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Inflammatory Markers of Pulmonary Expirate in Children with Airway Remodeling In Bronchial Asthma

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Abstract

Goal: of research is to study in the dynamics the informative value of the oxidatively modified proteins level in a pulmonary expirate of the school-age patients with bronchial asthma, depending on the degree of bronchial remodeling.

Material and methods: To achieve the goal children suffering from persistent bronchial asthma (BA) were examined in parallel groups selected by means of simple randomized sampling using the "experiment-control" method. According to given analysis results at the beginning of the observation patients were divided into two groups of comparison. The first (I) group consisted of 47 patients with VEGF level in sputum exceeding 80.0 ng/ml and reaching 193.71±12.94 ng/l on average. The second (II) group included 49 children with average VEGF level in sputum not reaching the indicated median (49.55±1.24 ng/ml). Condensate of pulmonary expirate was obtained using a patented condenser for 10-15 minutes of free breathing, the usual amount of condensate was 1.5-2.0 ml. The total protein level (g/l) in it was determined by the method of Lowry O.H., the levels of alkaline and neutral 2,4-dinitrophenylhydrazone derivatives of aldehydes and ketones – by the method of

Dubinina O.E. et al. According to the informed consent obtained from patients' parents, biochemical study of PE condensate was performed three times: at the beginning of the monitoring, after 1.5, and after 3 years of observation. The bronchi lability was assessed using graduated jogging with inhalation of 200 mkg of salbutamol test and further calculation of Bronchus Lability Index (BLI, %).

Results and discussion: In children from the I clinical group level of the extracellular eosinophilic cationic proteins in sputum averaged 2.78±0.24 pg/ml, while in the comparison group it was 1.77±0.21 pg/ml ($P<0.05$). Results of the dynamic evaluation of total protein level, aldehyde and ketone derivatives of dinitrophenylhydrazones levels in PE condensate of BA patients indicating the predominance of protein oxidative modification processes in patients with high level of bronchial remodeling markers in sputum. In the dynamics of anti-inflammatory treatment there were discordant changes in these indices of PE, which reflected the higher effectiveness of the standard anti-inflammatory therapy in children I group, possibly due to the eosinophilic nature of airway inflammation. However, in the dynamics of 3-year observation, activity of the oxidative processes in patients from the I group decreased, while in patients from the II group with normal indices in the condensate of RE at the beginning of observation, activity of the oxidative modification increased in the dynamics with the highest level after 1.5-2 years from the beginning of monitoring observation. At the same time, during the period of dynamic observation of the bronchial lability index gradually reduced from year to year only in children from the I group: at the initial examination – 22.14%, and at the final one – 13.28% and the representatives of the II clinical group had an average BLI of 18.55% and 23.89% respectively. Thus, it can be assumed that in children with high level of bronchial remodeling marker in sputum, despite the decrease in the activity of inflammatory process, apparently there was a realization of the risk of structural changes in the airways, as evidenced by the signs of protein release and formation of the bronchial wall rigidity.

Conclusions: Examination of the condensate of pulmonary expirate at the beginning of observation give reason to suggest that in children with high level of bronchial neoagnogenesis biomarker (VEGF) in sputum the standard control treatment causes a decrease in the severity of the protein oxidative modification, but at the same time protein release increases and bronchial lability decreases. In the process of dynamic observation in children with asthma from the reference by the parameters of bronchial remodeling group the activity of protein oxidation as well as bronchial lability increases in spite of the standard treatment.

Keywords : bronchial asthma, children, airways remodeling, sputum.

Introduction. As it is known, a bronchial epithelium extends to the alveolar part of the respiratory tract acting as the first barrier between the external and internal environments as well as a site of the first interaction between allergens and tissues of the child body, and following immune responses to that contact. In recent years, there is an increasing interest of researchers in the processes of the structural changes in mucous membrane of the respiratory tract [1] as a consequence (or precondition) of the chronic inflammatory process in bronchial asthma (BA) [2]. It is established that a persistent chronic inflammation in bronchi causes a graduate remodeling of airways [3], which main feature is heterogeneity of the processes, resulting from a prolonged and multicomponent alteration in response to epithelial-mesenchymal complex injury by allergens [4], as well as a participation of proteolytic enzymes and vascular growth

factors (neoangiogenesis).

