

Original Research Article

Evaluation of Serum Level of Neutrophil Elastase, Superoxide Dismutase and Nitric Oxide In COPD Patients: Correlation With Lung Function Test

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-antiprotease imbalance is the major hallmarks for the pathogenesis of COPD. The present study was planned to assess the correlation between serum neutrophil elastase, nitric oxide and superoxide dismutase with the markers of airflow obstruction in COPD patients.

Study design: Case Control Study.

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 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. No significant correlation was found in current study between neutrophil elastase, nitric oxide and superoxide dismutase with FEV₁%predicted, FVC % predicted and FEV₁/FVC ratio in control group.

Conclusion: : The finding of this study suggest that protease (neutrophil elastase), oxidant (nitric oxide) were increased and enzymatic antioxidant superoxide dismutase was decreased with decline of lung function parameter (FEV₁% predicted) in COPD patients. This results indicates that there is definite protease – antiprotease, oxidant-antioxidant imbalance in COPD patients with decline of lung function.

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.

17 1. INTRODUCTION

18

19 Chronic Obstructive Pulmonary Disease (COPD) is a major public health problem in India
20 and is projected to rank third leading cause of deaths globally by year 2030 [1]. COPD is a
21 chronic inflammatory disease of the lungs, that is characterized by progressive and
22 irreversible airflow limitation [2]. Tobacco smoking is the most common risk factor for
23 COPD. One puff of tobacco smoke contains 10^{17} free radicals. These free radicals increases
24 numbers of neutrophils in the airways, its activate neutrophils and this activated neutrophil
25 inactivate natural antiprotease system of lung , the most importantly, of them being $\alpha 1$
26 Antitrypsin. This inactivation enhances the progression of emphysema in lung[3].

27

28 Over the recent years, proteolysis has been recognised as a special form of physiological
29 regulation. Neutrophils contains high levels of proteolytic enzyme neutrophil elastase. This
30 enzymes are responsible for extracellular matrix degradation as well as it involving in
31 hypersecretion of mucus in both large and small airways in COPD. Therefore
32 determination of the serum level of neutrophil elastase is implicated in the pathogenesis of
33 lung disease[4].

34

35 Increase of oxidants, a decrease in antioxidants status and gene polymorphism may all
36 contribute to the development of COPD through various mechanisms such as inactivation of
37 antiproteases , alveolar epithelial injury and increased sequestration of neutrophils in the
38 pulmonary vasculatures[5].

39

40 $\text{NO}^\cdot$ is a short -lived, free radical [6]. $\text{NO}^\cdot$ is a marker of inflammation and nitrosative stress.
41 $\text{NO}^\cdot$ produced in human cells by three types of isoenzymes commonly known as NO
42 synthases (NOS) i.e. eNOS, nNOS and iNOS. This three isoforms of NOS catalyze the
43 conversion of L-arginine to $\text{NO}^\cdot$ and L- Citrulline [7]. $\text{NO}^\cdot$ has a dual role. Nitric oxide is
44 involved in the activation of soluble guanylate cyclase which is responsible for signal
45 transduction and it has physiological role. However excess of $\text{NO}^\cdot$ can exert cytotoxic effects.
46 eNOS and nNOS are constitutive and release little bit amount of $\text{NO}^\cdot$ for short periods after
47 receptor stimulation. In contrast to that, iNOS is formed in presence of proinflammatory
48 stimuli and produce large amount of $\text{NO}^\cdot$ for continuous time periods is occurs in most
49 chronic inflammatory disease conditions [8].

50

51 Increased oxidative stress is the major hallmark for the pathogenesis of obstructive lung
52 disease such as chronic obstructive pulmonary disease(COPD) [9]. Reactive oxygen species
53 and reactive nitrogen species plays a central role in COPD. The first reactive oxygen species
54 produced in the reduction pathway of conversion of oxygen to water is superoxide anion.
55 This superoxide anion takes part in the generation of toxic metabolites, most commonly
56 hydrogen peroxide, hydroxyl radical and peroxynitrite[10]. Lung tissue protected against
57 these toxic oxidants by a specific antioxidant mechanism among them the superoxide
58 dismutase (SOD) are the only ones antioxidant enzymes which converts superoxide radicals
59 to hydrogen peroxide[9]. SOD enzymes play an important role in protecting against the
60 progression of oxidant related lung diseases. SOD enzymes found in alveolar epithelium,
61 alveolar type II epithelial cells , alveolar macrophage, bronchial epithelium and in endothelial
62 cells of lungs[11].

