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Original Article

Inhibitory effects of catalpol coordinated with budesonide and their relationship with cytokines and Interleukin-13 expression

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Abstract: In this study, the molecular mechanism for inhibitory effect of Catalpol coordinated with Budesonide (BUD) on allergic airway inflammation was investigated. A total of 30 adult SD rats were randomly divided into five groups, namely the positive control group, the model group, the Catalpol group, the BUD group, and the Catalpol+BUD group with 6 rats in each group, respectively. The pathologic changes of lung tissue were observed by HE stain method. The lung function of rats, the cell count, and the cytokine concentrations in bronchoalveolar lavage fluid (BALF) were detected. The levels of cytokines [interleukin-4 (IL-4), interleukin-5 (IL-5), and interferon gamma (IFN-γ)] in BALF were measured using enzyme-linked immunosorbent assay method. The expressions of Interleukin-13 (IL-13) and Eotaxin in lung tissue were measured by RT-PCR method. The total number of cells in the BALF of the group treated with Catalpol and BUD was significantly lower than the model group. The cytokines IL-5 and IL-4 exhibited a similar tendency: the concentrations of IL-4 and IL-5 for the Catalpol group were dramatically decreased compared with the model group. However, the IFN-γ concentration for the Catalpol and BUD groups were higher than the model group. After treatment with Catalpol+BUD, the eosinophils and neutrophils of the rats were further reduced, asthma-associated inflammation was obviously inhibited, IL-4 level was further decreased and IFN-γ level was further increased comparing the Catalpol group and the BUD group. Moreover, IL-13 expression was positively correlated with Eotaxin expression. The results indicated that Catapol could inhibit the expression of IL-13 and Eotaxin in the lung of asthmatic rats, which also exhibited a synergistically inhibitory effect with BUD on airway inflammation. It is suggested that Catalpol+BUD might be an effective and potential treatment for the clinical therapy of asthma.

Keywords: Catalpol, budesonide, asthma, IL-13, cytokines

Introduction

Bronchial asthma, in short asthma, is a chronic inflammation of bronchi. Disrupted homeostasis of various immune cells, such as helper T cells, eosinophils, mast cells and neutrophils have been commonly observed in asthma [1-4]. The primary changes in pathology result from inflammatory reactions of bronchial mucous membrane, hyperkinesia of bronchial smooth muscle and a reversible block of bronchi resulting from secretion [5-9]. During asthma exacerbation, IgE produced by immunocyte has been considered as the most important factor of the disease when the immune system was activated by allergen [10, 11]. Inflammatory reactions of respiratory tract are the fundamentals of hypersensitivity. Many inflammatory cells such as neutrophil macrophage, eosinophil, T cell and B cell, inflammatory medium and cytokine participate in the inflammatory reactions [12-15]. Therefore, the methods of treatment of asthma should include the control of airway inflammation, the elimination of airway edema, the reduction of mucus secretion, and the improvement of the immune function of the body.

Catalpol ($C_{15}H_{22}O_{10}$) belongs to one of 70 monomeric compounds that have been separated from *Radix Rehmanniae Preparata* (Shudihuang) - a common traditional Chinese herbal medicine for nourishing the kidney according to the traditional Chinese medicine theory [16-

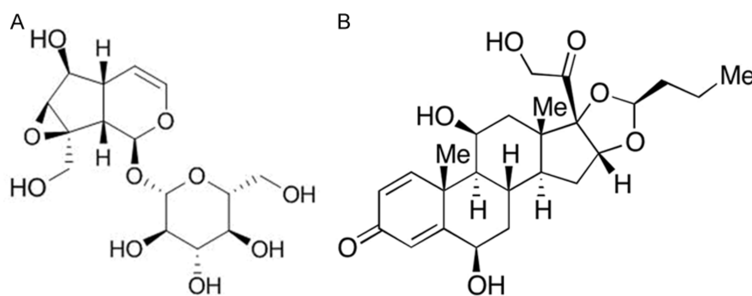

Figure 1. Chemical structures of (A) Catalpol and (B) BUD.

