Egypt J Pediatr Allergy Immunol 2003; 1(1): 62-67.

Original article

Assessment of hydrogen peroxide in breath condensate as an inflammatory marker in asthmatic children.

Background: *Asthma is a major global public health problem. Airway inflammation is the primary cause of development and progression of asthma. Activation of inflammatory cells induces a respiratory burst resulting in the production of reactive oxygen species, such as H_2O_2.*
Objective: *We sought to measure the concentration of H_2O_2 in exhaled breath condensate in asthmatic children and its correlation to asthmatic triggers, pulmonary function tests, treatment modalities and blood absolute white blood cell counts.*
Methods: *Fifty asthmatic and 35 healthy children were studied. Their ages ranged from 3-14 years. Exhaled H_2O_2 was measured using a colorimetric assay.*
Results: *In asthmatic children, there was a significant elevation of the mean H_2O_2 concentration compared to values in normal subjects($p<0.0001$). Bad housing showed significant correlation to asthma severity and to number of acute asthmatic attacks. Basal spirometric pulmonary function tests, mean values showed no significant correlation to the level of H_2O_2 nor to treatment with inhaled steroids. Similarly, neither asthma severity nor the intake of inhaled steroids did show any significant correlation with H_2O_2 level.*
Conclusion: *Exhaled H_2O_2 was found significantly elevated in asthmatic children. Measurement of exhaled H_2O_2 may help to assess airway inflammation and oxidative stress in asthmatic patients.*

Keywords: Hydrogen peroxide – H_2O_2 – exhaled breath condensate – asthma – children – oxidative stress – pulmonary function tests.

Laila Abdel Ghaffar, Malak A. Shaheen and Ahmed E. Shouman*

From the Departments of Pediatrics and Community Medicine, Ain Shams University, Cairo, Egypt.*

Correspondence:
Malak A. Shaheen, MD – Division of Pediatric Pulmonary Medicine and Pediatric ICU, Ain Shams University, Cairo – Egypt.
E-mail: childshaheen@yahoo.com

INTRODUCTION

Asthma is a leading cause of chronic illness in childhood. As many as 10-15% of boys and 7-10% of girls may have asthma at sometime during childhood. Current data support the hypothesis that inflammation underlies the pathophysiology of asthma [1]. Activation of inflammatory cells induces respiratory burst resulting in the production of reactive oxygen species, such as hydrogen peroxide [2].

Oxidative stress, defined as an increased exposure to oxidants and/or decreased antioxidant capacities, is implicated in inflammatory airway disease. Inflammatory cells, especially eosinophils which produce more superoxide anions (O_2) than neutrophils or macrophages, release several reactive oxygen radicals. Oxygen radicals are rapidly metabolized to form hydrogen peroxide (H_2O_2), which diffuses into the airway lining fluid and may evaporate into exhaled air [3].

Hydrogen peroxide in exhaled breath condensate is increased in chronic obstructive airway disease, adult respiratory distress syndrome, cigarette smoking, and asthma, and may be used as a non invasive marker of oxidative stress. However, the effect of corticosteroids on production of reactive oxygen species or H_2O_2 is not certain [4].

This study is aimed to assess hydrogen peroxide concentration in exhaled breath condensate as an inflammatory marker in bronchial asthma.

Hydrogen peroxide assessment may be used to monitor the inflammatory process in asthmatic patients and is to be correlated with the severity of airway obstruction.

METHODS

This study comprised 85 Egyptian children attending the Children's Hospital of Ain Shams University. They were 46 males and 39 females. They were classified into two groups:

Group I: It included 50 asthmatic children, 30 males and 20 females. Their ages ranged from 3-14 years. They were enrolled from the Chest Clinic, Children's Hospital, Ain Shams University, during the period from January to December 2002.

Criteria for diagnosis of asthma:

The diagnosis of asthma in those patients was based upon the criteria of the American Thoracic Society (ATS) [5] which defined diagnosis of asthma according to previous recurrent documented history of wheezing, shortness of breath and nocturnal cough. All the patients had airway obstruction by pulmonary function tests, that reversed by >15% either spontaneously or after administration of β_2 agonist inhalation**.**

Classification of asthmatic children:

According to National Heart, Lung and Blood Institutes (NHLBI)[6] asthmatic children were subclassified into:

1. Moderate asthmatic children: included 20 children, 14 males and 6 females their ages ranged from 3–14 years (mean ±SD: 9±3 years).
2. Severe asthmatic children: included 30 children, 17 males and 13 females their ages ranged from 3-14 years (mean ±SD: 9±3 years).

