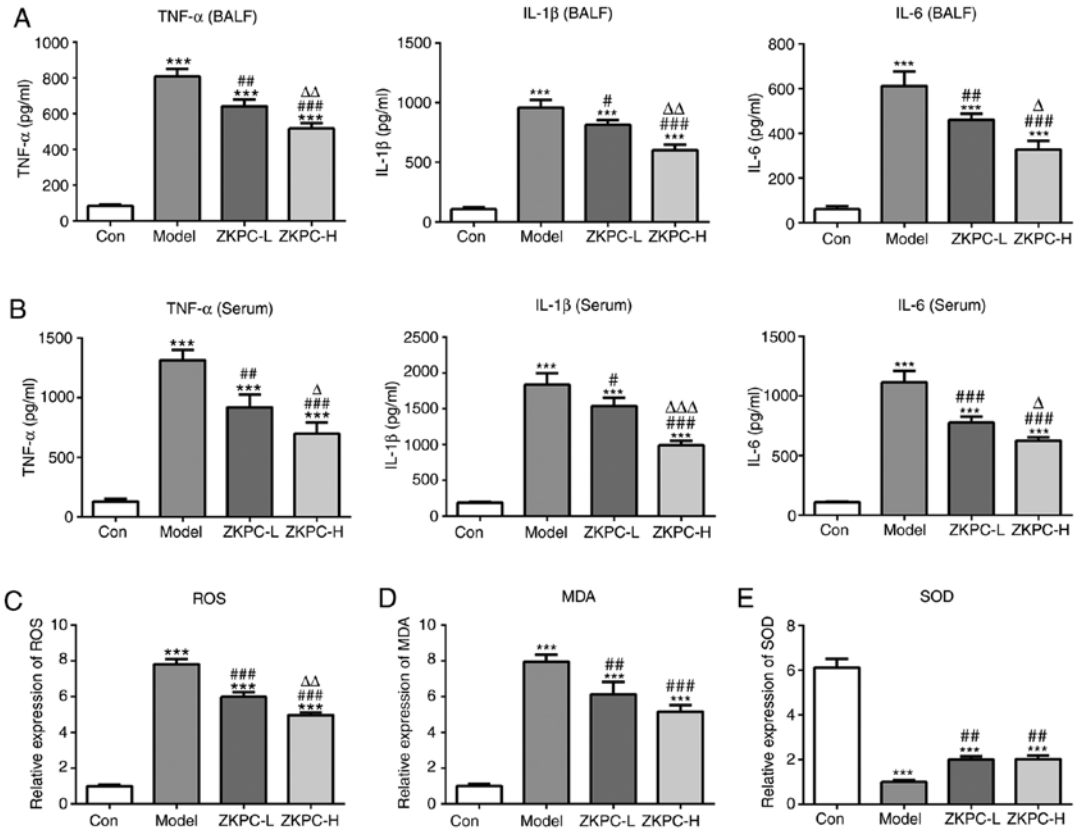

Figure 2. ZKPC improves inflammation and oxidative stress in bronchial asthmatic mice. The levels of IL-1β, TNF-α and IL-6 in (A) BALF and (B) serum were analyzed by ELISA. (C) ROS levels in lung tissues was detected using a ROS kit. (D) MDA levels in lung tissues was detected using an MDA kit. (E) SOD levels in lung tissues was detected using a SOD kit. ***P<0.001 vs. Con group. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. Model group. $^{\Delta}$P<0.05 and $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 vs. ZKPC-L group. n=10. BALF, bronchoalveolar lavage fluid; ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase; ZKPC, zhike pingchuan granules; L, low; H, high; Con, control.

compared with the ZKPC group. Co-treatment of ZKPC and fedratinib showed the highest improvement effects on lung tissues (Fig. 4B).

Fedratinib enhances the improvement effects of ZKPC on inflammation and oxidative stress in bronchial asthmatic mice. Fedratinib treatment downregulated the levels of IL-1β, TNF-α and IL-6, and co-treatment of ZKPC and fedratinib showed further inhibition effects on the levels of IL-1β, TNF-α and IL-6 in both BALF and serum (Fig. 5A and B). The levels of ROS and MDA decreased and SOD levels increased in lung tissues treated with fedratinib, and co-treatment of ZKPC and fedratinib further decreased levels of ROS and MDA, while SOD levels did not significantly change (Fig. 5C-E).