20]. In previous studies [21, 22], we have demonstrated that the use of large doses of Catalpol injection can significantly mitigate the symptoms of asthma in an OVA-induced rat model, Catalpol is able to inhibit eosinophil infiltration in the asthmatic rats, which may be considered as the anti-asthmatic effect of Catalpol. Furthermore, we showed that after Catalpol treatment, the level of IFN-γ the in the serum of rats significantly increased, but the level of IL-4 decreased, suggesting that Catalpol improved the balance of the ratio IL-4/IFN-γ in asthma. These results and findings indicate that Catalpol may become a promising drug for the treatment of asthma. However, there are little to no studies on the mechanism of synergistic effect of Catapol and other methods in the treatment of asthma.

Budesonide (BUD) is a glucocorticoid with high local anti-inflammatory effect [23-32]. BUD can enhance the stability of endothelial cells, smooth muscle cells and lysosome membranes, inhibit immune response and reduce antibody synthesis, thus reducing the release and activity of allergic active mediators such as histamine. BUD can also alleviate the enzymatic process stimulated by antigen-antibody binding, and inhibit the synthesis of bronchial contractile substances to release and alleviate contractile response of smooth muscle [33-35]. BUD has been clinically used in patients with glucocorticoid-dependent or non-dependent bronchial asthma and chronic asthmatic bronchitis [36-40]. The chemical structures of Catalpol and BUD are shown in **Figure 1**.

In this study, an asthmatic model was established using SD rats, a coordinated effect of Catalpol and BUD on asthmatic rats was investigated by cell classification and counting in bronchoalveolar lavage fluid (BALF) and serum ovalbumin (OVA). Interleukin 13 (IL-13) and Eotaxin are important factors in the pathogenesis of asthma. The expression of IL-13 and Eotaxin in the lungs of asthmatic rats and the changes of eosinophils in BALF were observed. The molecular mechanism of Catalpol combined with BUD in inhibiting airway allergic inflammation in bronchial asthma was probed and discussed. The present study demonstrates that the therapeutic effects of Catalpol on asthma can be significantly enhanced by coordination with BUD in a rat model of OVA-induced asthma.

Materials and methods

Experimental animals

Thirty SPF grade male SD rats (age: 5-6 weeks, weight: 170-200 g) were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The rats were divided into five groups-the positive control group, the model group, the Catalpol group, the BUD group, and the Catalpol+BUD group with 6 rats in each group. Temperature and humidity were 25 ± 1°C and 55%-60%, respectively. Illumination time and dark time were both 12 h. There were no restrictions on drinking water and feeding. The care and treatment of the animals were approved by the Animal Care and Use Committees of Heilongjiang University of Chinese Medicine.

Chemicals and reagents

OVA was purchased from Sigma-Aldrich (St. Louis, MO, USA), Catalpol injectable powder was purchased from Melone Pharmaceutical Co., Ltd (Dalian, China), BUD was manufactured by CR double-crane Pharmaceutical Co Ltd (Beijing, China), aluminum hydroxide (99.9%) was purchased from Sinopharm (Shanghai, China), and the corresponding enzyme-linked immunosorbent assay (ELISA) kits were purchased from USCN and Boster (Wuhan, China). All other chemicals or reagents were purchased from Sinopharm (Beijing, China) without further purification.

Establishment of asthma model

The asthma model was established by sensitization and challenge with OVA. Briefly, the rats were sensitized with 1% OVA (10 mg OVA and 200 mg aluminum hydroxide) in 1 mL of normal saline via intraperitoneal injection on Day 1 and Day 8. From Day 15 to Day 28, the rats were challenged with 1% OVA for 30 min every day using an ultrasonic nebulizer. The rats in the Control group received an equal amount of normal saline. From Day 15 to Day 28, the rats in the Catalpol group and the BUD group daily received a dose (10 mg/kg in normal saline) of Catalpol and a dose (1 mg/kg) of BUD, respectively, through intraperitoneal injection 0.5 h prior to each OVA challenge, and the rats in the Catalpol+BUD group received a joint treatment combining 10 mg/kg Catalpol and 1 mg/kg BUD via intraperitoneal injection 0.5 h prior to each OVA challenge. The rats in the control group and experimental groups received equal amounts of normal saline. On Day 29, the peripheral blood and BALF in each group were harvested, and the lung tissues were also collected.

