Original Research Article

Association between various antioxidants in normals and chronic obstructive pulmonary disease, their alteration and impact of smoking and disease on levels of antioxidants

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ABSTRACT

Background: There is total alteration of various antioxidants in response to the oxidative stress, which is one of the major patho-physiologic hallmarks in chronic obstructive pulmonary disease (COPD) development. This study aims to establish the correlation between different antioxidants in normals and COPD, study the alteration in the correlation due to COPD and smoking as well as the impact of COPD and smoking on antioxidants levels.
Methods: Study comprises of 96 normals as group I and 96 COPD patients as group II. The antioxidants albumin (Alb), bilirubin (Bil), uric acid (UA) ceruloplasmin (Cp), glutathione peroxidase (GSHPx), catalase (CAT) and superoxide dismutase 3 (SOD_3) were estimated.
Results: Significant lower serum Alb, UA, SOD_3 and increased serum Cp and GSHPx were found in Group II. Significant correlation was found between Alb and UA (r=0.24); Bil and UA (r=0.26); Alb and CAT (r=0.211) and SOD_3 and CAT (r=0.318) in normals. However, these correlations were altered in COPD where Alb correlates with Bil (r=0.235); UA with CAT (r=0.203) and SOD_3 with GSHPx (r=-0.27). The correlation between SOD_3 and CAT remained unaltered. Similar correlation of UA with Alb and Bil was observed in nonsmoker normals and between SOD_3 and CAT in smoker normals. In COPD, no correlation was seen in nonsmokers, while in smokers Alb correlates with Bil (r=0.316) and SOD_3 with CAT (r=0.317).
Conclusions: These alterations may have clinical ramifications in further understanding the pathogenesis of COPD and developing therapeutic approaches.

Keywords: Antioxidants, Albumin, Chronic obstructive pulmonary disease, Ceruloplasmin, Catalase, Uric acid

INTRODUCTION

Chronic obstructive pulmonary disease (COPD), a condition defined by virtue of chronic obstruction to airflow because of chronic bronchitis and /or emphysema, is the disease which has shown growing trend in mortality as well as morbidity and by 2020 is anticipated to be the third foremost cause of death in the world.[1] The colossal surface area of a lung is incessantly exposed to exogenous or endogenous oxidants and is protected

against the oxidative challenge by well-developed enzymatic antioxidants like catalase (CAT), glutathione reductase (GSHRx), glutathione peroxidase (GSHPx) ceruloplasmin (Cp), superoxide dismutase (SOD), etc. and non-enzymatic antioxidant such as bilirubin (Bil), uric acid (UA), albumin (Alb), α-tocopherol, glutathione, lipoic acid, ascorbate etc.[2] The diminution in antioxidant defenses and/or augments in pulmonary exposure to oxidative source lead to a stage of imbalance referred to as oxidative stress.[3] Oxidative stress has been implicated in the pathogenesis of COPD. These include various events like activation of the molecular mechanisms that initiate lung inflammation membrane lipid peroxidation, alveolar epithelial injury, remodeling of extracellular matrix, and oxidative inactivation of antiproteases and surfactants.[3,4]

Alb was reported as the most abundant plasma antioxidant which is continuously exposed to oxidative stress.[5] UA, an excellent scavenger of singlet oxygen, protects RBC membrane from peroxidation, was found effective in preventing lipid peroxidation at levels significantly below the plasma levels.[6,7] An important aspect of UA in-vivo function resides in its antioxidant activity.[7,8] Antioxidant effect of Bil has been demonstrated to surpass that of vitamin E towards lipid peroxidation and Bil concentration in serum is high enough to account for the total antioxidant capacity of serum significantly.[9]

Ceruloplasmin is the chief serum inhibitor of lipid peroxidation and has been widely reported as a key extracellular serum antioxidant.[10,11] It has been demonstrated to inhibit ferrous ion mediated lipid peroxidation thereby preventing lung injury.[12] An aberration of Cp oxidative inhibition may possibly be involved in COPD pathogenesis.[13]

