Eosinophil Chemokines and Clara Cell Protein 16 Production in Nasal Mucosa of Patients with Persistent Allergic Rhinitis

Persistan Alerjik Riniti Olan Hastaların Nazal Mukozalarında Eozinofil Kemokinler ve Clara Hücresi Protein 16 Üretimi

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ABSTRACT

Objective: Eotaxin-2 and regulated on activation normal T cell expressed and secreted (RANTES) are involved in the eosinophil trafficking in patients with persistent allergic rhinitis (PAR). Clara cell protein 16 (CC16) is an anti-inflammatory protein mainly produced by the epithelial non-ciliated Clara cells. The aim of this study was to investigate the production of CC16 and chemokines eotaxin-2 and RANTES in nasal mucosa of patients with PAR.

Materials and Methods: Twenty-one PAR patients and 20 healthy participants were included. CC16, eotaxin-2, and RANTES concentrations were measured in nasal secretions. PAR patients were administered fluticasone furoate nasal spray (220 µg daily for 14 days). We performed nasal cytology, symptom score assessment, and inflammatory mediator detection before and after the therapy.

Results: The level of CC16 in patients with PAR was lower than in the healthy subjects (p=0.023). The eosinophil counts and local concentrations of eotaxin-2 and RANTES were higher in patients with PAR in comparison with controls (p=0.008, p=0.001, p=0.031, respectively). We also found a negative correlation between the CC16 and eotaxin-2 levels in nasal secretions of PAR patients (r=−0.492, p=0.023). After corticosteroid therapy, the patients with PAR had lower nasal symptoms, eosinophil counts, eotaxin-2, and RANTES levels and higher levels of CC16 (p<0.001 for all parameters).

Conclusion: Our results suggest the presence of a negative correlation in production of CC16 and eotaxin-2 in nasal mucosa of patients with PAR. Intranasal corticosteroids have a suppressive effect on mucosal eosinophilic inflammation and a stimulating effect on local CC16 production.

Keywords: Allergic rhinitis, chemokines, eosinophils, glucocorticoids, inflammation mediators

ÖZ

Amaç: Eotaksin-2 ve RANTES (aktivasyonla regüle edilen, normal T hücresi ekspresyonu ve sekresyonu) persistan alerjik riniti (PAR) olan hastalarda eozinofil göçünde yer almaktadır. Clara hücresi protein 16 (CC16) çoğunlukla sil içermeyen epitelyal hücreler tarafından üretilirler. Bu çalışmanın amacı PAR hastalarının nazal mukozasında CC16 ve eotaksin-2 ve RANTES kemokinlerinin üretimini araştırmaktır.

Gereç ve Yöntemler: Çalışmaya 21 PAR hastası ve 20 sağlıklı katılımcı dahil edildi. Nazal sekresyonlardaki CC16, eotaksin-2 ve RANTES seviyeleri ölçüldü. PAR hastalarına flutikazon furoat nazal sprey uygulandı (14 gün boyunca günlük 220 µg doz). Tedavi öncesi ve sonrasında, nazal sitoloji, semptom skoru değerlendirmesi ve inflamatuar mediyatör taraması yapıldı.

Bulgular: CC16 seviyesi PAR hastalarında sağlıklı deneklere kıyasla daha düşük bulundu (p=0,023). Kontrollere göre, eozinofil sayıları ve lokal eotaksin-2 ve RANTES seviyeleri PAR hastalarında daha yüksekti (sırasıyla p=0,008, p=0,001, p=0,031). Ayrıca PAR hastalarının nazal sekresyonlarında CC16 ile eotaksin-2 düzeyleri arasında negatif bir korelasyon bulundu (r=-0,492, p=0,023). Kortikosteroid tedavisi sonrasında, PAR hastalarında daha düşük nazal semptomlar, eozinofil sayıları, eotaksin-2 ve RANTES seviyeleri ve daha yüksek CC16 izlendi (tüm parametreler için p<0,001).

Sonuç: Bulgularımıza göre, PAR hastalarının nazal mukozalarında CC16 ve eotaksin-2 üretiminde negatif bir korelasyon vardır. İntranazal kortikosteroidler mukozal eozinofilik inflamasyon üzerinde baskılayıcı bir etkiye ve lokal CC16 üretimi üzerinde de stimüle edici bir etkiye sahiptirler.