Cell count and classification of BALF

All the rats were executed after last OVA challenge. Normal saline was injected into the right ventricle to completely remove the residual blood in the lung. The lung was taken from the chest cavity after washing. A tube was inserted into the syringe to inject 1 mL of PBS solution for bronchoalveolar lavage three times. BALF was obtained, and the total number of cells was counted with 10 μL BALF. BALF was centrifuged for 10 minutes at 1500 rpm. The supernatant was extracted for cytokines detection, and the cells were classified and counted after precipitation and suspension to determine the percentages of neutrophils, eosinophils, lymphocytes, and macrophages.

HE staining

The right lung tissues were placed and fixed in 4% polyformaldehyde followed by dehydration using gradient ethanol. The tissues were embedded, fixed, sectioned then stained using HE method.

Determination of IgE and cytokines

The level of IgE in the BALF was determined using an IgE ELISA kit. The levels of IL-4, IL-5, and IFN-γ in the BALF were determined using the ELISA kits according to the manufacturer's instructions. In the measurement, the optical density (OD) values were recorded using a Biorad450 enzyme standard instrument at the wavelength of 450 nm. OD values more than twice of the negative control were defined as positive.

mRNA determination of lung tissue IL-13

The content of lung tissue was determined by reverse transcription-polymerase chain reaction (RT-PCR). The central tissue of left lung (100 mg) was taken, and the total RNA was extracted by adding Trizol. The purity and content of RNA were determined by UV spectrophotometer. RNA (5 μg) was used to synthesize cDNA under the presence of oligo-dT and M-Mulv. After amplification of 35 cycles according to their respective reaction conditions, PCR products were electrophoretic on 1% agarose gel, and β-actin was used as positive control. Imaging and strip density scanning were conducted by gel imaging analysis system and semi-quantitative analysis was performed with integral optical density ratio $IOD_{IL-13}/IOD_{\beta\text{-actin}}$.

Statistical methods

The experimental data were analyzed by SPSS17.0 software. Single-factor Analysis of Variance (ANOVA) was used to compare the difference between the groups. The least significant difference method was used for the comparison of two sets of data with the homogeneity of variance. Dunnett's t-test was used for heterogeneity of variance. Spearman's rank correlation analysis was used for correlation analysis. $P < 0.05$ indicated that there were no significant differences between the data. The results were expressed by $x \pm s$.

Results

Determination of IgE in BALF

The level of IgE in BALF was determined by the ELISA method. As shown in **Figure 2**, the level of IgE in BALF was increased in the model group, and it was significantly higher than that in the control group with $P < 0.01$. After Catalpol or BUD single treatment, the IgE level was dramatically decreased, and it was significantly lower than that in the model group with $P < 0.01$. It should be noted that after the combined treatment using Catalpol+BUD, the IgE

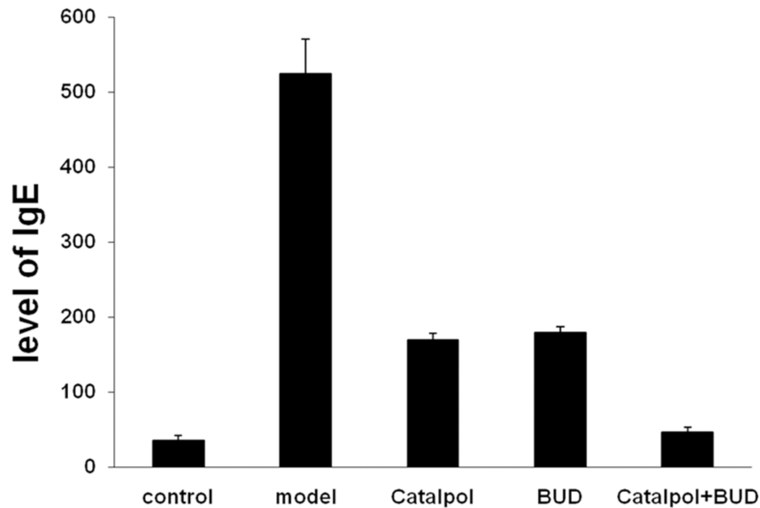

Figure 2. The level of IgE in BALF from each group.