SOD which catalyze the breakdown of the superoxide anion into O_2 and H_2O_2 are major defenses against oxidative damage caused by the superoxide anion $(O_2 \bullet)$.[14,15] Extracellular superoxide dismutase, or SOD_3 binds lung matrix components and inhibits their fragmentation in response to oxidative stress is the primary extracellular antioxidant enzyme in the lung protects the extracellular matrix during lung injury and accounts for the majority of SOD activity in airways and vessels.[16-19] CAT antioxidant activity is due to its powerful action to decompose H_2O_2 which otherwise generates very toxic hydroxyl radical.[20] GSHPx, a selenium-dependent enzyme is one of the major enzymes in the antioxidative defense mechanism. GSHPx competes with catalase for H_2O_2 as a substrate. Glutathione redox cycle is a major source of protection against mild oxidative stress, whereas catalase becomes increasingly important in protection against severe oxidative stress.[21,22]

However, in animal cells, and especially in human RBC, the principal antioxidant enzyme for H_2O_2 detoxification

has been considered to be GSHPx, as CAT has much lower affinity for H_2O_2 than GSHPx.[23] In COPD there is a total alteration of these antioxidants in response to the oxidative stress. We estimated the levels of nonenzymatic antioxidants bilirubin, albumin and urate and enzymatic antioxidants ceruloplasmin, catalase, glutathione peroxidase and superoxide dismutase in serum of smoker and nonsmoker COPD patients as well as in smoker and nonsmoker normal subjects, to find if any correlation exists between the various antioxidants in normal subjects and COPD patients thereby studying their alterations, effect of smoking on the correlation and to explore the impact of COPD and smoking on the levels of these antioxidants.

METHODS

Subject selection and procedure

Group I or Normals

This group comprising of 96 normals, consisted of 48 smokers and 48 nonsmokers, were taken from normal healthy individuals visiting Biochemistry Lab of the department for general checkup and annual medical board and other volunteers.

Group II or COPD patients: This group comprising of 96 patients of COPD diagnosed as per GOLD guidelines (GOLD report 2011), consisted of 48 smokers and 48 nonsmoker COPD patients. Subjects for this group were taken from the patients reporting with the symptoms of COPD to respiratory OPD of Cardio-Thoracic Centre, Pune. The study has Institutional Ethical Committee approval. Informed consent was taken from the subjects before drawing their blood specimen.

Criteria for exclusion of subjects for Group I and II

Asthma patients and patients of diffuse parenchymal lung disease, coronary artery disease (CAD), patients on long term oxygen therapy or those whose spirometry could not be performed were excluded from Group II. Similarly, normal subjects with any lung/ respiratory disease or past history of any respiratory problem were excluded from Group I. All patients were administered a questionnaire to collect demographic data, duration of symptoms, history of atopy or nasal allergies, episodic or progressive symptoms, chest examination, radiological investigations and whether spirometry was performed for initial diagnosis. From the questionnaire an initial clinical impression of the diagnosis was made. Subjects were asked not to use short acting bronchodilators within 04 hours of testing, long acting bronchodilators for 12 hours prior to testing and oral therapy with theophylline or slow release B-agonists for 24 hrs prior to the test. All patients underwent spirometry for confirmation of the diagnosis of COPD. The presence of post bronchodilator Forced expired volume in one second to Forced vital capacity ratio less than 0.70 was used to confirm COPD.[24] To

quantify smoking exposure among the smokers, smoking index (which is defined as average number of cigarettes or bidis smoked in one day multiplied by total duration of smoking in years) was used.[25]

Sample collection and storage

Fasting venous blood samples were collected in a 5 mL plain sterile clot activator gel vacutainer and in a 4 mL heparin vacutainer from all the subjects. Blood collected in heparin was immediately centrifuged at 2000 rpm for 5 minutes and plasma was separated, aliquoted and stored immediately at -700C to be used specifically for GSHPx. The clotted blood sample was centrifuged at 2000 rpm for 5 minutes and serum was separated and aliquoted in Eppendorf tube. Estimation of Cp, Alb, Bil and UA was done immediately while remaining aliquots were stored at -80°C until estimation of other remaining analytes.

