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Altered antioxidant enzyme activity with severity and comorbidities of chronic obstructive pulmonary disease (COPD) in South Indian population

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Abstract

Background: Oxidative stress has been suggested in the pathogenesis of Chronic Obstructive Pulmonary Disease (COPD) with an additional burden of diabetes, hypertension and cardiovascular disease. In the present study, we investigated erythrocyte antioxidant enzymes activities in correlation to COPD severity and COPD comorbidities.

Methods: One hundred twenty seven subjects with COPD and 59 healthy controls participated in this study. COPD severity was done based on the Global Initiative for Chronic Obstructive Lung Disease criteria. The erythrocytes enzyme activities of superoxide dismutase (SOD), catalase (CAT), glutathione-s-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR) and total antioxidant status (TAS) were measured with spectrophotometric method.

Results: COPD patients showed significant decrease in TAS ($p > 0.05$), GST ($p < 0.001$) and GPx ($p < 0.01$) activities with progression of the disease. In patients, FEV₁ was negatively correlated with SOD, GR and positively correlated with GST and GPx activities. Further, multivariate logistic regression analysis revealed GST (OR = 0.93; 95% CI = -0.10 – 0.01; $p < 0.01$), GPx (OR = 0.98; 95% CI = -0.03 – 0.00; $p < 0.05$) and TAS (OR = 0.95; 95% CI = -0.08 – 0.00; $p < 0.05$) were independently associated with FEV₁ in GOLD stage IV and GST (OR = 1.11; 95% CI = 0.04–0.18; $p < 0.001$) in GOLD stage II. Regression analysis confirmed a significant difference in GPx activity in COPD – type 2 diabetes (OR = 0.04; 95% CI = -6.60 – 0.53; $P = 0.09$), and GST activity in COPD – cardiovascular disease (OR = 2.51; 95% CI = 0.00 - 1.84; $p < 0.05$) patients when compared to patients without comorbidities.

Conclusion: A significant decline in lung function may be associated with altered antioxidant enzyme activity due to the strong correlation between GST and GPx with COPD severity. Our results also indicate that erythrocytes GST and GPx activities are significantly associated with comorbidities, but only in COPD patients with type 2 diabetes and cardiovascular disease.

Keywords: Erythrocyte, Antioxidant enzyme, COPD, GOLD stage classification, Lung function test, Forced expiratory volume in 1 s, Diabetes, Hypertension, Cardiovascular disease

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Background

Chronic obstructive pulmonary disease (COPD) is a disease characterized by irreversible and progressive airflow obstruction that is associated with lung inflammation, according to American Thoracic Society guidelines [1]. The most common causes of COPD are cigarette smoking, environmental exposure to dust particles and air pollutants such as allergens, bacterial and viral spores [2]. The lungs are the organs that are continuously exposed to oxidants, either generated from inside the body or from outside the body due to exposure to air pollution or cigarette smoke. These particles increase the levels of oxidants in the lungs, and have the potential to interact with biological systems to produce oxidative stress, which results in the destruction of alveoli, the tiny air sacs in the lungs where the exchange of oxygen and carbon dioxide takes place, and also results in narrowing of airway lumen diameters. Oxidative stress promotes inflammation of the airways of the lungs as seen in COPD patients.

This causes an irreversible decrease in reduction of forced expiratory volume in 1 s (FEV₁) as seen in COPD cases [3]. COPD is a major, ever increasing global health problem due to increase in smoking rates and lifestyle changes and is projected to be the fourth leading cause of death worldwide by 2030 [4]. A report submitted by National Commission on Macroeconomics and Health (NCMH), showed that the incidence of COPD in India may increase from 17.0 million (in the year 2006) to 22.2 million by 2016 [5].

Oxidative stress, being the major risk factor in the pathogenesis of COPD, is caused by the generation of free oxygen radicals (ROS) [6]. Excessive cigarette smoking or environmental exposure to smoke and air-pollutants has been shown to settle in the lungs, leading to increased production of ROS causing oxidative stress [7]. Another prominent source of ROS is either environmental derived or cell-derived, both of these are directly associated with many chronic respiratory lung diseases, including COPD [8, 9]. These ROS categories include superoxide anion (O₂⁻), hydroxyl radical (.OH), hydrogen peroxide (H₂O₂) and nitric oxide (NO.) radicals [10]. In order to maintain the balance of oxidative stress caused by ROS, the body has to balance between enzymatic and non-enzymatic antioxidants [11, 12]. The main function of antioxidant enzymes is to neutralize the free radicals and transform them into safer (O₂). Antioxidants enzymes, such as superoxide dismutase (SOD) which detoxifies the superoxide anion and converts into H₂O₂ and O₂, a less toxic product. Catalase (CAT) enzyme completes the detoxification process initiated by SOD via decomposition of H₂O₂ to water and oxygen [13]. Glutathione peroxidase (GPx) is a family of selenium-dependent and independent antioxidant enzymes [14]. There are six known isoforms of GPx and the most

abundant isoforms, GPx-1, is ubiquitously expressed in the cytoplasm of all mammalian cells [15, 16]. The main function of GPx is to detoxify hydrogen peroxide to H₂O and O₂. However, it also detoxifies the lipid peroxides to release water and alcohol [13]. Glutathione-s-transferase (GST) is major cytosolic phase II detoxification enzymes which inactivates reactive electrophiles by Glutathione (GSH) dependent mechanism [7, 8]. Glutathione reductase (GR) recycles the oxidized glutathione disulfide (GSSG) using NADPH as the reducing co-factor and thereby maintains an appropriate intracellular GSH level in the cell [17].

