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Oxidative Stress in Chronic Obstructive Pulmonary Disease Alters Ferroxidase Activity of Ceruloplasmin

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Authors' contributions

Author AS Concept, designed and supervised the study, author VA conducted the study, review of literature, and wrote the manuscript, author DB performed all the statistical analysis, author RT did the diagnosis of COPD patients and managed the sample collection. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: Oxidative stress is one of the major patho-physiologic hallmarks in the development of COPD. Ceruloplasmin, the major serum inhibitor of lipid peroxidation has been documented as a main extracellular antioxidant in serum and plays a role in preventing lung injury, and an abnormality in its oxidative inhibition could be involved in pathogenesis of COPD. This study aims to estimate levels of ceruloplasmin and its ferroxidase activity in COPD and compare with that in controls to explore their utility in COPD.
Study Design: Comparison study.
Place and Duration of Study: Department of Biochemistry, AFMC, Pune. Subjects for COPD were selected from the patients reporting with symptoms of COPD to respiratory OPD of Cardio Thoracic Centre, Pune, during Dec 2010 to Aug 2012.
Methodology: Study comprised of two groups: Group I of 77 normal as controls (61 men, 16 women; age range 27-90 years) and Group II of 92 COPD patients (70 men, 22

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women; age range 45 - 97 years). Both the groups were further divided into smoker and nonsmoker groups. Ferroxidase activity of ceruloplasmin was estimated by indigenous patented kinetic method of Somani and Ambade while ceruloplasmin was estimated by immunotubidimetric method using commercially available kit.
Results: Serum ceruloplasmin and ferroxidase activity were significantly higher in COPD patients as compared to normal controls. Mean ± SD in COPD versus controls respectively are ceruloplasmin: 45.84 ± 12.7 mg/dL versus 37 ± 9.7 mg/dL; ferroxidase: 1324.9 ± 278.53 IU/L versus 980.5 ± 202.3 IU/L, P< .001. Statistically significant & good correlation (r > 0.7) was found between ceruloplasmin and ferroxidase in controls, nonsmoker controls and smoker controls (r = 0.76, 0.71 and 0.79 respectively) while in COPD, COPD nonsmokers and COPD smokers, no correlation was found (r = 0.00, 0.29 and 0.09 respectively).
Conclusion: There is alteration in the ferroxidase activity of ceruloplasmin in COPD. Future studies with quantification of carbonyl residues or other groups in ceruloplasmin molecule leading to altered oxidative or ferroxidase activity of ceruloplasmin may provide further evidence to support a role of oxidative stress in COPD.

Keywords: Chronic obstructive pulmonary disease; antioxidants; ceruloplasmin; ferroxidase.

1. INTRODUCTION

Chronic obstructive pulmonary disease (COPD) a condition of chronic obstruction to airflow due to chronic bronchitis and /or emphysema is the only chronic disease that is showing progressive upward trend in both mortality and morbidity is expected to be the third leading cause of death by 2020 [1,2].

The lung which is continuously exposed to endogenous or exogenous oxidants [3,4,5] is protected against oxidative challenge by well-developed antioxidant systems. Increased oxidative stress not only produces direct injurious effects in the lungs, but also activates the molecular mechanisms that initiate lung inflammation [6] and may have a role in many of the processes thought to be involved in the complex pathological events that results in COPD. Oxidative stress is one of the major patho-physiologic hallmarks in the development of COPD. Ceruloplasmin, the major serum inhibitor of lipid peroxidation [7] has been documented as a main extracellular antioxidant in serum [8], inhibiting ferrous ion stimulated lipid peroxidation and is known to be involved in the decomposition of lipid peroxides [9]. Ceruloplasmin protects protease inhibitor from oxidative inactivation [10]. It has been reported that ceruloplasmin activity play a role in preventing lung injury and an abnormality of ceruloplasmin oxidative inhibition could be involved in the pathogenesis of COPD [10].

The present study was conducted with the aim of estimating the levels of ceruloplasmin as a molecule by immunoturbidimetric method, and its ferroxidase activity by kinetic enzymatic assay, in serum of COPD patients and normal subjects and correlated their levels to explore their utility in COPD.