Estimation of nonenzymatic antioxidants: albumin, bilirubin and urate

All the three nonenzymatic antioxidants were estimated by End-Point methods using kits from Transasia on a fully automated analyzer XL 600 from Transasia Mannheim GmbH, Germany. Albumin was estimated by BCG Dye Method, Bilirubin by Diazo Method, and urate by Modified - Trinder Method.

Estimation of enzymatic antioxidants: ceruloplasmin, glutathione peroxidase, superoxide dismutase and catalase

Cp was estimated using commercially available kit from Agappe Diagnostics (SensIT Ceruloplasmin) as per their instruction manual on a fully automated analyser XL600 from Transasia Mannheim GmbH, Germany. It is based on the principle that goat antihuman Cp of the reagent reacts with the Cp present in serum sample forming turbidity leading to increase in the absorbance. This change in absorbance is directly proportional to the concentration of Cp 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.

GSHPx was measured with the Glutathione peroxidase Assay Kit (Cayman Chemical Company, USA). GSHPx catalyzes the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH (nicotinamide adenine dinucleotide phosphate hydrogen) the oxidized glutathione (GSSG) is immediately reconverted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm is measured and is directly proportional to the GSHPx activity in the sample expressed as U/L.

SOD3 was estimated with the ELISA Kit for Human Superoxide Dismutase 3, Extracellular (SOD_3), from

Wuhan EIAAB Science Co Ltd, Wuhan China. This competitive ELISA employs microtitre plate pre-coated with an antibody specific to SOD_3. During incubation SOD_3 in standards/samples competes with the fixed amount of biotin-conjugated SOD3 for sites on a precoated monoclonal antibody specific to SOD_3. Excess conjugate and the unbound sample/standard were washed off. Avidin conjugated to Horseradish peroxidase HRP) was added followed by 3,3',5,5' Tetramethylbenzidine (TMB). Only in SOD_3 containing wells, biotin-conjugated antibody and HRP-conjugated Avidin exhibits a change in color which was later terminated by H_2SO_4. The color change at 450 nm is proportional to the levels of SOD_3 determined by standard curve in U/L.

CAT was estimated with the ELISA Kit for Human Catalase, from Wuhan EIAAB Science Co Ltd, Wuhan, China. Principle of this competitive ELISA is exactly similar to that of SOD_3 estimation. The levels of CAT are determined in U/L.

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 two tailed 't' test. Correlation between the variables was calculated using Pearson's coefficient. Cut-off point for determining statistical significance levels was p=0.05. Univariate Analysis of Variance (Two way ANOVA) was applied to all the analytes using the model: Intercept + COPD + Smoking + COPD*Smoking, to determine the interaction between smoking status and COPD status and to compare the levels of analytes between Group I and II with respect to their smoking status so as to assess the impact of COPD status and smoking status on the level of an analyte.

RESULTS

In ninety six subjects of Group I, Mean±SD of age was 59.82±12.17 years with range 27-90 years while in Group II, Mean±SD of age was 67.65±8.9 years with range 45-97 years. In both Group I and II there were 73 males and 23 females. The number of males and females within nonsmokers were 27 and 21 while in smokers, males and females were 46 and 2 respectively in both the groups.

Levels of all the analytes were compared between the smoker of Group I and II and nonsmoker of Group I and II using Levene's Test for Equality of Variances and unpaired t-test for Equality of Means and are shown in Table 1.

Correlation studies were conducted using Pearson Correlation to establish correlation between analytes in Group I and II and are shown in Table 2.

Correlation was also determined to establish correlation between analytes in smokers & nonsmokers of Group I as

well as in smokers and nonsmokers of Group II and is shown in Table 3 and 4 respectively.

