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Effect of smoking status, disease severity, exacerbations on Oxidative stress and hs-CRP levels in Chronic Obstructive Pulmonary Disease

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ABSTRACT

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease condition of narrowing of lung airways. Smoking is a well-known etiological factor to develop COPD. Oxidative stress and lung inflammation in COPD may be related to oxidant/antioxidant imbalance. The aim of the present study was to investigate effect of smoking status, disease severity, exacerbations on Oxidative stress and hs-CRP levels in Chronic Obstructive Pulmonary Disease.Total antioxidant capacity (TAC) and total oxidant status (TOS) were determined according to standard protocol. High sensitivity C- reactive protein (hs-CRP) was assessed by commercially available kit. All the statistical analysis was performed using SPSS v16.0. TAC was significantly low ($p<0.001$) in patients than controls whereas, no significant difference was observed for TOS. Oxidative stress index (OSI) and hs-CRP levels were significantly elevated in patients than controls. TAC revealed a significant positive association with weight, WC, BMI, WHR, WSR and FVC. OSI was negatively correlated with TAC and WHR. TAC was significantly ($p<0.05$) higher in non-smokers as compared to smokers. Significantly ($p<0.001$) high TAC was observed in ex-smokers than current smokers. TOS levels were significantly high ($p<0.05$) in non-smokers compared to ex-smokers and significantly high ($p<0.05$) in current smokers than ex-smokers. OSI was significantly high ($p<0.05$) in current smokers than non-smokers. OSI was significantly high ($p<0.001$) in current smokers than ex-smokers. Significant difference ($p<0.05$) for TAC was observed among all the four COPD stages. Significantly ($p<0.05$) elevated serum hs-CRP levels were observed in moderate COPD than very severe COPD. No statistical difference was observed for TAC, TOS, OSI and hs-CRP in patients with and without exacerbations. Our data provided further support to the fact that there is higher production of ROS in COPD leading to oxidative stress.

Keywords: *Antioxidants, hs-CRP, smoking, TOS, TAC*

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease condition of narrowing of lung airways due to exposure of harmful gases or particles (Pauwels et al., 2001; Barnes et al., 2003; GOLD, 2012). Prevalence of COPD is 4.1% in Indian population and is one of the major causes of morbidity and mortality worldwide (Jindal et al., 2006). Smoking had been shown to be a well known etiological factor to develop COPD (Snider, 1989). A cigarette smoke is a complex mixture of chemical compounds and has more than 10^{14} oxidants per puff and lots of them are fairly long-lived, which can produce $^{\cdot}OH$ and H_2O_2 resulting in increased oxidant burden in smokers (Nakayama et al., 1989; Pryor and Stone, 1993). Oxidants are also generated endogenously through various metabolic reactions. An imbalance between antioxidant capacity and reactive oxygen species directs to oxidative stress (Frank, 1991). The lung is particularly susceptible to damage caused by ROS due to its large surface area and blood supply with high-oxygen milieu. ROS production can lead to direct lung injury or initiates various cellular responses, which are directly linked to oxidation of proteins, DNA and lipids.

Furthermore, ROS also play a role in the modification of extracellular matrix and blood vessels, accelerated mucus secretion, apoptosis caused by inactivated antiproteases and cell proliferation regulation (Rahman and MacNee, 1996, 1999; Rahman and Adcock, 2006). Reduced antioxidant levels in smokers and COPD patients, signify the manifestation of systemic oxidative stress (Rahman et al., 2000; Rahman, 2005).

Inflammation is a well-known feature of COPD, as revealed by the presence of activated neutrophils and macrophages and, increased numbers of inflammatory mediators in the airways (Keatings and Barnes, 1997; Bhowmik et al., 2000). Various transcription factors including nuclear factor-kB (NF-kB) and activator protein 1 (AP-1) that are important for gene transcription of the inflammatory cytokines are oxidant sensitive (Rahman and MacNee, 2000). So, the balance of oxidant/antioxidant plays an important role in the disease etiology along with the role of inflammatory markers which trigger the inflammatory cascade. This indicates that lung inflammation and oxidative stress in COPD may be related. It is well documented that in addition to the local inflammation, systemic inflammation plays a key role in COPD (Agusti et al., 2003; Gan et al., 2004). Systemic inflammation is associated with increased mortality in COPD by loss of general body mass and lean body mass (Landbo et al., 1999; Agusti et al., 2002). This led to direct measures of systemic inflammation which might offer good markers of prognosis in COPD.

