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IN VIVO AND *IN VITRO* STUDIES ON MULTIDIRECTIONAL MECHANISM OF ANTI-ALLERGIC ACTIVITY OF BUDESONIDE

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Most studies on the effects of glucocorticosteroid therapy in rhinitis relate to their inhibitory effect on activation and the number of inflowing cells that are involved in the development and maintenance of inflammation. It is also very important to determine the range of effect of budesonide on residing cells (epithelial cells). The purpose of this study was to evaluate the effect of local budesonide therapy on the cytological image of the nasal mucosa, with attention paid to columnar cells in patients with rhinitis. The *in vivo* results obtained were analyzed in correlation with changes in normal CHO-K1 cells exposed to budesonide at concentrations falling within the pharmacological dose range. Fifty patients diagnosed with rhinitis with suspected allergic background without nasal polyps were included in clinical trials. The control group were 10 healthy people without clinical signs of rhinitis. Only in patients with homogeneous cytological picture, exfoliative cytology was performed before treatment and after 4 weeks of therapy with budesonide used in aerosol form. Papanicolaou and Pappenheim - stained smears were evaluated qualitatively and quantitatively for changes in nasal mucosal cells. The nasal mucosal image of the patients before treatment clearly indicated the pathological state confirmed by the presence of numerous neutrophils, eosinophils, abundant bacterial flora and goblet or epithelial cells prevalence. In contrast, in smears of patients post-treatment budesonide observed a clear improvement in their nasal mucosa by reducing inflammation. There was a significant increase in the number of columnar cells and the appearance of very numerous epithelial cells with increased cytoplasmic vacuolization and visible leucophagocytosis. *In vitro* studies were performed on normal CHO-K1 cells that were treated with budesonide at concentrations of 0.5 μM – 45 μM. After 48 hours of incubation with the test agent, the samples were prepared for optical microscopy using the H&E method and transmission electron microscopy. Comparison of cells exposed to budesonide with control cells (without addition of test agent) revealed vacuolization changes with autophagy. Apoptotic changes have also been demonstrated, which occured to a lesser extent than vacuolization. The changes observed after budesonide treatment in the cytological picture of patients with allergic rhinitis indicate the therapeutic effect of this drug. On the other hand, the changes observed in the cytoplasm of epithelial cells, such as autophagy (clearly promoted in CHO-K1 cells) and leucophagocytosis, may indicate an additional mechanism of action for budesonide.

Key words: *nasal rhinitis, local glucocorticosteroid therapy, exfoliative cytology of nasal mucosa, ultrastructural changes, vacuolization, apoptosis, leucophagocytosis*

INTRODUCTION

Glucocorticosteroids such as budesonide are undoubtedly powerful anti-inflammatory, antiallergic and immunosuppressive agents (1). Therefore, they are commonly used in the long-term treatment of inflammatory diseases, such as allergic (AR) and non-allergic rhinitis (NAR) (2). Local administration of these compounds in the form of aerosol or nasal drops made it possible to reduce the single dose to achieve a high drug concentration in the place of ongoing inflammatory process. This resulted in a high therapeutic effect and the removal of most clinical signs of rhinitis (3).

The action of glucocorticosteroids on the respiratory tract (including the nasal mucosa) results in clear changes in the structure of eosinophils (4), dendritic cells (5), T cells (6), mast cells (7), basophils (8) indicating the anti-inflammatory effect of the drug. The dominant effect of these compounds is the transrepression of many genes encoding cytokines, chemokines, adhesion molecules, inflammatory enzymes, receptors and proteins, the expression of which is specific for each cell in the inflammatory process (9). The reduced survival of some inflammatory cells (in asthma) is also explained by the inhibition of proliferation or stimulation of apoptosis caused by glucocorticosteroids (6).

Glucocorticosteroids also inhibit other cellular groups such as structural cells (10), for example bronchial epithelial cells, which act as inflammatory modulators, secreting i.a. endothelin-1 (11) and induced nitric oxide synthase (iNOS) (12). In addition to the protective action of glucocorticosteroids described in the literature, there are also

in vitro hypotheses for the induction of apoptosis in bronchial epithelial cells, which may explain one of the causes of persistent respiratory tract injury in asthma following treatment with these drugs (13).

Much less attention in the literature has been given to the precise mechanism of action of glucocorticosteroids on cells that build the proper epithelium of the nose. Their morphology can reflect the effects of the ongoing inflammatory process (14) or treatment (15). Only single reports of the effects of these compounds on fibroblasts derived from polyps of the nose are available. Some of them describe inhibitory effects of budesonide on the proliferation of these cells *in vitro* (16), and other studies suggest no effect of dexamethasone used at high doses on apoptosis of these cells, but *in vivo* studies indicate stimulation of this process (17).

Because of the lack of clear data on the effects of glucocorticosteroids (intranasally used) on the morphological profile of nasal mucosa, the purpose of the study was to answer the question of how standard treatment with budesonide affects the morphological image of nasal mucosa cells (especially columnar cells) in patients with clinical symptoms suggesting allergic rhinitis without nasal polyps. Clinical trials have been extended to assess the changes in normal cells from *in vitro* cultures exposed to budesonide in concentrations that were within the pharmacological range.

MATERIALS AND METHODS

In vivo study

1.1. Patients

The research was performed in cooperation with an allergist from the Allergy Clinic, Military Specialist Medical Clinic SP ZOZ in Kielce.

The study was accepted by the Jan Kochanowski University in Kielce Bio-ethical Committee (No. 45/2011).

The study group consisted of 50 patients (22 women and 28 men), aged 5 – 30 (median 11.5) diagnosed with rhinitis with suspected allergic background. The patients were qualified to participate in the study by a doctor based on clinically homogenous symptoms, such as runny and stuffy nose, sneezing and itching of nose.

The control group were 10 healthy people (5 women and 5 men) aged 5 – 22 (median 14.20 years) who were showing no symptoms of rhinitis.

1.2 Methods of smears preparation and assessment

The cytological sampling of the nasal mucosa was performed by means of an exfoliative technique. From each patient, two smears from the surface of the nasal mucosa membrane were taken, from the inferior nasal concha (1 cm from its front edge) with the use of a sterile cytology brush. The material thus obtained was spread by hand on the slide with a single movement parallel to the slide edges to obtain a thin layer. The obtained smears were stained by the Papanicolaou method to differentiate epithelial cells changes and May-Grunwald Giemsa method to differentiate inflowing cell changes (18, 19). Afterward the samples were analyzed by the blind method using the Nikon ECLIPSE 80i light microscope (Nikon Instruments) with a digital image analysis system Nikon Nis Elements D in the magnification of × 400. Changes in the nasal mucous membrane of patients with rhinitis were analyzed based on a control image obtained in smears taken from healthy people. The results of the control group corresponded to the description of healthy nasal mucosa (19). For the purpose of detecting all relevant cells for diagnostic purposes, the entire surface of the randomly selected fields of view of the microscope was read carefully. During the sample assessment, special attention was given to the morphological changes of epithelial cells. These concerned the morphological profile of the nucleus, cytoplasm and cell contours. In order to obtain the full picture, an assessment of the percentage of various types of cells in the smears was performed, according to the method of Tarchalska-Krynska (19). The inflammatory cells counted were neutrophils, eosinophils, basophiles, lymphocytes and monocytes, and among epithelial cells, columnar, goblet and squamous cells were differentiated. In the microscope field of view, all cell types (epithelial and inflammatory cells), as well as the cells with morphological changes, were counted together to a total of 500 cells in the preparation in three repetitions (total counted: 1.500 cells/preparation), and the final result was averaged. Determining cytograms for all patients made it possible to identify 10 ill people (5 women and 5 men) aged 5 – 20 (median 10.4) with a similar cytological image (*Table 1*), which became the basis for their classification into the treatment group.

1.3 Patients treatment

Patients applied to each nostril twice daily budesonide in aerosol form in a double dose for adults (total daily dose of 200 μg of active substance) and a single dose for children (total daily dose of 100 μg of active substance). Control cytology was performed after 4 weeks of treatment and compared to cytology taken from the same patients prior to treatment (baseline cytology was performed during assignment of patients to the study).

In vitro study

2.1. Cell culture and in vitro conditions

CHO-K1 (Chinese Hamster Ovary) normal cell line was used for the study, which were cultured in a plastic plates (Nunc)

Table 1. Patients characteristics.

	Patients with persistent rhinitis	**Patients with intermittent rhinitis**
n	**6**	**4**
typical nasal symptoms: stuffy and runny nose, sneezing, itching of nose	6	4
conjunctivitis	6	0
asthma	4	2
atopic dermatitis	2	0
positive prick test result with grass pollen allergen	0	2

containing DMEM medium supplemented with 10% fetal bovine serum and a mixture of antibiotics (Penicilin 10,000 U/ml, Streptomycin Sulphate 10 mg/ml, Amphotericin B 25 µg/ml). Reagents were purchased from PAA Laboratories (Austria). Cells were kept in an incubator (Thermo Scientific) at 37°C, in 5% CO_2 atmosphere and 100% humidity.

