

Original Article

Effects of Ligustrazine on Airway Inflammation in A Mouse Model of Neutrophilic Asthma*

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ABSTRACT **Objective:** To investigate the effects of ligustrazine (LTZ) on airway inflammation in a mouse model of neutrophilic asthma (NA). **Methods:** Forty healthy C57BL/6 female mice were randomly divided into 4 groups using a random number table, including the normal control, NA, LTZ and dexamethasone (DXM) groups, with 10 rats in each group. The NA mice model was established by the method of ovalbumin combined with lipopolysaccharide sensitization. At 0.5 h before each challenge, LTZ and DXM groups were intraperitoneally injected with LTZ (80 mg/kg) or DXM (0.5 mg/kg) for 14 d, respectively, while the other two groups were given the equal volume of normal saline. After last challenge for 24 h, the aerosol inhalation of methacholine was performed and the airway reactivity was measured. The bronchoalveolar lavage fluid (BALF) was collected. The Wright-Giemsa staining was used for total white blood cells and differential counts. The levels of cytokines interleukin (IL)-17 and IL-10 were detected by enzyme-linked immunosorbent assay. The pathological change of lung tissue was observed by hematoxylin eosin staining. **Results:** The airway responsiveness of the NA group was significantly higher than the normal control group ($P < 0.05$), while those in the LTZ and DXM groups were significantly lower than the NA group ($P < 0.05$). The neutrophil and eosinophil counts in the LTZ and DXM groups were significantly lower than the NA group ($P < 0.05$), and those in the LTZ group were significantly lower than the DXM group ($P < 0.05$). There were a large number of peribronchiolar and perivascular inflammatory cells in filtration in the NA group. The airway inflammation in the LTZ and DXM groups were significantly alleviated than the NA group. The infiltration in the LTZ group was significantly reduced than the DXM group. Compared with the normal control group, the IL-17 level in BALF was significantly increased and the IL-10 level in BALF was significantly decreased in the NA group ($P < 0.05$). LTZ and DXM treatment significantly decreased IL-17 levels and increased IL-10 levels compared with the NA group ($P < 0.05$), and the changes in the above indices were more significant in the LTZ group ($P < 0.05$). **Conclusion:** LTZ could alleviate the airway inflammation in the NA mice model through increasing the IL-10 level and decreasing the IL-17 level.

KEYWORDS ligustrazine, neutrophilic asthma, airway inflammation, cytokine

Bronchial asthma (asthma) is a chronic airway inflammation that involves multiple inflammatory cells [(eosinophils (EOSs), mastocytes, T lymphocytes, and neutrophils (NEUs)] and airway structural cells as well as several cytokines. Eosinophilic airway inflammation is the typical asthma type. However, an in-depth study showed that eosinophilic asthma (EA) and non-eosinophilic asthma (NEA) are different subtypes of asthmatic airway inflammation.⁽¹⁾ More than half of NEA cases involve neutrophilic asthma (NA).⁽²⁾ Moreover, neutrophilic airway inflammation is closely related to severe asthma and steroid-resistant asthma.⁽³⁾ Th17, a helper T cell that mainly produces interleukin 17 (IL-17), is important to the pathogenesis of asthma. Given that IL-17 is implicated in asthmatic airway inflammation, airway hyperresponsiveness,

and airway remodeling that involves NEUs, IL-17 is a potential target for asthma treatment.^(4,5)

Ligustrazine (LTZ) is the bioactive in gradient from the Chinese medicinal (CM) herb *Ligusticum wallichii*, which has been used for a long time in

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clinical practice. LTZ has multiple targets, few side-effects, and an exact curative effect in asthma treatment.⁽⁶⁻⁹⁾ Studies have shown that LTZ reduced IL-17 secretion in a classical asthma mouse model, and reduced airway inflammation and improved bronchial hyper-responsiveness in a classical asthma rat model.^(10,11) However, there are few reports on the therapeutic effects of LTZ on NA. This study aimed to observe the changes of airway inflammation and IL-17 and IL-10 levels in NA mice after LTZ intervention, and to explore the mechanism of LTZ on airway inflammation in NEUs.

