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Oxidative Stress Bio Markers and Antioxidant Status in Cigarette Smokers Compared to Nonsmokers

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Abstract

Oxidative stress plays an important role in the pathogenesis of some diseases such as lung cancer, chronic obstructive pulmonary disease, and atherosclerosis. Smoking may enhance oxidative stress not only through the production of reactive oxygen radicals in smoke but also through weakening of the antioxidant defense systems. Cigarette smoke may promote atherogenesis by producing oxygen-derived free radicals that damage lipids. The present study was conducted to determine the effect of cigarette smoking on changes in lipid profile, lipid peroxidation and antioxidant status in cigarette smokers. The study population consisted of 200 male subjects divided into two groups; 100 smokers and age- and sex-matched non-smokers 100 subjects were selected. The mean systolic and diastolic blood pressure values were found to be significantly higher for smokers than for non-smokers. Biochemical parameters such as cardiac markers, lipid profile, apolipoproteins B and A1 (Apo B and A1), lipid peroxidation thiobarbituric acid reactive substances (TBARS) and antioxidants (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH), vitamin A, vitamin C and vitamin E were measured. A highly significant increase in the levels of cardiac markers was found in smokers when compared with non-smokers. Enhanced lipid peroxidation with concomitant depletion of antioxidants was observed smokers as compared to non-smokers. The levels of serum total cholesterol, triglycerides, LDL and VLDL were found to be significantly high, while HDL was significantly low in smokers compared to non-smokers. In addition, the activity of enzymatic and non-enzymatic antioxidants were more significantly altered in smokers than non-smokers. These results suggest that the atherogenic effects of smoking are mediated in part by free radical damage to lipids and possible breakdown of antioxidant status in cigarette smoking.

Key words: Smoking, Lipids, Lipid peroxidation, Antioxidant status

Introduction

Cigarette smoking is a serious health problem and most important avoidable causes of death in world [1]. Smoking has been strongly implicated as a risk factor for chronic obstructive pulmonary disease, cancer and atherosclerosis etc [2-4]. The World Health Organization predicts that tobacco deaths in India may exceed 1.5 million annually by 2020 [4]. In recent years, large household surveys have shown that in middle age, more than one third of men and a few percent of women smoke tobacco and that there are about 120 million smokers in India [5,4]. The leading causes of death from smoking are cardiovascular diseases (1.69 million deaths), chronic obstructive pulmonary disease (0.97 million deaths) and lung cancer (0.85 million deaths) [6].

Cigarette smoke is a complex mixture of chemicals containing more than 4000 different constituents. In the last 30-40 years, a large body of knowledge has accumulated identifying the exact chemical composition of cigarette smoke both qualitatively and quantitatively. Some of the compounds identified include different pyridine alkaloids such as nicotine, ammonia, acrolein, phenols, acetaldehyde, N-nitrosamine; polycyclic aromatic hydrocarbons such as benzopyrine; combustion gases such as carbon monoxide, nitrogen oxides, hydrogen cyanide; trace metals, α-emitter radioactive elements such as polonium, radium, and thorium [7].

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Two major phases were identified in cigarette smoke: a tar phase and a gas phase; both phases are rich in oxygen-centered, carbon-centered and nitrogen-centered free radicals as well as non-radical oxidants. From the analysis of each phase, it was estimated that a single cigarette puff contains approximately, 10^{14} free radicals in the tar phase, and 10^{15} radicals in the gas phase. These include various compounds, which are capable of causing an increase in the generation of various reactive oxygen species (ROS) like superoxide ($O_2^{\bullet -}$) hydrogen peroxide (H_2O_2), hydroxyl ($OH^{\bullet}$) and peroxyl ($ROO^{\bullet}$) radicals. These reactive oxygen species in turn are capable of initiating and promoting oxidative damage in the form of lipid peroxidation [8].

Evidence suggests that reactive oxygen species (ROS) may play important roles in the pathogenesis in myocardial infarction. Following ischemia, ROS are produced during reperfusion phase. ROS are capable of reacting with unsaturated lipids and of initiating the self-perpetuating chain reactions of lipid peroxidation in the membranes [9] Numerous reports have demonstrated the increased risk of coronary problems in smokers [10,11]. Smoking is thought to have an influence on the prevalence of myocardial infarction by means of several mechanisms, including atherosclerotic injury, increase in platelet aggregation, increase in the levels of adhesion molecules and fibrinogen and vasoconstriction [12]. Cigarette smoking leads to the uptake of many hazardous compounds. Such compounds or their metabolites may be electrophilic and thereby able to react with biological macromolecules, or they may give rise to oxidative stress by formation of reactive species or the initiation of radical chain reactions [13].

