

Smoking induced oxidative stress in serum and neutrophil of the university students

Santanu Kar Mahapatra, Subhasis Das, Sankar K. Dey and Somenath Roy*

*Immunology and Microbiology Laboratory,
Department of Human Physiology with Community Health,
Vidyasagar University, Midnapore – 721 102,
West Bengal, India.*

Abstract: Cigarette smoking has been implicated as a significant risk factor for the establishment and progression of several diseases. The purpose of this study was to examine the effect of smoking in serum as well as in neutrophil oxidative damage and antioxidant status. Subjects were randomly chosen from male university students (22-25 years) in a rural community in the District of Midnapore, West Bengal, India. To understand status of oxidative damage, we measured the level of lipid peroxidation (MDA), activities of lactate dehydrogenase (LDH), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and reduced glutathione (GSH) level. The MDA level and the LDH activity in serum and neutrophil were higher in smokers than the non-smokers. The activities of CAT, SOD, GPx, GR and the level of GSH were significantly lower in smokers in comparison to the non-smokers in both serum and neutrophil. These findings may suggest that, students with a short smoking history have evidence of oxidative stress and an impaired oxidant defense system. Alterations observed in smokers that increased oxidative stress can represent a risk factor for the development of chronic disease in earlier future.

Key words: Smoking, serum, neutrophil, oxidative stress, university students

Introduction

Cigarette smoking, here after referred to as ‘smoking’, is the largest single risk factor for premature death in developed countries. The main addictive component of cigarette smoke is nicotine, which was first prescribed as medical drug to treat rodent ulcer and constipation. But now it is common knowledge that, nicotine does harm to our body. Smoking is an easy way to administer multiple doses of psychoactive drug nicotine. However, it leads to nicotine addiction, and is the most important cause of preventable death [1]. It is the chief, single avoidable cause of death in our society and most important health issue of our time [2]. Hence prevention and quitting smoking are major public health goals. It has been suggested that smoking is more addictive than nicotine alone due to the fact that tobacco or smoke seems to contain compounds, which are addictive in their own rights e.g., acetaldehyde [3] or increased the addictive potency of nicotine e.g., ammonium compounds [4].

Different prevalence survey indicate that, some demographic variables sex, age, ethnicity, and socioeconomic status are consistently associated with cigarette smoking, specially, male sex, younger age, lower socioeconomic status and lower educational attainment are positively associated with smoking. The main cause of smoking starts at the age of 17-20 years mainly due to absence of neighborhood disadvantages. Started smoking at younger ages score higher on psychological profiles of nicotine dependence [5] and are less likely to quite later in life [6] suggesting that younger particularly vulnerable to nicotine dependence.

Smoking has been implicated as a significant risk factor for the establishment and progression of several diseases. Although the underlying mechanisms involved in the pathogenesis associated with smoking is still an active debate. Free radical-induced oxidative damage has been suggested to play a major role in the pathogenesis of numerous smoking-related disorders [7]. Tobacco smoke is a rich source of oxidants. It has been argued that, the increased production of reactive oxygen species associated with smoking may exceed the capacity of oxidant defense system, resulting in oxidative damage [8, 9]. The potential damage that can be caused by free radicals is normally minimized by a combination of biological antioxidant systems including enzymatic and non-enzymatic reactions. Important antioxidant enzymes include superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase [10]. In adult smokers, several arms of the oxidant defense system have been reported to be impaired as compared with non-smokers. Huela et al., 1995 reported that adult smokers have significantly lower erythrocyte CuZn SOD and Se-GSH-Px activities than do non-smokers [11]. They suggested that one consequence of the lower activities of these enzymes in smokers is oxidative stress. Although smoking is recognized to be a significant health problem in older subjects, there are surprising shortages of information on the health consequences of smoking in teenagers. The frequency of tobacco use by teenagers is increasing annually, especially in developing countries [12].

Smoking is a major health problem risk factor worldwide and significantly increases the incidence of several diseases [13]. It is hypothesized that this increased disease susceptibility reflects cigarette smoke induced changes in the immune system [14] and chronic exposure to cigarette smoke suppress a wide range of immunological parameters in human and animal models [15, 13]. So, studies of effect of cigarette smoking in neutrophil and serum get an important impact. In light of these findings, the present study was conducted to obtain information on the effects of cigarette smoking on select makers of oxidative damage and antioxidant status in university students.

