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Full Length Research Paper

Effect of vitamin C on salivary superoxide dismutase activity in smokers

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This study was performed to elucidate the effect of ascorbic acid on salivary superoxide dismutase (SOD) activity in smokers. In this single blind, cross over clinical trial, whole unstimulated saliva of 30 smokers, who were randomly divided into two groups, was collected. In the first phase, patients of one group took 500 mg of vitamin C powder, for 3 weeks. Then saliva of all patients was collected. After a one- week wash-out period, vitamin C was given to the other group. Collection of saliva was done after 3 weeks. SOD activity was measured. Statistic evaluation was performed by Repeated Measured ANOVA, Independent sample T test and Paired T test. Variability of SOD activity without using vitamin C and after its use was not significant (p=0.639).The effect of vitamin C in saliva is not through enzymatic mechanisms. Oxidative stress from cigarette smoke may be decreased by other mechanisms.

Key words: Superoxide dismutase, ascorbic acid, smoking, antioxidant.

INTRODUCTION

Cigarette smoking has been associated with an increased incidence of cancer in different sites of body including oral cavity (Lee et al., 1998). Cigarette smoke is responsible for 50 to 90% of the cases (Greabu et al., 2007). The incidence of oral SCC in cigarette smokers is 4 to 7 times higher than in non smokers (Nagler and Reznick, 2004).

The World Health Organization (WHO) ranks tobacco smoking among the 10 greatest risks to health. Cigarette smoke contains more than 4000 chemical agents several types of toxic components including carbon monoxide, nicotine, aromatic hydrocarbons and specially free radicals and reactive oxygen (O^{2-}). One "puff" of a cigarette exposes the smokers to more than 10^{15} free radicals (Northrop-Clewes and Thurnham, 2007). In recent years, increasing evidence has supported the involvement of free oxygen radicals in several human diseases such as atherosclerosis, rheumatoid arthritis, ischemic heart disease and cancer (Garg et al., 2006).

According to field cancerization concept, there is a constant and direct attack of various cigarette smoke reagents on the oral epithelial cells, which gradually accumulate and cause stepwise malignant transformation. It has been suggested that free radicals, reactive oxygen species and reactive nitrogen species in the inhaled cigarette smoke induce this gradually evolving process, initially expressed by dysplastic lesions of the mucosa and eventually result in full blown infiltrating and metastasizing oral SCC (Nagler and Reznick, 2004).

Antioxidants are present in all body fluids and tissues and protect against endogenously-formed free radicals (Scully et al., 2002). Salivary antioxidants system consist of enzymatic (superoxide dismutase and peroxidase) and non enzymatic (uric acid) components (Nagler et al., 2002).

Superoxide dismutase (SOD) is found in all tissues and cells of the aerobic organisms. SOD is a key antioxidants enzyme that efficiently and specifically scavengers free radical of oxygen by catalyzing its dismutation to H_2O_2 and O_2 (Akalin et al., 2008; Perry et al., 2010). The reactive O_2 (O^{2-}) is converted to H_2O_2 by SOD and the antioxidants enzymes such as catalase, glutathione peroxidase and peroxidase can in turn convert H_2O_2 to mole-

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Abbreviation: SOD; superoxide dismutase.

cular oxygen and water that are harmless for body tissues (Kanehira et al., 2006).

Cigarette smoke may attack antioxidant enzymes rather than molecules. Salivary antioxidants activity in smokers cannot be protective against accumulative stresses and several studies have demonstrated a reduction in the activity of salivary antioxidants system in patients with SCC comparing to control group (Nagler and Reznick, 2004).

Vitamin C is able to scavenge free radical of both reactive oxygen group (super oxide and hydro peroxyl) and reactive nitrogen group (nitrogen dioxide and peroxynitrite) (Northrop-Clewes and Thurnham, 2007).Vitamin C may be capable of regenerating other antioxidants like vitamin E therefore prevents oxidative damages (Panda et al., 2001).

