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STUDY OF OXIDATIVE / NITROSATIVE STRESS, NON-ENZYMATIC ANTIOXIDANTS AND MARKERS OF AIRFLOW OBSTRUCTION (FEV1 % PREDICTED) IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) PATIENTS

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Abstract: Chronic Obstructive Pulmonary Disease (COPD) represents a major health problem. Its prevalence is increasing in worldwide. Oxidative stress is one of the major pathphysiologic hallmarks in the development of COPD. The aim of our study was to assess the relationship between the markers of oxidative / nitrosative stress (malondialdehyde and NO·) and the non-enzymatic antioxidants (vitamin E, vitamin C and GSH) with the marker of airflow obstruction (FEV1% predicted) in COPD patients. Study comprised of 185 stable COPD patients were divided into four stages according to GOLD guideline and 60 healthy controls were selected for the comparison. Pulmonary function test was done by using spirometer. Serum levels of MDA, NO·, vitamin E, vitamin C and erythrocyte GSH were estimated by spectrophotometric method. Lung function tests namely FEV1/FVC% ratio and FEV1 % predicted showed significant reduction in COPD patients as compared to healthy controls. Serum level of MDA and NO· shows increasing trend while serum levels of vitamin E, vitamin C and GSH shows decreasing trend across the stages of COPD. We found a significant negative correlation between NO· and MDA with the FEV1% predicted and positive correlation between vitamin C, vitamin E and GSH with FEV1% predicted in different stages of COPD patients. From this study we conclude that as the severity of disease increases FEV1 % predicted decreases. These changes are associated with an increase in oxidative/nitrosative stress and a concomitant decrease in non-enzymatic antioxidants in different stages of COPD patients studied.

Key Words: FEV1 - Force Expiratory Volume in one second, FVC- Force Vital Capacity, GOLD - Global Initiative for Obstructive Lung Disease, GSH- Reduced Glutathione, MDA-Malondialdehyde, NO· - Nitric Oxide

INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is the major health problem its prevalence and incidence is increasing in worldwide. COPD is characterized by slow, progressive airflow limitation which is largely irreversible and associated with the abnormal inflammatory response of the lungs to noxious particles and gases [1,2]. Tobacco smoking is the most common etiological factor for COPD. Tobacco smoke contains oxidants nicotine, carbon monoxide, nitrogen oxide, superoxide radicals, benzopyrene and hydroxyquinone. Increase of oxidative stress in patients with COPD results from the action of exogenous oxidants (such as air pollutant and tobacco components) as well as endogenous oxidants produced during inflammatory process [3,4,5]. Inflammation is to begin by the external noxious stimuli which remain in the lungs even after cessation of smoking, this persistence of inflammation leads to progression of the disease and destruction of lungs tissues, it results in impairments of respiratory function. This is measured in the term of markers of airflow obstruction (FEV1 % predicted) [6].

Oxidant and antioxidant imbalance play a vital role in the pathogenesis of COPD. Lung represents a unique tissue for oxidant stress among most organs because it is directly exposed to higher oxygen tensions [7]. Oxidative stress leads to increase in concentration of free radicals which can cause damage to the biomolecules (proteins, lipids and DNA) present in the cells. Polyunsaturated fatty acids lies on the cell membrane are highly susceptible to free radicals, which leads to lipid peroxidation resulting in membrane dysfunction and eventually cause cell death. Malondialdehyde (MDA) is the end product of lipid peroxidation and marker of oxidative damage in vivo [8].

NO· is a short-lived, reactive free radical. NO· is produced in mammalian cells by group of isoenzymes collectively termed as NO synthases (NOS). NOS exist as three distinct isoforms, namely, endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). All forms of NOS catalyze the conversion of L-arginine to L-citrulline and nitric oxide [9]. It is now well known that each of these isoforms may express in a different tissues and cell types. Nitric oxide has

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numerous functions in the airways including vascular tone regulation, anti and pro-inflammatory actions, neurotransmission and tumor cell lysis [10]. NO· is produced in the respiratory tract in respiratory epithelium, macrophages, vascular endothelium, neutrophils, smooth muscles cells, mast cells and platelets[11]. Nitric oxide is an important signaling molecule that acts in tissues to regulate a wide range of physiological processes.