The present study was designed to assess the oxidative stress and serum hs-CRP levels in COPD patients and healthy controls. Effect of smoking, disease severity and exacerbations was ascertained on these parameters.

MATERIALS AND METHODS

The present study recruited 200 COPD patients from various local hospitals of Amritsar (Punjab, India) during Jan, 2012 to Oct, 2014. The patients were diagnosed by the pulmonary function test (GOLD, 2012) and/or X-rays by a chest physician. Long-acting β_2-agonists and/or anti-cholinergic inhaled bronchodilator therapy was given to each patient at sampling time. Patients having chronic diseases such as pulmonary tuberculosis, bronchial asthma, HIV infection, interstitial lung disease, pulmonary hypertension and/or other chronic respiratory or other disorders were excluded from the study. Age- and gender-matched healthy subjects (n=203) without any present respiratory symptoms and with no previous medical history of respiratory problem as well as any chronic ailment were recruited as controls. Information regarding demographic variables including age, gender, dietary pattern, smoking status and disease history etc. was recorded from all the subjects. Patients (mean age 58.74±10.64y) and controls (mean age 54.97±10.52y) were age- and gender-matched. Smoking pattern was recorded as smokers (smoking at the time of sampling), non-smokers and ex-smokers (quit smoking for ≥1 month). Exacerbations in COPD patients were assessed by GOLD (2012) guidelines. Blood samples were collected from each subject after taking informed voluntary consent according to ICMR guidelines (2006) and ethical clearance was obtained from the institutional ethics committee.

Anthropometric Measurements

Anthropometric measurements including height (cm), weight (Kg), waist circumference (cm) and hip circumference (cm) were recorded using standard method of Weiner and Lourie (1981). Body Mass Index (BMI), waist hip ratio (WHR) and waist stature ratio (WSR) were determined from the above measurements. General obesity was assessed by BMI using WHO (2004) criteria. Abdominal obesity was also determined by WC and WHR using criteria of Snehalatha et al. (2003). WSR was assessed by criteria of Hsieh and Muto (2005).

Pulmonary Function Test

For the diagnosis of COPD patients, pulmonary function test (PFT) was carried out by computerized spirometer (Medicad Spiro Excel, Chandigarh, India) as per the guidelines of American Thoracic Society (ATS). Prior to test, the procedure of PFT was thoroughly explained to each subject. In this procedure, after forceful inhalation of air, forceful expiration was pursued by keeping the nostrils closed. Readings were taken thrice from each subject and the best reading was taken into account. Post bronchodilator test was also performed. Pulmonary parameters including forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), FEV_1/FVC and peak expiratory flows (PEF) were recorded. Based on disease severity, patients were classified into four categories named as mild, moderate, severe and very severe as per the GOLD (2012) criteria.

Biochemical Analysis

Peripheral blood samples (2 ml) without anticoagulant were collected from each study participant. For serum separation, blood samples were incubated for one hour at 37°C followed by 10 min centrifugation (Spinwin, Tarson, India) at 320 g. Serum was collected as supernatant and stored at -80°C till further use. These serum samples were processed for various biochemical analyses.

Assessment of Total Antioxidant Capacity

Serum Total Antioxidant Capacity (TAC) was assessed using method of Erel (2004) with slight modifications by using microplate method given by Gupta et al. (2009). For this, 5 µl of serum sample was added to each well of the flat bottom microplate (Tarson, India) except for the blank (0.01 M PBS) and standard wells. Then 200 µl of reagent I (acetic acid-sodium acetate buffer, pH 5.8) was added to each well. Absorbance was measured at 655 nm on an ELISA reader (BIO -RAD, iMark™ Microplate Reader, India). Afterward, 20 µl of reagent II ($ABTS^+$ in 30 mmol/l acetate buffer, pH 3.6) was added to each well and incubated at 37ºC for 5 min. Final absorbance was read at 655 nm. Trolox (20 mmol/l) was used as standard and the results were expressed in mmolTrolox equivalent/l. The assay was carried out in triplicates.

