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Original Research Article

Effects and mechanism of Chinese medicine Jiawei Yupingfeng in a mouse model of allergic rhinitis

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ABSTRACT

Objective: Chinese medicine has the potential to modulate allergic rhinitis (AR). There have been studies investigating the treatment efficacy of Yupingfeng San, alone or in combination with other ingredients, in AR, though few have studied the potential mechanisms of these drugs. In the present study, we measured the effects of Jiawei Yupingfeng (JWYPF), a traditional Chinese medicine formula, on mice with ovalbumin-induced AR and explored its underlying mechanism of action.

Methods: Forty BALB/c mice were randomly divided into normal control, allergy control and two treatment groups of ten mice each. In the normal control group, mice were sensitized and challenged with saline. The mice in the allergy control and treatment groups were sensitized and challenged with ovalbumin and aluminum hydroxide gel. The treatments of JWYPF and Nasonex were administered intranasally in the AR mice for one week. Several signs of allergic inflammation, such as nasal eosinophils and inflammatory cytokines, were measured to determine the underlying mechanisms.

Results: Mice in the JWYPF and Nasonex groups had significantly lower AR symptom scores than those in the allergy control group (the mean differences between JWYPF and the allergy control, and Nasonex and the allergy control were -2.00 ± 0.35 and -2.40 ± 0.32). After treatment with JWYPF and Nasonex, the levels of ovalbumin-specific IgE and histamine were significantly reduced, as were the levels of interleukin-4 and transforming growth factor- β , while interferon- γ levels were increased (all $P < 0.0001$, vs. allergy control). These two treatments also significantly inhibited eosinophil and mast cell infiltration into the nasal cavity but were not statistically different from one-another.

Conclusion: JWYPF has a potential therapeutic effect on AR via adjusting the rebalance of T helper 1 and T helper 2.

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1. Introduction

Allergic rhinitis (AR) is characterized by IgE-mediated mucosal inflammation, driven by T-helper 2 (Th2) cells [1]. It affects 23%–30% of the European population [2,3] and 12%–30% of the United States population [4]. In traditional Chinese medicine (TCM), AR belongs to the category of “Bi Qiu,” characterized by sudden nasal itching, sneezing, rhinorrhea and nasal blockage [5]. The prevalence of AR in China ranges from 4% to 38%, across urban and rural areas [6]. Given the high morbidity and serious impact of AR on

school and work performance, successful treatments for the condition are particularly important.

Pharmacotherapy for AR includes corticosteroids, antihistamines and leukotriene receptor antagonists, as recommended by the Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines, as first-line therapy [7]. According to the latest Chinese AR guidelines, in addition to the recommended treatments of the ARIA guidelines, it is recommended that Chinese AR patients seek TCM therapies, including Chinese herbal medicine (CHM), taken orally or applied externally [5]. Yupingfeng San (YPFS), one such CHM formula, an oral drug, was recommended by the Chinese medicine clinical practice guidelines for management of AR [8]. It contains three key herbs: Astragali Radix (Huangqi), Atractylodis Macrocephalae Rhizoma (Baizhu) and Radix Ledebouriae Divaricatae

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(Fangfeng) [9]. In practice, YPFS usually is used together with other herbs, including *Magnoliae Flos* (Xinyi), *Xanthii Fructus* (Cangerzi) and *Angelicae Dahuricae Radix* (Baizhi) [10]. In addition, for this study, we added *Menthae Haplocalycis Herba* (Bohe), which is an herb used frequently by ancient physicians to treat allergic conjunctivitis, to the YPFS. The formulation that we use in this study was Jiawei Yupingfeng (JWYPF), comprised of YPFS plus *Magnoliae Flos*, *Xanthii Fructus*, *Angelicae Dahuricae Radix* and *Menthae Haplocalycis Herba*. Recently, there have been studies investigating the treatment efficacy of YPFS alone and in combination with other ingredients for AR [10]; nevertheless, to our knowledge, only a few studied the potential mechanisms of these drugs. Therefore, in the present study we evaluate the effect and explore the mechanism of action of JWYPF with regard to various histological and biochemical parameters, including transforming growth factor- β (TGF- β) and interferon- γ (IFN- γ), in an AR mouse model; the study also compares JWYPF effects against Nasonex, a common pharmaceutical used to manage AR. Among the tested parameters, IFN- γ , secreted by T helper 1 (Th1) cells, mainly participates in cellular immunity and mediates delayed hypersensitivity, while IL-4, secreted by Th2 cells, plays a central role in the regulation of eosinophil and mast cell migration and plays an important role in the occurrence and development of AR [7]. Thus, a change in the ratio of IFN- γ and IL-4 is a proxy for the change of Th1/Th2 balance in the system we used to evaluate the effect of treatment in AR. Importantly, we used an intranasal dispersal mechanism, instead of the traditional oral route, to reduce potential systemic side effects.

2. Methods

2.1. Animals

Four-week-old BALB/c mice were obtained from the Experimental Animal Research Center of Hubei Province, with experimental animal production license number of SCXK (E) 2015-0018 and animal use license number of SYXK (E) 2013-0069. Experiments were conducted according to the guidelines of animal care at the Clinical Research Institute of Wangjing Hospital, China Academy of Chinese Medical Sciences, China.

2.2. Preparation of JWYPF

JWYPF was prepared by New Green Technology Development Company for use as a nasal spray and composed of *Astragali Radix* 15 g, *Atractylodis Macrocephalae Rhizoma* 30 g, *Radix Ledebourielae Divaricatae* 15 g, *Magnoliae Flos* 10 g, *Xanthii Fructus* 6 g, *Menthae Haplocalycis Herba* 6 g and *Angelicae Dahuricae Radix* 10 g, dissolving in 200 mL of 0.9% normal saline.

