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Effects of Yupingfeng nasal drops on serum cytokines, histopathology and eosinophil cationic protein in nasal mucosa of rats with allergic rhinitis

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Abstract: Allergic rhinitis (AR) is one of the most common atopic disorders, which seriously affects patients' quality of life. Yupingfeng (YPF) Power is a traditional Chinese herb formula, and its oral dosage form has been widely used for the treatment of AR in Asian countries. In this study, we investigated the effects of YPF nasal drops on ovalbumin (OVA) sensitized/stimulated allergic rhinitis in rats. A Sprague-Dawley (SD) rat model of OVA-induced AR was established and then treated with three doses of YPF nasal drops. Besides, histopathological features, eosinophil cationic protein (ECP) in the nasal mucosa, and expression of type 1 helper T (Th1)/type 2 helper T (Th2)-related cytokines in serum were analyzed. The results showed that YPF nasal drops alleviated the injury of nasal mucosal epithelial structure, promoted the recovery of ciliary morphology and function and reduced interstitial edema and inflammatory cell infiltration to some extent. Moreover, YPF nasal drops regulated imbalance in Th1/Th2 cells caused by AR via regulating downward the expression of interleukin 4 (IL-4) and adjusting upward the expression of interferon-γ (INF-γ) and interleukin 12 (IL-12). Furthermore, it inhibited the expression of ECP in nasal epithelial eosinophil-specific granules. The findings of this study provided a new perspective for the treatment of AR with YPF nasal drops based on Traditional Chinese Medicine.

Keywords: Allergic rhinitis, Yupingfeng nasal drops, histopathology of nasal mucosa, eosinophil cationic protein, serum cytokines, rats.

INTRODUCTION

Allergic rhinitis (AR) is an allergic disease caused by immunoglobulin E (IgE)-mediated immunoreaction and involves mucosal inflammation driven by type 2 helper T (Th2) cells (Wheatley *et al*., 2015; Kawauchi *et al*., 2019). It occurs after the action of allergens on atopic individuals in vitro. The typical symptoms of AR consist of sneezing, itching, nasal congestion, and watery rhinorrhea, and it affects 10% to 40% of the world's population (Licari *et al*., 2014). With increasing incidence, AR is closely related to conjunctivitis, secretory otitis media, chronic nasosinusitis, and adenoid hypertrophy (Brożek *et al*., 2017). It is also a significant risk factor for bronchial asthma (Guerra *et al.*, 2002; Baïz *et al.*, 2019). The number of patients with allergic rhinitis is estimated to be more than 400 million worldwide and 150 million in the Asia-Pacific region (Pawankar *et al.*, 2009). Patients have to endure nasal discomfort with a variety of accompanying symptoms, such as fatigue, insomnia, irritability, etc (Linneberg *et al.*, 2016). AR seriously affects the quality of life and brings enormous economic and psychological pressure to patients (Meltzer EO *et al*., 2012). AR has developed into a global public health problem (Postolache *et al*., 2008; Roger *et al*., 2016).

The main medications for the treatment of AR are oral H1 antihistamines and nasal corticosteroids which are often combined with immunotherapy. These medications can control this allergic disease (Bernstein *et al.*, 2016). There may be some side effects during treatment, such as lethargy, dizziness, fatigue, inattention, and arrhythmias (Bousquet *et al*., 2001). Given these side effects, many patients have received complementary therapies, such as Traditional Chinese Medicine (TCM) and acupuncture in the treatment of AR, which were used for less side effects and low toxicity (Kern *et al.*, 2014; Yonekura *et al.*, 2017). In recent years, TCM has accumulated a wealth of experience in treating allergic diseases. With the development of a large number of clinical studies, TCM has become a recommended medicine in diagnostic and therapeutic guidelines for AR in China (Cheng *et al*., 2018).