Assessment of Total Oxidant Status

Serum Total Oxidant Status (TOS) was assessed using method of Erel (2005) with slight modifications by using microplate method given by Gupta et al. (2009). For this, 35µl serum

sample was added to each well except standard (H_2O_2; 10mmol/l) and blank. Then 225 µl reagent I (xylenol orange 150 µM, NaCl 140 mM and glycerol 1.35 M in 25 mM H_2SO_4solution) was added to the each well. Absorbance was taken at 595 nm on an ELISA reader (BIO -RAD, iMark™ Microplate Reader, India). Then 11 µl reagent II (ferrous ion 5 mM and o-dianisidine 10 mM in 25 mM H_2SO_4 solution) was added to each well. It was incubated at 37^{o}C for 5 min and final absorbance was taken at 595 nm.

Oxidative stress Index

Oxidative stress index (OSI) was calculated by ratio of total oxidant status to total antioxidant capacity i.e. OSI (arbitrary unit) = TOS (µmol H_2O_2equivalent*/*l)*/*TAC (µmolTrolox equivalent*/*l).

Assessment of high sensitivity C-reactive protein

High sensitivity C-reactive protein (hs-CRP) concentration was assessed quantitatively using ELISA kit (Bioassay Technology Laboratory, China) according to manufacturer's instruction. On an ELISA plate pre-coated with hs-CRP monoclonal antibody, 40 µl of serum sample and 10 µl of hs-CRP were mixed with 50 µl of streptavidin-HRP except for standard well where 50 µl of standard (conc = 12.8 mg/L) was added instead of serum. Then microplate was covered with seal plate membrane and incubated at 37°C for 60 min after gentle mixing. After incubation seal plate membrane was removed carefully and the content was drained. Each well was filled with freshly prepared washing solution (1X) which was drained after 30 sec. This procedure was repeated five times to remove unbound enzyme from the microplate. Afterwards, 50 µl of chromogen solution A was added to the microplate followed by addition of 50 µlchromogen solution B. Gentle shaking was done. After incubation at 37°C for 10 min, 50 µl of stop solution was added. Then absorbance was taken at 450 nm using microplate ELISA reader (BIO -RAD, iMark™ Microplate Reader, India).

Statistical Analysis

Data was presented as Mean±SD. Comparison of two groups was done by Student's t-test and for more than two groups was done by one way ANOVA analysis. Association was checked by Pearson's correlation analysis. In post-hoc, tuckey and bonferroni test were performed. All the statistical analysis was done by SPSS version 16.0.

RESULTS

TAC was found to be significantly low ($p<0.001$) in patients than controls whereas, no significant difference ($p<0.05$) was observed for total oxidant status. OSI was significantly high ($p<0.05$) in patients than controls. The hs-CRP levels were found to be significantly high ($p<0.001$) in patients as compared to controls (Table 1). Total antioxidant status revealed a positive association ($p<0.05$) with weight, WC, BMI, WHR, WSR and FVC (Table 2). Oxidative stress index was negatively correlated with TAC ($p<0.001$) and WHR ($p<0.05$) whereas positively correlated ($p<0.001$) with TOS. However, no significant correlation of hs-CRP levels was observed with these studied variables. as

TAC was significantly ($p<0.05$) higher in non-smokers as compared to smokers (Table 3). Smokers were further categorized into current smokers and ex-smokers. Significantly ($p<0.001$) high levels of TAC were observed in non-smokers as compared to current smokers. However, no significant difference was observed between non-smokers and ex-smokers. Significantly ($p<0.001$) high TAC was observed in ex-smokers than current smokers. For TOS, no significant difference was observed in non-smokers and smokers as well as with current smokers. TOS levels were significantly high ($p<0.05$) in non-smokers compared to ex-smokers and significantly high ($p<0.05$) in current smokers than ex-smokers. Furthermore, no significant difference for OSI was observed in non-smokers and smokers. However, OSI was significantly high ($p<0.05$) in current smokers than non-smokers. No significant difference for OSI was observed in non-smokers and ex-smokers. OSI was significantly high ($p<0.001$) in current smokers than in ex-smokers. No significant difference was observed in serum hs-CRP levels between smokers and non-smokers and with smoking categories. A significant difference ($p<0.05$) for TAC was observed among all the four COPD stages but independently between any two COPD stages no statistical difference was observed (Table 4). No significant difference was observed for TOS and OSI in different COPD stages. Significantly ($p<0.05$) elevated serum hs-CRP levels were observed in moderate COPD than very severe COPD. No statistical difference was observed for TAC, TOS, OSI and hs-CRP when patients with and without exacerbations were compared (Table 5).