There is evidence that erythrocyte GPx activity is lower in smokers compared to non-smokers [18]. Few studies report a direct relationship between forced expiratory volume in 1 s (FEV₁) and pathogenesis of COPD [19, 20]. On the other hand, another study failed to find significant relationship between FEV₁ and plasma antioxidant capacity [21]. It has been reported that erythrocytes are more exposed to oxidative stress and studies have shown that these cells are highly susceptible to oxidative damage in the development and progression of various diseases. In view of such conflicting reports, we have taken up this study to investigate the role of antioxidants in diseased patients. It is also known that comorbidities in COPD are known to cause difficulties in diagnosis and cause economic burden to patients. Metabolic complications, namely diabetes and metabolic syndrome are the most common comorbidities in COPD. Besides, some reports have demonstrated that COPD patients have a relatively increased risk of developing lung cancer, pulmonary hypertension and cardiovascular disease [22]. There are evidences that cardiovascular disease and COPD patients share the same risk factors i.e. smoking and alcohol [23]. It is not surprising to note that pulmonary hypertension which results in type 2 diabetes could also lead to COPD with significant lower FEV₁ values than in non-diabetics [24]. However, it is not understood why COPD patients are at greater risk of type 2 diabetes as compared to non-diabetes subjects or vice-versa [25].

It has been a common practice to identify the antioxidant enzyme levels in plasma or serum. Furthermore, in the progression of COPD erythrocytes are highly susceptible to oxidative damage. Therefore, the present study aimed to investigate the erythrocyte antioxidant enzyme activities in COPD patients and correlate these levels with lung function test assessed as FEV₁. It is also possible that the comorbidities may further increase the risk of COPD severity. We are not aware of any previous studies that addressed the erythrocyte antioxidant enzyme activities in COPD patients with comorbid diseases such as type 2 diabetes, pulmonary hypertension and cardiovascular disease. Hence, the present study also aims to investigate the relationship between the antioxidant enzymes activities of COPD patients with comorbidities.

Methods

Demographic details of patients

One hundred twenty seven COPD patients and 59 healthy controls were participated in this study. The data regarding gender, age, body mass index (BMI) and smoking status were analyzed. All study subjects underwent a standardized clinical examination at the Outpatients clinic at Mahavir Hospital and Research Centre, Hyderabad, India. The COPD patients and controls were informed about the study and purpose; and gave written informed consent before inclusion in the study group. The study was conducted in accordance with the guidelines of Committee responsible for human studies as guided by the “Declaration of Helsinki” and good clinical practice. The Ethics Committee of Mahavir Hospital and Research Centre – Hyderabad approved the study.

Inclusion criteria: All patients recruited had confirmed COPD as determined by the Pulmonologist. The potential cases having symptoms such as chronic cough, breathing problem and production of mucus or sputum

Table 1 Association of significant demographic characteristics among study variables

Variables	Controls	COPD
	Mean ± SEM (n = 59)	Mean ± SEM (n = 127)
Age, years	51.02 ± 0.27	60.10 ± 0.31**
Male (n)	38 (64.40)	98 (77.16)
Female (n)	21 (35.59)	29 (22.83)
Weight (kg)	65.32 ± 1.50	61.69 ± 0.32**
Height (cms)	157.12 ± 1.04	158.50 ± 0.26
BMI (kg/m ²)	26.43 ± 0.65	24.43 ± 0.20**
Smoking Status		
Ex-smoker (n)	13 (22.03)	50 (39.37)
current smoker (n)	17 (28.81)	32 (25.19)
non-smoker (n)	29 (49.15)	45 (35.43)
Spirometry parameter		
FEV ₁ , % of predicted	71.20 ± 1.26	36.21 ± 0.40***
FVC, % of predicted	83.99 ± 1.06	50.15 ± 0.34***
FEV ₁ /FVC, % of predicted	97.56 ± 0.71	70.41 ± 0.46***
PaO ₂ (mmHg)		57.51 ± 0.11
SaO ₂ (%)		90.62 ± 0.19
COPD co-morbidities		
Type 2 diabetes (n)		41 (32.28)
Pulmonary hypertension (n)		52 (40.94)
Cardiovascular disease (n)		27 (21.25)

Variables are expressed as Mean ± SEM or number (%), standard error of the mean; Significance of difference to control: **p* < 0.05, ***p* < 0.001, ****p* < 0.001. COPD Chronic Obstructive Pulmonary Disease, BMI Body Mass Index, FEV₁ forced expiratory volume in 1 s, FVC force vital capacity, FEV₁/FVC forced expiratory volume in 1 s/forced vital capacity ratio, PaO₂ Partial pressure of oxygen, SaO₂ Arterial oxygen saturation, n number

were reviewed by respiratory physiologist. Each patient was checked 10–15 min after administration of 200 µg of salbutamol. Only patients who showed airway obstruction reversibility <12% of forced expiratory volume in 1 s (FEV₁) and or forced vital capacity FVC, were retained for the study. The predicted values for FEV₁, FVC and FEV₁/FVC were generated for COPD severity. The Pulmonary function test was performed using Mir Spiro Lab II Spirometer (MIR s.r.l, Roma, Italy). At least 3 acceptable and two reproducible curves (the two highest FVC and FEV₁ being within 200 ml of each other) were obtained. Patients which showed highest values of FEV₁ were selected for analysis. Spirometry measurements in COPD patients were performed as per Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [26]. The GOLD stage classification of disease severity was done as per Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [26]. GOLD stage I (mild): ≥ 80%; GOLD stage II (moderate): 50–70%; GOLD stage III (severe): 30–49% and GOLD stage IV (very severe) < 30% predicted.

Among the selected COPD study group, we found three types of comorbid diseases including type 2 diabetes, some cases with pulmonary hypertension and some cases with cardiovascular disease. These were noted for further analysis. Among the COPD patients comorbid disease analyses were limited to subjects in whom complete data on demographic factors, risk factors and baseline comorbid disease were available. The 59 volunteers selected as controls were recruited from the general population (Among these three were ex-smokers, 17 current smokers and 39 non-smokers) with

Table 2 Association of significant clinical characteristics of the COPD patients according to GOLD Stage classification using Spirometry parameters