Anahtar Kelimeler: Alerjik rinit, kemokinler, glukokortikoidler, eozinofiller, inflamasyon mediyatörleri

Introduction

Persistent allergic rhinitis (PAR) is a chronic, immunoglobulin E (IgE)-mediated, T helper 2 (Th2)-type immune response disease, histologically characterized by intense mucosal infiltration by eosinophils, mucosal hypersecretion, and tissue remodeling [1]. Eosinophils can directly damage the epithelium of the nasal mucosa by toxic products, such as eosinophil cationic protein (ECP),

mayor basic protein (MBP), and eosinophil peroxidases (EPOs) [2]. These enzymes stimulate the production of epidermal and neural growth factors resulting in hypertrophy and neuronal hyperreactivity of the nasal mucosa [3, 4].

The pathophysiology of mucosal hypereosinophilia in patients with PAR is not well-known. Previous investigations showed an increased chemokine-guided migration of eosinophils in all chronic upper airway eosinophilic inflammatory disorders [5, 6]. Eosinophil chemokines can be divided into non-selective and selective. Regulated on activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein-1 alpha (MIP-1α), MIP-1β, monocyte chemoattractant protein-1 (MCP-1), MCP-3, and MCP-4 are non-selective chemokines, and they attract not only eosinophils, but also monocytes, macrophages, and lymphocytes [7, 8]. Eotaxin-1, -2, and -3 are strong selective chemokines and they attract only eosinophils via specific CC chemokine receptor 3 (CCR3) [7].

Respiratory epithelial cells are the first-line defense barrier against microbes and allergens, as well as a very important source of inflammatory mediators. The non-ciliated secretory Clara cells are the part of upper and lower respiratory tract epithelium. These cells have an important function in modulation of immune responses by production of anti-inflammatory factors [9]. Clara cell protein 16 (CC16), named also "uteroglobin," is an anti-inflammatory protein of small molecular weight (16 kDa). This mediator is dominantly produced by Clara cells in nasal and bronchoalveolar epithelium [9]. Previous investigations suggest that CC16 has an important role in controlling and inhibition of oxidative stress and inflammatory response in nasal mucosa of patients with seasonal allergic rhinitis (SAR) [10, 11]. The anti-inflammatory effect of CC16 is thought to be dependent on the inhibition of pro-inflammatory enzymes phospholipase A2 (PLA2) and transglutaminase. Both these enzymes play a key role in arachidonic acid release during an allergic inflammation [9-11]. However, the role of CC16 in regulation of chronic inflammation in patients with PAR has not been fully investigated. Also, the relationships between the CC16 activity and local production of selective (eotaxin-2) and non-selective (RANTES) eosinophil chemokines are not well-known. The aim of this study was to analyze this relationship and to evaluate local production of CC16, eotaxin-2 (CCL24), and RANTES (CCL5) in nasal mucosa of patients with PAR, before and after nasal glucocorticoid administration.

Materials and Methods

Participants

Twenty-one (n=21) PAR patients and 20 control participants were enrolled in this cross-sectional and case–control study, which was performed between September 2014 and September 2015, in accordance with the Helsinki Declaration. The protocol of investigation was approved by the Ethics Committee of our institution (Approval number 22-05-2014)hospitals. Written informed consent was obtained from participants in both the PAR and control group. The control group consisted of 20 participants without symptoms and clinical findings of nasal inflammation and negative for allergy tests.

The diagnosis of PAR was done in accordance with the definition of PAR given in the Allergic Rhinitis and Its Impact on Asthma Guideline [12]. In the patients group, we included only the subjects with a history of "PAR," with the presence of nasal symptoms (nasal obstruction, rhinorrhea, itching, sneezing, and hyposmia) more than 4 days a week and for more than 4 weeks to avoid differences due to actual allergen exposure between co-seasonal and extra-seasonal patients. The PAR patients had sensitivity to at least one indoor allergen (house dust mite, animal dander, mold-like aspergillus, etc.), positive serological test (total serum IgE concentration >100 IU/mL), negative endoscopic findings for nasal polyps and infection (several mucosal edemas with mucopurulent discharge and/or purulent nasal crusting), and negative CT scan of paranasal sinuses for mucosal swelling.

