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The Effects of Smoking Cessation and Antioxidant Vitamins on Oxidative Stress

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In this study, the effects of smoking cessation and relative antioxidant activities on the oxidative stress were determined by using in vitro method. Thirty healthy smokers who were free of any disease and smoked more than 1 pack per day for the past 10 years participated in this study. For smoking cessation, smokers were asked to wear nicotine patch (21mg nicotine/ patch) everyday for 30 days and then to replace at the same time of the day. Smoking cessation program in conjunction with nicotine patch replacement was also conducted every week, one hour/each session, for 4 weeks. Canthaxanthin, β-carotene, and α-tocopherol were added into red blood cells at pre and post smoking cessation. As indicators of oxidative stress, hemoglobin degradation, lipid peroxidation, and percent hemolysis were determined at both pre and post smoking cessation. After 30 days of smoking cessation, the subjects gained an average of 5 pounds, varying 2 to 8 pounds, by suggesting that behavioral problems rather than nicotine itself are more important for gaining weight in ex-smokers. The total hemoglobin concentrations in blood were similar in pre and post smoking cessation, but smoking cessation resulted in a decrease in the percentage of methemoglobin from 0.96% to 0.85%. Smoking cessation also caused to decease malondialdehyde (MDA) values (26.7±7.8 vs. 23.6±4.5 (without oxidation), 179.3±21 vs. 161.2±28 nmol/ml (with oxidation) ($p<0.05$)), not percent hemolysis.

Various antioxidants with smoking cessation significantly decreased MDA values($p<0.05$), in contrast to marginal decrease of MDA in smoking cessation only. Three antioxidants used in this stu study were similarly effective in inhibiting MDA production, but relative effectiveness of canthaxanthin or α-tocopherol was greater than that of β-carotene ($p<0.05$), in case of oxidation induced. The percent hemolysis was greatly decreased when antioxidants were added into the blood of ex-smokers ($p<0.05$) but no statistical significance in relative effectiveness of antioxidants was observed.

Key words: Smoking cessation, Nicotine, Carotenoids, α-tocopherol, Oxidative stress, Lipid peroxidation

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INTRODUCTION

Cigarette smoking plays a role in many diseases which ultimately lead to death or disability. Most common deaths related to cigarette smoking are heart disease and lung cancer. Researchers worldwide have reported that there are twice more premature deaths from cardiovascular disease among smokers, compared to lung cancer deaths from smoking in the same year.[1] Smoking is also the main risk factor in the development of chronic obstructive pulmonary disease.[2]

Despite the fact that smoking is a major cause of chronic diseases, the mechanism by which cigarette smoking causes the diseases is not still clear. Oxidative stress is considered to be one of major pathological mechanism associated with smoking.[3-5] Inhaled smoke from cigarette contains over 4000 chemical compounds, several of which are known toxic free radicals. Cigarette smoke itself, both in particulate fraction and the gaseous phase, contains large amounts of free radicals. The presence of trace metals such as iron or copper in cigarette smoke is also one of the factors important for free radical formations.[6] Free radicals in cigarette smoking are produced by increasing the numbers of neutrophil and macrophases in the body and activating them to produce more free radicals.[6-7] When free radicals interact with polyunsaturated fatty acids or proteins, lipid peroxidation or degeneration of protein is occurred. These products damage a wide range of cells and membranes, leading to the cause of chronic diseases.[8]

Smoking cessation is the only effective treatment for avoiding or reducing the progression of this disease.

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Excess risk of heart disease caused by smoking is believed to be reduced by half after quitting smoking.[1] However it is not easy to quit smoking because it is addictive habit. One of the important barriers for quitting smoking is to gain weight after smoking cessation. Dale et al.[9] reported that nicotine replacement limited weight gain after smoking cessation. The nicotine patch delivers small doses of nicotine into the body, which allows ex-smokers to manage withdrawal symptoms and cravings. The nicotine patch contains none of the toxic compounds, such as tars, carbon monoxide and carcinogens, found in tobacco smoke.

