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Effects of Short-term Supplementation with Vitamin C on Lipid Peroxidation in Cigarette Smokers

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Abstract: *Background*:Cigarette smoke has been reported to result in a depletion of body antioxidant vitamins. This study evaluated the association between smoking, dietary antioxidants and indices of oxidative stress in male cigarette smokers. *Methods*: Blood samples were collected at a baseline and at the end of 5 days supplementation with 500 mg vitamin C (two dosages/day) from 36 smokers and 43 nonsmokers. Serum levels of total and reduced forms of vitamin C, retinol, α-tocopherol were measured as antioxidants and malondialdehyde as an oxidative stress index. *Results*: The study showed that cigarette smokers were at higher risk of marginal vitamin C deficiency. Smokers retained significantly higher concentrations of dehydroascorbate and malondialdehyde levels as compared to nonsmokers implying that smoking enhanced lipid peroxidation. Vitamin C supplementation improved lipid peroxidation index with 18% reduction in serum malondialdehyde of smokers ($P < 0.05$), but did not significantly change that of nonsmokers. *Conclusion*: Vitamin C supplementation attenuated lipid peroxidation of smokers, which may reflect its protective role against oxidative stress. This may suggest the need for either a more prolonged vitamin C supplementation or the requirement of more than one constituent of the antioxidant system.

Key words: Smoking, free radicals, lipid peroxidation, vitamin C, supplementation.

INTRODUCTION

Cigarette smoking have been implicated as a major risk factor for atherosclerotic vascular disease, including coronary heart disease and stroke and several others chronic diseases (He *et al.,* 2001; Ezzati and Lopez, 2003; Burke and Fitzgerald, 2003; Centers, 2004). Although the precise mechanisms involved in the pathologies associated with smoking is not completely understood, inflammatory response it induces and the presence of free radicals and reactive species are causative factors capable of damaging cellular macromolecules, in particular lipids, proteins and DNA (Pryor *et al.,* 1983; Church and Pryor, 1985; Pryor and Stone, 1993).

Over the past decade much attention has been focused on the antioxidant micronutrients as possible protective agents against smoking-related diseases by protecting low-density lipoproteins (LDL) from oxidative modification (Martin and Frei, 1997; Al Senaidy *et al.,* 1997). High dietary intake of vitamins E, C and β-carotene has been related to a lower prevalence of chronic bronchitis in smokers and a reduced risk of stroke or coronary artery disease in several observational investigations (Nyyssonen *et al.,* 1997; Anderson, 2001; Padrao *et al.,* 2007; Britton *et al.,* 1995; Kirkham and Rahmam, 2006). The association of smoking with lower serum levels of dietary antioxidants has been consistently reported in the literature. Several nutrition surveys indicate that smokers generally consume fewer fruits and vegetables and have lower serum antioxidant concentrations than do nonsmokers (Faruque *et al.,* 1995; Marangon *et al.,* 1998; Dyer *et al.,* 2003).

Vitamin C is the most essential water-soluble antioxidant in human serum. Recent reports have shown that the concentration of vitamin C is lower in smokers than non-smokers, which could be due to alteration in metabolism of this vitamin (Murata, 1991; Wei *et al.,* 2001). Decreased serum vitamin C is most likely associated with the inordinate high levels of free radicals in cigarette smokers that lead to increased turnover of the vitamin (Schectmin *et al.,* 1998; Cross *et al.,* 1993). In this context, Brown, (1996) reported that cessation of smoking can improve antioxidant concentrations within 84 hours with no change in food habits.

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The most important mechanism of tissue damage is the production of lipid peroxides acquired in cell membrane as a consequence of oxidative stress (Cross *et al.,* 1993; Morrow *et al.,* 1995). The antioxidant activity of vitamin C is considered to be the major defense mechanism, in the aqueous phase, against the harmful effect of free radicals (Chaudiere and Ferrari-lliou, 2005; Lykkesfeldt, 2005). In view of the antioxidant roles of vitamin C, we decided to investigate its effects on malondialdehyde (MDA) as an index of oxidative stress in cigarette smokers. In this study, we assessed serum vitamin C, α-tocopherol and retinol levels in smokers and nonsmokers and investigated the impact of daily supplementation of ascorbic acid on the levels of vitamin C and lipid peroxides.