The volume of JWYPF for mice was calculated as: $5.55 \times 3.33 \text{ mL/kg} \times 0.4 \text{ kg (per side)} = 7.4 \text{ mL}$; it is 370 times of 20 μL , and we hence concentrated the volume of decoction 370 times to 0.54 mL.

2.3. Mouse AR model and treatments

The sensitization and antigen challenge for the murine model of AR were performed as described by Kim et al. [11] with some modifications. The 4-week-old female BALB/c mice were randomly divided into four groups (ten per group) as follows: (1) normal control group; (2) ovalbumin (OVA)-sensitized (allergy) group; (3) JWYPF group; and (4) Nasonex group. After a one-week acclimatization, the mice were sensitized using an intraperitoneal injection of 50 mg OVA (grade V; Sigma, St. Louis, MO, USA) and 2 mg of aluminum hydroxide gel (grade V; Sigma) on days 0, 7 and 13. Start-

ing on day 21, once a day for 7 d, these mice were challenged with 20 μL of phosphate-buffered saline (PBS) solution, containing 50 $\mu\text{g/mL}$ of OVA; treatment solutions were introduced into both nasal cavities by instillation. From days 27–35, the mice in the control and allergy groups were given nasal drips of saline solution, while the treatment groups received JWYPF (20 μL per side, 2 times/d; New Green Pharmaceutical Technology Development Co., Ltd. Sichuan, China) or Nasonex (1.85 μg per side, 2 times/d, Schering-Plough Labo N.V., Brussels, Belgium) 1 h before the intranasal OVA challenge. One hour after the final administration of day 35, all mice were euthanized; routine blood samples were taken and paraffin specimens were prepared. The experimental protocol is shown in Fig. 1.

2.4. Symptom scores

The nasal symptoms of the mice in the normal control group and the model group were observed and scored, and the scores were statistically analyzed. The results showed that compared with the normal control group, the mice in the OVA model group scratched noses more frequently, sneezed more frequently, and their mucus production exceeded the anterior nasal passages; the total score of the model group was significantly higher than in the normal control group ($P < 0.01$). These results indicated that the mouse AR model had been successfully induced for the subsequent drug intervention and treatment research.

For each group, we recorded the symptom scores for the frequency of sneezing, nasal itching and rhinorrhea, within 30 min of administration of OVA and treatment.

Scoring criteria were as follows. I) nose scratching: 1 point, lightly rubbing the nose several times; 2 points, scratching the nose, rubbing elsewhere in the face. II) sneezing: 1 point, 1–3 times; 2 points, 4–10 times; 3 points, 11 times or more. III) rhinorrhea: 1 point, flow to the anterior of the nostril; 2 points, flow beyond the anterior nostril; 3 points, clear water over the entire face. The symptom scores were added by each symptom score.

2.5. Wright staining of nasal secretions for eosinophils

We used a small cotton swab to wipe the surface of the turbinates three times gently and applied the material to slides. The smear was placed flat on a staining wet box (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), and 3–5 drops of eosin Y were used to cover the nasal secretion, with staining for 1 min to fix the film. We then added 6–10 drops of methylene blue directly, gently shaking the slide and staining for 5–8 min. Finally, the slide was rinsed with running water for 30 s, allowed to dry and observed under a microscope (Nikon, Tokyo, Japan) at 400 $\times$ magnification.

2.6. Collections of serum and measurements of OVA-specific IgE

After the final treatment, blood was taken from the femoral artery. Blood samples were stored for 4 h at 4 $^{\circ}\text{C}$ and centrifuged to obtain serum. OVA-specific IgE levels were measured using a mouse anti-OVA IgE enzyme-linked immunosorbent assay (ELISA) kit (BD Pharmingen, San Diego, CA, US).

2.7. Tissue preparation and staining for mast cells

Extruded nasal tissue specimens were decalcified for 5 d and then sectioned from behind the upper incisor to 2 mm in front of the incisive papilla of the hard palate. A paraffin block was prepared and sliced into 4 μm thick sections. Each section was stained with toluidine blue (Shanghai Sinopharm Group, No. 71041284) to detect cellular infiltration. The numbers of inflammatory cells at

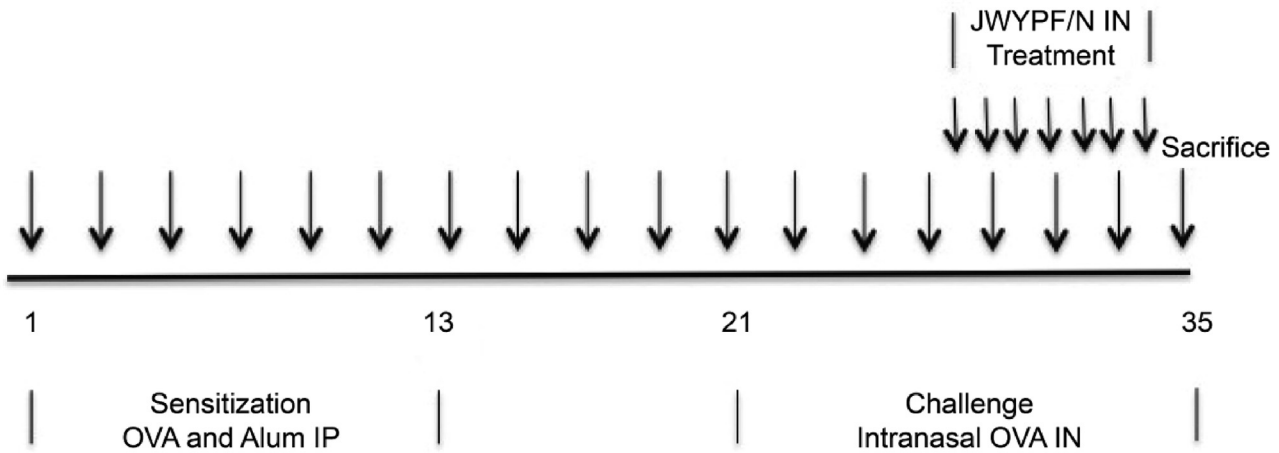


Fig. 1. Schematic representation of the experimental allergic rhinitis model and treatment protocol. BALB/c mice were sensitized with OVA and 2 mg of Alum on days 1, 3, 5, 7, 9, 11 and 13 (general sensitization). All groups except for control group received intranasal OVA from day 21 to day 35. In addition to sensitization and challenge, mice of treatment groups were treated with intranasal JWYPPF or Nasonex, 1 h before intranasal OVA challenge from day 28 to day 35. OVA: ovalbumin; Alum: aluminum hydroxide gel; JWYPPF: Jiawei Yupingfeng; IN: intranasal administration; IP: intraperitoneal administration; N: Nasonex. Units are days.

