Original Article J. Clin. Biochem. Nutr., **45**, 341–346, November 2009

Susceptibility to Oxidative Stress is Greater in Korean Patients with Coronary Artery Disease than Healthy Subjects

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Received 8 May, 2009; Accepted 17 June, 2009

Summary **There are some evidences that the increased oxidative stress and thus increased oxidizability of lipoproteins and DNA can contribute to the development of certain human diseases, such as cardiovascular disease. To confirm the association of DNA damage with cardiovascular disease, we investigated susceptibility of DNA to oxidation in lymphocytes and oxidative stress related parameters in blood of patients with coronary artery disease (CAD). Subjects were consisted of 42 patients (27 men, 15 women) with documented CAD and 49 apparently healthy subjects (33 men, 16 women) as controls. Cellular DNA damage induced by 100 µM H_2O_2 was measured using Comet assay and quantified by TL. There were no differences in age (61.4 ± 1.7 years vs 62.0 ± 2.2 years) between the two groups. All the findings were shown to be independent of either sex or smoking habit. The patients showed significantly higher TL (87.3 ± 1.6 µm) compared to the control (79.3 ± 1.7 µm, *p*<0.01). Plasma TRAP, vitamin C, γ-tocopherol, and α-carotene levels in patients group were lower than those of control groups, while erythrocytic catalase activity increased in patients group. In conclusion, we observed that reduced overall antioxidant status was closely connected to higher susceptibility of DNA damage in CAD patients.**

***Key Words*: CAD, Susceptibility of DNA to damage, oxidative stress, antioxidant status**

Introduction

As in other European and western countries, diseases in circulatory system in Korea have also become the leading cause of death followed by neoplastic diseases and accidents [*1*]. Korean Medical Insurance Corporation (KMIC) study reported that annual medical care costs in 1994 for treating cardiovascular disease accounted for more than 10% of its total medical expenses, and it's still on the rise [*2*]. The incidence of ischemic heart disease in 1998 has also risen 6 times over the past 10 years [*1*].

Somatic mutations accumulated for a long period of time may play a role in the pathophysiology of chronic-degenerative illnesses such as atherosclerosis, diabetes mellitus and neurodegenerative diseases. Oxidative stress to lipids and DNA caused by free radicals are known to be one of the several fundamental biological mechanisms that are responsible for developing atherosclerosis and cancer [*3*]. Some of the possible atherogenic mechanisms were suggested in a recent article: 1) endothelial dysfunction, 2) diminished release of nitric oxide (NO), 3) increasing reactive oxygen species (ROS) production, 4) potentiation of LDL oxidation and 5) stimulation of smooth muscle cell

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proliferation [4]. Biological oxidative stress of free radicals are readily controlled by our own endogenous antioxidants including the scavenger enzymes superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) and by exogenous dietary antioxidants, including vitamin E, C, and carotenoids [5].

Some studies reported that DNA damage, one of the more reliable markers to detect oxidative stress, was increased in patients with coronary artery disease (CAD) [6–9]. However, the majority of studies have thus far been conducted in western countries, with almost no published data available from Asia [10]. It would be interesting to see any difference between western and Asian populations which may have different dietary patterns and be subjected to different environmental factors.

Therefore, the aims of this study were to establish the level of overall antioxidant status and H_2O_2-induced DNA damage, a marker of DNA susceptibility to oxidation, in patients with CAD and to compare the level to those of healthy control subjects among Korean population. To give more correct information of the body antioxidant status, we measured endogenous antioxidant enzyme activities, exogenous dietary antioxidant vitamins, and DNA damage.

Methods

Subject selection and general assessments

Forty-two patients (angiographically documented CAD patients; ≥50% luminal blockage in at least one coronary artery) from local C hospital were participated in this trial. Patients included 19 patients with stable angina and 23 patients with history of acute myocardical infarction. Patient exclusion criteria were acute inflammation, diabetes mellitus, metabolic disease, history or presence of any neoplastic diseases, recent major surgical procedure. Forty-nine age-matched control subjects were recruited from a local community center and the health status were reviewed by a study coordinator. Subjects with no known history of heart disease, diabetes mellitus and hypertension were included in the study.