2.2. Reagent

Budesonide (purity ≥ 99%) was dissolved in DMSO (dimethylsulfoxide) (reagents were obtained from Sigma-Aldrich St. Louis, USA) to prepare a starting concentration of 10 mM and then in culture medium to obtain the desired concentration of 0.5 µM, 7 µM, 25 µM, 45 µM. The concentrations used are within the maximum local concentrations (10^{-8} – 10^{-6}M) obtained in the mucous membrane of the respiratory tract and tissues using budesonide at a therapeutic dose of 200 µg in *in vivo* studies (20).

2.3. Procedure for preparing and evaluating the material

2.3.A. Evaluation of ultrastructural changes in transmission electron microscopy

After reaching the confluence CHO-K1 cells were trypsinized and resuspended in a plastic plates (Nunc) in complete medium. After 24 hours of incubation the medium was removed and replaced with a fresh nutrient medium with budesonide. Control group did not contain studied drug. After 48 hours of incubation, preparations for electron microscopy were prepared according to the modified Marzella & Glauman's method (1980) (21). For this purpose, cells were collected and fixed in 3% glutaraldehyde (Serva Electrophoresis), then postfixed in 2% osmium tetroxide (SPI), dehydrated in increasing series of ethanol, immersed in propylene oxide and embedded in Epon 812 (Serva Electrophoresis). The samples were cut using ultramicrotome Leica EM UC7 (Leica Microsystems) into ultrathin slices, which stained with uranyl acetate and lead citrate and finally observed using a transmission electron microscope Tecnai G2 Spirit (FEI Company). The picture has been evaluated for changes in the cytoplasm of CHO-K1 cell line and in the morphological profile of individual cell organelles.

2.3.B. Analysis of morphological changes in the light microscope

After reaching the confluence cells were trypsinized and resuspended in a plastic plates with coverslips in complete medium. Then, after 24 hours, the medium was removed and replaced with a fresh nutrient medium with budesonide. Control group did not contain studied drug. After 48 hours incubation, cells were fixed in methanol and stained in hematoxylin & eosin. Afterwards the material was thoroughly dehydrated in ethyl alcohol and overexposed in xylene. In the obtained preparations, the morphological changes of the examined cells were assessed based on the analysis of photographs made using the Nikon Eclipse 80i light microscope with the Nikon NIS Elements D digital image analysis system. During observation, the occurrence of such changes as cytoplasmic vacuolization, presence of apoptotic cells, or perinuclear halo were analyzed.

Statistical analysis

Statistical significance (defined as $P < 0.05$) *in vitro* and *in vivo* study results was evaluated using chi-square (χ^2) test and z-test for differences in proportions, with Bonferroni correction for post-hoc multiple comparisons.

RESULTS

Analysis of smears obtained from patients with symptoms of allergic rhinitis before treatment with budesonide

Nasal epithelial cells, i.e. columnar cells and goblet cells, remaining in a 5:1 ratio, clearly dominated in the nasal mucosal control image (*Figs*. *1a*, *2a* and *3*).

Compared to the control image, the smears of patients with rhinitis showed significant changes in pseudostratified epithelium in the form of a statistically significant increase in the number of enlarged goblet cells to 92.39 (18.48%) ($z = 2.076$; $P < 0.05$) (*Figs*. *2b* and *3*), with a simultaneous, highly statistically significant decrease in the number of columnar cells to 50.00 (10%) ($z = 24.572$; $P < 0.001$) (*Fig*. *3*). Columnar cells showed morphological differences in the form of poorly colored cytoplasm and blurred contouring of the cytoplasm (*Figs*. *1b* and *2b*). Highly statistically significant changes concerned the cells of the squamous stratified epithelium. The number of cells with eosinophilic cytoplasm was increased to 75.14 (15.03%) ($z = 9.143$; $P < 0.001$) (*Figs*. *1c* and *3*). In a similar number 60.33 (12.07%) ($z = 6.232$; $P < 0.001$) (*Fig*. *3*) there were cells derived from deeper epithelial layers (basophilic cells), characterized by clear morphological differentiation on cells: intermediate (*Fig*. *2c*), metaplastic (*Fig*. *1c*), basal (*Fig*. *1c*) and parabasal (*Fig*. *1c*).

Among the analyzed images, the dominant change was a highly statistically significant increase in the number of neutrophils to 202.30 (40.46%) ($z = 9.969$; $P < 0.001$) (*Fig*. *3*), which was characterized by reduced coloration of cytoplasm and blurred outline of nucleus as well as the cells themselves, which gave the impression of naked nucleus (*Figs*. *1d* and *2d*). Neutrophils collected in clusters occupy very large surfaces of the preparation. Eosinophils were also highly statistically significant among the observed inflammatory cells (17.05, 3.41%) ($z = 4.079$; $P < 0.001$) (*Figs*. *1d*, *2d* and *3*), while lymphocytes, monocytes and basophils were few (0.55%) (*Fig*. *3*). The analyzed preparations show the presence of abundant bacterial flora, which were adhered to the surface of squamous epithelial cells (*Figs*. *1c* and *2c*). Another change, not occurring in the image of control cells, was the appearance of individual columnar cells with slight vacuolization changes in the cytoplasm (*Fig*. *1b*). Occasionally appeared squamous epithelial cell containing granulocyte within the vacuoles (*Fig*. *1c*). The total number of vacuolated cells was 3.27 (*Fig*. *4*).

Evaluation of smears obtained from patients with symptoms of allergic rhinitis following standard budesonide therapy

Preparations from patients treated with glucocorticosteroids had few cells. There was a statistically significant increase in the number of columnar cells to 239 (47.8%) ($z = 14.506$; $P < 0.001$) (*Fig*. *3*), which occurred in large aggregates and were characterized by strong staining and clearly contoured cytoplasm (*Figs*. *5a* and *6a*). Characteristic was the presence of binucleated columnar cells (*Figs*. *5b* and *6b*). In contrast, the number of goblet cells that did not exhibit strong enlargement characteristics (*Figs*. *5b, 5e* and *6a*) was statistically significantly reduced do 44.14 (8.83%) ($z = 4.488$; $P < 0.001$) (*Fig*. *3*). A highly statistically significant decrease in the number of acidophilic cells of the stratified squamous epithelium was demonstrated (31.33, 6.27%) ($z = 4.538$; $P < 0.001$) (*Figs*. *3*, *5e, 6b, 7b and 7c*) with a statistically significant increase of basophilic cytoplasmic cells up to 95.67 (19.13%) ($z = 3.095$; $P < 0.01$) (*Fig*. *3*). Among them, the presence of intermediate cells occurring in smaller numbers (*Figs*. *5d, 5f, 6e* and *6f*), basal cells (*Figs*. *5f* and *6f*) and metaplastic cells (*Figs*. *5e, 6e* and *6f*). Characteristic was the presence of cells with typical features of