Group (II): Controls (In H_2O_2 measurement) This group included 35 healthy children, age and sex matched, thoroughly examined, 16 males and 19 females their ages ranged from 4-15 years (mean ±SD: 9±4 years).

Methods: Asthmatic children in the study were subjected to the following

1. Full medical history: including
   - Frequency of attacks.
   - Nocturnal symptoms.
   - Seasonal variation.
   - Medications received, dose and frequency [100% used salbutamol, 12% budesonide, 80% fluticasone, 34% montelukast and 12% were also receiving other medications e.g.: cough sedatives, oral theophylline].
   - Allergic background (food allergy was verified from the history).
   - Other atopic disease (e.g. nasal, dermal).

   * Environmental conditions: (to define bad housing)
   - Socioeconomic standard.
   - Animal and bird contact.
   - Housing: old/ new, ventilation, sun exposure, humidity and exposure to fumes and dust
2. Complete clinical examination:
3. Laboratory investigations:
   a- Complete blood count (CBC) with calculation of absolute eosinophilic (AEC) and absolute neutrophilic counts (Coulter counter, MicroDiff 17, USA).
   b- Pulmonary function tests (PFTs): Spirometric pulmonary function tests were done, using a pocket spirometer, which can operate as a stand-alone pocket spirometer, or connected with a personal computer (MIR Spirobank, Ocean Co., Italy, Roma).

   Parameters measured
   - FVC: forced vital capacity
   - FEV1: volume expired in the 1st second of test
   - FEV1 %: (FEV1 / FVC x 100).
   - FEF25-75: average flow 25 - 75% of FVC.

Spirometry: starting testing:

Inserting the disposable mouth piece at the set and fitted well and fit the nose clip to the nose in order to ensure that no air can escape from the nostrils, then hold the spiroband facing the user then switch on the set to the testing pattern. Put the free end of mouth piece well into the mouth for at least 2 cm inside the mouth and close the side of mouth tightly around the mouth piece.

Start to breath into the machine according to the test (better in a standing position).

FVC test:

First, start the test by breathing at rest for a few moments, then when ready to start, inspire slowly, as much as possible (and this is made easier by holding the arms wide apart). Then, make a complete expiration as fast as possible, complete the cycle by inspiring again as quickly as possible.

The test may be repeated several times by repeating the cycle without taking the mouth piece out of the patient.

c- Hydrogen peroxide measurement: (Done for patients and controls) by using Dekhuijzen et al.[7] technique (calorimetric assay).

Reagents used:

1) 420 μ M tetra-methyl Benzidine dissolved in 0.42 M citrate buffer at pH 3.8.
2) Horseradish peroxides (HRP) (Sigma chemicals, Pole, UK).
3) 18 μ M sulphuric acid.

Instruments:

- Valve-mouth piece.
- Sterile plastic tube.
- Spectrophotometer.

Technique:

A patient was asked to breathe into a valve-mouth piece for 15` and air was directed into a vial kept at 10°C. The vial was cooled in ice and 1 ml condensate breath was collected and stored at 70° in a 2-mL sterile plastic. A 100 mL of 420 μ M, tetramethyl benzidine with 10 mL of enzyme stock solon was reacted for 20` at room temperature. The mixture was acidified to pH1 by 10 mL of 18 H_2O_4. The reaction production was measured spectro-photometrically at 450 nm [7].

Statistical Analysis:

Data collected is coded, tabulated and introduced to a PC for statistical analysis. Quantitative data are presented as mean ± standard deviation, while qualitative data are described as frequency table i.e., number and percentage. Two group comparison, for quantitative data was performed using student t-test, while chi-squared test was used to test association between 2 variables in case of qualitative data. Correction was done if the sample is less than 30 and Fisher Exact was performed if less than 2 expected observations were encountered. P value was set at 0.05. All statistical manipulations were performed using the 6th version of SPSS (Statistical Package for Social Sciences).

RESULTS

The results of the present study showed that asthmatic children had significant elevation of the mean H_2O_2 concentration in expired air (0.345±0.16 μM/L) compared to normal controls (0.137±0.04 μM/L) with p value <0.001 (Figure 1).

Study of asthma triggers, showed a significant increase in asthmatic attacks frequency and degree of severity with bad housing (Tables 1 and 2). However, all asthma triggers showed non significant correlation to the mean value of H_2O_2 concentration in expired air of asthmatic children.