Fedratinib enhances the inhibitory effects of ZKPC on cell apoptosis and the IL-6/JAK2/STAT3 pathway in lung tissue. The expression levels of IL-6, Bax and c-caspase-3 declined, while Bcl-2 expression levels increased in lung tissues of model mice treated with fedratinib. Meanwhile, the expression on IL-6, Bax, c-caspase-3 and Bcl-2 were further declined by co-treatment of ZKPC and fedratinib (Fig. 6A). Fedratinib treatment downregulated p-JAK2 levels, which was further suppressed by co-treatment of ZKPC and fedratinib. p-STAT3 levels slightly decreased in the Fedr group and further decreased in ZKPC + Fedr group (Fig. 6B).

Discussion

Asthma is presented with repeated and reversible airway obstruction, which is often associated with airway hyperresponsiveness and inflammation (26). Although glucocorticoid drugs are the preferred treatment for alleviating asthma, there are many adverse effects caused glucocorticoids, which makes a safe dose difficult to determine. In addition, asthmatic patients are unable to inhale glucocorticoids daily, limiting the curative effect of glucocorticoids in asthma therapy (27,28). Therefore, it is urgent to find newly identified drugs for the treatment of asthma that are highly efficacious, easily managed and with fewer adverse effects.

As a pre-inflammatory factor with multiple biological functions, IL-6 plays an important role in the proliferation and differentiation of immune-modulating inflammatory response cells, and is closely related to the incidence of inflammatory diseases, such as asthma and rheumatoid arthritis (29). IL-6 expression was shown to be high in the serum, lung tissues and BALF of asthmatic patients (30,31). Kuhn *et al* (32) found that in mouse experiments, significant expression of IL-6 resulted in the expansion of alveolar cavities, followed by the infiltration of peripheral tracheal monocytes, which gradually resulted in the thickening of the airway wall, airway epithelial fibrosis and airway remodeling. The present study found increased levels of IL-6, IL-1β and TNF-α in the serum and BALF of bronchial asthmatic mice. In addition, ZKPC