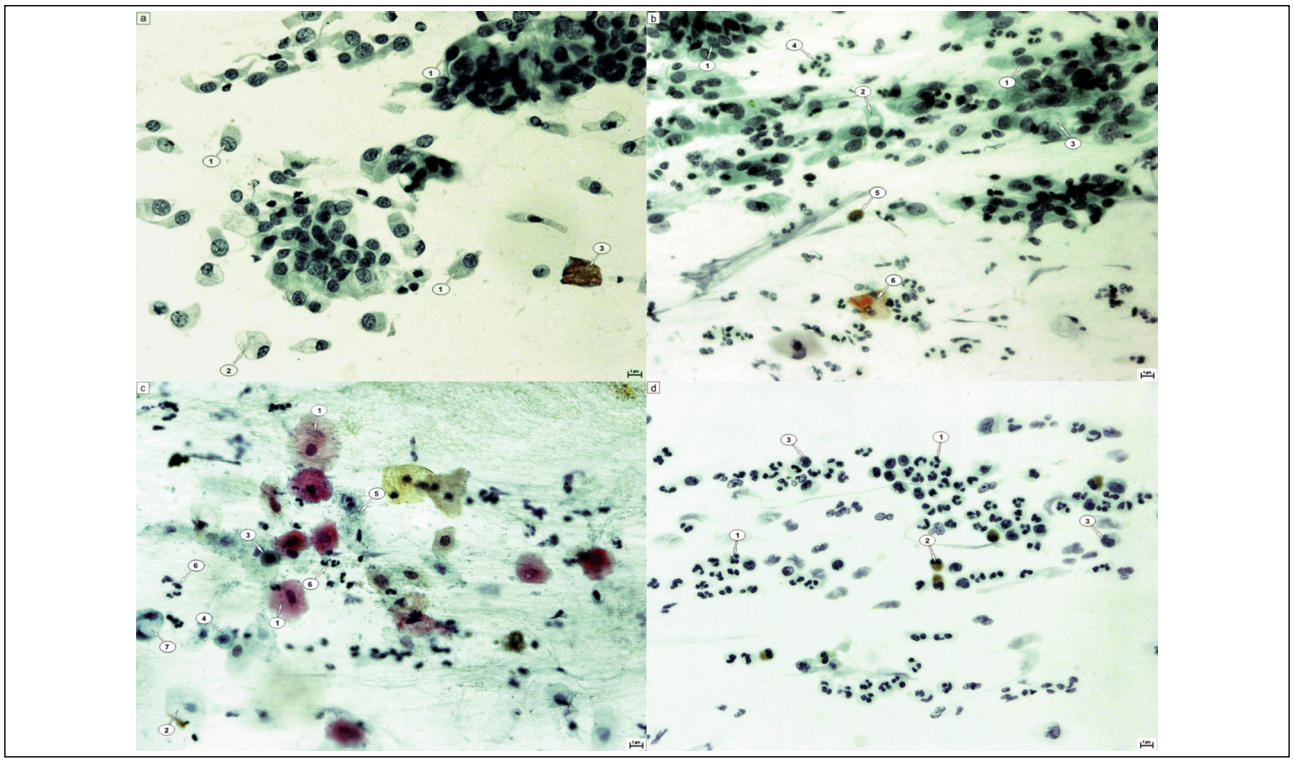

Fig. 1. The cytology of healthy controls (a) and patients with rhinitis before budesonide treatment (b, c, d) stained with Papanicolaou method under a light microscope (magnification × 400). (a): 1-columnar cells of the pseudostratified epithelium, 2-goblet cells, 3-acidophilic cell of the stratified squamous epithelium. (b) 1-columnar cells of the pseudostratified epithelium, 2-vacuolization in a columnar cell of the pseudostratified epithelium, 3-goblet cells, 4-neutrophils, 5-eosinophil, 6-acidophilic cell of the stratified squamous epithelium. (c) 1-acidophilic cells of the stratified squamous epithelium, 2-basophilic cell of the stratified squamous epithelium, 3-metaplastic cell, 4-basal cell, 5-bacteria, 6-neutrophils, 7- leucocyte in the cytoplasm of basophilic cell (parabasal cell). (d) 1-neutrophils, 2-eosinophils, 3-monocytes.

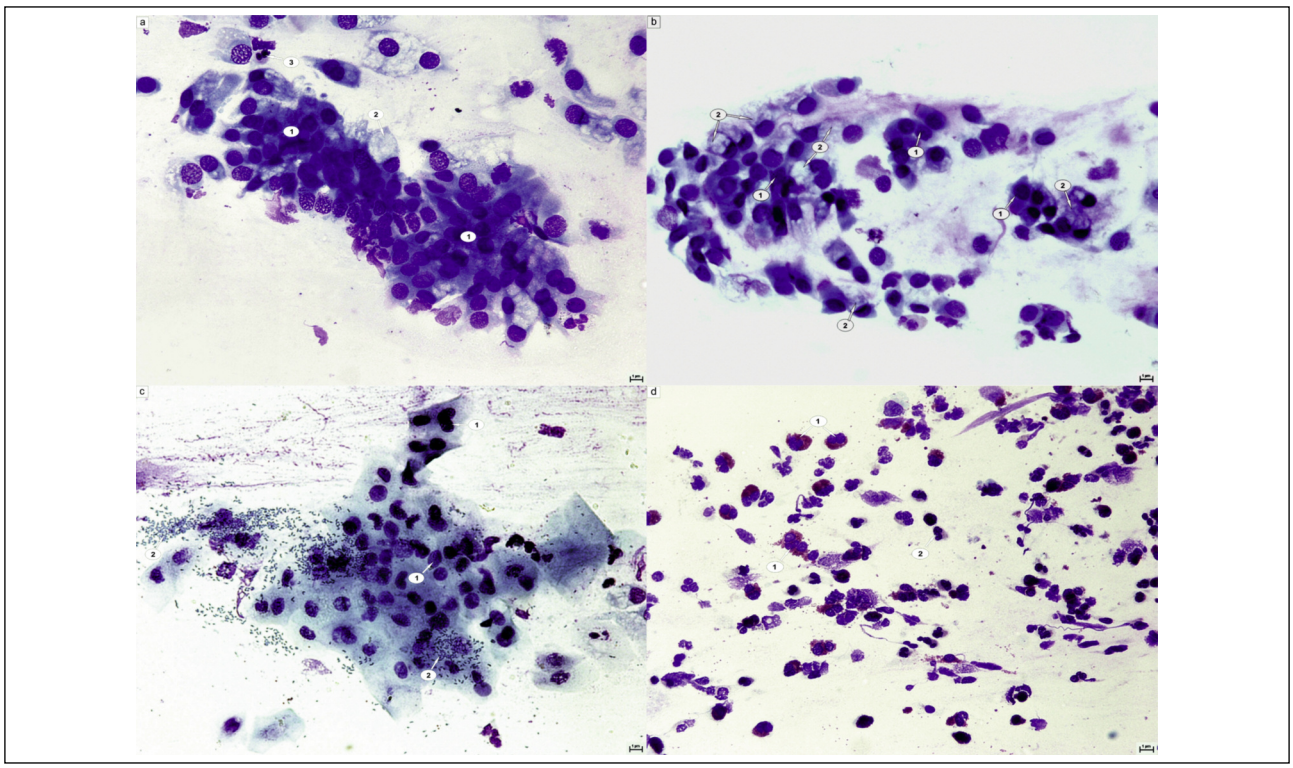

Fig. 2. The cytology of healthy controls (a) and patients with rhinitis before budesonide treatment (b, c, d) stained with Pappenheim method under a light microscope (magnification × 400). (a) 1-columnar cells of the pseudostratified epithelium, 2-goblet cells, 3-neutrophil. (b) 1-columnar cells of the pseudostratified epithelium, 2-goblet cells. (c) 1-basophilic cells of the stratified squamous epithelium (intermediate cells), 2-bacteria. (d) 1-eosinophils, 2-neutrophils.

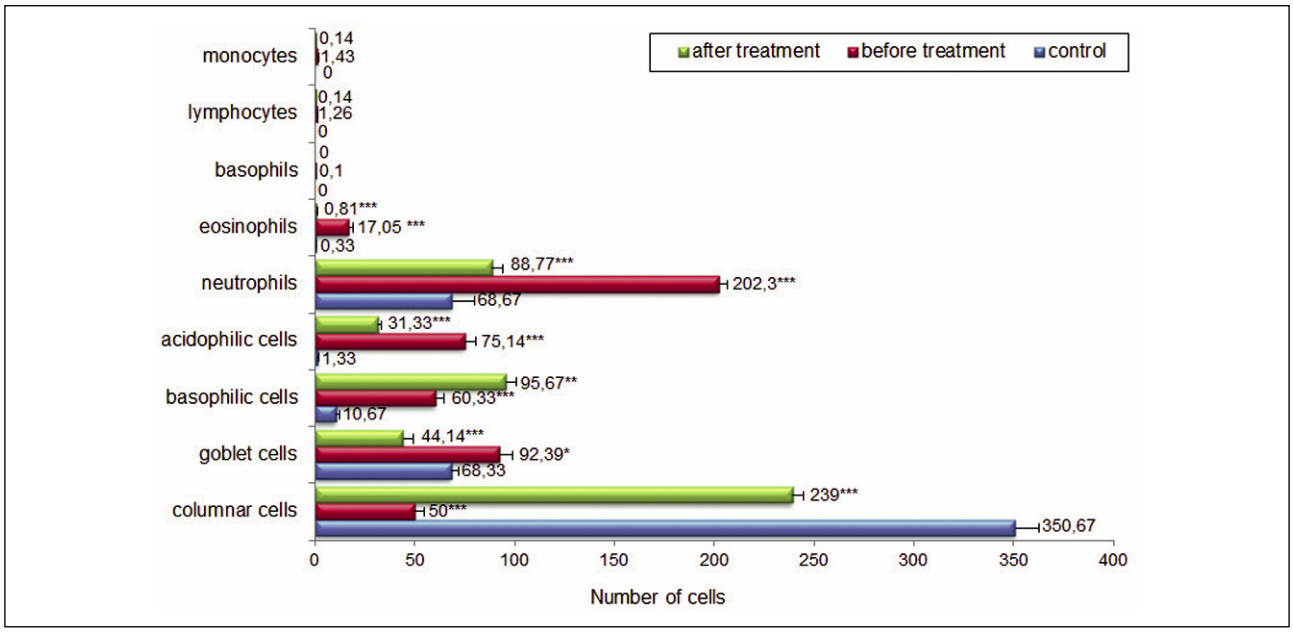

Fig. 3. Distribution of epithelial and inflammatory cells in smears of the nasal mucous membrane of patients with rhinitis before budesonide treatment compared to control and to post-treatment condition; ***P < 0.001, **P < 0.01, *P < 0.05 (two-proportion z-test with Bonferoni correction).

