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Original Article

The effect of Pingchuan decoction on EOS apoptosis and related factors Bcl-2, Bax, and IL-5 levels in the lung tissue of rat asthma model

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Abstract: Objective: This study is to identify the effect of Pingchuan decoction on the lung tissue morphology, Eosinophil (EOS) apoptosis, and association with factors Bcl-2, Bax and IL-5 expressions in rat asthma model. Methods: SD rats were randomly divided into normal group, model group, positive control group, and treatment group. Ovalbumin (OVA) was used to establish asthma model through sensitization and stimulation and was administered by gavage (ig) for 14 days. The general condition of the rats was observed. The lung morphology was observed by HE staining and the lung index was calculated. TUNEL was adopted to detect the *in situ* expression of EOS in lung tissue. qRT-PCR and Weston Blot methods were selected to detect Bcl-2 and Bax mRNA and protein expressions, correspondingly. ELISA was applied to measure IL-5 expression in lung tissue. Results: Compared with the normal group, evident pathological changes were found in the lungs tissue with significantly large amount of EOS in the model group ($P<0.01$). The mRNA and protein expressions of Bcl-2 were markedly upregulated, IL-5 were significantly increased, while Bax was obviously reduced in the model group compared to the normal group ($P<0.01$). Drug intervention significantly alleviated the asthmatic symptoms, improved the pathological damages of lung tissue, decreased the number of EOS, declined Bcl-2 mRNA and protein expressions, reduced IL-5 content, while upregulated Bax level ($P<0.01$). No statistical difference was observed between positive control group and the treatment group ($P>0.05$). Conclusion: Pingchuan decoction can promote airway inflammation regression, reduce airway epithelial cell injury, and improve the clinical symptoms by induction of EOS apoptosis and inhibition of EOS, which provide new insights for the asthma therapy.

Keywords: Pingchuan decoction, asthma, EOS apoptosis, Bcl-2, Bax, IL-5

Introduction

Bronchial asthma is one of the common chronic respiratory diseases. It is a heterogeneous syndrome featured by chronic airway inflammation participated by a variety of cells (such as eosinophils, mast cells, airway epithelial cells, etc.). The clinical symptoms contained recurrent wheezing, shortness of breath, chest tightness, or cough. Untimely diagnosis and treatment may lead to high mortality rate [1, 2]. In recent years, the prevalence and mortality of asthma keep increasing worldwide following the changes of lifestyle and the increase of environmental pollution, thus it seriously affects the physical and mental health of patients [3, 4]. Although effective, modern medical treatment on asthma is easy to relapse and causes drug side effects [5]. Traditional Chinese medicine has unique advantages in the prevention and treatment of bronchial asthma. Therefore, it is of great significance to combine the effect of traditional Chinese medicine with modern medicine for clinical application against asthma [6-8].

Pingchuan decoction develops with clinical experience based on Sanzi Yangqin decoction, combined with other types of therapeutic components. Previous studies demonstrated that this prescription can significantly alleviate the clinical symptoms caused by asthma, but the specific mechanism is still unclear [9, 10]. Airway inflammation is the essence of bronchi-

al asthma and poses as one of the main causes of airway hyperresponsiveness and recurrent attacks [11-14]. It is currently believed that although a variety of inflammatory cells are involved in the process of chronic airway inflammation, EOS infiltration and activation are frequently the ultimate common pathways [15-18]. Bcl-2 and Bax are the major related proteins of apoptosis pathway, while IL-5 is a key cytokine in the process of EOS maturation, endothelial adhesion and activation, which plays an important role in EOS infiltration and activity regulation in airway inflammation [19-22]. Therefore, this study will investigate the effect of Pingchuan decoction on asthma and determine its possible mechanisms by using a rat asthma model.

Materials and methods

Experimental animals

Forty eight healthy SD rats, aged 6-8 weeks and weighted 196.6±11.2 g, were fed with ovalbumin (OVA) at environmental temperature 21-25°C and humidity 52.1-64.2%. The rats were raised in the Experimental Animal Center of Yangzhou University. The experimental rats were purchased from the Experimental Animal Center of Nantong University [license number: SCXK (Su) 2014-0001]. All operations and protocols in this study were reviewed and approved by the Ethics Committee of the Zhangjiagang Hospital of Traditional Chinese Medicine Affiliated to Nanjing University of Chinese Medicine.

