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Evaluation of oxidant/antioxidant status and ECP levels in asthma

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Aim: Inflammatory and immune cells, such as eosinophils, macrophages, and neutrophils, generate more reactive oxygen species in patients with asthma than they do in healthy individuals, and oxygen radicals contribute to tissue injury in asthma. We aimed to measure total oxidant status (TOS) and antioxidant status (TAS) in order to assess oxidative and antioxidative capacity. Eosinophil cationic protein (ECP), total IgE, and eosinophils (%) were measured to evaluate the level of inflammation.

Materials and methods: The study included 56 non-smoking asthma patients that were followed-up at the respiratory disease and allergy outpatient clinics, where they received ongoing treatment for 5 months (May 2008-October 2008). Patients with malignancy or chronic diseases, such as DM, chronic renal disease, and rheumatoid arthritis, were excluded. The patients with asthma were divided into 2 subgroups according to the level of asthma control: controlled and partially controlled.

Results: Despite the absence of statistically significant differences in TAS and TOS in the asthma and control groups ($P > 0.05$), the levels of ECP, eosinophils, and total IgE were higher in the asthma patients (27.4-16 mg/dL, $P = 0.008$; 2.8%-1.7%, $P = 0.03$; 59-19.3 IU/L, $P < 0.001$, respectively); there were no statistically significant differences between the asthma subgroups.

Conclusion: Therapy administered to the asthmatic patients prevented generation of excess oxidants, although eosinophilic inflammation persisted.

Key words: Asthma, oxidative stress, total oxidant status, total antioxidant status, eosinophil cationic protein

Astımda oksidan/antioksidan durumları ve ECP seviyelerinin değerlendirilmesi

Amaç: Eozinofiller, makrofajlar ve nötrofiller gibi inflamatuvar ve immün hücreler sağlıklı bireylerden daha fazla astımlı hastalarda reaktif oksijen ürünleri meydana getirir; ve oksijen radikalleri astımdaki doku zedelenmesine katkıda bulunurlar. Biz oksidatif ve antioksidatif kapasitenin değerlendirilmesi için total antioksidan (TAS) ve total oksidan (TOS) seviyelerini astımlı hastalarda değerlendirmeyi amaçladık. ECP, total IgE ve (%) eozinofilleri gruplardaki inflamasyonun seviyesini değerlendirmek için ölçüldü.

Yöntem ve gereç: Solunum hastalıkları ve Alerji kliniğinde takip edilen astımlı sigara içmeyen (n = 56), 5 aydır (Mayıs 2008-Ekim 2008) tedaviye devam eden hastalar çalışmaya dahil edildi. Biz malignensili hastaları veya diabetes mellitus, kronik renal hastalık, romatoit artrit gibi kronik hastalıkları dışladık. Sonrasında, astımlı hastalar astım kontrol seviyelerine göre kontrollü ve kısmi kontrollü olarak iki subgruba bölündü.

Bulgular: Astım ve kontrol gruplarında TAS ve TOS seviyeleri arasında farklılık istatistiksel olarak anlamlı olmamasına rağmen ($P > 0,05$); ECP, eozinofil ve total IgE seviyeleri astım hastalarında daha yüksekti (27,4-16 mg/dL, $P = 0,008$; % 2,8-1,7, $P = 0,03$; 59-19,3 IU/L, $P < 0,001$, sırasıyla). Astımın subgrupları arasında farklılık gözlenmedi ($P > 0,05$).

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Sonuç: Astımlı hastalarda tedavinin aşırı oksidan oluşumunu engellediği fakat eozinofilik inflamasyonunun sebat ettiği sonucuna vardık.