Each participant's age, occupation, medical history, drinking, smoking, exercise habits were asked thoroughly. All subjects provided written informed consent before participation. Body weight was determined to the 0.1 kg using a balance scale and height (without shoes) was measured using a wall-mounted stadiometer. Waist and hip circumferences were measured by standard protocols [11]. The measurements were then used for calculating the body mass index (BMI), ideal body weight and waist/hip ratio. The subjects' diets were assessed using 24-h recall and a food frequency questionnaire. All patients gave written informed consent, and the Institutional Review Board at the Hannam University approved the study protocol.

Blood parameters

After an overnight fast, blood was drawn by a phlebotomist between 8:30–10:30 am. Plasma obtained from heparinized blood samples was centifugated at 3000 rpm for 15 min and the supernatant fraction was separated. The plasma was divided in appropriate aliquots and stored at −80°C until further analysis. Serum lipid profiles (total cholesterol, HDL-cholesterol, and triglycerides) were measured using assay kits from Bioclinical Systems (Anyang, Korea) and a photometric autoanalyzer (SEAC, CH-100 plus, Calenzano, Italy). Plasma LDL cholesterol levels were calculated using the formula developed by Friedewald *et al.* [12] and the atherogenic index was calculated according to the following formula: (total cholesterol-HDL cholesterol)/ HDL cholesterol.

Plasma ascorbic acid was measured photometrically by the method of Denson and Bowers [13]. The HPLC procedure developed by Jakob and Elmadfa was used to measure α-, γ-tocopherols, lycopene, α-, and β-carotene concentrations in plasma [14]. Tocopherols and carotenoids level were expressed as both uncorrected and corrected for the level of plasma triglycerides as suggested by Thurnham *et al.* [15]. Total radical-trapping antioxidant potential (TRAP) was analyzed using an inhibition assay described by Rice-Evans and Miller [16]. The SOD, GSH-Px, and catalase activities were assayed in erythrocyte-suspension by the procedure of Marklund and Marklund, Beutler, and Aebi, respectively [17–19].

Susceptibility of DNA to oxidation

The alkaline comet assay was conducted according to Singh *et al.* with little modification [20]. The isolated lymphocytes duplicated from one subject were subjected to oxidative stress by suspension in PBS with 100 μM H_2O_2 for 5 min on ice. The lymphocytes, were mixed with 75 μl of 0.7% low melting agarose (LMA), and added to the slides precoated with 0.5% agarose. The slide was then immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, and 1% sodium laurylasarcosine; 1% Triton X-100 and 10% DMSO) for 1 h at 4°C. The slides were next placed into an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2EDTA (pH 13.0) for 40 min. For electrophoresis of the DNA, an electric current of 25 V/300 ± 3 mA was applied for 20 min at 4°C. The slides were washed three times with a neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4°C, and then treated with ethanol for another 5 min before staining with 50 μl of ethidium bromide (20 μg/ml). Measurements were made by image analysis (Kinetic Imaging, Komet 4.0, Liverpool, U.K) and fluorescence microscope (LEICA DMLB, Wetzlar, Germany), determining the tail length (TL; 50 cells from each of two replicate slides).

Table 1. General characteristics of the subjects

Variables	Control ($n = 49$)	CAD ($n = 42$)	p value[1)]
Male/Female	33/16	27/15	NS[§]
Age (years)	62.0 ± 2.2[2)]	61.4 ± 1.7	NS[3)]
Height (cm)	162.3 ± 1.8	159.8 ± 1.5	NS
Weight (kg)	61.8 ± 1.7	62.9 ± 1.9	NS
BMI (kg/m^2)	23.4 ± 0.5	24.6 ± 0.6	NS
WHR	0.90 ± 0.01	0.91 ± 0.01	NS
Smoking habits			
Smoker (n (%))	25 (51.0%)	24 (57.1%)	NS[§]
Pack-years[4)]	8.8 ± 1.7	13.0 ± 2.3	NS
Drinking habits			
Drinker (n(%))	27 (55.1 %)	17 (40.5 %)	NS[§]
Alcohol consumption, (g/week)	89.2 ± 23.5	167.7 ± 51.5	NS

[1)] p values by Student's t test. [2)] Values are Mean ± S.E. [3)] Not significant. [4)] Pack years = (Cigarettes smoked/day × Years smoked)/20. [§] p values by χ^2-test. BMI (Body mass index), WHR (waist hip ratio).