Drugs

Pingchuan soup was composed of 10 g ramie, 10 g almond, 10 g medlar, 10 g perillaseed, 15 g semen raphani, 10 g white mustard, 10 g fructusaurantii, 6 g platycodon, 30 g earthworm, 10 g silkworm, and 6 g schisandra. The traditional Chinese medicine decoction pieces were provided by the Chinese Pharmacy of Zhangjiagang Traditional Chinese Medicine Hospital (Suzhou, Jiangsu, China). They were made into a sterilized concentrated reagent after the mixture was fried and sealed by the Chinese medicine preparation room and stored at 4°C. Dexamethasone tablet (0.75 mg/tablet) dissolved in normal saline was used for the positive control group. Dexamethasone was purchased from Chenxin Pharmaceutical Co., Ltd., National Pharmaceutical Standard (H37021-969) (Jining, Shandong, China).

Reagents and instruments

Reagents

TUNEL apoptosis kit (No.: 11684817910) was purchased from Roche (Indianapolis, IN, USA). Ovalbumin (OVA) was bought from Sigma (Temecula, CA, USA). Aluminum Hydroxide Gel (H41024119) was obtained from Yucheng Pharmaceutical Co., Ltd (Dezhou, Shandong, China). TRIzol™ Reagent (No.: 15596026) was purchased from Thermo Fisher (Waltham, MA, USA). ReverTra Ace® qPCR RT Master Mix (No.: FSQ-101) and SYBR® Green Realtime PCR Master Mix (No.: QPK-201T) were purchased from TOYOBO (Osaka, Japan). Radio Immunoprecipitation Assay (RIPA) Lysate (No.: P0013C) and bicinchoninic acid (BCA) protein concentration detection kit (No.: P0012) were acquired from Beyotime (Beijing, China). Anti-Bax Antibody (No.: BA0315), Anti-Bcl-2 Antibody (No.: A00040-2), Anti-β-Actin (ACTB) Antibody (No.: BM0626), and HRP-labeled goat anti-rabbit and goat anti-mouse IgG secondary antibodies (No.: BA1056) were purchased from Boster (Shanghai, China). IL-5 ELISA kit (ab100711) was purchased from Abcam (Cambridge, MA, USA). Slice paraffin was provided by Shanghai Xinran Biochemical Reagent Co., Ltd. (Shanghai, China). Eosin dyeing was purchased from Boster (Shanghai, China). Hematoxylin dyeing solution and neutral gum were purchased from KGI Biotech (Claremont, CA, USA).

Instruments

Ultra-clean workbench was purchased from Suzhou Sujing Group Antai Company (Suzhou, Jiangsu, China). Electronic balance was bought from Shanghai Second Balance Instrument Factory (Shanghai, China). Microscope Nikon Eclipse E200 was purchased from Nikon Corporation (Chiyoda, Japan). Adhesive Slide was purchased from Jiangsu Hengtai Experimental Equipment Co., Ltd (Jiangyin, Jiangsu, China). Push-pull Paraffin Slicer was purchased from Lycra Company (Wilmington, DE, China). Ultrasonic atomizer 403C, YZB/Su 0024-2012 was acquired from Puze Pharmacy (Shanghai, China). Optical microscope was purchased from OLYMPUS (Tokyo, Japan). Automatic microplate reader 680 was purchased from BIO-

RAD (Hercules, CA, USA). Fluorescence quantitative PCR Model 7500 was purchased from ABI (Foster City, CA, USA).

Methods

Modeling and grouping

The experimental animals were randomly divided into 4 groups with 12 in each group, including normal control group, asthma model group, Pingchuan decoction treatment group, and positive control group (dexamethasone). Ovalbumin (OVA) was used to establish asthma model. 20 μg OVA and 20 mg aluminum hydroxide gel dissolved in 200 μL normal saline was administered intraperitoneally on day 0, 7, and 14 for sensitization and stimulation [5]. The normal control group was intraperitoneally injected with the same volume of distilled water. In the asthma group and the treatment group, asthma was induced by inhalation of 5% OVA challenge solution daily for about 0.5 hour starting 21 days after modeling for 7 continuous days. The normal control group was aerosolized with 0.9% NaCl. The behavior changes were observed after the last spray challenge.