Materials and Methods

2.1. Selection of Subject

The subjects for this study were randomly selected from a population of male university students in a rural community in the District of Midnapore, West Bengal, India. All subjects were healthy and reported no use of illegal drugs (Except smoking in case of smoker). Subjects were interviewed for tobacco use and questioned on the number of cigarettes smoked on average per day and when they started smoking. Twenty smokers (age range 22-25 yrs) who had smoked more than 10 cigarettes per day continually for at least 4-5 years were identified; their smoking consumption (mean \pm standard error of the mean) was 18.5 ± 1.3 cigarettes/day. Twenty nonsmokers (age range 21-25 y) were identified who reported no previous smoking experience. The sample size in the present study was small because both groups indicated they did not like the idea of giving blood.

2.2. Chemicals and reagents

Histopaque 1077, dextran, 5, 5-dithio-bis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), NADPH, Na₄, NADPH, sodium pyruvate, 1,1,3,3-tetraethoxypropane were purchased from Sigma Chemical Co., USA. Heparin, sodium dodecyl sulfate were purchased from Himedia, and other chemicals were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India.

2.3. Collection of blood samples and separation of serum and neutrophil

The subjects were asked to attend the laboratory between 8.00-9.00 a.m. after overnight fasting. Fasting blood samples were drawn by medical officer, Vidyasagar University from nonsmoker and smoker individuals. The ethical committee of the Vidyasagar University approved the study, and informed consent of all the subjects was obtained. Serum was obtained by centrifugation at 1500× g for 15 min of blood samples taken with out anticoagulant. Serum was kept at -86°C for the biochemical estimation of different parameters. Heparinized blood samples were used for the separation of neutrophils. Neutrophils were isolated from blood using standard isolation techniques [16]. Briefly blood was diluted with PBS (pH = 7.0) buffer in equal ratio and then layered very carefully on the density gradient (Histopaque 1077, Sigma) in 1:2 ratio, centrifuged at 500 × g for 20 min. Neutrophils were isolated from buffy coat with RBC layer followed by dextran sedimentation and hypotonic lysis to remove red blood cells. The pellets of neutrophil were lysed in a hypotonic lysis buffer for 45 min at 37 °C and kept at -86 °C until biochemical estimations [17].

2.4. Biochemical estimation

2.4.1. Lipid Peroxidation

The extent of lipid peroxidation was estimated as the concentration of thiobarbituric acid reactive product malondialdehyde (MDA) by using the method of Ohkawa et al., 1979 [18]. 100µl of serum/neutrophil lysates were added to 100µl of double-distilled water and 50µl of 8.1% sodium dodecyl sulfate (SDS) and incubated at room temperature for 10 min. 375µl of 20% acetic acid (pH 3.5), along with 375µl of thiobarbituric acid (0.6%), was added to the sample solution and placed in a boiling water bath for 60 min. After incubation, 250µl of double-distilled water and 1.25 ml of 15:1 butanol-pyridine solution were added to the mixture and centrifuged for 5 min at 2000×g. The resulting supernatant was removed and measured at 532 nm with the use of the Hitachi U-2000 spectrophotometer. Malondialdehyde concentrations were determined by using 1,1,3,3-tetraethoxypropane as standard in terms of n mol/mg protein.

2.4.2. Lactate dehydrogenase

Lactate dehydrogenase (LDH) activity was determined according to King, 1959 & 1965 with some modification using lactate as substrate and measure the pyruvate formation [19, 20]. It was calculated according to standard curve prepared using sodium pyruvate.

2.4.3. Superoxide dismutase

Superoxide dismutase (SOD) activity was determined from its ability to inhibit the auto-oxidation of pyrogallol according to Mestro & McDonald, 1986 [21]. The reaction mixture considered of 50mM Tris (hydroxymethyl) amino methane (pH 8.2), 1mM diethylenetriamine pentaacetic acid, and 20-50 μ l of serum/ neutrophil lysate. The reaction was initiated by addition of 0.2mM pyrogallol, and the absorbance measured kinetically at 420nm at 25°C for 3 min. SOD activity in serum and neutrophil were expressed as unit/mg protein.