We excluded from our study all patients with non-allergic rhinitis with eosinophilia syndrome (NARES). As previously described [7, 8], these patients had typical persistent symptoms of rhinitis (nasal obstruction, rhinorrhea, itching, sneezing, hyposmia), typical cytological finding (profound eosinophilia in the nasal mucosa with more than 20% eosinophils in the total granulocytic or mononuclear cell population, excluding nasal epithelial cells), but negative all allergy tests, negative endoscopy for nasal polyps and infection, and negative CT scan of paranasal sinuses for mucosal swelling. Allergic status was evaluated in all study subjects in accordance with the presence of nasal symptoms and results of skin prick tests and serology tests. For skin prick tests, we used the standard battery of 15 respiratory allergens including, among others, house dust mites, fungus, dogs, and cats (Soluprick® SQ, Hørsholm, Denmark). Histamine dihydrochloride (1 mg/mL solution) was used as positive and saline as negative control. The size of the cutaneous reaction on the volar part of the forearm was measured after 15 min. The allergic response was considered positive when the mean wheal diameter was greater than or equal to the diameter of the histamine wheal. Total serum IgE was detected using the enzyme-linked immunosorbent assay (ELISA) kit (ELITechGroup, Milano, Italy). Subjects with serum IgE level >100 IU/mL were considered atopic.

Exclusion criteria were NARES, chronic rhinosinusitis with or without nasal polyps, asthma, reactivity to non-steroidal anti-inflammatory drugs, systemic diseases with nasal manifestations (Wegener's granulomatosis), and others. Also, we excluded the subjects with history of nasal surgery; cigarette smoking; acute lower and upper airway inflammation; and use of systemic or topical antihistamines, steroids, and antibiotics during the 3 weeks before the start of the study. Inclusion and exclusion criteria for the study group are presented in Table 1.

Drug administration

Fluticasone furoate aqueous nasal spray (Avamys®, Glaxo Smith Kline, Brentford, United Kingdom) in a daily dose of 220 µg (two morning applications in both sides of the nasal cavity) was used for 14 days to all patients with PAR. The patency of nasal cavities and ability to proper use of the spray device were verified in all PAR patients.

Symptoms

All the PAR patients were treated and followed up by the same rhinologist, before and within 2 days after the fluticasone furoate therapy. The patients assessed the severity of symptoms (nasal obstruction, rhinorrhea, itching, sneezing, and hyposmia) into the following categories: 0 for lack of symptoms, 1 for mild, 2 for moderate, and 3 for severe symptoms, as previously described [13]. The maximal nasal symptom score is 15.

Nasal cytology

Nasal leukocyte counts were performed in all participants, before and within 2 days after treatment with fluticasone furoate. The samples of mucosa tissue from inferior turbinate were obtained bilaterally by nasal curettes (RhinoProbe™ Nasal Cytology Curettes, ASI, Los Angeles, USA). After gently spreading on glass slides and fixation by 95% ethanol, the tissue samples were stained with May Grünwald–Giemsa method. The percentage of eosinophils was assessed blindly by an experienced cytologist by light microscopy. The number of eosinophils was expressed as a percentage of total

Table 1. Inclusion and exclusion criteria for the study group

Inclusion criteria for the study group
Nasal symptoms for more than 4 days for a week and longer than 4 weeks
Negative nasal endoscopy for polyps
No infection signs on nasal endoscopy
Negative CT scan of paranasal sinuses for mucosal swelling
Positive skin prick test for at least one indoor allergen, including house dust mite, animal dander or mold like aspergillus
Total serum IgE level >100 IU/mL
Exclusion criteria for the study group
Non-allergic rhinitis with eosinophilia syndrome (NARES)
Chronic rhinosinusitis with or without nasal polyps
Bronchial asthma
Sensitivity to non-steroidal anti-inflammatory drugs
Systemic diseases affecting the nose
History of cigarette smoking
Previous nasal/paranasal sinus surgery
Upper and lower respiratory tract infections
Use of antibiotics, antihistamines, and corticosteroids during the three weeks before the start of the study

Table 2. Sensitivities of detection, assay range, and coefficient of variation for investigated mediators

Mediator	Sensitivities of detection	Assay range	Coefficient of variation
CC16	0.61 ng/mL	1.56 ng/mL-100 ng/mL	10%
Eotaxin-2	24.9 pg/mL	62.5 pg/mL-4000 pg/mL	10%
RANTES	3.0 pg/mL	3.0 pg/mL-2000 pg/mL	6.8%

