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The Oxidative Stress in Cigarettee Smokers and Antioxidant Vitamins

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ABSTRACT

The purpose of this study was to find the extent of lipid peroxidation of erythrocytes in cigarette smokers, and to determine the relative effectiveness of β-carotene, canthaxanthin, and α-tocopherol as antioxidants. Thirty smokers and 30 nonsmokers participated in this study. No significant differences according to age, sex, and height were shown. Cigarette smokers in this study had higher hemoglobin concentrations and more oxidation of hemoglobin than nonsmokers. In addition, the erythrocytes of cigarette smokers had significantly higher MDA concentrations than erythrocytes of nonsmokers, which suggests that smokers may have erythrocytes under high oxidative stress. The antioxidant activities of carotenoids and α-tocopherol were studied in vitro by measuring the concentration of malondialdehyde(MDA) and percent hemolysis of erythrocytes. The addition of any antioxidant to erythrocytes significantly decreased MDA concentrations($p<0.05$) while antioxidants showed nonsignificant inhibition of hemolysis. Among the antioxidants used in this study, canthaxanthin showed the greatest inhibition of both lipid peroxidation and hemolysis. Meanwhile, α-tocopherol showed potent inhibition of lipid peroxidation, but not of hemolysis. (*Korean J Nutrition* 30(9) : 1102~1108, 1997)

KEY WORDS : cigarette smokers · carotenoids · α-tocopherol · lipid peroxidation · hemolysis.

Introduction

It is generally accepted that reactive free radical mediated oxidation of biological systems is related to various diseases, such as cancer, heart diseases, and aging[1-2]. Cigarette smoking is one of the major exogenous sources for production of free radicals. It has been reported that cigarette smokers are under high and sustained free radical stress[3-4]. The mechanism by which cigarette smoking increases the production of free radicals is not clear. Maybe cigarette smoking increase the numbers of neutrophils and macrophages and activates them to produce more free radicals[5-8]. Another study showed that cigarette smokers stored large amount of iron in their bodies, and the stored iron increased the risk of free radical formation[9].

When free radicals interact with polyunsaturated fatty acids or proteins, lipid peroxidation or protein degeneration occurs. These products damage a wide range of cells and membranes. Erythrocytes are particularly vulnerable to oxidative stress because they have high oxygen tensions, iron as prooxidants, and high polyunsaturated fatty acids(PUFA)[9]. Even though erythrocytes have been used as target cells for free radical attacks[10-11], only a few studies have determined the oxidative stress of erythrocytes in cigarette smoking[12-13].

Free radical damage to erythrocytes has been characterized by hemoglobin degradation and lipid per-

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oxidation, further causing hemolysis[14]. Lipid peroxidation occurs when free radicals interact with polyunsaturated fatty acids. Lipid peroxidation is accepted as a cause for the development of various chronic diseases[15-16]. Hemoglobin degradation is represented as the index of protein damage since most protein in erythrocytes is complexed with iron as hemoglobin[17-18]. Since mature erythrocytes cannot synthesize protein and thus cannot replace damaged components, oxidative stress may induce a fatal defect in these erythrocytes[7].

To decrease or prevent lipid peroxidation, antioxidants play an important role. Limited information regarding the effects of antioxidants and peroxidation of erythrocytes in cigarette smokers is available[12,19]. In both studies, supplementation with vitamin E to smoking-related groups was effective in decreasing oxidation of erythrocytes. Few studies have been reported concerning the effect of carotenoids on erythrocytes exposed to cigarette smoke components[20]. Even though there are few studies on the effects of carotenoids as antioxidants of erythrocytes in cigarette smokers, plasma concentration of β-carotene in cigarette smokers was shown to be significantly lower than in nonsmokers[21-23]. It has also been demonstrated that supplementation with β-carotene was effective for the treatment of free radical related diseases[20,24]. These studies strongly support the idea that carotenoids could play an important role in neutralizing oxidative stress in cigarette smokers.

Therefore, the purpose of this study is to determine the degree of lipid oxidation of erythrocytes in cigarette smokers and the effects of β-carotene, canthaxanthin, and α-tocopherol on lipid oxidation of erythrocytes in cigarette smokers by using in vitro methods.

Methods and Materials

1. Selection of the subjects

Thirty smokers and 30 nonsmokers, aged 28 to 67 participated in this study. All smokers were heavy smokers who smoked at least 1.5 – 2 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. Age-and sex-matched nonsmokers included faculty, staff, and students of Florida State University. This study was approved by the Human Subjects Committee at F.S.U.