Yupingfeng (YPF) formula, a classical prescription in TCM for strengthening healthy qi and eliminating pathogenic factors of the body, originating from the book Danxi Xinfa written by Zhu Zhenheng in Yuan Dynasty, has a history of more than 700 years. It is a famous TCM prescription of qi-boosting and exterior-securing of the body and is widely used in clinical practice. YPF consists of three Chinese herbs: Astragalus, Rhizoma Atractylodis Macrocephalae and Radix Saposhnikoviae. The theory of TCM holds that YPF has a function of enhancing and replenishing qi as well as strengthening the body to prevent perspiration. It has a good effect on enhancing

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immunity. Many studies showed that oral administration of YPF played a decisive role in improving the immunity of the body (Liu *et al*., 2017; Song *et al*., 2016; Huang *et al*., 2015). Increasing evidence indicated that YPF can bidirectionally regulate immune function, it is anti-allergy, anti-inflammatory, and has bacteriostatic effects (Cao *et al*., 2017). YPF is widely used in the prevention and treatment of inflammatory diseases, including allergic diseases and respiratory inflammatory diseases in China and other Asia countries (Song *et al.*, 2013; Shi *et al.*, 2018; Nishijima *et al.*, 2018). It has been proved that the notable advantages of oral YPF formula include relieving nasal symptoms, improving quality of life, reducing the risk of recurrence and alleviating the immunosuppressive effect of hormone drugs (Li *et al*., 2017). However, the underlying mechanism of YPF nasal drops on AR is still unknown and has not been reported yet.

TCM has been used for thousands of years for the prevention and treatment of multiple kinds of human diseases in China because of its low toxicity and few side effects. As an alternative or complementary therapy, TCM has also attracted the attention of western countries in recent years (Zhou *et al*., 2017). A combination of factors causes the occurrence and progression of the disease. The research of TCM is not only confined to the mode of action of multi-component and multi-target, but its mechanism of action is regarded as the interaction between two complex biosystems: A complex material system composed of active drug ingredients and a complex biological system composed of drug targets under pathological conditions. Chinese herbal compound formulas are complicated chemical cocktails with therapeutic properties that modern pharmaceuticals cannot reproduce. It is necessary to further study the mechanism and efficacy of TCM in the treatment of AR.

In this study, we aimed to evaluate the effect of YPF nasal drops on allergic inflammatory responses in the ovalbumin (OVA)-induced AR Sprague-Dawley (SD) rats model. The immune responses of Th1 and Th2 associated with AR were further detected to elucidate the possible therapeutic mechanism of YPF nasal drops.

MATERIALS AND METHODS

Sixty SD adult male rats (280±20g) were fed adaptively for a week at laboratory before the experiment began. The rats were kept in a temperature-controlled (24±2°C), humidity-controlled (60±10%) and light controlled (12: 12 dark/light cycle) experimental environment. All rats were placed in cages and were provided with water and standard rearing environment for rodent animals. The whole research process was conducted at the Experimental Animal Research Laboratory of Hospital of Chengdu University of TCM. This study was in line with the guidelines of the use and care of laboratory animals in the Declaration of Helsinki and was approved by the Experimental Animal Ethics Committee of the Hospital of Chengdu University of TCM.

The prescription of YPF nasal drops was made up of three kinds of herbs: Astragalus, Rhizoma Atractylodis Macrocephalae and Radix Saposhnikoviae. In this study, all herbs were purchased from Sichuan Neautus TCM Co., Ltd. (Chengdu, Sichuan, China), and the extract was prepared by the Pharmacy Department of the Hospital of Chengdu University of TCM. The composition and dosage of YPF were strictly controlled by Pharmacopoeia of the People's Republic of China and the ratio of it is 3:1:1. The nasal drops of YPF contained 60 copies of Astragalus, 20 copies of Rhizoma Atractylodis Macrocephalae and 20 copies of Radix Saposhnikoviae. Radix Saposhnikoviae was chopped for the extraction of volatile oil and the distilled aqueous solution was collected by separate apparatus. The other two herbs and the dregs of Radix Saposhnikoviae were boiled twice in water. The first time decoction cost 1.5 hours while the second time cost 1 hour. The decoction was mixed and filtered afterwards. The filtered decoction was concentrated to an appropriate amount and ethanol was added to precipitate it. The supernatant was removed to reduce pressure and ethanol was recovered. The decoction was then well stirred with water and was sat still. The supernatant of the above liquid was filtered and the filtrate was concentrated. Volatile oil and water were added into the filtrate. The relative density should not be lower than 1.16, with 4.0-5.5 potential of hydrogen (PH) value (Chinese Pharmacopoeia Commission, 2015).