METHODS

Animals and Grouping

Forty C57BL/6 mice (specific-pathogen-free grade, female, 6-week old, weighing 20 g) were provided by the Shanghai SLAC Laboratory Animal Co. Ltd. with production license No. SCXK (Hu) 2012-0002. Use of animals was in accordance with the regulations of the Ethic Committee of Medical College of Shandong University. These mice were free fed in different cages under a temperature range of 20–25 °C and relative humidity of 40%–70% and lighted 12 h per day. After acclimatization for 1 week, the animals were randomly divided into 4 groups using a random number table, including the normal control, NA, LTZ and dexamethasone (DXM) groups, with 10 mice per group.

Drugs, Reagents and Instruments

LTZ hydrochloride injection (batch No. 1411503B11) was purchased from Henan National Pharmaceutical Group Rongsheng Pharmaceutical Co., Ltd., China. DXM injection (batch No. 1501052621) was purchased from Chenxin Pharmaceutical Co., Ltd., China. Ovalbumin (OVA, batch No. SL82914V), lipopolysaccharide (LPS, batch No. 7391X15V), and methacholine (Mch, batch No. BCBG2402V) were purchased from the Sigma Company (USA). Mouse IL-10 (batch No. 221041232) and IL-17 (batch No. 221750312) enzyme-linked immunosorbent assay (ELISA) kits were provided by Hangzhou Affinity Biotechnology Co. Ltd., China. A full-wave length spectrometer (Spectra Plus 384, MD, USA) and small animal lung function detection system (GYD-003, Emka, France) were utilized.

Establishment of NA Mouse Model

To induce sensitization, 100 µg of OVA and

0.1 µg of LPS were dipped into the airways of anesthetized mice in the NA, LTZ and DXM groups for 0, 6 and 13 d. Aerosol inhalation of 1% OVA was conducted beginning on the 21st day once daily for consecutive 14 d. Each session lasted for 60 min.^(12,13) At 30 min before stimulation, mice in the LTZ and DXM groups received LTZ hydrochloride intraperitoneal injections (80 mg/kg) and DXM intraperitoneal injections (0.5 mg/kg) for 14 d, respectively, while mice in the normal control group were intraperitoneally injected with 0.8 mL normal saline.

Airway Responsiveness Detection

Twenty-four hours after the last OVA atomization stimulation, mice were made to inhale 0, 1, 2, 4, 8 and 16 mg/mL Mch successively followed by the airway responsiveness detection for 3 min. The noninvasive pulmonary function test was performed by small animal lung function detection system. The airway responsiveness monitoring index enhanced pause (Penh) was recorded.

Bronchoalveolar Lavage Fluid Collection

The mice were sacrificed with the method of cervical dislocation after detecting airway responsiveness. The right main bronchus was occluded. The trachea was cut open, and the corresponding catheter was inserted into the left main bronchus. A total of 0.3 mL saline was injected into the left lung through a catheter and washed. This procedure was repeated thrice. The sample recovery rate was 60%–70%. The samples were then centrifuged at 3,000 r/min for 10 min under 4 °C. The cells were recycled and smeared prior to Wright-Giemsa staining. The number of white blood cells (WBCs) was counted and classified. The supernatant was stored at –80 °C. Cytokines were detected in the samples.

Pulmonary Tissue Hematoxylin Eosin Staining

The right lung was dissected and fixed in 10% formalin solution after bronchoalveolar lavage fluid (BALF) collection. Conventional material collection, dehydration, paraffin embedding, slicing into 4-µm thick sections, and hematoxylin eosin (HE) staining were performed.

Detection of Cytokine Levels by ELISA

IL-17 and IL-10 levels in the collected BALF were measured following the instructions of the ELISA test kit.

Statistical Analysis

The data were analyzed with SPSS 18.0 statistical software. The measurement data were expressed as mean $\pm$ standard deviation ($\bar{x} \pm s$). Among-group and intra-group data comparisons were performed with the ANOVA and LSD-*t* tests. $P < 0.05$ indicated statistically significant difference.