GOLD stage classification of COPD patients			
Spirometry parameter	GOLD stage II Mean ± SEM (n = 28)	GOLD stage III Mean ± SEM (n = 42)	GOLD stage IV Mean ± SEM (n = 57)
FEV ₁ , % predicted	60.08 ± 0.38**	38.85 ± 0.31**	20.76 ± 0.25**
FVC, % predicted	85.91 ± 0.47**	58.32 ± 0.38**	42.18 ± 0.37**
FEV ₁ /FVC, % predicted	70.21 ± 0.46	67.18 ± 0.05**	50.55 ± 0.04**
PaO ₂ (mmHg)	57.08 ± 0.27	57.60 ± 0.20	57.60 ± 0.16
SaO ₂ (%)	87.33 ± 0.33**	89.92 ± 0.34**	92.23 ± 0.29**

Variables are expressed as Mean ± SEM, standard error of the mean; All *p* values were calculated using ANOVA with Bonferroni post hoc test. Non-significant *p* values, *p* > 0.05, ***p* < 0.01. FEV₁: GOLD stage II vs. III vs. IV, ***p* < 0.01; FVC: GOLD stage II vs. III vs. IV ***p* < 0.01; FEV₁/FVC: GOLD stage II vs III, *p* > 0.05; GOLD stage II vs. III vs. IV, ***p* < 0.01; PaO₂ (mmHg): GOLD stage II vs. III vs. IV, *p* > 0.05; SaO₂ (%): GOLD stage II vs III vs. IV *p* > 0.05; GOLD stage II vs IV, ***p* < 0.01 COPD Chronic Obstructive Pulmonary Disease, GOLD Global initiative for chronic Obstructive Lung Disease; GOLD stage classification: GOLD II- Moderate COPD, GOLD III - Severe COPD, GOLD IV - Very severe COPD; FEV₁ forced expiratory volume in 1 s, FVC force vital capacity, FEV₁/FVC forced expiratory volume in 1 s/forced vital capacity ratio; PaO₂ Partial pressure of oxygen, SaO₂ Arterial oxygen saturation

no respiratory problems. Inclusion criteria: **Exclusion criteria:** All those subjects with very severe conditions, pregnant women and children with severe cough were excluded from the study.

Blood sample collection and processing

Blood samples from COPD patients and controls (5 ml/individual) was collected in anticoagulant vacutainers and processed immediately for analysis. Blood samples were first centrifuged at 3000 rpm (5430R, Eppendorf, Germany) for 10 min and the plasma was removed. Then packed erythrocytes cells were washed three times with 0.9% NaCl hypotonic solution and washed erythrocytes were lysed using ice-cold distilled water and stored at -20 °C to determine the antioxidant enzyme estimation. The erythrocyte hemolysate was prepared as mentioned above using venous blood samples from the study group.

Estimation of antioxidant enzymes in erythrocytes

Erythrocyte hemolysates were used for the estimation of SOD, CAT, GPx, GST, GR and TAS. SOD (EC 1.15.1.1) activity was measured based on inhibition of pyrogallol oxidation by the method of Marklund and Marklund [27]. CAT (EC 1.11.1.16) activity was determined by the method of Aebi [28]. Glutathione-s-transferase [GST] (EC2.5.1.18) activity was assayed according to the method described by Beutler [29] via measurement of the conjugation product of GSH with 1-chloro-2, 4-dinitrobenzene (CDNB). Glutathione peroxidase (EC 1.11.1.7) activity was measured according to the method described by Flohe and Gunzler [30]. Glutathione reductase (EC 1.6.4.2) activity was measured by the method of Carlberg and Mannervik [31]. The total antioxidant power, expressed as the Ferric Reducing Ability of the Plasma (FRAP) in erythrocytes, was analyzed by the method described by Benzie and Strain [32]. The TAS was determined using a different concentration of Ferric (II) sulfate as standard. The results were expressed in μM Fe²⁺/L equivalent.

Statistical analyses

All the demographic data and spirometric parameters are presented as mean values ± Standard Error of the Mean (SEM) using non-parametric statistics. Normality distribution was analyzed using Shapiro-Wilk test and the two-tailed *t-test* was used to analyze the statistical significance for analyses between controls and COPD patients. Comparisons between erythrocyte antioxidant enzyme activities on disease severity GOLD stages I, II, III and IV and COPD comorbidities were tested by ANOVA; post hoc test for multiple comparisons using Bonferroni correction were performed using GraphPad Prism 6 (GraphPad Software Inc, San Diego, CA, USA). GOLD stage I was excluded from the study. The

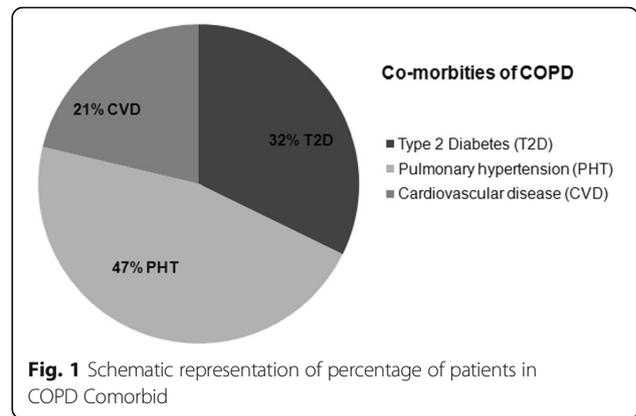


Fig. 1 Schematic representation of percentage of patients in COPD Comorbid

relationship between antioxidant enzyme activities and FEV₁ among COPD patients were evaluated using Pearson's coefficients of correlation. Furthermore, a multivariate logistic regression model was constructed to determine Odds ratios (OR), 95% confidence intervals (95% CI) and significant of severity of COPD and COPD comorbidities have been calculated in order to correlate the erythrocyte antioxidant enzyme activities were analyze using the XLSTAT statistical analysis (Addinsoft, New York, NY, USA). A *p*-value of less than 0.05 (*p* < 0.05) was considered as statistically significant.