2. MATERIALS AND METHODS

2.1 Subject selection and Procedure

The study subjects comprises of two groups:

i) Group I or Controls: Comprises of 80 normal, age and sex matched individuals. Subjects for this group were taken from normal healthy individuals coming to Department of Biochemistry for routine check up and annual medical board and other volunteers.

ii) Group II or COPD patients: Comprises of 100 patients of COPD diagnosed as per GOLD guidelines (GOLD report 2011). Subjects for this group were selected from the patients reporting with symptoms of COPD to respiratory OPD of Cardio-Thoracic Centre, Pune between Dec 2010 to Aug 2012.

Both the groups were further divided into smoker and nonsmoker groups.

The clinical assessment at the time of presentation, in terms of signs and symptoms and relevant investigation, were recorded in a proforma. Study was approved by the institutional ethical committee and informed consent was taken from the subjects before drawing their blood specimen.

2.2.1 Exclusion criteria for control and COPD patients

Patients with a diagnosis of asthma, coronary artery disease (CAD), diffuse parenchymal lung disease, patients on long term oxygen therapy or unable to perform spirometry were excluded from the Group II. Similarly, individuals with any respiratory or lung disease or past history of any lung/respiratory problem/disease were excluded from control group.

All patients were administered a questionnaire to collect demographic data (name, age, sex, address) duration of symptoms, history of allergic symptoms (history of atopy, nasal allergies), episodic or progressive symptoms, chest examination (wheeze), radiological investigations (CXR, CT Scan), basis of initial diagnosis (ascertained from follow up book) and whether spirometry was performed for initial diagnosis. From the questionnaire an initial clinical impression of the diagnosis was made. All patients underwent spirometry for confirmation of the diagnosis of COPD. The presence of post bronchodilator FEV1/FVC <0.70 was used to confirm presence of persistent airflow limitation and thus of COPD [11].

Smoking index was used to quantify smoking exposure among the study subjects. Smoking index is defined as number of cigarettes or bidis 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 [12]. Subjects who have quit smoking completely, uninterrupted for more than 15 years were considered as nonsmokers.

Group I, 3 subjects due to incomplete data, and 8 subjects in Group II due to CAD were excluded and accordingly 77 and 92 study subjects respectively were finally included in the study for statistical analysis.

2.2 Subject Collection and Storage

Fasting venous blood samples, about 5 mL, were collected in a plain sterile gel vacutainer from all the subjects. Serum was separated by centrifuging the blood sample at 2000 rpm for 5 minutes. The supernatant was separated into micro-centrifuge vials/Eppendorf tube. Estimation of ceruloplasmin and its ferroxidase activity was done immediately without any storage.

2.3 Estimation of Ferroxidase Activity

Ferroxidase activity was determined by indigenous kinetic method of Somani and Ambade (Govt of India Patent No192356), [13] on fully automated analyzer XL 600 from Transasia Mannheim GmbH, using the reagents as below: Reagent-1: Chromogen (0.5 mmol/L) was made by dissolving 159.65 mg of norfloxacin in 1000 mL of acetate buffer (0.45 mol/L, pH 5.4) containing 0.2% Triton X-100. Reagent-2: Substrate (2.04 mmol/L) was made by sequentially dissolving, 320 mg of DTT and 800 mg of ferrous ammonium sulphate, $Fe(NH4)_2(SO4)_2 \bullet 6H_2O$, in 1000 mL of distilled water. Both Reagent-1 and Reagent-2 were stable for more than 6 months at 4°C as well as at room temperature. The fully automated analyser was set at following parameters: Assay Type: Rate A; Wavelength: Primary = 376nm, Secondary = 0nm; Assay Points 0, 0, 15, 18 cycles; Sample volume: 10µL; Reagent 1: 200µL; Reagent 2: 30µL; Factor 2010.

This estimation is based on the principle that enzymatic oxidative property, that is, ferroxidase property oxidizes ferrous to ferric ion which then complexes with chromogen. The formation of this complex is measured kinetically at 376nm. The ferroxidase activity in serum was calculated from the factor and displayed directly by the fully automated analyser in IU/L.

