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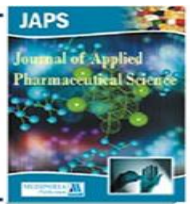

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Oxidative stress in bronchial asthma: A clinical study

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ABSTRACT

Bronchial asthma is a chronic inflammatory disorder of airways. Recently, oxidative stress has been implicated in the pathogenesis of the disease. Several studies have documented the total antioxidant activity (AOA) of saliva and serum as a potential biomarker for redox state of body. The present study has been designed to assess the redox status of patients, by determining the total AOA of the serum and saliva and correlating it with the disease status. The study was carried out on two groups: control (normal) healthy volunteers) and asthmatic patients attending OPD of Department of Pulmonary Medicine. Severity of asthma was graded using Global Initiative for Asthma (GINA) 2010 guidelines. AOA was assayed spectrophotometrically by measuring the inhibitory potential of saliva and serum against *in vitro* production of thiobarbituric acid reactive substances (TBARS). After drug treatment, patients were followed for a period of three months and AOA was measured again in saliva and serum. AOA in serum was significantly higher than that in saliva in the control subjects. Asthmatic patients exhibited a decrease in both serum and salivary AOA as compared to control. The decrease in AOA in saliva was more pronounced than that observed in serum. Significantly, the decrease in serum and salivary AOA correlated well with the severity of disease process. AOA in serum and saliva is a good indicator for assessing the severity and progress of bronchial asthma.

Keywords: Bronchial asthma, Oxidative stress, AOA, GINA, TBARS.

INTRODUCTION

Bronchial asthma is a chronic inflammatory disease of airways. It was earlier regarded to be a "reversible obstruction" of the airways. However, it is now viewed as an inflammatory illness with bronchial hyper reactivity resulting in bronchospasm. An increased number of inflammatory cells including eosinophils, macrophages and lymphocytes are present in bronchoalveolar lavage fluid from asthmatic patients (Caramori and Papi, 2004). The evaluation of airway inflammation associated with asthma may be done by examining spontaneously produced or hypertonic saline induced sputum for eosinophils and metachromatic cells. In addition, levels of exhaled NO or CO have been suggested as noninvasive markers of airflow inflammation in asthma. Measuring exhaled NO concentration appears to be useful noninvasive method to predict the the bronchial hyper-responsiveness, airway obstruction and inflammation in asthma and chronic cough patients (Nogami, Shoji and Nishima, 2003). Epidemiologic studies show that there is correlation between increasing immunoglobulin E (lgE) levels and prevalence of asthma (Corren *et al.*, 2003). When allergen cross links two lgE molecules, basophils and mast cells are activated and release a large number of inflammatory mediators. Immunological stimulation of basophils also leads to the synthesis of several pro-inflammatory mediators such as interleukin (IL)-4 and IL-13 (Schroeder and MacGlashan, 1997). Histamine and leukotrienes are released from mast cells in an acute reaction, whereas these mediators together with IL-4 and IL-13 are released from basophils in chronic disease.

The symptoms of asthma consist of a triad of dyspnoea, cough and wheezing. In its most typical form, asthma is an episodic disease and all three symptoms coexist. According to Global Initiative for Asthma (GINA) 2010 guidelines, measurement of lung function, particularly reversibility of lung function abnormalities, provide a direct assessment of airflow limitation. Lung function measurements that are most helpful for diagnosis of asthma include Forced expiratory volume in I second (FEV1), Forced vital capacity (FVC) and Peak expiratory flow (PEF) and airway hyper-responsiveness. Asthma is defined as heterogenous disease, Broadly, it is classified into two types 1. Allergic or extrinsic and 2. Idiosyncratic or intrinsic. Allergic asthma is often associated with a personal and/or family history of allergic disease such as rhinitis, utricaria and eczema. Patients produce abnormal amounts of lgE antibodies in response to contact with environmental allergens and are demonstrated by increased total or specific serum lgE and by a positive response to skin prick test using a battery of standardized allergens, specific to each geographic zone (Holgate, 1999).

Another group of patients with asthma is not associated with personal or family history of allergy, skin tests are negative and serum lgE levels are within normal limits. These patients are said to have idiosyncratic or intrinsic asthma.

In general, asthma that has its onset in early life tends to have a strong allergic component, where as asthma that develops late tends to be non-allergic.