five different sites were counted under 400 $\times$ magnification using a light microscope.

2.8. Immunohistochemistry (TGF- β)

Nasal mucosa was obtained as described above. The samples were soaked in xylene (Shanghai Sinopharm Group, No.

10023418) for 5 min, replaced with xylene for 5 min and then immersed for 5 min in absolute ethanol (Shanghai Sinopharm Group, No. 10009218); we then washed the tissue in ethanol of different concentrations. Antigen repair was performed and then washed again with PBS for 5 min each. The primary antibody-coated tissue (Omnim Abs No. OM108471) was added to each section and washed with PBS and 50 μ L of freshly prepared 3,3'-

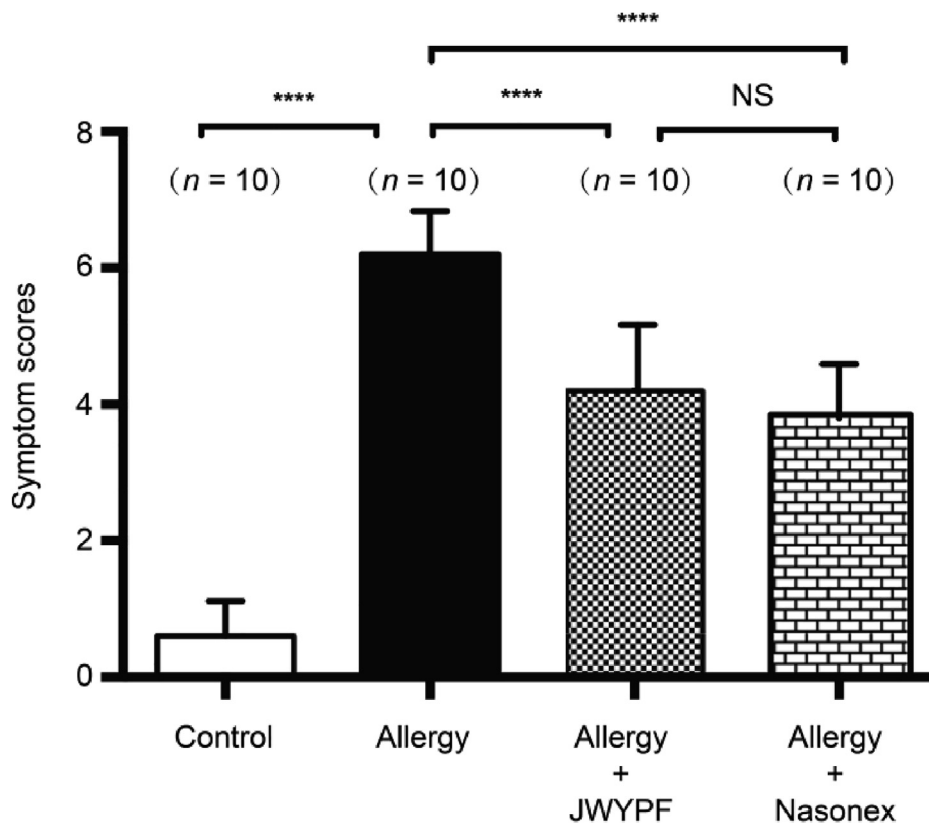


Fig. 2. Statistical results of nasal symptom scores in different groups. Data are expressed as mean $\pm$ standard error of the mean. Nasal symptom score was higher in the allergy group than in the control, while after one-week treatments, they reduced significantly in both JWYPPF and Nasonex groups. There was no difference between the treatment groups. **** P < 0.0001. JWYPPF: Jiawei Yupingfeng; NS: not significant.