2.4.4. Catalase

Catalase (CAT) activity was measured in the serum and cell lysates by the method of Luck, 1963 [22]. The final reaction volume of 3ml contained 0.05M Tris-buffer, 5mM ethylenediamine tetra acetic acid (EDTA) (pH 7.0), and 10mM hydrogen peroxide (H_2O_2) (in 0.1M potassium phosphate buffer, pH 7.0). About 50 μ l aliquots of the serum/lysates were added to the above mixture. The rate of change of absorbance per min at 240 nm was recorded. Catalase activity was calculated by using the molar extinction coefficient of 43.6M⁻¹cm⁻¹ for H_2O_2 and was expressed in terms of μ mol H_2O_2 consumed/min/mg protein.

2.4.5. Reduced Glutathione

Reduced glutathione (GSH) estimation in the serum and cell lysate were performed by the method of Moron et al., 1979 [23]. The required amount of the serum/cell lysates were mixed with 25% of trichloroacetic acid and centrifuged at 2000 \times g for 15min to settle the precipitated proteins. The supernatant was aspirated and diluted to 1ml with 0.2M sodium phosphate buffer (pH 8.0). Later, 2ml of 0.6mM DTNB was added. After 10 minutes the optical density of the yellow-colored complex formed by the reaction of GSH and DTNB was measured at 405 nm. A standard curve was obtained with standard GSH. The levels of GSH were expressed as μ g /mg protein.

2.4.6. Glutathione Peroxidase

The Glutathione Peroxidase (GPx) activity was measured by the method of Paglia & Valentine, 1967 [24]. The reaction mixture contained 50mM potassium phosphate buffer (pH 7.0), 1mM EDTA, 1mM sodium azide, 0.2mM NADPH, 1U glutathione reductase, and 1mM GSH. The sample, after its addition, was allowed to equilibrate for 5 min at 25°C. The reaction was initiated by adding 0.1ml of 2.5mM H_2O_2 . Absorbance at 340nm was recorded for 5 min. Values were expressed as nano moles of NADPH oxidized to NADP by using the extinction coefficient of 6.2 X 10³ M⁻¹ cm⁻¹ at 340 nm. The activity of GPx was expressed in terms of n mol NADPH consumed/min/mg protein.

2.4.7. Glutathione reductase

The activity of Glutathione reductase (GR) activity was measured by the method of Miwa, 1972 [25]. The tubes for enzyme assay were incubated at 37°C and contained 2.0ml of 9mM GSSG, 0.02ml of 12mM NADPH, Na₄, 2.68ml of 1/15M phosphate buffer (pH 6.6) and 0.1ml of serum/cell lysate. The activity of this enzyme was determined by monitoring the decrease in absorbance at 340nm. Values were expressed as nano moles of NADPH oxidized to NADP by using the extinction coefficient of 6.2 X 10³M⁻¹cm⁻¹ at 340 nm. The activity of GR was expressed in terms of n mol NADPH consumed/min/mg protein.

2.4.8. Protein estimation

Protein was determined according to Lowry et al., 1951 using bovine serum albumin as standard [26].

2.5. Statistical analysis

The data were expressed as mean ± standard error. Comparisons of the means of non-smoker and smoker were made by student's t-tests, P < 0.05 as a limit of significance.

Results

To investigate the effects of cigarette smoking in serum and neutrophil of university student, oxidative stress related biochemical parameters were studied in the present work. We also investigate some of their demographic and smoking history (Table 1).

Table. 1: Demographic and smoking history of the subject studied (n=20)

Characteristics	Smoker	Non-smoker
1. Subject (n)	20	20
2. Age (years)	21.35 ± 1.96	21.23 ± 1.82
3. Body weight (Kg)	54.6 ± 2.96	57.24 ± 2.28
4. Body height (cm)	165.23 ± 6.38	168.54 ± 6.74
5. Current number of cigarette smoking per day	18.5 ± 1.3	Nil
6. Current number of cigarette smoking per month	563.8 ± 23.29	Nil
7. Duration of smoking (years)	5.00 ± 0.92	Nil
8. Variation of socioeconomic status	Nil	Nil
9. Variation of calorie intake	Nil	Nil

3.1. Lipid peroxidation

The levels of lipid peroxidation in terms of MDA in smoker are shown in figure 1. The increases of MDA levels were higher in neutrophil rather than serum. The MDA

levels were increased significantly ($p < 0.05$) by 81% and 270% in serum and neutrophil respectively compared to their respective control.