RESULTS

Asthma Symptoms of Mice in All Groups

During the aerosol excitation process, mice in the NA group exhibited scratching, dysphoria, polypnea, hair color tarnish, and abdominal muscle convulsions, as well as audiblestridors. The mice in the LTZ and DXM groups exhibited similar, but milder symptoms. The normal control mice did not exhibit any symptoms.

Airway Responsiveness Detection of Mice in All Groups

At a Mch concentration of 16 mg/mL, the NA group had significantly higher airway responsiveness than the normal control group ($P < 0.05$). The LTZ and DXM groups had significantly lower airway responsiveness than the NA group ($P < 0.05$, Table 1).

Comparison of Total WBCs and Differential Counts in BALF of Mice among Groups

The NA group had obviously higher NEU and EOS counts than the normal control group ($P < 0.05$). Moreover, the NEU count was significantly higher than EOS count in the NA group ($P < 0.05$). The NEU and EOS counts in the LTZ and DXM groups were significantly decreased compared with the NA group ($P < 0.05$), and the LTZ group was significantly lower

Table 2. Total WBCs and Differential Counts in BALF of Mice in Each Group ($\times 10^5/\text{mL}$, $\bar{x} \pm s$)

Group	<i>n</i>	Total cell	NEU	EOS
Normal control	10	6.9 ± 2.5	1.9 ± 0.9	0.7 ± 0.2
NA	10	17.0 ± 4.1	$10.1 \pm 2.2^{*\Delta}$	$1.7 \pm 0.7^{\Delta}$
LTZ	10	12.1 ± 1.7	$5.2 \pm 1.0^{\Delta\circ}$	$0.7 \pm 0.5^{\Delta\circ}$
DXM	10	13.6 ± 1.0	$7.2 \pm 2.0^{\Delta}$	$1.6 \pm 0.4^{\Delta}$

Notes: $^*P < 0.05$ vs. EOS count in the same group; $^{\Delta}P < 0.05$ vs. normal control group; $^{\Delta}P < 0.05$ vs. NA group; $^{\circ}P < 0.05$ vs. DXM group

than the DXM group ($P < 0.05$, Table 2).

Pulmonary Tissue Pathology of Mice in All Groups

The normal control group had clear pulmonary bronchiolar and alveolar structures without any inflammatory cell infiltration. By contrast, the NA group had a large amount of bronchial and perivascular inflammatory cell infiltration that was mainly NEU infiltration, along with significantly thickened alveolar and bronchial walls. Compared with the NA group, the LTZ and DXM groups exhibited significantly alleviated bronchial and perivascular inflammatory cell infiltration, as well as alveolar and bronchial wall thickening. Inflammatory infiltration significantly decreased in the LTZ group compared with the DXM group (Figure 1).

Comparison of IL-17 and IL-10 Levels in BALF of Mice among Groups

Compared with the normal control group, the IL-17 level in BALF was significantly increased and the IL-10 level in BALF was significantly decreased in the NA group ($P < 0.05$). LTZ and DXM significantly

Table 1. Airway Responsiveness of Mice after Mch Stimulation ($\bar{x} \pm s$)

Group	<i>n</i>	Mch (mg/mL)					
		0	1	2	4	8	16
Normal control	10	0.95 ± 0.26	1.52 ± 0.47	1.94 ± 0.41	2.43 ± 0.71	2.72 ± 1.10	3.04 ± 0.83
NA	10	1.04 ± 0.05	1.59 ± 0.56	2.35 ± 1.10	3.69 ± 0.90	4.62 ± 0.78	$5.11 \pm 0.92^*$
LTZ	10	0.96 ± 0.24	1.45 ± 0.78	2.20 ± 0.99	2.58 ± 1.35	3.52 ± 1.86	$4.05 \pm 1.04^{\Delta}$
DXM	10	1.01 ± 0.06	1.53 ± 0.50	2.21 ± 1.01	2.97 ± 1.24	3.01 ± 2.26	$4.13 \pm 1.01^{\Delta}$