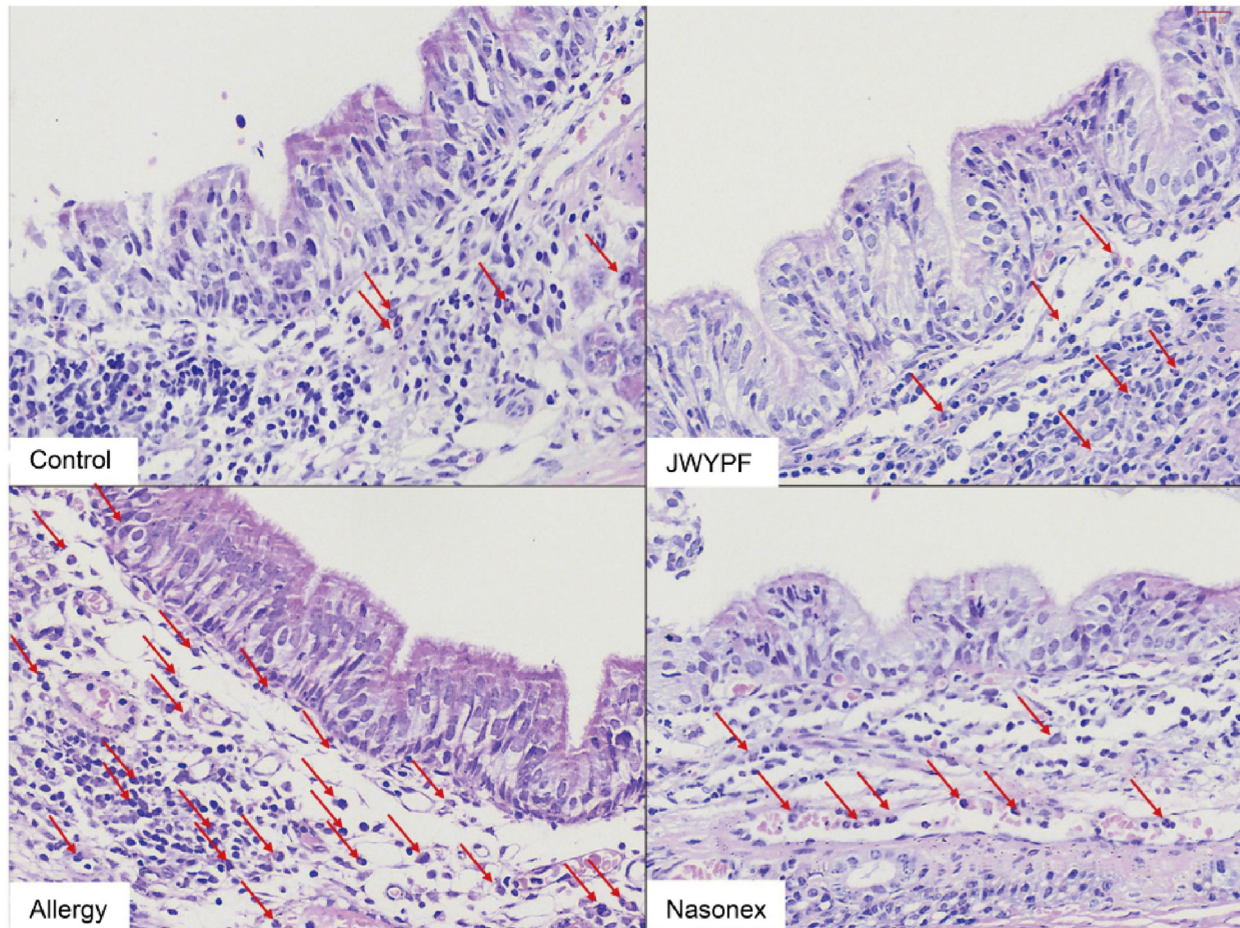


Fig. 3. Wright staining of eosinophils from nasal secretions of mice in the control, allergy, JWYPF treatment and Nasonex treatment groups. There were 10 mice in each group (red arrows in the figure indicate eosinophils under magnification 400 $\times$). The eosinophils were significantly more in allergy group than in control group, while they reduced in both JWYPF and Nasonex treatment groups. JWYPF: Jiawei Yupingfeng.

diaminobenzidine tetrahydrochloride solution. Finally, a microscope (Olympus No. IX51, 400 $\times$ magnification) was used to analyze the relevant parts of the sample.

2.9. ELISA for serum histamine, IL-4 and IFN- γ

Serum was collected the same as the way described in OVA-specific IgE section. Levels of IL-4 and IFN- γ were measured using ELISA kits (LiankeBio, MultiSciences, Hangzhou, China) according to the manufacturer's instructions.

2.10. Statistical analysis

Statistical analysis was performed using SPSS version 22.0 (IBM, Armonk, NY). Data are expressed as mean $\pm$ standard error of the mean. Differences among the four groups were compared using one-way analysis of variance and Bonferroni's *t* test was used to make pairwise comparison between different groups when the analysis of variance indicated significance among group differences. A value of $P < 0.05$ (two-tailed) was considered statistically significant.

3. Results

3.1. Symptom improvement

The nasal symptom scores in the allergy group were significantly higher than those in the control group (Fig. 2). Symptom

total scores of nasal itching, sneezing and rhinorrhea were lower in the JWYPF and Nasonex groups than in the allergy group; the difference between group means was -2.00 ± 0.35 between the allergy and JWYPF groups, and -2.40 ± 0.32 between the allergy and Nasonex groups. There was no difference between the JWYPF and Nasonex groups ($P = 0.313$).

3.2. Eosinophils in nasal mucus

Eosin Y red staining revealed markedly lower levels of eosinophil infiltration in the JWYPF and Nasonex groups than in the allergy group (Fig. 3).

3.3. Mast cell infiltration and histology of nasal mucosa

The mean $\pm$ standard error of the mean of mast cell counts in the allergy group was 62.33 ± 2.98 , which was significantly greater than that in the control group (6.40 ± 0.76 , $P < 0.0001$). Mast cell counts in the treatment groups of JWYPF and Nasonex were 22.13 ± 1.17 and 19.20 ± 1.28 , respectively, both significantly lower than in the allergy group (each, $P < 0.0001$; Fig. 4). The numbers of mast cells in the allergy group were significantly higher than the control group and were lowered in the JWYPF and Nasonex groups (Fig. 5).

