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Biomarkers of inflammation and oxidative stress in smokers and severe chronic obstructive pulmonary disease patients

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ABSTRACT

Introduction: Identification of biomarkers for the novel therapeutics targets of Chronic obstructive pulmonary disease (COPD) is an important area of current research. In this study, the level of inflammatory cytokines was investigated and correlated these levels with erythrocytic antioxidant activities in COPD patients with smokers and without smokers and parameters of severity.

Material and Methods: Plasma levels of Interleukin (IL)-6, IL-8 and IL-10 concentrations were assayed by means of Enzyme-linked Immunosorbent Assay (ELISA) and erythrocytic glutathione-s-transferase (GST) and glutathione peroxidase (GPx) activities were estimated by spectrophotometric method.

Results: Both IL-6 and IL-8 plasma levels showed a statistically significantly higher in COPD patients as compared to healthy controls ($p<0.001$; $p<0.05$). In contrast, the IL-10 was lower in COPD patients as compared to control group ($p<0.05$). FEV1 was significantly negatively correlated with plasma IL-6 ($r= - 0.565$, $p< 0.001$) and IL-8 ($r= -0.453$, $p <0.05$). Plasma IL-6 was found negative association with erythrocytic GST ($r= - 0.018$, $p>0.05$) and GPx activities ($r= - 0.080$, $p>0.05$). Similarly IL-8 was also found negative association with GST ($r= - 0.260$, $p>0.05$) and GPx activities ($r= - 0.268$, $p>0.05$). Whereas, a significant positive association was observed between IL-10 and erythrocytic GST ($r= 0.494$, $p <0.05$) and GPx activities ($r= 0.546$, $p < 0.001$).

Conclusion: In conclusion, Plasma levels of inflammatory cytokines IL-6 and IL-8 are related with severity of COPD and IL-10 and oxidative stress markers GST and GPx are co-dependent and strongly interrelated processes and may be used as a potential marker for the evaluation of COPD.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is described as a disease which is poorly reversible and progressive airflow obstruction that is typically associated with lung inflammation. COPD is a major, increasing global health problem and it is estimated that 64 million people are affected by COPD and it has been projected that COPD could be the 3rd biggest cause of mortality by 2030 worldwide (World Health Organization [WHO] statistics).[1] Tobacco smoking is the primary risk factor linked to COPD in prosperous Westernized countries and environmental pollution, especially indoor biomass smoke, is associated with increasing prevalence of COPD in the developing world.[2] It has long been proven that cigarette smoking is strictly correlated with the risk factor in the development of COPD.[3,4]

Oxidative stress can trigger various intracellular signaling pathways such as transcription factor, nuclear factor- kB (NF-κB) and activator protein (AP-1) that can trigger various inflammatory cytokines associated with airway inflammation in COPD. The inflammatory process of COPD is associated with cigarette smoking that initiates the key processes that cause airway inflammation

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and structural changes in COPD patients.[5] Inflammatory cytokines are the key biomarkers of circulating leukocytes. Upon activation, the circulating leukocytes secrete a cocktail of inflammatory molecules that initiate the process of inflammation locally and systemically.

Interleukin-6 (IL-6) is a pro & anti-inflammatory cytokine that is secreted by T cells and macrophages. Increased levels of IL-6 were found in the sputum, exhaled breath and plasma of patients with COPD, particularly during exacerbations Interleukin-8 (IL-8) is a pro-inflammatory cytokine, produced by macropahages/monocytes and other tissue cell types such as airway smooth muscle cells, epithelial cells, and endothelial cells. IL-8 is an important potent activator of neutrophils and it has been shown that a significant amount of neutrophils accumulate in a variety of lung diseases, including COPD.[6,7] A few studies have reported higher number of neutrophils and IL-8 levels in sputum and broncho-alveolar lavage of COPD patients.[8,9] Interleukin-10 (IL-10) is a major anti-inflammatory cytokine that inhibits the production of pro-inflammatory mediators. Therefore, IL-10 possesses anti-inflammatory effectsand can directly regulate the T-cell activation and differentiation in various tissues, resulting in reduced immunopathology.[10,11] Moreover, an increase in levels of the anti-inflammatory cytokine IL-10 has been observed in the alveolar macrophages of cigarette smokers and COPD patients.[12] In contrast, IL-10 level were reported to be lower in the sputum of COPD patients.[13]

