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Erythrocyte antioxidant enzymes activities and plasma carbonyl protein levels in smokers

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Smoking has been implicated in the pathogenesis of ischemic heart disease, chronic obstructive lung diseases and cancers. More than 4700 chemical compounds of cigarette smoke, including free radicals, oxidants and reducing agents have been identified. The aim of this study is to investigate the effect of cigarette smoking on the levels of erythrocyte antioxidant enzymes and plasma carbonyl protein which can be used as a marker of protein oxidation. Thirty-two smokers and 36 apparently healthy control subjects from staff of University of Medicine (1) and Department of Medical Research (Lower Myanmar) and blood donors of National Blood Centre, Yangon were studied. The subjects were males aged between 25-40 years. Erythrocyte superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities and plasma carbonyl protein levels were measured using a spectrophotometer. The erythrocyte CAT activity was significantly lower in cigarette smokers (264.57±108.37 U/g Hb) compared with non-smokers (473.59±267.87 U/g Hb) ($p<0.005$). There were no significant differences in erythrocyte SOD and GPx activities and plasma carbonyl protein levels between the two groups. There was a positive correlation ($r=+0.35$, $p<0.05$) between the number of cigarettes consumed per day and CAT activity in smokers. In conclusion, erythrocyte CAT activity was reduced in cigarette smokers due to the free radicals present in cigarette smoke.

INTRODUCTION

Cigarette smoking is the largest growing cause of death in the world [1]. Tobacco smoking is a risk factor for 6 of 8 leading causes of death including ischaemic heart diseases, cardiovascular diseases, lower respiratory infection, chronic obstructive pulmonary diseases, tuberculosis and trachea, bronchus and lung cancer in the world [2]. It has been estimated that a single puff of a cigarette contains as much as 10^{15} gas phase radicals and 10^{14} tar phase radicals potentially capable of modifying macromolecules including lipids, proteins and DNA [3].

The defense systems within the body limit the levels of reactive oxidants and the damage they inflict. The three main enzymes that control the biological effects of reactive oxygen species are superoxide dismutase (SOD), which catalyses dismutation of the superoxide anion into H_2O_2, catalase (CAT), which detoxifies H_2O_2, and glutathione peroxidase (GPx), which reduces organic peroxides to their alcohols [4]. However, when free radicals are generated in excess or the cellular antioxidant defense system is defective, they can stimulate chain reactions by interacting with proteins, lipids and nucleic acids, causing cellular dysfunction and even death [5].

Proteins are major targets for reactive oxidants in cells and oxidized proteins accumulate during aging, oxidative stress and in some pathological conditions. Exposure of proteins to reactive oxygen species results in modification of amino acid residues, which alters protein structure and function [6]. It is reported that in vitro exposure of plasma to gas-phase cigarette smoke leads to the rapid accumulation of plasma protein carbonyls [7].

The circumstantial data from in vitro studies and measurements of antioxidant levels in smokers suggested that cigarette smoke may cause oxidative injury. From the literature, the findings are in disagreement over the erythrocyte SOD, GPx and CAT activity in smokers. The present study was conducted firstly to find out the effect of cigarette smoking on erythrocyte antioxidant enzymes activities and a selected marker of oxidative damage, and secondly, to verify the possible correlation between the smoking status and the erythrocyte anti-oxidant enzymes activities due to cigarette smoking.

MATERIALS AND METHODS

A comparative, cross-sectional study was carried out on a total of 68 male subjects aged between 25-40 years (32 smokers and 36 non-smokers). Smokers were defined as those who, at the time of survey, smoked any tobacco product at least once a day daily for at least 3 year duration while non-smokers were those who do not smoke at all [8]. All subjects were apparently healthy volunteers recruited from the staff of University of Medicine (1), Department of Medical Research (LM) and blood donors at National Blood Centre, Yangon.

Informed consents were obtained from all subjects. The subjects were firstly explained the nature and procedure of the study. They were not suffering from any diseases, were not on any medication, including vitamins and were not taking excess alcohol. These facts and smoking status of the subjects were asked with questionnaires.

Venous blood was collected in tubes with EDTA and centrifuged at 3000 rpm, 30°C for 10 minutes. Erythrocytes were washed 3 times with 3 ml of cold isotonic saline solution. Then, the plasma and the packed cells were stored at -80°C until analysis. The plasma was used for the determination of carbonyl protein. The packed cells were lysed in 4 times of its volume of deionized water by vortex-mixing. Membrane-free haemolysate was obtained by centrifugation at 3000 rpm, 4°C for 10 minutes. The clear supernatant was collected as haemolysate to determine SOD, GPx, CAT activities and hemoglobin concentration.