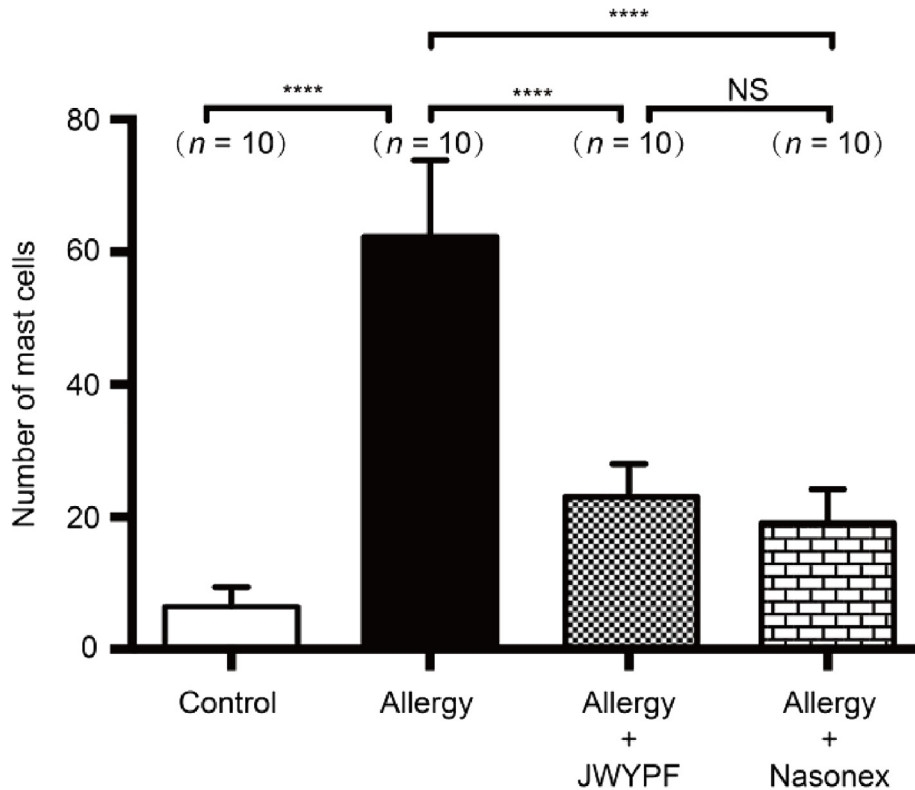


Fig. 4. Mast cell counts in different groups. Data are expressed as mean $\pm$ standard error of the mean. The count of mast cells was higher in the allergy group than in the control group. While after one-week treatments, they decreased significantly in both JWYPF and Nasonex groups, and there was no difference between them. **** $P < 0.0001$. JWYPF: Jiawei Yupingfeng; NS: not significant.

3.4. Expression of TGF- β in nasal mucosa

TGF- β levels, indicated by the density of brown color in the nasal mucosa tissue, were significantly higher in the allergy group than in normal control group, and reduced in the JWYPF and Nasonex groups, compared to the allergy group (Fig. 6).

3.5. ELISA for IgE and histamine

Serum OVA-specific IgE levels were significantly higher in the allergy group than in the control group; these levels were lower in the JWYPF and Nasonex groups, compared with the allergy group (Fig. 7A). Histamine levels were significantly higher in the allergy group than in the control group ($[19.79 \pm 1.30]$ ng/mL vs. $[5.85 \pm 0.27]$ ng/mL; $P < 0.0001$), consistent with the symptom scores and IgE levels. Histamine levels were significantly lower in the JWYPF and Nasonex groups than in the allergy group (differences between means were $-[7.81 \pm 1.46]$ ng/mL and $-[7.36 \pm 1.46]$ ng/mL, respectively), and there was no difference in histamine levels between the JWYPF and Nasonex groups ($P = 0.641$, Fig. 7B).

3.6. Serum cytokines

Serum IL-4 levels were significantly higher, while IFN- γ levels were significantly lower, and the ratio of IFN- γ /IL-4 was significantly lower in the allergy group, compared with the control group (differences between means were $-[87.72 \pm 8.47]$ pg/mL, $[61.40 \pm 5.28]$ pg/mL and $-[0.82 \pm 0.05]$, respectively; Fig. 8A–C). After one week of treatments, there was a significant trend toward higher IFN- γ , lower IL-4 and higher IFN- γ /IL-4 ratio in both the JWYPF and Nasonex groups, compared with the allergy group (all P values < 0.0001). There were no significant differences among the treat-

ment groups in terms of IFN- γ , IL-4 and IFN- γ /IL-4 ratio (P values were 0.546, 0.859 and 0.657, respectively).

4. Discussion

YPFS originates from the book *Experiences of Zhu Danxi*. Studies have shown that YPFS plays a role of anti-bacterial adhesion by protecting and restoring airway mucosal epithelium [12,13]. In a review of immunopharmacology research, it was shown that YPFS had extensive effects on the immune system, when used in clinical practice to prevent diseases of the respiratory tract [14] including wind-cold in Chinese medicine category, respiratory infections, bronchial asthma, and AR.

In the present study, the JWYPF was adopted for AR by adding *Magnoliae Flos* and *Xanthii Fructus*, two herbs that are commonly used for nasal congestion and runny nose; *Angelicae Dahuricae Radix*, to disperse wind-heat and relieve wind-evil on the face; and *Menthae Haplocalycis Herba*, which is used to evacuate wind-heat. The addition of these four drugs to YPFS has the potential to strengthen the effect of dispelling wind and clearing the nasal orifice and should also better reduce the symptoms of nasal congestion and runny nose. Also, in order to avoid potential systemic side effects, the mixture was prepared by New Green Technology Development Company for delivery as a nasal spray.

Nasal administration of corticosteroids is recommended as a first-line therapy for AR patients, especially for those with moderate-to-severe symptoms [15]. Therefore, we used Nasonex as a positive control, to investigate the effect and the putative immunologic mechanisms of short-term treatment of JWYPF in an OVA-induced AR mouse model.