CC16: Clara cell protein 16; RANTES: regulated on activation normal T cell expressed and secreted

Table 3. Baseline data

Parameters	PAR	Controls	p
Participants (n)	21	20	/
Male/female ratio	12/9	10/10	/
Age (years)*	41.85±10.35	41.15±11.35	/
Nasal symptom score*	7.26±1.23	0	/
Serum IgE (IU/mL)*	138.27±27.36	67.85±24.18	0.028
Eosinophils (%)*	31.56±2.13	4.25±1.25	0.008
CC16 levels (ng/mL)*	14.86±12.03	30.76±24.98	0.023
Eotaxin-2 levels (pg/mL)*	274.36±215.90	69.83±60.75	<0.001
RANTES levels (pg/mL)*	44.25±19.85	29.40±13.50	0.031

* Values are presented as mean±SD.
IgE: immunoglobulin E; CC16: Clara cell protein 16; RANTES: regulated on activation normal T cell expressed and secreted

number of inflammatory cells (mononuclear and granulocyte origin), excluding respiratory epithelium, at a magnification of ×400, as the mean percentage of the 10 examined fields.

Detection of mediators

Nasal fluid samples were obtained from all 41 subjects, that is, 21 with PAR and 20 healthy subjects, before and after fluticasone furoate administration by absorption method. After the insertion of cotton wool stick (Institute for Virology, Vaccines and Sera "Torlak," Belgrade, Serbia) into the nasal middle meatus for 5 min, as previously described [7], the stick watered with nasal fluid was put in a 2-mL tube, which contained 1 mL of transfer medium (two antibiotics and one antimycotic in phosphate-buffered saline). It takes about 30 min for the diffusion of mediators into the medium. After centrifugation of samples for 10 min and cell separation, the supernatants were frozen at −70°C, until mediator determination. The measurement of CC16 was done by using the human ELISA kit (Clara cell 16 kDa protein human ELISA kit, Cloud Clone Corporation, Houston, USA). The eotaxin-2 and RANTES concentrations were also measured by commercial human ELISA kits (Eotaxin-2 (CCL24) human ELISA kit, Cloud Clone Corporation, Wuhan, China; RANTES (CCL5) human ELISA kit, Bio Legend, San Diego, USA). The levels of CC16 were expressed in nanograms/milliliters (ng/mL), whereas the concentrations of eotaxin-2 and RANTES were expressed in picograms/milliliters (pg/mL). The sensitivities of detection, assay range, and coefficients of variation for investigated mediators are presented in Table 2.

Strength of the study and statistical analysis

The results of study by De Corso et al. [8] showed the highest statistical difference in the levels of eotaxin-2 (CCL24) in nasal secretions in patients with PAR in comparison to healthy participants (128.9±51.7 pg/mL in comparison to 16.4±10.7 pg/mL). With criterion that the expected strength of the effect was 0.4 (more than 30% of difference between the groups), a power analysis predicted that simple sizes of 16 patients in each group would be required to raise the power of the study of 80%. The type I error (α level) was set to 0.05. For calculation of the number of participants in each group, the G*Power 3.1.9 program (Heinrich Heine Univerität, Düsseldorf, Germany) was used. The parameters were expressed as mean±standard deviation. For comparison between the groups, a non-parametric Mann–Whitney U test was used. For paired comparisons in a group, we used the Wilcoxon's test. Assessment of the correlation between the different parameters was explored using the Spearman's test. p values <0.05 were considered significant. The analysis was done by using the Statistical Package for the Social Sciences, version 15.0 software (SPSS Inc.; Chicago, IL, USA).

Results

All participant's characteristics and baseline data are presented in Table 3.