The modern endoscopic methods to study the processes of bronchial remodeling (BR) are considered as standardized, safe [5] and allowing to clarify a clinical diagnosis as well as define the basic cellular, immunological and molecular modifications of the structural units of a bronchial wall in respiratory diseases [6]. However, these methods remain quite invasive, which severely limits their use in pediatric practice.

As an alternative to those methods is the study of the BR markers directly in locus minoris [7], particularly, in a mucospine of sputum or in a pulmonary expirate (PE) of patients. Rather promising in this regard is the vascular endothelial growth factor (VEGF) – the main regulator of both physiological and pathological neoanogeogenesis, which increase is observed in malignant neoplasms, inflammatory processes and traumatic tissue damage [8-9]. Consequently, the determination of the VEGF level directly at the site of chronic inflammatory process should be considered as a quite informative and non-invasive method. It may be used for a dynamic estimation the degree of inflammation control under the influence of basic treatment of asthma. Moreover, as the chronic allergic inflammation of the bronchial wall is the basis of BA pathogenesis, it is difficult to overestimate the value of determination the level of inflammatory markers directly at the site of pathological process as an effective method of the dynamic control over the degree of local inflammatory process in children with bronchial asthma [10].

Nowadays, measurement of the bronchial remodeling markers in mucospin of sputum and levels of oxidatively modified proteins in PE with the purpose of their use as the additional criteria to assess the effectiveness of the standard basic treatment of asthma is not sufficiently introduced and studied, while in pediatric pulmonology there are only isolated reports on topic.

The aim of research: To study in the dynamics the informative value of the oxidatively modified proteins level in a pulmonary expirate of the school-age patients with bronchial asthma, depending on the degree of bronchial remodeling.

Material and methods: A cohort of 96 school-age patients with BA was created by random method at the Pulmonology and Allergology Department of the Municipal Medical Establishment “Chernivtsi Regional Children's Clinical Hospital”. During a three-year observation patients have been subjected to an in-depth comprehensive clinical-paraclinical examination, which included, in particular, determination of VEGF in sputum and measurement of total protein and its oxidative modifications in a condensate of pulmonary expirate. The average age of the examined children reached 11.6 years, and the average duration of the course of disease was 4.9 years.

The degree of airway remodeling was evaluated by determination the level of VEGF (ng/ml) in the mucospin of patients sputum using a three-phase "sandwich" technique of the *solid-phase* enzyme immunoassay with mono- and polyclonal antibodies («VEGF-VectorBest» reagents, RF, registration number A-8784); and level of extracellular eosinophilic cationic proteins (pg/ml) – using reagents for immunoassay analysis in human biological fluids («VectorBest» reagents, RF, registration number A-8756). Immunological studies were carried out in the accredited laboratory of the Chernivtsi Regional Children's Clinical Hospital.

Condensate of PE was obtained using a patented condenser [11] for 10-15 minutes of free breathing, the usual amount of condensate was 1.5-2.0 ml. The total protein level (g/l) in it was determined by the method of

Lowry O.H., the levels of alkaline and neutral 2,4-dinitrophenylhydrazone derivatives of aldehydes and ketones – by the method of Dubinina O.E. et al.

According to the informed consent obtained from patients' parents, biochemical study of PE condensate was performed three times: at the beginning of the monitoring, after 1.5, and after 3 years of observation.

At the beginning of the monitoring observation a median of VEGF level in sputum of the examined patients was 80.0 ng/ml. According to given analysis results at the beginning of the observation patients were divided into two groups of comparison. The first (I) group consisted of 47 patients with VEGF level in sputum exceeding 80.0 ng/ml and reaching 193.71±12.94 ng/l on average. The second (II) group included 49 children with average VEGF level in sputum not reaching the indicated median (49.55±1.24 ng/ml).

At the beginning of the study two formed groups were also compared according to the basic clinical characteristics. Thus, the average age of patients in the I group was 12.04±0.42, in the II group – 11.48±0.50 years ($P<0.05$), and the average duration of the disease was 5.37±0.68 and 4.40±0.56 years respectively ($P<0.05$). The fraction of boys among the patients of the I group was 61.7%, and in the II group – 69.39% ($P<0.05$). 46.81% of patients from the I group were the city residents, 53.18% – villagers, in the II group the distribution was practically the same – 38.78% and 61.22% respectively ($P<0.05$). Early onset asthma (up to 3 years old) occurred in 25.53% of patients from the I group, and in 30.61% of children from the II group, while the late onset asthma (over 6 years of age) was diagnosed in 46.81% and 53.06% of patients, respectively ($P>0.05$).

