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Red blood cell susceptibility to oxidants in chronic cigarette smokers

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ABSTRACT

Smoking is an important preventable cause of mortality worldwide and has been implicated as a significant risk factor for the establishment and progression of several diseases. It has been argued that the increased production of reactive oxygen species associated with smoking may exceed the capacity of the oxidant defense system, resulting in oxidative damage. The aim of this study was to investigate a possible relationship between cigarette smoking and red cell destruction due to oxidative damage. Hundred (100) subjects were randomly chosen from a population of males (22 -38 years) within the Osogbo, Osun State metropolis. Blood samples were collected and drawn into an anticoagulated bottle with the aid of a 5ml sterile disposable syringe from one of the prominent veins of the cubital fossa of each of the subjects and used immediately for analysis. To understand the status of oxidative damage, we measured the level of lipid peroxidation using the malondialdehyde (MDA) method, activities of glutathione peroxidase (GPx) and reduced glutathione (GSH) in the plasma of both smokers and non smokers were also investigated. To establish a possible relationship between cigarette smoking and red blood cell destruction, the reticulocyte counts, haematocrit and total bilirubin levels of smokers and non-smokers was examined. MDA levels were significantly higher in the plasma of smokers. Activities of GPx and the level of GSH where markedly decreased in smokers when compared with the control group. There was also a significant increase in the total bilirubin levels and the reticulocyte count values of smokers when compared with those of the non smokers. Haematocrit values were slightly increased in smokers when compared with control. The present study thus concluded that an increased oxidative burden decreases the function of the antioxidant systems which can in a long run ultimately lead to red blood cell haemolysis.

Keywords: Smokers, Antioxidants, haemolysis, red blood cells, peroxidation, reduced glutathione

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INTRODUCTION

Cigarette 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 [1] Cigarette smoke contains a wide range of xenobiotics, including oxidants, and oxygen free radicals that can increase lipid peroxidation [2]. It has been hypothesized that many of the adverse effects of smoking may result from oxidative damage to critical biological substances. Such damage may be from the oxidants present in cigarette smoke and from the activation of phagocytic cells that generate reactive oxygen species [3].

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 [4]. 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. It has however been argued that the increased production of reactive oxygen species associated with smoking may exceed the capacity of the oxidant defense system [5]. Red blood cell (RBC) membrane lipids are rich in polyunsaturated fatty acids; therefore, the oxidative effects of oxygen on red blood cell membranes are greater than on other tissues. Moreover, red blood cells contain hemoglobin, which is one of the most potent catalysts of lipid peroxidation. The invasion of the red blood cell membrane by peroxidants, which occurs with hemoglobinopathies, radioactive radiation and the consumption of oxidative drugs such as cigarette, increases the levels of certain metals in the body and decreases the function of the antioxidant systems which can ultimately lead to red blood cell hemolysis [6]. In addition to causing lipid peroxidation, peroxidants can cause the oxidation of the -SH groups in proteins and red blood cell membranes. The –SH groups are highly reactive and can be a target during oxidative stress. Glutathione therefore directly protects membrane proteins and preserves their stability. Decreased levels of glutathione lead to a decrease in the -SH groups [7] which can result in the oxidization of membrane -SH groups and loss of membrane stability [8].

Reducing potential is required by the red cells to maintain the iron atoms of haemoglobin in the functional state and to counteract the presence of highly reactive free radicals which may be associated with increased cigarette smoking. This study was therefore conducted to obtain information on the effect of cigarette smoke on the cellular redox potential with response to increased oxidative burden. Hence, the present study examined the Red blood Cell Susceptibility to Oxidants in Chronic Smokers.

SUBJECTS AND METHODS

The subjects for this study were randomly selected from a population of male within the Oshogbo, Osun State metropolis, South Western Nigeria. All subjects were healthy and reported no use of illegal drugs (Except smoking in case of smokers). Subjects were interviewed

for tobacco use and questioned on the number of cigarettes smoked on average per day and when they started smoking. Fifty (50) smokers (age range 17-25 yrs) who had smoked more than 10 cigarettes per day continually for at least 4-5 years were identified; their smoking consumption (mean ± standard error of the mean) was 12.5 ± 1.3 cigarettes/ day. Fifty (50) 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.

Laboratory analysis of samples collected was conducted at the clinical Immunology laboratory of the University College Hospital Ibadan (UCH).