Hemoglobin concentration was determined by cyanmethaemoglobin method [9]. Erythrocyte catalase activity was determined with the method of Aebi [10]. The principle of the CAT assay was based on the determination of the rate constant (s^{-1}, k) of H_2O_2 decomposition rate. Rate constant, $k=(1/\Delta t)x\ln (A_1/A_2)$, where A_1 and A_2 were the absorbance values of H_2O_2 at times t_1 and t_2. The initial rate of disappearance of H_2O_2 (0-40 seconds) was recorded spectrophotometrically at a wavelength of 240 nm. One unit of catalase activity was defined as the rate constant of the first-order reaction. The catalase activity was expressed as unit per gram hemoglobin (U/g Hb).

Erythrocyte glutathione peroxidase activity was determined by using the method of Takahashi [11]. The GPx activity was determined spectrophotometrically in erythrocyte haemolysates using an indirect coupled assay. The GPx catalyzes the oxidation of glutathione by t-butyl hydroperoxide. In the presence of glutathione reductase and nicotiamide adenine dinucleotide phosphate (NADPH), the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The change in absorbance at 340 nm was measured. One unit of activity was defined as the number of NADPH oxidized per minute, using a molar coefficient of extinction of $6.22x10^6$ $mM^{-1}cm^{-1}$ for

NADPH. Erythrocyte GPx activity was expressed as U/g Hb.

Erythrocyte superoxide dismutase activity was determined according to the procedure of Suzuki [12]. The principle of SOD activity measurement was based on the inhibition of nitroblue tetrazolium reduction with the xanthine-xanthine oxidase system as a superoxide generator. Superoxide radicals produced reduce nitroblue tetrazolium and form formazan. SOD prevents this reaction, and its activity is inversely proportional to the absorbance value of formazan at 560 nm. One SOD unit was defined as the amount of the enzyme causing 50% inhibition of the nitroblue tetrazolium reduction rate. SOD activity was expressed in U/g Hb.

Plasma carbonyl protein was determined as described by Reznick and Packer [13]. Carbonyls were determined by using dinitrophenylhydrazine (DNPH) dissolved in HCl, accompanied by blank in HCl alone. After the DNPH reaction, proteins were precipitated with an equal volume of 20% (w/v) trichloroacetic acid and the pellets were washed once with 2 ml of 10% (w/v) trichloroacetic acid and 3 times with 2 ml of an ethanol/ethyl acetate mixture (1:1).

Washings were achieved by mechanical disruption of the pellets in washing solution and re-pelleting by centrifugation at 3000 rpm for 5 minutes. Finally, the precipitates were dissolved in 6M guanidine-HCl solution and the absorbance at 370 nm was determined. Protein contents were determined on the HCl blank pellets using a bovine serum albumin standard curve in guanidine-HCl and reading the absorbance at 280 nm.

All results are presented as mean±SD. Differences in means between groups were analyzed by un-paired 't' test. Correlation statistics between variables were assessed by calculating the Pearson coefficient. Differences were considered statistically significant at $p<0.05$.

RESULTS

Table 1 shows the smoking status of the studied population. They smoked 3-15 cigarettes per day (9.09±3.76 cigarettes/ day) and the time duration they smoked was 3-22 years (9.87 ± 5.62 years).

Table 1. Smoking status of the studied population (n=32)

	Range	Mean±SD
Age (years)	25-40	31.77 ± 4.67
No. of cigarettes/day	3 -15	9.09 ± 3.76
Duration of smoking (years)	3 -22	9.87 ± 5.62

Table 2 shows the erythrocyte antioxidant enzymes activities and plasma carbonyl protein levels in smokers and non-smokers. Erythrocyte catalase activity was significantly decreased ($p<0.001$) in smokers when compared with non-smokers. It was found that smokers had a lower erythrocyte SOD activity than non-smokers but this difference did not reach the level of significance. The mean erythrocyte gluthathione peroxidase level was higher in smokers than that in non-smokers but they were not significantly different. The mean plasma carbonyl protein levels in smokers and non-smokers were not also different.

Table 2. Erythrocyte antioxidant enzymes activities and plasma carbonyl protein levels in smokers and non-smokers

	Smokers (n = 32)	Non-smokers (n = 36)
Age (years)	31.77±4.67	31.96±5.50
Superoxide dismutase (U/gHb)	679.87±324.28	757.83±373.33
Catalase (U/gHb)	264.57±108.37*	473.59 ±267.87
Glutathione peroxidase (U/gHb)	76.65±27.86	68.74±23.02
Carbonyl protein (nmol/mg protein)	1.41±0.83	1.41±0.70

*$p<0.001$

The results of correlation analysis between the activities of the studied enzymes and the number of cigarettes consumed per day and smoking duration are given in Table 3.

Table 3. Correlation between smoking status and erythrocyte superoxide dismutase and glutathione peroxidase activities in smokers

Smoking status	Correlation coefficients of erythrocyte antioxidant enzymes	
	SOD	GPx
No. of cigarette per day (3-15 cigarettes/day)	-0.019	-0.141
Duration of smoking (3-22 years)	0.04	-0.155

Both the superoxide dismutase and glutathione peroxidase activities have negative correlation with number of cigarette smoked per day. The superoxide dismutase has positive correlation with the duration of smoking but glutathione peroxidase has negative correlation with the duration of smoking.