Notes: $^*P < 0.05$ vs. normal control group; $^{\Delta}P < 0.05$ vs. NA group

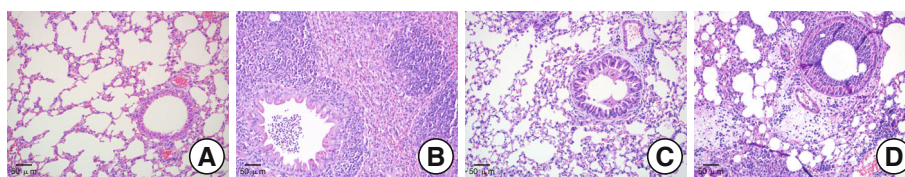


Figure 1. HE Staining of Lung Tissue in Mice of Each Group ($\times 200$)

Notes: A: normal control group; B: NA group; C: LTZ group; D: DXM group

decreased the IL-17 levels and increased the IL-10 levels compared with the NA group ($P < 0.05$), and the changes in the above indices were more significant in the LTZ group ($P < 0.05$, Table 3).

Table 3. Comparisons of IL-17 and IL-10 Levels in BALF of Mice among Groups (pg/mL, $\bar{x} \pm s$)

Group	<i>n</i>	IL-17	IL-10
Normal control	10	41.6 $\pm$ 7.5	118.0 $\pm$ 22.1
NA	10	83.0 $\pm$ 10.4*	31.5 $\pm$ 7.2*
LTZ	10	64.7 $\pm$ 5.2 [△]	71.3 $\pm$ 19.6 [△]
DXM	10	71.1 $\pm$ 4.1 ^{△▲}	55.3 $\pm$ 8.9 ^{△▲}

Notes: * $P < 0.05$ vs. normal control group; [△] $P < 0.05$ vs. NA group; [▲] $P < 0.05$ vs. LTZ group

DISCUSSION

Bronchial asthma is the most common chronic respiratory disease. Previous study showed that asthma is an "eosinophilic" inflammatory disease driven by Th2 cells. However, recent study has found that in addition to EA, other inflammatory phenotypes, such as NA, oligo asthma, and mixed asthma, also exist.⁽²⁾ Moreover, each inflammatory phenotype has different pathogenesis and treatment response. NA is characterized by a significant increase in NEUs in the airway of asthma patients and airway hyper-responsiveness.⁽³⁾ Meanwhile, EOS levels remain normal with poor hormonal therapeutic effect.^(14,15) Research on NA depends on a successfully established model. Airway inflammation and airway hyper-responsiveness are the two most important characteristics used to evaluate an asthma model. The present study showed that compared with the normal controls, mice in the NA group behaved differently during the excitation process. The NEU and EOS counts were higher in BALF from mice in the NA group than that from the normal control group. Furthermore, the NEU count was higher than EOS count in the NA group. This result suggested that airway inflammation appeared in mice and mainly NEU infiltration appeared in the NA group. In addition, after excitation by different Mch concentrations, airway responsiveness indices were higher in the NA group, suggesting that airway responsiveness increased in the NA group. In this study, the model mice exhibited airway inflammation and responsiveness, as well as mainly NEU infiltration, suggesting that the NA mouse model was successfully established.

The main component of LTZ is

2,3,5-tetramethylpyrazine, which belongs to the amide alkaloids. It can block calcium channels, prevent platelet aggregation, improve hemorheology, block ischemic reperfusion injury, regulate the immune system, and prevent tumor growth. The in-depth understanding of the pharmacological mechanism of LTZ has enabled its application in the treatment of partial respiratory system, digestive system, immune system, and urinary system diseases. Its effectiveness in asthma treatment has been confirmed by related study.⁽¹⁶⁾ Our previous study showed that LTZ aerosol inhalation had better clinical efficacy in the treatment of bronchial asthma. Its mechanism may be related to the inhibition of IL-4 synthesis, thereby inhibiting the pre-potent response of Th2 cell subsets and regulating the immune balance reaction.⁽¹⁷⁾