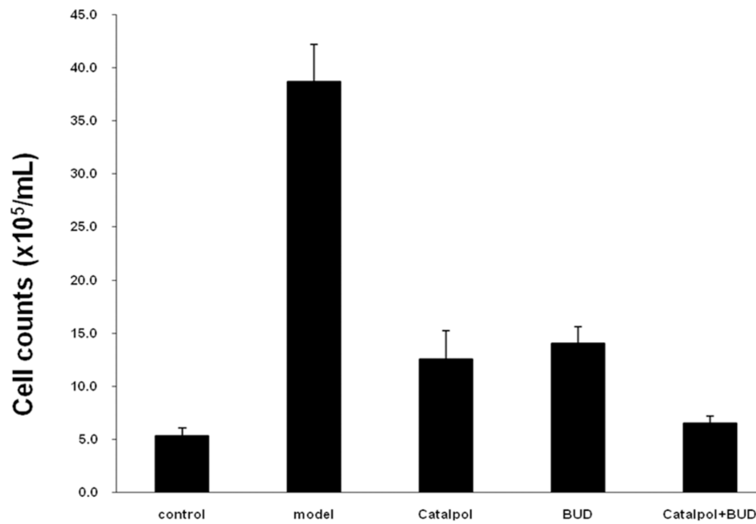

Figure 3. Total number of cells in BALF from each group.

level was further decreased, and it was significantly lower than that in the Catalpol or BUD group with P < 0.01.

Cell counts in BALF

After the sensitization and OVA-challenge, **Figure 3** shows that the total number of cells in the model group was significantly increased comparing the control group with P < 0.01. **Figure 3** also indicates that after the Catalpol or BUD treatment, the total number of cells was decreased comparing the model group with P < 0.01. In addition, after the combined treatment using Catalpol+BUD, the total number of cells was further decreased, and it was significantly lower than that in the Catalpol or BUD group with P < 0.01. **Figure 4** shows that the percentages of eosinophils and neutrophils in the model group were significantly increased comparing the control group with P < 0.01. **Figure 4** also indicates that after the Catalpol or BUD treatment, the percentages of eosinophils and neutrophils were decreased comparing the model group with P < 0.01. In addition, after the combined treatment using Catalpol+BUD, the percentages of eosinophils and neutrophils were further decreased, and it was significantly lower than that in the Catalpol or BUD group with P < 0.01.

Concentration of cytokines

The concentrations of cytokine in the BALF from each group are shown in **Figure 5**. The cytokines IL-4 and IL-5 in the model group were significantly increased comparing the control group with P < 0.05, and the cytokines IL-4 and IL-5 in the Catalpol or BUD group were significantly decreased comparing the model group with P < 0.05. Furthermore, after the combined treatment using Catalpol+BUD, the cytokines IL-4 and IL-5 were further decreased, and it was significantly lower than that in the Catalpol or BUD group with P < 0.01. However, the IFN-γ in the model group was decreased comparing the control group with P < 0.05, IFN-γ in the Catalpol or BUD group was increased comparing the model group with P < 0.05, IFN-γ in the catalpol+BUD group was further increased comparing the Catalpol or BUD group with P < 0.05.

Pathological changes of lung tissue

The inflammatory cells in the lung tissue of rats were observed with light microscopy with H&E

