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Evaluation of Serum Level of Neutrophil Elastase, Superoxide Dismutase and Nitric Oxide in COPD Patients and its Correlation with Lung Function Test

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Authors' contributions

Author SAA supervised the study, author RSP concept, designed, conducted the study, review of literature, wrote the manuscript and managed the sample collection. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: Chronic Obstructive Pulmonary Disease (COPD) is the fourth leading cause of death worldwide. Its prevalence is increasing in the world. Tobacco smoking is the major risk factor for COPD. Oxidant-antioxidant and protease – anti-protease imbalance is the major hallmarks for the pathogenesis of COPD. The present study was planned to assess the correlation between markers of airflow obstruction with the serum level of neutrophil elastase, nitric oxide and superoxide dismutase in COPD patients.
Study Design: Case Control Study.

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Place and Duration of Study: Department of Biochemistry, B. J. Govt. Medical College, Pune [Maharashtra]. The study period was in between Feb.2012 to Dec. 2013.
Methodology: Study comprised of 60 stable COPD patients and 60 healthy controls. COPD patients were selected as per the GOLD (Global Initiative for Obstructive Lung Disease) criteria with of aged between 40 to 75 yrs. Each subject undergone through the pulmonary function test by spirometry prior to enter in the study and predicted values of FEV1, FVC and FEV1/FVC were measured. Serum level of neutrophil elastase (NE) was analyzed using commercial available ELISA kits while serum level of nitric oxide and superoxide dismutase were measured by spectrophotometric methods. Statistical analysis was done by using SPSS software 17 version.
Results: In our study we observed significantly increased levels of serum neutrophil elastase and nitric oxide and decreased level of enzymatic antioxidant superoxide dismutase (SOD) in COPD patients as compared to healthy controls. We found significant strong inverse correlation between neutrophil elastase (r=-0.604, *P*<0.0001) and nitric oxide (r=-0.565, *P*<0.0001) with FEV1% predicted and positive correlation between superoxide dismutase and FEV1% predicted (r=+0.394, *P*<0.001) in COPD patients.
Conclusion: The present study demonstrates that the level of nitric oxide, superoxide dismutase and neutrophil elastase in serum might have played role in oxidative stress and inflammation in COPD patients. Hence, it can be concluded that the measurement of these biomarkers in serum may provide a good approach to assess the severity of the disease in COPD patients.

Keywords: COPD- Chronic Obstructive Pulmonary Disease; ELISA- Enzyme Linked Immunosorbant Assay, FEV1% predicted- Forced Expiratory Volume in one second % predicted, FVC- Forced Vital Capacity; GOLD-Global Initiative of Obstructive Lung Disease, NE- Neutrophil Elastase, NO• - Nitric oxide; SOD- Superoxide Dismutase.

1. INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is a major public health problem in India. It is projected to rank third leading cause of deaths globally by the year 2030 [1]. COPD is a chronic inflammatory disease of the lungs that is characterized by progressive and irreversible airflow limitation [2]. Tobacco smoking is the most common risk factor for COPD. One puff of tobacco smoke contains 10^{17} free radicals. These free radicals increase numbers of neutrophils in the airways. It activates neutrophils. These activated neutrophils inactivate natural antiprotease system of lung such as α1-antitrypsin. This inactivation enhances the progression of emphysema in lung [3].

Neutrophil elastase is a powerful protease released by neutrophils. This enzyme is responsible for extracellular matrix degradation as well as it is involved in hypersecretion of mucus in both large and small airways in COPD. Therefore determination of the serum level of neutrophil elastase is implicated in the pathogenesis of lung disease [4].

Cigarette smoke itself contains numerous oxidants. These oxidants are responsible for the inhibition of α1-antitrypsin. In addition to this release of neutrophil elastase and other protease from neutrophils causes increased total protease activity. This results in protease - anti-protease imbalance in COPD. The increase in oxidant and protease-antiprotease imbalance contributes to the development of COPD [5].

Oxidant present in cigarette smoke causes activation of neutrophils and macrophages which releases oxidants superoxide and nitric oxide ($NO^{\bullet}$) which contribute to oxidative burden [6]. $NO^{\bullet}$ is a short lived free radical. Excess of $NO^{\bullet}$ can exert cytotoxic effects by producing nitrosative stress [7,8]. So it was of interest to study the level of $NO^{\bullet}$ in serum of the COPD patients.