Collection of Blood Samples and Separation of Plasma

5ml of venous blood was drawn aseptically from the anti-cubital vein of subjects using a 5ml sterile disposable plastic syringe with a stainless needle and dispensed immediately into the various collection bottles. The ethical committee of the Ladoke Akintola University approved the study, and informed consent of all the subjects was obtained. Plasma was obtained by centrifugation of whole blood at 2000rpm for 15 min. Plasma samples was kept at -70ºC for the biochemical estimation of different parameters. Blood films were immediately made from Heparinized blood samples and used for reticulocyte count analysis also haematocrit estimation was done using standard techniques.

BIOCHEMICAL ESTIMATION

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., [9]. 100: l of lysates were added to 100: l of double distilled water and 50: l of 8.1% sodium dodecyl sulfate (SDS) and incubated at room temperature for 10 min. 375: l of 20% acetic acid (pH 3.5), along with 375: l of thiobarbituric acid (0.6%), was added to the sample solution and placed in a boiling water bath for 60 min. After incubation, 250: l 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.

Reduced Glutathione

Reduced glutathione (GSH) estimation in the plasma were performed by the method of Moron *et al.,* [10]. The required amount of the plasma/cell lysates were mixed with 25% of trichloroacetic acid and centrifuged at 2000×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 _g /mg protein.

Glutathione Peroxidase

The Glutathione Peroxidase (GPx) activity was measured by the method of Paglia & Valentine, [11]. 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 mins. 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.

Total Bilirubin

The Jendrassik and Groff method was used for the estimation of total bilirubin. In this method, Sulphanilic acid is diazotized by the nitrous acid produced from the reaction between sodium nitrite and hydrochloric acid. Bilirubin reacts with the diazotized Sulphanilic acid (diazo reagent) to form azobilirubin. Caffeine is an accelerator and gives a rapid and complete conversion to blue azobilirubin by an alkaline tartrate reagent and the absorbance of the blue-green solution is read with a spectrophotometer at wavelength 600nm. 1 ml of caffeine-benzoate reagent was placed into six different tubes and mixed, To tube S and SB 0.1ml standard serum was added, To tube C and CB 0.1ml control serum was added, To tube TB, 0.1ml plasma sample was added and mixed individually, 0.5 ml of diazo reagent was then added to each tubes S, C, and TS, respectively and mixed well. After mixing, 0.5 ml of sulphanilic acid, reagent was added to tubes SB, CB, TB. This was then mixed well. The samples were left at room temperature (20 – 28 ^{0}C) for 5 minutes. After which 1 ml of alkaline tartarate regent was added to each tube and mix well. The absorbance's of the solutions was then read immediately with a spectrophotometer set at wavelength 600nm. Readings of the blanks were subtracted from the readings of the standard, control and Test samples.

Reticulocyte Count

Reticulocyte was counted in the study after staining with a romanowsky stain. Two drops of whole blood was added to two drops of new methylene blue solution In a clean test tube, The solution was incubated in a water bath for 15 minutes at 37^{0}C, After incubation, the cells were re-suspended by gentle tapping of the bottom of the tubes. A thin film was made with the solution on a grease free clean glass slide. The slide was allowed to dry and examined microscopically under an oil immersion lens 1000 red cells were counted and the number of reticulocyte seen on each field was noted.

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$$\text{Reticulocyte Percentage} = \frac{\text{No of retics counted in 10 fields} \times 100}{\text{No of RBC counted} + \text{Retics}}$$

Red Cell Haematocrit

The red cell haematocrit was measured using the microhaematocrit method .Anti-coagulated blood was carefully mixed; a microhaematocrit tube was filled with the blood sample up to ¾ of its length by capillary action. One end of the tube was sealed with a sealer; It was then centrifuged at 10,000 RPM for five minutes, which separated the blood sample into distinct layers.

Statistical Analysis

Results obtained were expressed as mean and standard deviation, this was compared with the control value using the SPSS version 15 and significance was considered at $p<0.05$

RESULTS

To understand the status of oxidative damage; 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=50)

	Smokers	Non – Smokers
Subjects (n)	50	50
Age (years)	21.35 ± 1.96	21.23 ± 1.82
Body weight (kg)	62.88 ±2.96	68.50 ± 2.28
Body height (cm)	165.23 ± 6.38	168.54 ± 6.74
Cigarettes per day (no)	12.5 ± 1.3	Nil
Duration of smoking (yrs)	8.00 ± 0.92	Nil
Chronic Disease	Nil	Nil
Variation of socioeconomic status	Nil	Nil
Variation of calorie intake	Nil	Nil