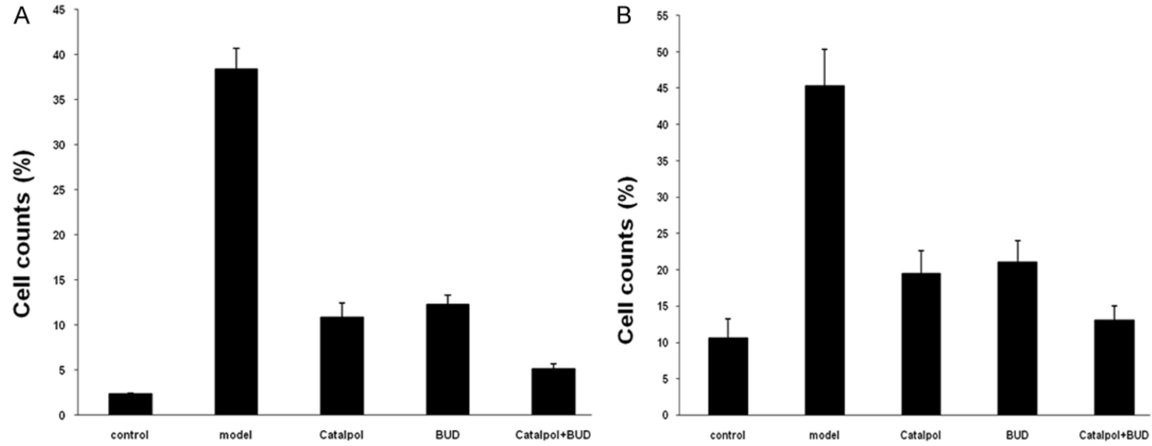

Figure 4. Percentages of (A) eosinophils and (B) neutrophils in BALF from each group.

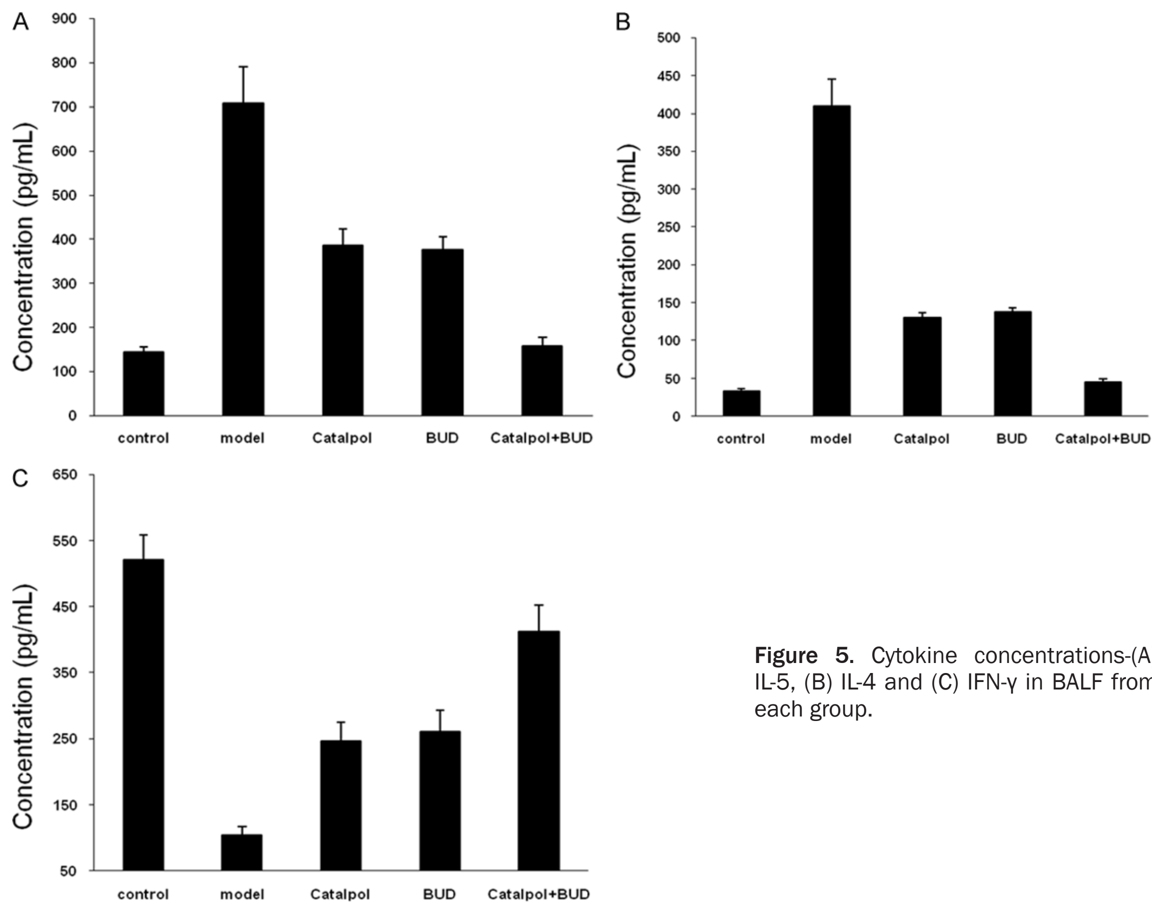

Figure 5. Cytokine concentrations-(A) IL-5, (B) IL-4 and (C) IFN-γ in BALF from each group.