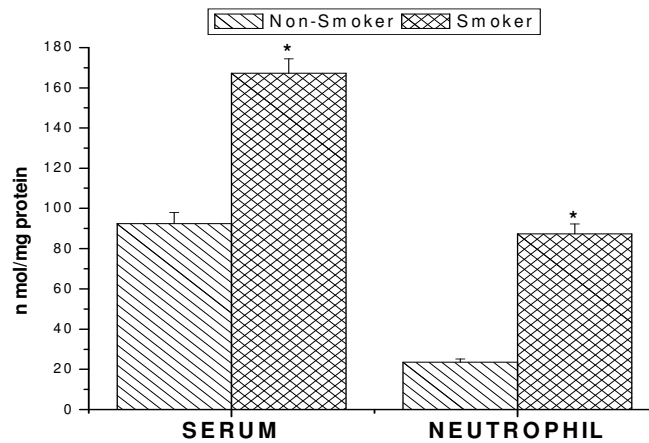


Fig. 1 MDA levels in serum and neutrophil of non-smoker and smoker. Data are expressed as mean ± SEM, n=20. * Represents the significant difference between non-smoker and smoker ($P < 0.05$).

3.2. Lactate dehydrogenase activity

LDH activity was generally higher in serum rather than blood cell in both smoker and non-smoker (Fig-2) and it was increased with short term smoking habit in both serum and neutrophil of our studied subjects. The rate of increased LDH activity was higher in serum rather than in neutrophil and the LDH activity was increased significantly ($p < 0.05$) by 223.15% and 60.9% in serum and neutrophil respectively compared to their respective control.

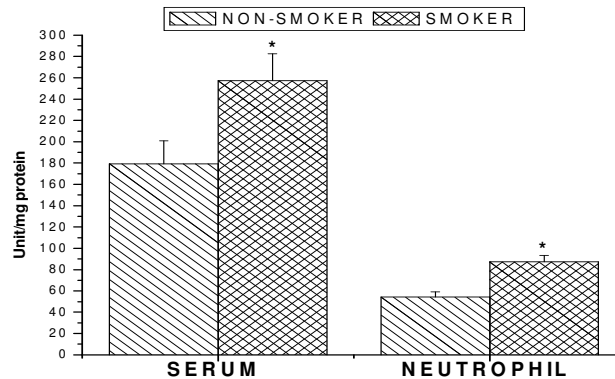


Fig. 2 LDH activity in serum and neutrophil of non-smoker and smoker. Data are expressed as mean ± SEM, n=20. * Represents the significant difference between non-smoker and smoker ($P < 0.05$).

3.3. Activity of Superoxide dismutase and Catalase

The enzymatic antioxidant levels were also decreased with the smoking habit. In our study, the SOD and CAT activity in serum and neutrophil of university students were decreased significantly ($p < 0.05$) in smoker compared with non-smoker (Fig-3, 4). In smoker student, serum SOD and CAT activity were decreased by 66.65% and 33.74%, and they were decreased 47.43% and 35.56% in neutrophil than non-smoker.

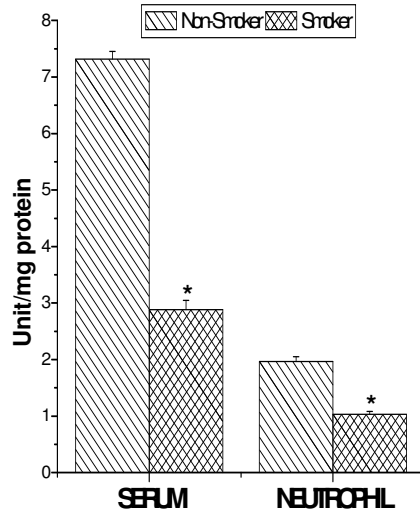


Fig. 3 SOD activity in serum and neutrophil of non-smoker and smoker. Data are expressed as mean \pm SEM, n=20. * Represents the significant difference between non-smoker and smoker ($P < 0.05$).

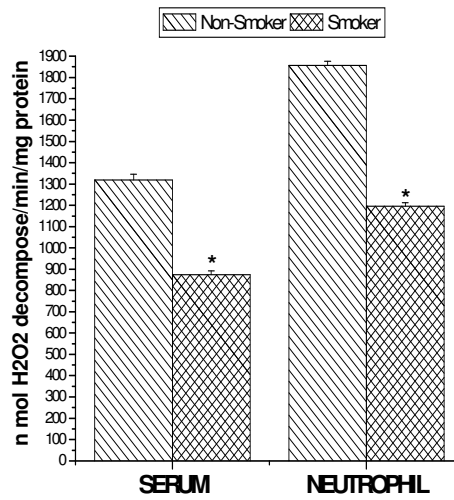


Fig. 4 Catalase activity in serum and neutrophil of non-smoker and smoker. Data are expressed as mean \pm SEM, n=20. * Represents the significant difference between non-smoker and smoker ($P < 0.05$).