2.4 Estimation of Ceruloplasmin

Ceruloplasmin was estimated using commercially available kit SensITCeruloplasmin (Code: No. 11813001) from Agappe Diagnostics as per their instruction manual on a fully automated analyser XL600 from Transasia Mannheim GmbH. This estimation is based on the principle that the reagent containing goat antihuman ceruloplasmin reacts with serum ceruloplasmin forming turbidity. The increase in the absorbance due to turbidity is directly proportional to the concentration of ceruloplasmin in the serum which was calculated from the multipoint standard curve derived from the calibrators provided in the kit and displayed directly by the analyser in mg/dL.

2.5 Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS version 17). Results have been expressed as mean ±SD. Mean differences were tested using unpaired t test, and correlation between the variables was calculated using Pearson's coefficient. Cut-off point for determining significance levels was $P = .05$.

3. RESULTS AND DISCUSSION

Seventy seven controls and ninety two COPD patients were statistically analysed. In seventy seven controls (Group I), Males to Females ratio was 61:16, Mean ± SD of age was 60.6 ±

12.7 years with range 27-90 years. In ninety two COPD patients (Group II), Males to Females ratio was 70:22; Mean ± SD of age 67.58 ± 9.12 years with range 45-97 years (Table 1).

Table 1. The age and sex distribution of study groups

Parameter	Controls (n=77)			COPD patients (n=92)		
	Males	Females	Total	Males	Females	Total
Number (%)	61 (79.2)	16 (20.8)	77 (100)	70 (76)	22 (24)	92 (100)
Mean (yrs)	60.5	61.2	60.6	69	63	67.58
SD (yrs)	14	5.36	12.7	8.89	8.5	9.12
Range (yrs)	27-90	54-77	27-90	45-97	45-76	45-97

Ceruloplasmin is a major extra cellular antioxidant in serum [8], inhibits ferrous ion stimulated lipid peroxidation [9] and plays a role in preventing lung injury and an abnormality of its oxidative inhibition could be involved in the pathogenesis of COPD [10]. The antioxidant action has been proposed as a crucial function of ceruloplasmin with multiple mechanisms [14,15]. Among various substrates the highest oxidizing activity has been found for Fe^{+2} and therefore the alternative name of ferroxidase (EC.1.16.3.1) has been proposed [16,17] and this study was undertaken with the objective of studying ferroxidase activity of ceruloplasmin by virtue of which it plays as an antioxidant role in COPD.

The serum levels of ceruloplasmin and ferroxidase activity were significantly higher in COPD patients (24% and 35% respectively) as compared to healthy controls. Their levels in COPD versus controls respectively are ceruloplasmin: 45.84 ± 12.7 mg/dL versus 37 ± 9.7 mg/dL; ferroxidase: 1324.9 ± 278.53 IU/L versus 980.5 ± 202.3 IU/L; (Table 2).This is in agreement with the study by Verrills et al [18] who found that a panel of four biomarkers (α(2)-macroglobulin, haptoglobin, ceruloplasmin, and hemopexin) was able to discriminate with statistical significance between the clinical groups of patients with asthma, patients with COPD, and control subjects and reported increased ceruloplasmin in COPD patients as compared to controls. Table 3 shows a comparison of levels of ferroxidase and ceruloplasmin between controls and COPD patients within smokers and nonsmokers group. When the levels of ceruloplasmin and ferroxidase activity were compared within the smoker and nonsmoker groups in COPD patients and controls, the levels in COPD were found significantly higher than those in controls ($P < 0.001$). The levels in nonsmokers COPD versus nonsmoker controls respectively are ceruloplasmin: 44.5 ± 13.8 mg/dL versus 35.24 ± 8.5 mg/dL; ferroxidase : 1312.3 ± 236.3 IU/L versus 964.1 ± 192.2 IU/L. Similarly, smoker COPD versus smoker controls respectively are ceruloplasmin: 46.4 ± 12.2 mg/dL versus 38.5 ± 10.5 mg/dL; ferroxidase : 1352 ± 296.9 IU/L versus 993.5 ± 211.2 IU/L..