2. Preparation of erythrocytes and antioxidants enriched erythrocytes[25-26]

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

All antioxidants were generously provided by Hoffman-La Roche(Nutley, N.Y). The proper concentration of β-carotene, canthaxanthin, and α-tocopherol was determined from the preliminary studies. After the last washing and centrifugation of blood samples, erythrocytes were suspended in K-R(Krebs-Ringer) phosphate buffer(PH 7.4) containing either various antioxidants(10uM) or control. After additions, wave completed the tubes were treated with a stream of nitrogen, sealed with stoppers, and incubated for 90minutes in a shaking water bath at 37℃. Each 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 antioxidant effects. After incubation, aliquots of each sample were stored for 24 hours. Oxidation, with 20nM butylhydroperoxide, was induced into erythrocytes containing various antioxidants or control After the oxidant was added into the erythrocytes, the samples were incubated in a water bath at 37℃ for 90min. Hemolysis and MDA formation were determined.

3. Measurement of total hemoglobin and hemoglobin derivatives

Total hemoglobin in 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[27]. For Oxyhemoglobin, after 30min of lysis, the absorbance of sam-

ples was measured at 620nm before and after the addition of 0.25M potassium ferricyanide(Sigma Chemical Co. St Louis, Mo). To calculate the concentration of oxyHb, an extinction coefficient of E=0.014 g/100ml[28] was used. For MetHb, each sample was treated with 0.25M of sodium azide(Sigma Chemical Co. St Louis, Mo). The absorbance of each sample at 620nm was measured before and after the addition of sodium azide. The concentration of MetHb was calculated as described in the study of Evelyn & Malloy[29].

4. Measurements of Lipid Peroxidation in erythrocytes

Antioxidant activity was assayed by determining the concentration of malondialdehyde(MDA) with the TBA test and bt 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[30]. The supernatant of each sample was mixed with 30% trichloroacetic acid and centrifuged at 2000×g for 15min. After centrifugation, the samples were mixed with 1% thiobarbituric acid in 0.05 M NaOH and then boiled for 15min. The absorbance of samples versus blank was measured at 535 nm 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 Miki, et al.[31]. After incubation of the samples, two sets were prepared for the hemolysis assay. In one set, the tubes contained 100ul of sample and 4ml saline, while the second set of tubes included 100ul of sample and 4ml of water to facilitate complete hemolysis. Ater 40min. 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 in the study of Miki, et al.[31].

• Statistics

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's t-test was also applied to determine statistical differences between groups.

Results and Discussion

1. Sample Characteristics and the relative percentage of hemoglobin degradation

Characteristics of the experimental subjects are presented in Table 1. The group of smokers consisted of 20 males and 10 females aged 28 to 68 with a mean age of 43 years. Nonsmokers included 20 males and 10 females, aged 27-63 with a mean age of 45 years. All subjects were white caucasians. No differences in sex, age, and height between groups were shown. The total hemoglobin concentration in blood was greater in smokers than in nonsmokers(15.7 vs 14.9g/100ml), but the differences were not statistically significant. The blood of smokers contained significantly higher levels of methemoglobin than that of nonsmokers(0.93% vs 0.83%).

About two-thirds of all iron is found in hemoglobin, and the erythrocytes contain large amounts of iron as hemoglobin contents. Hemoglobin in the presence of free radicals or oxygen is converted to methemoglobin. Some studies suggested that increased iron content in smokers caused oxidative damage to various biological systems, and the oxidation of oxyhemoglobin to methemoglobin involved the lipid peroxidation of erythrocytes[30]. Hem-

Table 1. The characteristics of experimental subjects and the percentage of hemoglobin degradation(Values are mean and standard deviation(S.D))

Variables	Nonsmokers	Smokers
Numbers	30	30
Sex	20 Male & 10 Female	20 Male & 10 Female
Age(years)	45 ±12.1	46.7 ±11
Weight(kg)	78.9±12.6	78.5 ±14.0
Height(cm)	170.4±12	174.4 ± 9
Hemoglobin(g/dl)	14.9± 1.9	15.7 ± 1.7
MetHemoglobin(%)*	0.8± 0.2	0.93± 0.2
OxyHemoglobin(%)	99.2± 1.2	98.7 ± 1.4

* : There are significant differences between two group, at $p<0.05$ level

oglobin degradation is also represented as an index of protein damage in erythrocytes because most protein in erythrocytes is complexed with iron as hemoglobin[31].

Therefore this study may suggests that higher methemoglobin levels in smokers contribute to increased lipid peroxidation and protein damage in erythrocytes. However further study is needed for the clear relationship between iron content in cigarette smokers and lipid peroxidation.