Results

Normality analysis showed that all the variables were normally distributed. The demographic characteristics of COPD patients and healthy controls are presented in Table 1. We found that the mean age of COPD patients was > 60 years, whereas the mean age of control group was >50 years old. The mean BMI was significantly lower than that of controls (*p* < 0.05). The smoking status in COPD patients in this study was as follows: there were 39.37% ex-smokers, 25.19% current smokers and 35.43% non-smokers, whereas in controls we had 22.03% ex-smokers, 28.18% current smokers and 49.15% non-smokers respectively, Table 1. Spirometry measurements in COPD patients as per GOLD stage classification of

Table 3 Significant association of antioxidant enzyme activities in cases and controls

Study parameters	Healthy control <i>n</i> = 59	COPD <i>n</i> = 127
Superoxide dismutase (U/mg protein)	87.56 ± 0.48	77.34 ± 0.48***
Catalase (U/mg protein)	98.34 ± 0.94	72.77 ± 0.60***
Glutathione s transferase (U/mg protein)	42.05 ± 0.56	21.30 ± 0.33***
Glutathione peroxidase (U/mg protein)	63.77 ± 0.44	59.43 ± 0.50***
Glutathione reductase (U/mg protein)	77.08 ± 0.96	61.97 ± 0.60***
Total antioxidant status (μM Fe ²⁺ /l)	46.19 ± 0.27	41.64 ± 0.30***

Variables are expressed as mean ± SEM. Significance of difference between COPD patients compared to controls: ****P* < 0.001

disease severity are presented in Table 2. There were no patients belonging to GOLD stage I, GOLD stage II had 22.04%, GOLD stage III had 33.07 and 44.88% were in GOLD stage IV. The pulmonary function test, assessed as FEV₁, FVC and the ratio of FEV₁/FVC, showed significantly decrease in the mean value from GOLD stage II to IV in COPD patients ($p < 0.001$; ANOVA followed by Bonferroni post hoc test) Table 2. No significant difference in PaO₂ between GOLD stages II to IV ($p > 0.05$) was observed Table 2. A similar examination of SaO₂ revealed a significant increase in levels from GOLD stage II to IV

($p < 0.05$). Comorbidities of COPD patients are summarized in Fig. 1. Of the 127 patients diagnosed with COPD, we found that 41 (52.07%) were Type II diabetic, 52 (74.93%) had pulmonary hypertension and 27 (34.29%) had cardiovascular disease.

A significant decrease in the first and second-step antioxidant enzymes SOD and CAT activities was found in COPD patients ($p < 0.001$). Similar significant decrease was also observed in the erythrocyte GST, GPx, GR and TAS in COPD patients compared to controls ($p < 0.001$), results are presented in Table 3. On

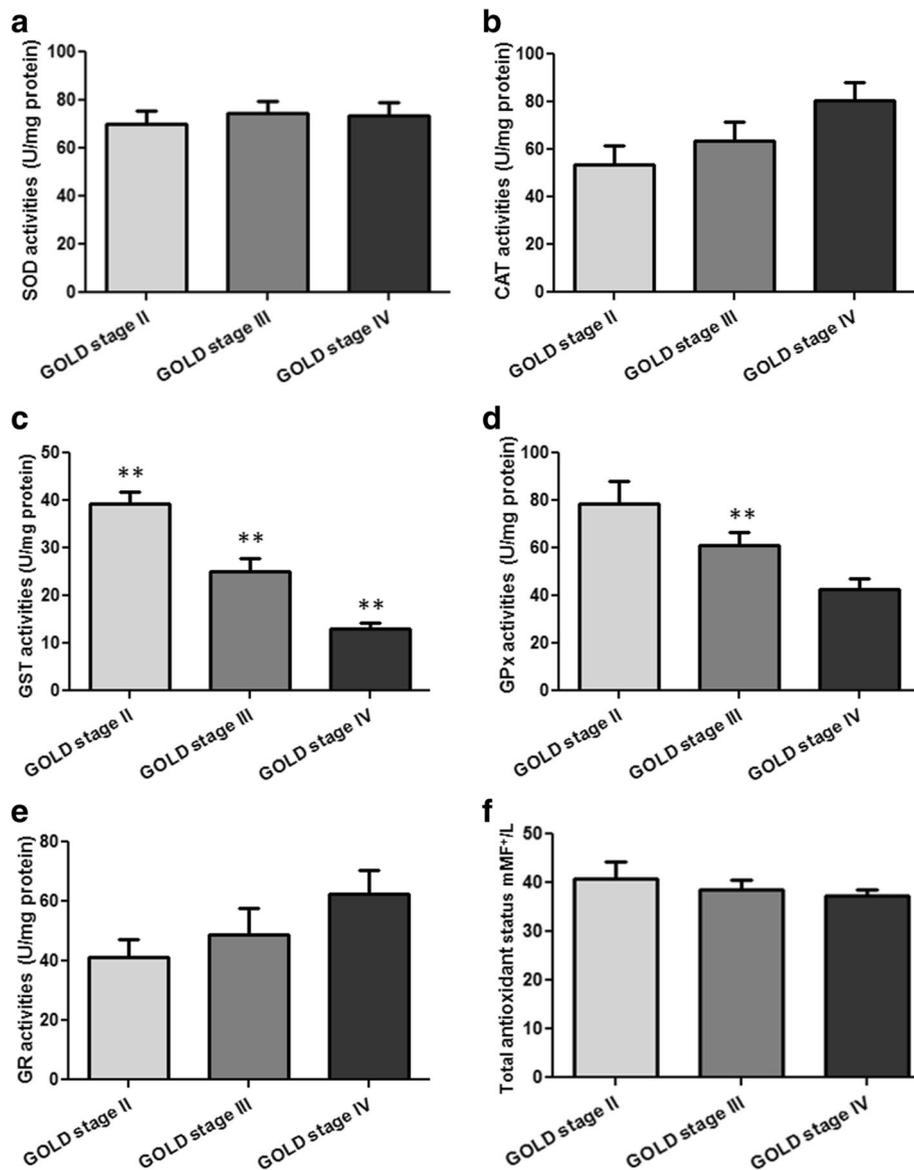


Fig. 2 Comparison of antioxidant enzyme activities according to severity of COPD using GOLD stage classification (GOLD stage II, III and IV): Erythrocytes (a) SOD, superoxide dismutase; b CAT, catalase; c GST, glutathione-s-transferase; d GPx, glutathione peroxidase; e GR, glutathione reductase; expressed as U/mg protein and (f) TAS, total antioxidant status; expressed as μM Fe²⁺/L. Data expressed as Mean ± SEM: standard error of the mean; Significant of differences and respective p values $**p < 0.001$ are mentioned on the graph using One-way ANOVA followed by Bonferroni's multiple comparison post hoc test

comparison of erythrocyte antioxidant enzyme activities with severity of COPD, we found an increase in the mean value of SOD and CAT in stages II to IV however, this increase was not statistically significant ($p > 0.05$; Fig. 2a and b). Conversely, a significant decrease in the mean value of GST ($p < 0.001$) and GPx ($p < 0.01$) was observed in disease stages II, III and IV respectively, (Fig. 2c and d). In contrast, the mean value of GR activity increased from stage II to IV ($p > 0.05$; Fig. 2e). A decrease in the mean value of TAS was observed in stages II, III and IV ($p > 0.05$; Fig. 2f).