There was no significant statistical variation in H_2O_2 concentration in expired air of asthmatic children with the grade of asthma severity (mean value in severe patients 0.321±0.16 μM/L, and in moderate patients 0.379 ± 0.16 μM/L).

Table (1): Average frequency of the attacks/month in relation to different precipitating factors.

		Mean ±SD of attacks frequency/month	p value
Bad housing			
- Present	28%	8.71±5.2	<0.01
- Absent	72%	4.0±2.5	Sig
Passive smoke			
- Present	80%	5.2±3.8	NS
- Absent	20%	5.8±5.0	
Upper respiratory tract infection			
- Present	82%	4.87±3.8	NS
- Absent	12%	7.33	
Food allergy			
- Present	38%	4.89±3.5	NS
- Absent	62%	5.58±4.3	
Family history			
- Present	8%	3.75±2.9	NS
- Absent	92%	5.45±4.1	
Animal contact			
- Present	8%	7.50±4.1	NS
- Absent	92%	5.13±4.0	
Other atopic conditions			
- Present	4%	8.0	NS
- Absent	96%	5.2	

Similarly, the values of spirometric PFTs (FVC, FEV1, FEV_1% and PEF) expressed as a percentage of predicted for age and sex (Table 3), did not significantly correlate to H_2O_2 concentration in expired air of asthmatic children (with r values respectively 0.173, 0.243, 0.050 and 0.183).

Mean value of H_2O_2 concentration in expired air of asthmatic children receiving inhaled steroid therapy (44 patients) was 0.343±0.1 μM/L, which was comparable to the mean value of asthmatic children not on inhaled steroids (0.355±0.1 μM/L).

Correlations of H_2O_2 concentration in expired air of asthmatic children with total leukocytic counts (r value 0.043), eosinophilic counts (r value 0.077) and neutrophilic counts (r value -0.038) were all non significant.

Table (2): Relation of grades of bronchial asthma to the presence and absence of different precipitating factors.

	Grade		
	Moderate No (%)	**Severe No (%)**	**p value**
Bad housing			
- Present	2(14.3)	12(85.7	0.04
- Absent	18(50.0)	18(50.0)	S
Passive smoke			
- Present	15(37.5)	25(62.5)	NS
- Absent	5(50.0)	5(50.0)	
Upper respiratory tract infection			
- Present	16(39.0)	25(61.0)	NS
- Absent	4(44.4)	5(55.6)	
Food allergy			
- Present	8(42.1)	11(57.9)	NS
- Absent	12(38.7)	19(61.3)	
Family history			
- Present	2(50.0)	2(50.0)	NS
- Absent	18(39.1)	28(60.9)	
Animal contact			
- Present	1(25.0)	3(75.0)	NS
- Absent	19(41.3)	27(58.7)	
Atopy			
- Present	-	2(100.0)	NS
- Absent	20(41.7)	28(58.3)	

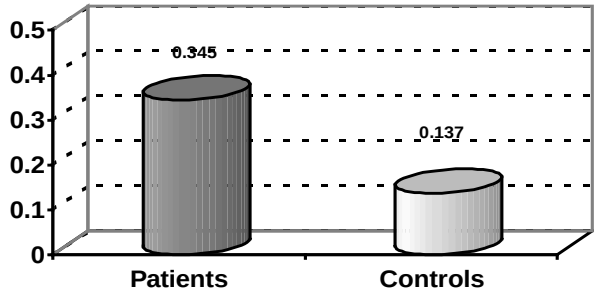
S= Significant, NS= non-significant.

Table (3): Pulmonary function tests (PFTs) parameters of bronchial asthma patients.

	Mean	SD	Range
FEV_1	80.9	21.8	33 - 166
FVC	81.7	20.7	35 - 154

FEV_1%	93.1	13.5	35 - 110
PEF	74.3	19.4	25 - 110

The values of PFTs are expressed as a percentage of predicted values for age and sex.



p value < 0.001

Fig. (1): Mean value of H2O2 level in expired air (μM/L) of asthmatic children compared to controls.

DISCUSSION

Asthma is a major chronic airway disorder that is a serious public health problem in countries through out the world. Asthma affects people of all ages, can be severe, and is sometimes fatal. Over 100 million people worldwide have asthma, and the prevalence is increasing especially among children [8].

In the present study 28% of the patients had bad housing environment (like poor ventilation, high humidity, overcrowding, poor exposure to the sun, low socio-economic status and bad housing hygiene).