Anahtar sözcükler: Astım, oksidatif stres, total oksidan status, total antioksidan status, eozinofil katyonik protein

Introduction

Bronchial asthma is a chronic inflammatory illness that affects the airway due to the effects on various cells, including mast cells, eosinophils, and T lymphocytes, and other cellular components. Eosinophils are the most important component of inflammation in asthma. Eosinophil cationic protein (ECP) is secreted by eosinophil granulocytes, has cytotoxic effects on bronchial epithelium cells, and causes lysis. ECP also complicates the pathogenesis of asthma by causing histamine secretion by mast cells and basophils. Eosinophils and the granule proteins they produce, such as ECP, are important in evaluating bronchial inflammation in asthma (1).

Free radicals are short-lived and independent chemicals that have at least one unpaired electron in their outer orbit. They form more stable structures by reacting with other molecules (2). Free radicals may be created via normal metabolic pathways or other factors (3). Free radicals formed within an organism are mostly derived from molecular oxygen. In certain pathological conditions the quantity of free radicals may increase due to the formation of a large number of free oxygen radicals or inability of an organism's defense system to cope. These radicals interact with various cellular components and macromolecules, and cause metabolic, structural, and functional damage that may lead to cell death. It is thought that cell damage caused by free oxygen radicals contributes to the pathogenesis of several chronic diseases, including asthma.

Inflammatory and immune cells, including macrophages, neutrophils, and eosinophils, produce more reactive oxygen species in asthma patients than in healthy individuals (3,4). Many characteristic traits of asthma occur due to the production of hyper-reactive oxygen species. Oxygen radicals are known to cause β-receptor dysfunction (3), bronchial smooth muscle contraction (3), bronchial hypersensitivity (3,5,6), increased mucin secretion (3,6), and a rise in vascular permeability (3,6). Numerous studies suggest that in asthma oxidative stress caused by overproduction of various free radicals or by an insufficient antioxidant defense system contributes to the tissue damage induced by inflammatory cells (3-5,7-12).

In the present study patients being treated for asthma were tested for total oxidant status (TOS) and total antioxidant status (TAS) to determine their oxidant-antioxidant state, and tested for ECP, as an inflammation marker. The aim of this study was to evaluate the levels of these parameters, and compare them with those in healthy controls.

Materials and methods

Patient and control groups

The present study included study 56 patients (43 female, 13 male) diagnosed with asthma at the İstanbul Education and Research Hospital Allergy Clinic between May and October 2008, and 22 healthy controls (15 female, 7 male). Asthma patients with additional conditions related to oxidative stress, such as cancer, eczema, and chronic illnesses (diabetes mellitus, chronic kidney disease, etc.), or chronic inflammation (rheumatoid arthritis, etc.) were excluded from the study. Additionally, cigarette smokers were excluded.

The level of control of asthma in the patient group was evaluated based on the *Global Strategy for Asthma Management and Prevention* (Global Initiative for Asthma [GINA] 2007) (13). Based on the severity of their condition, asthma patients consistently used low to moderate doses of inhaled corticosteroids and long-acting bronchodilators, or as needed, short-acting β2-agonists. The controls were not using any drugs. Detailed anamneses, physical examinations, and treatment plans for the asthma patients were noted. Fasting blood samples were collected from the patients and controls to measure TOS, TAS, ECP, and total IgE, and to obtain hemograms. The patients and controls underwent pulmonary function tests to measure lung function.

Collection and storage of samples

Serum samples were collected from the median cubital vein into test tubes without anticoagulants (Becton Dickinson Vacutainer® SST II Advance, BD Diagnostics, Franklin Dakes, USA) for TOS, TAS, ECP, and total IgE measurement. For TAS and TOS measurement the blood samples were centrifuged at 1660 ×*g*, and sera were stored in portions at –80 °C in Eppendorf tubes. The samples were brought back to room temperature before being tested in an Abbott Aeroset 2.0 analyzer (Abbott Laboratories, IL, USA). For ECP and total IgE measurement the blood samples were centrifuged at 1660 ×*g* for 10 min, and immediately analyzed. To obtain hemograms blood samples were collected from the median cubital vein into EDTA tubes, and then immediately analyzed.

