EFFECTS OF CIGARETTE SMOKING ON ERYTHROCYTE ANTIOXIDATIVE ENZYME ACTIVITIES AND PLASMA CONCENTRATIONS OF THEIR COFACTORS

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Abstract- Tobacco smoke contains numerous compounds, many of which are oxidants and capable of producing free radical and enhancing the oxidative stress. The aim of this study was to investigate the effect of cigarette smoking on the erythrocyte antioxidative enzyme activities and the plasma concentration of their cofactors. Sixty eight healthy men were enrolled, 32 of whom had never smoked and 36 had smoked at least 10 cigarettes per day for at least one year. Hemolysate superoxide dismutase (Cu-Zn SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) activities were measured using spectrophotometer. Plasma copper, zinc and selenium concentrations were determined using atomic absorption spectrophotometer. Plasma iron concentration was determined by colorimetric method. We found that erythrocyte Cu-Zn SOD activity was significantly higher in tobacco smokers compared with non-smokers (1294 ± 206.7 U/gHb in smokers vs. 1121.6 ± 237.8 U/gHb in non-smokers, $P < 0.01$). While plasma selenium concentration was significantly lower in tobacco smokers (62.7±14.8 µg/L in smokers vs. 92.1 ± 17.5 µg/L in non-smokers, $P < 0.01$), there were no significant differences in erythrocyte GSH-Px and CAT activity and plasma copper, zinc and iron concentrations between the two groups. It seems that cigarette smoking can alter antioxidative enzymes activity and plasma concentration of some trace elements.

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INTRODUCTION

Cigarette smoking has been implicated as a significant risk factor for the establishment and progression of several diseases, including atherosclerosis, cancer and emphysema (1). Although the underlying mechanisms involved in the pathogenesis of diseases associated with smoking are not well understood, tobacco smoke contains numerous compounds, many of which are oxidants and pro oxidants capable of producing free radical and enhancing the oxidative stress *in vivo* (2, 3). Each puff of a tobacco contains 10^4 oxidants in the tar phase and 10^5 in the gas phase. It has been demonstrated that one of the prominent risk factors for increased lipid peroxidation is smoking (3).

There are many intrinsic radical scavenger systems which involve enzymatic and non enzymatic reactions. Copper-zinc superoxide dismutase (Cu-Zn SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) are important components of the enzymatic

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antioxidative systems. Cu-Zn SOD catalyzes dismutation of the superoxide anion (O°_2) into hydrogen peroxide (H_2O_2), which is then deactivated to H_2O by catalase and GSH-Px. GSH-Px also reduces organic peroxides into their corresponding alcohols (3, 4). In some studies it has been shown that tobacco smoking can alter trace elements metabolism (5). Since trace elements are required in small concentrations as essential component of antioxidative enzymes (cytoplasmic Cu-Zn SOD contains copper and zinc metals as cofactors, GSH-Px enzyme contains selenium and CAT contains iron), tobacco smoking can affect antioxidant enzyme activities, thereby indirectly affecting trace element metabolism.

The present study was conducted to obtain information on the effects of cigarette smoking on selected markers of oxidative damage, and plasma mineral concentrations and to investigate the causes of alterations in antioxidative enzyme activities and their cofactor concentrations.

MATERIALS AND METHODS

Subjects

Sixty eight men were enrolled among the subjects referred to a district blood transfusion station in Tehran, 32 of whom had never smoked, and 36 of whom had smoked at least 10 cigarettes per day for at least one year. Ex-smokers or subjects with discontinuous smoking were excluded from either the smokers group or the non-smokers group. None of the subjects had any history of cardiovascular, endocrine or gastrointestinal disorders and none were receiving medication or taking any nutritional supplements. The general data of the study subjects are summarized in table 1. We obtained informed consent from all patients.

Sample preparation

All of the materials (glass and plastic) employed were thoroughly cleaned with a hot solution of nitric acid (20% v/v) for 48 h and rinsed tree times with ultra deionized water. In total, 10 mL of heparinized venous blood was drawn after overnight fasting and centrifuged at 3000 rpm for 10 min to separate the plasma from the erythrocytes. Plasma was stored at -80 °C to determine selenium, copper, zinc and iron concentration. To obtain packed erythrocytes, the remaining erythrocytes washed repeatedly with an isotonic solution of NaCl (0.9%) until a colorless supernatant was observed. To obtain erythrocyte hemolysate, 500 µl packed erythrocyte were destroyed by addition of four volumes of cold redistilled water. The resulting suspension was centrifuged twice to eliminate all of the cell membranes: first for 10 min in the tube centrifuge at 3500 rpm at 4°C, then in an Eppendorf centrifuge at 7800 rpm for 5 min at 4°C (6). Clear supernatant was obtained as hemolysate to determine Cu-Zn SOD, GSH- Px and CAT activity.