Sixty rats were randomly divided into six groups: Control group, AR model group, group with high concentrations of YPF nasal drops, group with middle concentrations, group with low concentrations (crude drug concentrations were 0.54g/ml, 0.27g/ml, 0.1g/ml) and mometasone furoate aqueous nasal spray (MSD Belgium BVBA/SPRL, Brussels, Belgium) group (MF group). There were ten rats in each group. The standard protocol was used to establish an AR rats model (Long R *et al*., 2015). From the first day, 0.3mg OVA used as antigen and 30mg $Al(OH)_3$ used as adjuvant and mixed with 1ml saline had been taken as the configuration of suspensions, and all the model rats had been sensitized by intraperitoneal injection every other day for a total of seven times. From the fourteenth day, the rats had received an intranasal challenge with 50μl of 5% OVA saline solution once a day for seven days in each nasal cavity. On days 21-34, rats in each group were treated one hour before the intranasal challenge of OVA. The AR model group was treated with 10μl saline nasal drops. MF group accepted MF, 5μg/kg/d, in both nasal cavities once a day. The groups of high, medium and low concentrations were given YPF nasal drops with a corresponding concentration of 10μl twice a day for two weeks respectively.

Six rats were randomly selected from each group and were given the intraperitoneal injection of 3% pentobarbital sodium (30mg/kg). The nasal mucosal tissues were fixed with 4% paraformaldehyde and were then dehydrated, pruned, embedded, sliced, dyed, sealed by an automatic dehydrator and finally observed under a microscope.

Blood samples collected from the abdominal aorta of rats in each group and the supernatant were isolated. The biotin double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was used for the detection of interleukin 4 (IL-4), interferon-γ (IFN- γ) and nterleukin 12 (IL-12) secretion in serum. The steps were as follows: (1) rewarming: adjusting all reagents to room temperature; (2) diluting standard products; (3) adding samples; (4) matching solutions; (5) washing; (6) color development; (7) termination: 50μL per pore plus termination solution; (8) determination: adjusting the blank to zero and detecting the absorbance of each pore (OD value) at 450nm wavelength in turn.

The expression of eosinophil cationic protein (ECP) in the nasal mucosa of rats was observed under the microscope after immunohistochemical staining, and the location and intensity of ECP expression were determined. (1) The dewaxed slices were placed in a dyeing cylinder at room temperature for 10min with 3% methanol hydrogen peroxide;(2) the slices were washed with PBS for 3 times with 5min for each time; (3) the slices were immersed in 0.01m citrate buffer solution (PH 6.0) and were heated with high temperature; the process was repeated for one time 5min later; the slices were cooled and were washed twice using PBS with 5 minutes for each time; (4) drops of goat serum blocking solution were added with 20min standing time at room temperature; (5) drops of first antibody were added and the solution was sat still at 4ºC all night long; (6) drops of biotinylated secondary antibody were added with 30min standing time at 37ºC; (7) the slices were washed with PBS for 3 times with 5min for each time; (8) DAB coloration. The above specimens were tackled according to the SOP procedure of pathological examination. The integrated optical density (IOD) and the area of ECP protein expression of all acquired images were measured using an Image-Pro Plus 6.0 image analysis system. The mean OD of each image was calculated, and the OD of each sample was obtained by using the mean OD of three images.

STATISTICAL ANALYSIS

All data was presented as the means ± standard ($\overline{X}$ ±SD) and was analyzed by SPSS 20.0 software. One-way ANOVA was used to compare the differences among all groups, and the Student-Newman-Keuls test was used to compare the differences between the two groups. P-values below 0.05 indicated statistical significance.