2. **MDA concentration and hemolysis in erythrocytes of smokers**

As seen in Fig. 1, before the induction of oxidation, the MDA concentration of smokers(2.6±0.5nmol/ml) was higher than that of nonsmokers(1.8±0.3nmol/ml)($p<0.05$). In contrast to the results of the MDA test, no differences in hemolysis were observed between smokers and nonsmokers before oxidation induction. When oxidation was induced in samples, MDA concentration and hemolysis were significantly increased. Smokers showed higher concentrations of MDA(18.0nmol/ml vs 16.1nmol/ml)($p<0.05$) and a higher percent hemolysis(85.0% vs 81.0%) than nonsmokers.

The TBA test was used as an index of lipid peroxidation in this study because it is a common, simple method that provides good recovery[32-33]. The TBA test measures the concentration of malondiadehyde (MDA) produced when polyunsaturated fatty acids are degraded[32].

In this study, cigarette smokers showed higher MDA formation($p<0.05$) and a higher percentage hemolysis of erythrocytes. Since oxidative stress of erythrocytes is characterized by hemolysis and and lipid peroxidation[16], this study suggests that smokers have higher oxidative stress than nonsmokers. In addition, erythrocytes are partly involved in the contact between macrophage and activated granulocytes[34], and thus it is possible that oxidative damage to erythrocytes in cigarette smokers may lead to incre-ased oxidative damage to other tissue.

The effects of antioxidants on the lipid peroxidation of erythrocytes

As shown in Table 2, all antioxidants, regardless of group, were effective in decreasing MDA concentration in erythrocytes($p<0.05$). However the inhibition of MDA production by carotenoids in smokers was more effective than in nonsmokers. MDA production in smokers was significantly decreased by canthaxanthin and α-tocopherol($p<0.05$), and the

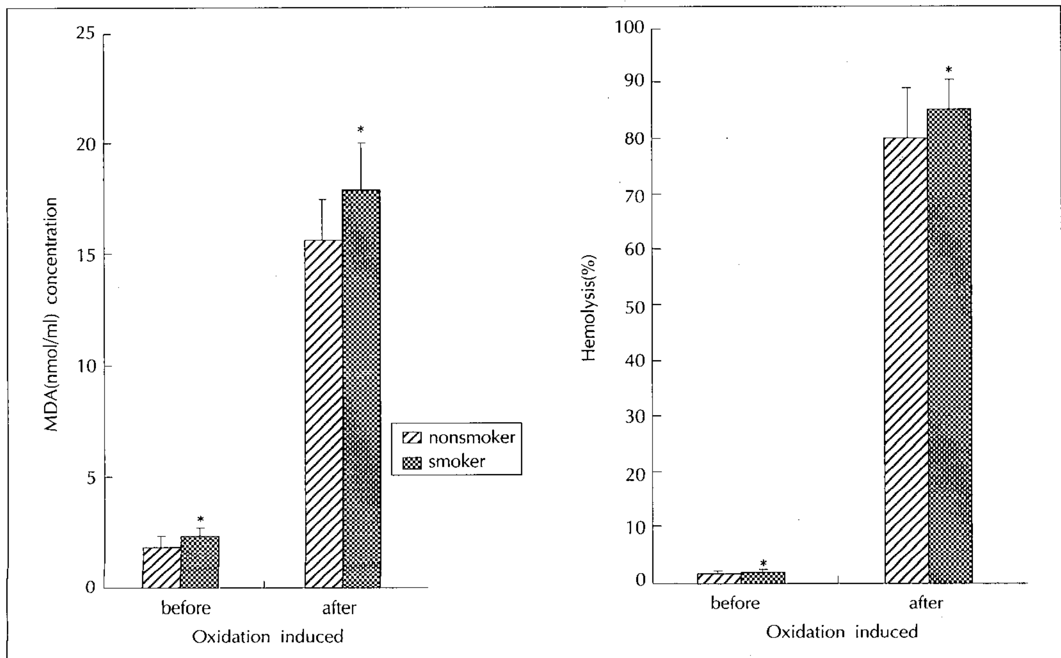

Fig. 1. The MDA(nmol/ml) concentrations and the percent hemolysis of erythrocytes in msokers and nonsmokers.