Samet et al. [9] reported that levels of respirable particulates, nicotine, polyaromatic hydrocarbons, CO, NO and many other pollutant substances increase with the indoor smoke, the intensity of smoking, and air exchange rate inside houses. This usually occurs in families of low socio-economic status and in overcrowded homes.

Studies done by Andriessen et al. [10] revealed that there is a significant relation between dampness and moulds in the house and the presence of asthmatic symptoms and cough in children. In the present study there was a significant positive correlation between bad housing environment and the number of asthmatic attacks/month. Similarly, there was a significant positive correlation between bad housing and the degree of severity of asthma. This stresses the importance of bad housing environment in relation to asthma pathogenesis.

Bronchial asthma is characterized by reversible lower airway obstruction and pulmonary function tests (PFTs) are considered a useful way to objectively measure the degree, location and reversibility of lung compromise in asthmatic children [11].

Spirometric parameters in this study showed average mean values of forced vital capacity (FVC) and forced expiratory volume in 1st second (FEV_1) compared to predicted values for age and sex of examined patients (either value was more than 80% of predicted). Nevertheless, the mean value of peak expiratory flow (PEF) was slightly lowered than the average value predicted for age and sex, it was 74% and normal average value more than 80% [12]. This signified an obstructive lung disease; however this was mild, and could be explained by the fact that our patients were regularly on asthma controller and reliever medications.

The mean value of FEV_1/FVC ratio (FEV_1%) was higher than 90% in our asthmatic children. This could be explained by the fact that younger children often have an FEV_1/FVC ratio of more than 90%, and even FEV_1 was nearly equal to FVC. This might be explained by physiological and anatomical features of developing chest in children which expire almost all the FVC in 1st second**.** Accordingly, the occurrence of airway obstruction in the pediatric age groups, may not be accompanied by a significant decrease in FEV1% [11].

The present study showed that there was no significant relation between the different medications used and the pulmonary function test parameters. It was observed that 100% of asthmatic children needed to use asthma relievers (e.g. salbutamol) on different occasions and 92% needed to use inhaled steroids as a controller medication (80% used fluticazone and 12% used budesonide) regularly.

The dose of fluticazone ranged from 50μg-500μg/day. Budesonide dose ranged from 200 μg - 400 μg/day. 34% of our patients were on regular oral intake of a leukotriene modifier (montelukast) as an asthma controller. Other drugs (oral theophylline, cough sedative, and expectorant) were used regularly in 12% of patients.

A study done by Barnes [13] reported that the regular treatment with inhaled steroids diminished asthma symptoms, decreased asthma exacerbations and decreased the need for bronchodilator. Russell and Ninan [14] reported that inhaled steroids are effective in children of all ages and even in younger children, it can improve symptoms score. Unfortunately, corticosteroids do not appear to cure asthma. Its withdrawal is accompanied by rapid return of airway hyper-responsiveness and symptoms [15].

Airway inflammation is important in the development and progression of asthma [16]. Oxidative stress contributes to airway inflammation; activation of inflammatory cells such as eosinophils and neutrophils induces a respiratory burst resulting in production of superoxide anion (Ò2). This oxidant species undergoes spontaneous or enzyme catalized dismutation to form

hydrogen peroxide (H_2O_2). Hydrogen peroxide is one of the most stable of the reactive oxygen metabolites. Due to lack of charge, it may easily penetrate cellular membranes and may generate hydroxyl radicals in presence of iron cations (Fenton reaction). H_2O_2 and hydroxyl radicals are able to react with membrane and lipid components of bronchial lining fluid and cause their peroxidation. Similarly, they diffuse into the airway lining fluid and may evaporate into exhaled air[17]. We hypothesized that expired breath H_2O_2 concentration may serve as a simple, non invasive method to document airway inflammation.

In the present study, the hydrogen peroxide (H_2O_2) in expired breath condensate was significantly higher in asthmatic cases compared to the controls. This agrees with previous studies [1,2,18-21]. They reported that the level of exhaled H_2O_2 was elevated significantly in asthmatic patients compared with normal control subjects. There was no other single reported study of H_2O_2 in asthmatic children to show normal values of H_2O_2 in expired air. This could be explained by the fact that asthmatic patients have an enhanced number and activity of inflammatory cells specially neutrophils. This induces a respiratory burst resulting in generation of cytotoxic oxygen metabolites such as superoxide radicals and hydrogen peroxide. Sufficient quantities of hydrogen peroxide may be utilized by myeloperoxidase to mediate cytotoxicity via hypochlorous acid [22]. H_2O_2 appears to be an important reactive oxygen species causing cellular injury, perhaps via further reactions leading to more reactive species such as hydroxyl radicals and lipid peroxidation products [23].