The present study investigated the effects of LTZ on NA airway inflammation and its immune-modulatory mechanism. The results showed that the NEU and EOS counts in BALF and pulmonary tissue inflammatory cell infiltration in the treatment group were significantly decreased or alleviated. These results suggested that LTZ and DXM can alleviate neutrophilic airway inflammation, while partially alleviate airway inflammation in NA mice. The changes in the LTZ group were more significantly than the DXM group, suggesting that LTZ has a stronger anti-inflammatory effect on the NA mice model than DXM. In addition, LTZ is an effective component from Chinese herb, which lacks hormonal side effects or hormonal resistance. Although its therapeutic mechanism needs further study, it has the potential for further development and utilization in asthma, especially in refractory and hormone-dependent asthma.

Abnormal immune-reaction is the key to asthma occurrence.⁽¹⁸⁾ Previous study showed that the Th1/Th2 cell imbalance is the immunological basis for asthma.⁽¹⁹⁾ Recent study found that Th17, regulatory T cells (Treg), and its representative cytokines IL-17 and IL-10 were involved in the pathogenesis of asthma.⁽²⁰⁾ IL-17 has a strong pro-inflammatory effect and drives immune cells. IL-17 can bind its receptor, induce vascular endothelial cells, fibroblasts, and epithelial cells to release IL-8, IL-6, and other inflammatory cytokines, induce the secretion of chemokines, adhesion molecules (intercellular cell adhesion molecule-1), and matrix metallo-proteinases, and drive and activate the partial infiltration and destruction of NEUs in the

airway.⁽²¹⁾ The effect of Th17 cells in mediating partial NEU infiltration may be related to the occurrence of neutrophilic airway inflammation in asthma.⁽¹⁴⁾ Moreover, Th17 cell-mediated airway inflammation and airway hyper-responsiveness are insensitive to hormone therapy.⁽²²⁾ IL-10 is a multifunctional negative regulator with strong immuno-suppressive and immune-regulatory effects. Moreover, it is an important immune-regulation medium. Treg, a type of T cell subset with immune suppressive action, can negatively inhibit the helper T cell subset, thus preventing the occurrence of allergic and autoimmune diseases.⁽²³⁾ Chen, et al⁽²⁴⁾ found that there existed imbalance of IL-17 and IL-10 in patients with chronic persistent asthma and it took part in the occurrence and development of asthma. It also implied that the imbalance of Th17/Treg cells was one of the immunological mechanism of asthma.⁽²⁵⁾ IL-17 may inhibit the level of IL-10 and there exist antagonistic function between these two factor.

The receptor of IL-10 was expressed on Th17 cell surfaces. The T cells with specific knockdown of the IL-10 receptor increased, and the IL-10 receptor was expressed on Th17 cells. Treg can secrete IL-10 to inhibit Th17 cells.⁽²⁶⁾ This study showed that after LTZ treatment, the secretion of IL-10 was significantly increased, whereas that of IL-17 was significantly decreased. LTZ might promote the secretion of IL-10, which combines with the Th17 cell surface receptor to inhibit the immune response of Th17, reduce the secretion of IL-17 in the airway, and alleviate neutrophilic airway inflammation. The changes in the above indices were more significant in the LTZ group, suggesting that LTZ can inhibit Th17 immunoreaction in the NA mice model and strongly reduce IL-17 secretion compared with DXM.

In summary, LTZ can promote the secretion of inflammation suppression factor IL-10, inhibit the secretion of pro-inflammatory cytokine IL-17, and alleviate airway inflammation in the NA mice model, thereby playing a role in treatment of NA.

Conflict of Interest

The authors claimed no potential conflicts of interest relevant to this article.

Author Contributions

Che XW and Liu XM conceived and designed the

experiments; Wang YB and Wu Q performed the experiments; Bian ZR analyzed the data. All authors read and approved the final manuscript.

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