Original Article

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Biochemistry Section

Erythrocyte Antioxidant Defenses Against Cigarette Smoking in Ischemic Heart Disease

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ABSTRACT

Background: Cigarette smoke promotes atherogenesis by producing oxygen-derived free radicals.

Aim: The present study was conducted to determine the effect of cigarette smoking on lipid peroxidation and erythrocyte antioxidant status in ischemic heart disease (IHD).

Materials and Methods: A total of 327 male subjects were enrolled for this study, divided into two groups consisting of 200 patients, who were consecutively admitted for IHD in the intensive cardiac care unit (ICCU) of a Government Hospital and 127 age matched male healthy subjects. Both the groups were subsequently categorised into smokers and non smokers sub groups depending upon the smoking history {>/= 20 pack years of smoking; (20 cigarettes per day for one year constitutes one pack year)}. All 327 subjects were investigated for lipid profile, malondialdehyde (MDA) levels and the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX).

Statistical Analysis: The differences in the parameters between the groups were tested for significance by one way ANOVA using the SPSS software version 19. A p-value of < 0.001 was considered to be significant statistically. Multiple comparisons were made between all the four groups by Post Hoc Tukey test.

Results: There was highly significant difference ($p<0.001$) observed in GPX activity, in comparison to CAT and SOD ($p=0.032$, $p=0.009$) between smokers vs non smokers in control group as well as patient group. The plasma MDA levels were found to be increased significantly ($p<0.001$) in IHD patients, who smoked as compared to those who did not.

Conclusion: Chronic smoking enhances erythrocyte lipid peroxidation in IHD patients with concomitant failure of both plasma and erythrocyte antioxidant defense mechanisms. Along with conventional lipid markers and plasma MDA levels, the erythrocyte GPX activity was observed to be a better marker of oxidative stress, in chronic smokers, who are at risk of developing IHD.

Keywords: Antioxidant enzymes, Oxidative stress

INTRODUCTION

Smoking is one of the major lifestyle factors influencing the health of human beings [1]. Cigarette smoke is a complex milieu possessing an array of free radicals namely hydroxyl, peroxyl, nitric oxide, and superoxide radicals [2,3]. Smoking causes pro-oxidant / antioxidant imbalance which elevates oxidative stress, accompanied by increase of lipid peroxidation and vasomotor dysfunction for the initiation and progression of atherosclerosis [4]. A large body of literature has linked oxidative stress with hypertension, atherosclerosis and ischemic heart disease [5,6].The quinone-hydroquinone radical complex from the cigarette tar causes redox cycling and generates copious superoxide radicals, which further produces hydrogen peroxide and hydroxyl radicals [7]. Human erythrocytes are important targets for the biological oxidative effects of free radicals as they are rich in polyunsaturated fatty acids, contain hemoglobin which can function as an oxidase as well as a peroxidase and one of the most potent catalysts of lipid peroxidation [8]. The sustained release of reactive free radicals from the tar and gas phases of cigarette smoke imposes an oxidant stress on the circulating erythrocytes [9]. The invasion of the erythrocyte membrane by peroxidants, alter the cellular metabolic function and cause red blood cell (RBC) hemolysis [8]. Since the RBC has limited biosynthesis capacity, it is completely dependent on antioxidant defensive components throughout its 120 days of life span [7]. The RBC antioxidant defense system consists of superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) [9]. SOD converts superoxide radicals into hydrogen peroxide,which is degraded to water and oxygen by GPX at physiological concentrations [10]. When hydrogen peroxide concentration increases to toxic levels, CAT also contributes to degradation metabolism of H_2O_2 [10,11]. In this way, they play an important role in the protective mechanisms against exposure to oxidative stress. Various studies in the past have reported the oxidative stress in smokers and in acute myocardial infarction [12,13]. however, very few have been undertaken to assess the impact of smoking on the antioxidant enzymes in IHD.The present study aims to investigate potential changes in the erythrocyte antioxidant status induced by chronic cigarette smoking in ischemic heart disease patients compared to healthy subjects, assess the utility of these markers for estimating smoking-induced harm and probability of ischemic heart disease incidence in healthy subjects.