The mean serum IgE level was higher in PAR patients than in the control group (p=0.028). We found significantly higher concentration of CC16 in nasal fluid in healthy participants than in the PAR group (p=0.023). In contrary, significantly higher concentrations of chemokines

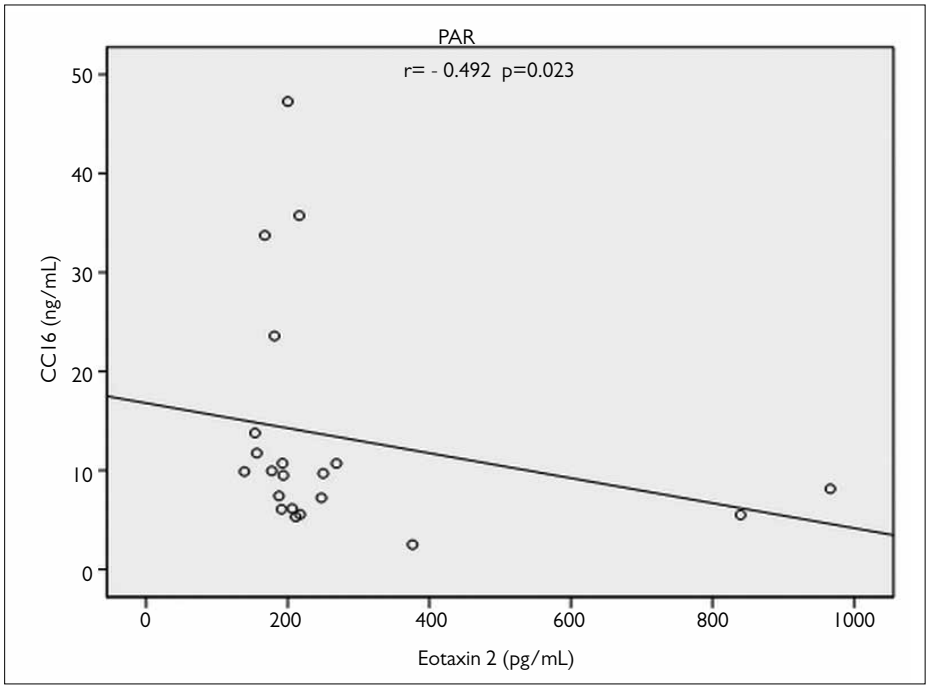

Figure 1. Inverse correlation between the concentration of CC16 and eotaxin-2 in nasal secretions in patients with PAR

Table 4. Data before and after nasal fluticasone furoate administration in patients with PAR

Parameters	Before treatment	After treatment	p
Nasal symptom score *	7.26±1.23	2.27±0.76	<0.001
Eosinophils (%) *	31.56±2.13	22.36±4.06	<0.001
CC16 levels (ng/mL) *	14.86±12.03	33.85±14.81	<0.001
Eotaxin-2 levels (pg/mL) *	274.36±215.90	144.11±61.95	<0.001
RANTES levels (pg/mL)*	44.25±19.85	26.12±13.22	<0.001

* Values are presented as mean±SD
CC16: Clara cell protein 16; RANTES: regulated on activation normal T cell expressed and secreted

eotaxin-2 and RANTES in nasal secretions were measured in patients with PAR than the control group (p<0.001, p=0.031, respectively). Mean eosinophil percentage in the cytological findings was higher in PAR patients than in control group (p=0.008).

Our results showed a negative correlation between the CC16 and eotaxin-2 levels in nasal secretions in PAR patients (r=−0.492, p=0.023) (Figure 1). We found no other correlations (positive or negative) between the investigated parameters.

After the nasal treatment, we demonstrated significantly decreased nasal symptom score (p<0.001), eosinophil counts (p<0.001), local eotaxin-2 (p<0.001), and RANTES (p<0.001) levels in patients with PAR. On the other hand, nasal fluid CC16 concentration was significantly increased in PAR patients after the fluticasone furoate therapy (p<0.001) (Table 4).

Discussion

Previous investigations showed a suppressive effect of allergic inflammation on CC16 production [9, 11, 14]. The levels of CC16 are lower in serum and nasal secretions of allergic children in comparison with healthy ones [14]. The concentrations of CC16 in nasal secretions of patients with seasonal allergic rhinitis are lower during the pollen season [11]. The results of one study concerning the role of CC16 in nasal inflammation in patients with PAR showed a positive correlation between the nasal fluid concentrations of CC16 and nasal production of nitric oxide (NO) [15]. This study also demonstrated a negative correlation between these biomarker concentrations in nasal secretions and the levels of mucosal infiltration by mast cells [15].

Our results showed significantly lower concentrations of CC16 in nasal secretions of PAR patients than in the nasal fluid of healthy participants. On the other hand, the local number of eosinophils in the nasal mucosa is significantly higher in patients with PAR. These findings suggest that allergic inflammation has an inhibitory effect on local production of CC16.