Table 2. The effects of antioxidants on MDA products(nmol/ml) with induced oxidation(means and standard diviation)

Treatment	Group		
	Nonsmokers(n=30)	Smokers(n=30)	Total N*(n=60)
No-addition	16.7±1.7	18.4±2.4[a]	17.2±2.1[a]
β-carotene	14.8±2.4	14.9±2.4[b]	14.8±2.4[b]
cathaxanthin	15.2±1.8	13.6±2.4[b]	14.4±2.0[b]
α-tocopherol	14.3±2.2	13.5±2.7[b]	13.9±2.5[b]

a,b : values with different superscripts in column are significantly different($p < 0.05$)

potency of tocopherol as an antioxidant was similar to that of canthaxanthin. The antioxidant effect of canthaxanthin on MDA concentration in erythrocytes seems to be greater than that of β-carotene. However, this data does not show statistically different values for these antioxidants.

This study showed that the additions of carotenoids or α-tocopherol erythrocytes of smokers was effective in reducing MDA concentration($p<0.05$). Canthaxanthin was an especially potent antioxidant in smokers. This suggests that carotenoids play important roles as antioxidants in reducing lipid peroxidation of erythrocytes in smokers.

Carotenoids stop the oxidative chain reaction through the highly unsaturated isoprenoid units of β-carotene which are very reactive with various types of free radicals[35]. Unlike beta-carotene, canthaxanthin contains a keto group with the addition of long conjugated double bonds that could give more potent antioxidant activities[36-38]. However, there is no study regarding the effect of canthaxanthin on lipid peroxidation of erythrocytes in cigarette smokers, and the mechanism by which carotenoids, especially canthaxanthin, function as antioxidants in erythrocytes is not known at present. Since this is in vitro study, further in vivo studies are needed.

Fig. 2 illustrates the changes in the percent hemolysis, before and after oxidation was induced. Without induced oxidation, the addition of antioxidants showed significant inhibition of hemolysis. When oxidation was induced, percent hemolysis was markedly increased, and the overall effectiveness of carotenoids as antioxidants in reducing hemolysis appeared greater than that of tocopherol, but there was no significant statistical difference. Canthaxanthin showed the least hemolysis followed by β-carotene and alpha-tocophrol without statistically signiticance.

Niki, et al.[33] and Yamamoto, et al.[16] reported that

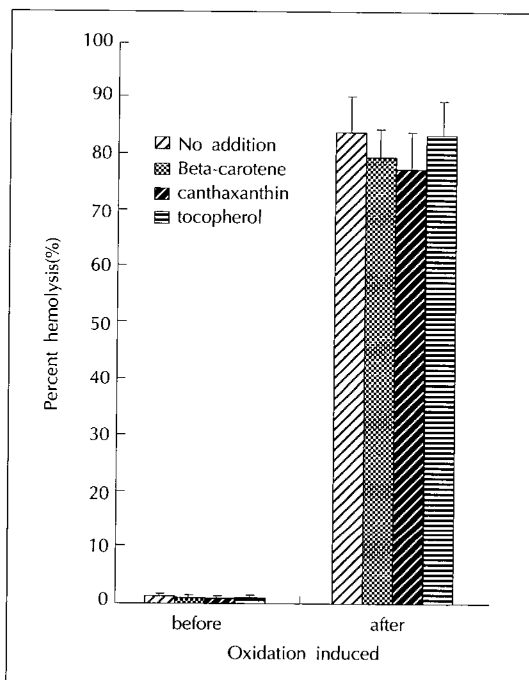

Fig. 2. The effects of antioxidants on the hemolysis of erythrocytes.

without antioxidants, more free radicals are produced in the body. These free radicals promote denaturation of the structure of hemoglobin, which then results in hemolysis. However in this study, although the addition of antioxidant vitamins to erythrocytes decreased hemolysis, no statistically significant differences were shown. In addition, among the antioxidants used in this study, the effect of α-tocopherol on hemolysis was not notable although it was effective in reducing MDA formation. Many earlier studies showed that tocopherol was effecive in reducing hemolysis while Miki, et al.[31] observed no differences in inhibition of hemolysis in the presence or absence of tocopherol. Since not many studies regarding antioxidant vitamins and hemolysis exist, the relationship is not clearly known at present. In order

to determine whether or not hemolysis is a good indicator of the activities of antioxidant vitamins, more reseach is needed. However this study may suggest that supplementation with vitamin E as a treatment for hemolysis should be approached with caution.

Conclusion

This study suggest that smokers may be at a high risk of erythrocyte lipid peroxidation as shown by higher hemoglobin degradation and MDA concentrations. The antioxidants β-carotene, canthaxanthin, and tocopherol, were apparently effective inhibitors of MDA formation in erythrocytes. The mechanism by which this occurs is not known at present. This in vitro study also showed that the addition of carotenoids decreased lipid peroxidation in smokers, which suggest that the supplementation of carotenoids in smokers could play an important role. However more in vivo research is needed to establish a clear relationship between carotenoids and smoking.

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