The idea of using vitamin C to treat and prevent cancer was first proposed in 1949. Cameron et al showed that administration of high dose ascorbic acid improved survival of patients with terminal cancer (Li and Schellhorn, 2007). Recent studies have shown that Plasma/serum vitamin C concentrations up to 40% lower in smokers than non smokers (Northrop-Clewes and Thurnham, 2007). Four hours after administration of 500 mg vitamin C, the mean urinary excretion of ascorbate was 35.4 mg in non smokers and 14.5 mg in smokers, a highly significant difference. In comparison to non smokers, smokers need higher levels of vitamin C to defend against oxidetive damages.

A recommended dietary allowance derived from direct antioxidants function of vitamin C could not be calculated because of the lack of a quantative relationship between any antioxidants function and health related end point (Northrop-Clewes and Thurnham, 2007).

This study was performed to elucidate the effect of ascorbic acid on salivary superoxide dismutase (SOD) activity in smokers.

MATERIALS AND METHODS

This study was designed as a single blind randomized cross over clinical trial. We used enzyme activity assessment package to determine level of the enzyme in collected saliva. Patients were selected by simple non randomized sampling method. Thirty (30) smokers who had been referred to dental faculty of Shaheed Beheshti University of medical sciences (Tehran, Iran) were included in this study. Smokers with the history of 5 or more pack/year of smoking (number of daily cigarette divide to 20, multiply by the number of years with history of cigarette smoking) were included in this study. They had no systemic disease, no history of chemotherapy or radiotherapy, did not take any medications continuously during 3 past months, did not take alcohol and supplements, and did not have nephrolithiasis. They also had no oral pathology based on clinical examination. Patients were informed about the whole procedure and consent form was filled up by each patient. Then they were randomly divided to two groups A and B.

At least 1 ml of each patient's unstimulated saliva was collected in an upright position, between 9 and 12 o'clock in the morning. They had been ordered to avoid eating, drinking, smoking one hour before saliva collection. We used 50 ml falcon tubes as saliva containers.

Collected saliva was transferred to laboratory and was placed in specific micro tubes immediately. To separate squamous cells and derbies, samples were centrifuged (5000 g, 4°C) for 15 min (Hetich, Germany). Then centrifuged samples were preserved at -70°C to be assessed later.

Each groups (A, B) had different instruction for using vitamin C. Patients in group A received 500 mg of solved powder of vitamin C daily (Osveh, Iran) for 3 weeks. In the first phase patients of group B took no vitamin C and they were asked to follow their routine diet. After 3 weeks, unstimulated saliva samples were collected again.

After a week period of wash out, patients of group B were ordered to take vitamin C as mentioned before and cases of group A took no vitamin C. After 3 weeks, unstimulated saliva samples were collected for the third time. These cases, who had forgotten to take vitamin C more than two times in a week, were excluded from the study.

Superoxide dismutase (SOD) activity in collected samples was evaluated using enzyme activity assessment kit (Cayman chemical, Cat No.706002, USA). This assay kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radical.

The operator was an experienced technician blind about cases. Each sample was assessed two times to be more accurate. We used ELISA Reader machine (Anthoos, 2002) to read the results. Data was statistically analyzed by computer SPSS 15 software. Repeated Measured ANOVA, Independent Sample T test and Paired T test were used as statistical tests.

RESULTS

Thirty (30) cases between ages 24 to 70 years participated in this study. The mean activity of SOD enzyme in baseline samples was 3.70 u/ml. Table 1 demonstrates changes of enzyme activity with and without vitamin C. Enzyme activity increased in 16 cases (53%) after taking vitamin C. But according to paired sample T test results, SOD enzyme activity did not change significantly after taking vitamin C (Table 2). Correlation coefficient between pack/year of smoking and enzyme activity showed that spearman correlation coefficient before vitamin C was $p=0.846$, $r=0.037$ and after its administration this coefficient was $p= 0.339$, $r= 0.181$. According to these results, correlation between pack/year of smoking and enzyme activity increased after taking vitamin C but this amount is not statistically significant.

DISCUSSION

In this study, we used whole saliva as samples. When conducting analysis of saliva for antioxidants, whole saliva is more relevant as it contains gingival crevicular fluid, immune cells and tissue metabolites (Scully et al., 2002).