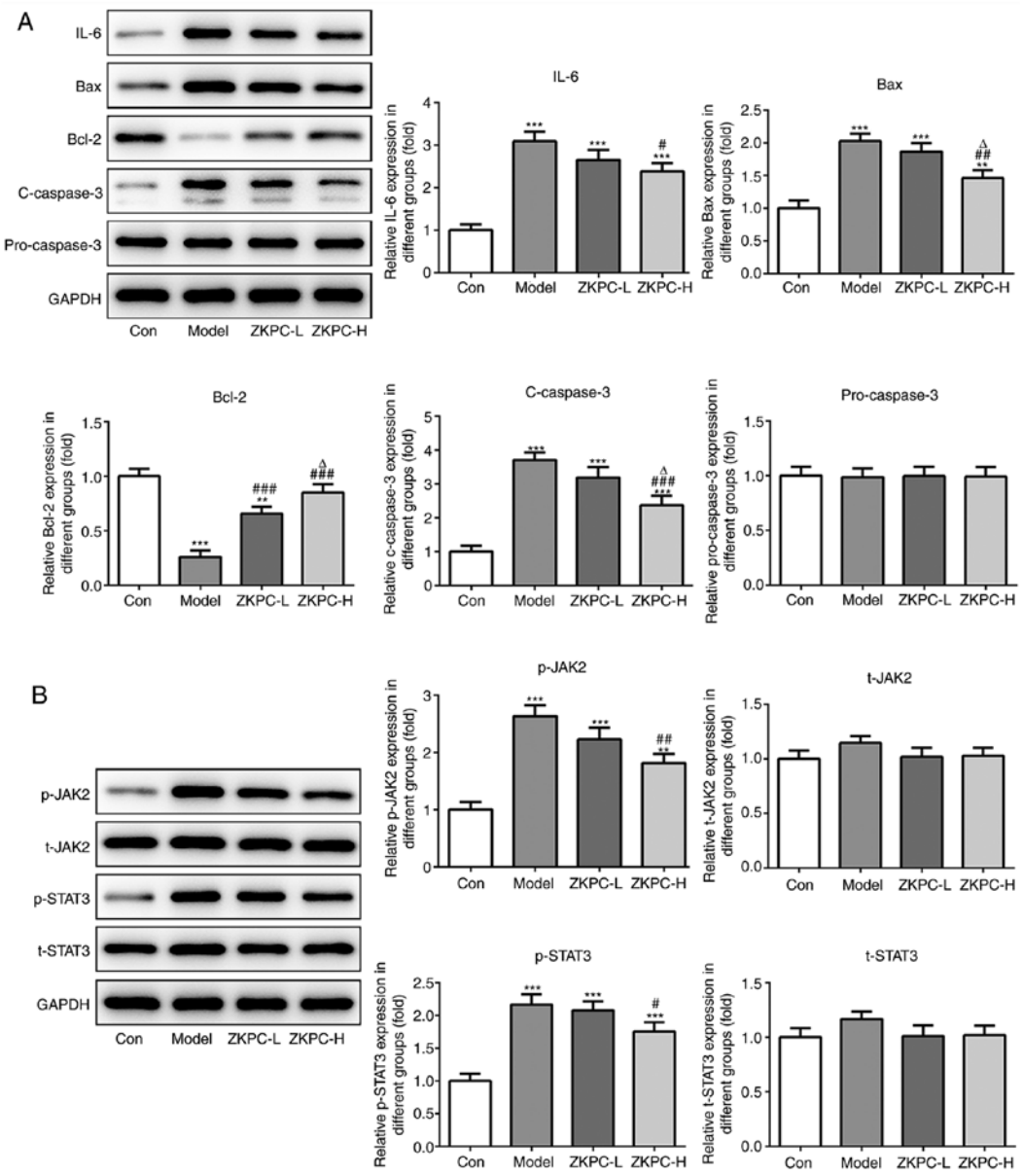

Figure 3. ZKPC inhibits cell apoptosis and the IL-6/JAK2/STAT3 pathway in lung tissue. (A) The expression of IL-6, Bax, Bcl-2, c-caspase-3 and pro-caspase-3 in lung and (B) levels of t-JAK2, p-JAK2, t-STAT3 and p-STAT3 in lung tissues were determined by Western blot analysis. **P<0.01 and ***P<0.001 vs. Con group. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. Model group. $^{\Delta}$P<0.05 vs. ZKPC-L group. n=10. c, cleaved; t, total; p, phosphorylated; ZKPC, zhike pingchuan granules; L, low; H, high; Con, control.

treatment decreased the levels of IL-6, IL-1β and TNF-α in serum and BALF.

A study showed that STAT3 mediated the occurrence and development of wheezing and could reduce airway inflammation and remodeling by inhibiting the activation of STAT3 signaling (20). Gavino *et al* (33) found that STAT3 activation could lead to the accumulation of local inflammatory factors in the airway, hence aggravating the original airway inflammation. This indicated that inhibiting STAT3 expression could block the transmission of relevant inflammatory signals, effectively treat airway inflammation, control repeated wheezing and result in the prevention of asthma. Endogenous STAT3 could promote the ability of smooth muscle cells to generate blood vessels by activating endothelial growth factors in asthmatic patients, which could be a possible target for asthma treatment (34). JAK2 is demonstrated to be important in the pathogenesis of asthma (35). Gong *et al* (36) indicated that kaempferol suppressed eosinophil infiltration and airway inflammation in mice with allergic asthma by inhibiting JAK2. Nakano *et al* (37) found that niflumic acid alleviated IL-13-induced asthma in mice by suppressing JAK2. IL-6 can combine with soluble IL-6R to activate glycoprotein 130 of the cell membrane surface, thus activating JAK2/STAT3 signaling pathway (38). The present study confirmed that the expression levels of IL-6, p-JAK2 and p-STAT3 in lung tissues of bronchial asthmatic mice were significantly increased, which were downregulated by ZKPC treatment. Fedratinib, a JAK2 inhibitor, further enhanced the improvement effects of ZKPC on bronchial asthmatic mice.

Various traditional Chinese medicines related to pingchuan have been demonstrated to improve asthma. Liu *et al* (39) indicated that pingchuan formula alleviated asthma in mice by restoring the balance of the T-helper cell17/regulatory T cell