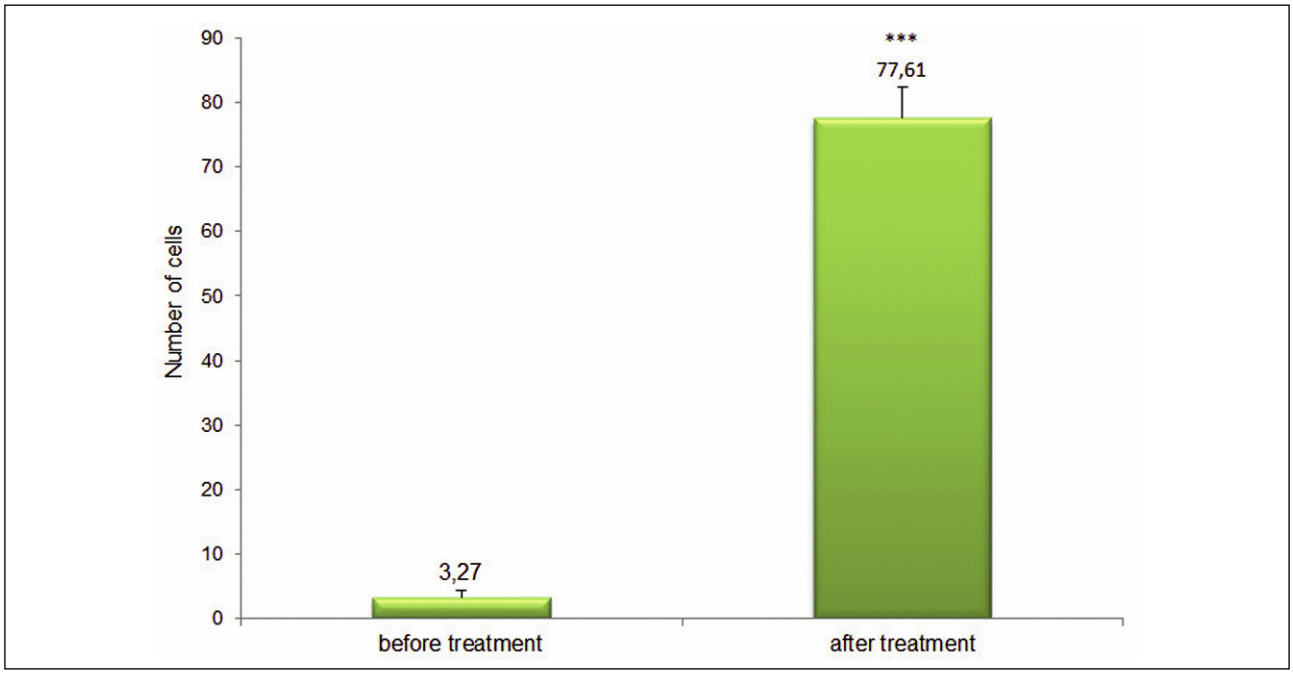

Fig. 4. The number of cells with vacuolization in smears from nasal mucous membrane from patients before and after budesonide treatment; ***P < 0.001 compared to pre-treatment condition (χ^2 test).

apoptotic death (*Figs*. *5d* and *6d*). Statistically significant changes were also observed in inflammatory cells. There was a clear reduction in the number of neutrophils to 88.77 (17.75%) (z = 8.162; P < 0.001) (*Figs*. *3*, *5d, 6e* and *6f*) and eosinophils to 0.81 (0.16%) (z = 3.907; P < 0.001) (*Figs. 3*, *6a* and *7b*). Neutrophils in the clusters were confined to individual spaces in the preparation (*Fig*. *7f*). The number of remaining inflammatory cells did not change significantly (*Fig. 3)*.

It should be noted that some granulocytes exhibited deep disintegration properties (*Figs*. *6f* and *7a*), as well as apoptotic changes in the form of nuclear decay (*Figs*. *5c* and *6c*).

A constant feature of these smears was the appearance of epithelial cells with increased vacuolization changes in the cytoplasm. These changes were largely related to columnar cells, but to a lesser extent were observed in squamous cells. One of them was characterized by the presence of one vacuole (*Figs*. *5d*, *5f* and *6f*) or many small vacuoles in the form of empty spaces in the cytoplasm (*Figs*. *5e*, *7a* and *7d*), others characterized the perinuclear vacuolization ('halo effect') (*Figs*. *5b*, *6c, 7a-7c*). Attention is paid to the presence of vacuole with a clearly visible content i.e. with apoptotic leukocytes in squamous cells (*Fig*. *7f*) and columnar cells (*Fig*. *7e*). The total number of vacuolated

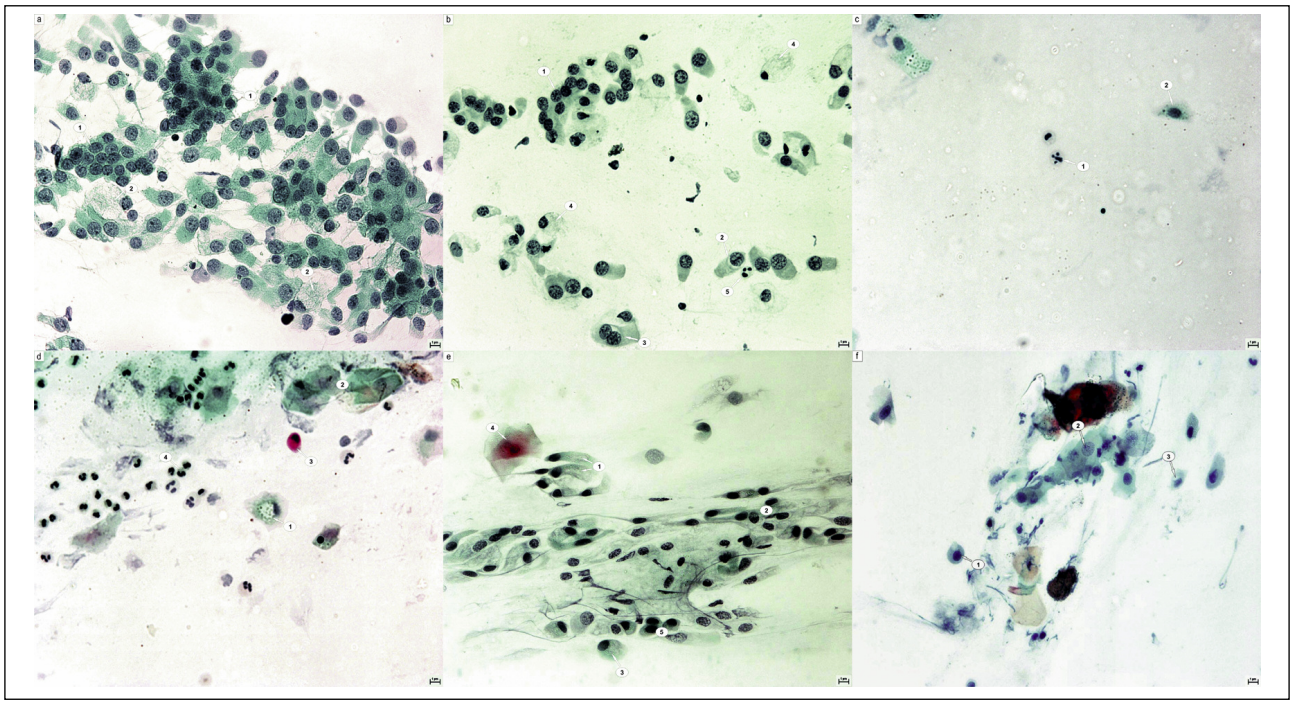

Fig. 5. The cytology of the nasal epithelium of patients with rhinitis after budesonide treatment stained with Papanicolaou method under a light microscope (magnification × 400). (a) 1-columnar cells of the pseudostratified epithelium, 2-goblet cells. (b) 1-columnar cells of the pseudostratified epithelium, 2-columnar cell with a perinuclear halo, 3-binucleated columnar cell with a perinuclear halo, 4-goblet cells, 5-neutrophil. (c) 1-neutrophil apoptosis, 2-basophilic cell (intermediate cell) of the stratified squamous epithelium. (d) 1-vacuolization in squamous cell (intermediate cell), 2-basophilic cells (intermediate cells) of the stratified squamous epithelium, 3-apoptotic eosinophilic epithelial cell, 4-neutrophils. (e) 1-columnar cells with cytoplasm vacuolization, 2-columnar cells, 3-goblet cell, 4-acidophilic squamous cell with pyknotic nucleus, 5-metaplastic cells. (f) 1-vacuolization changes in basal cell, 2-basophilic cells (intermediate cells) of the stratified squamous epithelium, 3-vacuolization in squamous cell (intermediate cell).