Table 1: Mean and SD of analytes in smokers and non-smokers of group I and II.

| Analyte | Nonsmokers | | | | | Smokers | | | | |
| | Group I | | Group II | | p Value | Group I | | Group II | | p Value |
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
Alb (g/dL)	4.29	0.24	3.82	0.49	0.00	4.39	0.34	3.78	0.52	0.00
Bil (mg/dL)	0.76	0.33	0.87	0.49	0.21	0.77	0.33	0.76	0.33	0.86
UA (mg/dL)	4.99	1.34	4.49	1.26	0.07	5.09	1.36	4.72	1.47	0.2
Cp (mg/dL)	32.90	6.61	43.80	10.91	0.00	36.72	8.40	48.24	12.83	0.00
CAT (U/L)	52.77	22.37	53.06	23.54	0.95	52.52	24.57	55.67	31.61	0.59
SOD3 (U/L)	264.18	75.07	173.21	59.61	0.00	225.41	57.03	162.2	58.20	0.00
GSHPx(U/L)	47.05	13.29	88.00	30.21	0.00	54.86	16.30	91.46	25.46	0.00

(Alb=Albumin; Bil = Bilirubin; UA= Uric Acid; Cp = Ceruloplasmin; CAT= Catalase; SOD_3 = Superoxidase dismutase 3; GSHPx = Glutathione peroxidase)

Table 2: Pearson Correlation with level of Sig. (2-tailed) between analytes in Group I and Group II.

| | GROUP I | | | | | | GROUP II | | | | | |
	ALB	BIL	UA	CP	CAT	SOD₃	ALB	BIL	UA	CP	CAT	SOD₃
BIL	-0.027						0.235*					
	0.795						0.021					
UA	0.242*	0.260*					0.036	0.019				
	0.018	0.010					0.729	0.853				
CP	-0.030	-0.145	-0.087				-0.005	-0.125	-0.079			
	0.771	0.160	0.401				0.964	0.225	0.445			
CAT	0.211*	-0.023	0.176	-0.114			0.060	-0.095	0.203*	-0.073		
	0.039	0.822	0.086	0.268			0.561	0.358	0.048	0.479		
SOD₃	-0.070	0.037	0.022	-0.130	0.318**		-0.132	-0.177	0.104	-0.185	0.271**	
	0.499	0.723	0.828	0.207	0.002		0.200	0.084	0.313	0.071	0.008	
GSHPx	0.013	0.048	-0.127	-0.029	-0.023	-0.056	0.120	0.003	0.037	0.088	-0.095	-0.270**
	0.897	0.646	0.218	0.781	0.825	0.585	0.245	0.979	0.724	0.394	0.359	0.008

Alb=Albumin; Bil = Bilirubin; UA= Uric Acid; Cp = Ceruloplasmin; CAT= Catalase; SOD_3 = Superoxidase dismutase 3; GSHPx = Glutathione peroxidase; *indicates statistical significant, $p<0.05$; ** indicates statistical significant, $p<0.01$.

Table 3: Pearson Correlation with level of Sig. (2-tailed) between analytes in non-smokers and smokers of Group I.

| | Nonsmokers of Group I | | | | | | Smokers of Group I | | | | | |
	Alb	TBil	UA	Cp	CAT	SOD₃	Alb	Bil	UA	Cp	CAT	SOD₃
TBil	-0.078						0.029					
	0.597						0.844					
UA	0.45**	0.37**					0.080	0.177				
	0.001	0.010					0.588	0.228				
Cp	0.106	-0.12	-0.10				-0.126	-0.141	-0.111			
	0.475	0.428	0.520				0.393	0.340	0.453			
CAT	0.187	0.004	0.145	-0.044			0.234	-0.043	0.191	-0.177		
	0.202	0.977	0.324	0.768			0.110	0.774	0.194	0.228		
SOD3	0.022	0.084	0.076	-0.058	0.188		-0.165	-0.065	-0.009	-0.165	0.44**	
	0.884	0.570	0.606	0.698	0.200		0.262	0.663	0.950	0.261	0.002	
GSHPx	-0.031	0.157	-0.18	0.151	-0.039	-0.098	0.068	-0.118	-0.091	-0.236	-0.016	0.006
	0.835	0.287	0.226	0.305	0.794	0.506	0.648	0.426	0.541	0.106	0.915	0.965