Lung tissue contains an enzyme superoxide dismutase [9]. This enzyme protects the lung tissue against oxidative stress produced by superoxide radicals [10]. Enzyme SOD is found in alveolar epithelium, alveolar type II epithelial cells, alveolar macrophage, bronchial epithelium and in endothelial cells of lungs [11].

As all these parameters are involved in one way or other in the pathogenesis of COPD. The present study was undertaken to evaluate the levels of neutrophil elastase, nitric oxide and superoxide dismutase in COPD patients and to find out the correlation of these parameters with FEV1% predicted in COPD patients.

2. MATERIAL AND METHODS

2.1Selection of Cases and Control

This case control study was carried out in Department of Biochemistry and Department of Pulmonary Medicine, B. J. Government Medical College and Sassoon General Hospital, Pune [Maharashtra], India. The study period was in between Feb.2012 to Dec. 2013. The study was approved by Institutional ethical committee [Ref. no. BJMC/ IEC/Pharmac/D1210133-35]. Informed consent was obtained from each subject prior to the study. Study comprised of two groups Group I and Group II.

a) Group I/COPD patient group: Group I consist of 60 patients with stable COPD of aged in between 40-75 yrs. All COPD patients were undergone through lung function test by spirometry prior to enter in the study. COPD was diagnosed according to the criteria recommended by (GOLD) Global Initiative for Obstructive Lung Disease. Chronic airflow obstruction was defined as forced expiratory volume in one second/forced vital capacity (FEV1/FVC) <70 after using an inhaled bronchodilator. Bronchodilators reversibility test showed an increase in FEV1 <12% above the pre-bronchodilator FEV1 after inhaled of 400µg of salbutamol.
b) Group II /Control group: Control group consisted of 60 age and sex-matched healthy volunteers with no history of COPD, confirmed by spirometric tests performed during medical examination prior to the study.

2.1.1 Exclusion criteria for control and COPD patients group

Patients who were suffering from or who were known to have tuberculosis, pneumonia, asthma, bronchiectesis, lung cancer, interstitial lung diseases, respiratory failure, cardiac failure, diabetes mellitus, hepatic disease, renal disease and who had any recent surgical intervention and who were unable to performed lung function test were excluded from our COPD patients group. Healthy individual with any past history of lung/respiratory disease or with abnormal lung function test were excluded from Control group.

2.2 Collection of Blood Samples

Under aseptic condition and with prior consent of the subject, 5ml of blood was drawn from large peripheral vein, after overnight fasting. The blood was collected in a plain bulb and allowed to clot for 1hr. Serum was separated by centrifugation at 3000rpm for 10min. at room temperature, separated serum was aliquot and stored at -80ºC until the analysis and was used for the estimation of serum neutrophil elastase (NE), nitric oxide ($NO^{\bullet}$) and superoxide dismutase (SOD).

2.3 Estimation of Serum Neutrophil Elastase (NE)

Serum Neutrophil Elastase was determined by ELISA method (Welldone Biotech., EIAab Science Co. Ltd, U.S.A). This method is based on the principle that the microtiter plate provided in the kit has been pre-coated with antibody specific to neutrophil elastase. Standards or samples are then added to the appropriate microtiter plate wells with biotin–conjugated polyclonal antibody preparation specific for Neutrophil elastase (NE) and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each well and incubated. Then a TMB substrate solution is added to each well. Only those wells that contain Neutrophil elastase biotin conjugated antibody and enzyme-conjugated avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometricaly at wavelength of 450nm. The concentration of Neutrophil elastase in the samples is then determined by compared the O.D. of the samples to the standard curve. It was expressed as pg/ml.

2.4 Estimation of Serum Nitric Oxide ($NO^{\bullet}$)

Serum nitric oxide was determined by Cortas and Wakid method (1990). This method based on the principle that serum was deproteinized first and nitrate was reduced to nitrite by cadmium granule reduction method. Which then coupling with N-napthyethylenediamine to give pink colour complex which is measure at 540nm on spectrophotometrically. $NO^{\bullet}$ was determined by use of standard curve and it was expressed as µmol/L [12].

2.5 Estimation of Serum Extracellular Superoxide Dismutase (SOD)

Serum extracellular superoxide dismutase (SOD) was determined by Marklund and Marklund method (1974). This method is based on the

principle that superoxide anion is involved in the auto-oxidation of pyrogallol at alkaline pH (8.5). The superoxide dismutase (SOD) an enzyme from the serum inhibits auto oxidation of pyrogallol, which can be determined as an increase in absorbance at 420nm. SOD was expressed as U/L [13].