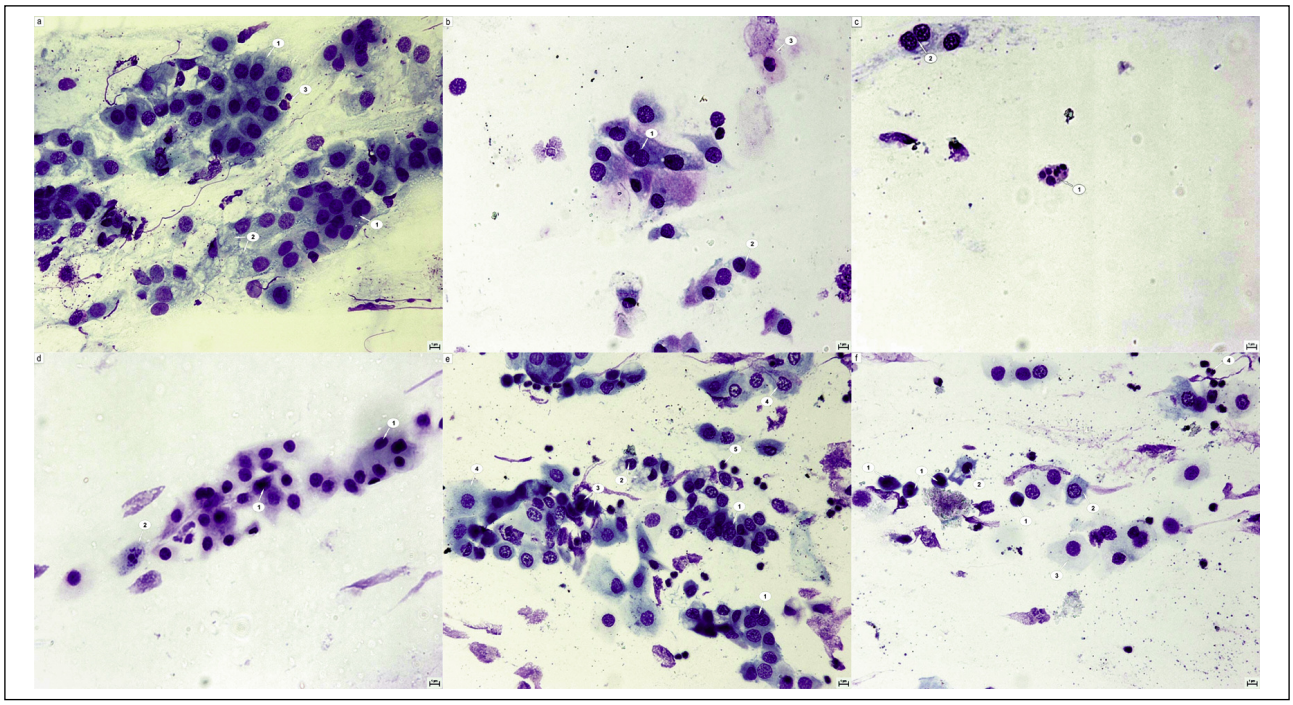

Fig. 6. The cytology of the nasal epithelium of patients with rhinitis after budesonide treatment stained with Pappenheim method under a light microscope (magnification × 400). (a) 1-columnar cells of the pseudostratified epithelium, 2-goblet cells, 3-eosinophil. (b) 1-binucleated columnar cell, 2-columnar cells of the pseudostratified epithelium, 3-acidophilic cell of the stratified squamous epithelium. (c) 1-neutrophil apoptosis, 2-binucleated columnar cell with perinuclear halo. (d) 1-columnar cells of the pseudostratified epithelium, 2-apoptotic epithelial cell. (e) 1-columnar cells of the pseudostratified epithelium, 2-goblet cells, 3-metaplastic cells, 4-basophilic cells of the stratified squamous epithelium (intermediate cells), 5-neutrophils. (f) 1-metaplastic cells, 2-vacuolization changes in basal cells, 3-basophilic cell of the stratified squamous epithelium (intermediate cell), 4-neutrophils.

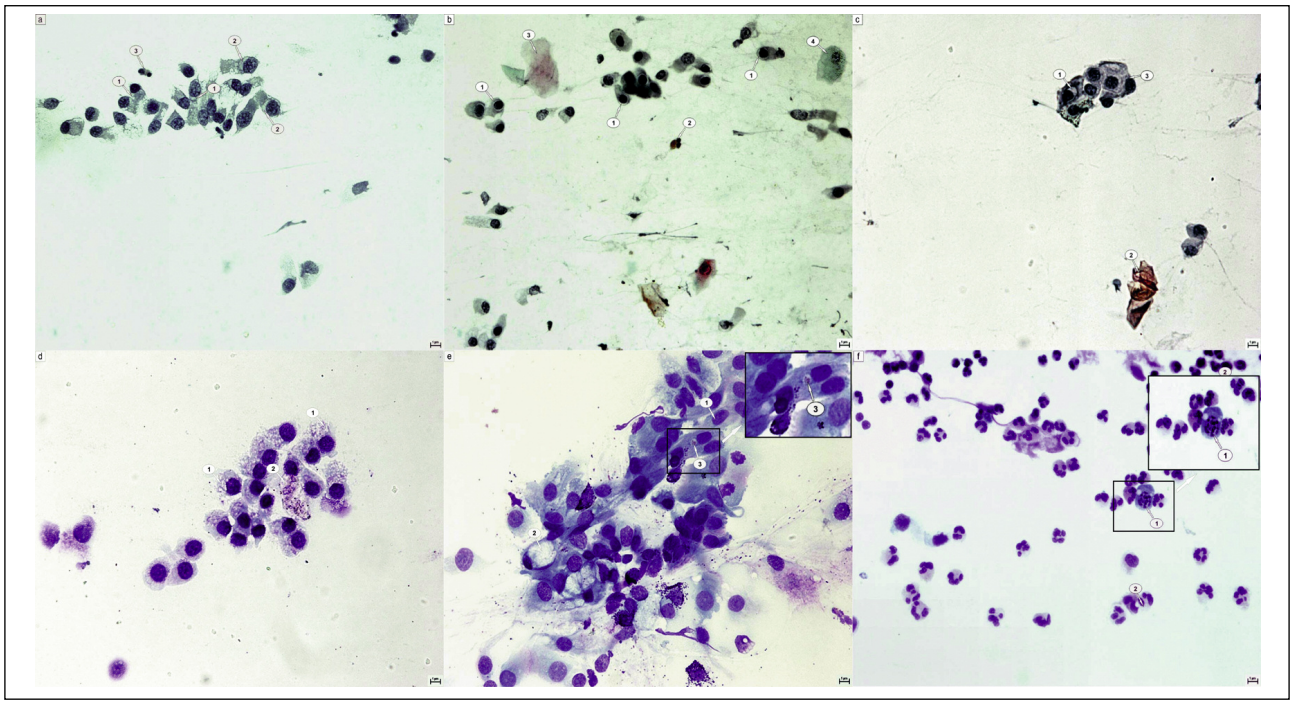

Fig. 7. The cytology of the nasal epithelium of patients with rhinitis after budesonide treatment under a light microscope (magnification × 400). Stained with the method: Papanicolaou (a, b, c) and Pappenheim (d, e, f). (a) 1-columnar cells with intensified cytoplasm vacuolization, 2-columnar cells with a perinuclear halo, 3-neutrophil. (b) 1-perinuclear halo in columnar cells, 2-eosinophil, 3-acidophilic cell of the squamous epithelium, 4-basophilic cell of squamous epithelium (intermediate cell). (c) 1-squamous cell with perinuclear halo, 2-eosinophilic squamous cell, 3-columnar cells with cytoplasm vacuolization. (d) 1-columnar cells with intensified cytoplasm vacuolization, 2-goblet cell. (e) 1-columnar cell of the pseudostratified epithelium, 2-goblet cells, 3- columnar cell with a large vacuole with visible content (leucophagocytosis in magnification). (f) 1-leucophagocytosis in squamous epithelial cell, 2-neutrophils.

cells after budesonide therapy was 77.61, which was a highly statistically significant change compared to the pre-treatment condition ($\chi^2 = 75.57$; $P < 0.001$) (*Fig. 4*).

Ultrastructural changes in CHO-K1 line exposed to budesonide

In the cytoplasm of the control cells was observed the organelles of normal size and structure such as the nucleus, mitochondria, and channels of the rough endoplasmic reticulum (*Fig. 8a*). The result of exposure of cells to budesonide at concentrations of 0.5 μM – 45 μM was mainly a cytoplasmic vacuolization that intensified with the increase in budesonide concentration. Changes also affected other organelles.

At a concentration of 0.5 μM, single small vacuoles were observed, as well as expanded Golgi apparatus occurring in the form of significantly enlarged cisternae and numerous vesicles near the nucleus (*Fig. 8b*). As a consequence of the action of budesonide at concentrations 7 μM (*Fig. 8c*) and 15 μM (*Fig. 8d*), there were numerous autophagic vacuoles of varied sizes and shapes containing material at different stages of degradation. Characteristic changes also affected the cell nucleus that was distorted (*Fig. 8c*) or fragmented with local chromatin condensation (*Fig. 8d*). Golgi apparatus exhibited strong reduction features (*Fig. 8d*). Cell exposure to budesonide at 25 μM and 45μM (*Figs. 8e* and *8f*) resulted in intensification of changes in the lysosomal compartment, as primary lysosomes were present in the cytoplasm of the cells and numerous and/or strongly expanded autophagy vacuoles with clear digested material. Quite commonly the change was a cell nucleus with fragmentation features and with local chromatin condensation (*Fig. 8f*).