MATERIALS AND METHODS

Subjects:

Seventy nine male students from King Saud University (Riyadh, Saudi Arabia) aged 19-26 years participated in this study. The study included 36 cigarette smokers and 43 non-smoking volunteers who were never smokers. Smokers were defined as those who have been smoking more than 10 cigarettes per day for at least 1 year before the time of entry into the study. All subjects were seemingly healthy, were not taking any vitamin supplements or medication, and none was on any special diet.

Subjects were excluded if they reported a history of any major chronic illness including diabetes and respiratory diseases or if the complete blood count revealed anemia. Each subject signed an informed consent before participating in the vitamin C supplementation trial.

Study subjects at the start of the study reported for collection of dietary information and an initial (Day 0) blood sample. Fasting venous blood samples were drawn, maintained protected from light and serum samples were used for baseline concentrations of vitamin C, retinol, α-tocopherol, and MDA. After that, all participants, who upheld their regular diet without vitamin supplementation or medication, were provided with ascorbic acid tablets (Hoffman–LaRoche) and were instructed to take two doses of 500 mg each in the morning and in the evening for 5 days. They were encouraged not to change their dietary habits. From each subject blood samples were again collected on the first (base line), third, fourth, and the sixth day of vitamin C supplementation period. Serum was separated from whole blood by centrifugation (2000 x g, 10 min at 4°C), portioned into 1 to 2 ml samples and stored at -70°C until analysis was conducted for vitamin C, and MDA.

Analysis:

Serum levels of α-tocopherol and retinol were estimated by a normal phase HPLC method as already described by Al Senaidy *et al.,* (2001). These measurements were conducted in HPLC with Varian 5000 pump, pulls damper and Rheodney 7125 injection port connected into MCH-5 OD reverse phase column (150 x 4.6 mm), Samples were eluted isocratically with a mobile phase of acetonitrile/methanol/water (60:20: 20 v/v/v) at a flow rate of 1.0 ml/min and detected at 300 nm. Internal standards of retinol acetate and α-tocopheryl acetate were added to each specimen for determination of sample concentration.

Serum total vitamin C (ascorbic acid) was determined after stabilization with meta-phosphoric acid by the dinitrophenylhydrazine method (McGown *et al.,* 1982). Briefly, protein-free supernatant was incubated with dinitrophenylhydrazine-thiourea-copper reagent at 37°C for 3 hours. The color was stabilized by adding 2 ml of 12 M cold sulfuric acid and keeping the mixture at room temperature for 20 min. The absorbance was measured at 520 nm. Concentration was calculated from a calibration curve obtained by plotting the absorbance of a series of ascorbic acid standard solutions. Dehydroascorbate level (oxidized form) was estimated using the above method but without the oxidizing agent (copper solution). The level of the reduced form of vitamin C was calculated as the difference between the total vitamin C and the oxidized form.

The malondialdehyde level in serum of smokers and nonsmokers was estimated using the fluorometric method of Yagi (1976). Fluorescence measurements were conducted using a Perkin-Elmer Model 203 fluorometer. Lipid peroxides were expressed as µmoles of malondialdehyde using a standard absorption curve of 1, 1, 3, 3-tetraethoxypropane.

Total cholesterol, triglycerides and HDL-Cholesterol were determined enzymatically using a commercial kit from Boehringer Mannheim (Mannheim, Germany). LDL-Cholesterol concentration was estimated by using the Friedewald equation.

Methods precision was investigated using pooled human serum divided into twelve portions. The coefficient of variation within run assays was 0.7% for ascorbic acid and 6.1 % for malondialdehyde. The between run CV values did not exceed 1.2% and 13% for ascorbic acid and malondialdehyde assays respectively. To minimize variability between assays, every patch of samples was run together for both assays for smokers and non-smokers.