We found that mice sensitized to OVA and subsequently challenged developed characteristic symptoms of AR, including sneez-

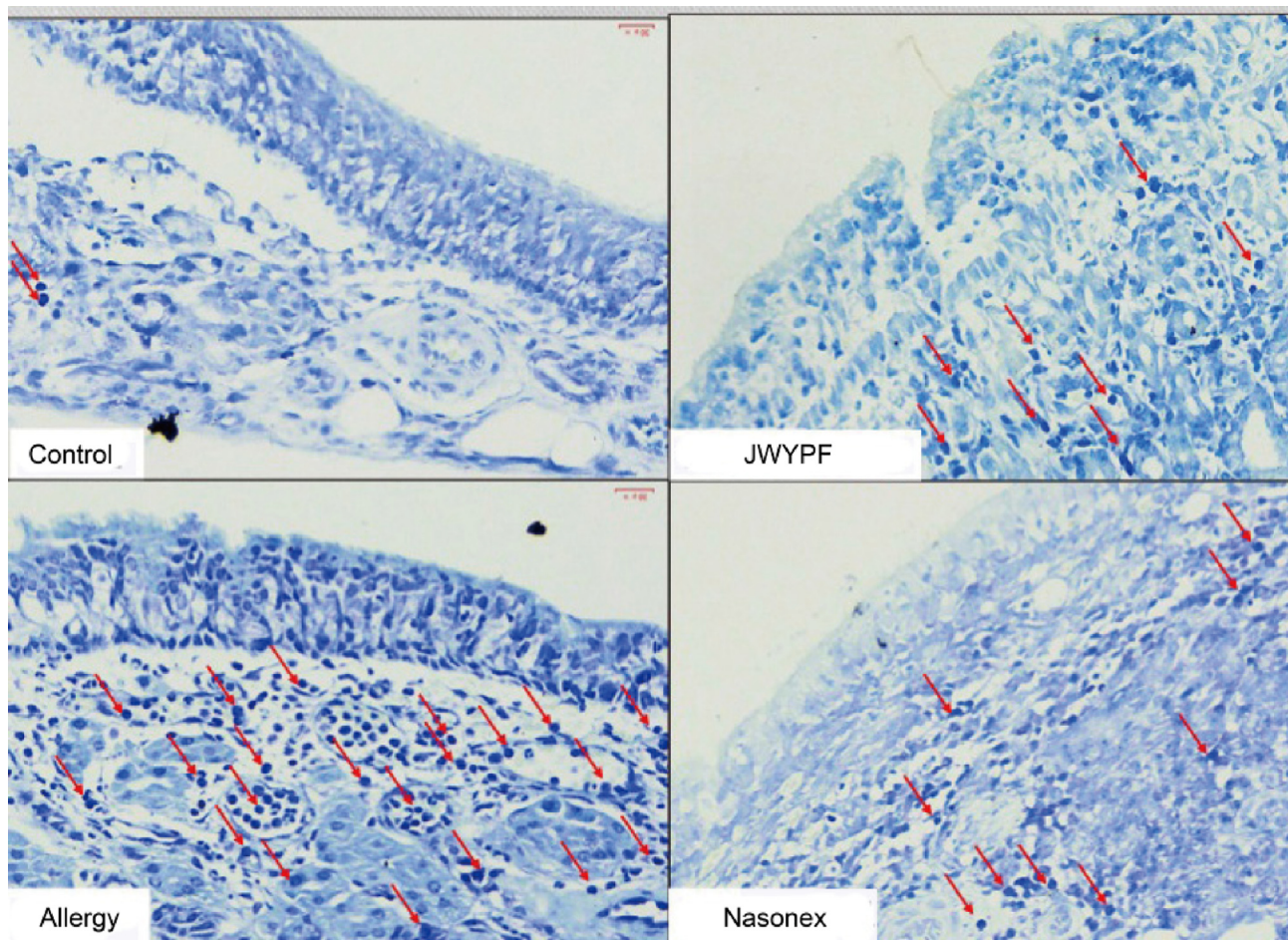


Fig. 5. Representative toluidine blue staining images of nasal mucosa for each treatment group (red arrows in the figure indicate mast cells under magnification 400 $\times$; 10 mice per group). There were significantly more mast cells in the allergy group than in the control group. Mast cell numbers were reduced in both JWYPF and Nasonex treatment groups, relative to the allergy group. JWYPF: Jiawei Yupingfeng.

ing and nasal rubbing [16]. The symptom scores were significantly lower after a one-week treatment with nasal JWYPF. This finding agreed with the study by Li et al. [17] who found Yupingfeng granule reduced the symptom scores of nasal rubbing, sneezing and rhinorrhea after two weeks of treatment. There were no differences in nasal allergic symptom relief, compared with the Nasonex treatment, suggesting that nasal JWYPF produced no less clinical therapeutic effectiveness than the nasal steroid.

We further studied the changes in inflammatory cells and cytokines that are important in the pathogenesis of AR. To demonstrate the pathology of AR, we measured infiltration of inflammatory cells in the nasal cavity, as has been described for previous mouse AR models [16]. We found that JWYPF acted by inhibiting the migration of eosinophils and mast cells into the local nasal cavity. The decrease in eosinophils and mast cells after a one-week treatment was consistent with results of previous studies that showed that YPFS significantly reduced the number of eosinophils in nasal secretions of mice with AR [18]. Furthermore, JWYPF prevented the release of histamine, probably as a result of reduced counts of mast cells and eosinophils, as well as of cytokines, that were necessary for the migration of effector cells into nasal epithelial tissues (explained in more detail below). We speculate that the reduction of inflammatory cells might be due to the physiological integrity of the nasal mucosa being maintained by JWYPF; we will study this phenomenon in the future.

As with histamine, JWYPF decreased serum IgE titers in the AR mouse model. This was probably caused by reduced release of

inflammatory cytokines that switched plasma cells into IgE from IgA or other forms. Yang et al. [19] found that YPFS could inhibit airway inflammation, reduce airway hyperresponsiveness, enhance the immune function of cough variant asthma (CVA) patients during remission, and reduce the recurrence rate of CVA, by reducing the level of serum IgE, increasing the level of IgG and IgA, and regulating T lymphocyte subsets. Reduced IgE levels, in turn, caused less release of histamine from degranulation of mast cells, by reducing the sensitized IgE, combined with eosinophils and mast cells.