Table 2. Plasma lipid profiles in the subjects

Variables	Control ($n = 49$)	CAD ($n = 42$)	p value[1)]
TC (mg/dl)	161.9 ± 4.4[2)]	165.4 ± 5.8	NS[3)]
LDL-c (mg/dl)	96.6 ± 4.7	106.0 ± 5.2	NS
HDL-c (mg/dl)	35.2 ± 1.3	29.2 ± 1.3	0.002
TG (mg/dl)	141.2 ± 12.6	150.8 ± 11.0	NS
AI	3.9 ± 0.2	5.1 ± 0.4	0.004

[1)] p values by Student's t test. [2)] Values are Mean ± S.E. [3)] Not significant. TC (Total Cholesterol), LDL-c (Low density lipoprotein cholesterol), HDL-c (High density lipoprotein cholesterol), TG (Triglyceride), AI (Atherogenic Index).

Statistical analysis

Data were analyzed using SPSS for Windows (Version 10). Values were expressed as mean ± standard error (SE) and differences were considered significant at $p<0.05$. Statistical comparisons were analyzed by using Student's t test and Pearson's correlation coefficient.

Results

Twenty-seven men and 15 women in CAD patients (mean age 61.4 ± 1.7 years) and 33 men and 16 women (mean age 62.0 ± 2.2 years) in normal controls participated in this study. The basic characteristics of the subjects are summarized in Table 1. No significant differences were found in age, anthropometric characteristics, smoking and drinking habit between control and CAD patients. The number of CAD patients were composed of one vessel disease ($n = 26$, 62%), two vessel disease ($n = 14$, 33%) and three vessel disease ($n = 2$, 5%) (data not shown).

Serum HDL cholesterol level in CAD patients was significantly lower than in the control group, while the atherogenic index was significantly higher in the patient group (Table 2).

The H_2O_2 stimulated DNA damage in lymphocytes was expressed as TL and is presented in Fig. 1.

The patients showed significantly higher TL (87.3 ± 1.6 μm) compared to the control (79.3 ± 1.7 μm, $p<0.01$). Plasma TRAP was significantly lower in patients than those of the control group (Fig. 2).

In addition, plasma vitamin C, γ-tocopherol, and α-carotene levels in patients group were lower than those of control groups, while erythrocytic catalase activity was increased in patients group (Table 3).

The relationship between DNA damage and antioxidant parameters is shown in Table 4 and Fig. 3. A significant negative correlation was found between TL and plasma levels of γ-tocopherol ($r = -0.257$, $p = 0.018$), α-carotene ($r = -0.217$, $p = 0.048$), β-carotene ($r = -0.273$, $p = 0.012$), and TRAP ($r = -0.27$, $p = 0.011$). There was a positive correlation between erythrocytic GSH-Px activity and TL ($r = 0.276$, $p = 0.01$).

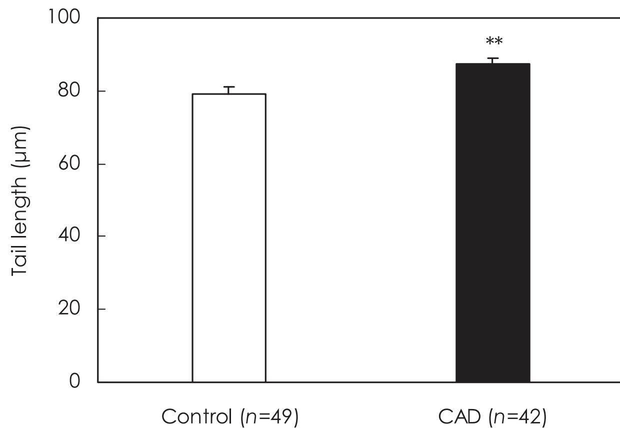

Fig. 1. DNA damage expressed as tail length in controls and CAD patients. Values are mean ± SE. ** significantly different at $p<0.01$ by Student's t test.