However excess of NO can exert cytotoxic effects. This may involve the interaction of NO· with free radicals like superoxide ion to form peroxynitrite, is a strong oxidizing agents, its causes cell damage[12]. iNOS is expressed in response to proinflammatory stimuli and produces large amount of NO for sustained time periods in chronic inflammatory lung disease conditions [10].

The deleterious effects of these free radicals are balanced in the cell by the scavenging action of both enzymatic and non-enzymatic antioxidants [13]. Vitamin E is major lipophylic antioxidants, it protect polyunsaturated fatty acids (PUFA) from oxidative damage and thus its role in maintaining the integrity and fluidity of plasma membranes. Beside its antioxidant property it has an anti-inflammatory function [14]. Vitamin C is a water soluble antioxidant, present abundantly in epithelial lining fluid of the lungs. It scavenges the superoxide, peroxyl and the hydroxyl radicals by electron transfer reaction. Ascorbic acid donates its electron to the highly reactive free radical species and converting them into the less reactive form thus prevent the cell from oxidative damage [15,16,17]. GSH is the most abundant intra and extra cellular antioxidant, capable of protecting cell against oxidants and toxic xenobiotics [18,19]. GSH in epithelial lining fluid of the lungs provides sensor system for the production of lung surfactant proteins [20,21,22,23].

Increase in oxidative stress in the circulation causes a fall in the serum non-enzymatic antioxidants (vitamins E, C and reduced glutathione) in smoking people was observed in previous reports [24, 25]. Study of non-enzymatic antioxidants in patients suffering from lung disease opens a promising field in prevention of oxidative stress related complications.

There are many studies available which uses biological fluids bronchoalveolar lavage, exhaled breath condensate, sputum and urine for the determination of oxidant and antioxidant levels in COPD patients but only few studies has use windows of serum for the determination of oxidant and antioxidants levels and compared it with lung function which may be important in deciding the severity or recurrence of the disease.

Keeping this situation in mind we have decided to find the relation between lung function test and marker of oxidative / nitrosative stress and non-enzymatic antioxidants in serum in different stages of COPD patients.

MATERIALS AND METHODS

This case control study was conducted 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 and September 2013. Study comprises of two groups:

1. Control group / Group I: 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. COPD patients group/ Group II: COPD diagnosis for all patients included in the study was made by the evaluation of pulmonary function tests by using spirometer. Study subjects were aged in between of 40-75 yrs. Patients with all stages of COPD were included if they had a post-bronchodilator forced expiratory volume in one seconds (FEV1)/force vital capacity (FVC) <70% after 400 mg of inhaled salbutamol. 185 stable COPD patients were classified into four stages according to GOLD (Global Initiative for Obstructive Lung Disease) guidelines based on the post- bronchodilator values of FEV1% predicted with FEV1/FVC % ratio <70% after performing lung function test, these are as follows:

   a) Stage I COPD: (n=33, post-bronchodilator FEV1≥ 80%, FEV1/FVC % ratio <70%),
   b) Stage II COPD: (n=52, post-bronchodilator FEV1 ≥50% and <80%, FEV1/FVC % ratio <70%),
   c) Stage III COPD: (n=57, post-bronchodilator FEV1≥30% and <50%, FEV1/FVC % ratio <70%),
   d) Stage IV COPD: (n=43, post-bronchodilator FEV1<30%, FEV1/FVC % ratio <70%).

Both written and verbal informed consent was obtained from each participant prior to the study entry and the study protocol was approved by the ethics committee of the institute [Ref. No. BJMC/ IEC/ Pharmac/ D1210133-35].

Exclusion criteria of both patients and control group were: We excluded patients and control with history of asthma, tuberculosis, bronchiectasis, malignancy, ischemic heart disease and patients with history of active infection.

Collection of Blood samples

Under aseptic condition and with prior consent of the subject, 7ml of blood was collected from large peripheral vein, after overnight fasting. Out of which 2ml was taken in an EDTA bulbs for the estimation of whole blood reduced glutathione (GSH) and 4ml blood was collected in a plain bulb, 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^{o}C until the analysis and was used for the estimation of serum MDA, nitric oxide, vitamin E and vitamin C.