Changes in morphological profile of CHO-K1 line exposed to budesonide

The morphology of approximately 99.5% of CHO-K1 control cells (*Fig. 9a*) was defined as normal because it was characterized by uniform pink cytoplasm and a nucleus with one or more nuclei. The rest was a small number (11) (*Fig. 10*) of small shrunken cells with a pyknotic nucleus (apoptotic cells) and occurring in a similar number (12) (*Fig. 10*) cells with slight vacuolization changes in the cytoplasm, i.e. with the presence of a few, clearly separated from the cytoplasm, uncolored spaces.

Compared to the image of control cells, morphology of cells exposed to 48 hours incubation with budesonide at a concentration range of 0.5 μM – 45 μM showed mainly changes related to the presence of vacuolated cells (including perinuclear halo) and apoptotic cells, the number of which increased significantly with increasing the concentration of budesonide in the medium. At a concentration of 0.5 μM, the number of vacuolated cells reached 41 (0.82%) ($\chi^2 = 15.95$; $P < 0.001$) and apoptotic cells 79.34 (1.59%) ($\chi^2 = 51.84$; $P < 0.001$) (*Figs. 9b* and *10*). As a consequence of the action of concentrations of 7 μM and 15 μM, the number of vacuolated cells definitely increased accordingly to 3443.40 (68.87%) ($\chi^2 = 5205.75$; $P < 0.001$) and 3734.67 (74.69%) ($\chi^2 = 5915.81$; $P < 0.001$), as well as apoptotic cells attaining value 689.66 (13.79%) ($\chi^2 = 707.27$; $P < 0.001$) and 945.67 (18.91%) ($\chi^2 = 1010.18$; $P < 0.001$) (*Figs. 9c*, *9d* and *10*).

Exposure of CHO-K1 cells to the highest concentration of 25 μM and 45 μM budesonide resulted in a marked increase in vacuolization changes, the number of cells with such changes was 3986 (79.72%) ($\chi^2 = 6581.38$, $P < 0.001$) and 4051.67 (81.03%) ($\chi^2 = 6765.74$, $P < 0.001$) (*Figs. 9e*, *9f* and *10*). The

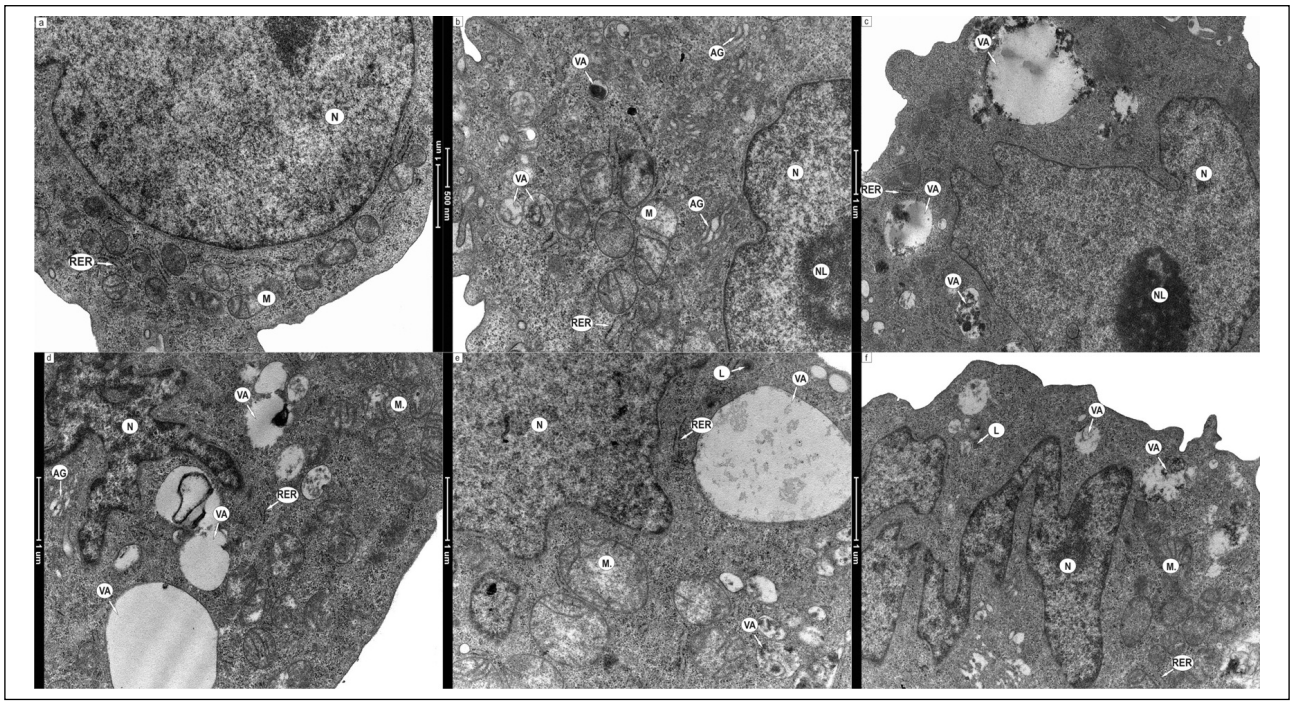

Fig. 8. Fragment of CHO-K1 line cell: from control group after 48 hours incubation in basal medium (a) and after 48 hours exposure to budesonide at 0.5 μM (b), 7 μM (c), 15 μM (d), 25 μM (e), 45 μM (f). In the cytoplasm visible: N, nucleus; NL, nucleoli; M, mitochondria; L, lysosomes; AG, Golgi apparatus; VA, autophagic vacuoles; RER, rough endoplasmic reticulum.

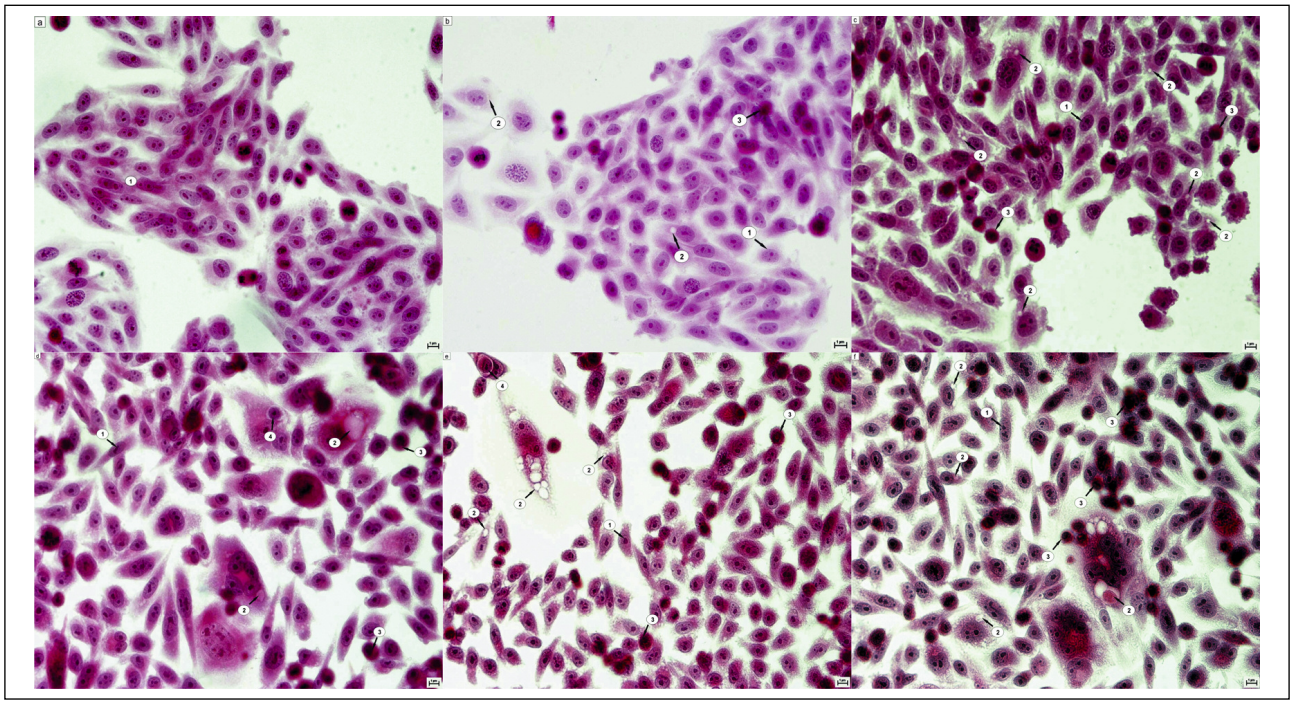

Fig. 9. CHO-K1 cell lines (H & E stained): 48 hours post-incubation in basal medium (a) and after 48 hours exposure to budesonide in concentration at 0.5 μM (b), 7 μM (c) 15 μM (d), 25 μM (e), 45 μM (f). Visible: 1-cell in the interphase, 2-cell with intensified cytoplasm vacuolization, 3-apoptotic cells, 4-cell with perinuclear halo. Magnification × 400.

apoptotic cells were significantly smaller, but their number increased by both concentrations to 832.67 (16.65%) (χ^2 = 874.37; P < 0.001) at 25 μM and 868.33 (17.37%) (χ^2 = 916.07; P < 0.001) at 45 μM (*Figs*. *9e*, *9f* and *10*). It is important to emphasize the cells with characteristic clearness around the nucleus (perinuclear halo) that are observed among those which became vacuolated (*Figs*. *9d* and *9e*).