DISCUSSION

To measure the extent of oxidative stress and inflammation in COPD patients and controls the present study, investigated the total antioxidant and oxidants capacity alongwith hs-CRP. Previous studies have revealed the role of oxidant/antioxidant imbalance in the COPD patients (Rahman and MacNee, 1996; MacNee and Rahman, 1999; Stanojkovic et al., 2011, Tavilani et al., 2012, Pirabbasi et al., 2013). Some studies have proposed the effect of oxidative stress in systemic circulation and lung of COPD patients (Barnes et al., 2003; van der Vaart et al., 2004). Several studies are in concordance with our findings where antioxidants levels were reduced in COPD patients than controls (Tavilani et al., 2012; Pirabbasi et al., 2013). However, the present study did not find the statistical difference in total oxidant status. CRP is primarily produced by stimulatory responses of IL-6 from hepatocytes and plays a protective role in innate immune responses against apoptotic cells and bacteria. Increased number of alveolar macrophages, activated epithelial cells and other inflammatory cells in COPD may release IL-6 into the circulation (Yoshida et al., 1995; Park et al., 2000). This can results in stimulation of an acute-phase response and elevated the level of CRP. Some previous studies also reported the higher levels of hs-CRP in COPD patients than controls (Pinto-Plata et al., 2006; Dahl et al., 2007; Aksu et al., 2013). In the present study, non-smoker patients had significantly high TAC as compared to smokers. However, pattern of similar TOS and OSI in non-smokers as well as smokers can be attributed to the fact that most of the patients belonging to non-smoker group had occupational exposure to coal press, biomass fuel etc (Jindal et al., 2006). Furthermore, ex-

smokers had significantly higher antioxidant capacity than current smokers. This was in consonance with a previous study which proposed that during smoking antioxidant capacity was decreased in smokers and solved quickly after smoking cessation (Rahman, 2005). Also antioxidant capacity decreased for many days after the onset of exacerbation and return to normal at the time of recovery. Higher antioxidant capacity in non-smoker than smokers in the present study was in concordance with earlier study (Rahman, 2005; Tavilani et al., 2012). Lack of TAC, TOS and OSI difference among patients at different stages of disease can be attributed to small sample size in each category.

CONCLUSION

Oxidative stress index as well as serum CRP was found to be significantly high in COPD patients than controls. Furthermore, current smokers had highest OSI as compared to non-smokers and ex-smokers. Our data provide further support to the fact that there is higher production of ROS in COPD leading to oxidative stress. These findings necessitate the inclusion of antioxidants in the management of COPD.

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Table 1. Comparison of oxidative stress variables and hs-CRP in COPD patients and controls

Variables	Total Antioxidant Capacity (TAC)	Total Oxidant Status (TOS)	Oxidative Stress Index (OSI)	High sensitivity- C Reactive Protein (hs-CRP)
Patients (N=200)	0.941±0.22	17.838±9.88	0.019±0.01	2.091±0.88
Controls (N=203)	1.344±0.48	19.329±9.67	0.015±0.01	0.598±0.63
p value	**0.000****	0.127	**0.034***	**0.000****

Values in bold are significant; *p<0.05; **p<0.001

Table 2. Correlation analysis of oxidative stress variables and hs-CRP with confounding parameters in COPD patients