The role of oxidative stress as a potential contributor to the pathophysiology of bronchial asthma has been less explored, and a few studies have been undertaken in this regard. An increased oxidative stress in patients with asthma is derived from increased burden of inhaled oxidants, and from the increased amounts of reactive oxygen species (ROS) like superoxide radial, hydroxyl radical and hydrogen peroxide generated by several inflammatory, immune and structural cells of the airways (Rahman, 2002). ROS might play an important role in the modulation of airway inflammation. There is evidence of an oxidant-antioxidant imbalance in asthma (Nadeem *et al.*, 2003). This concept is supported by high levels of ROS and oxidatively modified proteins in airways of patients with asthma. Concomitant with increased oxidants, antioxidant protection of lower airway is decreased in asthmatic lungs, Oxidative stress associated with enhanced lipid peroxidation and decreased antioxidant glutathione is implicated in the pathophysiology of asthma, Innate and acquired immune systems both have the potential to activate inflammatory cells and release ROS that may overwhelm the host antioxidant defenses and induce lipid peroxidation.

There are two major antioxidant defense systems in body to counter the deleterious effects of ROS: one enzymatic and the other non-enzymatic. Enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase catalase and glutathione S- transferase etc. Among non- enzymatic antioxidants reduced glutathione, beta-carotene, alpha-tocopherol and ascorbate are few. Uric acid is a predominant salivary antioxidant, with albumin and ascorbate providing minor contributions (Sculley and Langley-Evans, 2002). According to Meucci *et al.*, (1998), total protein concentration and uric acid level show a good correlation with saliva total antioxidant (AOA) capacity. Another study reports that the total AOA of saliva correlates with the concentration of uric acid, which contributes more than 70% of the total AOA. Stimulation of salivary flow is associated with an increased production of antioxidants (Moore *et al.*, 1994). Serum proteins (e.g. albumin), iron and copper binding proteins e.g. transferrin and ceruloplasmin, glutathione, uric acid ascorbic acid etc contribute to the antioxidant activity of serum.

The present study has been designed to assess the redox status of patients suffering from asthma and to compare it with that of normal healthy subjects, by determining the total AOA of their serum and saliva and correlating it with their disease state.

MATERIAL AND METHODS

Patients, aged between 20-50 years, of either sex, with bronchial asthma attending the outpatient department (OPD) of the department of TB and Respiratory Diseases, Dr. ML Chest Hospital GSVM medical college, Kanpur were investigated. Normal healthy volunteers were included in the study as control subjects. Both patients and healthy volunteers were explained the procedure and purpose of the study prior to their inclusion in it and a verbal consent was taken from them.

Evaluation of bronchial asthma

Patients included in this study comprised both newly diagnosed and follow up cases of bronchial asthma registering in the out patients department of TB and Respiratory Diseases. A complete clinical check-up of the patient was done, Information regarding details of chief complaints, history of present illness, family history, personal history and socioeconomic status was obtained and physical examination was done.

Severity of asthma in the patients was graded according to the criteria laid down by GINA (2010). According to their symptoms and pulmonary functions test (PFT), asthmatic patients were categorized into four categories:

a. Intermittent
b. Mild persistent
c. Moderate persistent
d. Severe persistent

Subjects of study

	Groups		No. of subject (n)
	Control		25
Bronchial Asthma (n=43)	Untreated (n=31)	A	11
		B	8
		C	12
		D	0
	Follow up (n=12)	A	4
		B	4
		C	4
		D	0

Where,

A--- Intermittent Asthma
B--- Mild Persistent Asthma
C--- Moderate Presistent Asthma
D--- Severe Persistent Asthma

Once the diagnosis was established, treatment was started. Unless otherwise specified, the medication, in general included either/or salbutamol, theophylline, a combination of salmeterol and fluticasone, prednisolone and a chemotherapeutic agent, gatifloxacin. These patients were followed up for a minimum period of three months from the initiation of drug therapy and samples of saliva and serum were taken when these patients came to attend the OPD.

Collection of samples

Samples of saliva and blood were collected in sterile sample vials. Patients were asked to rinse properly and oral intake of any kind was discouraged 20 minutes prior to sample collection. The samples of saliva were stored in a fridge at 4-80C. Blood samples were also preserved in fridge at 4-80C. The inhibitory potential of serum and saliva against TBARS products was determined and was taken as total antioxidant activity of that sample. All chemical measurements were made within twenty-four hours of sample collection.

