Measurement of the total antioxidant potential in chronic obstructive pulmonary diseases with a novel automated method

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ABSTRACT

Objectives: To determine the oxidative and antioxidative status of plasma of patients with chronic obstructive pulmonary disease (COPD) and to compare these values with healthy smokers and healthy non-smokers control subjects using a more recently developed automated measurement method.

Methods: This study involved 40 COPD patients, 25 healthy smokers, and 25 healthy non-smokers who attended the Chest Diseases Outpatient Clinic in Harran University Research Hospital, Turkey during the period between March 2006 and June 2006. We calculated the total antioxidant potential (TAOP) to determine the antioxidative status of plasma, and we measured the total peroxide levels to determine the oxidative status of plasma.

Results: The TAOP of plasma was significantly lower in patients with COPD than in healthy smokers and healthy non-smokers ($p<0.001$). In contrast, the mean total peroxide level of plasma was significantly higher in COPD patients than in healthy smokers and healthy non-smokers ($p<0.001$).

Conclusion: We found a decreased in TAOP COPD patients using a simple, rapid and reliably automated colorimetric assay, which may suitable for use in routine clinical biochemistry laboratory, and considerably facilitates the assessment of this useful clinical parameter. We suggest that this novel method may be used as a routine test to evaluate and follow-up the levels of oxidative stress in COPD.

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Reactive oxygen species (ROS) produce many metabolic and physiological processes, and harmful oxidative reactions may occur in organisms, which remove ROS via enzymatic and non-enzymatic anti-oxidative mechanisms. Under some conditions increases in oxidants and decreases in antioxidants cannot be prevented, and the oxidative/antioxidative balance shifts towards the oxidative status. Oxidative stress, which has been implicated in over a hundred disorders, develops in consequence.[1] The most important factor causing COPD is cigarette smoking which causes increased oxidative and nitrosative stress in this disease.[2-5] Cigarette smoke contains around 1017 oxidant molecules per puff, and this, with a large body of evidence demonstrating increased oxidative stress in smokers with and without COPD, has led to the proposed role of oxidant/antioxidant imbalance in the pathogenesis of this condition.[6] Various methods have been developed for the measurement of total antioxidant status. However, there is not yet an accepted "gold standard" reference method, and decisions concerning standardization, and the terms and units used for the measurement of TAOP have not yet been made.[7] This implies that this topic needs to be studied further.[8] The most widely used methods for TAOP measurement are colorimetric, or involve either fluorescence or chemiluminescence.[9-11] The fluorescence and chemiluminescence methods require sophisticated techniques, and these improved systems are not present in many routine clinical biochemistry laboratories. However, even when these technologies are available, their routine use is limited.[7] In this study, we aimed to measure both the levels of individual antioxidant components and the TAOP values in plasma samples from COPD patients, healthy smokers, healthy non-smokers to evaluate their antioxidant status using a novel automated method.[8] As a reciprocal measure, the total peroxide levels of the same plasma samples were also measured. The percent ratio of the total plasma

peroxide level to the plasma TAOP level was regarded as the oxidative stress index (OSI).[12]

Methods. This study involved 40 COPD patients who attended the Chest Diseases Outpatient Clinic, Harran University, Research Hospital during the period between March 2006 and June 2006. Twenty-five healthy smokers and 25 healthy non-smokers were selected as control groups. Healthy smokers group had a history of 32 pack-years. None of the COPD patients had been exposed to smoke, during the last 5 years. Chronic obstructive pulmonary disease was diagnosed on the basis of history, physical examination and spirometric data, according to American Thoracic Society guidelines.[13] All the patients included in the study had moderate COPD with airways obstruction shown by a forced expiratory volume in one second (FEV1) 50-80% of predicted, a FEV1/forced vital capacity (FVC) ratio of ≤70% that did not change markedly for >2 months, a history of chronic progressive symptoms such as dyspnea and coughing, and a history of smoking. The control group consists of 50 subjects (25 healthy smokers and 25 healthy non-smokers) shown to have normal pulmonary function (FVC >80%, FEV1 >80%, FEV1/FVC >70%). Patients with COPD were clinically stable. Chest radiography was carried out to exclude the other respiratory diseases. Patients with systemic diseases, malignancy, vascular disease, thrombosis, alcoholism, renal disease and hepatic disease were also excluded from the study. All the patients with COPD were treated with inhaled glucocorticoids (namely budesonide, 0.8mg/d; or fluticasone, 1 mg/d). Inhaled beta-2 agonists and anticholinergics were also used. This study was performed according to the European regulations under the supervision of a bioethics consultant, and ethical approval was obtained. None of our patients were treated with drugs that have anti-oxidant potency such as Theophylline, systemic steroid, N-acetyl-cysteine, vitamin C or vitamin E.

Blood samples were obtained before any medication was taken and before the onset of labor. Blood samples were taken in the smokers group 2 hours after smoking. Samples were withdrawn from a cubital vein into heparinised tubes and immediately stored on ice at 4°C. The plasma was then separated from the cells by centrifugation at 3000 rpm for 10 min, and the plasma samples were stored at -80°C until analysis.