3.4. Glutathione level

The non-enzymatic antioxidant like GSH level was decreased significantly ($p < 0.05$) in both serum and neutrophil in smoker subject (Fig-5). GSH level was decreased 21.76% in serum and 35.21% in neutrophil compared to non-smoker.

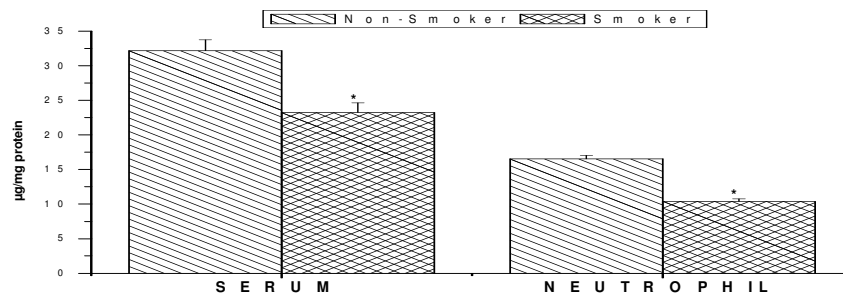


Fig. 5 GSH level in serum and neutrophil of non-smoker and smoker. Data are expressed as mean \pm SEM, n=20. * Represents the significant difference between non-smoker and smoker ($P < 0.05$).

3.5. Glutathione peroxidase and glutathione reductase activity

In the present study, we have also studied the glutathione dependent antioxidant enzymes profile through estimation of GPx and GR activity in serum and neutrophil. Both of the enzymes activities were decreased (Fig-6, 7) in smoking habited students. GPx and GR activity were decreased significantly ($p < 0.05$) by 27.8%, 18.37% in serum and 37.61%, 32.07% in neutrophil.

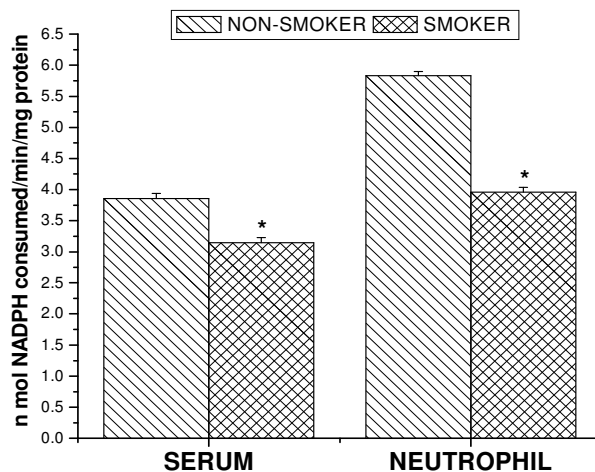


Fig. 7 GR activity in serum and neutrophil of non-smoker and smoker. Data are expressed as mean \pm SEM, n=20. * Represents the significant difference between non-smoker and smoker ($P < 0.05$).

Discussion

Cigarette smoke is an important variable of the student's life in our society. It may be inappropriate to make simple dichotomous divisions between smokers and non-smokers. The result of the present study suggested that, smoking habit is associated with the free radical scavenger system in serum and neutrophil. A limitation of the present investigation was that the smoking dose determinations might be imprecise because the authors depend only on their verbal information. Specifically other relevant factors of smoking were not considered including smoking technique, cigarette brand.

In the present study, cigarette smoking was not associated with a reduction in height, although body weights tended to be lower in the smokers than in the non-smokers. The slightly lower body weights of the smokers were probably secondary to a lower caloric intake in this group than in the non-smoking group. Although we attempted to obtain accurate diet records from all the students, the individuals who were in the smoking group reported markedly lower caloric intakes.

Smoking is associated with a variety of diseases. Cigarette smokes contain a range of xenobiotics, including oxidants, and oxygen free radicals that can increase lipid peroxidation [27, 28, 29]. Its toxicity may be further enhanced by the stimulation of reactive oxygen species (ROS) production by neutrophil [30]. One estimate suggests cigarette smoke contains on the order of 10^{14} free radicals per inhalation [31]. We revealed that antioxidant status in serum and neutrophil were diminished in student smokers and simultaneously lipid peroxidation was increased compared with non-smokers.