RESULTS

Nasal mucosa histopathology

In this study, the section of nasal mucosa revealeds intact architecture in the control group. The cells in each layer of mucosal epithelium arranged neatly, and there were no edema and degeneration in the stroma (fig. 1A). Nasal epithelial tissue destruction, mucosal epithelial cell degeneration and proliferation, cytoplasmic dissolution, unclear edge, mucosal epithelial necrosis, blurred structural boundaries, increased submucosal tissue permeability, and edema degeneration were observed in the nasal mucosa sections of AR rats model (fig. 1B) In addition, groups administered with YPF nasal drops and MF group, especially group with high concentrations of YPF nasal drops (0.54g/mL) (fig. 1C) and MF group (fig. 1F) showed that the mucosal structure was relatively complete with smooth surface, the cilia arranged neatly, and there was no significant edema, degeneration as well as inflammatory cell infiltration in the stroma. By contrast, group with middle concentrations of YPF (0.27g/mL) (fig. 1D) and group with low concentrations (0.135g/mL) (fig. 1E) had a different degree of nasal mucosa injuries.

Effect of YPF nasal drops on Th1 and Th2-associated cytokines in serum and ECP in the nasal mucosa

As shown in (fig. 2A), the mean levels of serum IL-4 in the AR model group increased very highly significantly ($P<0.01$) compared with the control group, whereas the levels of IL-4 in the serum of rats in group with high concentrations of YPF and MF group decreased significantly ($P<0.05$) compared with the model group. There were no significant differences in the serum IL-4 among other groups. The serum of IL-12 and IFN-γ in the AR model group significantly dwindled compared with that in the control group ($P<0.01$). The levels of the serum IL-12 in the MF group were significantly different from that in the model group ($P<0.01$). Moreover, compared with the model group, the serum IL-12 in group with high concentrations of YPF nasal drops ($P<0.05$) and MF group ($P<0.01$) increase significantly (fig. 2B). Besides, the content of IFN-γ in group with high concentrations of YPF and MF group grew significantly compared with the model group ($P<0.05$) (fig. 2C). There were no significant differences in IFN-γ and IL-12 in the serum of other groups. The levels of ECP in the nasal mucosa of the AR model group rised significantly ($P<0.01$) compared with the control group. The ECP levels in the nasal mucosa of rats in the group of high concentrations and the MF group declined ($P<0.05$) compared with the control group (fig. 2D) The positive cells were yellow or brownish yellow, and the ECP positive products mainly distributed in nasal cilia (fig. 3).