During the observation period the basic anti-inflammatory therapy of BA was used in both clinical groups in accordance with the protocol approved by the order No. 868 of the Ministry of Health of Ukraine and recommendations of the international coordination guidelines (GINA and its subsequent versions). The Pearson correlation analysis was used to assess the reciprocal influence of the main factors on the formation of airway remodeling. The complex laboratory and instrumental examination of patients was conducted during non-exacerbation periods.

The obtained results were analyzed using Statistica 6.0 StatSoft Inc. and Microsoft Excel XP for Windows software using parametric and non-parametric methods.

To determine the informative significance of the results of a comprehensive examination, the frequency of positive results (test sensitivity – TSn) and negative results (test specificity – TSp) was calculated, the probability of the event for the positive and negative test results was estimated (expected value of positive – EVPR, and negative result of the test – EVNR). The ability of the positive test result to confirm the existence of an event (the credibility ratio with a positive test result – CR+) and its absence with a negative test result (the credibility ratio with a negative test result – CR–) was determined. The probability of an increase in the detection of an event upon the positive test results (posttest probability of an event with a positive test result – PP+) and decreased probability of its denying with the negative test value (posttest probability with a negative test – PP-) was calculated.

To assess the risk of an event, the absolute (AR) and relative (RR) risks, as well as the odds ratio (OR) were determined. When evaluating the diagnostic value of the results of a comprehensive examination their 95% confidence intervals (95% CI) were taken into consideration.

Results and discussion: As it is shown in a paper, groups of schoolchildren, formed depending on the level of remodeling factor VEGF in sputum, did not differ

significantly in the severity of the BA course. Thus, the mild persistent form of disease occurred in 25.53% of the I group patients, and in 20.33% of the comparison group children, the moderate severity asthma was registered in 27.66% and 26.61% of patients respectively, and severe asthma was diagnosed in 46.81% and 53.06% of cases respectively (in all cases P>0.05). Thus, the amount of the basic control treatment in both clinical groups may also be considered comparable.

The absence of differences by the clinical assessment of the severity of BA between the children of the experimental groups indicates that these criteria do not reflect the processes of neoanogiogenesis in the bronchial wall, which occurs as a result of the chronic inflammatory process in the respiratory tract. Therefore, the standard basic therapy, focused on the severity of the disease, does not control the processes of remodeling. Besides, a significant correlation between the severity of disease and level of VEGF in mucospin of sputum (R=0.47, P=0.008) in the I clinical group emphasizes the need for correction of the standard BA therapy, taking into account the level of the biomarker of bronchial neonagiogenesis.

According to obtained results, it may be assumed that there is a connection between the intensified bronchial remodeling processes and the eosinophilic nature of the inflammatory process, since differences in the level of the extracellular eosinophilic cationic proteins in sputum of patients of the experimental groups have been detected. Thus, in children from the I clinical group this biomarker averaged 2.78±0.24 pg/ml, while in the comparison group it was 1.77±0.21 pg/ml (P<0.05).

The level of the extracellular eosinophilic cationic proteins in sputum of patients, exceeding the value of 2.0 pg/ml, as a test for detection of neoangiogenesis in the bronchial walls in BA patients, had a satisfactory TSn (71.43%: 95% CI 56.74-83, 42)%, but low TSp (46.81%: 95% CI 32.11-61.92)%, EVPR (58.33%: 95% CI 44.88-70.93)% and EVNR – (61.11%: 95% CI 43.46-76.86)%. The posttest probability of this test was: PP(+) – 7.32% and PP(-) – 12.1%. The odds ratio for the presence of bronchial remodeling at a positive test results reached 2.20 (95% CI 1.0-5.12) with the values of RR – 1.5 (95% CI 1.1-2.07) and AR – 0.2. Thus, it is more effective to use this test to disprove than to confirm the occurrence of neoangiogeogenesis in the bronchial wall of patients with BA.