Estimation of serum Malondialdehyde [MDA]

Serum malondialdehyde was determined by Buege and Aust method (1978). It was expressed as nmol/ml [26].

Estimation of serum Nitric Oxide

Serum nitric oxide was determined by Cortas and Wakid method (1990). It was expressed as µmol/L [27].

Estimation of Whole Blood reduced Glutathione

Total blood reduced glutathione (GSH) was determined by Ernest Beutler *et al.*, Method (1963). GSH was determined by use of standard curve and was expressed as mg/dl [28]

Estimation of Serum Vitamin C

Serum vitamin C was determined by Ayekyaw method (1996). It was expressed as mg/dl [29].

Estimation of Serum Vitamin E

Serum Vitamin E was measured by Baker and Frank method (1968). It was expressed as mg/dl [30].

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.

Statistical analysis: Statistical analysis was carried out 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 between two groups. One way analysis of variance (ANOVA) was use to compare mean values in all groups followed by multiple comparisons by *Tukey post hoc* tests. *P* value of <0.05 was considered as statistical significant. Pearson correlation was used to analyze the relation between biochemical and lung function parameters.

RESULTS

Table 1 show that irrespective of the sex in healthy volunteers the normal FEV1% Predicted, FVC% Predicted and FEV1/FVC% ratio was observed, which decreases with the advancement of the stage.

Table 1: Demographic Data and Pulmonary Function Tests for patients with different COPD severities and Healthy Volunteers.

Parameters	Healthy Controls (n=60)	COPD patients			
		Stage I (n=33)	Stage II (n=52)	Stage III (n=57)	Stage IV (n=43)
Age (yrs)	54.93±9.01	59.26±9.92	63.18±8.32	61.43±7.60	61.55±8.28
Sex (M/F)	49/11	27/6	44/8	51/6	39/4
Smoking History (Pack years)	----------	52.1±4.74	52.36±7.25	53.56±8.26	55.16±8.97
FEV1% Predicted	109.03±14.90	85.36±6.87[a]	64.15±7.98[b]	41.12±5.83[c]	21.83±4.36[d]
FVC % Predicted	110.47±9.77	97.3±12.49[a]	66.30±8.01[b]	63.96±8.72[c]	48.34±8.63[d]
FEV1 / FVC % ratio	103.33±10.40	65.09±4.64[a]	63.21±4.58[b]	60.86±5.90[c]	58.86±10.32[d]

FEV1% Predicted: Forced expiratory volume in one second, FEV1/FVC% ratio: Forced Expiratory volume in one second/forced vital capacity % ratio. Values are expressed as mean± SD

[a]*P*<0.001: statistically significant as compared to healthy controls

[b]*P*<0.001: statistically significant as compared to stage I COPD patients.

[c]*P*< 0.001: statistically significant as compared to stage II COPD patients.

[d]*P*< 0.001: statistically significant as compared to stage III COPD patients.

Table 2 shows the descriptive statistics and Table 3 shows the multiple comparison of serum MDA, nitric oxide, vitamin C, vitamin E and GSH levels in the different studied groups. One way analysis of variance (ANOVA) was use to compare mean values in all groups followed by multiple comparison *Tukey Post hoc tests.*

In our study, serum MDA level was increased in stage I, II, III and stage IV of COPD and this increment was statistically significant on comparison with control group. The statistical significant difference was found in serum MDA level between all groups (Table 2 and 3). Similarly, the nitrosative stress marker serum nitric oxide level was increased in stage I, II, III and stage IV of COPD patients, this difference was statistically

significant on comparison with control group. While in stage I COPD the serum level of nitric oxide was increased but this increment was not statistically significant when we compared it with stage II, stage III and stage IV of COPD patients by using *Tukey Post hoc test*. Serum level of nitric oxide was also increased in stage II COPD when we compared it with stage III and stage IV COPD but this enhancement was not statistically significant by using *Tukey Post hoc test.* Serum level of nitric oxide was also increased in stage IV COPD but this increase was not statistically significant when we compared it with stage III COPD by *Tukey Post hoc test.* (Table 2 and 3).