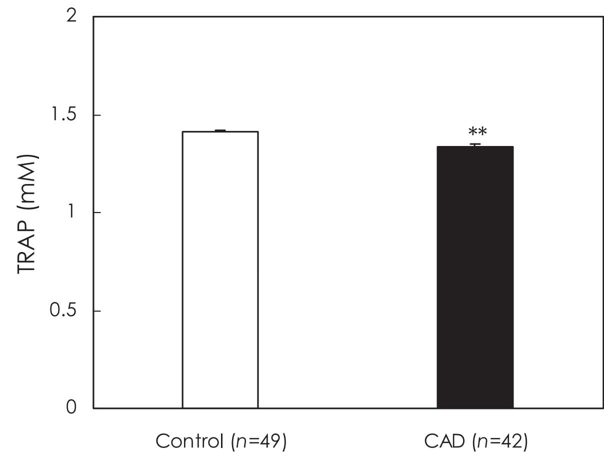

Fig. 2. Level of TRAP in controls and CAD patients. Values are means ± SE. ** significantly different at $p<0.01$ by Student's t test.

Table 3. Plasma antioxidant vitamin levels and Erythrocyte antioxidant enzyme activities in the subjects

Variables	Control (n = 49)	CAD (n = 42)	p value[1)]
Plasma			
Vitamin C (mg/dl)	1.38 ± 0.1[2)]	0.92 ± 0.1	0.0001
α-Tocopherol/TG (μg/mg)	19.4 ± 1.2	21.4 ± 1.6	NS[3)]
γ-Tocopherol/TG (μg/mg)	1.9 ± 0.1	1.1 ± 0.1	0.0001
α-Carotene/TG (μg/mg)	0.05 ± 0.01	0.04 ± 0.00	0.049
β-Carotene/TG (μg/mg)	0.27 ± 0.03	0.23 ± 0.03	NS
Lycopene/TG (μg/mg)	0.08 ± 0.01	0.08 ± 0.01	NS
Erythrocytes			
GSH-px (U/g Hb)	19.1 ± 0.6	19.6 ± 0.9	NS
SOD (U/g Hb)	2090.5 ± 29.4	2037.7 ± 28.9	NS
Catalase (K/g Hb)	37.0 ± 0.9	40.4 ± 1.4	0.044

[1)] p values by Student's t test. [2)] Values are Mean ± SE. [3)] Not significant. TG (triglyceride), GSH-px (Glutathione peroxidase), SOD (Superoxide dismutase).

Discussion

A major development in cardiovascular disease research is the finding that oxidation reaction plays a pivotal role in atherogenesis and many epidemiological studies have found that cardiovascular disease is associated with low levels of vitamin C, tocopherol, and carotenoids [*21*, *22*]. In agreement with those findings, the result of the present study clearly demonstrated that plasma concentrations of vitamin C, lipid standardised γ-tocopherol and α-carotene were lower in patients with CAD than those in healthy controls. Moreover, plasma TRAP was significantly reduced in patients as compared with healthy subjects, that is in accordance with earlier studies of Nojiri *et al.* [*23*] and Demirbag *et al.* [*9*]. We have also found that patients with CAD had significantly higher level of hydrogen peroxide induced DNA damage in peripheral lymphocytes, as measured by comet assay. The susceptibility of DNA to oxidation was negatively correlated with plasma γ-tocopherol, α-carotene, and β-carotene. A negative correlation of DNA damage in peripheral lymphocytes with plasma level of antioxidant vitamins might suggest that the higher the level of these micronutrients in plasma, the lower the level of DNA damage. This result is in agreement with the report of Staruchova *et al.* who demonstrated negative correlation between oxidized pyrimidines in lymphocytes DNA and plasma level of β-carotene and xanthophylls [*24*]. Demirbag *et al.* reported that the level of DNA damage in lymphocytes is increased and TAC level is decreased in CAD and DNA damage is negatively correlated with

Table 4. Pearson's correlation coefficients between DNA damage[1)] and antioxidant parameters in the subjects

Variables	$r^{2)}$	p value[3)]
Vitamin C	−0.199[2)]	0.061
α-Tocopherol/TG	0.157	0.154
γ-Tocopherol/TG	−0.257	0.018
α-carotene/TG	−0.217	0.048
β-carotene/TG	−0.273	0.012
lycopene/TG	−0.212	0.053
GSH-Px	0.276	0.01
SOD	−0.18	0.093
Catalase	0.197	0.061

[1)] Expressed as Tail length. [2)] Pearson's correlation coefficients. [3)] p values by Pearson's correlation test. TG (triglyceride), GSH-px (Glutathione peroxidase), SOD (Superoxide dismutase).