Table 2. Comparison of levels of ferroxidase and ceruloplasmin between study groups

Bio marker	Controls (n=77)			COPD patients (n=92)			t-test and significance	
	Mean	SD	Range	Mean	SD	Range	|t| Value	P Value
Ferroxidase (IU/L)	980.5	202.3	445.6-1464.3	1324.9	278.5	692.8-1932	9.29	<.001
Ceruloplasmin (mg/dL)	37	9.7	17.2-63.8	45.8	12.7	24.3-86	4.98	<.001

However, when the levels of ceruloplasmin and ferroxidase were compared between smokers and nonsmokers in controls and COPD then not much difference was found, though smokers were having slightly more ceruloplasmin and ferroxidase than nonsmokers in controls (993.5 IU/L & 38.5mg/dL as compared to 964.1 IU/L & 35.24 respectively (Table 3). This is in agreement with the study of Tavilani et al [19] who reported no statistically significant difference in levels of ceruloplasmin in smokers and healthy nonsmokers group.

Table 3. Comparison of levels of ferroxidase and ceruloplasmin between controls and COPD patients within smoker and nonsmokers group

Bio marker	Controls (n=77)		COPD patients (n=92)		
	Mean	SD	Mean	SD	p Value
Nonsmoker controls (n=34)			Nonsmokers COPD (n=29)		
Ferroxidase (IU/L)	964.1	192.2	1312.3	236.3	<.001
Ceruloplasmin (mg/dL)	35.24	8.5	44.5	13.8	.009
Smoker controls (n=43)			Smokers COPD (n=63)		
Ferroxidase (IU/L)	993.5	211.2	1352	296.9	<.001
Ceruloplasmin (mg/dL)	38.5	10.5	46.4	12.2	.004

To assess the correlation between increased ceruloplasmin and increased ferroxidase in COPD, correlation studies were conducted. Statistically significant and good correlation was found between ceruloplasmin and ferroxidase in controls, nonsmoker controls and smokers control group (r= 0.76, 0.71 & 0.79 respectively as shown in Figs. 1, 2, 3). However, in COPD, nonsmokers COPD and smoker COPD groups no correlation was found between ceruloplasmin and its ferroxidase activity (r = 0.00, 0.29 & 0.09 respectively as shown in Figs. 4, 5, 6). In COPD, there is an increase in ceruloplasmin by 24 % and ferroxidase activity by 35% over the controls but the correlation between ceruloplasmin and its ferroxidase is lost in COPD. To determine the effect of smoking index on the levels of ceruloplasmin and ferroxidase, correlation studies between ceruloplasmin and smoking index and between ferroxidase and smoking index were performed. No correlation was found between smoking index and ceruloplasmin or between smoking index and ferroxidase activity in any of the groups (Figs. 7, 8, 9, 10).