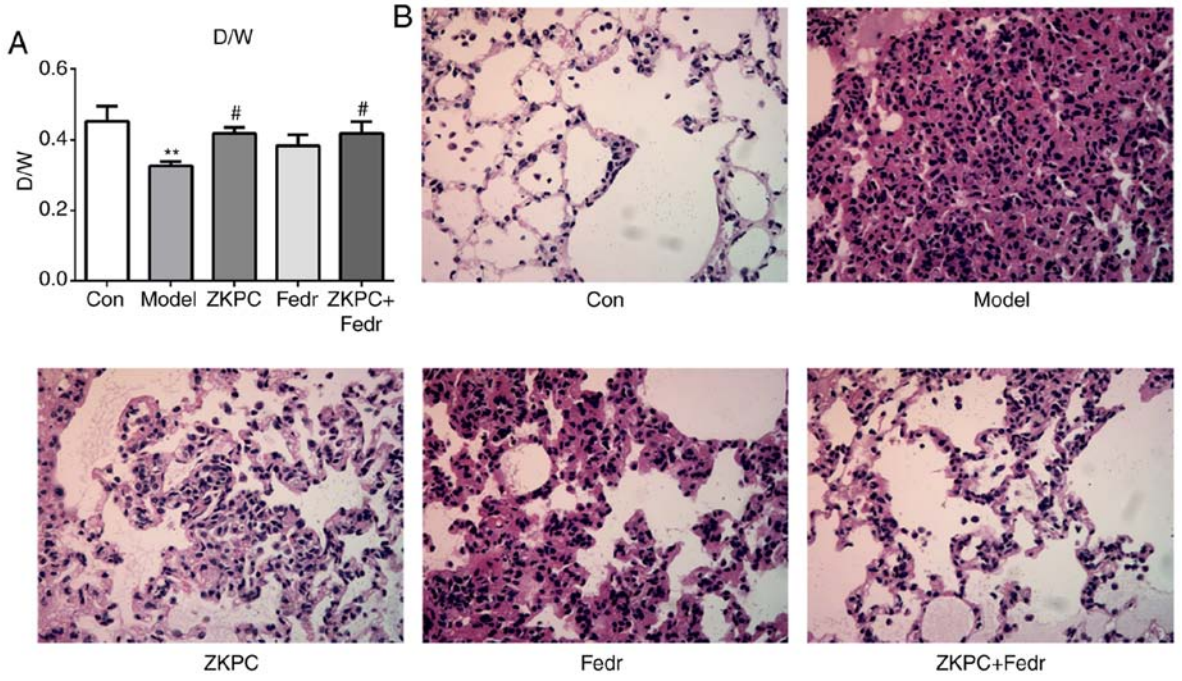

Figure 4. Fedratinib enhances the improvement effects of ZKPC on bronchial asthma-induced lung injury. (A) The D/W ratio of lung tissues was calculated after weighing. (B) Lung pathomorphology was observed by hematoxylin and eosin staining (magnification, x400). **P<0.01 vs. Con group. $^{\#}$P<0.05 vs. Model group. n=10. Fedr, fedratinib; Con, control; ZKPC, zhike pingchuan granules; L, low; H, high; D/W, dry/weight.