Determination of antioxidant activity

Principle

The method used in our study is based on the principle that Fe-EDTA complex reacts with hydrogen peroxide (H_2O_2) by a Fenton-type reaction generates hydroxyl radicals (OH^-) and superoxides (O_2^-). These reactive oxygen species degrades benzoate, resulting in the release of thiobarbituric acid reactive substances (TBARS). The inhibitory potential of saliva/serum against TBARS production was determined spectrophotometrically at 532nm as an antioxidant activity.

Analytic Procedure

The reaction mixture containing 0.5 ml of phosphate buffer (100 mmol/l, pH 7.4), 0.5 ml of sodium benzoate (10 mmol/l) 0.2 ml of Fe-EDTA (2 mmol/l EDTA+2 mmol/l of Fe [NH4]2SO4, 0.2 ml of H2O2 (10mmol/1), and 0.01 ml of saliva was incubated for 60 minutes at 37°C. The reaction was then stopped by addition of 1 ml of 20% acetic acid. 1ml of thiobarbituric acid (TBA) solution (0.8% in 50 mmol/l NaOH) was added and the solution was heated for 10 min at 100ºC. The absorbance of the pink colour thus formed was estimated spectrophotometrically at 532 mm. 0.01 ml of uric acid (1 mmol/l in 5 mmol/l NaOH) was used as standard antioxidant for determining the AOA activity of unknown samples. Proper blanks Ko, Ao and UAo representing control, unknown and uric acid sample, respectively, were run under similar experimental conditions.

Calculation

The AOA of each sample was calculated as follows: AOA (mmol/l= (CU) (K-A)/ (K-UA).

Where,

* K= absorbance of control (K-Ko)
* A= absorbance of sample (A1-Ao)
* UA= absorbance of uric acid solution (UA1-UAo)
* CU= concentration of uric acid (mmol/l)

For each sample, two tests were made in order to estimate its AOA. The mean of these two readings was taken as the AOA level in that sample.

Chemicals

The following chemicals were used in the study, Sodium dihydrogen orthophosphate dihydrate and sodium benzoate (Thomas Baker, India), di sodium hydrogen phosphate (Sarabhai M. chemicals, India), ferrous ammonium sulphate (BDH, Glaxo laboratories Ltd., India). thiobarbituric acid (Sigma Chemicals Company, St. Louis, MO,USA),acetic acid glacial 100% and Ethylene diamine tetracetic acid disodium (Merck India Ltd. ,India), sodium hydroxide (Qualigens Fine Chemicals, Glaxo India Ltd., India), All chemicals were of analytic and all regents were prepared in double distilled water.

STATISTICAL ANALYSIS

The means with standard error (SE) of means for each group were determined Comparison between the different groups was made by using Students't' test.

RESULTS

The present study was undertaken to evaluate the redox status of patients with bronchial asthma, by monitoring the total antioxidant activity of saliva and serum as a biomarker. Serum sample from all control and asthmatic subjects were not available for the study due to lack of patients' compliance. Attempts were also made to assess the effect of drug treatment on clinical and redox status of a group of patients different from those included in the un-treated patients' group. The AOA of saliva and serum from the patients was compared with the control values.

AOA in control subjects

The average constitutive level of AOA in saliva and serum of controls (n=25) was 1.48 $\pm$ 0.05 mmol/1 and 1.67 $\pm$ 0.08 mmol/l (Table I, II), respectively. The AOA of serum was significantly higher than that of saliva. There were 13 males and 12 females in this group. In male, the total AOA in saliva and serum was found to be 1.4 $\pm$ 0.07 mmol/l and 1.46 $\pm$ 0.04 mmol/l, respectively (Table III). In female total AOA in saliva and serum were 1.56$\pm$ 0.07 mmol/l and 1.61$\pm$0.08 mmol/l, respectively.