Nicotine replacement therapy, therefore, is accepted as a strategy for smoking cessation. Research has shown that chances of successful quitting were greatly increased when smokers actively participated in smoking cessation while using nicotine.[10] However, nicotine is believed to be one of smoking related components that enhance oxidant production.[11] Smoking cessation is involved in decreasing oxidative stress and normalized antioxidant system, but the effect of smoking cessation with nicotine on oxidative stress is not well demonstrated.[12-14] Therefore, the purpose of this study was to determine the effect of cessation of smoking with nicotine on lipid oxidation and degradation of protein. This study also determined the effect of antioxidant activity of cantha-xanthin, β-carotene, and α-tocopherol on oxidative stress in ex-smokers.

MATERIALS AND METHODS

1. Selection of the Subjects

Thirty smokers who had high anxiety to stop smoking, aged 35 to 55 participated in this study. All smokers were heavy smokers who had smoked more than 1 pack per day for the past 10 years. They were reported to be healthy and free of chronic disease such as heart diseases and diabetes. A cigarette delivers roughly 1 mg of nicotine, and thus subjects in this study used 21 mg nicotine patch/day for 30 days. Smokers were asked to wear nicotine patch for twenty-four hours at a time, and then to replace at the same time every day. Smoking cessation program in conjunction with nicotine patch replacement was also conducted every week, one hour/each session, for 30 days. Each week, the subjects came to the session to learn how to change their smoking related behaviors, and the investigators checked their smoking status to make sure the subjects not to smoke. After 30 days, it was revealed that three smokers smoked 1-3 cigarettes occasionally and two quitted because of family business, thus 5 subjects were excluded from this study. This study was approved by the Human Subjects Committee at F.S.U. and consented to this aspect of the study. Blood was collected before and after 6 weeks of smoking cessation program. When the blood was collected, the height and weight were also measured.

2. Preparation of Erythrocytes and Antioxi-dants Enriched Erythrocytes[15-16]

Subjects fasted for at least ten hours before blood was collected. The blood from each subject was drawn into heparin coated tubes (Fisher Chemical Co.) and erythrocytes were separated from plasma by centrifugation (1500×g, 15 minutes). After centrifugation, the supernatant was discarded and then washed 3 times with phosphate buffered saline, followed by centrifugation.

All antioxidants were generously provided by Hoffman-La Roche (Nutley, N.Y). From the preliminary studies, the proper concentrations of β-carotene, cantha-xanthin, and α-tocopherol were determined. After the last washing of blood samples, followed by centrifugation, erythrocytes were suspended in K-R (Krebs-Ringer) phosphate buffer (pH 7.4) containing either various antioxidants (10 uM) or control. After completing the additions, the tubes were treated with a stream of nitrogen, sealed with stopper, and incubated for 90 minutes in a shaking water bath at 37 ℃.

The sample was again suspended in phosphate buffered saline to give a final volume of 3.3% (v/v) erythrocytes suspension to measure lipid peroxidation and the antioxidant effects. After incubation of samples treated with various antioxidants or control, aliquots of each sample were stored for 24 hours. Oxidation, with 20 nM butylhydroperoxide, was induced into erythrocytes containing various antioxidants or control. After the oxidant was added into erythrocytes, the samples were incubated in a water bath at 37 ℃ for 90 min. Hemolysis and MDA formation were determined.

3. Measurement of Total Hemoglobin and Hemoglobin Derivatives

Total hemoglobin in the blood was measured using total hemoglobin assay kits from Sigma chemical Co. (St. Louis, Mo) and determined from a standard curve constructed with increasing concentrations of cyanomethemoglobin standard solution. Hemoglobin degradation was analyzed by a modification of the procedure of Harley & Mauer.[17] For oxyhemoglobin, after 30 min of lysis, the absorbance of samples was measured at 620 nm

before and after the addition of 0.25 M potassium ferricyanide (Sigma Chemical Co. St Louis, Mo). To calculate the concentration of oxyHb, the extinction coefficient of E=0.014 g/100 ml[18] was used. For MetHb, each sample was treated with 0.25 M of sodium azide (Sigma Chemical Co., St Louis, Mo). The absorbance of each sample at 620 nm was measured before and after the addition of sodium azide. The concentration of MetHb was calculated, as described in the study of Evelyn & Malloy.[19]

4. Measurements of Lipid Peroxidation in Erythrocytes

Antioxidant activity was assayed by determining the concentration of malondialdehyde (MDA) using the TBA test and measuring the extent of hemolysis in red blood cells.