Evaluation of lung function

Lung function was measured using a Koko Legend portable spirometer (Ferraris Respiratory Inc., Louisville, CO, USA).

Measurement of TAS

Measurement of TAS was performed using an Aeroset 2.0 analyzer and a total antioxidant status kit (Rel Assay Diagnostic, Turkey). With this kit the reduced ABTS (2,2'-azinobis(3-ethylbenzo-thiazoline-6-sulfonate)) molecule is oxidized to $ABTS^{\bullet+}$, using hydrogen peroxide alone in an acidic medium (acetate buffer 30 mmol/L; pH 3.6). In the acetate buffer solution the concentrated (deep green) $ABTS^{\bullet+}$ molecules remain more stable for a long time. While it is diluted with a more concentrated acetate buffer solution at high pH (acetate buffer 0.4 mol/L; pH 5.8), the color is spontaneously and slowly bleached. Antioxidants present in the sample accelerate the bleaching rate to a degree proportional to their concentrations. This reaction can be monitored spectrophotometrically and the bleaching rate is inversely related to the total antioxidant capacity (TAC) of the sample. The reaction rate is calibrated with Trolox, which is widely used as a traditional standard for TAC measurement assays, and the assay results are expressed as mmol Trolox equivalent/L (14).

Measurement of TOS

Measurement of TOS was performed using an Aeroset 2.0 analyzer and a TOS kit (Rel Assay Diagnostic, Turkey). With this kit oxidants present in the sample oxidize the ferrous ion–o-dianisidine complex to ferric ions. The oxidation reaction is enhanced by glycerol molecules, which are abundant in the reaction medium. The ferric ions make a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total quantity of oxidant molecules in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed as the micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 Equiv./L) (15).

Measurement of ECP

ECP was measured in an Immulite 1000 analyzer (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA) using an Immulite 1000 ECP kit and the chemiluminescent immunometric method.

Measurement of total IgE

Measurement of total IgE was performed in a Beckman Coulter IMMAGE Immunochemistry Systems analyzer (Beckman Coulter Inc., Fullerton, CA, USA), using Beckman Coulter's reagent and the turbidimetric method.

Measurement of eosinophil (%)

Eosinophil (%) levels were measured using hemogram tests, a Coulter GEN·S hematology analyzer (Beckman Coulter Inc., Fullerton, CA, USA) and a Coulter SCATTER PAK and LYSE S III Diff lytic reactive kit.

Statistical analysis

Statistical analysis was conducted using SPSS for Windows, v.11.5 (SPSS Inc., Chicago, IL, USA). The Kolmogorov-Smirnov test was used to compare the distribution of TAS, TOS, ECP, total IgE, and eosinophil (%) in the patient and control groups. As the group distribution of TOS, ECP, total IgE, and eosinophil (%) values was skewed, the Mann-Whitney U-test was used to compare the groups. Student's t-test was used for statistical evaluation of TAS, which was normally distributed.

Group distribution of the patients according to the level of control of asthma (determined by GINA) was evaluated using the Kolmogorov-Smirnov test. As the group distribution of TOS, TAS, ECP, total IgE, and eosinophil (%) values was skewed, the Mann-Whitney

U-test was used for between-group evaluation of the level of asthma control. Spearman's rho was used for correlation analyses in the asthmatic group. The level of statistical significance was accepted as $P \leq 0.05$.

Results

Mean age of the 56 patients was 36.2 ± 13.8 years, versus 32.3 ± 8.54 for the 22 controls. As expected, the predicted FEV_1 was higher in the control group (106 ± 14.3) than in the patient group (90.7 ± 19.07) (Table 1). There were no statistically significant differences in gender, age, or serum TOS and TAS levels between the patient and control groups ($P > 0.05$). A statistically significant difference was observed in serum ECP ($P = 0.008$), serum total IgE ($P < 0.001$), and serum eosinophil (%) levels ($P = 0.03$) between the patient and control groups (Table 2).