2.6 Smoking History

Smoking index was used to quantify smoking exposure among the study subjects. Smoking Index is defined as number of cigarettes or biddies smoked per day multiplied by the total duration of smoking in years. It is a better index to quantify smoking in Indian context as compared to pack years.

2.7 Pulmonary Function Test

Pulmonary Function test was done by using Spirometer. Measurement of Forced Vital Capacity and Forced Expiratory Volume was done in First seconds. The FEV1/FVC is calculated using the maximum FEV1 and FVC from the technically acceptable, though not from the same curves. The Data was obtained from the printer, attached to spirometer.

2.8 Statistical Analysis

Statistical analysis was performed by using Statistical Package for Social Sciences (SPSS) 17 version software. The data for biochemical analysis was expressed as mean ± SD. The statistical significance of the results was analyzed by using unpaired "t" test. *P* value of <0.05 was considered statistically significant. Pearson correlation was used to analyze the relationship between biochemical and lung function parameters.

3. RESULTS

The demographic characteristics of COPD patients and controls are depicted in Table 1. The groups were well matched with respect to age and sex. By definition of GOLD (Global Initiative for Obstructive Lung Disease), lung function parameters were normal in healthy controls and significantly decreased in patients with COPD. Severity of the disease is increased with the advancement of the age. In the present study only 9 female patients were included, as the disease prevalence is greater in male than female (Table 1).

Significantly high levels ($p<0.001$) of serum protease neutrophil elastase (NE) and oxidant serum nitric oxide ($NO^{\bullet}$) were observed in COPD patients as compared to healthy controls. The mean serum level of superoxide dismutase (SOD) ($P<0.001$) was lower in patients with COPD than controls (Table 2).

Table 3 showed the correlation analysis of the studied parameters neutrophil elastase (NE), nitric oxide ($NO^{\bullet}$) and superoxide dismutase (SOD) with pulmonary function tests markers , namely FEV1% predicted, FVC % predicted and FEV1/FVC % ratio in patients with COPD and healthy controls. A significant negative correlation was observed between serum neutrophil elastase (NE) and serum nitric oxide ($NO^{\bullet}$) with FEV1% predicted ($r = -0.604$, $P<0.0001$ and $r = -0.565$, $P<0.0001$) and FVC % predicted ($r = -0.345$, $P = 0.006$ and -0.329, $P<0.01$) in COPD patients respectively. Whereas there was no significant correlation of serum level of neutrophil elastase (NE) and nitric oxide ($NO^{\bullet}$) with FEV1/FVC % ratio ($r = -0.105$, $P>0.05$ and $r = 0.073$, $P>0.05$) in patients with COPD respectively. In present study we observed significant positive correlation between serum level of superoxide dismutase (SOD) and FEV1% predicted ($r = +0.394$, $P<0.001$) in COPD patients. We do not found significant correlation of superoxide dismutase with FVC% predicted and FEV1/FVC% ratio in COPD patients. No significant correlation was observed between serum level of neutrophil elastase (NE), nitric oxide ($NO^{\bullet}$) and superoxide dismutase (SOD) with the lung function parameters: FEV1% predicted, FVC% predicted and FEV1/FVC% ratio in control groups (Table 3).

4. DISCUSSION

Tobacco smoking is the main risk factor in Chronic Obstructive Pulmonary Disease (COPD). Tobacco smoke contains a large numbers of oxidants such as nicotine, semiquinone, carbon monoxide, nitrogen oxide and some toxic carcinogenic compounds. In India smoking habit is mostly seen in males than females. Hence there was a predominance of male patients in COPD patients. Our data suggest that the severity in the COPD patients increases with the advancement of age. In our study we observed reduction in pulmonary function tests namely: FEV1% predicted, FVC % predicted and FEV1/FVC % ratio in COPD patients as compared to healthy controls ($P<0.001$, Table 1). This is supported by the study of Garcia JM, et

al. [14]. They reported that mean values of FEV1% predicted were significantly lower in smokers with airflow obstruction than in those without obstruction [14]. This reduction in lung function might be due to persistent exposure tobacco smoke, which results in infiltration of inflammatory cells, mucus hypersecretion, thickening of airway wall, narrowing of the lumen of small airways as well as destruction of alveolar structure. This leads to increase in airflow obstruction in COPD patients [15].