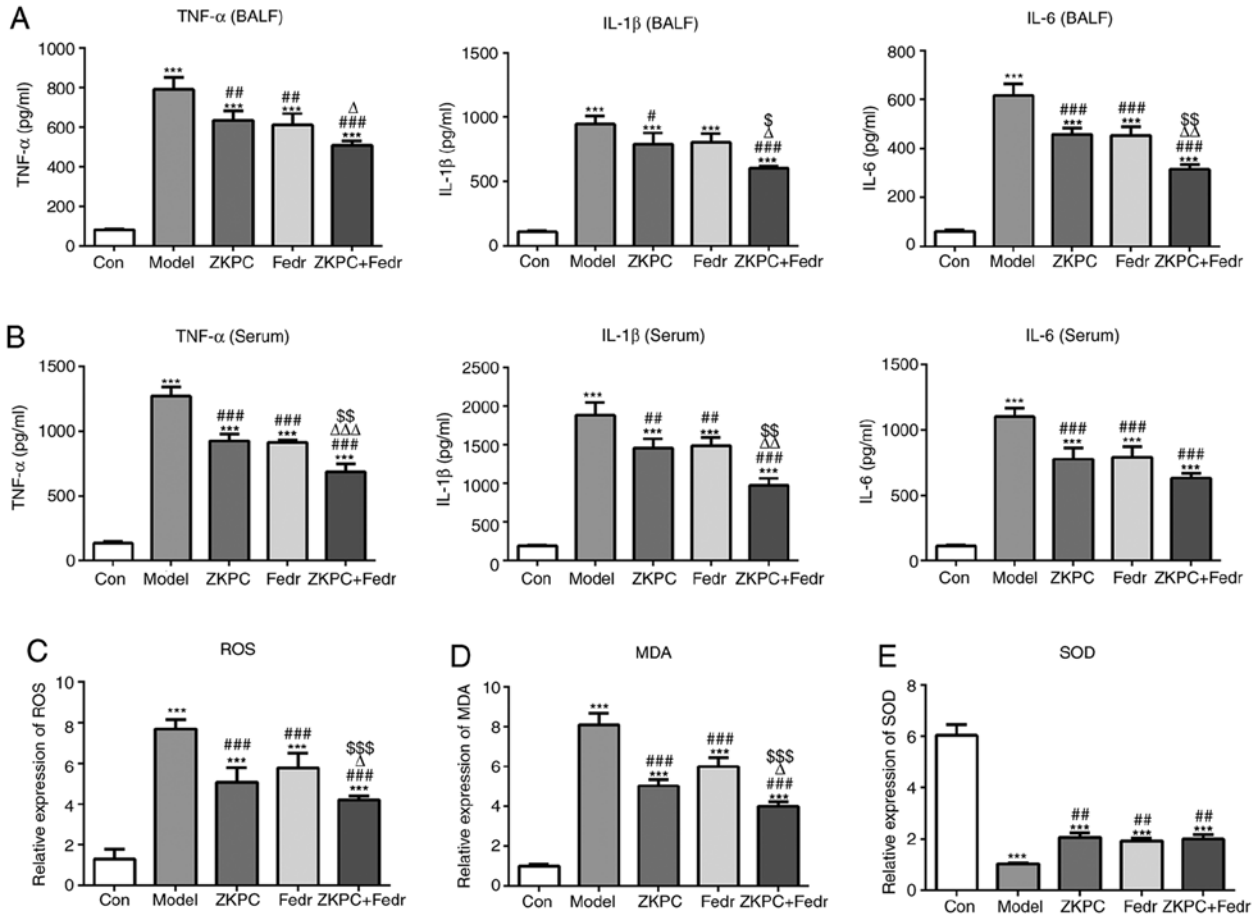

Figure 5. Fedratinib enhances the improvement effects of ZKPC on inflammation and oxidative stress in bronchial asthmatic mice. (A) The levels of IL-1β, TNF-α and IL-6 in BALF and (B) serum were analyzed by ELISA. (C) ROS levels in lung tissues were detected using a ROS kit. (D) MDA levels in lung tissues was detected using an MDA kit. (E) SOD levels in lung tissues was detected using a SOD kit. n=10. ***P<0.001 vs. Con group. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. Model group. $^{\Delta}$P<0.05, $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 vs. ZKPC group. $^{\$}$P<0.05, $^{\$\$}$P<0.01 and $^{\$\$\$}$P<0.001 vs. Fedr group. BALF, bronchoalveolar lavage fluid; ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase; ZKPC, zhike pingchuan granules; L, low; H, high; Con, control; Fedr, fedratinib.