1) The TBA Assay

The concentration of MDA (nmol/ml) was assayed by a modification of the method of Stocks & Dormandy.[20] The supernatant of each sample was mixed with 30% trichloroacetic acid and centrifuged at 2000×g for 15 min. After centrifugation, the samples were mixed with 1% thiobarbituric acid in 0.05 M NaOH and then boiled for 15 min. The absorbance of samples versus blank at 535 nm was measured with a spectrophotometer. Solution concentration was calculated by using extinction coefficient of 1.56×10^{-5} M.

2) Hemolysis

Hemolysis was measured with a modification of the method of Niki et al..[21] After incubation of the samples, two sets were prepared for the hemolysis assay: in one set, the tubes contained 100 ul of sample and 4 ml saline, while the second set of tubes included 100 ul of sample and 4 ml of water to facilitate complete hemolysis. After 40 min. the absorbance of two sets of samples against a blank was measured at 540 nm with a spectrophotometer, and the percent hemolysis was calculated as the study of Niki et al..[21]

5. Statistical Analysis

The data obtained in this study were analyzed by using multiple analysis of variances (MANOVA) test and analysis of variance (ANOVA) with SPSS/PC+ at F.S.U. was performed to compare the relative effectiveness among treatments and groups. Student t-test was also applied to determine statistical differences between groups.

RESULTS AND DISCUSSION

1. Sample Characteristics

Characteristics of the experimental subjects are presented in Table 1. The group of smokers consisted of 7 males and 18 females aged 35 to 55 with a mean age of 43 years. All subjects were white Caucasian. After 30 days of smoking cessation, the subjects gained an average of 5 pounds, varying 2 to 8 pounds. Several studies reported that the weight gain over a 10-year period with the cessation of smoking was 4.4 kg for men and 5.0 kg for women.[22-24] Jesssen et al.[10] suggested that weight gain after smoking cessation resulted from the withdrawal of nicotine and the subsequent reduction in metabolic rate, and that 1 mg of nicotine gum showed profound thermogenic effect.

Most ex-smokers in this study who used nicotine patch (21 mg/day) gained weight, varied 2-8 pounds. Recently, Bamia et al.[24] reported that the tendency to gain weight after smoking cessation mostly came from behavioral problems rather than tobacco-related pharmacological roots. From this study, to prevent weight gain after smoking cessation, behavior programs including weight management will be helpful for smokers to quit smoking. Behavioral program for smoking cessation should focus on avoiding the substitution of high calorie foods for cigarettes, managing withdrawal symptoms with proper food choices and exercise.

2. The Relative Percentage of Hemoglobin Degradation

The total hemoglobin concentrations in blood were similar in pre and post smoking cessation. Only after 30 days of smoking cessation, the percentage of oxyhemoglobin was increased from 98.5% to 99.5% (Table 1). Smoking cessation also resulted in a decrease in the percentage of methemoglobin from 0.96% to 0.85% ($p < 0.05$).

Table 1. The characteristics of subjects and the percentage of hemoglobin degradation

Variables	smoking cessation (30 days)	
	pre	post
Numbers	25	25
Sex	7 Male & 18 Female	7 Male & 18 Female
Age(years)	42.8 ± 9.5	42.8 ± 9.5
Weight(lbs)	174.7 ± 31	179.0 ± 31
Height(inches)	68.1 ± 3.7	68.1 ± 3.7
Hemoglobin(g/dl)	15.2 ± 1.6	15.0 ± 1.8
MetHemoglobin(%)	0.96 ± 0.2*	0.85 ± 0.3
OxyHemoglobin(%)	98.5 ± 0.7	99.5 ± 0.2

All data shows mean and standard deviation (Mean±SD)
* Significantly different between groups (pre and post) at $p<0.05$

Hemoglobin is a major protein in erythrocytes and thus hemoglobin degradation is also represented as the index of protein damage in erythrocytes. Free radicals inactivate red cell enzymes and denature the hemoglobin molecules.