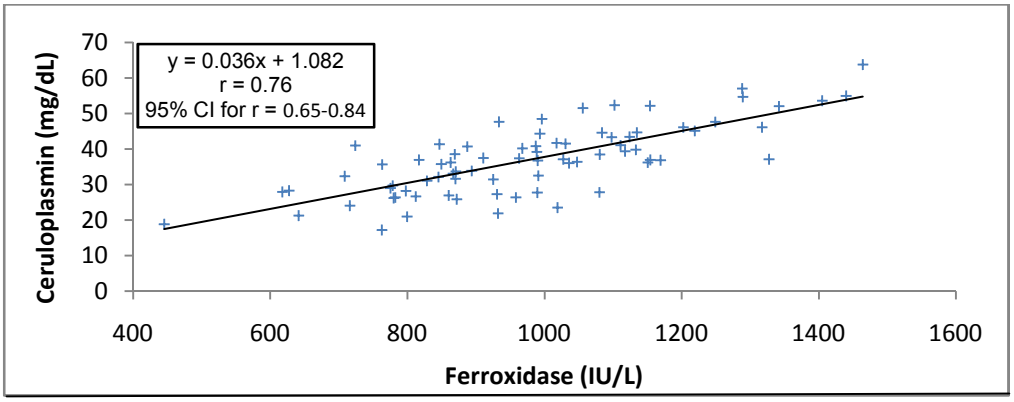

Fig. 1. Correlation between ferroxidase and ceruloplasmin in controls

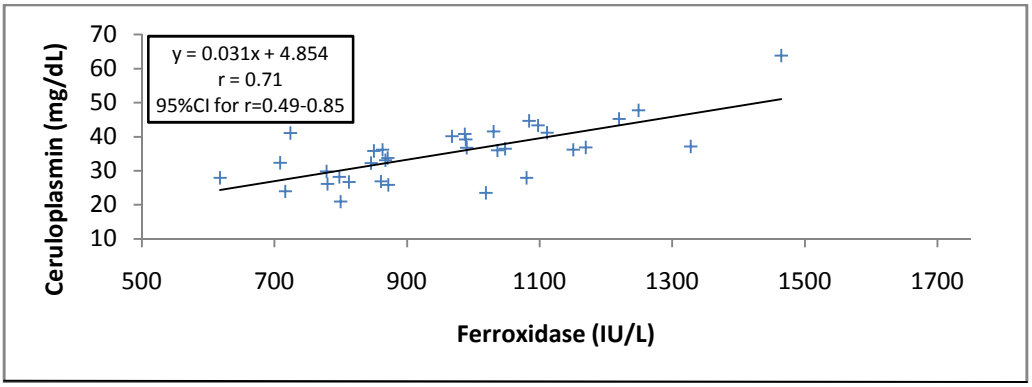

Fig. 2. Correlation between ferroxidase and ceruloplasmin in nonsmoker controls

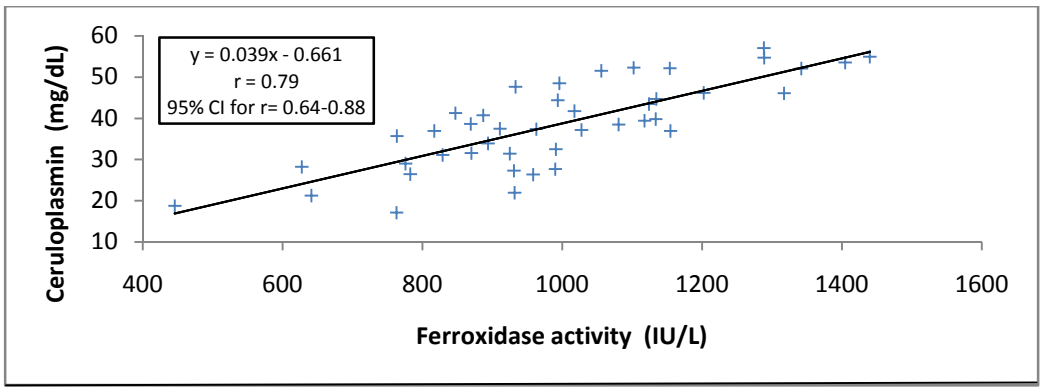

Fig. 3. Correlation between ferroxidase and ceruloplasmin in smoker controls

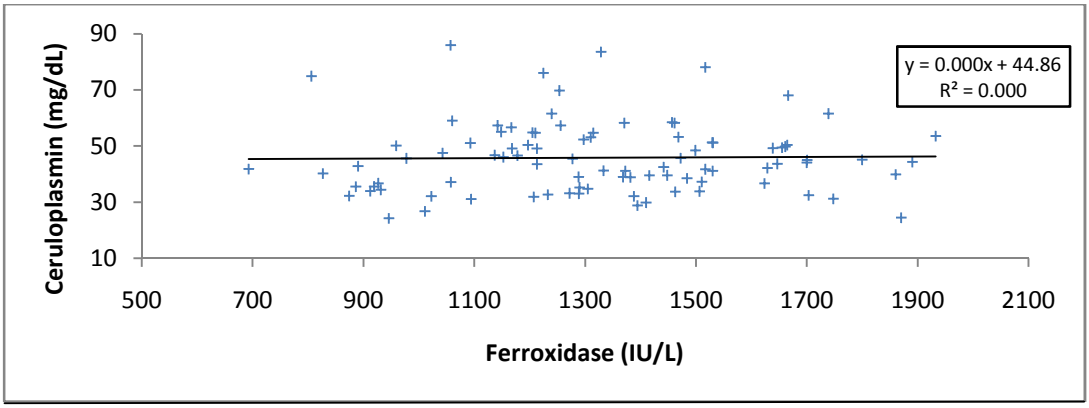

Fig. 4. Correlation between ferroxidase and ceruloplasmin in COPD

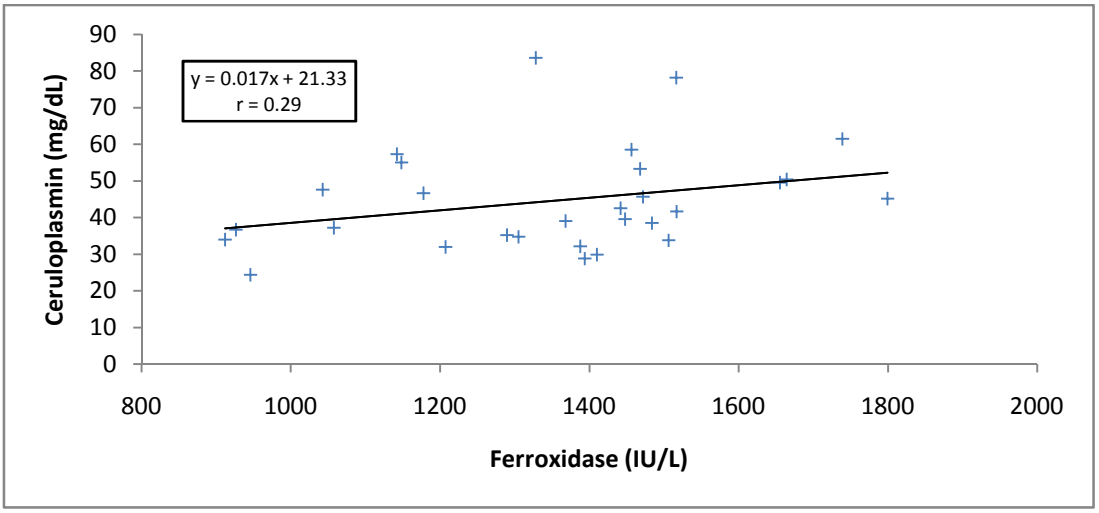

Fig. 5. Correlation between ferroxidase and ceruloplasmin in COPD nonsmokers

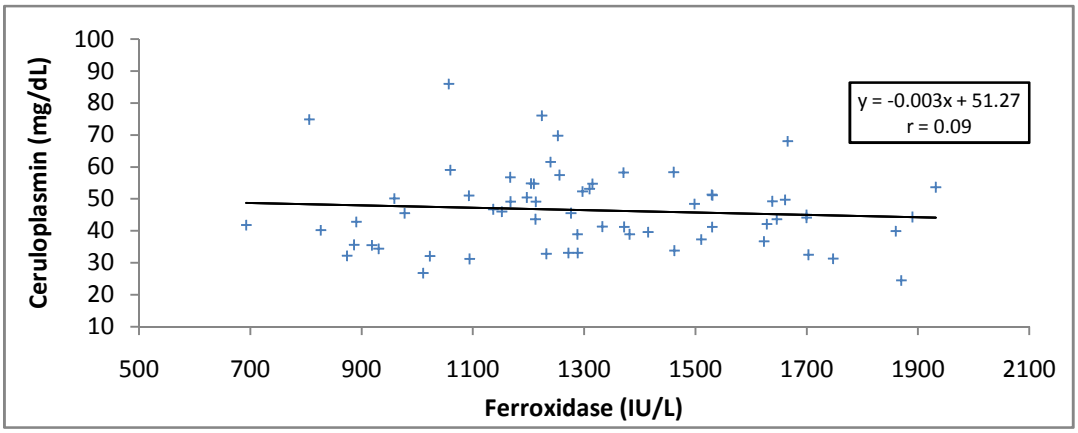

Fig. 6. Correlation between ferroxidase and ceruloplasmin in COPD smokers

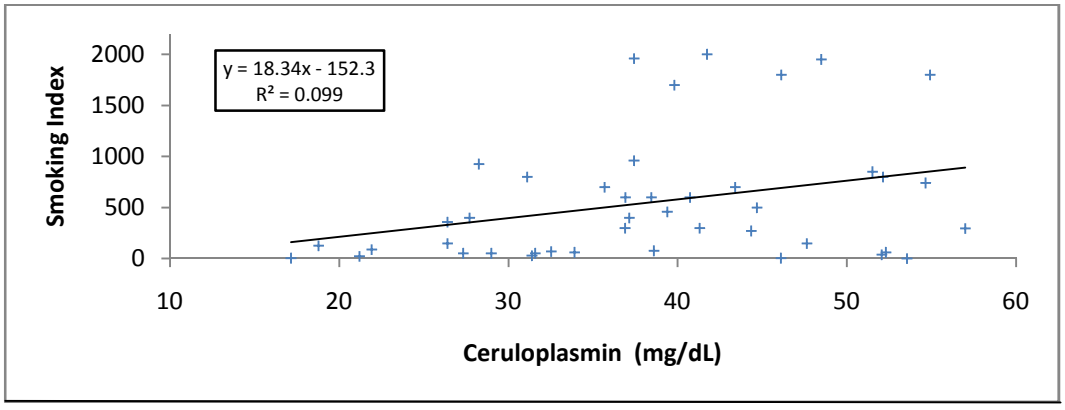

Fig. 7. Correlation between ceruloplasmin and smoking index in smoker controls

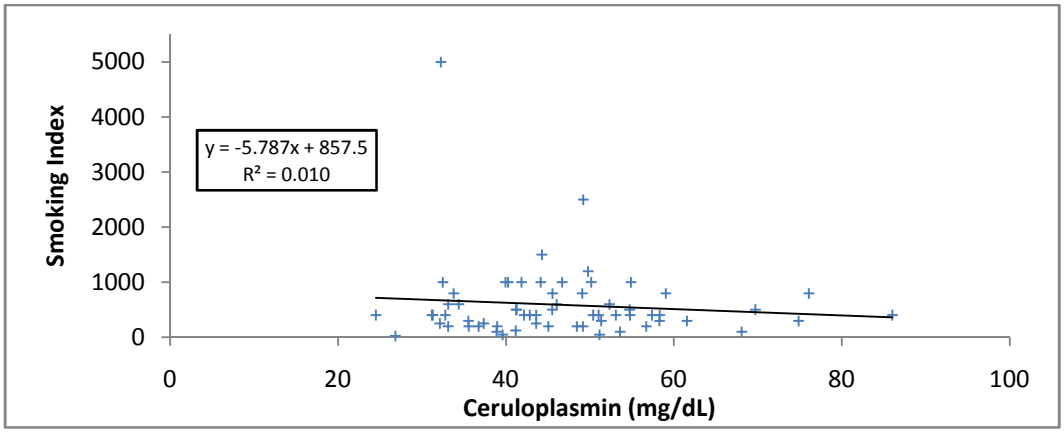