Serum level of non-enzymatic antioxidants vitamin C and vitamin E were significantly decreased in stage I,II,III and in stage IV COPD patients as compared to control. Serum level of vitamin C was decreased in stage IV COPD as compared to stage III and stage II of COPD but this reduction was not statistical significant by using *Tukey Post hoc test.* Serum level of vitamin C was decreased in stage III when we compared it with stage II COPD but these decreased was not statistically significant by using multiple comparison *Tukey Post hoc test* (Table 2 and 3).

Table 2: Descriptive statistic (mean ± S.D.) of serum Malondialdehyde (nmol/ml), Nitric Oxide (µmol/L), Whole Blood reduced Glutathione (mg/dl), Serum Vitamin C (mg/dl) and Vitamin E (mg/dl) levels in healthy controls and patients with different COPD severities.

S.No.	Parameters	Healthy Controls (n=60)	COPD patients			
			Stage I (n=33)	Stage II (n=52)	Stage III (n=57)	Stage IV (n=43)
1	Serum (MDA) Malondialdehyde (nmol/ml)	4.43±1.77	6.15±0.85	7.78±1.59	9.79±1.041	11.64±1.268
2	Serum Nitric Oxide (µmol/L)	76.47±7.67	115.96±41.79	121.08±39.06	136.72±49.59	136.79±47.17
3	Whole Blood reduced Glutathione (mg/dl)	33.49±5.3	28.96±7.0	22.32±4.5	18.60±6.42	14.49±3.5
4	Serum Vitamin C (mg/dl)	1.49±0.55	0.93±0.29	0.61±0.20	0.56±0.13	0.47±0.16
5	Serum Vitamin E (mg/dl)	1.48±0.36	1.00±0.32	0.86±0.32	0.63±0.13	0.47±0.12

Values are expressed as mean± SD

Table 3: *Tukey Post Hoc Test*: Multiple Comparison between different groups and parameters:

Dependant Variable	Group (X)	Group (Y)	Mean Difference between Group (X-Y)
Serum (MDA) Malondialdehyde (nmol/ml)	Control	Stage I	-1.71*
		Stage II	-3.34*
		Stage III	-5.35*
		Stage IV	-7.20*
	Stage I	Stage II	-1.63*
		Stage III	-3.64*
		Stage IV	-5.49*
	Stage II	Stage III	-2.01*
		Stage IV	-3.86*
	Stage III	Stage IV	-1.85*
Serum Nitric Oxide (µmol/L)	Control	Stage I	-39.49*
		Stage II	-44.61*
		Stage III	-60.25*
		Stage IV	-60.32*
	Stage I	Stage II	-5.12
		Stage III	-20.76
		Stage IV	-20.83
	Stage II	Stage III	-15.64
		Stage IV	-15.71
	Stage III	Stage IV	-0.07
Serum Vitamin C (mg/dl)	Control	Stage I	+0.56*
		Stage II	+0.88*
		Stage III	+0.93*
		Stage IV	+1.02*
	Stage I	Stage II	+0.32*
		Stage III	+0.37*
		Stage IV	+0.46*
	Stage II	Stage III	+0.05
		Stage IV	+0.14
	Stage III	Stage IV	+0.09

Serum Vitamin E (mg/dl)	Control	Stage I	+0.48*
		Stage II	+0.62*
		Stage III	+0.85*
		Stage IV	+1.01*
	Stage I	Stage II	+0.14
		Stage III	+0.37*
		Stage IV	+0.53*
	Stage II	Stage III	+0.23*
		Stage IV	+0.39*
	Stage III	Stage IV	+0.16*
Reduced glutathione (GSH) (mg/dl)	Control	Stage I	+4.53*
		Stage II	+11.17*
		Stage III	+14.89*
		Stage IV	+19.00*
	Stage I	Stage II	+6.64*
		Stage III	+10.36*
		Stage IV	+14.47*
	Stage II	Stage III	+3.72*
		Stage IV	+7.83*
	Stage III	Stage IV	+4.11*

*****This indicates mean difference between the groups is significant at p<0.05*

Serum vitamin E was decreased in stage II COPD when we compared it with stage I of COPD but this reduction was not statistical significant by using *Tukey Post hoc test.* (Table 2 and 3).