Variations in Pearson's coefficients of correlation values between erythrocyte anti-oxidation enzyme activities with FEV₁ (% predicted) in COPD patients are presented in Table 4. We observed negative correlation between erythrocyte SOD, CAT and TAS with FEV₁ in COPD patients ($r = -0.182$, $r = -0.263$ and $r = -0.183$; $p < 0.05$) whereas GR was not statistically significant ($r = -0.094$; $p > 0.05$). GST and GPx only entered as a model of antioxidant enzymes, showed a significant positive correlation with FEV₁ in COPD patients ($r = 0.630$ and $r = 0.030$, respectively) (Table 4). Further, correlation analysis was performed with severity of COPD using GOLD stage classification between erythrocyte antioxidant enzyme activities with FEV₁ is presented in Table 5. In COPD patients, FEV₁ showed a significant negative correlation with GST GOLD stage II ($r = -0.735$, $p < 0.001$) and a positive correlation with GOLD stages III and IV ($r = 0.098$ and $r = 0.102$, respectively). FEV₁ correlated negatively with GR activity in GOLD stages II and III ($r = -0.474$, $p < 0.05$ and $r = -0.315$, $p < 0.05$; respectively) and border line negative significant with GOLD stage III ($r = -0.242$, $p < 0.07$), TAS also showed a significant negative correlation with FEV₁ ($r = -0.383$, $p < 0.05$) in GOLD stage III, Table 5. To clarify whether the antioxidant enzyme activity and the severity COPD were independently related, a multivariate logistic regression analysis was performed (Table 6). Antioxidant enzyme activities were log transformed prior to analysis. Study variables with a Pearson correlation of

Table 4 Coefficients of correlation values between erythrocyte anti-oxidation enzyme activities with FEV₁ (% predicted) in COPD patients

Study parameters	Correlation coefficients (r)*
Superoxide dismutase (U/mg protein)	-0.182*
Catalase (U/mg protein)	-0.263*
Glutathione s transferase (U/mg protein)	0.630**
Glutathione peroxidase (U/mg protein)	0.030
Glutathione reductase (U/mg protein)	-0.094
Total antioxidant status (μM Fe ²⁺ /l)	-0.183*

Significance of correlation between erythrocyte antioxidant enzyme activities with FEV₁ (% predicted). * $p < 0.05$, ** $p < 0.001$, 'r' denotes Pearson correlation coefficient

Table 5 Coefficients of correlation values between erythrocyte anti-oxidation enzyme activities with FEV₁ (% predicted) in COPD patients according to GOLD stage classification

GOLD stage classification of COPD			
Study parameters	GOLD stage II coefficient (r)	GOLD stage III coefficient (r)	GOLD stage IV coefficient (r)
Superoxide dismutase (U/mg protein)	0.20	0.00	-0.21
Catalase (U/mg protein)	-0.09	-0.21	0.00
Glutathione s transferase (U/mg protein)	-0.73**	0.09	0.10
Glutathione peroxidase (U/mg protein)	0.24	0.02	-0.11
Glutathione reductase (U/mg protein)	-0.47*	-0.31*	-0.24 [†]
Total antioxidant status (μM Fe ²⁺ /l)	-0.07	-0.38*	0.20

Significance of correlation between erythrocyte antioxidant enzymes activities with FEV₁ (% predicted) according to GOLD stages:[†]
 $p < 0.07$, Border line significance, * $p < 0.05$; ** $p < 0.001$, 'r' denotes Pearson correlation coefficient

Table 6 Multivariate logistic regression analysis adusted odds ratio (95%CL) for erythrocyte anti-oxidation enzyme activities with severity of COPD according to GOLD stage classification, GOLD stage II, III and IV

Variables	GOLD stages	OR	95% CI	p-values
Superoxide dismutase (U/mg protein)	II	0.97	-0.05 – 0.01	0.173
	III	0.99	-0.02 – 0.01	0.686
	IV	1.00	-0.00 – 0.02	0.292
Catalase (U/mg protein)	II	0.99	-0.02 – 0.00	0.321
	III	0.99	-0.01 – 0.00	0.551
	IV	1.00	-0.00 – 0.01	0.849
Glutathione s transferase (U/mg protein)	II	1.11	0.04 – 0.18	<0.001
	III	1.01	-0.01 – 0.05	0.284
	IV	0.93	-0.10 – 0.01	<0.006
Glutathione peroxidase (U/mg protein)	II	1.02	-0.00 – 0.05	0.082
	III	1.00	-0.01 – 0.01	0.825
	IV	0.98	-0.03 – 0.00	<0.034
Glutathione reductase (U/mg protein)	II	0.98	-0.04 – 0.00	0.088
	III	0.99	-0.01 – 0.00	0.507
	IV	1.00	-0.00 – 0.01	0.313
Total antioxidant status (μM Fe ²⁺ /l)	II	0.99	-0.08 – 0.07	0.886
	III	0.97	-0.07 – 0.02	0.274
	IV	0.95	-0.08 – 0.00	<0.049

Significance of difference between erythrocyte antioxidant enzymes activities with FEV₁ (% predicted) $p < 0.05$; OR odds ratio, CI confidence interval

coefficient less than 0.11 that did not show multicollinearity with COPD severity: GOLD stage II, III and IV. A multivariate logistic regression analysis confirmed significant independent association between GST (OR = 0.93; 95% CI = -0.10 – 0.01; $p < 0.00$), GPx (OR = 0.98; 95% CI = -0.03 – 0.00; $p < 0.05$) and TAS (OR = 0.95; 95% CI = -0.08 – 0.00; $p < 0.05$) and FEV₁ in GOLD stage IV. A significant association was observed with GST (OR = 1.11; 95% CI = 0.04 – 0.18; $p < 0.001$), whereas, a border line significant of GPx (OR = 1.02; 95% CI = -0.00 – 0.05; $p < 0.08$) and GR (OR = 0.98; 95% CI = -0.04 – 0.00; $p < 0.08$) activities was observed in GOLD stage II, Table 6.