Intervention

The rats were intragastrically administered from the first asthma challenge (model at 21st day) after atomization: a 19.2 g/kg drug solution was administered to the treatment group and 0.001 g/kg dexamethasone was administrated to the positive control. The normal control group and the asthma model group were given an equal volume of normal saline once a day for 14 days. The behavioral changes were observed again at the end of the last dosage. After the last administration for 24 hours, the body weight was weighed and the rats were sacrificed by cervical dislocation. The thoracic and abdominal cavity was opened, and the lung tissue was taken out under aseptic condition. The lung wet mass was weighed, and the appearance was observed. The lung tissue was rinsed with pre-cooled sterile PBS and fixed in 10% formalin for histopathological analysis. The left lung tissue was used to extract RNA and protein.

Index observation

Pathological observation: The lung tissue was sectioned and stained by HE. After sealing, the pathological morphology of lung tissue was observed under light microscope and quantitatively analyzed for OD value.

Pulmonary index measurement: Pulmonary index = lung wet mass/rat body mass after experiment.

EOS in situ expression detection: After paraffin tissue was sliced with a Lycra slicer (5 μm), EOS counts were detected by TUNEL method according to the instructions. After the slice was sealed, EOS counts were observed under a light microscope and quantitatively analyzed for OD value.

qRT-PCR: The lung tissue was added with 600 μL TRIZOL and ground by a tissue homogenizer. After lysed at room temperature for 10 min, the tissue was extracted by chloroform, precipitated by isopropanol, washed by 75% ethanol, and solved in DEPC water. After quantification and purification, 1 μg RNA was reverse-transcribed into cDNA according to the ReverTra Ace® qPCR RT Master Mix kit procedure. 2 μL cDNA was used as a template, β-actin was used as an internal reference, and Bcl-2 and Bax mRNA expressions were detected according to the SYBR® Green Realtime PCR Master Mix kit procedure. Bcl-2 primers, F: 5'-TTCTTTGAGTTCGGTGGGGTC-3', R: 5'-TGCATATTTGTTTGGGGCAGG-3'; Bax primers, F: 5'-TCCACCAAGAAGCTGAGCGAG-3', R: 5'-GTCCAGCCCATGATGGTTCT-3'; β-actin primers, F: 5'-TGAGCGAGGCTACAGCTT-3', R: 5'-TCCTTGATGTCGCGCACGATTT-3'.

Western blot: Total protein was extracted by RIPA on ice for 15 min and centrifuged at 4°C and 12000 g for 30 min. The supernatant was moved to a new Ep tube for quantification. A total of 50 μg protein was separated by 12% SDS-PAGE at 40 V for 4.5 h and transferred to NC membrane at 60 V for 2 h. Next, the membrane was blocked in 5% skim milk at room temperature for 1 h and incubated in primary antibody at 4°C overnight (Bcl-2, Bax, and β-actin at 1:500). Then the membrane was incubated in secondary antibody for 1 h. At last, the protein expression was detected by ECL chemiluminiscence.

IL-5 detection: The IL-5 ELISA kit was equilibrated for 15 to 30 min at room temperature. IL-5 expression level was detected by ELISA according to the instructions provided in the kit.

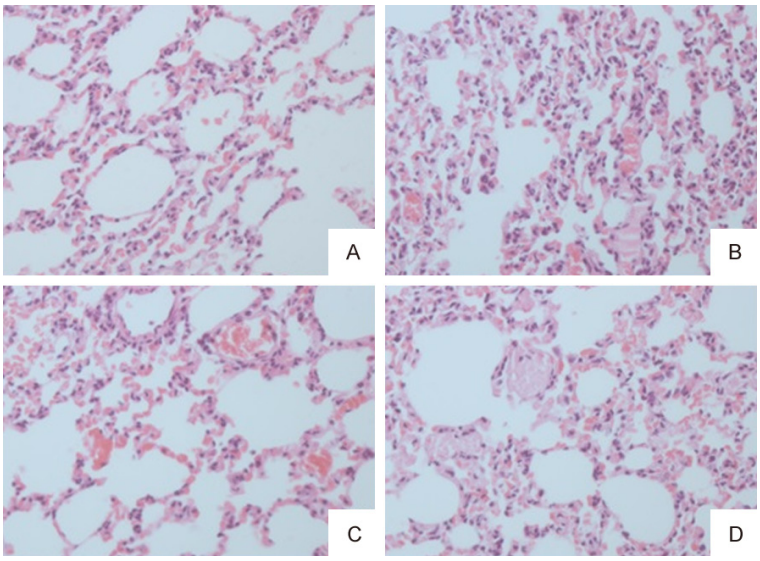

Figure 1. Pulmonary pathology (HE, 40×). A. Normal control; B. Model group; C. Positive control group; D. Treatment group.