Determination of plasma selenium concentrations

Plasma selenium determination was performed by Thermo Jarrel Ash Smith-Hieftje, Atomic Absorption spectrophotometer with a hydride generation system (Thermo Jarrel Ash atomic vapor accessory 440). Buck, Scientific, U.S.A). Hollow cathode lamps were employed at the 196 nm wavelength and 1.0 nm band pass. Selenium concentration was determined by an internal standard addition method, as previously described (7). Plasma was diluted (1:10) with redistilled deionized water. All determinations were run in duplicate and individual values were averaged. Accuracy and precision of the procedure was regularly checked with commercial samples with recommended selenium contents (Seronorm Serum, Nycomed AS, Oslo, Norway).

Table 1. Characteristics of the study subjects*

Subjects (n)		Age (year)		Number of cigarette smoked per day	Duration of smoking (year)
Smokers	Non-smokers	Smokers	Non-smokers		
36	32	41 ± 8	37 ± 11	12.8 ± 2.4	17.7 ± 8.7

* Data are given as mean ± SD.

Determination of plasma copper, zinc, and iron

Plasma samples were diluted with deionized, distilled water for copper and zinc measurements. Copper and zinc were determined by atomic absorption spectrometer (Thermo Jarrel Ash Smith-Hieftje). Plasma copper and zinc values were expressed in µg/dl. Iron concentration was determined by colorimetric method with a commercial kit (ZiestChem diagnostics cat, no. II-514). Hemoglobin (Hb) concentration of hemolysate was measured by the cyanomethemoglobin method (8). All enzyme activities were measured using the some hemolysate.

Determination of erythrocyte antioxidative enzymes activities

Hemolysate antioxidant Cu-Zn SOD and GSH-Px enzyme activities were measured using commercially available kits (Randox, Lab. Ltd. Ireland Cat. No. SD125 and RS505, respectively). Measurement of Cu-Zn SOD enzyme activity was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase , which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetra-zolium chloride (INT) to form a red formazan dye (9). The Cu-Zn SOD activity was expressed as U/gHb. One unit of Cu- Zn SOD was assigned to that amount of enzyme necessary to produce 50% inhibition in the INT reduction rate. Cu-Zn SOD activity was expressed as units per gram Hb.

Measurement of GSH-Px activity was based on the following principle: GSH-Px catalyzes the oxidation of glutathione by cumene hydroperoxide (10). In the presence of glutathione reductase and nicotinamide adenine dinucleotide phosphate (NADPH) the oxidized glutathione is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm was measured. Erythrocyte GSH-Px activity was expressed as U/gHb. The enzyme unit of GSH-Px was defined as the number of reduced NADP oxidized per minute at 37°C by 1 g of Hb under standard conditions. Measurements were carried out at 37°C and according to the Randox application procedure. CAT activity in hemolysate was assayed by a method described by Aebi (11). From the hemolysate, an aliquot of 10 µl was diluted with phosphate buffer (50 mmol/L, pH 7.0) to a final volume of 5.0 mL. CAT activity was measured by adding 1.0 mL H_2O_2 (30 mmol/L, Merck, Germany) to 2.0 mL diluted hemolysate and reading the decrease in absorbance at 240 nm between 20 and 30 seconds with a spectrophotometer (Cecil 9000, England) against a blank consisting of 2.0 mL diluted hemolysate and 1.0 mL deionized water at 37°C. One unit of CAT activity was defined as the amount of enzyme that degrades one mmol H_2O_2/min at an initial concentration of 10 mmol/L at pH of 7.4 and temperature of 37°C. Each sample was assayed twice, and the mean of the first order rate constants (k) was calculated. The final results corrected for the dilution and related to the Hb concentration, the enzyme activity was expressed as (k/gHb) as described in the literature (11). Results are expressed as mean ± standard deviation. Differences between data were calculated with Student's *t* test. All statistical analyses were performed with the program Statistical Package for the Social Sciences (SPSS) for windows, version 11.5 (12). The level of significance for tests was set at $P < 0.05$.

RESULTS

According to results presented in tables 2 and 3, it is evident that the erythrocyte Cu-Zn SOD activity was significantly higher in tobacco smokers than in nonsmokers ($P < 0.01$). However, plasma selenium concentration was significantly lower in tobacco smokers than in nonsmokers ($P < 0.001$). CAT and GSH-Px activity and copper, zinc and iron concentrations did not differ significantly between two groups ($P > 0.05$).

Table 2. Comparison of erythrocyte parameters of tobacco smokers and non-smokers

Variable	Smokers (n= 36)	Non-smokers (n= 32)	*P*
SOD, U/gr Hb	1294.6 ± 206.7	1121.6 ± 237.8	0.002
GSH-Px, U/grHb	39 ± 6.7	40.79 ± 7.1	0.385
CAT, k/grHb	322.96 ± 84.2	298.4 ± 93.2	0.258

Abbreviations: SOD, copper-zinc superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase.