Rice-Evan et al.[25] supported the idea by demonstrating the autooxidation of oxyhemoglobin to methemoglobin involved in the lipid peroxidation of red blood cells by generating superoxide radicals. When this superoxide radical interacted with prooxidants, hydroxyl radical was also produced. This hydroxyl radical has been shown to be a potent indicator of lipid oxidation.[20] In addition of supporting the fact that smokers have more oxidation of hemoglobin in red blood cells,[5-6] this study also suggests that smoking cessation with nicotine patch decreases the degradation of hemoglobin and thus prevents possible formation of free radicals and lipid peroxidation by smoking.

3. MDA Concentration and Hemolysis in Erythrocytes of Smokers & Ex-smokers

After quitting smoking, their MDA values were decreased from 26.7±7.8 to 23.6±4.5 nmol/ml (without oxidation) (Fig. 1). When oxidation was induced into red blood cells, the MDA concentration was increased 7 to 8 folds. The effect of cessation of smoking on the inhibition of MDA was more clearly demonstrated, and it was significant (MDA:179.3±21 vs 161.2±28 nmol/ml, $p<0.05$). In hemolysis (Fig. 2), as an another indicator of oxidative stress in red blood cells, no differences between pre and post study of smoking cessation were observed, in case of either with or without oxidation induced, although minimal decrease of hemolysis after smoking cessation was shown.

Several studies reported that the cessation of smoking decreased the oxidative stress in smokers. Isoprostanes (IP) have been identified as reliable markers of in vivo oxidation injury.[12] When IP values of vascular tissue and blood as well as urine in smokers and nonsmokers were measured, IP values in smokers were significantly increased. Refraining from cigarette smoking for a few days resulted in a significant drop of oxidation of blood and reached a steady state at about 4 weeks after quitting cigarette smoking.[12] Smoking is also associated with significant decreases in serum paraoxonase (PON) activities.[13] PON has antioxidant activities of the protection of LDL from oxidation. Lower serum paraoxonase is linked to more severe coronary artery disease and this PON concentration is normalized within a relatively short time of cessation of smoking. Those results suggested that ex-smokers may rapidly recover

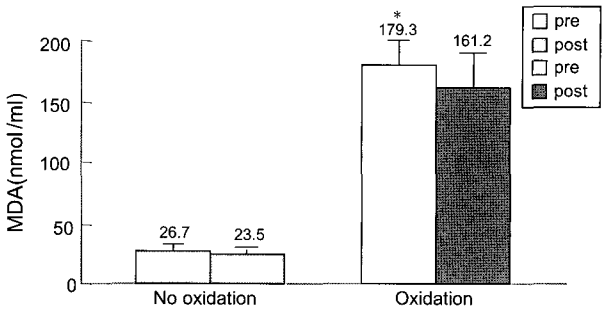

Fig. 1. The effect of smoking cessation on MDA concentration
* : $p<0.05$

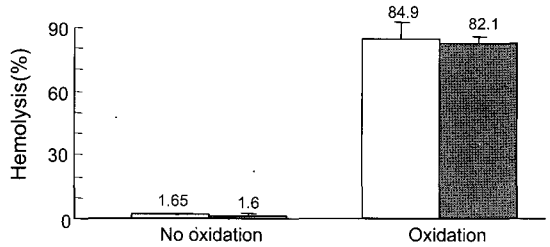

Fig. 2. The effect of smoking cessation on hemolysis of red blood cells

from their enhanced in vivo oxidation.

This study also showed the reduction of MDA after cessation of smoking, but statistical significance showed only in case of oxidation induced into the bloods of ex-smokers (Fig. 1). There are two possibilities that might affect the results of this study. First, the ex-smokers in this study used nicotine patch everyday and the amount of nicotine (21 mg) from nicotine patch equals to the amount of one pack of cigarettes. Nicotine is one of the major components that enhance oxidant production. In Jay's study,[11] various concentrations of nicotine added to isolated neutrophilis resulted in increased release of superoxide ions. Thus there is a possibility that the use of nicotine for smoking cessation may affect the results of oxidative stress in this study. In order to declare whether the use of nicotine patch retards the lipid oxidation in ex-smokers or not, the comparison between pre and post smoking cessation with and without nicotine patch need to be studied. Second, we did not control the dietary factor although smokers were advised to control their dietary habits during smoking cessation. Smokers in this study gained weight after smoking cessation. Considering smoker's eating habit,[14,26] food preference for fatty foods and relatively low preference for fruits and vegetables, there is a possibility that their eating habits also affect the value of oxidative indicators in this study.