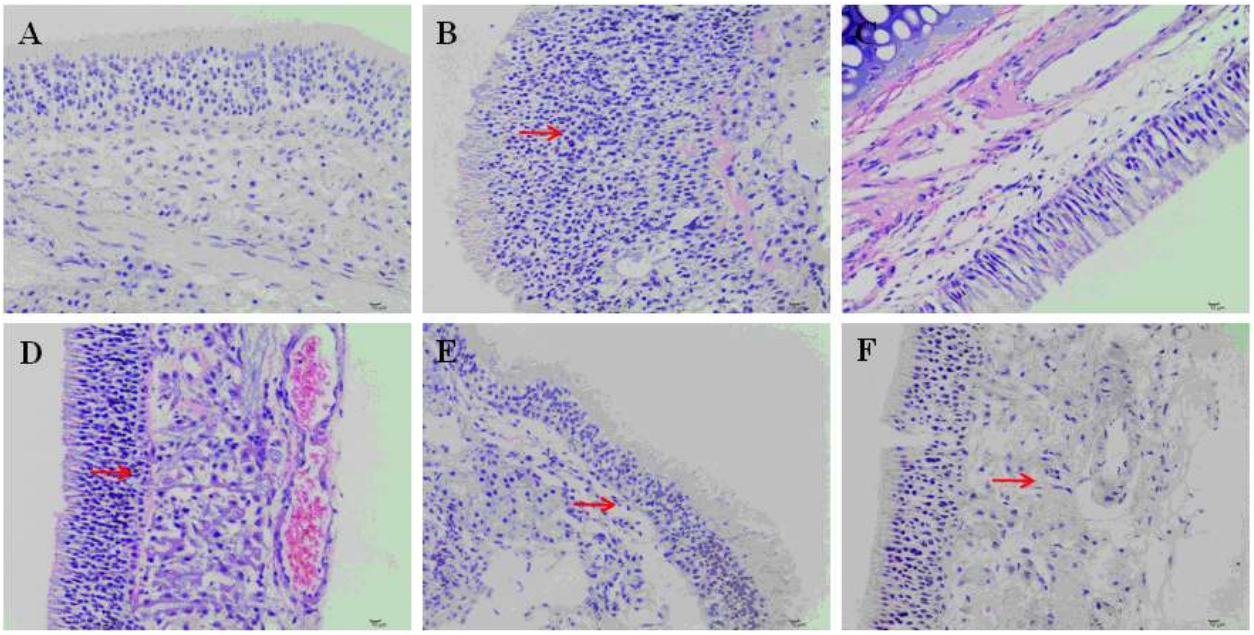

Fig. 1: Nasal mucosa section of (A) control group, (B) OVA-induced AR model group, (C) YPF nasal drops with high concentrations group (0.54g/mL), (D) YPF middle concentrations group (0.27g/mL), (E) YPF low concentrations group (0.135g/mL), (F) MF group. (H&E, × 400).

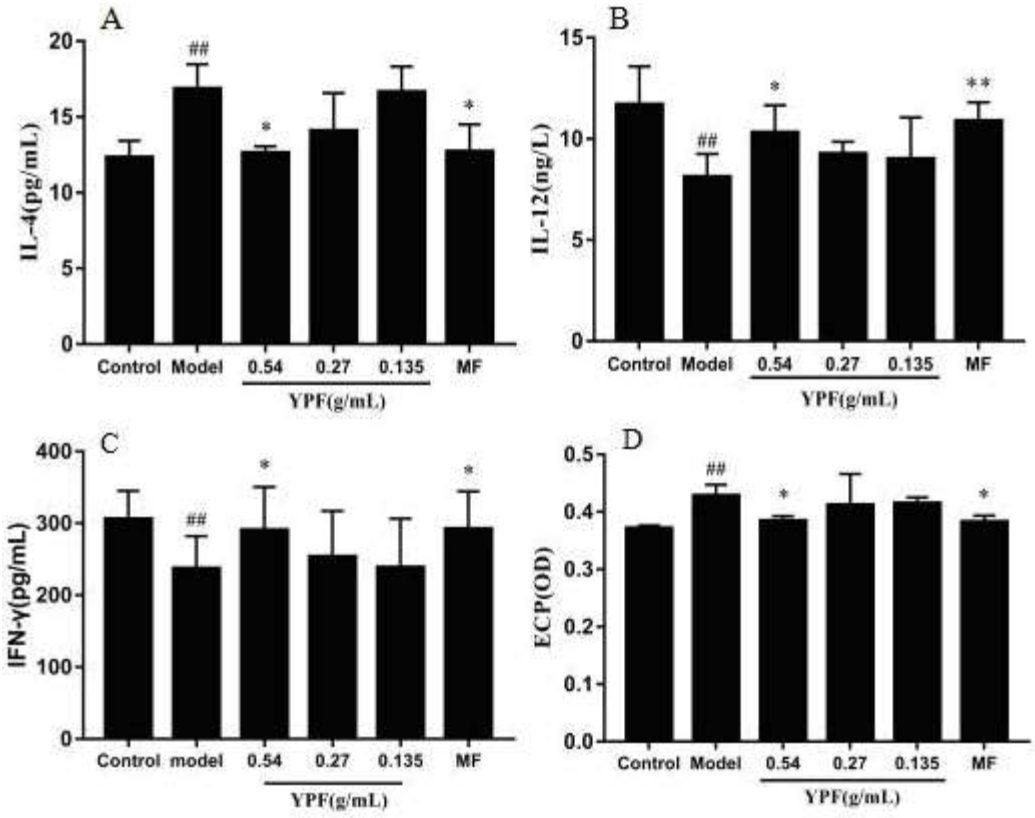

Fig. 2: Effect of YPF nasal drops on serum IL-4 in OVA-induced rats of AR (A). Effect of YPF nasal drops on serum IL-12 in OVA-induced rats of AR (B). Effect of YPF nasal drops on serum IFN-γ in OVA-induced rats of AR (C). Statistical results of ECP mean optical density in the nasal mucosa of rats (D). $^{\#}P < 0.05$ and $^{\#\#} P < 0.01$ vs Control, $^{*} P < 0.05$ and $^{**} P < 0.01$ vs Model, N=6.