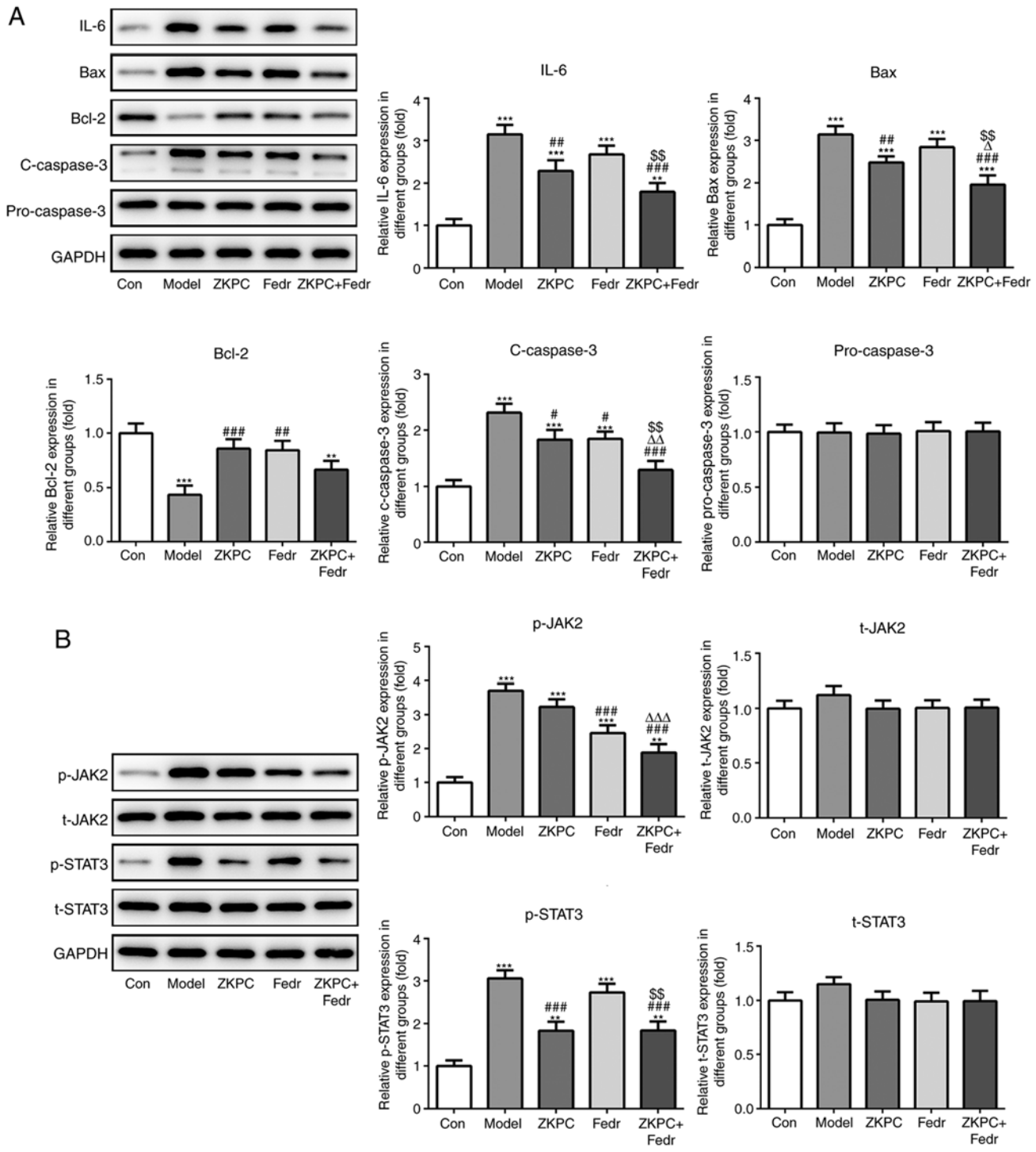

Figure 6. Fedratinib enhances the inhibitory effects of ZKPC on cell apoptosis and the IL-6/JAK2/STAT3 pathway in lung tissue. (A) The expression of IL-6, Bax, Bcl-2, c-caspase-3 and pro-caspase-3 and (B) levels of t-JAK2, p-JAK2, t-STAT3 and p-STAT3 in lung tissues were determined by western blot analysis. **P<0.01 and ***P<0.001 vs. Con group. $^{###}$P<0.001 vs. Model group. ^{ΔΔΔ}P<0.001 vs. ZKPC group. $^{$$}$P<0.01 vs. Fedr group. (n=10) **P<0.01 and ***P<0.001 vs. Con group. $^{#}$P<0.05, $^{##}$P<0.01 and $^{###}$P<0.001 vs. Model group. ^{Δ}P<0.05, ^{ΔΔ}P<0.01 and ^{ΔΔΔ}P<0.001 vs. ZKPC group. $^{$$}$P<0.01 vs. Fedr group. c, cleaved; t, total; p, phosphorylated; ZKPC, zhike pingchuan granules; L, low; H, high; Con, control.

ratio. Pan *et al* (40) found that Yanghe Pingchuan granules could ameliorate airway remodeling in a asthma rat model. Wang *et al* (41) indicated that Wenyang Yiqi Pingchuan improved lung pathomorphology and decreased the contents of nitric oxide and endothelin-1 in lung tissues of rats with bronchial asthma. These compounds contain ingredients which are also found in ZKPC. The present study found that ZKPC could alleviate lung pathomorphology, decreased the levels of oxidative stress and suppressed cell apoptosis in lung tissues of bronchial asthmatic mice.

In conclusion, ZKPC partially reversed the D/W ratio and improved the lung pathomorphology of bronchial asthmatic mice. ZKPC inhibited inflammation, oxidative stress levels and cell apoptosis by suppressing the IL-6/JAK2/STAT3 pathway. In addition, the effects of ZKPC on bronchial asthma was further promoted by fedratinib treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YM designed the experiments and revised the manuscript. YR and YL performed the experiments and wrote the manuscript. YR, YL, SW, ZL, YY, XG, JH, SZ, HS, XT, QW, CC and YZ analyzed and interpreted the data. YR, YL, SZ and HS confirmed the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by the Animal Experimental Ethics Committee of Henan University of Traditional Chinese Medicine (approval no. 20190412WZ).

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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