We used powder formulation of vitamin C. Several researches have revealed that different forms of vitamin C have no effect on the rate of its absorption or its efficacy (Choi et al., 2009).

Table 1. Statistical variables of enzymatic activity in samples.

Parameter	Change in enzyme activity	
	Before using vitamin C	After using vitamin C
Number of samples	30	30
Minimum	0	1.69
Maximum	6.42	6.65
Mean	4.04	4.17
SD	1.80	1.50
Standard error	0.33	0.27

Table 2. Paired T test results to determine the significance of vitamin C effect

T test	Degree of freedom	Significance Test
0.474	29	0.639

We have reported before that cigarette smoke leads to an elevation in salivary superoxide dismutase activity (Baharvand, et al., 2010). Here, we demonstrated roll of vitamin C on SOD activity. Many studies have investigated the effect of vitamin C on saliva in smokers and they had various results (Lee et al., 1998; Panda et al., 2001; Moller et al., 2004; Schneider et al., 2001).

Panada et al. (2001) have shown that tar phase cigarette smoking oxidative damage is almost completely prevented by vitamin C (Panda et al., 2000, 2001).

Lee et al. (1998) used 500 mg of vitamin C to investigate its inhibitory effect on oxidative damage in smokers (Lee et al., 1998). According to a review article, those studies that investigate anti cancerous effects of vitamin C used at least 80 to 110 mg of vitamin C daily (Carr et al., 1999). Considering the lack of *in vivo* study about vitamin C effects on saliva and also access to 500 mg vitamin C powder, we decided to use this product. This amount is less than tolerable upper intake level for vitamin C which is 2000 mg/day for adults (Weinstein et al., 2000).

In our study, we included smokers with the history of 5 or more pack/year of smoking according to Moller (Moller et al., 2004; Hamo Mahmoud et al., 2007).

In the research into oxidative stress, direct study of increased reactive oxygen species (ROS) is very difficult as their half life is very short, in the range of milliseconds. Therefore, it is generally studied by monitoring enzymatic antioxidants related to ROS reacting (Washio et al., 2008). Enzymes are the primary defense system against oxidative damages that mostly restrict free radicals (Abdollahi et al., 2004).

We measured SOD activity in this study. The level of SOD was measured in saliva of smokers in many studies (Nagler, 2007; Kanehira et al., 2006). Kanehira et al. (2006) concluded that measurement of SOD in human saliva might be useful for estimating the level of oxidative stress on smoking habits.

Our results showed that the use of vitamin C for 3 weeks in smokers does not change SOD enzyme activity significantly which was in consistent with the result of Washio and Klein's study. Klein designed an *in vivo* study to investigate the effect of cigarette smoke on oral peroxidase activity in human saliva. They demonstrated that exposure to cigarette smoke causes a 70% loss of enzyme activity. Several antioxidants agents (including ascorbic acid) were exposed to cigarette smoke and it was shown that they had no protective effect on oral peroxidase (Klein et al., 2003).

In another study, Washio et al. (2008) investigated the effect of vitamin C supplementation on the level of SOD in hemodialysis patients. They showed an increase in plasma level and concentration of vitamin C. Nor the amount neither the activity of SOD had significant changes.

In this study, we investigated the effect of vitamin C on SOD as an indicator of its effect on salivary oxidative damages. It is possible that antioxidant effect of vitamin C on saliva results from its effect on non enzymatic system such as uric acid molecules. Greabu et al. (2007) studied the effect of vitamin C on salivary antioxidants and showed that cigarette smoke decreases salivary uric acid level and amylase and lactate dehydrogenase. Addition of 10mg/dl vitamin C to saliva was not able to maintain/ restore the original uric acid level but it increased uric acid level significantly. So, vitamin C had a protective effect on salivary uric acid level. They suggested that an adequate intake of antioxidants may help smokers to avoid cigarette smoke-induced oxidative damage.

Conclusion

We concluded that vitamin C does not increase SOD activity significantly. It is possible that smoke–induced oxidative stresses may decrease after vitamin C intake and SOD detection is not able to determine it.

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