Statistical Analysis:

Results are expressed as mean ± SD. Statistical comparison between smokers and non-smokers was performed by Wilcoxon-Mann- Whitney U test. Differences within the two groups after supplementation were tested by the Student's t-test. All statistical tests were two-tailed, and P value lower than 0.05 was considered significant.

RESULTS AND DISCUSSION

All subjects, smokers and non-smokers, showed similar mean age and body mass index. (Table 1). Body weights tended to be lower in smokers than in non-smokers, but this difference was not statistically significant. On the average, lipid profile (triacylglycerol and total, LDL, and HDL cholesterol) for the subjects were not different between the 2 groups. However, smokers had significantly higher serum concentration of malondialdehyde suggesting a higher level of oxidative stress among cigarette users. Mean serum concentrations of retinol and α-tocopherol in smokers were lower than those of non-smokers, but the difference was not significant.

Table 2 compares the various forms of vitamin C levels in smokers and nonsmokers. Serum vitamin C concentration was 28.9% lower in smokers than non-smokers ($P < 0.01$). A more revealing aspect is comparison between reduced and oxidized forms of vitamin C. Smokers had 33.2 % lower amount of reduced form and 61.5 % higher oxidized form of ascorbic acid compared to nonsmokers ($P < 0.01$). In general about 13 % of smokers subjects have mean serum vitamin C level below 11 mM thus indicating hypovitaminosis C (serum concentrations < 11.4 µM) (Fig. 1)

Table 1: Characteristics of the study subjects (Mean ± SD).

Variable	Nonsmokers (n = 43)	Smokers (n = 36)
Age (y)	21.5 ± 1.6	22.3 ± 1.5
Weight (Kg)	73.2 ± 3.6	69.4 ± 2.7
BMI (Kg/ m^2)	24.2 ± 1.4	25.1 ± 1.1
Cigarettes per day	-	17.0 ± 3.6
	Serum concentration	
Total cholesterol (mmol/L)	5.70 ± 0.4	6.13 ± 0.4
LDL Cholesterol (mmol/L)	3.12 ± 0.4	3.49 ± 0.4
HDL Cholesterol (mmol/L)	1.11 ± 0.2	1.65 ± 0.2
Triglycerides (mmol/L)	2.40 ± 0.3	2.60 ± 0.4

Table 2: Antioxidants and MDA serum levels in the study subjects (Mean ± SD).

Variable	Nonsmokers (n = 43)	Smokers (n = 36)
α-Tocopherol (µmol/L)	18.9 ± 1.5	17.6 ± 1.1
Retinol (µmol/L)	1.4 ± 0.1	1.2 ± 0.1
Total Ascorbic acid (µmol/L)	39.7 ± 4.8	30.8 ± 4.2**
Ascorbic acid (Reduced form) (µmol/L)	34.6 ± 4.5	23.1 ± 3.1**
Dehydroascorbicate (Oxidized form) (µmol/L)	5.2 ± 0.1	8.4 ± 0.3**
Malondialdehyde (µmol/L)	1.5 ± 0.4	3.1 ± 0.5**

$P<0.01$, $^{**}P<0.001$

Effect of Vitamin C supplementation:

Figure 2 summarizes the changes in serum vitamin C and MDA levels after daily doses of one gram of ascorbic acid for a period of five days. Although the serum level of vitamin C continued to increase in both groups, smokers always had lower values than nonsmokers. Nevertheless, the level of lipid peroxides in the serum of smokers remained higher than in nonsmokers throughout the experiment.

Nonsmokers showed a more rapid response to ascorbic acid doses than smokers. Serum level of vitamin C increased by 51% after three days of supplementation in nonsmokers in comparison with only 34% increase in smokers. After five days of vitamin C supplementation (Fig.3A) vitamin C level increased by 47.3% and 61% for smokers and nonsmokers, respectively. At the same time smokers serum MDA level significantly ($P < 0.05$) decreased to 2.05 ± 0.096 µM, which means a reduction of 18 % (Fig. 3B), while nonsmokers serum MDA showed no notable changes.