MATERIALS AND METHODS

The present study included 327 male subjects, consisting of 127 healthy subjects and 200 consecutively admitted IHD patients in the intensive cardiac care unit of Cardiology Department (ICCU, Gandhi Medical College and Hospital, Secunderabad, India) over the period September 2012 to December 2013. All the IHD patients were diagnosed according to the following diagnostic criteria: chest pain lasting for > 3 h, ECG changes (ST elevation > 2mm in at least two leads) and elevation of enzymatic activity of serum creatine phosphokinase and asparatate aminotransferase. The group of healthy subjects were recruited from the patient relatives and from outpatient wards of other clinical departments, after they were screened for hypertension, diabetes, renal or neurological conditions that might interfere with our study. Each of the main groups were subsequently categorized as smokers and non-smokers based on the history of smoking. (>/= 20 pack years of smoking i e 20 cigarettes per day for one year constitutes one pack year) (Prignot) [14]. The group of IHD patients were divided into 2

sub groups consisting of 100 nonsmokers and 100 smokers, the healthy group consisted of 64 smokers and 63 non-smokers. The mean ages of investigated human groups were sufficiently close. The healthy smokers and non-smokers were of mean age 48 ± 1.5 y; the sub-group of smoking IHD patients was 54 ± 2.5 y and group of non-smoking patients was 50.4 ± 2.1 y of age. The study was approved by institutional ethics committee. Informed consents were obtained from all the patients or relatives before collection of blood sample. Patients with renal disease, hepatic disease and any other neurological disorders were excluded from the study. Clinical parameters were documented in a well designed proforma prior to collection of 10 ml of fasting venous blood from each of these patients, for carrying out biochemical investigations.

Erythrocyte antioxidant defenses: Heparinised blood was centrifuged at 1,000 g for ten minutes at 4⁰C, the buffy coat was discarded, and the isolated RBC pellet was hemolysed in four times its volume of ice-cold high performance liquid chromatography (HPLC)-grade water and again centrifuged at 4⁰C. The erythrocyte lysate was then used to evaluate the CAT, SOD and GPX activity.

Glutathione peroxidase assay: GPX activity was determined with Cayman kits (Item no703102, Ann Arbor ,MI 48108,USA) at 25⁰ C by colorimetry at 340 nm, based on the method of Paglia and Valentine, which recquires cumene hydroperoxide as a substrate [15]. Before analysis the erythrocyte lysates were diluted to 20 fold with sample buffer.The final concentration of reagents in the assay were those recommended by the manufacturer. The GPX activity was measured in IU/g of Hb.

Superoxide dismutase assay: SOD activity was determined with Cayman kits (item no 706002, Ann Arbor, MI 48108,USA) at 25⁰C by colorimetry at 340 nm, based on the method of Marklund, (1980) [16]. This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(-4-idophenyl)-3-(4-nitrophenol)-5-phenyl tetrazolium chloride to form red formazone dye.The SOD activity is then measured by degree of inhibition of this reaction. SOD units were obtained from standard curve expressed in IU/g of Hb.

Catalase assay: CAT activity was assayed based on the method of Johansson and Borge, using the Cayman kits (item no 707002, Ann Arbor, MI 48108, USA) [17]. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2.The formaldehyde produced was measured colorimetrically with purpald as the chromogen at 340 nm.CAT activity was calculated as the amount of enzyme that would cause the formation of 1.0 nmol of formaldehyde per minute expressed in nmol/min/ml.

Thiobarbituric acid reactive substances(TBAR) assay: Plasma malondialdehyde (MDA) levels were determined by the method of Richard et al., using the Cayman kits (item no.10009055, Ann Arbor, MI 48108, USA) [18].In this method after centrifuging the blood at 1,000 g for 10 min at 4⁰C, the top yellow plasma layer was pippetted off. The MDA-TBA adduct formed by the reaction of MDA and TBA under high temperature (90-100⁰C) and acidic condition was measured colorimetrically at 530-540 nm.

Lipid profile: Serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL), low density lipoprotein(LDL) were done by auto-analyser (Hitachi 912). Very low density lipoprotein (VLDL) was calculated by Friedewald´s equation [19].