Hemolysis is the breakage of the red blood cell's

Table 2. The Effects of antioxidants and smoking cessation on the MDA Products with and without oxidation

Treatment	Cessation of smoking			
	no oxidation		oxidation	
	pre	post	pre	post
No treatment	29.3 ± 6.7[a]	25.1 ± 5.4[a]	190.1 ± 24[a*]	171.2 ± 27[a]
+ β-carotene	18.4 ± 4.8[b]	17.8 ± 3.3[b]	148.9 ± 20[b]	136.7 ± 26[b]
+ cathaxanthin	17.1 ± 6.4[b]	15.6 ± 6.0[b]	136.5 ± 21[ab]	127.7 ± 24[ab]
+ α-tocopherol	19.2 ± 5.1[b]	14.7 ± 4.7[b]	135.4 ± 27[ab]	121.4 ± 25[ab]

All data shows mean and standard deviation (Mean±SD)
[a,b] Values with different superscripts within column are significantly different (p<0.05)
* Significantly different between groups (pre and post) at p<0.05
MDA: malondiadehyde(nmol/ml) as an index of lipid peroxidation

Table 3. The Effects of antioxidants and smoking cessation on the percent hemolysis with and without oxidation

Treatment	Cessation of smoking			
	no oxidation		oxidation	
	pre	post	pre	post
No treatment	2.75 ± 1.3[a]	2.36 ± 1.7[a]	95.9 ± 6.8[a]	91.2 ± 9.2[a]
+ β-carotene	1.31 ± 0.9[b]	1.31 ± 1.2[b]	84.1 ± 10.2[b]	80.6 ± 11.0[b]
+ cathaxanthin	1.10 ± 0.8[b]	1.32 ± 0.9[b]	81.5 ± 7.1[b]	78.2 ± 7.6[b]
+ α-tocopherol	1.20 ± 0.9[b]	1.30 ± 0.7[b]	80.5 ± 9.2[b]	78.6 ± 9.6[b]

All data shows mean and standard deviation (Mean±SD)
* Significantly different between groups (pre and post) at p<0.05
[a,b] values with different superscripts within column are significantly different (p<0.05)

membrane, causing the release of hemoglobin and other internal components into the surrounding fluids. Hemolysis can occur in a blood sample due to improper blood collection, blood processing and storage, or transport.[18] Because of this fragility, the blood sample in this study was collected by well-experienced nurse, and the hemolysis measurement was carried out on the same day as the blood samples were collected.

In case of hemolysis (Fig. 2), no difference between pre and post smoking cessation was shown. Only a minimal decrease of hemolysis after smoking cessation appeared. Goldberg and Stern[27] reported that a suspension of human red cells which were exposed to superoxide anion had rapid breakdown of the cellular hemoglobin to methemoglobin and resulted in increased osmotic fragility of the cell. When the effect of superoxide and hydrogen peroxide was generated enzymically on erythrocytes, these two free radicals participated in generating hydroperoxides and then directly oxidized the unsaturated fatty acid in red blood cells, resulting in hemolysis.[28] However Clemens MR[29] reported that hemolysis appeared at later stage in oxidative damage to red blood cells. Thus, the results of this study may be interpreted as the period of smoking cessation didn't enough to change hemolysis of red blood cells.

4. Antioxidants and the Cessation of Smoking on Oxidative Stress of Erythrocyte

When various antioxidants were added into the bloods of ex-smokers, the MDA values were significantly decreased ($p<0.05$), in contrast to marginal decrease of MDA in smoking cessation only (Table 2). Three antioxidants used in this study were similarly effective in inhibiting MDA production both in pre- and post- smoking cessation. Canthaxanthin and α-tocopherol seemed to decrease MDA more effectively than β- carotene, but no significant difference was shown. After inducing oxidation, the antioxidant effects of tocopherol and canthaxanthin were more clearly demonstrated. Cantaxanthin decreased MDA more effectively than β- carotene, and this was significant ($p<0.05$). The potency of canthaxanthin as an antioxidant was similar to that of α-tocopherol.