Alb=Albumin; Bil = Bilirubin; UA= Uric Acid; Cp = Ceruloplasmin; CAT= Catalase; SOD_3 = Superoxidase dismutase 3; GSHPx = Glutathione peroxidase; *indicates statistical significant, $p<0.05$; ** indicates statistical significant, $p<0.01$.

Two way ANOVA was applied to all the analytes to determine the interaction between smoking status and COPD status, and to compare the levels of analytes between Group I and II with respect to their smoking

status so as to assess the impact of COPD status and smoking status on the level of an analyte, and is shown in Table 5.

Table 4: Pearson Correlation with level of Sig. (2-tailed) between analytes in non-smoker Group II.

	Nonsmoker Group II						Smoker Group II					
	Alb	Bil	UA	Cp	CAT	SOD3	Alb	Bil	UA	Cp	CAT	SOD3
Bil	0.128						0.316*					
	0.385						0.029					
UA	-0.160	-0.11					0.161	0.146				
	0.279	0.446					0.273	0.323				
Cp	0.097	-0.15	-0.19				-0.128	-0.117	-0.018			
	0.510	0.296	0.199				0.385	0.427	0.905			
CAT	0.210	-0.07	0.146	-0.144			-0.032	-0.118	0.254	-0.024		
	0.153	0.643	0.322	0.329			0.827	0.423	0.081	0.869		
SOD3	-0.153	-0.19	0.182	-0.274	0.260		-0.040	-0.174	0.043	0.023	0.317*	
	0.298	0.198	0.215	0.060	0.074		0.787	0.237	0.770	0.878	0.028	
GSHPx	0.126	0.002	0.249	0.027	-0.026	-0.224	0.056	-0.003	-0.150	0.026	-0.149	-0.214
	0.393	0.988	0.088	0.856	0.861	0.127	0.707	0.982	0.308	0.862	0.311	0.144

Alb=Albumin; Bil = Bilirubin; UA= Uric Acid; Cp = Ceruloplasmin; CAT= Catalase; SOD_3 = Superoxidase dismutase 3; GSHPx = Glutathione peroxidase; *indicates statistical significant, $p<0.05$.

Table 5: ANOVA Test of between-subjects effects.

Source	Type III Sum of Squares	Mean Square	F	p-value
Dependent Variable: Albumin (Alb)				
Corrected Model	14.320	4.773	27.807	0.000
Intercept	3180.542	3180.542	18527.503	0.000
COPD	14.056	14.056	81.881	0.000
Smoking	0.044	0.044	0.253	0.615
COPD *Smoking	0.221	0.221	1.286	0.258
Dependent Variable: Total Bilirubin (Bil)				
Corrected Model	0.405	0.135	0.957	0.414
Intercept	120.634	120.634	854.385	0.000
COPD	0.111	0.111	0.784	0.377
Smoking	0.124	0.124	0.875	0.351
COPD *Smoking	0.171	0.171	1.211	0.273
Dependent Variable: Uric acid (UA)				
Corrected Model	10.452	3.484	1.890	0.133
Intercept	4467.516	4467.516	2423.387	0.000
COPD	8.942	8.942	4.851	0.029
Smoking	1.336	1.336	0.725	0.396
COPD *Smoking	0.174	0.174	0.095	0.759
Dependent Variable: Ceruloplasmin (Cp)				
Corrected Model	6856.738	2285.579	22.966	0.000
Intercept	313546.848	313546.848	3150.541	0.000
COPD	6032.886	6032.886	60.619	0.000
Smoking	819.227	819.227	8.232	0.005
COPD *Smoking	4.625	4.625	0.046	0.830
Dependent Variable: Catalase (CAT)				
Corrected Model	306.743	102.248	0.154	0.927
Intercept	549625.699	549625.699	827.285	0.000
COPD	142.226	142.226	0.214	0.644
Smoking	66.848	66.848	0.101	0.751
COPD *Smoking	97.669	97.669	0.147	0.702