Blood pressure : The systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded in the morning hour, after collection of blood sample.

Body mass index (BMI): The body mass index of all the subjects was calculated by the accepted formula weight (kg)/ {height(meter)^2}.

STATISTICAL ANALYSIS

All the data obtained was presented as mean ± SD. Any differences in the parameters between the groups were tested for significance by one way ANOVA test. Comparisons were made between the following groups such as control nonsmoker vs control smoker, control nonsmoker vs patient nonsmoker, control smoker vs patient nonsmoker , patient smoker vs patient nonsmoker by Post Hoc Tukey test using the SPSS software version 19. A p-value of < 0.001 was considered to be significant statistically.

RESULTS

All the 327 samples were analysed for MDA, lipid profile and antioxidant enzymes.The baseline data and lipid profile is given in [Table/Fig-1].The parameters of antioxidant defenses and MDA levels are given in [Table/Fig-2]. The multiple comparison of the parameters of lipid profile revealed significant difference (p<0.001) in all lipid parameters except HDL among nonsmokers vs smokers in patient and control groups. The difference in plasma MDA levels and GPX activity were highly significant (p<0.001), while comparing between smokers vs non smokers in control group as well as in patient group.

	Controls		IHD Patients		p-values
	Non smokers n=63	Smokers n=64	Non smokers n=100	Smokers n=100	
BMI (kg/m²)	19.79± 1.78	19.21± 1.13	23.43± 1.89	18.92± 1.1	*0.035, †0.001, ‡0.001, §0.001
SBP (mm of Hg)	128 ± 12.59	130.81± 20.1	128.78± 10.31	131.34± 19.93	*0.33, †0.765, ‡0.202, §0.266
DBP (mm of Hg)	80.25± 4.39	79.34± 6.95	80.14± 4.22	79.66± 6.7	*0.368, †0.901, ‡0.517, §0.551
MABP (mm of Hg	96 ± 6.09	95.83± 9.35	96.29± 13.53	96.14± 9.17	*0.926, †0.862, ‡0.935, §0.916
TC(mg%)	162.65± 16	235.1± 33.23	209.45± 18.44	236.5± 29.42	*0.001, †0.001, ‡0.001, §0.001
HDL(mg%)	46.07± 6.98	36.48± 6.16	43.93± 5.85	36.22± 5.38	*0.001, †0.001, ‡0.001, §0.654
LDL(mg%)	97.1± 10.7	189.49± 27.95	156.09± 34.08	151.04± 28.93	*0.001, †0.001, ‡0.001, §0.001
VLDL(mg%)	22.73 ± 5.72	31.63± 3.79	25.83± 3.83	32.29± 3.79	*0.001, †0.001, ‡0.001, §0.001
TG (mg%)	115.04 ± 26. 57	158.45± 18.91	130.1± 18.53	161.39± 18.82	*0.001, †0.001, ‡0.001, §0.001

[Table/Fig-1]: Comparison of baseline parameters and lipid profile across the groups
*Control nonsmoker vs Control smoker, †Control nonsmoker vs Patient nonsmoker, ‡Control smoker vs Patient nonsmoker, §Patient smoker vs Patient nonsmoker

	Controls		IHD Patients		p-values
	Non smokers n=63	Smokers n=64	Non smokers n=100	Smokers n=100	
CAT (n moles/ min/ ml)	552.85± 77.25	406.45± 73.52	385.99± 54.89	406.99± 73.18	*0.001, †0.001, ‡0.001, §0.032
SOD (IU/g of Hb)	156.06± 132.74	1294.26± 190.5	1330.11± 112.21	1272.26± 177.9	*0.001, †0.001, ‡0.001, § 0.009
GPX (IU/g of Hb)	51±10.21	37.05±4.13	47.88±7.08	37.38 ± 4.02	*0.001, †0.004, ‡0.001, §0.001
MDA (nmol/ dl)	2.4±0.73	6.03±0.72	4.2±0.59	6 ± 0.72	*0.001, †0.001, ‡0.001, §0.001