There was no statistically significant difference in TAS, TOS, ECP, total IgE, or eosinophil (%) levels ($P > 0.05$) between the controlled and partially controlled patients (Table 3). Additionally, there was a weak positive correlation ($r_s = 0.392$, $P < 0.001$) between ECP and total IgE levels, and a moderately positive correlation ($r_s = 0.560$, $P < 0.001$) between ECP and eosinophil (%) levels (Table 4).

Discussion and conclusion

Bronchial asthma is an inflammatory disease characterized by activation and accumulation of inflammatory cells in the airway. This inflammation causes tissue damage, resulting in the pathological manifestations of the disease, including airflow obstruction, airway hyperresponsiveness, and permanent structural changes that include airway

Table 1. Demographic data-respiratory functions tests.

	ASTHMA	HEALTHY CONTROL
N	56	22
Age (years)[a]	36.2 ± 13.8	32.3 ± 8.54
Gender (female/male)	43/13	15/7
predicted FEV_1[a] %	90.7 ± 19.01	106 ± 14.3

[a] The data are expressed as mean values ± standard deviation.

Table 2. Study parameters based on asthma-healthy control group.

	ASTHMA	HEALTHY CONTROL	P value
TOS[a] (μmol H_2O_2 Equiv./L)	3.31 (1.45-15.67)	2.99 (2.14-11.1)	P > 0.05
TAS[b] (mmol Trolox Equiv./L)	1.34 ±0.13	1.32±0.20	P > 0.05
ECP[a] mg/dL	27.4 (6-200)	16 (5-83)	P = 0.008
Eosinophil (%)[a] eosinophil/leukocyte* 100	2.8 (0-51)	1.7 (0-7)	P = 0.03
Total IgE[a] IU/L	59 (5-805)	19.3 (5-111)	P < 0.001

[a] The data are expressed as median values. Mann–Whitney U-test was used for statistical analysis.
[b] The data are expressed as mean value ± standard deviation. Student's t-test was used for statistical evaluation.

Table 3. Study parameters based on asthma control levels.

	CONTROL LEVEL		
	Controlled (n = 29)	Partly controlled (n = 22)	P value
TOS (μmol H_2O_2 Equiv./L)	3.31 (1.45-15.6)	3.25 (1.63-6.57)	P > 0.05
TAS (mmol Trolox Equiv./L)	1.33 (1.12-1.54)	1.32 (1.06-1.62)	P > 0.05
ECP mg/dL	31 (6-200)	26 (6-94)	P > 0.05
Eosinophil (%) eosinophil/leukocyte × 100	3.4 (0-50)	2.8 (0.8-11)	P > 0.05
Total IgE IU/L	58.5 (5-805)	77.5 (5-742)	P > 0.05

The data are expressed as median values.

Table 4. Results of correspondence analysis based on Spearman's rho in the asthma group.

	TOS	TAS	Total IgE	Eosinophil (%)
ECP	0.118 - P > 0.05	0.048 - P > 0.05	0.392 - P < 0.001	0.560 - P < 0.001
TOS		0.264 - P < 0.05	-0.57 - P > 0.05	0.085 - P > 0.05
TAS			0.019 - P > 0.05	-0.25 - P > 0.05
Total IgE				0.465 - P < 0.001

The data are expressed as r_s - statistical significance.

remodeling. Various bioactive mediators and cytokines are involved in the pathogenesis of asthma. Additionally, free oxygen radicals have negative effects that result in the tissue damage associated with asthma (7). It is well known that oxidative stress is an important component in airway inflammation (16).

As in other inflammatory conditions, oxidative burst in asthma is a non-specific event in which numerous inflammatory processes are simultaneously activated. Asthma mediators, such as lipid mediators, chemokines, adhesion molecules, and eosinophil granulocytes, are potential stimulators of oxidant production (17) and increase reactive oxygen species (ROS) production (9). In vitro exposure of structural and inflammatory cells of the lungs to oxidants stimulates the production of such proinflammatory mediators as cytokines, chemokines and their receptors, growth factors, arachidonic acid metabolites, adhesion molecules, and ligands (3,9).