In addition to this, blood level of GSH was significantly decreased in stage I, II, III and in stage IV of COPD compared to control. The statistical significant difference was found in blood level of reduced glutathione (GSH) between all groups (Table 2 and 3).

Table 5: Correlation between studied parameters and FEV1 in patients with different stages of COPD r values.

CORRELATION	Stage I	Stage II	Stage III	Stage IV
MDA-FEV1 % Pred.	-0.193	-0.308*	-0.428*	-0.655*
NO-FEV1 % Pred.	0.077	-0.057	-0.192	-0.335*
VitaminC-FEV1% Pred.	0.125	0.213	0.339*	0.507*
Vitamin E-FEV1 % Pred.	0.102	0.156	0.176	0.283*
GSH-FEV1% Pred.	0.109	0.252*	0.407*	0.503*

*****P<0.05 consider as statistically significant.*

Table 5 showed the correlation analysis between studied parameters and pulmonary function tests, there was significant negative correlation between MDA and FEV_1 % predicted in stage II, III and in stage IV COPD patients (r =-0.308, r = -0.428 and r = -0.655, Table no.5) respectively. No significant correlation was obtained between MDA and FEV1% predicted in stage I COPD patients (r=- 0.193, Table no.5).

In our study, we also found a significant negative correlation between serum nitric oxide and FEV1 % predicted only in stage IV COPD patient (r = -0.335, *P*<0.05). No significant correlations were observed in present study in stage I, II and stage III COPD patients between serum nitric oxide and FEV1% predicted (r= 0.077, r= -0.057 and r=-0.192 respectively, Table 5). In contrast to that, we found significant positive correlation between non-enzymatic antioxidants vitamin C with FEV1 % predicted in stage III and stage IV COPD patients (r = +0.339 and r = +0.507, Table 5) respectively. No significant correlation was found between FEV1 % predicted and serum vitamin C in stage I and stage II COPD patients (r =+0.125 and r=+0.213 respectively, Table 5). We also found a significant positive correlation between vitamin E and FEV1% predicated (r = +0.283) in stage IV COPD patients. No significant correlations were obtained between vitamin E and FEV1% predicted in stage I, II and stage III COPD patients (r=+0.102, r = +0.156 and r= +0.176 respectively, Table 5)

In addition to this, we obtained a significant positive correlation between reduced glutathione and FEV1 % predicted in stage II, III and stage IV COPD patients (r = + 0.252, r = + 0.407 and r = +0.503) respectively. No significant correlation was found between GSH and FEV1% predicted in stage I COPD patients (r=+0.109, Table 5).

DISCUSSION

Tobacco smoke contains free radicals that directly penetrate into respiratory tract system and reach the lung alveoli in order to generate ROS and other oxidants or free radicals is the main factor for the COPD [31]. Oxidant cannot only damage DNA, lipids and proteins but also modulate some process that leads to the development of COPD for eg. Increased production of mucus, impairment of cilia function, loss of elastic recoil and increased airway resistance [32]. All these factors are responsible for decrease in lung function so we did pulmonary function test in COPD patients. In present study, we observed lung function parameters namely FEV1 % predicted and FEV1/FVC % ratio were significantly decreased in all four stages of

COPD patients (P<0.001, Table 1). These findings were supported by the study of Thomason MJ and Strachan DP who reported a reduction of FEV1 and FEV1% in COPD patients [33]. In the study conducted by Daphne CR *et al.,* he reported FEV1 % decline in COPD groups as compared to healthy controls [34]. This decline in FEV1% predicted and FEV1/FVC % ratio occurs might be due to the structural changes in airways and alveoli of lungs of COPD patients this includes inflammation in lungs tissues, airway remodeling, bronchospasm, mucus hyper secretion, increased airway resistance and loss of elastic recoil, resulting in progressive reduction in the expiratory airflow. This reduction in airflow obstruction causes generation of reactive oxygen species and free radicals [3]