Table 1. Pulmonary index comparison

Group	n	Pulmonary index ($\times 10^{-3}$)	F	P
Normal control	12	5.71±0.109	1542.44	<0.001
Model	12	8.69±0.105*		
Positive control	12	6.38±0.118*,#		
Treatment	12	6.42±0.126*,#		

*P<0.05, compared with normal control; #P<0.05, compared with model group.

Statistical analysis

All data were analyzed by GraphPad Prism5 software package and SPSS16.0 system software. The results were expressed as mean ± standard deviation ($\bar{x}$ ± sd) and compared by one-way analysis of variance. Pairwise comparison was performed by Bonferroni method. P<0.05 was considered as statistical significance.

Results

Behavior observation

Compared to the rats in normal, the behavior of rates in the model group, positive control group, and treatment group changed significantly after modeling of OVA, along with poor mentality, irritability, shaking hair, scratching nose, nodding breath, shortness of breath, unstable standing, abdominal muscle twitching, and incontinence, suggesting successful modeling. After drug intervention, the above symptoms of the modeling rats in the positive control group and the treatment group were alleviated to a different extent. No rats died during the experiment.

The impact of Pingchuan decoction on pulmonary pathology

The lung tissue of normal rats showed normal appearance, with no inflammatory cell infiltration, intact bronchial epithelium, and no obvious abnormalities in alveolar structure. However, the rats in model group were found with hyperemia and enlarged lung, bronchial mucosa hyperemia, lumen stenosis and thickening, with eosinophils, mast cells, and lymphocytes infiltrated in the wall. Ciliated epithelial cells were shed, and red blood cells and mucus were visible in the alveolar space. The pathological damages such as bronchial wall thickness and inflammatory cell infiltration in the positive control group and the treatment group were evidently alleviated compared to those in the model group (**Figure 1**).

The influence of Pingchuan decoction on pulmonary index

The pulmonary index of the model group, the positive control group, and the treatment group was significantly higher than that of the normal group (P<0.05). There was no statistical difference between the positive control group and the treatment group (P>0.05, **Table 1**).

The effect of Pingchuan decoction on EOS in situ expression

TUNEL assay demonstrated that the amount of EOS in the cytoplasm of the intrinsic cells of the normal group was extremely low. By contrast, the counts of EOS in the model group were significantly enhanced as shown with brown particles. The number of EOS in the positive control group and treatment group was significantly reduced than that in the model group, but statistically higher than that in the normal group

Table 2. EOS expression level detected by TUNEL assay ($\bar{x}$ ± s, n=8)

Group	Dose (g·kg^{-1})	20×	40×
Normal control	19.2	2340.1±203.6$^{\#\#}$	5837.7±708.2$^{\#\#}$
Model	19.2	11187.1±1072.1**	30213.4±1203.8**
Positive control	1×10^{-3}	4372.8±280.8$^{*,\#\#}$	10253.9±643.0$^{*,\#\#}$
Treatment	19.2	5234.8±1710.8$^{**,\#\#}$	19670.0±1345.6$^{**,\#\#}$

*P<0.05, **P<0.01, compared with normal control; $^{\#\#}$P<0.01, compared with model group.

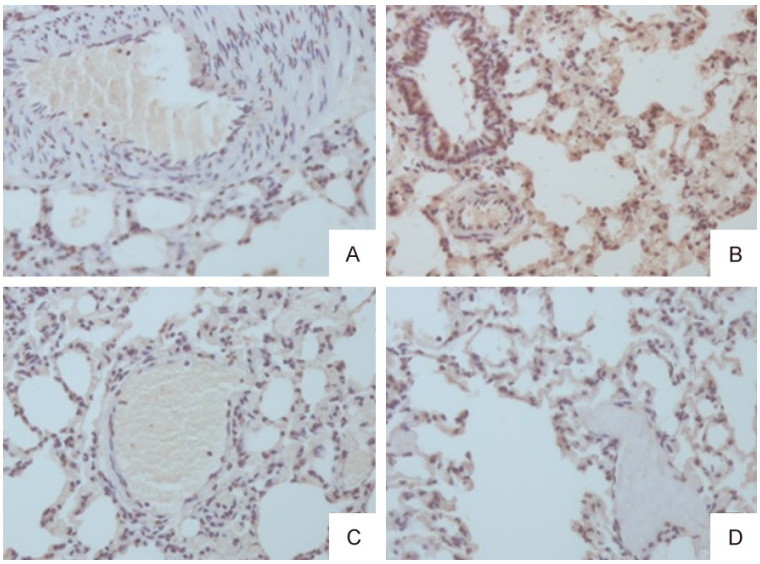

Figure 2. EOS in situ expression in lung tissue detected by TUNEL assay (40×). A. Normal control; B. Model group; C. Positive control group; D. Treatment group.