Numerous studies have shown that oxidative stress and inflammation play a pivotal role in the pathogenesis of COPD.[14,15] 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.[16] Recently, Sadowska et al, demonstrated a significant relationship between GPx in blood and serum IL-8 in patients with stable COPD.[17] Another study demonstrated an inverse relationship between the circulating inflammatory cells and erythrocyte GPx activity.[18]

Since these data suggest that there may be a link between oxidative stress and inflammation; therefore, it is of great interest to study the roles of inflammatory cytokine levels and correlate with the antioxidant enzyme activity in the pathogenesis of COPD. Our study is the first of its kind in South Indian population that examined the role of distinct biological processes such as imbalance of antioxidant enzymes activities and levels of inflammatory cytokines in correlation to COPD pathophysiology and has the potential for identification of novel targets and biomarkers that may lead to better treatment of COPD.

2. Material and Methods

The study population included 127 COPD patients and 59 healthy controls. The average age of the study group ranged from 50 - 60 years. The patients and controls were informed about the study and purpose, and gave written informed consent before inclusion in the study group and were willing to donate the blood samples. All study subjects underwent a standardized clinical examination at the Outpatients clinic at Mahavir Hospital and Research Centre, Hyderabad, India. The study was conducted in accordance with the guidelines of the 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.

2.1. 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 were reviewed by the respiratory physiologist.

2.2. Exclusion criteria

All those subjects with very severe conditions, pregnant women and children with severe cough were excluded from the study.

2.3. Pulmonary function tests

All study subjects underwent pulmonary function test using the Spirometer EasyOne® (Fleximed, and Medizintechnik AG, Zürich, Switzerland) and only patients who showed airway obstruction reversibility <12% of forced expiratory volume in 1 s (FEV1), checked 10-15 min after administration of 200μg of salbutamol, were retained for the study. The methods used were recommended by ATS.[19] COPD patients were required to have FEV1/FVC ratio<70%, and COPD staging was done based on FEV1 values according to Global Initiative for Chronic Obstructive Lung Disease guidelines (GOLD 2018).[20] The stages were: GOLD stage I (mild): $\geq$ 80%; GOLD stage II (moderate): 50-70%; GOLD stage III (severe): 30-49% and GOLD stage IV (very severe) < 30% predicted.

2.4. Blood Sample collection and processing

The blood was collected in vacutainer tubes (BD Vacutainers® Blood Collection tubes, USA) containing Potassium Ethylenediaminetetraacetic acid (K2 EDTA), as anticoagulant. Five ml of intravenous blood was collected into EDTA tubes with disposable needles and the blood samples were transferred from the site of collection to the research lab in thermocol boxes with ice packs. Blood samples containing tubes were first centrifuged at 3000

rpm (5430R, Eppendorf, Germany) for 10 min and the plasma was removed into cryovials. The obtained plasma was aliquot and stored at -20°C until analyses. The packed erythrocytes cells were washed three times with 0.9% NaCl hypotonic solution and washed erythrocytes were lyses 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. Protein quantification in erythrocytes hemolysates was carried out by the method specified by Lowry et al.[21]

2.5. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is a method for the quantitative or qualitative measurement of analytes (in our case, the cytokines). We used sandwich-ELISA kit (Peprotech, USA), which is based on the specific binding between an antigen and its antibody, and is therefore highly specific. The sandwich ELISA kit was used to measure the pro/anti-inflammatory cytokines (IL-6, IL-8 and IL-10) levels in the plasma of study subjects. According to the manufacturer's instructions, the plasma samples were assayed on 96-well plates (Tarson, India) using matched antibody against human IL-6, IL-8 and IL-10. The limits of detection were 32-2000pg/ml for IL-6, 16-1000pg/ml for IL-8 and 39–2500 pg/ml for IL-10.