Table 3 demonstrated the effect of smoking cessation with antioxidant vitamins on percent hemolysis. Like the results of MDA study, the percent hemolysis was greatly decreased when antioxidants were added into the blood of ex-smokers, compared to the hemolysis of ex-smokers without antioxidants ($p<0.05$). It seemed that α-tocopherol or canthaxanthin was more effective than β-carotene, but no statistical significance in relative effectiveness of antioxidants was observed.

To decrease oxidative stress in smokers, either smoking cessation or antioxidant supplementation plays important roles. Several studies showed that smokers have depleted antioxidant system[30-34] and antioxidant supplementations decreased oxidative stress in smokers.[35-39] The addition of dietary antioxidants such as tocopherol[33] and ascorbate[35] has been shown to be effective in reducing oxidative stress in smokers. Supplementing smokers' diets with carotenoids rich juice for 4 weeks resulted in significantly increased conjugated diene (CD) lag time, by suggesting that carotenoids protect against diseases related to oxidative stress.[36] Handelman et al.[37] reported that lycopene was rapidly depleted and tocoperol was lastly depleted when cigarette smoke and antioxidants were exposed to directly human plasma. Romanchik et al.[38] reported that the addition of carotenoids protected LDL oxidation *in vitro* study.

Smoking cessation is another way to decrease oxidative stress in smokers, and thus to improve antioxidant system in the body. Brown[40] AJ reported that acute smoking cessation in smokers showed significant increases in total plasma vitamin C and β-carotene levels. Polidori et al.[41] reported that 4 weeks of smoking cessation resulted in a markedly increase in plasma antioxidant concentrations and plasma resistance towards

oxidative challenge. Since the smoking cessation or antioxidant supplementation is generally accepted as a good way to decrease oxidative stress in smokers, it will be hypothesized that antioxidants in addition to smoking cessation will double the antioxidant-effect in ex-smokers. Our study showed that in case of smoking cessation only, the MDA values were marginally decreased, maybe because of nicotine, but the addition of antioxidants to smoking cessation greatly improved oxidative stress in ex-smokers.

Carotenoids and tocopherol function as chain-breaking antioxidants in various ways due to different chemical structures. Tocopherol is a well known antioxidant in red blood cells, and its antioxidant activity is well reported. Carotenoids consist of 8 isoprenoid units and two beta-ionone rings. Depending on the structure, carotenoids are largely divided into two groups, hydrocarbon carotenoids and oxygenated carotenoids. Hydrocarbon carotenes include beta-, alpha- and gamma- carotenes. Oxygenated carotenoids, also called xanthophylls, include cantha-xanthin, zeaxanthin, and lutein.[42] Canthaxanthin found in edible mushrooms, sea trout, algae, and some microorganisms has keto-groups on the beta-ionone ring. Substitution of hydrogen in the beta-ionone ring for the oxo-group in the case of xanthophylls resulted in increased quenching ability due to the two free electron pairs which might affect the electron resonance of conjugated double bond.[43] The antioxidant activity of canthaxanthin has been demonstrated in *in vivo* and *in vitro* studies.[43-46] From the results of this study, when smokers participate in smoking cessation with nicotine patch, the supplementation of antioxidants, especially, canthaxanthin and α-tocopherol, may be beneficial to decrease oxidative stress in ex-smokers.

CONCLUSION

We found that smoking cessation with nicotine patch (21 mg/day) for 30 days caused weight gain, an average of 5 pounds (about 2.5 kg), by suggesting that the behavioral problem rather than tobacco-related components, such as nicotine withdrawal, is an important factor for weight-gain in ex-smokers. With nicotine patch, smoking cessation causes to decrease hemoglobin degradation and lipid peroxidation. The addition of various antioxidant vitamins to the blood of ex-smokers was more effective in decreasing oxidative stress than the treatment of smoking cessation alone. This study also showed that canthaxanthin is very effective antioxidant to recover oxidative stress in both smokers and ex-smokers. In smoking cessation program with nicotine-products, the antioxidant vitamin supplementation may be beneficial to decrease possible oxidative stress from nicotine.

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