staining, as shown in **Figure 6**. There were no inflammatory changes in the lung tissue in the control group and significant inflammation changes in the model group. Moderate to mild inflammation changes in the Catalpol or BUD group and mild inflammation changes in the Catalpol+BUD group were observed, which were basically close to the normal group, indicating efficient treatment using the selected drugs.

Expression of IL-13 mRNA

Figure 7 shows the expression of IL-13 mRNA obtained by RT-PCR method. The expression in the model group was significantly enhanced

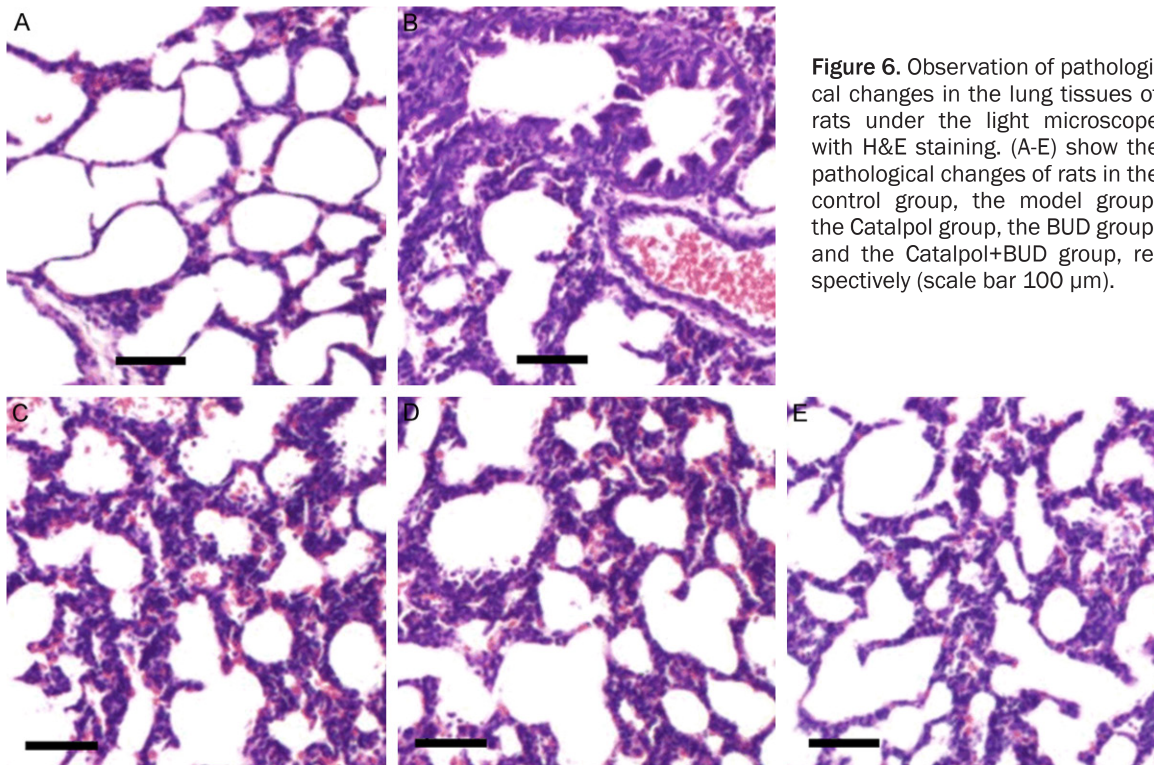

Figure 6. Observation of pathological changes in the lung tissues of rats under the light microscope with H&E staining. (A-E) show the pathological changes of rats in the control group, the model group, the Catalpol group, the BUD group, and the Catalpol+BUD group, respectively (scale bar 100 μm).