(Table 5)

Source	Type III Sum of Squares	Mean Square	F	p-value
Dependent Variable: Superoxide Dismutase (SOD$_3$)				
Corrected Model	324920.723	108306.908	27.368	0.000
Intercept	8164264.876	8164264.876	2063.037	0.000
COPD	285851.033	285851.033	72.232	0.000
Smoking	29925.437	29925.437	7.562	0.007
COPD *Smoking	9144.253	9144.253	2.311	0.130
Dependent Variable: Glutathione peroxidase (GSHPx)				
Corrected Model	73909.692	24636.564	49.201	0.000
Intercept	949970.393	949970.393	1897.173	0.000
COPD	72158.348	72158.348	144.106	0.000
Smoking	1524.448	1524.448	3.044	0.083
COPD *Smoking	226.895	226.895	0.453	0.502

Model: Intercept+COPD+Smoking+COPD *Smoking; R Squared (Adjusted R Squared) for Alb=0.307(0.296); Bil=0.015(-0.001); UA=0.029(0.014); Cp=0.268(0.257); CAT=0.002(-0.013); SOD$_3$=0.304 (0.293) and GSHPx=0.440(0.431)

DISCUSSION

The incidence of COPD has been reported to be growing in both mortality and morbidity, and by the year 2020 is likely to be the third leading cause of death globally. Dwindling in the levels of antioxidant defenses and/or increase in pulmonary exposure to oxidative source, leading to oxidative stress has been implicated in the pathogenesis of COPD.[5] This indicates explicit alteration in the levels of antioxidants in the body and this study was thus undertaken. Various antioxidants in serum were estimated, and the correlation between the different antioxidants in normal subjects and COPD patients were established. The alteration in the correlation between the different antioxidants due to COPD and smoking as well as the impact of COPD status and smoking status on the levels of these antioxidants were studied.

Statistically significant low levels of serum Alb, UA, SOD3 were found in Group II (COPD) while levels of serum Cp and GSHPx were found increased (p<0.001) as compared to Group I (controls). In both Bil and CAT the difference was statistically not significant. Levels of Alb, UA, SOD3, GSHPx and Cp in Group I versus Group II respectively were Alb: 4.34±0.3g/dL versus 3.8±0.5g/dL; UA: 5.04±1.34mg/dL versus 4.61±1.37mg/dL; Cp 34.81±7.76 mg/dL versus 46.02±12.06mg/dL; SOD3: 244.79±69.12 U/L versus 167.62±58.87 U/L; GSHPx: 50.95±15.30 U/L versus 89.73±27.84 U/L; (p <0.01). Similar decreased serum Alb, UA, SOD$_3$ and increased levels of Cp and GSHPx were found in Group II within the nonsmokers and smokers as shown in Table 1.The serum levels of Alb, Cp, SOD3, GSHPx, in nonsmokers of Group I were found significantly different from the nonsmokers of Group II. Similarly, these analytes in smokers of Group I were also significantly different from smokers of Group II (Table 1).