Free radicals are highly reactive molecules generated by biochemical redox reactions that occur as a part of normal cell metabolism. The human body has an inherent synergistic and multilevel defense mechanism, which comprise of two major classes of cellular protection against ROS [14]. Free radical scavenger enzymes namely SOD, CAT and GPx represent the enzymatic part. The non-enzymatic part includes a large number of natural and synthetic antioxidant compounds (GSH and vitamins) that have the ability to inhibit oxidative stress by scavenging the highly destructive free radicals. The deleterious effects of the free radicals are kept under check by a delicate balance between the rate of their production and the rate of their elimination by these defense systems. When there is an excessive addition of free radicals from exogenous sources added to the endogenous production, the available tissue defense system becomes overwhelmed resulting in oxidative damage to the tissues. The present study was therefore undertaken to assess the extent of lipid peroxidation and the status of antioxidants in cigarette smokers.

Materials and Methods

Study population

The study population consisted of 200 male (age-matched) subjects divided into two groups viz. 100 smokers and age- and sex-matched non-smokers (healthy volunteers) 100 subjects were selected. Participants in this study were selected from Kovai Medical Centre and Hospital (KMCH) & K.G. Hospital and Post Graduate Medical Institute, Coimbatore, Tamil Nadu, India, during the period January 2008 to January 2009.

Demographic and smoking habits information

Each subject was interviewed and asked to provide demographic and smoking habit information. The demographics included age, martial status (married and not married) and education status (low-illiterate or elementary, medium -intermediate or secondary and high- college or higher). Smoking habits included smoking period (years) and number of cigarettes smoked

daily. A written informed consent was taken from the subjects or the parents/guardians of the subjects prior to enrolment into the study. The protocol of this study was approved by the Institutional Human Ethics Committee (K.G. Hospital and Post Graduate Medical Institute, Tamil Nadu, India).

Blood collection and hemolysate preparation

Blood samples were collected by venous puncture in heparinized tubes and the plasma was separated by centrifugation at 1000 g for 15 min. After centrifugation, the buffy coat was removed and the packed cells were washed three times with physiological saline. A known volume of the erythrocytes was lysed with hypotonic phosphate buffer (pH 7.4). The hemolysate was separated by centrifugation at 2500× g for 15 min at 2°C.

Estimation of lipids and cardiac markers

Total cholesterol, triglycerides, HDL, LDL, specific apolipoproteins B and A1 (Apo B and A1) levels were determined by fully automated clinical chemistry analyzer (Hitachi 912, Boehringer Mannheim, Germany). VLDL level was calculated according to Friedewald *et al*. [15].

Estimation of lipid peroxidation

Lipid peroxides were estimated by measurement of thiobarbituric acid reactive substances (TBARS) in plasma by the method of Yagi[16]. The pink chromogen produced by the reaction of thiobarbituric acid with malondialdehyde, a secondary product of lipid peroxidation was estimated. TBARS concentration was expressed as nmoL mL/plasma

Assay of enzymatic antioxidants

SOD was assayed utilizing the technique of Kakkar *et al.* [17] based on inhibition of the formation of nicotinamide adenine dinucleotide, phenazine methosulfate and amino blue tetrazolium formazan. A single unit of enzyme was expressed as 50% inhibition of NBT (Nitroblue tetrazolium) reduction min/mg/Hb. CAT was assayed colorimetrically at 620 nm and expressed as μmoL of H_2O_2 consumed min/mg/Hb as described by Sinha [18]. The reaction mixture (1.5 mL) contained 1.0 mL of 0.01 M pH 7.0-phosphate buffer, 0.1 mL of hemolysate and 0.4 mL of 2 M H_2O_2. The reaction was stopped by the addition of 2.0 mL of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio).

GPx activity was measured by the method described by Rotruck *et al*. [19] with modifications. Briefly, reaction mixture contained 0.2 mL of 0.4 M Tris-HCl buffer pH 7.0, 0.1 mL of 10 mM sodium azide, 0.2 mL of hemolysate, 0.2 ml glutathione and, 0.1 mL of 0.2 mM H_2O_2. The contents were incubated at 37°C for 10 min. The reaction was arrested by 0.4 mL of 10% TCA, and centrifuged. The supernatant was assayed for glutathione content by using Ellmans reagent (19.8 mg of 5,5'-dithiobisnitro benzoic acid (DTNB) in 100 mL of 0.1% sodium nitrate). GPx activity was expressed as μmoL of GSH consumed min/g/Hb.