Several mediators are considered as eosinophil attractants in the airway mucosa; in particular, interleukin-5 (IL-5); leukotriene B4 (LTB4); chemokines, including RANTES, MCP-1, MCP-3, and MCP-4; and, finally, the recently discovered eotaxin-1, -2, and -3. Previous investigations have established that eotaxins induce maximal transendothelial eosinophil migration, with respect to the other chemokines in the following order of strength: eotaxin-2>RANTES>MCP-4>IL-5>LTB4 [16-19]. Many chemokines act by binding to the several receptors, whereas all eotaxins operate by stimulation of the same receptor, CCR3 [20]. Previous studies indicated epithelial cells of the nasal mucosa as the primary source of eotaxin-2 and RANTES [21, 22]. However, during immunological activation, the main sources of these mediators became fibroblasts and activated eosinophils [21, 22]. Our study demonstrated higher concentrations of eotaxin-2 and RANTES in nasal discharge of PAR patients suggesting the presence of strong eosinophilic inflammation in these patients. Our very interesting finding is a negative correlation between the concentrations of CC16 and eotaxin-2 in nasal secretions. However, we found no similar correlation between the concentration of RANTES and CC16. RANTES is a strong inducer of eosinophil migration. However, according to previous investigations, eosinophilic infiltration in the nasal mucosa correlates strongly with eotaxin mRNA mucosal expression than RANTES mRNA expression [23]. Eosinophils are attracted to the site of inflammation mainly by eotaxins and, in the smaller part, by other eosinophil chemokines. These attracted and activated eosinophils attract new eosinophils by the production of new and higher amounts of eotaxins by their autocrine action [23]. In the late phase of allergic inflammation, eosinophils release high amounts of toxic mediators (ECP, MBP, EPOs) that affect nasal respiratory epithelium, including non-ciliated Clara cells, resulting in lower production of CC16. On the other hand, CC16 has a suppressive effect on Th2 cytokine (IL-4, IL-5, and IL-13) and eosinophil chemokine production. The results of an animal investigation with experimentally induced asthma in mice showed that CC16 downregulates infiltration and activation of eosinophils in the bronchoalveolar mucosa [24]. According to the "united airway" concept, the similar effect of CC16 on local eosinophil inflammation can be presented in the nasal mucosa of patients with PAR. In fact, our results suggest that in chroni-

cally inflamed nasal mucosa, the local production of eotaxin-2 and CC16 exists in the status of a dynamic balance.

Our results also demonstrated that nasal corticosteroid administration decreases eotaxin-2 and RANTES concentrations and increases CC16 concentrations in nasal secretions of patients with PAR. The corticosteroid action may be the result of multifactorial effects on several levels of the inflammatory reaction [25]. On the mucosal level, corticosteroids reduce the number of antigen-presenting cells, lymphocytes, eosinophils, and mast cells. Prolonged corticosteroid administration reduces the eosinophil infiltration in nasal mucosa by suppressing the production of eosinophil chemokines (RANTES, eotaxins, etc.) [25]. Disturbance of eosinophil infiltration and activation leads to the reduction in release of toxic enzymes (ECP, MBP, EPOs), resulting in lower degree of Clara cell injuries.

The limitations of our investigation were relatively small number of participants included in the study and the absence of immunohistochemical and Polymerase Chain Reaction detection of inflammatory mediator production in the nasal mucosa. We did not investigate the relationship between mucosal production of CC16 and chemokines in patients with intermittent allergic rhinitis because of strong variations in inflammatory mediator levels in nasal secretions of these patients depending the presence of specific seasonal allergens in the air [9, 11].

In conclusion, our results showed decreased CC16 and increased eotaxin-2 and RANTES production in patients with PAR in comparison with subjects with healthy nasal mucosa. To the best of our knowledge, in PAR patients, the local concentration of CC16 is inversely correlated with the concentration of eotaxin-2, implying the presence of a dynamic balance between the local production and release of these inflammatory mediators. We also demonstrated that intranasal corticosteroid administration has a suppressive effect on eosinophilic infiltration in the nasal mucosa and local release of chemokines in nasal secretions and a stimulating effect on local CC16 production.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Military Medical Academy School of Medicine (MFVMA 06/16-18).

Informed Consent: Written informed consent was obtained from all patients who participated in this study.

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