2.6. Measurement of Erythrocytes antioxidant enzymes activities

Routine biochemical analyses were performed using standard spectrophotometric techniques. GST activity in erythrocytes was assayed according to the method described by Beutler.[22] The changes in absorbance were recorded at 340 nm and the enzyme activity was calculated as μmol CDNB conjugate formed/minute/mg protein using a molar extinction coefficient of 9.6 mM$-$1 cm$-$1. GPx activity in erythrocytes was measured according to Flohe and Gunzler.[23] Absorbance was measured at 420 nm. The activity was expressed in Unit/ minute/mg protein.

2.7. Statistical analyses

All the study parameters are presented as mean values $\pm$ 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. This analysis was performed using the XLSTAT software (Addinsoft, New York, NY, USA). Comparisons between smokers with and without COPD and severity of COPD and controls were assessed by ANOVA, followed by post hoc test for multiple comparisons using Tukey's Multiple Comparisons Test using GraphPad Prism 6 (GraphPad Software Inc, San Diego, CA, USA). As the distributions of IL-6, IL-8, IL-10, GST and GPx levels were all skewed, we used the log-transformed values of these variables in regression analysis. The relationship between selected variables were expressed as medians with interquartile range and a p-value of less than 0.05 ($p < 0.05$) was considered as statistically significant.

3. Results

3.1. Demographic characteristics of COPD patients and healthy controls

Normality analysis showed that all the variables were normally distributed. The demographic characteristics of COPD patients and healthy controls are presented in Table 1. The mean age of COPD patients was >50 years; the mean body mass index (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, respectively, whereas in controls, 22.03% were ex-smokers, 28.18% current smokers and 49.15% non-smokers, respectively. Patients with COPD showed significant lower mean values for pulmonary function test, assessed as FEV1, FVC and the ratio of FEV1/FVC.

3.2. Measurement of inflammatory cytokines in plasma of COPD patients and healthy controls by ELISA

To confirm the elevated level of inflammatory cytokines in plasma, the ELISA method was used to quantify the levels of inflammatory cytokines in COPD patients and healthy controls. As shown in Figure 1A & B, COPD patients had significantly higher levels of IL-6 (429.49 $\pm$ 1.81 pg/ml) compared with healthy controls (260.38 $\pm$ 2.23 pg/ml; $p<0.001$). Similarly, the plasma level of IL-8 was significantly higher in COPD patients (127.58 $\pm$ 0.76 pg/ml) as compared to control group (58.17 $\pm$ 1.11 pg/ml; $p<0.05$). In contrast, the plasma level of IL-10 was lower in COPD patients (99.92 $\pm$ 0.84 pg/ml) as compared to control group (125.11 $\pm$ 1.68 pg/ml; $p<0.05$) (Figure 1C).

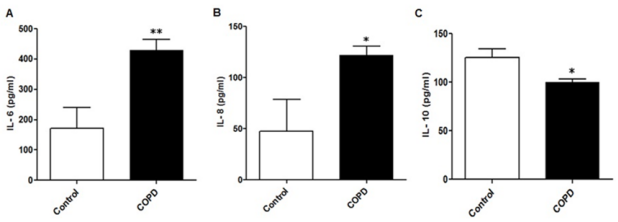

Fig. 1: Inflammatory cytokine levels (pg/ml) in individuals with COPD patients and the control group. **(A)** IL-6 plasma levels, **p < 0.001; **(B)** IL-8 plasma levels, *p < 0.05; and **(C)** plasma levels of IL-10, *p <0.05 was considered significant. IL-6, interleukin-6; IL-8, interleukin-8; IL-10, interleukin-10