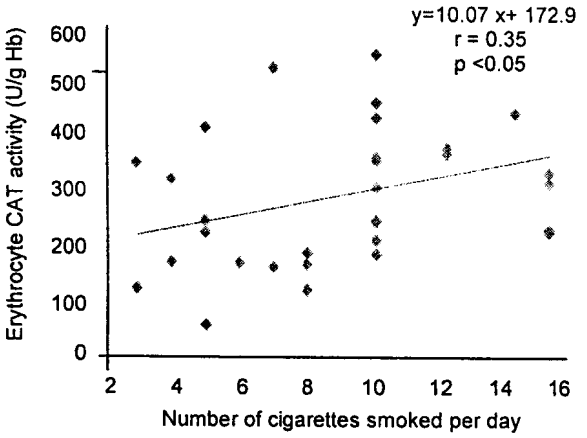

Fig. 1. Correlation between numbers of cigarettes smoked per day and erythrocyte catalase activity among smokers

There was significant positive correlation between the number of cigarettes smoked per day and erythrocyte CAT activity ($r=0.35$, $p<0.05$) in smokers. Figure 1 shows the moderate association between number of cigarettes smoked per day and erythrocyte CAT activity in smokers.

DISCUSSION

Cigarette smoke contains many oxidants, free radicals and carcinogenic substances that are capable of reacting with, or inactivating, the essential cellular constituents. These potentially toxic substances are inactivated or scavenged by the antioxidants before they can inflict damage to lipids, proteins or nucleic acids. Superoxide dismutase, catalase and glutathione peroxidase are generally believed to play a vital role in protecting the body against the toxic effect of oxidants [14].

In the present study, erythrocyte CAT activity was significantly decreased in smokers. The decrease in enzyme activity most probably reflects the increased oxidative stress thought to occur in smokers. This may be due to excess oxygen free radicals and other free radicals which oxidize, decompose and destroy the –SH group of antioxidant constituents, thereby weakening their activities [15]. The catalase is widely distributed in all animal tissues and high activity is found in red blood cells. Catalase has been suggested to play an important role in the protection of the erythrocyte against oxidative stress [16]. This may be the reason of no significant differences in erythrocyte SOD and GPx activities between the smokers and non-smokers in present study.

Among smokers, erythrocyte CAT activity increased with the number of cigarettes consumed per day. This may explain that increase CAT activity exerts a protective effect from subsequent oxidative stress. From these findings, it can be hypothesized that reactive oxygen species (ROS) produced from cigarette smoking reduces the activity of erythrocyte CAT. However, when the ROS reach to a certain level by smoking, there is increased erythrocyte CAT activity probably due to induction of the enzyme. This needs to be elucidated by studying the CAT expression by various smoking status.

Reactive oxygen species can damage all types of biological molecules. Oxidative damages to proteins, lipids or DNA may all be seriously deleterious and may be concomitant [17]. However, proteins are possibly the most immediate vehicle for inflicting oxidative damage. Some studies

showed higher plasma carbonyl level in smokers than in non-smokers [18, 19, 20]. However, a study showed that there was a trend towards higher carbonyl concentration in smokers than that in controls but they were not significantly different. The plasma carbonyl concentration in smokers was significantly higher than that in controls after oxidation of the plasma [21].

One study found out that carbonyl levels were higher among current smokers, but these differences did not attain statistical significance, nor did differences by urine cotinine levels, pack-years, pack/ day among current smokers and smoking duration [22]. There was no significant difference in plasma carbonyl protein levels between smokers and non-smokers in the present study. This may be due to the fact that the proteolytic enzymes are degrading and eliminating the oxidatively altered proteins effectively, thus preventing accumulation of altered and damaged proteins.

It is noteworthy that the higher carbonyl protein levels was independently associated with increasing age [19, 21, 23]. Age related accumulation of carbonyl protein in different human tissues was studied [24] and it was found out that there was little or no change in the level of protein carbonyls during the first 45 years of life but increased abruptly in linear fashion after age of 60 years. Therefore, the apparent effect of smoking on plasma carbonyl protein level is more marked in elderly subjects.

In conclusion, cigarette smoke causes oxidative stress. One of oxidative defense enzymes, erythrocyte catalase activity, was significantly decreased in smokers aged between 25-40 years consuming 3-15 cigarettes per day. Among smokers, the catalase activity increased according to the numbers of cigarettes consumed per day and further studies need to investigate the pattern of catalase enzymes responses to the oxidants of cigarette smoke in various smoking status. Concerning the marker of oxidative damage, plasma carbonyl protein was not obviously affected by cigarette smoking in this study. This may be probably due to the effective proteolytic enzymes, relative young age in the present study population and relative less pack-years than that of the other previous studies.

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