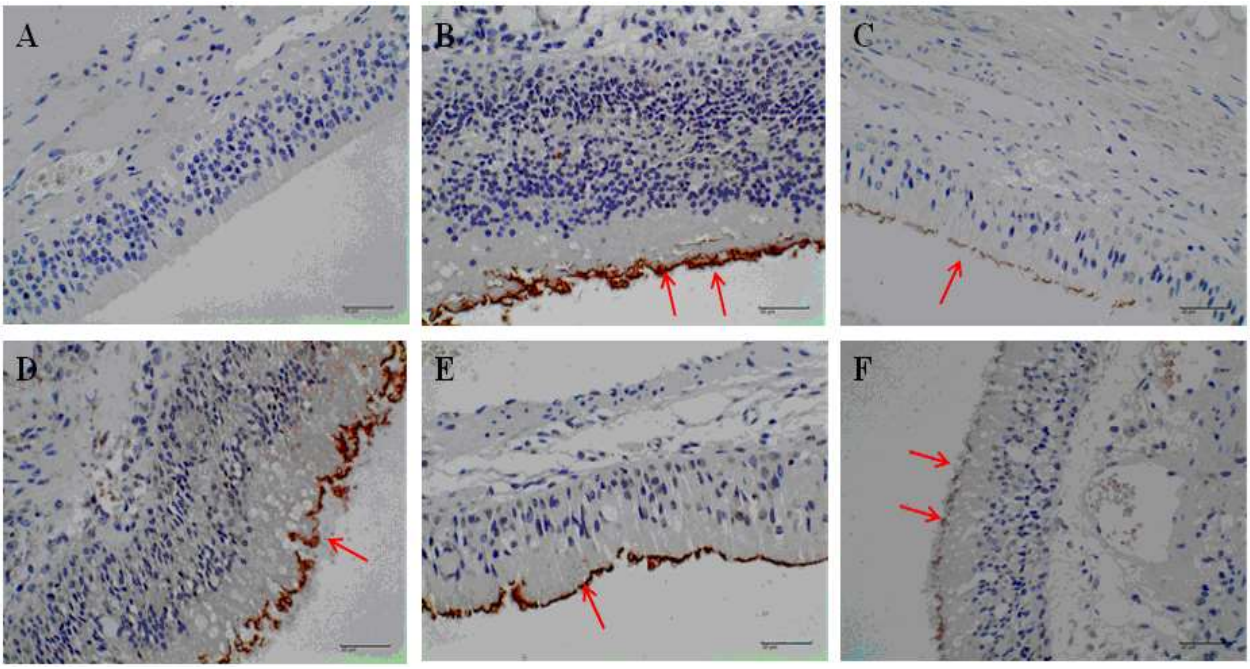

Fig. 3: Effect of YPF nasal drops on ECP of nasal mucosa epithelium in AR rats. (A) control group, (B) OVA-induced AR model group, (C) YPF nasal drops with high concentrations group (0.54g/mL), (D) YPF middle concentrations group (0.27g/mL), (E) YPF low concentrations group (0.135g/mL), (F) MF group (H&E, ×400).

DISCUSSION

AR is a significant chronic health problem worldwide. With the increasing incidence, AR is called the epidemic of the 21st century (Zvezdin *et al*., 2015). The animal model of AR can be successfully established by using OVA as an allergen and Al $(OH)_3$ as an adjuvant in previous studies (Vanacker *et al*., 2001). We successfully established an AR rats model via intraperitoneal injection of OVA, Al $(OH)_3$ suspension and nasal drip of 5% OVA. In the AR model group, the rats displayed significantly increased serum levels of IL-4 and decreased serum levels of IFN-γ and IL-12. In addition, the nasal mucosal epithelium of rats AR model was necrotic, the structural boundary was blurred, the permeability of submucosal tissue increased, and the edema and degeneration were observed in the AR rat's model. These results showed that the AR rat's model was built successfully and showed an obvious AR response.