DISCUSSION

Continuous increase of allergic diseases morbidity is a big challenge for modern science and medicine. Co-occurring rhinitis and asthma are nowadays very popular among children and adults (22). The progress in the diagnosis and the therapeutic effectiveness of allergic diseases depends primarily on the

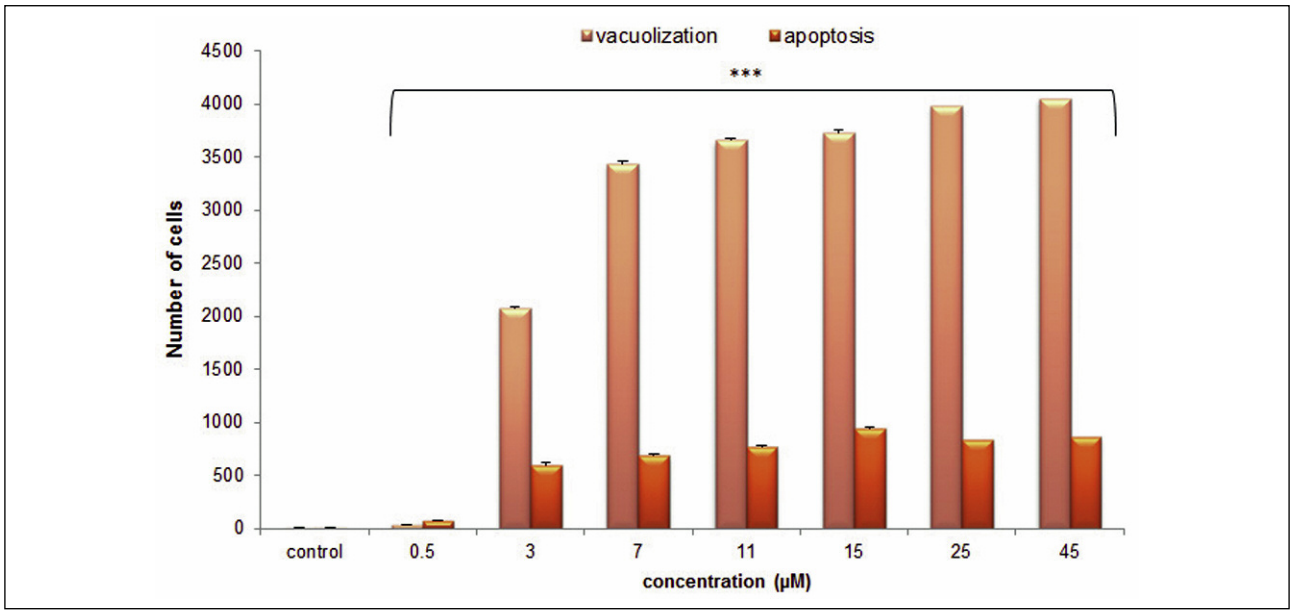

Fig. 10. Comparison of apoptotic and vacuolization changes in CHO-K1 cells after 48-hour exposure to differentiated concentrations of budesonide. Differences statistically confirmed at ***P < 0.001 compared to control (χ^2 test). The number of cells tested was 5000.

precise knowledge of the mechanisms of these diseases or the development of new effective drugs. It may also be important to modify and refine existing drugs with the possibility of extending their spectrum (23).

An effective form of treatment for nasal mucous membrane diseases, including allergic rhinitis and non-allergic forms, are glucocorticosteroids applied locally to the nose (24). The literature describes many different mechanisms of action of these compounds, but many of them remain completely unexplained. It has been shown, i.a., that glucocorticosteroids often have an adversarial effect (25), which can be determined by the type of tissue, cell, or cell cycle phase (26-28). Glucocorticosteroids applied locally to the nose may change the cytological image of the mucosa, as is the case with oral medications (29). According to research, these changes may be beneficial, and at the same time may negatively affect the condition of the patient's nasal mucosa (30).

It needs to be emphasized, that when using topical steroid preparations in a variety of respiratory tract disorders, including the nasal mucosa, attention is usually focused on the reduction of typical clinical symptoms (31). Attention should also be paid to the anti-inflammatory properties of direct or indirect inhibitory effects on the survival and function of inflammatory cells, mainly eosinophils (32, 33). It has been found that compared to the combined effect of antihistamines and anti-leukotrienes drugs, local glucocorticosteroids remain the most effective treatment for eosinophil rhinitis in patients with allergic rhinitis (34).

Budesonide used at doses that do not cause side effects, has a beneficial effect on most symptoms of rhinitis (35). The therapeutic efficacy of budesonide is achieved by local action on the nasal mucosa through intracellular glucocorticosteroid receptors. It has been shown that the concentration of intranasal glucocorticosteroids is very high on the surface of epithelial cells, so the clinically beneficial effects of their action should be associated with receptors present on these cells (36). The available literature suggests that the response of nasal mucosa to glucocorticosteroid therapy may vary and depend on the drug used (37).

Exfoliative cytology is a simple and safe study that allows for the penetration into the pathomechanism of processes that take place in the mucous membrane of the nose (38, 39). This method proves to be a reliable tool in diagnosing and differentiating rhinitis (38, 40), planning a targeted therapeutic strategy and monitoring the effects of treatment (41, 42). Cytological examination of the nasal mucosa allows to judge precisely the epithelial and inflammatory cells in the material being evaluated (41, 43).

Cytological evaluation in patients with chronic symptoms of rhinitis prior to treatment with budesonide showed a significant difference in comparison to the smears of the nasal mucous membrane obtained from control group patients. The cytogram determined as normal in infants, children and adults should contain mainly columnar and goblet cells and single neutrophils and bacteria (39, 44). In this study, nasal mucosal reorganization has been demonstrated, the revealed changes indicate a pathological state. Significant change in the material was the predominance of neutrophils (*Fig. 3*), which were characterized by reduced coloration and blurred outline (*Figs. 1d* and *2d*). Such a cell picture indicated a chronic inflammation (45). In turn, the presence of eosinophils (*Figs. 1d* and *2d*) suggested the allergic background of rhinitis, as these cells are the most important cells in the late allergic reaction (46, 47). As compared to neutrophils, the lower eosinophil number in the smear (*Fig. 3*) was the consequence of chronic exposure of the nasal mucosa to low allergen concentrations (36).

The pathological state is also indicated by an increase in the number of goblet cells relative to columnar cells (*Fig. 3*), while in normal cytograms the number of those two should be 4 – 5 times higher (43, 44). The dominant mechanism of the increase in the number of the secretory cells is metaplasia, i.e. the conversion of columnar cells to goblet cells (48, 49). In turn, the effect of this change was the structure of the secretory cells observed simultaneously in smears (*Fig. 2b*), showing that they were filled with a large amount of mucus. The demonstrated characteristics of columnar cells, including poorly stained cytoplasm and blurred cell contours (*Figs. 1b* and *2b*), and minor vacuolization changes in cytoplasm (*Fig. 1b*) are typical for degenerative changes (50, 51), often associated with impaired biocenosis (45, 52). Confirmation of disturbed biocenosis of the nasal mucosa is also the presence of very abundant bacterial

flora (*Figs*. *1c* and *2c*) - a result of improper mucociliary clearance and mucus congestion (39, 53, 54).

Manifestation of pathological state is demonstrated in the smears by the prevalence of squamous cells (*Fig. 3*) at various stages of development, including parabasal and basal cells (*Fig. 1c*), which should be associated with increased epithelial exfoliation and upper epithelial layers damage (55). Ciliary epithelial damage may also be affected by metaplasia (49), which was confirmed by the presence of metaplastic cell in the smears (*Fig. 1c).* Epithelial damage is a consequence of eosinophilic and neutrophil infiltrates in the smears, whose products interact with each other and exert a toxic effect on the respiratory epithelium (56). The sporadically observed leucofagocytosis of squamous cells (*Fig. 1c*) and the vacuolization of columnar cells (*Fig. 1b*) are examples of degenerative changes of these cells in inflammatory conditions (50).

The consequence of budesonide was a marked reduction in inflammation of the nasal mucosa (as compared to the pre-treatment state, the preparations were 'rarer'), which was confirmed primarily by bacterial deficiency, double reduction in neutrophil counts, and almost complete reduction of eosinophils (*Fig. 3).* According to numerous literature data, the presence of this type of inflammatory cells determines not only the effectiveness of glucocorticosteroids therapy, but allows to prolong the clinical improvement of the patient (57-59). According to research, including these, one of the mechanisms of action of glucocorticosteroids applied locally to the nose may be, inter alia, the induction of apoptosis (60), as indicated in studied smears by the presence of neutrophils with characteristic nuclear disintegration (*Figs*. *5c* and *6c*).