Oxidative stress is the result of an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant system in favour of the former. So, intensity of oxidative stress is determined not only by the free radicals production but also by antioxidants (enzymatic and non-enzymatic) defense [32]. Oxidative stress generates free radicals, which result in lipid peroxidation and destruction of lipid components of biological membranes that have been suggested to play a role in the carcinogenesis [33, 34]. In our previous report, MDA levels were increased in oral cancer patient and nicotine induced rat tissue [35, 36]. Nicotine, a major toxic component of cigarette smoke, is a well established procarcinogen [37]. However, it has been reported that nicotine disrupts the mitochondrial respiratory chain leading to an increased generation of superoxide anion and hydrogen peroxide [38]. This may lead to oxidative damaged macromolecules including lipid, DNA, RNA, antioxidant enzyme in subsequent cell through disruption of cellular functions and integrity [39]. According to Yildiz D et al., (1999) LDH activity was increased due to nicotine induced oxidative stress. In our present investigation, the increased level of lipid peroxidation (Fig.1) and increased LDH activity (Fig.2) in serum and neutrophil with smoking habit in university students may due to the increased generation of reactive oxygen species by smoking that leads to cell damage and also indicated the lower capacity to combat against ROS [38].

Glutathione being an important cellular reductant, involved in protection against free radicals, peroxides and toxic compounds [40]. Depletion of GSH is one of the primary factors that permit lipid peroxidation [41]. In our present study, the GSH levels were decreased (Fig.5) in serum and neutrophil with smoking history of university students. The decreased GSH level may be due to increase level of lipid oxidation products which may be associated with the less availability of NADPH required for the activity of glutathione reductase (GR) to transform oxidized glutathione to GSH [42] due to the increased production of ROS at a rate that exceeding the ability to regenerate GSH with 4 to 5 years smoking history. The decreased GSH level in association with decreased GR activity (Fig.7) may support the explanation as evidence.

Antioxidant defense system protects our body from the deleterious effect of reactive oxygen metabolites. Catalase act as preventive antioxidants and superoxide dismutase (SOD), a chain breaking antioxidant, play an important role in protection against the deleterious effect of lipid peroxidation [43]. In relation to the activity of the antioxidant enzyme SOD, there was decreased activity (Fig.3) in serum and

neutrophil of smoker. Smoking is associated with lower antioxidants concentrations increased oxidative stress, and damage, and increased risk of several chronic diseases [44]. Where the SOD stops its action, there the catalase exerts its function. The primary role of catalase is to scavenge H_2O_2 that has been generated by free radicals or by SOD in removal of superoxide anions and to convert it to water [45]. In our present study, the decreased in catalase activity (Fig.4) may be related to excess H_2O_2 production from smoking or SOD inhibition [46]. The decreasing activity of antioxidant enzymes i.e. SOD, CAT and decreasing level of GSH in serum and neutrophil due to increased utilization to scavenge the free radical generation along with cigarette smoking.

Glutathione peroxidase (GP_x) works nonspecifically to scavenge and decompose excess hydro peroxides including H_2O_2 , which may prevalent under oxidative stress [47, 48, 49]. In this study, decreased GP_x activity seems to indicate the susceptibility of neutrophil to smoking induced oxidative stress. The decreased level of GSH and activity of GSH- dependent enzymes i.e. GP_x , GR (Fig.6, 7) in serum and neutrophils of smoker university students may be due to increased utilization to scavenge the free radical generation along with smoking.

The university student smokers examined in the present study displayed evidence of oxidative stress and a diminished antioxidant defense system. The impaired oxidant-antioxidant balance is representing as a risk factor for the development of chronic disease. The oxidative damage observed in smokers can be associated with the direct effects of oxidants in cigarette smoke and the consequences of lower antioxidant status associated with smoking. The best medical advice for this population is to stop smoking. Campaigns aimed at improving the antioxidant status of this group should be mounted in conjunction with anti-smoking campaigns.

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*All Correspondence: Prof. S.Roy, Immunology and Microbiology Laboratory, Department of Human Physiology with Community Health, Vidyasagar University, Midnapore – 721 102, West Bengal, India. E-mail: somenathroy@hotmail.com