Fig. 8. Correlation between ceruloplasmin and smoking index in COPD smokers

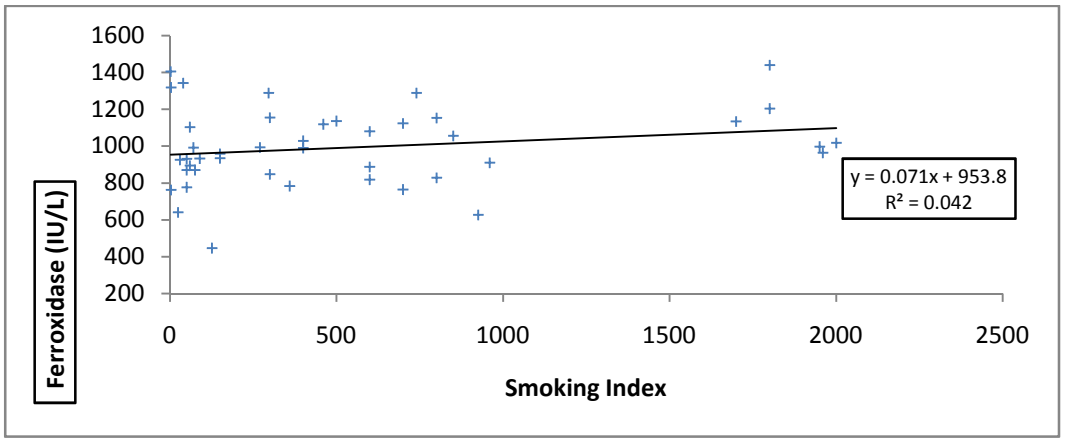

Fig. 9. Correlation between ferroxidase and smoking index in smoker controls

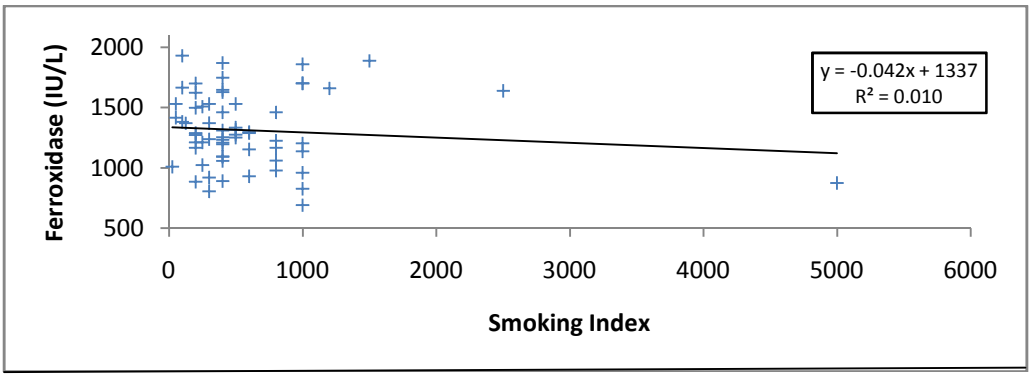

Fig. 10. Correlation between ferroxidase and smoking index in COPD smokers

It appears that in some patients with COPD an increase in ceruloplasmin is not associated with a corresponding increase in ferroxidase activity, while in other patients there is increase

in ferroxidase activity linearly. Thus, the increase in ceruloplasmin in COPD patients might not always guarantee the proportionate increase in its ferroxidase activity. This could be due to the fact that all COPD patients are not subjected to same level of oxidative stress. As a response to COPD, there might be more synthesis of ceruloplasmin in the body, but different levels of oxidative stress might be modifying the ceruloplasmin molecule accordingly, thereby altering its ferroxidase enzymatic property.

