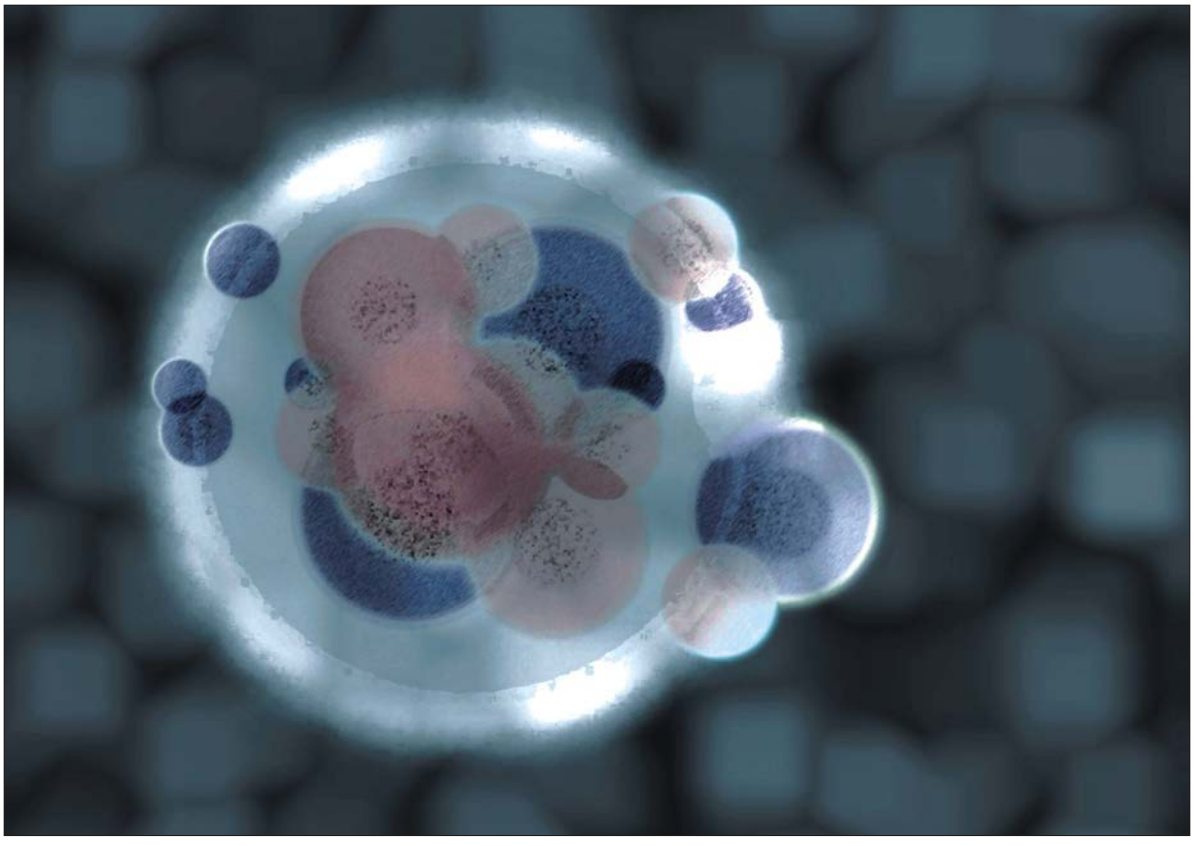

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Serum Angiotensin Converting Enzyme Activity, Total Antioxidants and Ascorbic Acid in Iranian Patients with Coronary Artery Disease

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Abstract: Angiotensin-Converting Enzyme (ACE) is a dipeptidyl carboxypeptidase (EC: 3.4.15.1) that catalyzes the conversion of angiotensin I to the potent vasoconstrictor angiotensin II. Angiotesin II is responsible for an increase in blood pressure and maintenance of hypertension through the stimulation of oxidative stress. The relationship between Coronary Artery Disease (CAD), Angiotensin-Converting Enzyme (ACE) activity, ascorbic acid and serum antioxidant status in patients with coronary artery disease. A group of 65 patients with angiographically defined Coronary Artery Disease (CAD) and 60 normal control subjects were examined. The activity of Angiotensin-Converting Enzyme (ACE) was determined by the reversed-phase High Performance Liquid Chromatography (HPLC) to separate and quantify Hippuryl-Histidyl-Leucin (HHL) and Hippuric Acid (HA). Ferric Reducing Ability of Plasma (FRAP Assay) as a measure of antioxidant power was used. Serum ascorbic acid concentration was determined photometrically. The results demonstrated significant differences in ACE activity, antioxidant and ascorbic acid between CAD cases and normal controls. Increased levels of ACE activity in serum have been related to coronary artery disease. Serum ascorbic acid concentration (25.6±3.8 mg dL^{-1}) and total antioxidant capacity (475.5±18.51 μM L^{-1}) were significantly ($p<0.05$) decreased in CAD patients compared with controls.