63

64 However , presently only few studies are available that have evaluated the levels of serum
65 neutrophil elastase , nitric oxide and SOD in COPD patients. Therefore we aimed to
66 investigate the correlation between markers of airflow obstruction (FEV1 % predicted) with
67 the serum level of NE, $\text{NO}^\cdot$ and SOD in COPD patients.

68

2. MATERIAL AND METHODS

2.1 Selection 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. Bronchodilator reversibility test showed an increase in FEV1 <12% above the prebronchodilator 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.

2.2.1 Exclusion criteria for Control and COPD patients group

Patients who were suffering from or who were known to have tuberculosis, pneumonia, asthma, bronchiectasis, 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 1 hr. Serum was separated by centrifugation at 3000 rpm for 10 min. at room temperature, separated serum was aliquot and stored at -80°C until the analysis and was used for the estimation of serum neutrophil elastase (NE), nitric oxide (NO[•]) 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

116 TMB substrate solution is added to each well. Only those wells that contains Neutrophil
117 elastase biotin conjugated antibody and enzyme-conjugated Avidin will exhibit a change in
118 color . The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution
119 and the color change is measured spectrophotometrically at wavelength of 450nm. The
120 concentration of Neutrophil elastase in the samples is then determined by compared the
121 O.D. of the samples to the standard curve. It was expressed as pg/ml .

122

123 **2.4 Estimation of Serum Nitric Oxide (NO[•])**

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

129

130 **2.5 Estimation of Superoxide Dismutase(SOD)**

131 Serum superoxide dismutase (SOD) was determined by Markund and Markund method
132 (1974).This method is based on the principle that superoxide anion is involved in the auto-
133 oxidation of pyrogallol at alkaline pH (8.5). The superoxide dismutase (SOD) inhibits auto
134 oxidation of pyrogallol , which can be determined as an increase in absorbance at 420nm.
135 SOD was expressed as U/L [13].

136

137 **2.6 Pulmonary Function Test**

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

142

143 **2.7 Statistical analysis**

144 Statistical analysis was performed by using Statistical Package for Social Sciences (SPSS)
145 17 version software. The data for biochemical analysis was expressed as mean $\pm$ SD. The
146 statistical significance of the results was analyzed by using unpaired "t" test. *P* value of
147 <0.05 was considered statistically significant. Pearson correlation was used to analyze the
148 relationship between biochemical and lung function parameters.

149

150 **3. RESULTS**

151

152 The demographic characteristics of COPD patients and controls are depicted in Table no.1.
153 The groups were wellmatched with respect to age,sex and smoking history. By definition of
154 GOLD (Global Initiative for Obstructive Lung Disease), lung function parameters were
155 normal in healthy controls and significantly decreased in patients with COPD (Table no.1).

156

157 **Table No.1. Demographic Characteristic of patients with COPD and Control groups.**

158

Variables	Healthy Controls (n=60)	COPD Patients (n=60)
Age	54.93 $\pm$ 9.01	63.18 $\pm$ 8.32
Sex (M/F)	49/11	51/9

Smoking History	-----	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

Results of the current study showed significant high levels ($p < 0.001$) of serum protease neutrophil elastase (NE) and oxidants serum nitric oxide (NO*) in cases with COPD as compared to healthy controls. The mean serum levels of superoxide dismutase (SOD) ($P < 0.001$) were lower in patients with COPD than controls (Table no.2).