It is known that structural proteins of bronchial wall can be damaged by free radicals, formed due to activity of phagocytic cells (eosinophils, neutrophils, etc), and under the influence of various cytokines [12], as well as from lipid peroxidation process [13], when reactive oxygen species cause oxidative modification of proteins and formation of aldehydes as lipid peroxidation by-products. As a result of these reactions carbonyl groups (aldehydes and ketones) can enter the proteins in various ways, so their appearance is believed to be a reliable conformation of the oxidative modification of proteins. Analysis of the carbonyl groups in proteins provides a convenient method for the detection and quantification of protein oxidative modification by reacting carbonyl groups with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone.

The table summarizes results of the dynamic evaluation of total protein level, aldehyde and ketone derivatives of dinitrophenylhydrazones levels in PE condensate of BA patients from clinical comparison groups. Represented results demonstrate the composition of PE in children of the comparison groups at the initial examination, indicating the predominance of protein oxidative modification processes in patients with high level of bronchial remodeling markers in sputum. In the dynamics of anti-inflammatory treatment there were discordant

changes in these indices of PE, which reflected the higher effectiveness of the standard anti-inflammatory therapy in children from the I group, possibly due to the eosinophilic nature of airway inflammation. However, our attention is drawn to the fact, that after 3 years of catamnestic observation the total protein level in PE of patients from the I group was significantly increased, apparently pointing to its release due to the active neoangiogenesis in the airway mucosa.

Table

Since the derivatization reaction with 2,4-dinitrophenylhydrazine is considered an informative marker of protein oxidative modification [14], and also based on the obtained results represented in the table, we can assume that at the beginning of observation in children from the I group there was an active inflammatory process in the respiratory tract, caused by an intense oxidative stress among other mechanisms, as evidenced by an increased level of both alkaline and neutral aldehyde and ketone dinitrophenylhydrazones. At the same time, as it was shown in the dynamics of 3-year observation, activity of the oxidative processes in patients from the I group decreased, while in patients from the II group with normal indices in the condensate of RE at the beginning of observation, activity of the oxidative modification increased in the dynamics with the highest level after 1.5-2 years from the beginning of monitoring observation.

Based on the available signs of a predominant activity of eosinophilic leukocytes degranulation in patients from the I group, such changes are explained by the effectiveness of the basic control therapy, which is mainly directed on the eosinophilic inflammatory process in bronchi. However, it can be assumed that the decrease in the intensity of the inflammatory process does not preclude a progression of the organic reorganization of bronchial wall, which, in turn, reduces the lability of the airways, producing the clinical effect of false "well-being" [15].

This assumption was confirmed by the results of spirographic bronchomotor tests using metered physical load with subsequent inhalation of a short-acting β2-agonist and calculation of the bronchial lability index (BLI). It was shown that during the period of dynamic observation BLI gradually reduced from year to year only in children from the I group: at the initial examination – 22.14%, and at the final one – 13.28%. At the same time, the representatives of the II clinical group had an average BLI of 18.55% and 23.89% respectively. Thus, it can be assumed that in children with high level of bronchial remodeling marker in sputum, despite the decrease in the activity of inflammatory process, apparently there was a realization of the risk of structural changes in the airways, as evidenced by the signs of protein release and formation of the bronchial wall rigidity.

The diagnostic value of the initial high level of total protein (> 4.0 g/l) and alkaline AKDNPH (> 60.0 mmol/g of protein) in PE of patients with non-invasive markers of bronchial remodeling was investigated. Thus, for the high level of total protein the following was established: TSn 52.6% (95% CI 42.3-62.7)%, TSp – 58.3% (95% CI 48.0-68.1)% , EVPR – 55.8% (95% CI 45.2-66.0)%, EVNR – 55.2% (95% CI 45.1-64.0)%, CR(+) – 1.3, CR(-) – 0.8. To use with this purpose the increased level of protein oxidative modification products (alkaline AKDNPH >60,0 mmol/g protein), the following indices of diagnostic informativeness of the test were: TSn – 31,2% (95% CI 22.3-41.3)%, TSp – 73.3% (95% CI 63.5-81.7)%, EVPR – 53.9% (95% CI 40.2-67.1)%, EVNR – 51,6% (95% CI 43,0-60,6)%, CR(+) – 1.2, CR(-) – 0.9. Use of the above tests to detect children with existing bronchial remodeling increased PP with a positive test result: for a total protein level of more than 4.0 g/l by 5.8%, for markers of alkaline