In the present study, we found no significant relation between the levels of H_2O_2 in expired air of asthmatic patients and the degree of severity of asthma. This could be explained by the fact that moderate asthmatics as well as severe asthmatics still have significant on-going inflammatory process in airways.

Sznajder et al. [24] reported that the level of H_2O_2 in expired air was elevated in patients with mild asthma. However, a study done by Horvath et al. [20] revealed that the level of H_2O_2 in expired air was also elevated in severe unstable asthmatic patients. This was explained by the fact that the patients with severe asthma who are still symptomatic presumably have ongoing high degree of inflammation.

Our present study showed no significant correlation between levels of H_2O_2 in expired air of asthmatic patients and the spirometry test parameters for age and sex. This was in accordance with Emelyanov et al. [2] who reported that expired H_2O_2 levels were elevated in asthmatic patients with FEV_1 >80% of predicted values. However, there was a significant correlation between the elevated H_2O_2 level and inhalation provocation test using histamine. This could be explained by the fact that increased oxidative stress in asthmatic patients is directly correlated with airway hyperresponsiveness as manifested by bronchial challenge test. This denotes that even with mild asthmatic symptoms and normal spirometric values there is an ongoing airway inflammation with increased oxidative stress.

In contrast, Loukides et al.[1] found that in asthmatic patients there was significant correlation between H_2O_2 concentration in expired breath condensate and lung function impairment. In that study there was a positive correlation between H_2O_2 in expired air and peak expiratory flow rate (PEFR) variability; also H_2O_2 inversely correlated with FEV_1% predicted. Another study done by Sont [25] reported that there was a significant negative correlation between exhaled H_2O_2, and FEV_1% predicted in asthmatic patients.

We did not observe a significant correlation between the level of H_2O_2 in expired air in asthmatic patients and the eosinophilic and neutrophilic counts in peripheral blood. Vachier et al.[26] found a significant correlation between eosinophil and neutrophil counts in induced sputum and the H_2O_2 concentrations in expired breath condensate of asthmatic patients. Another study done by Ronchi et al[27] reported that there was a significant correlation between exhaled H_2O_2 in asthmatic patients and the eosinophil counts in induced sputum. Emelyanov et al.[2] found that there was elevated number of total leukocytes especially mast cells, activated eosinophils and T-cells in bronchial biopsy specimen from an asthmatic patient and it was significantly correlated with the elevated level of expired H_2O_2. Sputum and lung biopsy cell counts are thus considered more accurate in the assessment of the local inflammation cell burden.

Further studies for H_2O_2 in asthmatic children are recommended and are to be correlated to sputum absolute counts of eosinophils and neutrophils.

Finally, we can conclude that the hydrogen peroxide in exhaled breath condensate is an easy marker to assess the ongoing inflammatory process in asthmatic patients. Although the patients were on controller medications and the pulmonary function tests parameters were within the average mean values for age and sex, the H_2O_2 level in expired air of these asthmatic patients showed highly significant elevation compared to the level in normal control subjects. This suggests that the oxidative stress might not be well controlled by the medications; so we may consider a new policy for the management of asthma including using higher inhaled steroid doses, antioxidant therapy and/or anti-inflammatory drugs other than steroids.