The cigarette smoke not only causes airflow obstruction but it is also responsible for altering the activity of neutrophil elastase, superoxide dismutase and levels of nitric oxide. In our study we observed the increased levels of serum neutrophil elastase (NE) in COPD patients as compared to healthy controls (P<0.001, Table 2). The increased levels of neutrophil elastase in COPD might be due to deleterious effects of free radicals, toxic agents and pollutants on lung tissues [16].

This causes increased number of neutrophils in lungs which secretes neutrophil elastase. Neutrophil elastase increases its proteolytic activity leading to the destruction of pulmonary tissue and the elastic fibers [17]. In addition to this, neutrophil elastase increases oxidative stress in lung cells [18]. Oxidants released from activated neutrophils inactivate antiprotease α1-antityrpsin [19], which controls the activity of protease neutrophil elastase.

Table 1. Demographic Characteristic of patients with COPD and control groups

Variables	Healthy Controls (n=60)	COPD Patients (n=60)
Age	54.93±9.01	63.18±8.32
Sex (M/F)	49/11	51/9
Smoking History (pack years)	---------	53.56±8.26
FEV1 % predicted	109.71±14.90	47.4±12.27*
FVC % predicted	110.97±9.7	55.93±12.14*
FEV1/FVC% predicted	103.58±10.40	63.11±6.30*

Values are expressed as Mean± S.D., FEV1 % predicted: - forced expiratory volume in one second % predicted, FVC% predicted:- forced vital capacity % predicted.
** This indicates P< 0.001: statistically significant when compared to healthy controls*

Table 2. Serum level of Neutrophil Elastase, Nitric Oxide and SOD in COPD patients and healthy controls

Biochemical Parameters	Healthy Controls (n=60)	COPD Patients (n=60)
Serum Neutrophil Elastase(NE)(pg/ml)	287.07±70.63	363.26±96.85*
Serum Nitric Oxide($NO^{\bullet}$) (µmol/L)	76.47±7.67	145.23±37.44*
Serum extracellular SOD(U/L)	3.40±1.29	2.10±0.96*

Values are expressed as Mean± S.D., SOD- superoxide dismutase.
** This indicates P<0.001: statistically significant when compared to healthy controls*

Table 3. Correlation of Neutrophil Elastase (NE), Nitric Oxide ($NO^{\bullet}$) and Superoxide Dismutase(SOD) with Pulmonary function tests markers(FEV1% Predicted, FVC %Predicted and FEV1/FVC % ratio) in COPD patients and Controls

Correlation	Healthy Controls (n=60)		COPD Patients (n=60)	
	r values	P values	r values	P values
Neutrophil Elastase-FEV1% Predicted	0.147	0.262	-0.604	0.000
Neutrophil Elastase-FVC % Predicted	0.083	0.528	-0.345	0.006
Neutrophil Elastase-FEV1/ FVC% ratio	0.090	0.494	-0.105	0.426
NO- FEV1% Predicted	-0.001	0.993	-0.565	0.000
NO - FVC% Predicted	0.052	0.693	-0.329	0.010
NO -FEV1/FVC% ratio	-0.038	0.773	0.073	0.579
SOD-FEV1% Predicted	0.006	0.963	0.394	0.001
SOD-FVC% Predicted	-0.096	0.465	0.107	0.415
SOD-FEV1/FVC % ratio	0.027	0.837	0.069	0.600

This results in increased activity of neutrophil elastase in COPD patients. Toxic effect of this neutrophil elastase is mostly enhanced by increased oxidative stress leading to destruction of elastin and loss of elasticity of the lung alveoli [20]. This is in accordance with study of Vaguliene N et al. who found increased level of serum neutrophil elastase in COPD patients as compared to the healthy controls [21]. There was a strong inverse correlation observed between neutrophil elastase and FEV1% predicted (r = -0.604, P<0.001, Table 3) in COPD patients. This indicates that as the air flow decreases there is increase in the activity of neutrophil elastase. This is supported by the work of Bizeto L et al. who found negative correlation between serum neutrophil elastase (NE) and FEV1 in COPD patients [22].