The levels of lipid peroxidation in terms of MDA in smoker are shown in figure 1. MDA in the plasma of smokers were increased significantly ($p < 0.05$) by 81%

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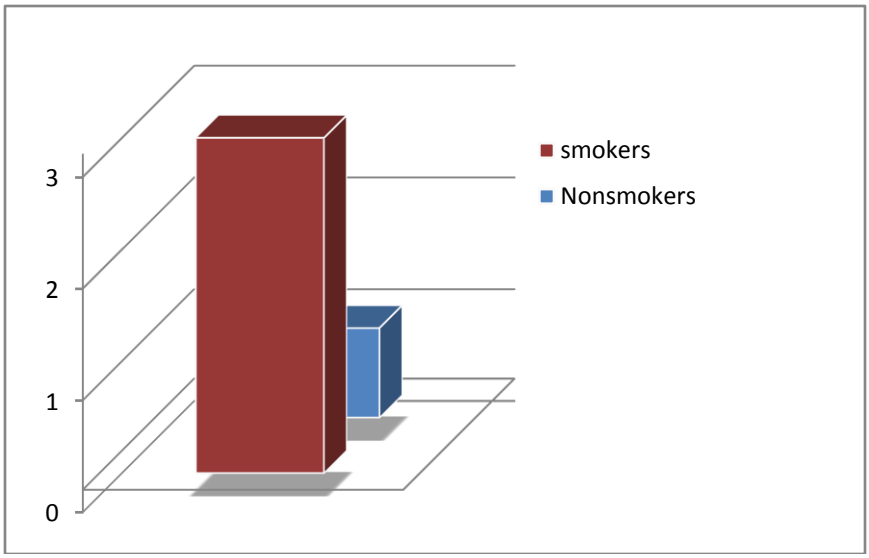

Fig. 1 MDA levels in plasma of non-smoker and smoker.

Data are expressed as mean ± SEM, n=50. * Represents the significant difference between smoker and non-smokers (*P<0.05*).

The non-enzymatic antioxidant GSH level was decreased significantly (*P <0.05*) in smoking subject (Fig-2). GSH level was decreased 21.76% compared to non-smoker.

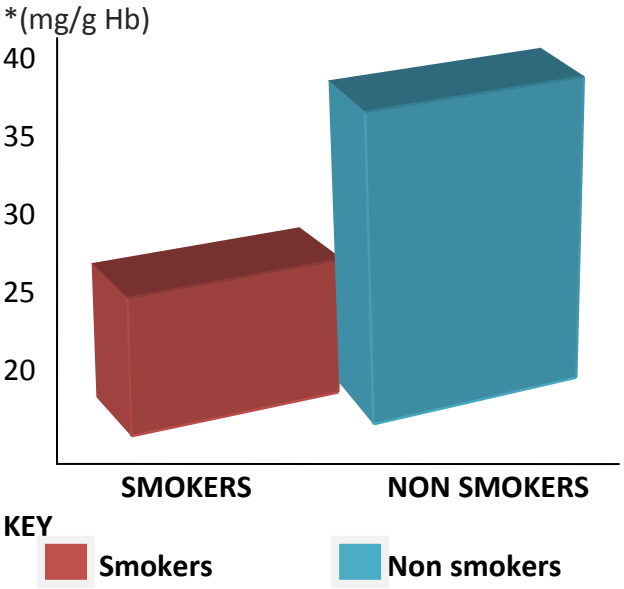

Fig. 2 GSH level in plasma of smoker and non-smoker.

Data are expressed as mean ± SEM, n=50. * Represents the significant difference between smoker and non-smoker (*P<0.05*).

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In the present study, we also studied the glutathione dependent antioxidant enzymes profile through estimation of GPx activity in plasma. The enzymes activity was decreased (Fig-3) in smoking habited individuals. GPx was decreased significantly ($p < 0.05$) 37.61% in plasma.

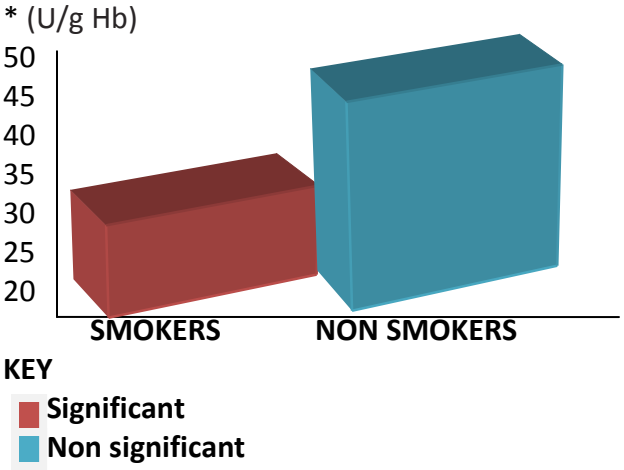

Fig. 3 GR activity in plasma of smoker and non-smoker.