Estimation of non-enzymatic antioxidants

Plasma GSH level was determined by the method of Ellman [20]. 1.0 mL of plasma was treated with 0.5 mL of Ellmans reagent and 3.0 mL of phosphate buffer (0.2 M, pH 8.0). The absorbance was read at 412 nm. GSH level was expressed as mg /dL.

Plasma vitamin A was estimated by the method of Bradly and Hombeck [21]. Proteins were precipitated with ethanol and the carotenes were extracted into light petroleum. The intensity of the yellow color due to carotene was read directly at 450 nm using a violet filter. Vitamin E was measured by the method of Baker *et al.* [22] on the basis of the reduction of ferric ions to ferrous ions by vitamin E and the formation of a red colored complex with 2.2'-dipyridyl at 520 nm. Vitamin C was estimated by the method of Roe and Kuether [23]. This involves oxidation of ascorbic acid by

copper followed by treatment with 2,4-dinitrophenylhydrazine that undergoes rearrangement to form a product with absorption maximum at 520 nm.

Statistical analysis

All data were expressed as mean ± SD. The statistical significance was evaluated by Student's t test using Statistical Package for the Social Sciences (SPSS Cary, NC, USA) version 10.0.

Results

Information about the demographic characteristics of the study population is shown in Table 1. The mean age, body weight, height and Body Mass Index of the subjects were statistically similar between smokers and non-smokers. Diastolic and systolic blood pressure was significantly higher in smokers than in non-smokers.

Table 2 shows the extent of fasting glucose, lipids and specific apolipoproteins B and A1 in non-smokers and smokers. Total cholesterol, TG, LDL, VLDL and apolipoproteins B levels were significantly increased ($p<0.001$), while the HDL and apolipoproteins A1 level was significantly decreased in smokers compared with non-smokers but fasting glucose did not differ significantly.

The level of plasma TBARS and enzymic and non- enzymic antioxidant in smokers compared with non-smokers was depicted in Table 3. The extent of lipid peroxidation in smokers was significantly increased compared to non-smoking subjects. A significant decrease in the activities of SOD, CAT and GPx in erythrocyte lysate was seen in smokers compared with non-smoking subjects. However significant decrease in activities of SOD, Catalase and GPx of the smoking subjects suggesting pro-oxidant role of oxidative stress, which was confirmed by the decreased activities of all the enzymes in the smokers when compared to the non-smoking group. On the other hand, the level of non-enzymic antioxidants (β-carotene, vitamin C, vitamin E and GSH) significantly decreased in smokers compared with non-smokers.

Discussion

Cigarette smoking is a serious health problem and most important avoidable causes of death in world. The risk of disease increases with increasing intensity and duration of smoking. The data in this study shows that there were no significant differences in weight and body mass index between smokers and non-smokers. The present study shows that systolic and diastolic blood pressure was significantly higher in cigarette smokers than in non-smokers.

Analysis of TBARS in plasma is a widely used method for the evaluation of lipid peroxidation. The concentration of TBARS in plasma was higher in smokers than in non-smoking control subjects. These results are consistent with previous studies [26-28], which reported that the oxidative stress biomarker (MAD) was significantly higher in smokers than in non-smokers. It appears that a smoking plays a major role in lowering plasma antioxidants and exhibit a greater degree of lipid peroxidation in smokers.

Malondialdehyde cross-linking and lipid peroxidation have been suggested to play a role in the immunological destruction of plasma and erythrocyte antioxidants. The decrease in plasma and erythrocyte antioxidant status in smokers may be a consequence of enhanced lipid peroxidation. Cigarette smoking significantly increases serum LDL and its specific apolipoprotein B, but significantly decreases HDL and its specific apolipoprotein A1 concentration. We mentioned that smoking decreased vitamin C concentration, which led to a significant increase in LDL and its apo B concentrations and a decrease in HDL-cholesterol and its specific apo A1 concentrations [31,32]. The risk of cigarette smoking increased due to greater synthesis of LDL apolipoprotein B. Consequently, the result significantly increases of LDL [33].