Key words: ACE, antioxidants, CAD, FRAP, oxidative stress

INTRODUCTION

Coronary Artery Disease (CAD), known as atherosclerotic heart disease, is a leading cause of morbidity and mortality throughout the world (Dubbert *et al*., 2002; Grech, 2003). Hypertension is the most common cardiovascular disorder with unknown mechanism which has a major contribution in coronary artery disease, heart failure, renal insufficiency and stroke. The renin-angiotensin system plays a key role in regulation of blood pressure, fluid and electrolyte balance in mammals (Unger, 2002). Renin-Angiotensin System (RAS) comprises a cascade of enzymatic reactions resulting in the formation of angiotensin II from the substrate angiotensinogen (Sturrock *et al*., 2004). Angiotensin-converting enzyme (EC3.4.15.1, a dipeptidyl peptidase) is an important enzyme of renin-angiotensin system. It belongs to the M2 family of zinc metallopeptidase and catalyses the hydrolysis of dipeptides from the carboxyl terminus of a variety of oligopeptides. One of its key actions is the regulation of blood pressure together with water and salt metabolism, since it cleaves angiotensin I into the potent vasopressor angiotensin II by removal of the C-terminal His-Leu. Angiotensin-converting enzyme also inactivates the vasodilator peptides bradykinin and kallidin (Turner and Hooper, 2002; Sentandreu and Toldra, 2005; Diet *et al*., 1996). Angiotensin II induced hypertension has been shown to alter the redox status of endothelial cells, resulting in increased concentration of oxygen free radicals. These reactive oxygen species inactivates endothelium derived nitric oxide and have pleiotropic effects on the vasculature, which may contribute to the initiation, maintenance and destabilization of atherosclerotic lesions (Dexler, 1999). Oxidative stress has been implicated as an important etiologic factor in atherosclerosis and vascular dysfunction. Antioxidants are one element of a collection of process that retards *in vivo* free radical oxidation. The serum contains many different antioxidants that may be important for general health maintenance. These include ascorbic acid, α-tocopherol, β-carotene, uric acid, bilirubin and albumin. In addition, trace amounts of antioxidant enzymes such as glutathione peroxidase and super oxide dismutase are

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found in serum to a lesser extend (Wassmann *et al*., 2004; Chapple, 1997). Among the endogenous plasma antioxidants ascorbic acid is particularly important in inhibiting lipid peroxidation induced by many different types of oxidative stress. Ascorbic acid, an important aqueous antioxidant which is the first line of defense against free radicals of aqueous origin, may inhibit atherosclerosis and thereby prevent the clinical complications of the disease such as CAD (Frei, 1999; Thomas, 2000; Carr *et al*., 2000).

The aim of this study was to compare the serum total antioxidant capacity, ascorbic acid and ACE activity of coronary artery disease patients documented by coronary angiography and controls. We also evaluated the relationship between the total antioxidant status, ascorbic acid and ACE activity in patients with coronary artery disease.

MATERIALS AND METHODS

Subjects: In this study, 65 male patients had angiography proven CAD were compared with 60 male control subjects that were selected from healthy population with a normal medical history. Data collection was conducted in surgery section of Tehran Rajai Cardiovascular Center and Participants were asked to fast overnight before their blood samples. The CAD group were further divided into one, two or three involved coronary arteries that had >70% diameter narrowing. All subjects were examined by a cardiologist and information on medical histories, age, weight, height, BMI, systolic and diastolic pressure, cigarette smoking, habits and medications were obtained via questionnaire and patients medical records. Patients with renal, liver, thyroid, gout, diabetes, or malignant disease were excluded from the study. Informed consent was obtained from all subjects enrolled in the study.

Blood pressure, height and weight measurement: For each participant, blood pressure was measured by nurses on the right arm using a mercury sphygmomanometer. Body weight and height were measured by a standard protocol. Weight was recorded on a firm, level surface and height was measured at a 90° angle against a wall. Body Mass Index (BMI) was calculated as body weight (in kg) divided by height (in Meters) squared.

Samples: Venous blood samples were drawn between 8:00 and 10:00 am in the fasting state in non additive tubes placed on ice and protected from light. Blood samples were left to clot and rapidly separated by centrifugation (2000 rpm for 10 min). Samples were divided into 500 μL portions and kept at -20°C for determination of total antioxidant capacity and ACE activity at Biochemistry Laboratory, Department of Biochemistry, Iran University of Medical Sciences. Ascorbic acid concentration was determined immediately.