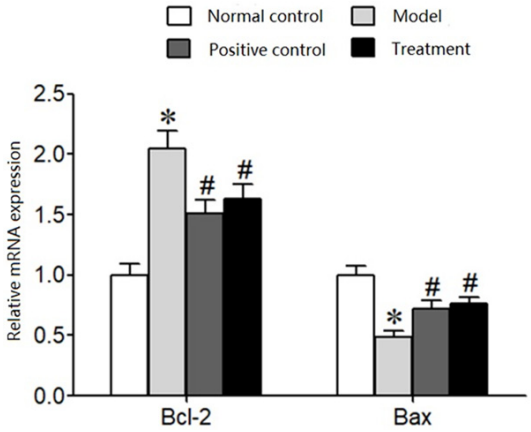

Figure 3. qRT-PCR detection of mRNA expression in the lung tissue. *P<0.05, compared with normal control; $^{\#}$P<0.05, compared with model group.

(P<0.05). The difference between the positive control group and treatment group was not significant (P>0.05, **Table 2**; **Figure 2**).

The influence of Pingchuan decoction on Bcl-2 and Bax mRNA expressions in asthma rat

Compared with the normal control, Bcl-2 mRNA expression was significantly increased, while Bax mRNA level was noticeably reduced in the lung tissue of the model group (P<0.05). There was no significant difference between the positive control group and the treatment group. However, Bcl-2 mRNA expression was apparently declined and Bax mRNA expression was significantly upregulated in the two groups compared with the model group (P<0.05, **Figure 3**).

The effect of Pingchuan decoction on Bcl-2 and Bax protein expressions in asthma rat

Compared with the normal control, Bcl-2 protein expression was significantly increased, while Bax protein level was markedly reduced in the lung tissue of the model group (P<0.05). But no significant difference between the positive control group and the treatment group was found. However, Bcl-2 protein expression was significantly declined and Bax protein expression was statistically upregulated in the two groups compared with those in the model group (P<0.05, **Figure 4**).

The impact of Pingchuan decoction on IL-5 level

OVA modeling significantly elevated IL-5 content in the lung tissue compared to that in the normal group (P<0.01). Of note, the use of Dexamethasone or Pingchuan decoction markedly down regulated the IL-5 compared to model group (P<0.01). No statistical difference of IL-5 level was found between the groups treated with Dexamethasone and Pingchuan decoction (P>0.05, **Table 3**).

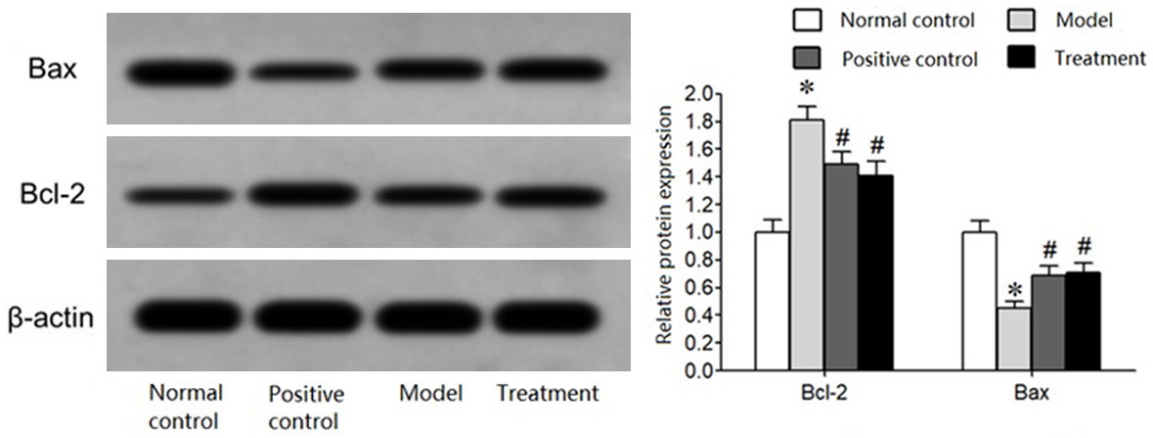

Figure 4. Western blot detection of protein expression in the lung tissue. *P<0.05, compared with normal control; #P<0.05, compared with model group.