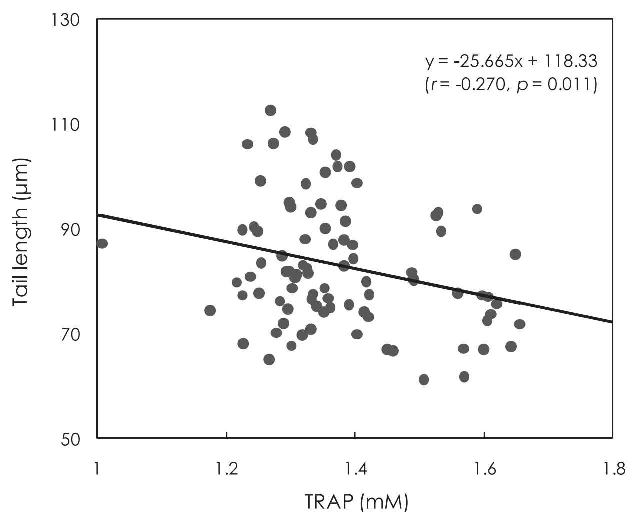

Fig. 3. Pearson's correlation coefficients between TL and TRAP in controls and patients.

levels of TAC [*9*]. It has been reported that increased level of endogenous DNA damage or oxidized purines and pyrimidines in lymphocyte DNA in patients with CAD mostly in western population [*6–9*]. The significance of our study is to suggest the possible fundamental role of DNA damage in the pathogenesis of this disease among Korean population, located in northeastern Asia where the race, dietary pattern and environmental factor are different from the western population. It is well-known that DNA damage frequently occurs in cells exposed to ROS [*6*, *25*], and over production of ROS plays an important role in the pathogenesis of atherosclerosis [*26*]. Recent studies suggest that the presence of somatic DNA instability in cells and tissue collected from patients with ischemic heart disease, suggesting that alterations at the DNA level may be involved in the development and the progression of the disease [*6*, *27*]. Free radical-scavenging enzymes such as SOD, catalase, and GSH-Px are the first line of cellular defense against oxidative injury, decomposing $O_2^{\bullet-}$ and H_2O_2 before interacting to form the more reactive hydroxyl radical (·OH) [*28*]. Some investigators have found reduced activities of the antioxidant enzymes in CAD patients [*29–31*], although conflicting results are available [*32*, *33*]. In this study, we observed increased erythrocytic catalase activity in patients with CAD and a positive correlation of DNA damage with erythrocytic GSH-Px activity. Bor *et al.* [*32*] and Zachara *et al.* [*33*] reported increased activity of erythrocytic GSH-Px in patients with acute myocardial infarction (AMI) and they interpreted this as an antioxidant defense against the chronic oxidant stress present before AMI, presumably due to the process of coronary atherosclerosis. A positive correlation of oxidized pyrimidines in lymphocytes DNA with GSH-Px was also reported by Staruchova *et al.*, although more data are needed to provide sufficient explanation [*24*].

In conclusion, the results of our study confirm that plasma vitamin C, γ-tocopherol and α-carotene, and plasma TRAP is decreased and H_2O_2 induced DNA damage is increased in CAD patients compared with normal controls. In addition, decrease in plasma antioxidant vitamins and total antioxidant potential seems to be responsible for the higher susceptibility of lymphocytic DNA to oxidation in CAD patients.

Acknowledgment

This research was supported by grants from the Korea Science (R04-2000-00066) and Engineering Foundation and Hannam University in 2009.

Abbreviations

KMIC, Korean Medical Insurance Corporation; TL, tail length; TRAP, total radical-trapping antioxidant potential; NO, nitric oxide; ROS, reactive oxygen species; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; CAD, Coronary artery disease; BMI, body mass index; WHR, waist hip ration; LMA, low melting agarose; TAC, total antioxidant capacity; AMI, acute myocardial infarction.

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