Table 1: Demographic characteristics of the study groups

Characteristics	Controls Mean ± SEM (n=59)	COPD Mean ± SEM (n=127)
Age, years	51.02 ± 0.27	60.10 ± 0.31**
Male/Female (n)	38/21	98/29
Weight (kg)	65.32 ± 1.50	61.69 ± 0.32**
Height (cm)	157.12 ± 1.04	158.50 ± 0.26
BMI (kg/m2)	26.43 ± 0.65	24.43 ± 0.20**
Smoking Status		
Ex-smoker/current smoker/non-smoker (n)	13/17/29	50/32/45
Spirometry parameter		
FEV1, % of predicted	71.20 ± 1.26	36.21 ± 0.40***
FVC, % of predicted	83.99 ± 1.06	50.15 ± 0.34***
FEV1/FVC, % of predicted	97.56 ± 0.71	70.41 ± 0.46***
PaO2 (mmHg)		57.51 ± 0.11
SaO2 (%)		90.62 ± 0.19

Variables are expressed as Mean ± SEM, standard error of the mean; Significance of difference compared 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 second; FVC, force vital capacity; PaO2, partial pressure of oxygen; SaO2, arterial oxygen saturation; n, number of patients

3.3. Measurements of antioxidant enzyme activities in COPD patients and the control group

A significant decrease in the mean value of erythrocytic GST (21.30 ± 0.33 U/mg protein vs. 42.05± 0.56 U/mg protein; p < 0.001) and GPx (59.43 ± 0.50 U/mg protein vs. 63.77 ± 0.44 U/mg protein; p < 0.001) activities were observed in COPD patients compared to healthy control group (Figure 2A &B)

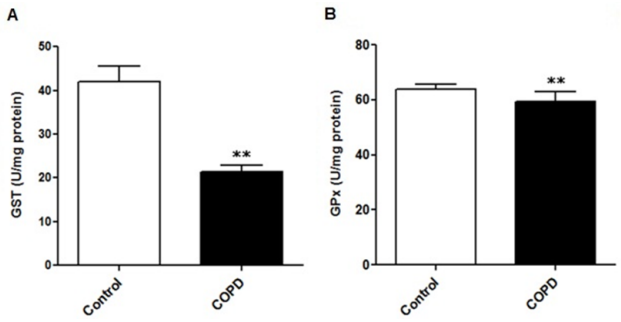

Fig. 2: Significance of difference between erythrocyte antioxidant enzyme of GST and GPx activities in COPD patients with control group: **p < 0.001. GST, glutathione-s-transferase; GPx, glutathione peroxidase

3.4. Comparison of inflammatory biomarkers levels in plasma of COPD patients with and without smokers and severity of COPD

Inflammatory cytokines levels were measured in plasma of COPD with non-smokers and smokers along with patients with different severities of COPD (GOLD stages II & GOLD stage III-IV). Significantly higher levels of plasma IL-6 were detected in COPD patients with smokers (p< 0.001) than in COPD non-smokers and control group. IL-6 levels were higher in the plasma of COPD patients with GOLD stage III-IV as compared to GOLD stage II and control group (p > 0.05) (Figure 3A). The levels of IL-8 in COPD patients with smokers and non-smokers (p< 0.001, p< 0.001, respectively) and COPD patients with GOLD stages II and GOLD stage III-IV (p< 0.001, p< 0.001, respectively) were significantly higher than in control group (Figure 3B). The levels of IL-10 in COPD patients with non-smokers were significantly higher than those in control group (p< 0.001); however they were generally low and were not significantly different in the COPD patients who were smokers and among patients with different severities (Figure 3C).

3.5. Comparison of antioxidant enzyme activities in smokers and non-smokers with COPD and with severity of COPD

Healthy controls had considerably higher erythrocytes GST activity than those COPD smokers and non-smokers and among patients with different grades of severity (Figure 4 A). Erythrocytes GST activity in patients with smokers with COPD (p< 0.001)were significantly lower than in healthy controls but were still higher than in non-smokers with COPD (p< 0.001), with an even greater significant reduction observed in GOLD stage III-IV COPD patients (p< 0.001) than those in GOLD stage II (p> 0.05). Similarly, there was a significant decrease in the erythrocyte GPx activity from healthy controls to non-smokers with COPD (p> 0.05) and smokers with COPD (p< 0.001). GPx activity significantly decreased in COPD patients with GOLD stage II (p> 0.05) and GOLD stage III-IV (p< 0.001) compared to control group (Figure 4B).