[Table/Fig-2]: Comparison of MDA and antioxidant enzymes levels across the groups
*Control nonsmoker vs Control smoker, †Control nonsmoker vs Patient nonsmoker, ‡Control smoker vs Patient nonsmoker , §Patient smoker vs Patient nonsmoker

DISCUSSION

Cigarette smoke exposure is an important cause of cardiovascular morbidity and mortality. In the present study we observed significantly lower BMI in smoker patients in comparison to non smoker patient (p=0.001), whereas the difference in BMI among the control nonsmoker vs control smokers even though low, was not highly significant(p=0.035) [Table/Fig-1]. Chiolero et al., have reported that, mean BMI tends to be lower in smokers than nonsmokers in health as well as disease [20]. This may be due to the metabolic and possible acute anorexic effects induced by nicotine [21,22]. We did not observe any significant difference in systolic, diastolic and mean arterial blood pressure in the study groups, as the IHD patients were already on anti hypertensive medication at the time of participation in the study and were constantly monitored for stable blood pressure in the ICCU.

While comparing the lipid profile, except HDL, all other lipid parameters such as TC,LDL,VLDL,TG were significantly high among the smokers in comparison to nonsmokers in control group as well as in patient group (p<0.001) [Table/Fig-1]. We did not observe any significant difference in HDL levels among the smoker patients vs nonsmoker patients, which may be due to other associated lifestyle factors like diet, physical activity, exercise, etc influencing the lipid metabolism [23]. Our findings are concurrent with that of previous studies suggesting that cigarette smoking promotes atherosclerosis and dyslipidemia [24,25].

The plasma MDA levels were observed to be increased significantly (p<0.001) [Table/Fig-2] in smokers as compared to non-smokers in the control group as well as in the patient group, indicative of enhanced lipid peroxidation in smokers. Our findings are concurrent with that of Lykkesfeldt et al., who have reported that, smoking induces lipid peroxidation significantly and thereby increases the plasma levels of MDA in comparison to non smokers [26]. The lipid peroxidation in smoker patients further gets augmented by myocardial ischemia followed by reperfusion in IHD [27]. In a similar study by Kashinakunti et al., increase in plasma lipid peroxidation products (MDA, TBARS) was observed in patients of stable ischemic heart disease [28].

While analyzing the erythrocyte antioxidant defenses, we observed significantly lower erythrocyte CAT, SOD, GPX activity in smokers than non smokers in control group as well as patient group. While comparing the control smokers vs patient smoker, even though there was a difference in CAT and SOD activity, it was not highly significant (p=0.032, p=0.009). However, the GPX activity was found to be highly significant (p<0.001) among smokers, in comparison to nonsmokers in both patient and control group. We suggest that erythrocyte GPX activity is a better and more sensitive indicator of erythrocyte antioxidant defenses against cigarette smoking than CAT and SOD in IHD. The protective effect of GPX activity on lipid peroxidation is reinforced by the fact that, this enzyme not only detoxifies the H_2O_2 produced by SOD action, but also converts lipid hydroperoxide to nontoxic alcohols,thus acting as a chain-breaking antioxidant [7,12]. Siddiqui et al., have reported significantly lower activity of CAT, SOD and GPX, in their study of waist hip ratio correlation with oxidative stress in patients of acute myocardial infarction [29]. Our observations are in line with Muzakova et al., who stated that erythrocyte SOD and GPX activities are crucial in assessing the antioxidant defenses in smoking and myocardial infarction [30]. However, our findings differ from that of Dutta et al., who have reported significantly lower GPX but higher SOD activity in smokers with IHD [31]. This may be due to the differences in the inclusion criteria of subjects in the previous study, such as duration of smoking, dyslipidemia status, diet, exercise, etc. Therefore the present study states that, chronic smoking enhances erythrocyte lipid peroxidation in IHD patients with concomitant failure of both plasma and erythrocyte antioxidant defense mechanisms. The erythrocyte GPX enzyme activity appears to be more sensitive indicator than SOD and CAT while assessing chronic smokers for risk of IHD.

CONCLUSION

Along with conventional plasma lipid markers and MDA levels, the erythrocyte GPX activity can be considered as a potential marker of oxidative stress, while assessing for antioxidant defenses in smokers suffering from IHD.

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