Increased serum Cp and decreased Alb in Group II as compared to controls in this study is similar to that reported by others studies of Verrills NM et al Moison RMW et al.[26,27] In Alb and UA, statistically no significant difference was found between smoker and nonsmoker group in either Group I or Group II, and is in agreement with other reported data by Lykkesfeldt J et al.[28]

Correlation between the variables indicated positive, poor but statistically significant correlation between Alb and UA (r=0.24, p=0.018); Bil and UA (r=0.26, p=0.01), Alb and CAT (r=0.211, p=0.039) and SOD3 and CAT (r=0.318, p=0.002) in the normal individuals (Group I) irrespective of their smoking habits (Table 2). The positive significant correlation between Alb and UA in this study is in total agreement with that reported by Ramsay et al, who reported a positive correlation between plasma uric acid and plasma Alb (r=0.43, p<00.005, n=49) in healthy male subjects.[29]

However, in COPD patients, there was considerable alteration in the correlation. Significant correlation between Alb and UA; Bil and UA; Alb and CAT; that was observed in the normal individuals was found lost in COPD patients while the correlation between SOD$_3$ and CAT remained unaltered (Table 2). In COPD patients, positive significant correlation between Alb and Bil (r=0.235, p=0.021); UA and CAT(r=0.203, p=0.048), and negative correlation between SOD3 and GSHPx (r=-0.27, p=0.008) was found. The positive correlation between SOD$_3$ and CAT found in this study is in disagreement with that reported by Bogdanska et al, who reported SOD correlated negatively with CAT (r=-0.199) in normal healthy subjects.[30] This does not appear logical as CAT acts on the product of SOD(H_2O_2), so increased SOD logically be proportional with increased CAT. The difference might be due to the difference in the methodology as we have estimated SOD3 instead of total SOD and that SOD$_3$ was estimated by ELISA.

When correlation studies were conducted in smokers and nonsmokers separately, then in nonsmokers of Group I,

similar correlation was observed between Alb and UA (r=0.454, p=0.018) and Bil and UA (r=0.37, p=0.01) but no correlation between SOD_3 and CAT, while in smokers of Group I, similar correlation was observed between SOD_3 and CAT (r=0.435, p=0.002) as shown in Table 3.This suggests that in healthy nonsmokers, just Alb, UA, Bil might be sufficiently handling the oxidative system in the body. However, during smoking even in healthy individuals, the oxidative stress might be eliciting changes in SOD_3 and CAT levels due to which there occurs a good significant correlation (r=0.435, p=0.002) between SOD_3 and CAT. Cigarette smoke has been reported to contain 1014 free radicals per 'puff', and therefore remains the most important etiological factor for the development of COPD.[31] These free radicals might be detoxified by SOD_3 by dismutation of superoxide radicals (O_2) which eventually generates H_2O_2. The H_2O_2 then was detoxified to H_2O and O_2 by CAT. This might be the reason for developing a good correlation between SOD_3 and CAT in smokers. No correlation was seen between any analyte in nonsmokers of Group II, while in smokers of Group II similar positive and significant correlation was found between Alb and Bil (r=0.316, p=0.029) and SOD_3 and CAT (r=0.317, p =0.028) as shown in Table 4.

The impact of COPD and smoking status on the levels of each analyte was determined by two way ANOVA. For Alb, there was no interaction between smoking and COPD status as indicated by COPD *Smoking p value of 0.258 (Table 5). ANOVA indicated significant difference in Alb levels between COPD and normal (p <0.001) but not between smokers and nonsmokers (p=0.615).Thus if any individual suffers from COPD then it causes significant impact on Alb levels, leading to decrease in Alb levels, while smoking was not found to have that impact on Alb as was shown by COPD. Thus, COPD has statistically significant impact on the levels of Alb, while smoking has no significant impact. ANOVA for UA also no indicated no interaction between smoking and COPD status. Significant difference in UA levels between COPD and normal (p=0.029) but not between smokers and nonsmokers (p=0.396) was seen. Thus, COPD has statistically significant impact on the levels of UA, while smoking has no significant impact. These findings in UA are exactly similar to that of Alb. Even though COPD has similar impact on both Alb and UA, the correlation between UA and Alb is lost in COPD. This might be due to different impact on Alb and UA in different COPD patients.