The proportion balance of Th1/Th2 is an essential link to the immune response. This dynamic balance maintains normal cellular and humoral immune defense function of the body. AR is a chronic inflammatory disease of nasal mucosa caused by the imbalance of Th1 and Th2 immune response as a result of the action of environmental factors in vitro (Han *et al*., 2010). Cytokines are essential factors in the detection of immune system regulation. Due to their sensitivity, they can be regulated with lower doses medicine (Shen *et al*., 2001). IL-4 is a necessary factor for the transformation of Th2 cells. It can promote the proliferation of thymus-derived cells and bone marrow-derived cells, the expression of adhesion molecules in vascular endothelial cells, and the infiltration of inflammatory cells, thus aggravating symptoms of AR (Baumann *et al*., 2013). IL-4 can induce the production of Th2 cytokines, nitrous oxide, and some chemokines in Th0 cells and also can inhibit the activation of helper T cells. It promotes the production of IgE, the formation of IgE based infiltration of eosinophils (EOS), and mast cells and mediated immune response. It is the autocrine growth factor of Th2 cells, which is the main factor of Th2 differentiation and can promote the immune response of Th2 cells. IFN-γ can be activated by Th1 cells. It can enhance the regulation of immune T cells, inhibit the inflammatory response, and then inhibits the overactivation of Th2 cells in order to maintain the balance of Th1 and Th2. IFN-γ plays a protective role in the occurrence of allergic diseases. IL-12 is an essential link between innate immunity and adaptive immunity, and a proinflammatory cytokine with a variety of immunomodulatory functions. As the most active natural killer cell (NK) activating factor and T lymphocyte inducer, IL-12 can elicit the immune response of Th1 and Th2 cells, and participate in the immune response of the organism. It has biological effects of inducing CD_4 + initial T cells to differentiate into Th1 cells, inhibiting the synthesis of IL-4 by Th2 cells and selectively inhibiting the synthesis of IgE induced by IL-4 (Chen *et al*., 2014; Hemdan, 2008*)*. It has been proved that a low level of IL-12 is closely related to the pathogenesis, curative effect, and prognosis of bronchial asthma (Guo *et al*., 2014). Th1 cell secretes IFN-γ whereas IL-12 promotes the activation of Th1 cells and inhibits the activity of Th2 cells. The

main role of IFN-γ and IL-12 is to inhibit the homologous transformation of B cells to produce IgE, which is the cause of allergic reaction (Natarajan *et al*., 2014). Increasing evidence showed that TCM stimulates humoral and cellular immunity by regulating the proliferation of immune cells and the expression of enzymes, receptors and cytokines related to immune response (Ma *et al.*, 2013). In this study, YPF nasal drops also played a regulatory role in Th1/Th2-related cytokines.

As a highly basic toxic protein, ECP is synthesized by EOS and is stored in the cytoplasmic granules of EOS. ECP is one of the four major eosinophilic proteins. It is an essential inflammatory marker to reflect the degree of EOS activation. The concentration of EOS can reflect the degree of increased EOS in tissue and the severity of disease inflammation. The study of serum ECP in patients with AR showed that there was no significant difference in the content of serum ECP between the patients with AR and the healthy controls (Chen, 2006). Besides, the serum ECP levels in AR patients were significantly higher than that in healthy subjects (Sin *et al*., 1998; Marcucci *et al.*, 2001). The above research results showed that the role of ECP in peripheral blood of patients with AR has not been fully elucidated. The expression of ECP in peripheral blood is more complex, and it may relate to a variety of factors. Based on pure Chinese medicine YPF nasal drops were used to treat rats with OVA-induced AR in the nose. We directly observed the expression and intensity of ECP in nasal mucosa through the direct administration site of the model animal. This study demonstrated that the expression of ECP in nasal mucosa was positively correlated with the expression of serum IL-4.

CONCLUSION

Histological examination showed that YPF nasal drops could reduce the injury of the nasal mucosa of rats with AR to some extent. Meanwhile, it inhibited the expression of IL-4 in serum and ECP in nasal epithelial cells and increased the levels of INF-γ and IL-12 in serum. The results showed that YPF nasal drops might improve inflammatory response by regulating the immune imbalance of Th1/Th2 cytokines in the treatment of AR. Different from the traditional Chinese oral medicine used in the treatment of AR, YPF nasal drops used in the nasal cavity provides a new idea for the treatment of AR with TCM. This study laid a foundation for further research on the development and application of YPF nasal drops as a supplementary alternative therapy.

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