Table 3. IL-5 content detection ($\bar{x}$ ± s, n=8)

Group	Dose ($g \cdot kg^{-1}$)	IL-5 ($ng \cdot L^{-1}$)
Normal control	19.2	4.16±0.89
Model	19.2	50.72±21.37**
Positive control	1×10^{-3}	9.25±1.42**,##
Treatment	19.2	9.82±1.37**,##

**P<0.01, compared with normal control; ##P<0.01, compared with model group.

Discussion

Asthma is an airway chronic inflammatory disease caused by a variety of inflammatory cells, inflammatory mediators, and cytokines [23-25]. Recent evidence demonstrates that, as complementary and alternative medicines, Traditional Chinese Medicine Pingchuan decoction plays an essential role in the alleviation of chronic obstructive pulmonary disease (COPD). It can modulate inflammation factors and protease molecules, as well as the activation of the Keap1/Nrf2 signaling pathway. It has been further reported to present therapeutic effect on OVA-induced allergic asthma mice [26, 27]. In this scenario, our study investigated the effect of Pingchuan decoction with a rat asthma model to explore its potential mechanism associated with EOS and inflammation.

Our result showed that the treatment of OVA led to poor mentality, irritability, shaking hair, scratching nose, nodding breath, shortness of breath, unstable standing, abdominal muscle twitching, and incontinence in rats, which were similar to previously reported, suggesting successful modeling [28]. It has been indicated that Dexamethasone prevented OVA-induced airway contraction and histopathological damage, and contributed to amelioration of bronchial asthma [29, 30]. In line with that, we utilized Dexamethasone in modeling rats as a positive control to compare with the effect of Pingchuan decoction. We then found that Pingchuan decoction significantly relieved the damage of airway inflammation as Dexamethasone did.

EOS apoptosis is the main pathway for EOS regression from the airway [22]. Current research suggested that EOS is a key effector cell that causes airway inflammation in asthma. Moreover, the infiltration degree of EOS in the airway was closely related to the severity of asthma [31]. Therefore, it was proposed that the reduction of EOS infiltration in the lung is one of the important ways to relieve airway inflammation in bronchial asthma [32]. In this study, the amount of EOS in the lung tissue of the asthma model group was significantly higher than that of the normal group, while the EOS infiltration in the positive control group and the treatment group was lower than that in the model group but still higher than the control group, indicating that Pingchuan decoction can reduce EOS infiltration in lung tissue of asthma rats.

The Bcl-2 family is one of the key factors in regulating apoptosis. Bcl-2 is a representative anti-apoptotic factor, while Bax is a representative pro-apoptotic factor. Bax overexpression can enhance apoptosis by antagonizing Bcl-2. Apoptosis is increased, and the balance between the two has important regulatory func-

tions for apoptosis [33, 34]. In addition, IL-5 is also considered to be one of the important targets for the regulation of EOS-mediated asthmatic inflammatory response [35]. In EOS *in vitro* experiment, elimination of IL-5 and other cytokines can cause rapid apoptosis [19, 36]. Compared with the normal group, Bcl-2 mRNA and protein levels and IL-5 content were significantly upregulated, while Bax expression was obviously declined in the lung tissue of the model group. However, compared with the model group, Bcl-2 mRNA and protein expressions and IL-5 content were reduced, while Bax expression was enhanced in the two groups. The results suggested that Pingchuan decoction may induce EOS apoptosis and inhibit EOS infiltration in asthmatic lung tissue by downregulating cytokines IL-5 and Bcl-2 levels, and promoting Bax protein expression, thus regress airway inflammation, alleviate airway epithelial cell damage, and improve clinical symptoms in asthmatic patients. However, the limitation in this study still exists that the clinical effect of Pingchuan decoction requires further evaluation within a large group of patients. Also, the molecular mechanisms of Pingchuan decoction in asthma, such as signaling pathways, as well as the combined therapy with current medicines, still need further investigation.

Conclusion

In conclusion, Pingchuan decoction can alleviate airway hyperresponsiveness and improve lung pathology, with similar effect to dexamethasone. It can induce EOS apoptosis and inhibit EOS infiltration to promote airway inflammation regression, reduce airway epithelial cell injury, and improve the clinical symptoms by downregulating IL-5 and Bcl-2 expressions and promoting proapoptotic protein Bax expression, which provides basis for the further treatment against asthma.

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Disclosure of conflict of interest

None.

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