Table 2: Linearrelationships between FEV1 (% predicted) and erythrocytes GST and GPxactivities and log-transformed plasma IL-6, IL-8 and IL-10 in COPD patients

Study parameters	FEV1 (% predicted)	GST (U/mg protein)	GPx (U/mg protein)
IL-6	- 0.565**	- 0.018	- 0.080
IL-8	- 0.453*	- 0.260	- 0.268
IL-10	0.131	0.494*	0.546**

A significance linear relationship between plasma IL-6, IL-8 and IL-10 levels with severity of COPD with lung function test assessed as FEV1 (% predicted) and erythrocyte GST and GPx enzymes activities.*p < 0.05, **p < 0.001, 'r' denotes Pearson correlation coefficient

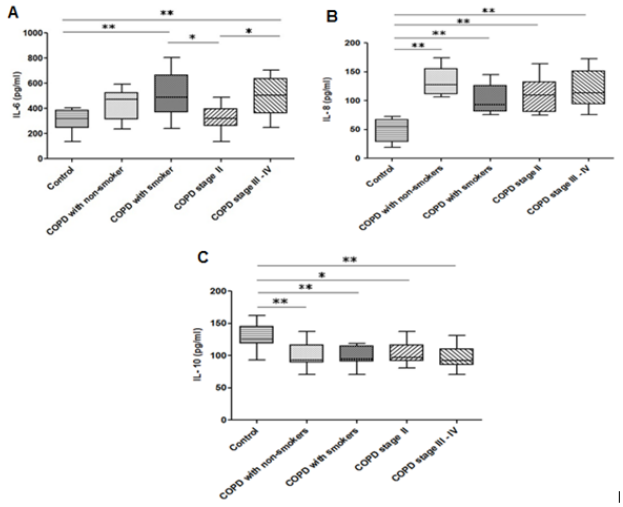

Fig. 3: Plasma levels of **(A)** IL-6 **(B)** IL-8 and **(C)** IL-10 in smokers, non-smokers and with severe COPD (GOLD stage I-II & III-IV) and controls. Box-and-whisker plot: The centre box represents the interquartile range (25–75%) and whiskers represent the highest and the lowest values. On top of the whisker, the horizontal lines indicate statistically significant differences between the groups. IL, indicates interleukin. Significant differences and respective p values*p < 0.05, **p < 0.001are mentioned on the graph using one-way ANOVA followed by Tukey's Multiple Comparisons post hoc test.

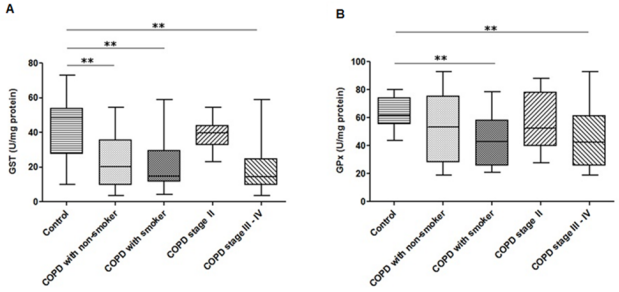

Fig. 4: Erythrocyte **(A)** GST and **(B)** (GPx) activities in smokers and non-smokers and with severe COPD (GOLD stage II & III-IV) and controls. Box-and-whisker plot: The centre box represents the interquartile range (25–75%) and whiskers represent the highest and the lowest values. On top of the whisker, the horizontal lines indicate statistically significant differences between the groups. Significant differences and respective p values *p < 0.05, **p < 0.001 are mentioned on the graph using one-way ANOVA followed by Tukey's Multiple Comparisons post hoc test.