	hs-CRP†		**TAC**		**TOS**		**OSI**	
	r	p	r	p	r	p	r	p
HEIGHT	0.011	0.926	-0.013	0.856	0.026	0.711	0.053	0.457
WEIGHT	0.063	0.584	0.147	**0.037***	0.057	0.426	-0.055	0.439
BMI	0.048	0.675	0.152	**0.031***	0.039	0.586	-0.083	0.244
WC	-0.025	0.825	0.145	**0.041***	-0.010	0.885	-0.113	0.112
WHR	-0.098	0.395	0.208	**0.003***	-0.115	0.105	-0.184	**0.009***
WSR	-0.032	0.780	0.140	**0.048***	-0.020	0.778	-0.124	0.081
FVC	-0.095	0.409	0.160	**0.043***	0.052	0.515	-0.042	0.599
FEV_1	-0.100	0.382	0.145	0.067	0.050	0.525	-0.046	0.566
FEV_1/FVC	-0.073	0.528	-0.019	0.813	-0.019	0.813	-0.030	0.702
PEF	-0.037	0.747	0.048	0.548	-0.037	0.645	-0.009	0.907
TAC	-	-	-	-	-0.179	**0.011***	-0.591	**0.000****
TOS	-	-	-0.179	**0.011***	-	-	0.675	**0.000****
OSI	-	-	-0.591	**0.000****	0.675	**0.000****	-	-

Values in bold are significant; *p<0.05; **p<0.001; †hs-CRP assessed in 78 COPD patients

BMI- Body mass index, WC- Waist circumference, WHR- Waist hip ratio, WSR- Waist stature ratio, FVC- Forced vital capacity, FEV_1- Forced expiratory volume in one second, PEF- Peak expiratory flow, TAC- Total Antioxidant Capacity, TOS- Total Oxidant Status, OSI- Oxidative Stress Index, hs-CRP- High sensitivity- C Reactive Protein

Table 3. Comparison of oxidative stress variables and hs-CRPin different smoking stages in COPD patients

	Smokers (N=128)	**Non-Smokers (N=72)**	**p-value**	**Current Smokers (N=73)**	**Ex-Smokers (N=55)**	**p-value**
TAC	0.921±0.25	0.977±0.13[a]	**0.043***	0.832±0.24[a,b]	1.040±0.21[b]	**0.000****
TOS	17.282±10.09	18.826±9.47[c]	0.290	19.758±10.89[d]	13.995±7.87[c, d]	**0.002***
OSI	0.024±0.03	0.019±0.01[e]	0.145	0.031±0.03[e, f]	0.009±0.001[f]	**0.000****
†hs-CRP	2.034±0.87	2.269±0.80	0.247	1.910±1.06	2.224±0.40	0.223

Values in bold are significant; *p<0.05; **p<0.001;†hs-CRP assessed in 78 COPD patients

[a,b,f]p=0.000, [c] p=0.015, [d] p=0.003, [e] p=0.009

TAC- Total Antioxidant Capacity, TOS- Total Oxidant Status, OSI- Oxidative Stress Index, hs-CRP- High sensitivity- C reactive protein

Table 4. Comparison of oxidative stress variables and hs-CRP in different COPD stages

	Mild (N=7)	**Moderate (N=29)**	**Severe (N=66)**	**Very Severe (N=59)**	**p-value**
TAC	1.053±0.09	0.967±0.17	0.919±0.19	0.859±0.24	**0.033***
TOS	20.959±11.87	19.611±10.87	18.406±10.11	17.515±9.97	0.735
OSI	0.019±0.01	0.021±0.01	0.023±0.02	0.027±0.03	0.728
†hs-CRP	-	2.526±0.76[a]	2.042±0.81	1.866±0.86[a]	**0.014***

Values in bold are significant; *$p<0.05$[a] p=0.012, †hs-CRP assessed in 78 COPD patients, Values in bold are significant
TAC- Total Antioxidant Capacity, TOS- Total Oxidant Status, OSI- Oxidative Stress Index, hs-CRP- High sensitivity- C Reactive Protein

Table 5. Comparison of oxidative stress variables and hs-CRP in COPD patients with and without exacerbations

	COPD without Exacerbation (N=92)	**COPD with Exacerbation (N=108)**	**p-value**
TAC	0.938±0.22	0.944±0.22	0.849
TOS	17.033±9.25	18.523±10.37	0.289
OSI	0.022±0.02	0.023±0.02	0.734
†hs-CRP	2.152±0.86	2.073±0.85	0.685

†hs-CRP assessed in 78 COPD patients
TAC- Total Antioxidant Capacity, TOS- Total Oxidant Status, OSI- Oxidative Stress Index, hs-CRP- High sensitivity- C Reactive Protein