Measurement of plasma total antioxidant potential. The total antioxidant status of the plasma was measured using a novel automated colorimetric measurement method for the TAOP developed by Erel.[8] In this method the hydroxyl radical, the most potent biological radical, is produced by the Fenton reaction, and reacts with the colorless substrate *O*-dianisidine to produce the dianisyl radical, which is bright yellowish-brown in color. Upon the addition of a plasma sample, the oxidative reactions initiated by the hydroxyl radicals present in the reaction mix are suppressed by the antioxidant components of the plasma, preventing the color change, and thereby providing an effective measure of the total antioxidant capacity of the plasma. The assay results are expressed as mmol Trolox eq./L and the precision of this assay are excellent, being lower than 3%.[14]

Measurement of plasma total peroxide concentration. Total peroxide concentrations were determined using the "FOX2" method with minor modifications.[15] The FOX2 test system is based on oxidation of ferrous ion to ferric ion by various types of peroxides contained within the plasma samples, to produce a colored ferric-xylenol orange complex whose absorbance can be measured. The FOX2 reagent was prepared by dissolving ammonium ferrous sulphate (9.8 mg) in 250 mM H_2SO_4 (10 ml) to give a final concentration of 250 mM ferrous ion in acid. This solution was then added to 90 ml high performance liquid chromatography (HPLC)-grade methanol containing 79.2 mg butylated hydroxytoluene (BHT). Finally, 7.6 mg xylenol orange was added with stirring to make the final working reagent (250 mM ammonium ferrous sulphate, 100 mM xylenol orange, 25 mM H_2SO_4, and 4 mM BHT in 90% vol/vol methanol in a final volume of 100 ml). The blank working reagent contained only ferrous sulphate. Aliquots (200 mL) of plasma were mixed with 1800 mL FOX2 reagent. After incubation at room temperature for 30 min, the vials were centrifuged at 12000 g for 10 min. Absorbance of the supernatant was then determined at 560 nm. Total peroxide content of plasma samples was determined as a function of the absorbance difference between test and blank tubes using a solution of H_2O_2 as standard. The coefficient of variation for individual plasma samples was less than 5%.

Oxidative stress index. The ratio of total peroxide to total antioxidant potential was the oxidative stress index; an indicator of the degree of oxidative stress.[12]

Statistical analysis. Data were presented as mean ± SD or percentages. Categorical variables were analyzed with contingency tables using the student t test, chi-square test and the Fisher's exact test when appropriate. Comparisons among the groups were performed by using Analysis of Variance test. A p-value of <0.05 was considered statistically significant. Analysis was carried out using the Statistical Package for Social Sciences for Windows Release 11.5 (SPSS Inc.) statistical software.

Results. Demographic and clinical data of the subjects are shown in **Table 1**. As seen in **Table 1**, the

COPD and matched controls (healthy smokers and healthy non-smokers) were similar ranges in terms of age, height, body weight and body mass index (BMI) (p>0.05). As seen in **Table 2**, plasma TAOP levels of patients with COPD were significantly lower than those of healthy smokers and healthy non-smokers (p<0.001). Plasma total peroxide levels were significantly higher in COPD patients than in healthy smokers and healthy non-smokers (p<0.001). Oxidative stress index was significantly higher in COPD than in controls (p<0.001) (**Table 2**).

Discussion. In the present study, we found that the oxidative/antioxidative balance shifted towards oxidative status, namely increased oxidative stress was present in patients with COPD compared with healthy smokers and non-smokers control subjects. There is now overwhelming evidence for the presence of increased oxidative stress in smokers and patients with COPD.[16-18] Direct measurements of specific markers of oxidative injury resulting from excessive free radical activity can be made by electron spin resonance, which cannot be applied to the study of tissues at present. Most studies have therefore relied on indirect measurements of free radical activity in biological fluids. Although these markers suggest that oxidative stress has occurred, they do not indicate that this event is necessarily involved in the pathogenesis of the condition that is being studied. Markers of oxidative stress have been shown to occur in the epithelial lining fluid, in the breath, and in the urine in cigarette smokers and patients with COPD.[5] A recent study directly examined the balance between oxidants/antioxidants in smokers and patients with acute exacerbations of COPD by measuring changes in the antioxidant capacity in the blood. Rahman et al[19] found that the plasma antioxidant capacity was significantly decreased in smokers one hour after smoking and in patients with acute exacerbations of COPD, when compared with plasma from age-matched nonsmoking control subjects. The decrease in plasma antioxidant capacity in smokers may be due to a profound depletion of plasma protein sulfydryls as demonstrated following cigarette smoke exposure in vitro.[20,21] Thus, there is clear evidence that oxidants in cigarette smoke, either in vitro or in vivo, markedly decrease low molecular plasma antioxidants both in vitro and in vivo. Depletion of plasma antioxidants reduces the protection against cigarette smoke-induced plasma membrane peroxidation.[5] Likewise, investigators have measured the major plasma antioxidants in smokers.[22-24] These studies show a depletion of ascorbic acid vitamin E, beta-carotene, and selenium in the serum of chronic smokers.[24,25] Moreover, decreased vitamin E and vitamin C levels were measured in leukocytes from smokers.[26] However, circulating red blood cells from cigarette smokers contained increased levels of super oxide dismutase and catalase, despite similar activity of glutathione peroxidase, and are better able to protect endothelial cells from the effects of hydrogen peroxide, when compared with cells from non-smokers.[27] Plasma ascorbate may be a particularly important antioxidant

Table 1 - Demographic and clinical data of the patients.