Reactive nitrogen species (RNS) also play an important role in inflammation. The nitric oxide ($NO^{\bullet}$) plays an important role in the physiological regulation of airways and is implicated in the pathophysiology of airway disease especially Chronic Obstructive Lung Disease (COPD) [23]. In current study we observed increased level of serum nitric oxide ($NO^{\bullet}$) in COPD patients as compared it with healthy controls (P<0.001, Table 2). An inducible form of the nitric oxide synthase enzyme (iNOS) is expressed in inflammatory diseases of airway such as COPD [24,25]. The increased level of nitric oxide in serum might be due to a pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) increase the expression of iNOS in airway epithelial cells it leads to increase level of $NO^{\bullet}$ in COPD patients [26]. The raised serum level of nitric oxide ($NO^{\bullet}$) has ability to react with superoxide to generate powerful oxidant peroxynitrite ($ONOO^{-}$). Peroxynitrite causes lungs cell death [27]. We postulate from our results that increased $NO^{\bullet}$ levels observed might be responsible for forming peroxynitrite. Thus our results are in agreement with the study of Ahmad A et al. [28] who found increased level of exhaled nitric oxide ($NO^{\bullet}$) in COPD patients.

In our study we found inverse correlation between serum nitric oxide with FEV1 % predicted (r = -0.565, *P*<0.001, Table 3) and FVC% predicted (r = -0.329, *P*=0.01, Table 3) in COPD patients respectively. Similar results were reported by the study of Maziak W et al. who found inverse correlation between FEV1% predicted and exhaled $NO^{\bullet}$ in COPD patients[29]. Mccurdy MR et al. [30] and Ahmad A et al. [28] found increased nitric oxide ($NO^{\cdot}$) production with increased airway obstruction in severe COPD

patients. In contrast to our study, Doruk S, et al. did not found any correlation between exhaled NO levels and pulmonary function test in COPD patients [31].

Oxidative stress is produced by the components of cigarette smoke. These toxic components inhibit the activity of superoxide dismutase in COPD patients. This inhibition is reflected by the observed significant decreased in the SOD activity in present study as compared to healthy controls (P<0.001, Table 2). Similar findings were reported by studies of Shetekar N. et al. [32], Rai RR, et al. [33], Kirkil G, et al. [34]. Yun LL et al. [35] and Wassem AM, et al. [36] also reported decreased levels of serum SOD in stable and acute exacerbated COPD patients. In study conducted by Daga MK, et al. who found that the serum SOD levels were lower in smoker with COPD, compared to controls [37]. Similarly, Kondo T, et al. found that smoking in elderly men reduces antioxidants in alveolar macrophages with increase in the level of oxygen radicals species. He observed decrease in the levels of SOD in alveolar macrophages from elderly chronic smokers [38]. Jain A et al. studied antioxidant status in cigarettes and biddy smokers. The authors found in their study lower level of erythrocyte SOD in biddy smokers than cigarette smokers and healthy controls [39]. In the study of Gavali Y, et al. the author found decreased level of serum SOD in smoker COPD patients as compared to healthy controls [40].

We found a significant positive correlation between superoxide dismutase (SOD) and FEV1% predicted (r = +0.394), P<0.001, Table 3) in COPD patients. Thus SOD activity is directly related with the decrease in airway obstruction. Our results are supported by study of Kluchova et al who found the positive correlation between serum SOD and severity of COPD [41]. In the present study no correlation was found between SOD with FVC% predicted (r =+0.107, P>0.05) and FEV1/FVC% ratio (r = +0.069, P>0.05) in COPD patients (Table 3).

5. CONCLUSION

Smoking causes inflammation of lung tissue (increase in neutrophil elastase) and also responsible for producing oxidative stress which results in imbalance between oxidant and antioxidants present in lung tissue. Decline in

lung function (FEV1% predicted) is a result of this imbalance and inflammation. Hence it can be concluding that measurement of these (neutrophil elastase, superoxide dismutase and nitric oxide) biomarkers in serum may provide a good approach to assess severity of the disease in COPD patients.

CONSENT

Declare that written informed consent was obtained from the patients.

ETHICAL APPROVAL

The study protocol was examined and authorized by the Medical Research and Ethics Committee of the B. J. Govt. Medical College and Sassoon General Hospital in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki on biomedical research on human subjects. Obtained necessary Institutional ethical approval [Ref. No. BJMC/ IEC/ Pharmac/ D1210133-35]

STRENGTH AND LIMITATIONS OF THE STUDY

Strength: We are the first here to report the correlation between serum level of Neutrophil Elastase and Nitric Oxide with the marker of airflow obstruction (FEV1% predicted) in COPD patients.

Limitations: The present study was carried out with a relatively small number of subjects. It would be better to perform this study with a greater number of subjects in order to determine the difference between the analysis of biochemical and lung function parameters of COPD patients and control groups more clearly.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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