protein peroxidation higher than described above – by 3.9%, and with a negative test result the probability of the structural rearrangement of bronchial wall was reduced by 5.2% and 1.6%, respectively. The risk indices of the structural alteration of bronchi due to the increased neoanogeogenesis with an increase in the total protein level in PE were: OR – 1.6 (95% CI 0.9-2.7), RR – 1.2 (95% CI 0.9-1.7), AR – 0.01; and for the neutral AKDNPH (>60.0 mmol/g protein): OR – 1.3 (95% CI 0.7-2.3), RR – 1.1 (95% CI 0.7-1.7), AR – 0.06.

The obtained data suggest that none of the proposed tests for the detection of bronchial structures alterations did not possess a sufficient diagnostic value to detect bronchial remodeling with a positive result and, moreover, to exclude the risk of this event with a negative test result. Consequently, these non-invasive methods of research should be used either in the complex (in parallel), or in the dynamics (consistently).

Conclusions

1. The presence of a statistically significant correlation between the severity of the persistence of bronchial asthma and level of VEGF in mucospin of sputum (R=0.47, P=0.008) determines the need for correction of the standard therapy of the disease based on the level of this bronchial neonagiogenesis biomarker.
2. In the detection of the processes of bronchial mucosa neoagnogenesis the increase in the level of extracellular eosinophilic cationic proteins in mucospin of sputum (> 2.0 pg/ml) should be acknowleged as the most sensitive test (TSn=71.43%; 95% CI 56.74-83.42)%, and the increase in the level of protein oxidative modification products in the condensate of pulmonary expirate (alkaline AKDNPH >60.0 mmol/g of protein) as the most specific (TSp=73.3%, 95% CI 63.5-81.7)%, but due to insufficient value of credibility ratio and moderate values of an increase or decrease in the posttest probability of the event, use of these laboratory tests alone to determine the bronchial remodeling does not seem appropriate.
3. Examination of the condensate of pulmonary expirate at the beginning of observation give reason to suggest that in children with high level of bronchial neoagnogenesis biomarker (VEGF) in sputum the standard control treatment causes a decrease in the severity of the protein oxidative modification, but at the same time protein release increases and bronchial lability decreases (by 8.86%).
4. In the process of dynamic observation in children with asthma from the reference by the parameters of bronchial remodeling group the activity of protein oxidation as well as bronchial lability increases (+ 5.34%) in spite of the standard treatment.
5. The established significant cross-group differences in the level of extracellular cationic proteins in sputum of patients at the initial examination (2.78±0.24 vs 1.77±0.21 pg/ml respectively, P<0.05), and higher efficacy of anti-inflammatory corticosteroid therapy in patients with signs of bronchial remodeling according to the final examination, provides a substantiation to connect the processes of alteration and structural rearrangement of the bronchial wall with the eosinophilic nature of the inflammatory process. The latter responds well to prescribed therapy, but further it does not determine the respiratory reactivity in a bronchomotor test with metered physical load and inhalation of a short-acting β2-agonist.

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Table: Results of the study of protein oxidative modification in the condensate of pulmonary expirate in patients from the comparison groups during the monitoring observation

Groups of patients	Period of observation	Markers of protein oxidative modification		
		Total protein (g/l)	Alkaline AKDNPH (mmol/g of protein)	Neutral AKDNPH (mmol/g of protein)
I group	Initial observation	3.91±0.45	67.33±9.07*	7.87±1.44*
II group		4.11±0.30	45.73±5.90	4.76±0.51
I group	After 1.5 years	3.91±0.32	40.94±9.92*	4.69±0.70*
II group		3.51±0.47	70.23±13.06	7.40±1.20
I group	After 3 years	4.72±0.47*	33.95±4.15* **	3.65±0.55* **
II group		3.45±0.45	54.56±6.90	6.31±0.45 **
*P<0.05 I:II; **P<0.05 initial examination VS after 3 years				

Note: AKDNPH – derivatives of 2,4-dinitrophenylhydrazine