Table No.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*) (µmol/L)	76.47±7.67	145.23±37.44*
Serum 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 no. 3 showed correlation analysis of between studied parameters neutrophil elastase (NE), nitric oxide (NO) 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. In our study we found significant negative correlation between serum neutrophil elastase (NE) and serum nitric oxide (NO) 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. In current study we do not found significant correlation between serum level of neutrophil elastase (NE) and nitric oxide (NO) 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 superoxide dismutase (SOD) and FEV1% predicted ($r = +0.394$, $P < 0.001$) in COPD patients. We do not found significant correlation between superoxide dismutase with FVC% predicted and FEV1/FVC% ratio in COPD patients. No significant correlation was observed between serum level of neutrophil elastase(NE) and nitric oxide (NO) and superoxide dismutase (SOD) with the lung function parameters: FEV1% predicted, FVC%predicted and FEV1/FVC% ratio in control groups(Table no.3).

Table No.3. Correlation of Neutrophil Elastase (NE), Nitric Oxide (NO) 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

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 our study we observed reduction in level of pulmonary function test parameters namely : FEV1% predicted, FVC % predicted and FEV1/FVC % ratio in COPD patients as compared to healthy controls ($P<0.001$, Table no.1). This is in accordance with the study of Garcia JM. et al. who found mean values of FEV1% predicted was significantly lower in smoker with airflow obstruction than those without obstruction [14]. This reduction in lung function is might be due to persistant exposure of tobacco smoke 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 of airflow obstruction in COPD patients[15].

The study of the level of neutrophil elastase (NE) enzymes is rapidly evolving field of increasing intrest due to their application in the process of connective tissue destruction in chronic lung disease patients. Neutrophil elastase plays a dual role. On the one hand neutrophil elastase may play a role in bacterial killing. On the other hand neutrophil elastase induces expression of several cytokines including IL-8 and TNF- α and initiate the process of inflammation in chronic lung disease[16].

In our study we observed increased the serum level of netrophil elastase (NE) in COPD patients when we compared it with healthy controls($P<0.001$, Table no.2).The increased level of neutrophil elastase in COPD might be due to deleterious effects of free radicals, toxic agents and pollutants on lung tissues causes increase numbers of neutrophil in lungs which secretes neutrophil elastase. Neutrophil elastase enzyme increases its proteolysis activity leading to the destruction of pulmonary tissues and the elastic fibre [17]. In addition to this, neutrophil elastase increases oxidative stress in lung cells [18]. Oxidants released from activated neutrophils it inactivates antiprotease α 1-antityrpsin [19]. Therefore the function of antiprotease alpha-1 antitrypsin to control activity of protease neutrophil elastase is hampered 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 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].

In our study we observed strong inverse correlation between neutrophil elastase and FEV1% predicted ($r = -0.604$, $P<0.001$, Table no.3) in COPD patients. Our results are consistence with study 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. There is increasing evidence that endogenous nitric oxide ($\text{NO}^{\cdot}$) play a 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 ($\text{NO}^{\cdot}$) in COPD patients when we compared it

with healthy controls ($P < 0.001$, Table no.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 is might be due to a proinflammatory cytokine tumor necrosis factor alpha (TNF- α) increase the expression of iNOS in airway epithelial cells it leads to increase level of NO $^{\cdot}$ in COPD patients [26]. The raised serum level of nitric oxide (NO $^{\cdot}$) has of its most studied effects is its ability to react with superoxide to generate powerful oxidant peroxynitrite (ONOO $^{\cdot}$), its causes lungs cell death [27]. Our study is in agreement with the study of Stanescu et al [28] who found increased level of nitric oxide (NO $^{\cdot}$) in COPD patients.

In our study we found inverse correlation between serum nitric oxide with FEV1 % predicted ($r = -0.565$, $P < 0.001$, Table no.3) and FVC% predicted ($r = -0.329$, $P = 0.01$, Table no.3) in COPD patients respectively. This is in accordance with the study of Maziak W et al. who found inverse correlation between FEV1% predicted and exhaled NO $^{\cdot}$ in COPD patients [29]. Keating et al [30] and Stanescu et al [28] found increase 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 NO levels and pulmonary function test in COPD patients [31].