Data are expressed as mean ± SEM, n=50. * Represents the significant difference between smoker and non-smoker (*P<0.05*).

The present study attempted to understand the effect of reduced antioxidant status on the red blood cell by examining the Haematocrit, Reticulocyte counts and Total bilirubin levels of smokers and non smokers, Haematocrit values were significantly reduced (46.38 ±4.5) while the Reticulocyte counts (1.432 ±1.1) and total bilirubin (18.040 ±4.5) levels showed a significant increase when compared with the control group.

Table 2 Shows the mean, standard deviation and p values of haematocrit, reticulocyte and bilirubin which revealed a statistical difference at P<0.005

Group		Mean + SD	P Value
MDA (nmol/mg)	TEST CONTROL	2.81 • ± 0.14 0.81 • ± 0.53	0.001*
GSH (mg/g Hb)	TEST CONTROL	25.38 ± 6.62 35.55 ± 10.8	0.000*
GPX (U/g Hb)	TEST CONTROL	31.36 ± 3.63* 45.10 ± 10.34	0.000*
HAEMATOCRIT (%)	TEST CONTROL	48.16 ± 6.30 42.40 ± 3.74	0.003*
RETICULOCYTE (%)	TEST CONTROL	1.64 ± 1.09 0.86 ± 0.52	0.001*
TOTAL BILIRUBIN (mg/ml)	TEST CONTROL	11.73 ± 5.84 5.00 ± 5.84	0.000*

***statistically significant**

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DISCUSSION

Cigarette smoke is an important variable in our society. It may be inappropriate to make simple dichotomous divisions between smokers and nonsmokers. The result of the present study suggested that, smoking habit is associated with the free radical scavenger system in the plasma and red cell.

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 and cigarette brand. In this study, 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. Glutathione is an important cellular reductant, involved in protection against free radicals, peroxides and toxic compounds [12]. Depletion of GSH is one of the primary factors that permit lipid peroxidation [13]. In our present study, the GSH levels were decreased (Fig.2) in plasma of individuals with smoking history. The decreased GSH level may be due to increased levels of lipid oxidation products which may be associated with the lesser availability of NADPH, a cofactor required for the activity of glutathione reductase (GR) to transform oxidized glutathione to GSH [14] due to the increased production of reactive oxygen species (ROS) at a rate that exceeds the ability to regenerate GSH. The decreased GSH level in association with decreased GR activity obtained from this study (Fig.3) may support the explanation as evidence.

Glutathione peroxidase (GPX) works nonspecifically to scavenge and decompose excess hydro peroxides including H_2O_2, which may be prevalent under oxidative stress [15]. In this study, decreased GPX activity seems to indicate the susceptibility of the red cells to smoking induced oxidative stress. The decreased level of GSH and activity of GSH- dependent enzyme i.e. GPX, (Fig.3) in plasma of smoking individuals may be due to their increased utilization in scavenging smoking induced free radicals.

A low antioxidant capacity in plasma suggests an increased oxidant burden in the blood. Many researchers have reported increased levels of superoxide anion release from circulating neutrophils [1, 15] and increased lipid peroxidation products in the plasma of smokers [16]. This supports the concept of systematic oxidative stress in these individuals. It is however unclear, if the oxidative stress occurring in smokers is as a result of the direct effect of the oxidants/free radicals present in cigarette smoke or due to an endogenous inflammatory response [17].

The slightly increased Total bilirubin levels observed in this study when a comparison was made with results obtained from the control group, might have resulted from red cell destruction occurring at a very reduced pace; this however further explains the mild rise in the reticulocyte counts and haematocrit levels, possibly resulting from increased production of erythropoietin and consequent rise in the erythrocyte level (erythrocytosis) following

inadequate oxygenation of blood circulation through the lungs due to tissue hypoxia. This inference is in agreement with previous studies carried out by Sagone *et al* [18].

CONCLUSIONS AND RECOMMENDATIONS

The present study confirms results obtained from previous studies which highlighted the destructive and oxidative effects of cigarette smoke on the cell membrane and other body tissues. The impaired oxidant/antioxidant balance may however represent a risk factor for the development of chronic diseases. 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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