Free radicals directly attack on polyunsaturated fatty acids (PUFA) which lies on cell membrane and initiate the process of lipid peroxidation, which may cause direct lung injury [35]. So in the present study we estimated MDA as a marker of lipid peroxidation, it indicates presence of oxidative stress related damage in vivo. In our study we found that serum MDA level was significantly raised in stage IV COPD patients as compared to other stages of COPD patients (Table 2 and 3). The increase in lipid peroxidation product in serum of COPD patients support the hypothesis of oxidative stress associated with the severity of the disease. This increase might be due to stage IV COPD patients having more severe lung function impairment, poor quality of life and more serious systemic dysfunction [36, 37]. This finding is in accordance with the study of Daga M.K. *et al.,* [38], Kirkil G. *et al.,* [39], Isik B. *et al.,* [40] Lee S.I. [41] and Rahman I. *et al.,* [42]. Yessica D *et al.,* and Menon B. *et al.,* reported an increase in MDA level in all stages of COPD severity as compared to controls. Our finding is in agreement with report of Yessica D *et al.,* and Menon B. *et al.,* [43, 44]

In our study we observed serum nitric oxide levels was also significantly increased in stage IV COPD patients as compared to other stages and healthy controls (Table 2 and 3). We observed a significant increase in nitric oxide with the advancement of the stage but the increase observed was less as compared to MDA. The increased level of serum nitric oxide in COPD patients was also reported in the study of Rout. A. *et al.,* [45]

Inflammation is a most critical event in the pathogenesis of COPD. Nitric oxide oxidation products are important inflammatory mediators in COPD [46]. In our study we found significantly high level of nitric oxide metabolite in serum from COPD patients compared to controls and high level of nitrite in COPD correlated with the severity of the disease. This might be due to in patients with COPD peripheral airway is the predominant site of obstruction and inflammation. In COPD patient's oxidative stress occurs along with inflammation. It induces the release of pro-inflammatory cytokines and it activates inducible NOS enzymes in lung neutrophils, macrophages and airway epithelium which lead to increased production of nitric oxide in COPD patients [47].

We found significant inverse correlation between MDA and FEV_1 % predicted in stage II, III and in stage IV COPD patients (r =-0.308, r = -0.428 and r = -0.655 respectively, Table 5). No significant correlation was obtained in our study between MDA and FEV1% predicted in stage I COPD patients (r= - 0.193, Table 5). In the study conducted by Schunemann H J *et al.,* [48], Arpana V *et al.,* [49] who showed that in COPD patients FEV1 % predicted decreases as the mean value of MDA increases. In our study, we also found a significant negative correlation between serum nitric oxide and FEV1 % predicted only in stage IV COPD patient (r = -0.335, *P*<0.05). This is in accordance with study of Ahmad *et al.,* who found inverse correlation between exhaled nitric oxide with FEV% in COPD patients [50]. In current study we did not find any significant correlation in stage I, II and stage III COPD patients between serum nitric oxide and FEV1% predicted (r= 0.077,r= - 0.057 and r= - 0.192 respectively, Table no.5). Our study is consistent with the study of Calikoglu M. *et al.,* who did not find any significant correlation between serum nitric oxide and FEV1% in COPD patients [51].

These results shows that the levels of serum malondialdehyde and nitric oxide increases with the severity of the disease and related to decrease in lung function test.

Under normal condition, oxidative stress is counterbalanced by efficient antioxidant system in the body. Antioxidant defense in the lungs are provided by enzymatic antioxidant system and non-enzymatic antioxidant compounds. Lung cells contains variety of antioxidants: Enzymatic antioxidants includes superoxide dismutase, catalase, glutathione peroxidase and glutathione oxidase and non-enzymatic antioxidants includes: vitamin C, vitamin E, uric acid, albumin, bilirubin and reduced glutathione. In the present study we studied few non-enzymatic antioxidants such as reduced glutathione, Vitamin C and Vitamin E [52].