No interaction between smoking status and COPD status for Cp was observed. Significant difference in Cp levels between COPD and normal (p<0.001) as well as even between smokers and nonsmokers (p=0.005) were found. Thus, if an individual suffers from COPD or even if becomes smoker, then it causes significant impact on Cp levels, leading to increase in Cp levels to handle the oxidative stress. The increased level of Cp in both COPD and smoking might be due to increased free radicals damage resulting into increased oxidative stress seen in both the

COPD and smoking conditions as Cp is the most abundant plasma antioxidant and is an acute phase reactant.

Neither COPD nor smoking status was found to have any impact on the levels of Bil and CAT. The Levene's Test for Equality of Variances and unpaired t-test for Equality of Means as shown in Table 1 also indicated the same result for Bil and CAT. There was no interaction between smoking and COPD status in SOD_3. ANOVA also indicated significant difference in levels of SOD_3 between COPD and normal (p<0.001) as well as between smokers and nonsmokers (p=0.007). Thus, both smoking status and COPD has statistically significant impact on the levels of SOD_3. The levels of SOD_3 in COPD and smoking condition might have been increased to counter the increase oxidative stress in these conditions. The impact of smoking and COPD status on SOD_3 was thus similar to that of Cp. However, no correlation was found between SOD_3 and Cp in COPD (Table 3). This might be due to different impact on the levels of SOD_3 and Cp in different COPD patients.

For GSHPx, no interaction was found between smoking status and COPD status. Significant difference in GSHPx activity between COPD and normal (p<0.001) but not between smokers and nonsmokers (p=0.083) was established. Thus, COPD has statistically significant impact on the activity of GSHPx, while smoking has no significant impact. The effect of smoking status and COPD status on the levels of GSHPx was thus similar to that seen on the levels of Alb and UA (Table 5).

Thus, COPD was found to have significant impact on the levels of Alb, UA, Cp, SOD_3 and GSHPx of which smoking has been found to have impact on the Cp and SOD_3 activity. Though the ANOVA studies indicated that the impact of smoking status and COPD status on the levels of Alb, UA and GSHPx as well as on the levels of Cp and SOD_3 was similar, but the Pearson correlation studies did not indicate the expected correlation amongst these analytes in COPD condition. Thus this study clearly demonstrated that though COPD condition has a definite impact on the levels of Alb, UA, Cp, SOD_3 and GSHPx but the extent of impact is diverse in different COPD patients resulting into loss of any correlation between various antioxidants.

Trials aimed by means of single antioxidants showed almost no effect on Forced expired volume in one second, indicating a single antioxidant not efficient in affording protection against the oxidative stress in COPD and consequently combination of antioxidants were recommended and approach based on targeting NRF2, which up-regulates an extensive range of antioxidants genes, was suggested as a more efficient way in COPD therapy.[3]

CONCLUSION

This study indicated significant alteration in the serum levels of Alb, Cp, SOD_3, GSHPx, in nonsmokers and smokers of

Group II as compared to nonsmokers and smokers respectively of Group I. Correlation which was found between Alb and UA, Bil and UA and Alb and CAT in normals was altered in COPD where correlation was seen between Alb and Bil, UA and CAT and between SOD_3 and GSHPx. The correlation between SOD_3 and CAT was found unaltered. COPD was found to have significant impact on the concentration of Alb, UA, Cp, SOD_3 and GSHPx of which smoking has been found to have impact on the Cp and SOD_3 activity. The ANOVA studies indicated the similar impact of smoking and COPD status on the levels of Alb, UA and GSHPx as well as on the levels of Cp and SOD_3, but the correlation studies did not indicate the expected correlation amongst these analytes thereby demonstrating definite impact on the levels of antioxidant but totally diverse in different COPD patients resulting into loss of any correlation between the various antioxidants. These alterations may have clinical ramification in further understanding of the pathogenesis of COPD and developing therapeutic approaches. Our study further enhances the understanding about the association between different antioxidants, alteration in the correlation due to COPD and smoking as well as the impact of smoking and COPD status on the levels of enzymatic and nonenzymatic antioxidants.

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