The decrease in the mean value of SOD, CAT in patients of COPD with type 2 diabetes, pulmonary hypertension and cardiovascular disease compared to healthy controls are shown in (Fig. 3a and b). However, this decrease was not statistically significant ($p > 0.05$). Similar result was also noticed in GST and GR activities in COPD patients with type 2 diabetes, pulmonary hypertension and cardiovascular disease when compared to healthy controls (Fig. 3c and d). A significant decrease in the GPx activity ($p < 0.001$; Fig. 3d) and TAS ($p < 0.05$; Fig. 3f) in COPD patients with type 2 diabetes were observed compared to healthy controls. No significant differences of GPx and TAS were observed in COPD patients with pulmonary hypertension and cardiovascular disease. However, in COPD comorbidities multivariate logistic regression analysis confirmed a border line significant difference in GPx activity in COPD with T2D (OR = 0.04; 95% CI = -6.60 - 0.53; $p = 0.09$), and GST activity in COPD - CVD (OR = 2.51; 95% CI = 0.00 - 1.84; $p < 0.05$) patients when compared to non-comorbid patients, Table 7.

Discussion

It is now evident that COPD is a leading cause of hospitalizations in adults, particularly older people. In fact, recent medical research indicates that antioxidants eventually could represent a promising therapy for COPD. Further, comorbidities have been a common cause, contributing to many of these ICU hospitalizations. This investigation has determined important risk factors in COPD namely the role of erythrocyte antioxidants related to disease severity. An important feature in the pathogenesis of COPD is oxidative stress. The relationship between COPD and oxidative stress parameters has been extensively studied in different populations across the world [33, 34], but very few studies are available in the Indian population, especially in south India [35]. This study showed that COPD patients had decreased SOD activity as compared to controls, which has been one of the most consistent findings across several studies in many countries [36]. In the present study, CAT activity was found to be reduced in COPD patients. Consistent with our result, a study in

erythrocyte red blood cells showed decreased CAT activity in patients with COPD [35, 36]. In contrast, a study on single nucleotide polymorphism in CAT gene (-262 C > T) showed that erythrocyte catalase activity was higher in COPD patients with the C/C genotype, and with the combined C/T and T/T genotypes, than in healthy controls [37]. Our study supports the finding of Tavailani et al., which reported an increase in SOD and CAT activities in alveolar macrophages of elderly smokers [38]. Kirkil et al. reported that the plasma levels of SOD and CAT decreased in the COPD group [39]. However, we found a dramatic increase in SOD and CAT activities among patients with more severe COPD. It was found that erythrocyte SOD and CAT activities gradually increased from GOLD stages I to IV in COPD patients, which is supported by previous reports [36–41]. Indeed, various studies [40–42] except one study [7] have suggested that certain markers of oxidative stress may be related to tobacco smoking or to the severity of COPD.

In the present study, we found that erythrocyte GST activity significantly decreased in COPD patients. A possible explanation for this decrease in activity of GST could be that in COPD patients the increase in toxic substrates in the body, reduces the GST activity which was inversely proportional to the increased FEV₁. Also it is known that GST is influenced by nuclear factor, erythroid-derived 2, like 2 (Nrf2), a transcription factor that contributes to the induction of several protective enzymes during oxidative stress [43]. Another possible explanation could be due to the high levels of GSH in the epithelial lining of the lungs, which regulate GSH homeostasis by the GST enzymes that participate in the transport and detoxification mechanism.

Hence it is suggested that there may be a strong association of GST with lung function at the molecular level [44–48]. Interestingly, in this investigation, we also found GST activity was not only lower in COPD but also progressively decreased with the severity of COPD disease. Our study observed not only lowered level of GPx activity in COPD patients, but also a uniform decrease in GPx activities from GOLD stage II to IV. These findings suggest that the lower activity of GPx in erythrocytes may be associated with the severity of COPD. However, there is recent evidence suggesting that transgenic GPx-1 mice protect lungs from cigarette smoke-induced lung inflammation [49]. Our present study found a significant positive correlation between GPx and FEV₁ in COPD patients.

It was observed that erythrocyte GR activity was lower in COPD patients, similar findings were also reported by a few authors [38, 50] whereas, Gosker et al. reported an increase glutathione activity in skeletal muscle of patients with COPD [51]. This investigation supports the fact that in COPD patients oxidative stress deactivates