The lungs possess an advanced antioxidant system that functions to protect them from exposure to harmful oxidants (3,4,12); however, the oxidant-antioxidant balance is disturbed in asthma (10,12). In asthma free oxygen radicals are produced in excess during oxidative stress, and cause cellular dysfunction via lipid, protein, and DNA oxidation or nitration (3-5,10-12). Many characteristic traits of asthma appear due to the overproduction of reactive oxygen species.

We used TOS to evaluate the oxidant state, and TAS to evaluate the antioxidant state in patients with mild asthma. Zeyrek et al. studied patients with mild asthma and reported that the oxidant markers plasma TOS and total peroxide, and the antioxidant marker TAS were higher in the asthmatic group than in the

healthy control group (18). In the present study we did not observe a significant difference in the TOS level, taken as an oxidant marker, between the mild asthmatic patients and controls. Although we used the same method, our results contradict those of Zeyrek et al. (18).

The lack of a significant difference in TOS between our asthmatic patients and controls might have been due to the fact that the patients were stabilized with inhaled steroids. Studies show that steroid treatment slows oxidant production in asthma patients (9). Hanazawa et al. reported that inhaled steroid treatment impedes inflammation of the airway, reduces oxidative load, and causes a decrease in the nitric oxide and nitrotyrosine present in tidal breath (19). Other studies reported that steroid treatment decreases MDA, H_2O_2, and NO levels (20). Similarly, inhaled glucocorticoids decrease the total nitrate and nitrite levels in tidal breath and sputum (9).

The results of studies conducted on the antioxidant state based on antioxidant markers vary. For example, some studies reported elevated erythrocyte and leukocyte superoxide dismutase (SOD) levels, others reported decreased erythrocyte SOD levels, and some reported that SOD levels in erythrocytes (21), bronchoalveolar lavage (BAL) fluid, and bronchial epithelium (22) do not change. Likewise, there are studies that reported decreased glutathione peroxidase (4,6,23) or vitamin C (4,23) levels, no change in glutathione peroxidase or vitamin C (21,24), and studies that report increased extracellular glutathione peroxidase in the BAL fluid (4).

The lack of a significant difference in the level of the oxidant marker TOS between our patient and control groups shows that our patients maintained an oxidant-antioxidant balance and did not have high oxidant load. This might be why we did not observe activation of compensatory mechanisms during high oxidant load, an increase in antioxidant levels, or a decrease in antioxidant consumption due to oxidative load.

We chose to classify our patients according to the level of asthma control instead of the severity of asthma, because we think that classification based on severity at the time of presentation would have been ineffective. Classification based on the severity of asthma is useful for initial evaluation. However, the parameters for determining severity during initial evaluation, such as nighttime and daytime symptoms, limitation of activity, and the number of attacks, can improve with treatment and might not reflect a patient's current condition. Therefore, it is more useful and valuable to evaluate the level of control of asthma (13).

ECP and eosinophil levels were higher in the patient group than in the control group, and there was a positive correlation between ECP, and total IgE and eosinophil (%) levels. These findings indicate that inflammation due to eosinophil was present, but the similarity in the TOS level between the groups suggests that there was a lack of oxidative stress due to overproduction of oxidants in the patient group.

Inflammation in asthma is a complex condition that results from the interaction of numerous factors. Inflammatory mechanisms, including overproduction of oxidants, harm asthma patients and create the observed symptoms; however, the present study's findings suggest that inflammation persisted, even in the absence of oxidant overproduction. We did not include newly diagnosed patients that had not received treatment, which could be considered a limitation of the present study. Another limitation is the lack of evaluation of dietary intake of antioxidants. We think that the evaluation of the oxidant-antioxidant state and ECP levels in newly diagnosed untreated asthma patients would provide data that warrant additional research.

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