REFERENCES

1. **Loukides S, Georgios P, Nikolaos M.** The relationships among hydrogen peroxide in expired breath condensate, airways inflammation, and asthma severity. Chest 2002; 121: 339-46.
2. **Emelyanov A, Fedoseev G, Abulimity A.** Elevated concentration of exhaled hydrogen peroxide in asthmatic patients. Chest 2001; 120(4): 1136-9.
3. **Silkof PE, Sylvester JT, Zamel N, Permutl S.** Mechanism of elevated exhaled No concentration in asthma. Am J Respir Crit Care Med 1999; 85: 653-66.
4. **Antczak A, Kurmanowska Z, Kasielski M.** Inhaled glucocorticoids decrease hydrogen peroxide level in expired air condensate in asthmatic patients. Resp Med 2000; 94(5): 416-21.
5. **ATS "American Thoracic Society".** Guidelines for the evaluation of impairment disability in asthmatics. Am Rev Resp Dis 1993: 149;1056.
6. **Paton JY.** Management of asthma in children. In: O'Byrne PM, Thomson NC, editors. Manual of asthma management. 2nd ed. Philadelphia: WB Saunders; 1997. p. 432.
7. **Dekhuijzen RPN, Aben KKH, Aekker I.** Increased exhalation of H_2O_2 in patients with stable and obstructive pulmonary disease. Am J Resp Crit Care Med 1996; 154:813-6.
8. **GINA (Global Initiative for Asthma).** Workshop report on global strategy for asthma management and prevention (WHO updated March 2002).
9. **Samet JM, Marbury MC, Spengler JD.** Health effects and sources of indoor air pollution. In: Stephen T, Homer A, Leonardo M, editors. Difficult asthma. 1st ed. Philadelphia: WB Saunders; 1999. p. 58-9.
10. **Andriessen TW, Brunekreef B, Roemer W.** Home dampness and respiratory health status in European Children. Clin Exp Allergy 1998; 28(10): 1191-200.
11. **Voter KZ , McBride JT.** Pulmonary function testing in childhood asthma. In: Pediatric asthma. Immunology and allergy and exposure to cockroach allergen in causing morbidity among inner-city children with asthma. N Engl J Med 1998; 336: 1356-63.
12. **Castile RG.** Pulmonary function testing in children. In: Chemick V, Boat TH, Kending T, editors. Kending's disorders of the respiratory tract in children. 6th ed. Philadelphia: WB Saunders; 1998. p. 196.
13. **Barnes PJ.** Corticosteroids. In: O'Byrne PM, Thomson NC, editors. Manual of asthma management. 2nd ed. Philadelphia: WB Saunders; 1997. p. 219.
14. **Russell G, Ninan TK.** Inhaled corticosteroid toxicity, growth and asthma. In: David TJ, editor. Recent advances in pediatrics. 17th ed. London: Churchill Livingstone; 1999. p. 1-15.
15. **Woolcock A, Lundback B, Ringdal N.** Comparison of addition of salmetrol to inhaled steroids with doubling the dose of inhaled steroids. Am J Resp Crit Care Med 1996; 153: 1481-91.
16. **Dohlman AW, Black HR, Royall A.** Expired breath hydrogen peroxide is a marker of acute airway inflammation in pediatric patients with asthma. Am Rev Respir Dis 2002; 148: 955-60.
17. **Kharitonov SA, Yates DH, Barnes PJ.** Inhaled glucocorticoids decrease nitric oxide in exhaled air of asthmatic patient. Am J Respir Crit Care Med 2001; 153: 454-7.
18. **Kanazawa H, Kazuto H , Borish L.** The role of free radical in airway obstruction in asthmatic patients. Chest 1991; 100: 1319-22.
19. **Dohlman WA, Black G, Hugh A.** Expired breath hydrogen peroxide is a marker of acute airway inflammation in pediatric patient with asthma. Am Rev Respir Dis 1993; 148:955-60.
20. **Horvath I, Donnelly LE, Kharitonow SA.** Non-invasive markers of airways inflammation in asthmatic patients. Int Rev Allergol Clin Immunol 1998; 3(Suppl.1): 17.
21. **Dworski R.** Oxidant stress in asthma. Thorax 2000; 55(Suppl. 2): S51-S3.
22. **Samuel T, Stephen J, Weiss V.** Quantitative and temporal characterization of the extracellular H_2O_2 pool generated by human neutrophils. J Biol Chem 2002; 10: 399-405.
23. **Hoffner TE, Repine J, Gofi B.** Pulmonary strategies of antioxidants deference. Am Rev Respir Dis 1998; 140: 531-54.
24. **Sznajder JI, Fraiman A, Hall B, Saunder W.** Increased hydrogen peroxide in the expired breath in patient with asthma. Am J Respir Crit Care Med 1998; 158: 1042-6.
25. **Sont JK.** The relationship between the inflammatory infiltrate in bronchial biopsies and clinical severity of asthma in patients treated with inhaled steroids. Chest 2001; 120: 1136-9.
26. **Vachier I, Channez P, Le Dougen C.** Enhancement of reactive oxygen species formation in stable and unstable asthmatic patients. Eur Respir J 2002; 7: 1585-92.

27. **Ronchi MC, Piragion E, Rosi L, Stendardi A.** Do sputum eosinophil and ECP relate to the severity of asthma. Eur Respir J 1998; 10: 1809-13.