The effective anti-inflammatory effect of budesonide in topical administration associated with the reduction of infiltration of inflammatory cells has also been confirmed in studies on the lower airway epithelium using an animal model. Mikolka's team (61) showed clearly caused by the action of budesonide in combination with an exogenous surfactant a reduced neutrophil leak into the alveolar compartment in oxidative damage caused by meconium. A similar therapeutic effect was obtained in the saline-lavage model of acute lung injury, when a single intratracheal administration of budesonide reduced the migration of inflammatory cells, mainly neutrophils, to the lung tissue (62).

The anti-inflammatory activity of budesonide was also confirmed in the morphology of both columnar cells (clear green coloration and well-contoured cytoplasm) (Figs. *5a* and *6a*) as well as goblets (no strong magnifying features) (*Figs*. *5b*, *5e* and *6a*), and in normalizing their quantity (reduction of goblet cells in favor of columnar cells) (*Fig. 3*). Visible aggregates of columnar cells as well as the presence of binucleated forms among them speak for the repair processes (63). The inhibitory effect of glucocorticosteroids on goblet cells was demonstrated by the team of Lin (37), Mygind (64) and Sorensen (65), while the stimulating effect on the columnar cells was demonstrated by Tarchalska-Krynska (30) and Meltzer (59). The obtained changes in columnar cells are very beneficial especially when considering the aspect of therapy duration. Under conditions of frequent, chronic inflammation, the normal ratio between the different cell types in the nasal mucosa is unlikely (66). In our study only 4 weeks of treatment with budesonide showed an important therapeutic effect, which is in addition to effective control of inflammation, as well as stimulation of regenerative mechanisms (67). At the same time, it must be noted that this effect is only possible with the proper application of the drug at the correct dose, because wrong use of steroid implies the risk of mucosal damage and nose bleeding (68).

The beneficial effect of budesonide was also achieved in studies by reducing the number of surface cells of the stratified squamous epithelium (*Fig*. *3*), this indicates a normalization of the exfoliation process that was too intense prior to the treatment. The reduction in the number of these cells was due to stimulation of apoptosis (*Figs*. *5d* and *5d*). Similar results have been reported in previous studies using fluticasone (69), but when compared to both glucocorticosteroids, it should be emphasized that the more potent action was caused by budesonide.

It is difficult, however, to have a clear assessment and specific conclusion regarding basophilic cells, whose numbers have clearly increased after treatment with budesonide (*Fig. 3*). However, the basal cells present in the cytological picture should be taken into account (*Figs*. *5f* and *6f*). These are stem cells for goblet and columnar cells, so their presence suggests the renewal of the nasal mucus after the infection (19, 55, 70). The above data leads to the conclusion that the increase in the number of basophilic cells resulting from the treatment with budesonide does not indicate a damaging effect of the drug because at the same time a significant increase in the number of columnar cells has been demonstrated. Thus, the presence of metaplastic cells (*Figs*. *5e, 6e* and *6f*) was also not a degenerative change, which can be supported by studies with other glucocorticosteroid - beclometasone dipropionate (64). According to Stankiewicz (71), squamous metaplasia may be accompanied by nasal mucosal regenerative changes, but other data (72, 73) in healthy people (without symptoms of rhinitis), images on the border of pathology are often observed, with the presence of squamous cells. In addition, it should be emphasized that squamous cells accounted for only 25% of the analyzed cells. For comparison, in occupational rhinitis, which is a consequence of anti-allergic drugs action, the presence of squamous cells in the amount of more than 30% was confirmed (70). These cytological results confirm the safety of locally applied drugs and are referred to in other available studies that confirm the absence of a damaging effect of budesonide (74). Many authors emphasize this beneficial effect also referred to inhaled glucocorticosteroids, describing the influence on improving epithelial condition in respiratory remodeling (75). As a consequence of the action of budesonide, increased cytoplasmic vacuolization appeared, mainly in columnar cells (*Figs*. *5e*, *7a* and *7d*) and visible in the cytoplasm of these cells (*Fig. 7e*) and squamous cells (*Fig. 7f*) - phagocytosis of apoptotic leucocytes: so-called leucophagocytosis.

The cytoplasmic vacuolization, mainly of columnar cells, may indicate autophagy clearly promoted by budesonide. According to the literature, this may be a process not associated with immune cells (76), which is important in the elimination of bacteria and viruses (77, 78), pathogens of key importance in the development of rhinitis. Mechanism of stimulation of phagocytosis as an apoptotic cell elimination route has so far been assigned to dexamethasone (79, 80) and hydrocortisone (81). According to literature data, non-professional phagocytes such as dendritic cells and structural cells (fibroblasts, hepatocytes) also play an important role in the recognition and removal of apoptotic cells alongside professional phagocytes-macrophages (82). Sexton's team (80) described deaxamethasone-induced phagocytosis of apoptotic eosinophils by bronchial epithelial cells, whereas the team of Giles (79) describes deaxamethasone-induced phagocytosis of apoptotic neutrophils by macrophages. In contrast, our studies show inclusion of also the columnar cells of the nasal mucosa in the leucophagocytosis process, which was clearly stimulated by budesonide action. Simultaneous stimulation of phagocytosis can be associated with modulation by budesonide cytoskeleton activity involved in this process. The effect of budesonide on the epithelial cell cytoskeleton was also confirmed by the perinuclear halo showed in the study (*Figs*. *5b*, *6c* and *7a-7c*). This change is due to shrinkage of the caryoplasms and evidence

of cytoskeletal damage, but according to the literature it is completely reversible (50, 52).

The ability of the glucocorticosteroid-receptor complex to reorganize the cytoskeleton is highlighted in their reports by team of Liu (81) and Wu and Horwitz (83), referring to the programmed cell death as well as Giles's team (79), justifying its importance for the efficient phagocytosis process. Vacuolization changes have also been reported in previous studies in patients with persistent rhinitis after fluticasone therapy (69). However, these changes were stronger, as was the case with the reduction in the number of surface cells. The results of our study have been confirmed in previous reports (84), which showed that budesonide was more effective than fluticasone (used in equal doses) in the treatment of all symptoms of rhinitis. The authors explain this, inter alia, the ability of budesonide to form conjugates with fatty acids, the intracellular formation of its magazines, which contribute to prolongation of action, and thus higher efficacy of drug (31). In correlation to the results of the clinical study remain the results of *in vitro* studies. Clearly increasing with budesonide concentrations (0.5 μM to 45 μM) the vacuolization changes in CHO-K1 line cells were demonstrated by both hematoxylin and eosin method (*Figs*. *9b-9f*) and electron microscopy technique (*Figs*. *8b-8f*), which unambiguously confirmed the induction of autophagy. Also demonstrated ultrastructural changes, such as the presence of highly reduced Golgi apparatus (*Fig*. *8d*), lysosomes (*Fig*. *8e* and *8f*), and very numerous, giant vacuoles (*Figs*. *8c-8e*), with clearly marked contents (*Fig*. *8d*) indicated to the overwhelming macroautophagy. Clear vacuole content was also observed in H&E staining (*Fig*. *9f*). Macroautophagy, a form of autophagy, is responsible for adjusting the overall level of intracellular proteins to the current needs of the cell, e.g. under conditions of stress, hunger or in response to a damaging effect of the drug (84, 86). The effect of budesonide was also the presence of CHO-K1 cells with clear apoptotic changes, whose numbers increased proportionally to the steroid concentration, reaching the highest value at high concentrations (*Figs*. *9b-9f* and *10*). In the ultrastructure of cells the apoptosis was confirmed by the presence of strongly deformed cell nuclei (*Figs*. *8c* and *8e*), also fragmented with local chromatin condensation (*Figs*. *8d* and *8f*). However, apoptotic changes were much less severe. For example, about 870 cells were shown at concentration of 25 μM and 45 μM, while the number of vacuolated cells was approximately 4000 (*Fig*. *10*), suggesting the promotion of autophagy by budesonide. The appearance of these budesonide concentrations of apoptotic cells can be explained by excessive and unregulated autophagy (87). Confirmation of this suggestion is the presence of cells in which simultaneous features of increased autophagy (such as extensive vacuolization) and apoptosis (irregular nucleus with chromatin condensation) clearly indicate the possibility of going from autophagic pathway to apoptotic (*Figs*. *8d* and *8f*).

An important aspect of *in vitro* studies was the confirmation of perinuclear halo (*Figs*. *9d* and *9e*), which suggests the participation of budesonide in modulating cytoskeleton activity.

Many important therapeutic aspects of budesonide have been confirmed in our clinical studies, including stimulating epithelial cells (increased number of columnar cells and stimulation of regenerative mechanisms), as well as activation of apoptosis in cells with pro-inflammatory relevance. However, an important and underestimated aspect of anti-inflammatory properties in the treatment of persistent rhinitis, as in the treatment of asthma (80), is the induction of leucophagocytosis and autophagy in epithelial cells, especially those building the proper epithelium of the nose, demonstrated in this study. The promotion of autophagy was clearly highlighted in *in vitro* studies. In view of recent research on the role of autophagy in numerous diseases, the possibility of pharmacological modulation of this process is admissible (88) in order to develop alternative therapies, for example when eosinophilia apoptosis is blocked (e.g. in asthma) (89). Taking into account the fact that this occurrence may also be associated with non-allergic diseases, the obtained results are of broader relevance.

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