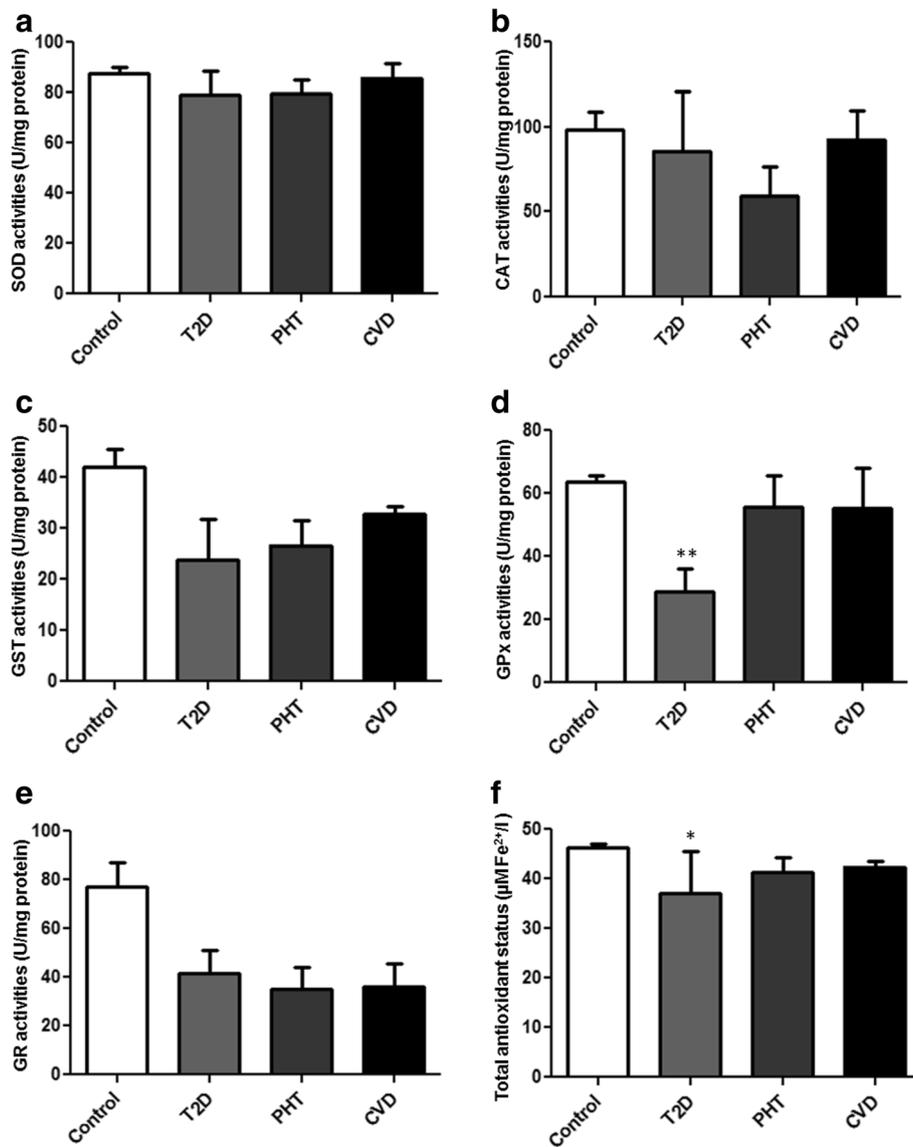


Fig. 3 Antioxidant enzyme activities in COPD patients with T2D, PHT and CVD and compared with the healthy controls. Erythrocytes (a) SOD, superoxide dismutase; (b) CAT, catalase; (c) GST, glutathione-s-transferase; (d) GPx, glutathione peroxidase; (e) GR, glutathione reductase; expressed as U/mg protein and (f) TAS, total antioxidant status; expressed as $\mu\text{M Fe}^{2+}/\text{l}$. Data expressed as Mean \pm SEM: standard error of the mean; Significance of difference between COPD and type 2 diabetes (T2D), pulmonary hypertension (PHT) and cardiovascular disease (CVD) to control: Significant differences and respective p values * $p < 0.05$, ** $p < 0.001$ are mentioned on the graph using one-way ANOVA followed by Bonferroni's multiple comparison post hoc test

the regulatory enzymes such as GPx, GST and GR involved in the GSH/GSSG redox system which may be involved in the protection of cellular and mitochondrial functions in the lungs. Activity of GR increased from GOLD stage II to IV, TAS in erythrocytes was lower in COPD patients. A decrease in TAS suggests that there was an increase in oxidative stress in COPD patients; Rahman et al [52] also reported similar findings. With the increase in severity of COPD from GOLD stage II to IV we observed a significant decrease in the level of TAS. Correlation analysis revealed a significant negative

correlation between erythrocyte SOD, CAT, GR and TAS with FEV₁ in COPD patients. Previously a significant positive correlation between SOD and CAT with FEV₁ was described in erythrocytes of COPD patients [35, 36]. One reason for failing to find a significant positive correlation between erythrocyte SOD or CAT and FEV₁ may be due to the phenomenon that various enzymatic systems differ substantially in their responses to smoking-induced increases in oxidative stress [53].

In this study, of all the enzymes assayed, only GST and GPx showed a significant positive correlation with FEV₁

Table 7 Multivariate logistic regression analysis adjusted odds ratio (95%CL) for antioxidant enzyme activities with COPD comorbidities: type 2 diabetes, pulmonary hypertension and cardiovascular

Variables	COPD co-morbidities	OR	95% CI	p-values
Superoxide dismutase (U/mg protein)	T2D	1.99	-1.11 – 2.49	0.453
	PHT	1.61	-0.57 – 1.53	0.374
	CVD	2.32	-0.35 – 2.04	0.169
Catalase (U/mg protein)	T2D	1.98	-0.62 – 2.00	0.306
	PHT	0.80	-1.14 – 0.70	0.642
	CVD	1.77	-0.30 – 1.45	0.199
Glutathione s transferase (U/mg protein)	T2D	0.90	-1.39 – 1.18	0.873
	PHT	1.41	-0.57 – 1.26	0.462
	CVD	2.51	0.00 – 1.84	<0.049
Glutathione peroxidase (U/mg protein)	T2D	0.04	-6.60 – 0.53	<0.096
	PHT	0.82	-1.12 – 0.72	0.677
	CVD	0.63	-1.52 – 0.59	0.392
Glutathione reductase (U/mg protein)	T2D	0.58	-1.76 – 0.70	0.400
	PHT	0.52	-1.67 – 0.40	0.228
	CVD	0.57	-1.60 – 0.49	0.299
Total antioxidant status ($\mu\text{M Fe}^{2+}/\text{l}$)	T2D	1.04	-1.07 – 1.16	0.934
	PHT	1.26	-0.61 – 1.08	0.589
	CVD	1.69	-0.45 – 1.50	0.291