Oxidative stress, occurs by increased production of reactive nitrogen species (RNS) especially NO $^{\cdot}$ and peroxynitrite (ONOO $^{\cdot}$) and decreased concentration of antioxidant enzymes superoxide dismutase (SOD) is now discovered to be nearly in all pathological conditions, especially in the inflammatory process [32,33]. These oxidants damages tissue either by oxidation of biomolecules (lipid peroxidation, DNA damage) or by alteration in transcription factors such as NF-KB, SPI or AP-1 [34]. Fore most among the lines of biological defenses are SOD [27]. In our study we found decreased level of serum SOD in COPD patients as compared to healthy controls ($P < 0.001$, Table no.2). This may be due to the alteration in activity of SOD enzyme in COPD patients. The impairment of serum SOD activity in COPD patients might be due to increased production of ROS (Reactive Oxygen Species) leading to increase body compensate to antioxidant enzymes. This is in accordance with studies of Shetekar N. et al. [35], Rai RR et al [36], Kirkil G. et al [37]. Yun LL et al. [38] and Wassem AM et al. [39] also reported decreased levels of serum SOD in stable and acute exacerbated COPD patients. In study conducted by Daga M K. et al who found that the serum SOD levels were lower in smoker with COPD, compared to controls [40]. 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 [41]. Jain A et al studied antioxidant status in cigarettes and bidi smokers he found in their study lower level of erythrocyte SOD in bidi smokers than cigarette smokers and healthy controls [42]. In the study of Gavali Y. et al who found decreased level of serum SOD in COPD patients as compared to healthy controls [43].

In our study we found a significant positive correlation between superoxide dismutase (SOD) with FEV1% predicted ($r = +0.394$), $P < 0.001$, Table no.3) in COPD patients. This is in accordance with the study by Kluchova et al who found the positive correlation between serum SOD and severity of COPD [44]. In the present study we do not found any correlation 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 no.3).

5. CONCLUSION

Finding of this study suggest that serum protease neutrophil elastase (NE) and oxidant nitric oxide (NO $^{\cdot}$) were increases and enzymatic antioxidant superoxide dismutase (SOD)

decreases with declined of lung function parameter (FEV1% predicted) in COPD patients. The clinical importance of the present results suggests that free radicals inhaled in of tobacco smoke are highly toxic, it impaired protease-antiprotease and oxidant-antioxidant balance with decline in respiratory capacity and increases risk of COPD patient to lung cancer.

CONSENT

Declare that written informed consent was obtained from the patient.

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 NE, NO and SOD 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 parameters of COPD patients and control groups more clearly.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

- 1.Koul PA. Chronic obstructive pulmonary disease: Indian guidelines and the road ahead. Lung India.2013;30,3,175-177.
- 2.Rovina N, Konstsonkon A, Koulouris NG. Inflammation and immune response in COPD: Where do we stand? Mediators of Inflammation. 2013; 13, 9
3. Saetta M, Turato G, Maestrelli P, Mapp CE, Fabbri LM. Cellular and structural bases of COPD. Am. J. Respir. Crit. Care Med. 2001; 163,1304-1309.
4. Thorley AJ,Tetley TD. Pulmonary epithelium, cigarette smoke and chronic obstructive pulmonary disease. Int. J. Chron. Obstruct. Pulmon. Dis. 2007;2,4,409-428