AOA in untreated asthma patients

Intermittent asthma patients

The mean salivary AOA of in the group of patients (n=11) was 1.25 $\pm$ 0.10 mmol/l, which was found to be significantly lower ($p<0.05$) than that of control group (Table I). The mean AOA of serum from intermittent asthma (n=6) was estimated to be 1.53

±0.03 mmol/l, which was significantly higher ($p<0.05$) than that of saliva. There was a slight but significant decrease in the serum AOA of these patients when compared to control saliva levels (Table II).

Mild persistent asthma patients

This group comprised 8 cases. The average AOA of saliva was 0.86± 0.14 mmol/l, which was found to be significantly lower ($p<0.01$) than that of controls (Table I).

Out of these 8 cases, 4 patients were examined for serum AOA. The mean AOA was 1.37± 0.08 mmol/l, which was found to be significantly higher ($p<0.05$) than that of saliva. The level of serum AOA in this group of patients was significantly lower than that of control subjects (Table II).

Moderate persistent asthma patients

12 patients having moderate persistent asthma were analyzed for salivary AOA and their mean AOA was found to be 0.57 ± 0.04 mmol/l. The salivary AOA was considerably lower ($p<0.001$) than that recorded in the control group (Table 1). In line with the decreasing pattern of salivary AOA , the serum AOA in the group of patients was reduced maximally compared to other groups (Table II).

Table I. Antioxidant activity (AOA)in the saliva of bronchial asthma patients in different groups.

Groups		N	AOA± SE (mmol/l)	Percent Difference
	Control	25	1.48±0.05	
Asthma Patients	Intermittent	11	1.25±0.10*	15.5
	Mild persistent	08	0.86±0.14**	41.8
	Moderate persistent	12	0.57±0.04***	61.4

Values are the average ± SE. *p<0.05; **p<0.05; ***p<0.001 as compared to control group.

Table II. Antioxidant activity (AOA)in the serum of bronchial asthma patients in different groups.

Groups		N	AOA± SE (mmol/l)	Percent Difference
	Control	25	1.67±0.08	
Asthma Patients	Intermittent	11	1.53±0.03*	15.5
	Mild persistent	08	1.37±0.08**	41.8
	Moderate persistent	12	1.15±0.11***	61.4

Values are the average ± SE. *p<0.05; **p<0.05; ***p<0.001 as compared to control group.

Table III. Sex-Related Changes in Saliva and Serum of Controls.

Groups	N	AOA± SE (mmol/l)	
		Saliva	Serum
Males	13	1.40±0.07	1.46±0.04
Females	12	1.56±0.07*	1.61±0.08**

Values are the average ±SE. *p>0.05; **p>0.05 as compared to males.

AOA in follow-up asthma patients

Intermittent asthma patients

Four patients taking medication were follow-up minimum for a period of 3 months to examine improvement in their clinical and redox status. The pre-treatment values of AOA in saliva and serum were 1.23 ± 0.09 mmol/l and 1.52 ± 0.07 mmol/l (Table IV). After drug treatment , the activities were found to be 1.33 ± 0.09 mmol/l and 1.58 ± 0.07 mmol/l , respectively, but the difference between the respective pre- and post treatment AOA values was not statistically significant ($p>0.05$).

Mild persistent asthma patients

This group comparised 4 patients who had been taking drugs for 3 months. The pre-treatment values of AOA in saliva and serum were 0.74 ± 0.05 mmol/l and 1.30 ± 0.26 mmol/l (Table V). After treatment, the AO activities were found to be 0.93 ± 0.03 mmol/1 and 1.40 ± 0.22 mmol/1, respectively. The increase in AOA of saliva following drug treatment was significant ($P<0.01$),. There was no significant change in the serum AOA under these conditions.

Moderate persistent asthma patients

Four patients suffering from moderate persistent asthma and taking treatment for 3 months were followed to assess their post treatment clinical and redox status. The pretreatment values of AOA in saliva and serum were 0.38 ±0.07 mmol/l and 1.16 ±0/08 mmol/l, respectively, and after drug treatment, the AOA restored to 0.62 ±0.06 mmol/l in saliva only (Table VI). The AOA of serum remained unaffected.

Table IV. AOA in the Pre-treatment and Post treatment Follow-up Group of patients with intermittent Asthma.

Groups		N	AOA± SE (mmol/l)	
			Saliva	Serum
Control		9	1.35±0.07	1.67±0.08
Asthma Patients	Pre-treatment	4	1.23±0.09	1.52±0.07
	Post-treatment	4	1.33±0.09	1.58±0.07

Values are the average ±SE.