TGF- β plays a key role in tissue repair and remodeling [20]. Previously, TGF- β had been recognized as playing an essential role in the repression of inflammation; however, recent studies have shown the positive roles of TGF- β in inflammatory responses [21–23] driving inflammation by inducing inflammatory mediator release, and accelerating the progress of inflammation. A study showed that TGF- β over-expression led to significant inflammation in transgenic mice [24]. It follows that modulation of TGF- β signaling might alleviate inflammation. In the present study, we found that TGF- β levels were greater in OVA-sensitized and challenged mice; however, after treatment with JWYPF, levels were lower in nasal mucosa tissues, as measured using immunohistochemistry.

In the present study, changes in the pathology of AR included higher IL-4 levels and lower IFN- γ levels, as well as lower ratios of IFN- γ /IL-4 in the serum of OVA-sensitized/challenged mice, while JWYPF reversed the tendency of pathologic changes and rebalanced the Th1/Th2. This result agrees with the results of

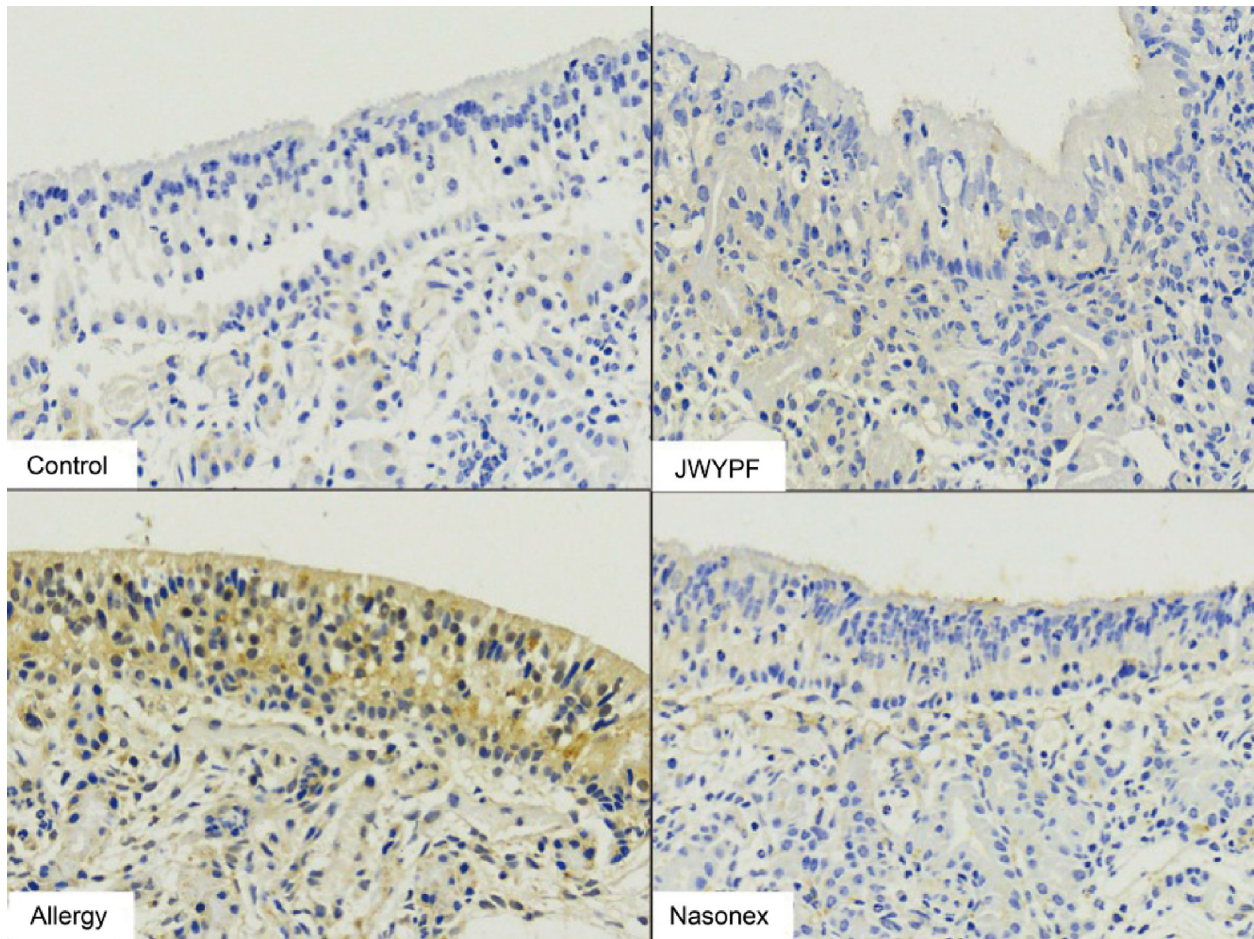


Fig. 6. Immunohistochemical staining of TGF- β of nasal mucosa in each group (brown coloration indicates positive TGF- β staining; magnification 400 $\times$). TGF- β : transforming growth factor- β ; JWYPF: Jiawei Yupingfeng.