- 346 5. Oudijk EJD, Lammers JWJ, Korenderman L. Systemic inflammation in chronic obstructive
347 pulmonary disease. *Eur. Respir. J.* 2003;22,46,5S-13S.
- 348 6. Palmer RMJ, Ashton DS, Moncada S. Vascular endothelial cell synthesize nitric oxide from
349 L-arginine. *Nature*. 1988;333,664.
- 350 7. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease.
351 *Physiol. Rev.* 2007;87,315-424.
- 352 8. Barnes PJ, Bevis MG. Nitric oxide and lung disease. *Thorax* 1993;48,1034-43
- 353 9. Kinnula VL, Crapo JD. Superoxide dismutase in the lung and human lung disease. *Am. J.*
354 *Respir. Crit Care Med.* 2003;167,1600-19
- 355 10. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. Oxford :Clarendon
356 Press. 1996
- 357 11. Pietarinen RP, Lakari E, Raivio KO, Kinnula VL. Expression of antioxidant enzymes in
358 human inflammatory cells. *Am. J. Physiol. Cell Physiol.* 2000 ; 278,C118-C125
- 359 12. Cortas NK, Wakid W. Determination of inorganic nitrate in serum and urine by kinetic
360 cadmium – reduction method. *Clin. Chem.* 1990, 3618, 1440-1443.
- 361 13. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation
362 of pyrogallol and a convenient assay for superoxide dismutase *Eur. J. Biochem.* 1974; 47,
363 469-474. SOD
- 364 14. Garcia JM, Hernandez JR, Martinez MA, Sanchez AA, Marron MG, Monte C, Martinez J,
365 Escudero C. *Respiration.* 1996;63,199-204
- 366 15. Wright JL, Lawson LM, Pare PD, Wiggs BJ, Kennedy S, Hogg JC. Morphology of
367 peripheral airways in current smokers and ex-smokers. *Am. Rev. Respir. Dis.* 1983;127,474-
368 477
- 369 16. Greene CM, McElvaney NG. Protease and antiproteases in chronic neutrophilic lung
370 disease-relevance to drug discovery. *Br. J. Pharmacol.* 2009;158(4),1048-58
- 371 17. Vasilyeva EM, Zykova KA, Bakanov MI, Bogatyreva AO, Rvacheva AV, Beilina VB,
372 Kuznetsova TV, Simonova OI, Solovjeva YV. The system of neutrophil elastase and plasma
373 level of MMP7 in children with pulmonary arterial hypertension and chronic cor pulmonale.
374 *Intr. J. of Biomedicine.* 2013;3(4),269-73
- 375 18. Aoshiba K, Uasuda K, Yasui S, Tamaoki J, Nagai A. Serine protease increase oxidative
376 stress in lung cells. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2001; 281,L556-64
- 377 19. Boudier C, Bieth JG. Oxidized mucus proteinase inhibitor: a fairly potent neutrophil
378 elastase inhibitor. *Biochem J.* 1994 ; 303,61-8
- 379 20. Zeiher BG, Matsuoka S, Kawabata, Repin JE. Neutrophil elastase and acute lung injury:
380 prospects for sivelestate and other neutrophil elastase inhibitors as therapeutic. *Crit Care*
381 *Med.* 2002;30,S281-7
- 382 21. Vaguliene N, Lavinskiene S, Zemaitis M, Miliauskas S, Sakalauskas R. Neutrophil
383 elastase levels is higher in patients with lung cancer than chronic obstructive pulmonary
384 disease. *European Respiratory Journal.* 2011, 38,55,2793.
- 385 22. Bizeto L, Mazzolini AB, Ribeiro M, Stelmach R, Cukier A, Nunes MPT. Interrelationship
386 between serum and sputum inflammatory mediators in chronic obstructive pulmonary
387 disease. *Brazilian J. of Medi. and Biolo. Reseach.* 2008,41,193-198.
- 388 23. Barnes PJ, Kharitonov SA. Exhaled nitric oxide: a new lung function test. *Thorax.* 1996 ;
389 51,233-237
- 390 24. Barnes PJ. Nitric oxide and airway disease. *Ann. Med.* 1995;27,91-97
- 391 25. Nathan CF, Hibbs JB. Role of nitric oxide synthesis in macrophage antimicrobial activity.
392 *Curr. Opin. Immunol.* 1991;3,65-70
- 393 26. Robbins RA, Barnes PJ, Springall DL. Expression of inducible nitric oxide synthase in
394 human bronchial epithelial cells. *Biochem. Biophys. Res. Commun.* 1994;209-218