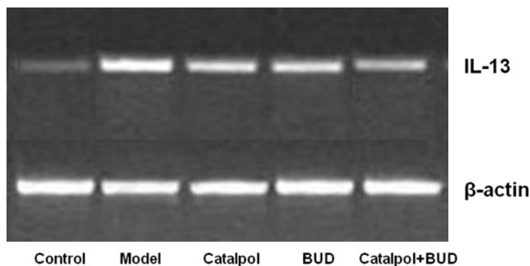

Figure 7. The protein level of IL-13 for each group was detected by PCR method with β-actin as the internal reference.

comparing the control group with $P < 0.01$. After treatment by Catalpol or BUD, the expression was significantly reduced compared with the model group with $P < 0.05$, The expression in the Catapol+BUD group was significantly reduced comparing the Catalpol group ($P < 0.05$) or BUD group ($P < 0.01$).

Discussion

Bronchial asthma is a chronic airway inflammatory disease caused by a variety of cells, cytokines and inflammatory mediators, which is characterized by eosinophil infiltration and airway hyperresponsiveness (AHR) and reversible airway obstruction [1-3]. Cytokines and chemokines play an important role during the formation of asthma inflammation [4-6]. EOS infiltration is the main feature of airway pathology. EOS interaction in T lymphocyte is the central link in the pathogenesis of asthma. The main target cells are Th2 (CD4+) cells to induce EOS inflammation [1-6]. IL-13 is the most important Th2 cytokine that has been investigated in the past five years [7-9]. It has been considered as the most directly related Th2 factor in the pathogenesis of asthma. IL-13 acts as a central function in the pathogenesis of allergic asthma [10-13] and regulates eosinophilic inflammation, mucus secretion and airway hyperresponsiveness. Eotaxin has specific chemotaxis to EOS, such chemotaxis is stronger than other chemotaxis factors [14, 15]. Eotaxin is an important pathogenic factor in patients with bronchial asthma [16]. It tends to recruit EOS and other inflammatory cells in bronchial and lung tissues. The expression level of Eotaxin in serum is closely related to the clinical severity of asthma. It has been reported that the expression level of Eotaxin in sputum of asthmatic patients was related to the acute attack of asthma [17-19]. IL-13 and Eotaxin interact with each other in peripheral blood and tissues to

regulate the recruitment of EOS and make great contribution to the pathogenesis of asthma [23, 24]. IL-13 is a strong inducer of Eotaxin, which can up-regulate the expression of Eotaxin mRNA and protein. IL-13 also stimulates Eotaxin release through mitogen-activated protein and signal transduction activator-6 (STAT6) signal transduction pathway in airway smooth muscle [25, 26]. Blocking IL-13 or Eotaxin pathway can be used to reduce the number of EOS and this method exhibits therapeutic effect on bronchial asthma.

Although glucocorticoid such as BUD has been considered as the most effective drug in the treatment of asthma, the current studies showed that BUD was able to down-regulate the expression of IL-13 and Eotaxin, which has significant effects on reducing AHR and inhibiting airway allergy. However, the toxicity and side effects of large doses of hormones were too significant to adopt a long-term treatment. Therefore, an integration of traditional Chinese medicine and western medicine could improve the curative effect by combining their advantages to promote the total therapeutic effects.

Conclusions

In this study, the asthmatic rat model was established, and the total number of white blood cells and EOS in the Catalpol group were significantly reduced comparing the model group after intervention with Catalpol injection and BUD injection. The total number of leukocytes and EOS in BALF of the Catalpol+BUD group were significantly lower than the Catalpol group and the BUD group. These results suggest that Catalpol injection can inhibit airway inflammation in asthmatic rats and enhance the anti-inflammatory effect of BUD. The expression of IL-13 and Eotaxin in lung tissue of Catalpol group was significantly lower than that of model group. The expression of IL-13 and Eotaxin in lung tissue of the Catalpol+BUD group was significantly lower than that of Catalpol group and BUD group, and the expression of IL-13 and Eotaxin was positively correlated. Therefore, it can be speculated that Catalpol injection can combine with BUD to result in a synergistic effet, which down-regulate the expression of IL-13 and Eotaxin in the lung tissue of asthmatic rats and further inhibit the airway inflammation of asthma.

Disclosure of conflict of interest

None.

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