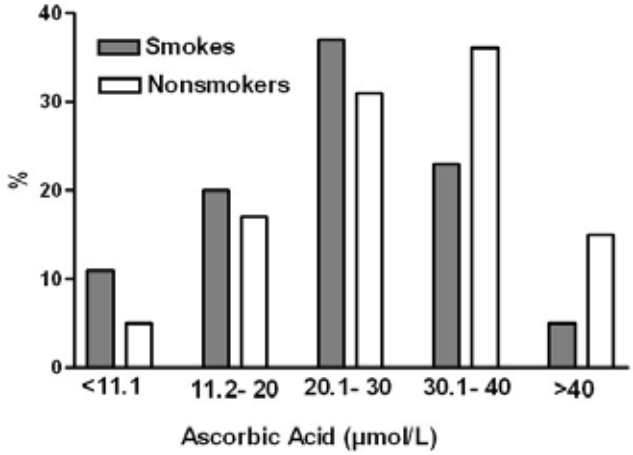

Fig. 1: Distribution of total vitamin C in serum of smokers and nonsmokers.

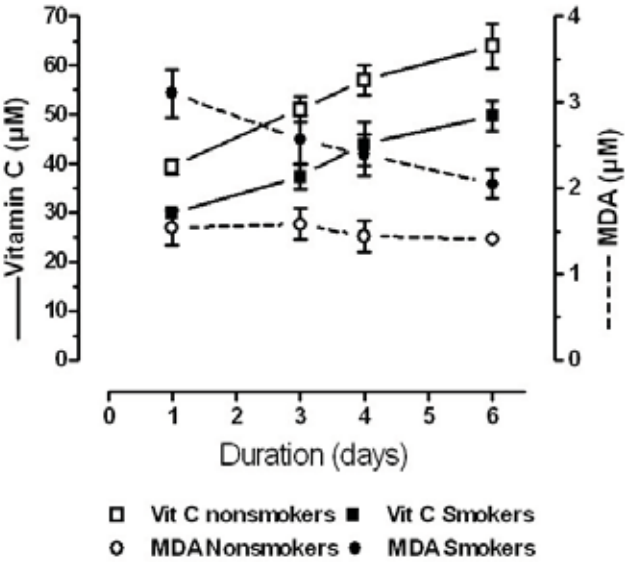

Fig. 2: Vitamin C and MDA levels of smokers and nonsmokers serum during the supplementation study (Mean ± SD).

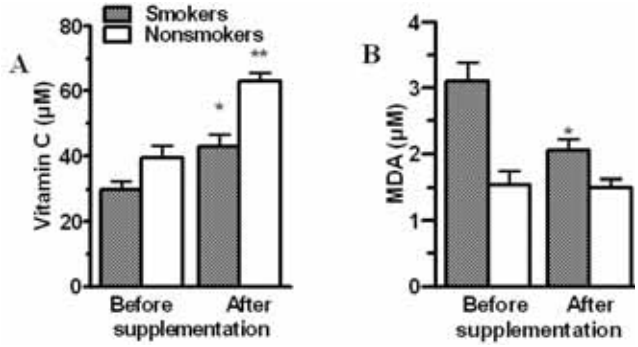

Fig. 3: Vitamin C (A) and serum MDA (B) of smokers and nonsmokers supplemented with vitamin C. **P*<0.05 and ***P*<0.01 vs. corresponding value before supplementation.

Discussion:

This study demonstrated that, healthy young adult male smokers had significantly lower serum vitamin C level. The total vitamin C concentration was about 22% lower in smokers, a result that is consistent with those reported in literature(Murata, 1991; Duthie, 1992). Several other investigators have reported that serum ascorbic acid concentrations are lower in cigarette smokers than in non-smokers (Schectman *et al.,* 1998; Brown, 1996; Jain *et al.,* 2009).

The extent of lipid peroxidation was found to be higher in smokers than in non-smokers, as shown by the significantly higher levels of serum MDA. Higher oxidative stress exerted by cigarette smoke was also manifested by marked difference in dehydroascorbate levels between smokers and nonsmokers (Cross *et al.,* 1993; Morrow *et al.,* 1995).