4. CONCLUSION

Thus this study indicated that there is alteration in the ferroxidase activity of ceruloplasmin in COPD which may be due to any reason like modification in the structure of ceruloplasmin due to the oxidative stress in COPD. It is reported widely that free radical-initiated reactions leads to the formation of carbonyl groups on amino acid residues [20,21]. In fact the oxidative modification of proteins and lipids has been implicated in the etiology of a number of diseases [22,23]. Oxidized human serum albumin has been reported as a reliable marker of oxidative stress [24]. Ischemia modified albumin, an altered type of serum albumin that is formed under conditions of oxidative stress has been reported as a new marker of oxidative stress in thalassemic patients [25]. Recently, Olivieri S et al. [26], hypothesized that increased oxidative stress in Parkinson's disease modifies ceruloplasmin molecule by its oxidation thereby altering its ferroxidase activity and suggested that oxidized ceruloplasmin in Parkinson's disease cerebrospinal fluid might be used as a marker for oxidative damage and might provide new insights into the underlying pathological mechanisms in Parkinson's disease. Quantification of carbonyl residues or other groups in ceruloplasmin molecule leading to altered oxidative capabilities or altered ferroxidase activity of ceruloplasmin may provide further evidence to support a role of oxidative stress in COPD pathology.

CONSENT

Declare that written informed consent was obtained from the patient for sample.

ETHICAL APPROVAL

Obtained necessary Institutional ethical approval.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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