Significance of difference between erythrocyte antioxidant enzyme activities with COPD comorbidities: type 2 diabetes (T2D), pulmonary hypertension (PHT) and cardiovascular disease (CVD) $p < 0.05$; Border line significance: if $p < 0.09$; OR odds ratio, CI confidence interval

in COPD patients. Previous reports have also found a significant positive correlation between erythrocytes GST activity [35] and GPx [36] with lung function, reflected by FEV₁. In agreement with the positive correlation of GST and GPx in erythrocyte of COPD patients, a recent study has shown a significant direct relationship between FEV₁ and erythrocyte GPx activity [48]. To our knowledge, there are no previous studies which have evaluated the erythrocytes antioxidant enzyme activities correlating with disease severity using pulmonary function test assessed as FEV₁ in COPD. We found that SOD and GPx activities were positively correlated with FEV₁. Conversely, CAT, GST, GR and TAS showed significant negative correlation with FEV₁. The risk conferred by GST and GPx towards progression of the COPD severity was further evaluated using multinomial logistic regression analysis by taking GOLD stages as reference category. Increase in GST and GPx activities was found to confer greater risk of progression to GOLD stage II and IV. Kluchová et al [48] reported a similar trend. In their study, the authors measured and compared GPx activity in patients with moderate and severe COPD. They found that a significant direct relationship

between FEV₁ and erythrocyte GPx activity with severity of COPD. Our findings extend those of Duthie et al. who reported that erythrocyte GPx activity is associated not only with smoking status but also with severity of COPD [54]. The significant correlation found between the enzymes and FEV₁ suggests that decrease in antioxidant enzyme activities might be the result of greater airway obstruction in COPD patients.

An interesting finding in the current analysis was the antioxidant enzyme activities in COPD comorbidities. We found no significant variations in erythrocyte SOD, CAT, GST and GR activity in COPD patients with type 2 diabetes, pulmonary hypertension and cardiovascular disease compared to control group. Whereas, erythrocyte GPx activity and TAS showed a significant decrease in the mean value of COPD patients with type 2 diabetes. Joppa et al. also reported a significant decrease in the GPx activity in erythrocyte of COPD patients with and without pulmonary hypertension [55]. Some of the previous finding of Orhan et al. showed a significant increase in plasma GPx activity in hypertensive pre-eclamptic pregnancy and also in insulin dependent diabetic pregnancy [56]. Further, it was observed that the standard dietary treatment for type 2 diabetic patients produced an increase of the SOD and GPx activities [57].

However, this may suggest that the COPD comorbidities are an independent risk factor for the development of COPD. This finding was substantiated by multivariate logistic regression analysis, which demonstrated that the GPx activity was significantly independently association of COPD – type 2 diabetes. Whereas, our present study showed that there was no statistically significant independent association of antioxidant enzyme activity in COPD – pulmonary hypertension. Similar finding was report by Al Shebly et al [58]; this study found no statistically significant difference in plasma GSH-Red activity during labor in diabetic, hypertensive, and control women. More recently, it was clarified that a significant increase of GPx and GSH activities in hypertensive disorders of pregnant women.

Logistic regression analysis of comorbidities, in our study, showed significant difference in GST activity between COPD - cardiovascular disease patients and GPx activity in COPD - type 2 diabetes patients and patients without comorbidities, suggesting that these enzymes could serve as a critical marker to assess oxidative damage in COPD patients with cardiovascular disease and type 2 diabetes. Conversely, there was no significant difference in the activities of the other antioxidant enzymes in patients with and without comorbidities, which could indicate that these comorbidities may not confer significant additive oxidative stress in the COPD patients. Therefore, we propose that elevated level of GPx activity in COPD patients with type 2 diabetes may be a useful

biomarker linked to an increase in oxidative stress. Surprisingly, our present study showed no significant enzyme activities in COPD patients with pulmonary hypertension. However, there are a number of conflicting reports as well [59, 60]. Hence, our study may have useful clinical implications in view of increased understanding of COPD risk parameters with comorbid diseases.

Conclusions

The present study provides a strong evidence for the oxidant-antioxidant imbalance in the pathogenesis of COPD. Our study is the first, to our knowledge, to examine the correlation between FEV₁ and antioxidant enzyme activities among the different stages of COPD and highlights a strong correlation between FEV₁, GST and GPx enzyme activity with severity of the COPD disease. This study also demonstrated for the first time that there exists an oxidative stress in COPD patients with comorbidities such as type 2 diabetes, pulmonary hypertension and cardiovascular disease. Further, it is likely that antioxidants may have in future combination therapies for COPD patients. This study may have useful clinical implications in view of increased understanding of COPD risk parameters. It has also been recognized that additional burden of comorbidities has negative impact on economics and also patient related outcomes.

Abbreviations

CAT: Catalase; COPD: Chronic obstructive pulmonary disease; CVD: Cardiovascular disease; FEV₁: Forced expiratory volume in 1 s; FEV₁/FVC: Forced expiratory volume in 1 s/forced vital capacity ratio; FVC: Force vital capacity; GOLD: Global initiative for chronic obstructive lung disease; GPx: Glutathione peroxidase; GR: Glutathione reductase activities; GST: Glutathione-s-transferase; NCMH: National commission on macroeconomics and health; PaO₂: Partial pressure of oxygen; PHT: Pulmonary hypertension; SaO₂: Arterial oxygen saturation; SOD: Superoxide dismutase; T2D: Type 2 diabetes; TAS: Total antioxidant status

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author.

Authors' contributions

MA and KJ designed the research project. MA, GV, MSA, SVR and KJ analyzed the data. All authors contributed in writing, revising and approving the manuscript for submission. All authors read and approved the final manuscript.

Authors' information

GV, SVR and KJ are professor. MSA is senior pulmonologist and MA is Assistant Director.

Competing interests

The authors declare that they have no conflict of interest.

Consent for publication

The patients and controls were informed about the study and purpose; and gave written informed consent before inclusion in the study group.

Ethics approval and consent to participate

The study was conducted in accordance with the guidelines of Committee responsible for human studies as guided by the "Declaration of Helsinki" and good clinical practice. The study was approved by the Ethics Committee of Mahavir Hospital and Research Centre – Hyderabad.

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