A number of evidence supports the hypothesis that cigarette smoke may contribute to cardiovascular disease, and that antioxidants may protect against such condition. Consumption of antioxidant vitamins has been associated with a reduced lipid peroxides and decreased rates of ischemic heart disease. A minimum plasma level of 12 µmol/L of vitamin C was suggested as necessary to avoid increased risk of myocardial infarction (Nyyssonen *et al.,* 1997). Although all subjects of the present study had similar characteristics and eating habits, the results suggested that smokers were at higher risk of marginal vitamin C deficiency than nonsmokers; more than 10% of smokers were below the minimal level compared to 4.7% of nonsmokers. Either inadequate intake or higher metabolic turnover of vitamin C are among the most probable explanation for the difference in vitamin C levels between smokers and nonsmokers.

The present study suggested that lower vitamin C level of smokers may not be solely caused by poor vitamin C intake, since supplementation of smokers with vitamin C did not elevate serum vitamin C level to that of nonsmokers. This conclusion is consistent with previous studies of vitamin C dietary intakes (Scjectman *et al.,* 1989). The latter studies demonstrated an inverse association between smoking and serum vitamin C level, and that was independent of dietary vitamin C intake. We observed a steady increase in vitamin C serum level, to a varying degree, in smokers and nonsmoker after short duration of vitamin C supplementation. The maximum serum vitamin C level observed (45.4 and 63.9 µmol/L for smokers and nonsmoker, respectively) was far less than the renal clearance value of 80 µM; (Nyyssonen *et al.,* 1997). Thus, smokers may require continuous supply of ascorbic acid, via natural sources or by supplementation, to maintain serum vitamin C level similar to that of nonsmokers. It was concluded that although smoking is associated with dietary intake of vitamin C, inflammatory changes increase its turnover so that blood concentrations are still lower in smokers than non-smokers even when there is control for dietary differences.

It has been reported that cessation of smoking led to rapid improvement in the level of plasma vitamin C (up to 23.3%) Brown, 1996; Lykkesfeldt *et al.,* 1996). Interestingly, this improvement value is equivalent to the difference in vitamin C levels between smokers and nonsmokers after short term of vitamin C supplementation as shown in the present study. Vitamin C interacts with the plasma membrane and protects α-tocopherol by donating electrons to α-tocopheroxyl radicals (Chaudiere and Ferrari-llou, 2005). Therefore, it is possible that the requirement of smokers for vitamin C, and probably other nutritional antioxidants, is greater than that of nonsmokers. Higher turnover rate of vitamin C seems as a more plausible explanation for the reduced concentration of vitamin C in smokers of this study.

The oxidative stress of smoking was also manifested by higher levels of circulating lipid peroxides expressed as MDA. While supplementation of smokers with vitamin C significantly reduced peroxides level, nevertheless, MDA remained higher than the corresponding values of nonsmoker. Improvement of lipid peroxidation index seems to be dependent on the efficiency of all members of antioxidant system rather than on vitamin C alone.

Previously, we observed that smokers retained lower levels of β-carotene, α-tocopherol and γ-tocopherol than nonsmokers (Anderson, 2001). Among antioxidants, plasma ascorbic acid was the first to be depleted after plasma exposure, in vitro, to cigarette smoke (Cross *et al.,* 1993). Therefore, vitamin C may spare the degradative metabolism of α-tocopherol when vitamin E is limited, and this may lead to greater vitamin C requirement of smokers. Moreover, smokers have higher alveolar concentration of vitamin C than nonsmokers, a process that may occur as a response to higher levels of oxidative stress in the lungs of smokers (Bui *et al.,* 1992). Thus, ascorbic acid may be mobilized from circulation to other body organs that are under persistent oxidative stress, such as the lungs of smokers.

In conclusion, short term vitamin C supplementation to smokers induced elevation in the level of serum ascorbic acid and to some improvement in lipid peroxidation index. However, smokers seem to require continuous supply of vitamin C in order to maintain adequate level needed to counter the sustained free radical load of cigarette smoking.

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