Characteristics	COPD (n=40)	Healthy Smokers (n=25)	Healthy Nonsmokers(n=25)	*P*-value
Female gender [n (%)]	18 (45)	11 (44)	12 (48)	x >0.05
Age (years)	54.1 ± 9.5	52.4 ± 11.6	53.6 ± 9.8	ANOVA >0.05
Body mass index (kg/m^2)	23.8 ± 3.1	24.5 ± 2.8	25.2 ± 2.7	ANOVA >0.05
FVC, predicted (%)	89.3 ± 13.2*	90.22 ± 11.27	92.15 ± 12.32	ANOVA >0.05
FEV_1, predicted (%)	58.9 ± 10.2*	88.32 ± 15.31	91.41 ± 11.15	ANOVA <0.001
FEV_1/FVC (%)	64.2 ± 11.4*	96.8 ± 14.61	98.27 ± 12.21	ANOVA <0.001

*p<0.001versus healthy smokers and healthy smokers. COPD - chronic obstructive pulmonary disease
FEV_1 - forced expiratory volume in one second, FVC - forced vital capacity, ANOVA - analysis of variance

Table 2 - Plasma indicators of oxidative stress in patients with COPD and healthy non-smokers and healthy smokers.

Plasma indicators	COPD	Healthy Smokers	Healthy Non-smokers	*P*-value
Total antioxidant potential (µmol Trolox equiv./L)	0.9 5± 0.43	1.45 ± 0.35	1.83 ± 0.53	ANOVA <0.001
Total peroxide (µmol H_2O_2/L)	8.74 ± 4.1	5.38 ± 1.67	5.14 ± 2.31	ANOVA <0.001
Oxidative stress index (arbitrary unit)	11.26 ± 8	3.67 ± 1.37	2.89 ± 0.9	ANOVA <0.001

ANOVA - analysis of variance, COPD - chronic obstructive pulmonary disease

in the plasma because the gas phase of cigarette smoke induces lipid peroxidation in plasma in vitro that is decreased by ascorbate.[28] Inhalation of nitric oxide (NO) from cigarette smoke, as well as NO and O_2- released by activated phagocytes react to form peroxynitrite, which is cytotoxic. Peroxynitrite has recently been shown to decrease plasma antioxidant capacity by rapid oxidation of ascorbic acid, uric acid, and plasma sulfydryls.[29] Evidence of NO/peroxynitrite activity in plasma has been demonstrated in cigarette smokers. Nitration of tyrosine residues or proteins in plasma leads to the production of 3-nitrotyrosine.[29] Petruzzelli et al[30] demonstrated the presence of 3-nitrotyrosine in plasma in smokers, which were possibly in higher levels than in a small group of non-smokers. They also confirmed low levels of antioxidant capacity in smokers, which were negatively correlated with the levels of 3-nitrotyrosine.[30] The levels of antioxidant capacity in the plasma have a negative correlation with the increased release of oxygen radicals from circulating neutrophils in patients with exacerbations of COPD.[5,19] Plasma concentrations of antioxidants can be measured separately in the laboratory, but these measurements are time-consuming, labor intensive and costly. Since the effects of the antioxidant components in plasma are additive, measurement of the total antioxidant response accurately reflects the redox status of the plasma.[31] Thus, instead of measurement of individual antioxidant components of plasma as single tests, the TAOP may be more useful and practical to evaluate the antioxidant status of plasma. The most widely used methods for measurement of plasma TAOP are either colorimetric, fluorescence-based, or chemiluminescence[9-11] is not appropriate for routine usage. However, as yet there is no accepted, "gold standard" reference method but the novel assay we report here have several major advantages over the other techniques currently available. It is simple and inexpensive, and can easily be fully automated. Accurate measurements of the total serum antioxidant response can be obtained in as little as 10 min, making this assay eminently suitable for the clinical biochemistry laboratory. In this study, we have used this novel measurement method to evaluate the extent of oxidative stress in COPD patients, and compared these values with healthy smokers and healthy non-smokers. It provides a useful method for the rapid evaluation of the TAOP, a parameter valuable not only in the diagnosis of this condition, but also for other disorders involving oxidative stress. In terms of COPD, however, routine measurement of the TAOP may provide a useful tool to aid in the assessment of the patient's oxidative stress status, and in the determination of an appropriate treatment management plan. Routine screening of the TAOP healthy smokers may also prove useful in terms of early recognition evaluation of COPD, which could then be treated with appropriate dietary supplementation, and more careful follow-up of patients at risk of developing COPD.

In conclusion, detecting plasma TAOP levels in healthy smokers as a routine and rapid test may be useful in the management of disorders coexisting with oxidative damage such as COPD.

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