3.6. Relationship between plasma inflammatory cytokines with FEV1and erythrocyte GST and GPx activities

In this study, we evaluated the correlations between plasma cytokines levels of COPD patients with FEV1 and antioxidant enzyme activities (Table 2). In COPD patients, a significant negative correlation was found between plasma IL-6 levels and FEV1 (r= - 0.565, p< 0.001) (Figure 5 A). Also, a negative correlation was found between IL-6 and erythrocytic GST (r= - 0.018, p>0.05) and GPx activities (r= - 0.080, p>0.05). The plasma IL-8 levels showed significant negative correlation between IL-8 and FEV1 (r= -0.453, p <0.05) (Figure 5B). A negative correlation was also found between IL-8 and erythrocytic GST (r= -0.260, p>0.05) and GPx activities (r= - 0.268, p>0.05. In contrast, the plasma IL-10 levels showed a positive correlation between IL-10 and FEV1 (r= 0.131, p>0.05), However, the decrease in plasma level of IL-10 was not statistically significant. A significant positive correlation was observed between IL-10 and erythrocytic GST (r= 0.491, p <0.05) and GPx activities (r= 0.546, p < 0.001). However, the decrease in plasma level of IL-10 was statistically significant (Figure 5C & D).

4. Discussion

COPD is characterized with the abnormal activation of inflammatory cells and increase in circulating pro- and anti-inflammatory cytokines.[24] The inflammatory cytokines are currently the most used parameter for the evaluation of the risk of the development of COPD. Therefore, the current study provides a novel observation of the inflammatory marker released in plasma of COPD patients with non-smokers and smokers along with patients with severity of COPD (GOLD stages II & GOLD stage III-IV). In addition, our study extended the novel observation by demonstrating the relationship between systemic inflammatory markers and the antioxidant activities of COPD.

COPD patients had significantly higher level of plasma IL-6 and IL-8 compared with healthy controls. These results extend the previous findings and found increased levels of plasma IL-6 in COPD patients compared to healthy

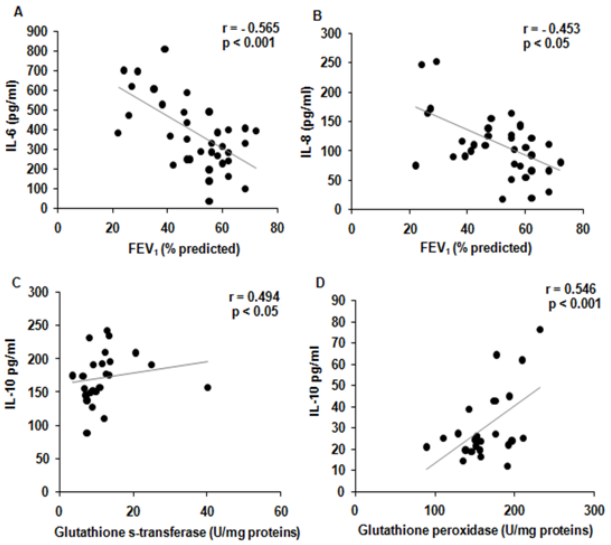

Fig. 5: The scatter plot showing the relationship between plasma. **(A)** IL-6 (r= -0.565, p < 0.001) and, **(B)** IL-8 (r= -0.453, p < 0.05) and FEV1 in COPD patients. Relationship between erythrocytic, **(C)** GST activity and log-transformed of plasma IL-10 (r = 0.494, p < 0.05) and, **(D)** GPx activity and log-transformed of plasma IL-10 (r = 0.546, p < 0.001) in patients with COPD. Line through the scatter points is regression based fit line showing the trend of the data. The r value indicates a correlation coefficient.

controls.[25,26] Bhowmik et al, found an increased level of IL-6 and IL-8 in sputum of stable COPD subjects who had frequent exacerbations. Previous studies have also shown that IL-6 level in bronchoalveolar fluids fluid was higher in COPD patients than in healthy control.[27–29] IL-6 concentration directly participates in inflammation, and it may be a good parameter for the assessment of COPD. IL-8 is the main mediator of neutrophil chemotaxis at the site of inflammation and was observed to be released in greater amount in patients with COPD using both induced sputum and bronchoalveolar lavage.[30,31] In contrast, the plasma level of IL-10 was lower in COPD patients compared to the control group. IL-10 inhibits pro-inflammatory cytokines, which is mediated by specific cell surface receptor complex and lower levels of IL-10 were associated with development of COPD.