We observed a significant decrease in the level of GSH from stage I to stage IV in COPD patients (Table 2 and 3). This observed decrease may be due to the utilization of GSH in order to overcome oxidative stress thereby depleting total available GSH pool [53]. The

decreased level of GSH was also observed in COPD patients in the study of Nagraj *et al.*, [54]

The other antioxidants vitamin C and E levels was also observed to be decreased. Vitamin E is a lipid soluble, chain breaking antioxidant. It converts superoxide, hydroxyl and lipid peroxyl radicals to less reactive form thus prevent the cell from oxidative damage [55, 56]. In present study vitamin E significantly lowered in all stages of COPD compared to controls (Table no 2 and 3). Rout A. *et al.*, also observed a significant decreased level of vitamin E in serum of COPD patients [45].

The stage wise decrease was observed in vitamin C levels as the stage advances (Table 2 and 3). The decrease observed may be due two reasons first is the gas phase of tobacco smoke stimulate lipid peroxidation in lung that is reduced by vitamin C and second is vitamin C is also required to regenerate vitamin E, once the vitamin C is converted to tocopheroxyl radical. These loss of serum antioxidants may indicate the ongoing biological oxidative stress. We observed decreased level of vitamin C in COPD patients, this is in accordance with studies of Sargeant L.A. *et al.*, [57], Rai R. *et al.*, [58] and Calikoglu M. *et al.*, [59].

In our study we found significant positive correlation between non-enzymatic antioxidants vitamin C with FEV1 % predicted in stage III and stage IV COPD patients (r = +0.339 and r = +0.507, Table no.5) respectively. This is in accordance with the study of Schwatz J *et al.*, [60] who found positive correlation between FEV1 and vitamin C in chronic bronchitis patients. In present study no significant correlation was observed between FEV1 % predicted and serum vitamin C in stage I and stage II COPD patients (r =+0.125 and r=+0.213 respectively, Table no.5). We also found a significant positive correlation between vitamin E and FEV1% predicated (r = +0.283) in stage IV COPD patients. This is in accordance with the study of Jadhav BS *et al.*, [61] who has found significant positive correlation of vitamin E with FEV1% predicted in COPD patients. In current study no significant correlations were obtained between vitamin E and FEV1% predicted in stage I, II and stage III COPD patients (r=+0.102, r = +0.156 and r= +0.176 respectively, Table 5). In addition to this, we obtained a significant positive correlation between reduced glutathione and FEV1 % predicted in stage II, III and stage IV COPD patients (r = + 0.252, r = + 0.407 and r = +0.503) respectively. No significant correlation was found between GSH and FEV1% predicted in stage I COPD patients (r=+0.109, Table no.5). Our study is in contrast to the study of Balcom HO *et al.*, [14] who found a statistically significant inverse association of glutathione and FEV1 in COPD patients. They suggest that this inverse association of GSH to FEV1% predicted may be due to body compensate level of glutathione in tissue during more severe disease.

CONCLUSION

COPD patients are more vulnerable to oxidative stress and have impaired status of antioxidants. In our study, we observed the remarkable decrease in lung function test with the severity of the disease. The levels of oxidative stress markers (malondialdehyde and nitric oxide) were increases with increasing severity of COPD. In addition to this when we studied non-enzymatic antioxidants (reduced glutathione, vitamin E and vitamin C) a concomitant decreased was observed. The correlation is an ample testimony of their effectual involvement in COPD. The coordination between oxidative stress markers and antioxidant status in COPD patients might be useful in the diagnosis and prognosis of the disease.

This observation led us to conclude that imbalance in oxidative status and low antioxidants level may have role in disease severity as observed by their correlation with lower FEV1% predicted.

So if we try to decrease oxidative stress with the additional supplementation of dietary antioxidants and GSH might results in alleviation of the disease.

Strength and limitations of the study:

Strength:

1) We are the first to report the levels of oxidative / nitrosative stress markers (MDA and nitric oxide) and non-enzymatic antioxidant (vitamin E, vitamin C and reduced glutathione) in different stages of COPD patients.
2) We correlated pulmonary function test markers namely: FEV1% predicted with the markers of oxidative / nitrosative stress (MDA and nitric oxide) and non-enzymatic antioxidants (vitamin E, vitamin C and reduced glutathione) in different stages of COPD patients.

Limitations: Considering the prevalence of the COPD patients this work has to be done with the larger sample size for to confirm the results.

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