Table V. AOA in the Pre-treatment and Post treatment Follow-up Group of patients with mild persistent Asthma.

Groups		N	AOA± SE (mmol/l)	
			Saliva	Serum
Control		9	1.35±0.07	1.67±0.08
Asthma Patients	Pre-treatment	4	0.74±0.05*	1.30±0.26
	Post-treatment	4	0.93±0.03**#	1.40±0.22

Values are the average ±SE. *p<0.01; **p<0.01 as compared to control group. #p<0.01 as compared to pre-treatment group

Table VI. AOA in the Pre-treatment and Post treatment Follow-up Group of patients with moderate persistent Asthma.

Groups		N	AOA± SE (mmol/l)	
			Saliva	Serum
Control		9	1.35±0.07	1.67±0.08
Asthma Patients	Pre-treatment	4	0.38±0.07*	1.16±0.08***
	Post-treatment	4	0.62±0.06**#	1.21±0.21

Values are the average ±SE. *p<0.01; **p<0.01; ***p<0.05 as compared to control group. #p<0.01 as compared to pre-treatment group

Correlation between Antioxidant Potential and Clinical Status of Patients with Bronchial Asthma

In the follow-up group of asthmatic patients AOA was measured in the saliva and serum of patients taking medication for 1-2 months and compared with the pretreatment values. A correlation between clinical improvement and redox status was also evaluated. The patients showed variable clinical improvement following drug treatment. The depressed saliva and serum AOA of intermittent asthma patients did not recover significantly after the drug treatment. However, more serious patients having mild

persistent and moderate persistent asthma responded well to drug treatment and there was substantial improvement in their salivary AOA and clinical status, as judged by the consultant physician. Remarkably, the serum AOA remained unaltered following the medication.

DISCUSSION

Bronchial asthma is a chronic, episodic, inflammatory condition of the airways characterized by recurrent episodes of respiratory symptoms, variable airflow obstruction and presence of airway hyper-reactivity. Several mediators including leukotrienes, prostaglandins and ROS have been implicated in the pathophysiology of bronchial asthma. Oxygen free radicals are known to play an important role in cell degeneration in tissues under oxidative stress and have also been implicated in the causation of bronchial asthma. High levels of ROS and oxidatively modified proteins have been measured in the airways of patients with asthma (Nadeem *et al.*,2003). Another reactive species , nitric oxide (NO), has been found to be increased in airway of asthmatic patients (David *et al.*,2003). Kavuru *et.al.*, (2005) have reported abnormalities of NOS -I and NOS-II genotype and their expression in patients of bronchial asthma . An oxidized product of arachidonic acid, 8- isoprostane, which is PGF2u analogue,is increased 3-4 fold in patients with asthma compared to that observed in healthy subjects (Kavuru *et al.*,2005) .Further support to the above hypothesis comes from the fact that many antioxidants have been found to have beneficial effects in patients suffering from bronchial asthma (Rahman, 2002; Caramori and Papi, 2004; Romieu, 2004).

AOA in different body fluids has been measured by various techniques. All of these techniques use the measurement of either one or the other parameter and deduce the results in terms of total AOA. We have, instead, measured the total AOA in the body fluids, which gives more accurate information regarding the total anti-oxidant potential of that sample. The results of the present study exhibited a difference between the AOA levels in serum and salivary controls. The AOA levels in serum were found to be significantly higher than in saliva in the healthy persons. Our results are in accordance with those of Koracevic *et al.*, (2001) who have also reported a higher value of the serum antioxidant activity as compared to the salivary antioxidant activity in human subjects. However, Ziobro and Bartosz (2003) have reported that the levels of AOA are higher in saliva as compared to those of serum in human subjects. The reason for these contradictory results is not clear and may, in part be attributed to the measurement techniques.

CONCLUSION

Our study demonstrates a definite relationship between the total AOA and the disease process. It was established that there is an increase in the level of oxidants as deduced from the decreased AOA levels in patients of bronchial asthma. The current study suggests (a) role of oxidative stress in the pathogenesis of bronchial asthma and (b) salivary and serum AOA as a potential biomarker for oxidative stress. AOA in serum and saliva is a good indicator for assessing the severity and progress of bronchial asthma.

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