Table 1. Demographic characteristics of non-smokers and smokers

	Non-Smokers	Smokers
Total number of subjects (n)	100	100
Sex (male)	100 %	100 %
Mean age (mean ± SD; years)	43.3 ± 9.7	40.1 ± 10.3*
Body Weight (kg)	81± 9.5	75± 12.5 NS
Body mass index (mean ± SD; Kg/m^2)	26.15 ± 1.03	25.82 ± 0.96 NS
Risk factors		
Systolic blood pressure (mm of Hg)	119 ± 3	137 ± 5***
Diastolic blood pressure (mm of Hg)	80 ± 2	90 ± 4***
Smoking status		
Smokers	-	100 %
Smoking Period (Year)	-	12 ± 5
No. of. Cigarettes Smoked per day	-	20 ± 5

Values are given as mean ± S.D from hundred subjects in each group.
Smokers compared with Non-smoking subjects
(*P<0.05, ***p<0.001, NS-Not significant)

Table 2. Levels of serum lipids and specific apolipoproteins B and A1 in non-smokers and smokers subjects

Parameter	Non-Smokers	Smokers
Fasting Glucose (mg /dl)	97.8 ± 7.03	100.4 ± 10.1 NS
Total cholesterol (mg/ dl)	154.2 ± 10.7	235.2 ± 17.8***
Triglyceride (mg/dl)	99.3 ± 12.18	195.6 ± 12.25***
HDL-cholesterol (mg /dl)	45.8 ± 5.37	31.8 ± 5.15***
LDL-cholesterol (mg /dl)	76.6 ± 9.84	147.0 ± 10.90***
VLDL-cholesterol (mg /dl)	27.7 ± 3.00	42.7 ± 7.40***
Apolipoprotein-A-l (mg/dl)	142.1± 22.0	92.5± 17.3***
Apolipoprotein-B (mg/dl)	99.5 ± 17.1	167.3± 15.2***

Values are given as mean ± S.D from hundred subjects in each group.
Smokers compared with Non-smoking subjects (***p<0.001, NS-Not significant)

Table 3: Circulatory lipid peroxide and antioxidant status in non-smokers and smokers subjects

Parameter	Non-Smokers	Smokers
TBARS (nmole/ml)	2.56 ± 0.21	4.72 ± 0.35***
SOD (Unit[a] mg/Hb)	4.30 ± 0.40	2.71 ± 0.29***
CAT (Unit[b] mg/Hb)	61.5 ± 7.60	49.2 ± 5.10***
GPx (Unit[c] mg/Hb)	7.31 ± 1.23	4.51 ± 0.95***
GSH (mg/dl)	40.71 ±1.70	22.02 ± 2.54***
Vitamin A (mg/dl)	0.99 ± 0.07	0.51 ± 0.09***
Vitamin C (mg/dl)	1.31 ± 0.31	0.77 ± 0.23***
Vitamin E (mg/dl)	1.35 ± 0.23	0.83 ± 0.19***

Values are given as mean ± S.D from hundred subjects in each group.
Smokers compared with Non-smoking subjects (***p<0.001)
a- One unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in one minute.
b - µmole of H_2O_2 consumed/minute.
c - µg of GSH consumed/min.
d - µmole of CDNB–GSH conjugate formed/min.

Antioxidants constitute the foremost defense system that limit the toxicity associated with free radicals. It is known that plasma antioxidant capacity decreases and oxidative/antioxidative balance shifts to the oxidative side in smokers. A reason for increased lipid peroxidation in plasma of smokers may be a poor enzymatic and non-enzymatic antioxidant defense system. SOD along with CAT and GPx, the preventive antioxidants, plays a very important role in protection against lipid peroxidation. In this study, SOD, CAT and GPx activities were significantly lower smokers than innon-smokers.

SOD is the first enzyme in antioxidant defense that scavenges superoxide radicals to form H_2O_2 and hence diminishes the toxic effects of the radical. Decreased activity of SOD has been reported in pathological conditions. The quinone-semiquinone radicals from the tar phase of cigarette smoke are capable of reducing molecular oxygen to superoxide radicals whose excessive generation inactivates this enzyme. Hence, a decrease in SOD activity upon smoke exposure could have resulted from its inactivation by tar phase oxidants. CAT is involved in the detoxification of high concentrations of H_2O_2. CAT has been suggested to play an important role in the protection of the erythrocyte against oxidative stress [34]. The presence and production of the free radicals from smoke lower this enzyme, leading to accumulation of H_2O_2 and lipid hydroperoxides further worsening the damage. A marked decrease in the activity of CAT in patients suffering from MI in the present study suggests the inability of host antioxidant defense to meet the oxidative stress following chronic exposure to cigarette smoke.