- 395 27. Radi R, Beckman JS, Bush KM. Peroxynitrite oxidation of sulfhydryls the cytotoxic
396 potential of superoxide and nitric oxide. *J. Biol. Chem.* 1991; 266,4244-4250
- 397 28. Stanescu D, Sanna A, Veriter C, Kostianev S, Calagni PG, Fabbri LM, Maestrelli P.
398 Airway obstruction, chronic expectoration and rapid decline of FEV1 in smokers are
399 associated with increased levels of sputum neutrophils. *Thorax.*1996; 51,267-271
- 400 29. Maziak W, Loukides S, Culpitt S, Sullivan P,Kharitonov SA, Barnes PJ. Exhaled nitric
401 oxide in chronic obstructive lung disease. *Am. J. Respir. Crit. Care Med.*1998;157,998-1002
- 402 30. Keating VM, Collins PD, Scott DM, Barnes PJ. Differences in interleukin-8 and tumor
403 necrosis factor- α in induced sputum from patients with chronic obstructive pulmonary
404 disease or asthma. *Am. J. Respir.Crit.Care Med.*1996;153,530-534
- 405 31. Doruk S, Ozyurt H, Inonu H, Erkorkmaz U, Saylan O, Seyfikli Z. Oxidative status in the
406 lungs associated with tobacco smoke exposure. *Clin Chem Lab Med.* 2011; 49, 2007-12].
- 407 32. Mccord JM. Oxygen derived radicala : A link between reperfusion injury and
408 inflammation. *Fed. Proc.*1987;46,2402-2406
- 409 33. Mccord JM. Superoxide radical: controversies,contradictions and paradoxes. *Proc. Soc.*
410 *Expla. Med. Biol.* 1995;1-6
- 411 34.Toledano MB, Leonard WJ. Modulation of transcription factor NF-kappa-B binding activity
412 by oxidation reduction in vivo. *Proc. Natl. Acad.Sci USA.*1991;4328-4250
- 413 35.Shetekar N, Pyati A , Murthy S. Oxidative stress and antioxidant status in COPD patients.
414 *Intr. J of Pharm. and Biol. Sci.* 2011; vol 1, issue4, 447-456
- 415 36. Rai RR, Phadke MS. Plasmaoxidant-antioxidant status in different respiratory disorders.
416 *Indian Journal of Clinical Biochemistry*, 2006; 21,2, 161-164.
- 417 37. Kirkil G ,Muz. MH, Seckin D, Sahin K, Kucuk O. Antioxidant effect of Zinc picolinates in
418 patients with Chronic Obstructive Pulmonary Disease. *Respir. Med.* 2008; 102:840-844.
- 419 38.Yun LL, Mian Z, Canmao XE. Oxidative stress status in patients with COPD and its
420 relation to glucocorticoid receptor levels. *Journal of South Med.Univ.*2008;28(6),992-6
- 421 39.Waseem SMA,Hussain M, Islam N. Oxidative stress in mild and moderate COPD :
422 Assessment of oxidant antioxidant status. *Biomed. Research.*2014;25(1),115-119
- 423 40.Daga MK, Chhabra R, Sharma B, Mishra TK. Effects of exogenous vitamin E
424 supplementation on the levels of oxidant and antioxidants in Chronic Obstructive Pulmonary
425 Disease. *J Biosci.* 2003; 28,1,7-11
- 426 41.Kondo T , Tagami S, Yohioka A, Nishumare M ,Kawaskami Y. Current smoking of elderly
427 men reduces antioxidants in alveolar macrophages; *Am. J. Respir. Crit. Care Med.* 1994;
428 149. 178–182.
- 429 42. Jain A, Agrawal B K, Varma M, Jadhav A A.Antioxidant status and smoking habits:
430 relationship with diet. *Singapore Med J* 2009; 50(6): 624-627.
- 431 43. Gavali Y, Deore D.,Surwase SP, Zingade U. Study of the serum superoxide dismutase
432 levels in smoking and non-smoking patients with COPD.*Intr. J. Recent Trade in Sci. and*
433 *Technology.*2013; 5, 3, 121-126
- 434 44. Kluchova Z, Petrasova D, Joppa P, Dorkova Z, Tkacova R. The Association between
435 Oxidative Stress and Obstructive Lung Impairment in Patients with COPD.*Physiol. Res.*
436 2007; 56, 51-56.
- 437