Cigarette smoking is the most common cause of COPD. We observed that the levels of IL-6 and IL-8 in COPD patients who were smokers and non-smokers were significantly higher than in control group. The results of the current study are consistent with previous findings wherein an increased level of IL-8 was observed in COPD patients who were smokers compared to healthy smokers.[32] We also observed that the circulating level of plasma IL-6 and IL-8 were higher in GOLD stage III-IV as compared to GOLD stage II in COPD patients and control group. The results of the present work are consistent with the study of WS El-Shimy et al, who declared that serum and bronchoalveolar fluids of IL-6 and IL-8 level increases with increasing severity of COPD.[33] Our results suggest that IL-6 and IL-8 are mainly involved in the occurrence of chronic inflammation in the airway of COPD, which indicates that systemic inflammatory activity exists even in stable COPD patients.

COPD patients who were non-smokers showed significantly higher level of IL-10 than those in control group and were generally lower and not significantly different between smokers and with severity (GOLD stages II & GOLD stage III-IV) of COPD patients. In contrast, using ELISA technique Zhang et al showed that IL-10 was significantly higher in the serum of healthy nonsmokers compared to healthy smokers and severity of COPD patients (GOLD stages I-IV).[34] The elevated signaling of IL-10 inhibit the production pro-inflammatory cytokines such as IL-1β and TNFα[35] and chemokines. IL-10 immunosuppressive activity is regulated by the specific cell surface membrane receptor complex, and decrease level of IL-10 was associated with the progression of COPD.[36,37] This may suggest that low levels of IL-10 may reflect the up-regulation of the inflammatory response.[38]

Similarly, the erythrocyte GPx activity significantly decreased in non-smokers and smokers with COPD compared to healthy controls. GPx activity also reduced in patients with GOLD stage I-II and GOLD stage III-IV compared to control group. A similar finding was also reported and showed that GPx activity was reduced in patients with moderate and severe COPD.[38] There is some evidence showing that reduced levels of GPx in plasma of COPD patients.[39] Decrease in GST and GPx activities was found to confer greater risk of progression COPD.

This study analyzed the correlation between inflammatory cytokines and oxidative stress markers. To our knowledge, only few studies have investigated the plasma level of inflammatory cytokines and erythrocytes antioxidant markers in COPD. In our study, we demonstrated that IL-6 and IL-8 were significantly negatively related to log-transformed FEV1. Similar negative correlation was observed in induced sputum of COPD patients.[40] In contrast, plasma IL-6 and IL-8 levels were positively related to erythrocytic GST and GPx activities in patients with COPD. The results presented here suggest that decrease in antioxidant activities is related to increase production of pro-inflammatory markers IL-6 and IL-8 in COPD patients. A possible explanation for the significant decrease in GST and GPX activities could be that in COPD patients, the increase in toxic substrates in the body reduces the antioxidant activities, which was inversely proportional to the elevated level of IL-6 and IL-8. Moreover, no significant was observed between plasma IL-10 level and FEV1. It is suggested that the accentuated inflammatory status, partially due to low levels of IL-10

which support worsen of inflammatory response leading to more severe level of COPD.

5. Conclusions

In conclusion, our study provides evidence that plasma inflammatory cytokines IL-6, IL-8 and IL-10 could be potential markers for the evaluation of both smokers with and without COPD and with severity of COPD. Secondly, our study demonstrated that GST and GPx activities progressively decrease in COPD patients with increasing severity which could be associated with loss of IL-10 secretion. These suggested that antioxidant activities and inflammatory cytokines are co-dependent and strongly interrelated processes in the pathogenesis of COPD. Future studies aimed towards understanding the mechanistic relationship between the systemic inflammatory markers and antioxidant enzymes in pathogenesis of COPD would help in designing targeted therapeutic approaches.

6. Acknowledgement

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7. Source of Funding

None.

8. Conflict of Interest

None.

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