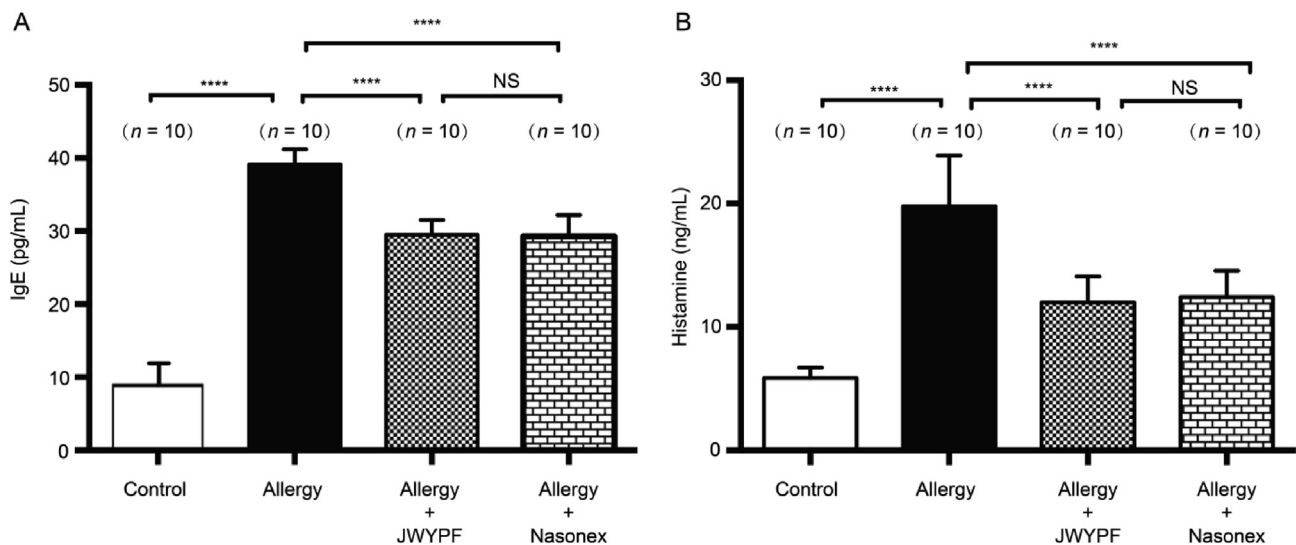


Fig. 7. Levels of IgE and histamine in serum samples from each study group. Panel A shows the results of serum IgE in the various groups. Panel B shows the levels of histamine in the blood in the various groups. Data are expressed as mean $\pm$ standard error of the mean. **** $P < 0.0001$. JWYPF: Jiawei Yupingfeng; NS: not significant.

Wang et al. [25] who found that YPFS markedly reduced IL-4 levels and consequently increased the ratio of IFN- γ /IL-4. IgE production was significantly dependent on the ratio of IL-4 and IFN- γ after

exposure to allergen [26]. These results further suggest that JWYPF inhibits IgE production by regulating IL-4/IFN- γ ratios in allergic responses.

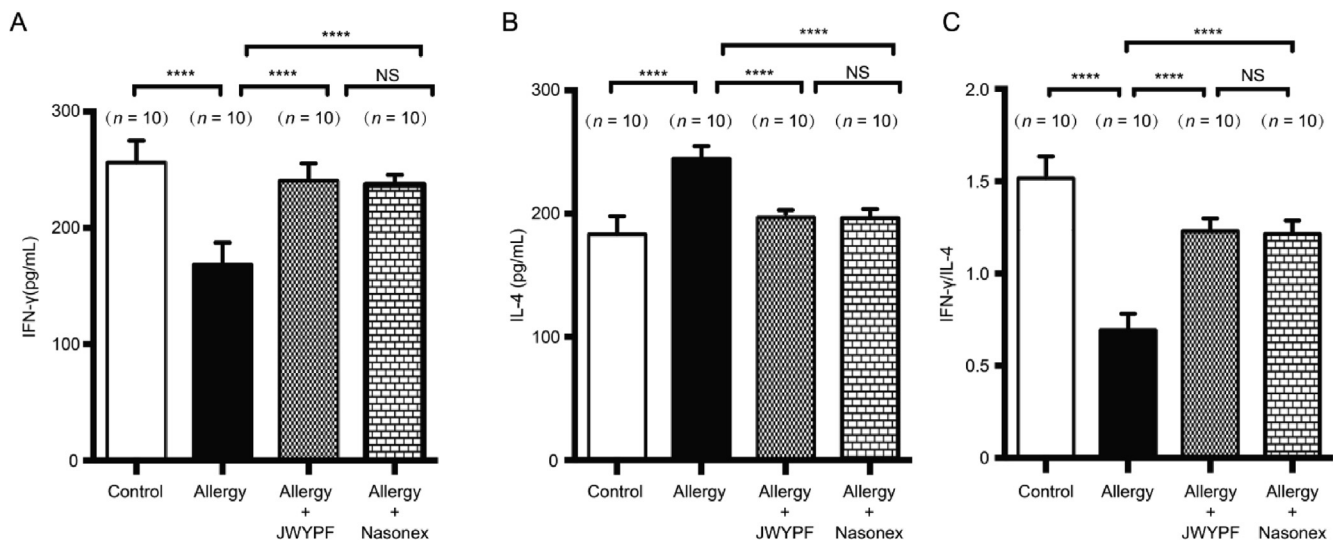


Fig. 8. Cytokine levels in the treatment groups. Panels A and B show the results of serum IFN- γ and IL-4. Panel C shows the ratio of IFN- γ to IL-4. Data are expressed as mean $\pm$ standard error of the mean. **** $P < 0.0001$. JWYPF: Jiawei Yupingfeng; NS: not significant; IFN- γ : interferon- γ ; IL-4: interleukin-4.

5. Conclusion

JWYPF inhibited some allergic parameters in a mouse model of AR. JWYPF inhibited migration of eosinophils and mast cells as well as degranulation in the nasal mucosa; it also down-regulated TGF- β expression, rebalanced cytokine release from T cells, and reduced the OVA-specific IgE secretion from B cells. Taken together, the data suggest that Chinese herbal preparation JWYPF should be considered as a treatment for AR.

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Authors' contribution

HC and WF contributed equally to this study. WF and YL designed the hypotheses and the experiments. All authors participated in data interpretation and manuscript review. HC was responsible for preparation of the tables and figures and manuscript writing. All authors contributed to the scientific discussion of the data and of the manuscript.

Conflicts of interest

All authors declare no conflicts of interest.

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