Table 3. Comparison of plasma parameters of tobacco smokers and non-smokers

Variable	Smokers (n= 36)	Non-smokers (n= 32)	*P*
Cu µg/dl	101.4 ± 18	94.1 ± 17.3	0.103
Zn µg/dl	69.1 ± 13.8	71.5 ± 13	0.484
Se µg/l	62.7 ± 14.8	92.1 ± 17.5	0.00
Fe µg/dl	106.2 ± 35	113.1 ± 38.9	0.443

DISCUSSION

Cu-Zn SOD, GSH-Px and CAT are among most important antioxidative enzymes in free radical scavenger system in normal conditions. Trace elements like Cu, Fe, Se and Zn play key roles as cofactors for enzymatic activity of above mentioned enzymes in the process of oxygen-free radical formation and can regulate anti oxidative activity of Cu-Zn SOD, GSH-Px and CAT.

In this study, activities of erythrocyte Cu-Zn SOD, GSH-Px and CAT and plasma concentrations of copper, zinc, iron and selenium in male smokers and non-smokers are investigated and compared.

We have demonstrated that erythrocyte Cu-Zn SOD activity was significantly higher in smokers compared with nonsmokers. This observation suggests oxidative stress induction following cigarette smoking. However, it is not exactly clear that which constituent of the cigarette smoke involves in this phenomenon. In some studies nicotine has been shown to be involved in oxidant production following cigarette smoking (13). In other studies, cadmium has been shown to be involved in free radical production (14) . On the other hand, it has been shown that cigarette smoking causes stimulation of inflammatory response (15) which leads to increased Cu-Zn SOD activity (16). Kocyigit *et al.* also showed increased erythrocyte Cu-Zn SOD activity following cigarette smoking (3).

We did not find significant differences in erythrocyte glutathione peroxidase activities between smokers and nonsmokers which are similar to findings in some studies (1, 17, 18). However, in some other studies both decreased (4) and increased (14) activities of this enzyme have been reported. These conflicting observations may be attributed to several factors: some studies have been done on rats exposed to cigarette smoke and increased activity of GSH-Px have been reported (19) and assumed that cigarette smoke induces antioxidative enzyme activities as a self-defence mechanism. In some other studies erythrocyte GSH-Px activities in groups of female smokers have been investigated and decreased activities have been reported (4). Other factor which can partly contribute in conflicting results on GSH-Px activity is methodological details in determination of GSH-Px-activity. In some studies GSH-Px activity was determined using tert-butyl-hydroproxide as substrate which allows the measurement of both seleno- dependent and non-seleno-dependent GSH-Px activity (Total GSH-Px) , or by using H_2O_2 as substrate. We assessed the activity of this enzyme by using Cumen hydroperoxide which only allows the measurement of seleno-dependent GSH-Px.

There was not significant differences in erythrocyte catalase activities between smokers and non-smokers that is similar to findings in the literature (1, 3, 20). It seems that cigarette smoking has no considerable effects on the activity of this enzyme. In contrast, decreased activity of this enzyme has been reported (1). In fact, smoker group in that study was chosen among females. So this inconsistency could be attributed partly to the function of sex or life-style differences compared to men. Inactivation of enzyme, as a result of oxidative stress stroke has also been considered for decreased activity of catalase in smokers (21).

We found that there is no significant difference in copper plasma concentrations between smokers and non-smokers. In contrast to our findings, Kocyigit *et al.* have reported increased copper plasma concentration in smokers compared to nonsmokers (3). They suggested that induced inflammatory response following smoking is responsible for increased copper plasma concentration. However, the mechanism underlying and the type of cigarette which can induce this inflammatory response have not been mentioned. It is worth mentioning that in some other studies copper concentration has been determined in serum while we determined it in plasma.

We did not find significant differences in zinc plasma concentrations between smokers and non-smokers as Kocyigit *et al.* found (3), while Dubick *et al.* (5) and Kim *et al.* (4) have reported increased zinc

concentrations in smokers. In Dubick *et al.* study, subjects were exposed to nicotine, and zinc concentrations have been determined in serum samples (5). In Kim *et al.* study, zinc concentrations were also determined in serum samples and subjects were chosen among teenage girls (4).

According to our findings, there is no significant differencesin iron plasma concentrations between smokers and non-smokers. Most of researchers did not investigate on the iron concentration in their studies.

Our results shows that selenium plasma concentrations in cigarette- smokers are significantly lower than non- smokers. Kocyigit *et al.* (3) and Ellis *et al.* (17) also found same results in their studies. Decreased concentration of selenium may be related to several factors. First, cadmium and arsenic, the natural components of tobacco smoke, stimulate liver excretion of selenium and decrease bioavailability of this element.

Second, decreased concentration of selenium may be due to inflammatory response following cigarette smoking. It has been demonstrated that stimulation of inflammatory process cause increased expression of inflammatory mediators such as interleukins (22). It has also been demonstrated that IL-8 levels are inversely correlated with selenium plasma concentration (23).

Regarding to relationship between plasma concentrations of trace elements and erythrocyte enzyme activities, it is shown that copper plasma levels tend to increase in parallel with Cu-Zn SOD activity; and GSH-Px activity tends to decrease in parallel with selenium concentration. Although studies have shown that inflammatory and oxidation processes play critical roles in several events followed by cigarette smoking, considerable uncertainties are remained. These include precise constituents of cigarette smoking which are responsible for such events after smoking and the intermediate pathways through which smoking related events occur, and roles of heredity, environment and life style of smokers.

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