GSH, a widely distributed cellular reductant is a metabolic regulator and putative indicator of health. Blood glutathione levels are believed to be predicators of morbidity and mortality [35]. GSH plays a key role in protecting cells against electrophiles and free radicals. GSH can act directly as a free radical scavenger by neutralizing hydroxyl radicals, or indirectly by repairing initial damage to macromolecules inflicted by hydroxyl radicals. It is essential in the maintenance of protein and non-protein SH group in reduced form [36]. Smoking-induced depletion of GSH level has also been reported. This depletion was directly associated with elevation in lipid peroxidation which could be attributed to its protection against ROS generated by smoke, besides its consumption by the antioxidant enzymes GPx. Acetaldehyde, a major aldehyde from the smoke has been shown to deplete the cells of their GSH by conjugating with it, which further makes the cells more vulnerable to peroxidative damage. GPx, catalyses the reduction of H_2O_2 and organic hydroperoxides with simultaneous oxidation of GSH [36]. Absence of an augmentation in GPx activity upon smoke exposure in this study has been hypothesized to arise from a decrease in the levels of GSH that is essential for the conjugation of lipid peroxides. Low GSH levels and the decreased activity of GPx found in patients suffering from MI in the present study supports the hypothesis that smoking leads to a greater oxidative burden and depletion of antioxidant defences.

In the present study, we found a significant inverse correlation between the concentration of serum MAD and β-carotene in the smokers and non-smokers. Moreover, these relations were strong in smokers than in non-smokers and also stronger between MAD and lycopene than between MAD and β-carotene while α-tocopherol shows a weak inverse correlation with MAD levels in both smokers and non-smokers respectively. In the same respect, Sobczak *et al.* [29] found a weak inverse relation only in women and concluded that cigarette smoking had no effect on plasma α-tocopherol concentration while Farchi *et al.* [30] found a significant inverse relation between exposure to smoke and plasma β-carotene. Thus may be more effective to scavenge free radical and prevent

peroxidation in smokers than β-carotene and α-tocopherol.

Vitamin C is the first strong reductant in the aqueous phase that readily reacts with cigarette smoke oxidants and affords considerable protection to the cells. Studies involving different types of oxidative stress have shown that under all types of oxidative stress, ascorbic acid successfully prevents detectable oxidative damage and therefore it would be helpful in prevention of diseases in which oxidative stress plays a causative or exacerbation role [37]. Vitamin E, an important lipophilic antioxidant has an effective role in maintaining the cell structure against oxidative damage through blocking the chain reaction of free radicals. Vitamin E reacts with peroxyl radicals present in the smoke and terminates lipid peroxidation and vitamin A effectively quenches singlet oxygen [37]. Hence, the decrease in GSH levels could possibly be related to the inability of host tissue to synthesize GSH that is reflected from decrease in vitamin C, E and A. GSH and these vitamins are tightly linked to each other in a way that it helps to replenish vitamin C which in turn regenerates vitamin E and A. However, smokers are constantly overexposed to free radicals through inhalation of long-lived carbon- and oxygen-centered radicals that subsequently deplete the plasma and tissue stores of these micronutrients [37,38]. *In vitro* exposure of plasma to cigarette smoke resulted in the destruction of tocopherols, carotenoids and retinal [39]. The present study also revealed depletion in the levels of non-enzymatic antioxidants such as vitamin C, E and in plasma of smokers as compared to non-smokers.

Cigarette smoking is one of the most important exogenous factors, which cause 3-fold higher incidence of oxidative stress in smokers. Free radical-mediated oxidative stress appears to play a central role in cigarette smoking-mediated athero-thrombotic diseases. The results of present study clearly show that cigarette smoking induces an oxidative stress in smoking by augmenting lipid peroxidation and diminishing both enzymatic and non-enzymatic antioxidant status. The above findings also support the hypothesis that the atherogenic effects of smoking are mediated in part by free radical damage to lipids. The low antioxidant status of smokers may predispose them to oxidant- and cytokine-inflicted tissue damage and disease, which may manifest itself as coronary heart disease, atherosclerosis and cancer.

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