Laboratory assays: Total and HDL cholesterol and serum triglycerides were analyzed enzymatically (Allain *et al*., 1974). LDL-C was calculated by using Friedewald formula. Na^+ concentration was determined using flame photometry.

Serum ascorbic acid levels were detected using spectrophotometer (Burtis *et al*., 1999) (intra-assay CV 6%, inter-assay CV 8.3%). The antioxidant capacity (intra-assay CV 2.1%, inter-assay CV 5.6%) of each sample was estimated using FRAP assay described by Benzie and Strain (1996). In order to measure antioxidant capacity 30 μL of serum of each sample were added to 900 μL of FRAP reagent containing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 m $FeCl_3.6H_2O$ solution. The FRAP method uses the ability of antioxidants to reduce a Fe^{III}-TPTZ complex to its blue colored Fe^{II} form. The change in absorbance at 593 nm after a reaction time of 5 min is due to the combined activity of all the reacting antioxidants present in the sample.

The ACE (intra-assay CV 3%, inter-assay CV 6%) activity was determined by High-Performance Liquid Chromatography (HPLC) on a symmetry C_{18} reversed-phase column (46× 250 mm, 10 μm, Waters). Briefly, 40 μL of borate buffer (pH 8.4) containing 3.5 mM p-benzoyl-L-glycyl-L-leucine (Hip-His-Leu) as substrate was added to 10 μL of serum. The mixture was incubated at 37°C for 30 min with constant shaking. The reaction was stopped by addition of 150 μL methaphosphoric acid (3%) and then was centrifuged at 1500 g for 5 min. Twenty microliter of supernatant was injected into the column and the amount of Hippuric Acid (HA) liberated from the substrate were detected at 228 nm. The column was eluted (1 mL min^{-1}) with 1:1 methanol and 10 mM KH_2PO_4 pH adjusted to 2 using ortho-phosphoric acid. One unit of activity is defined as the amount of enzyme catalyzing the release of 1 μM of hippuric acid from Hip-His-Leu per minute at 37°C under standard assay conditions (Horiuchi *et al*., 1982; Schnaith *et al*., 1994; Wu *et al*., 2002).

Statistical analysis: All statistical analysis were performed using SPSS 13. Data were tested for normal distribution with the Kolmogorov-Smirnov test. Differences were compared using student-test and ANOVA for parameters which showed a normal distribution and Mann-Whitney test for parameters which did not show normal distribution. Relationships between

parameters were determined by Pearson's correlation coefficient. The values of ($p<0.05$) were taken as significant.

RESULTS AND DISCUSSION

The study subjects included 60 controls and 65 CAD patients. There were no significant differences in age, BMI and blood pressure between the groups. The groups were generally well matched with respect to age and sex. The clinical characteristic of the groups are summarized in Table 1. Total serum Cholesterol, LDL-C and HDL-C concentrations did not differ significantly between patients and controls (Table 2).

Comparison between patients and controls showed that CAD was associated with significantly increased serum ACE activity and decreased serum total antioxidants capacity (Table 3). In the current study, serum ascorbic acid concentration in patients with coronary disease (25.6±3.8) was lower than those in controls (35.3±2.1) and the differences were significant. As expected a significant correlation between ascorbic acid and antioxidant capacity (r-0.365 and p value of 0.005) was observed among controls (Fig. 1) but was not seen in patients. There were no significant correlations in blood pressure and serum ACE activity. Serum Na^+ concentrations did not differ significantly between patients and controls (Table 3).

This study was carried out to find out whether there is a relation between ACE activity and antioxidant capacity in patients with CAD. Our interest in ACE was stimulated by the very wide clinical application of its inhibitors in high blood pressure and in some diseases of the heart and kidney. The relation among angiotensin II, hypertension, elevated levels of superoxide anions and endothelial dysfunction has been explored by Rajagopalan *et al*. (1996). The objective of this study was to compare ACE activity, antioxidant status and serum ascorbic acid concentration in controls and patients with CAD. There was no statistically significant relationship between ACE activity and blood pressure. However, the role of ACE in blood pressure control remains an important unresolved question in hypertension study.

The results of this study demonstrate increased ACE activity in patients with coronary artery disease. Several studies indicate that ACE-activity is related to atherosclerosis (Stamloae *et al*., 2003).

The association between ACE activity and ACE gene polymorphism and found a relation. In another study increased ACE expression was found in human atherosclerotic coronary artery disease (Martinez *et al*., 2000). We assume that the increased ACE activity

Table 1: Characteristics of control subjects and patients

Characteristics	Control subjects N = 60 (Male)	PatientswithCAD N = 65 (Male)
Age (year)	53.09±9.5	51.54±10.4
BMI (kg M^{-2})	26.70±5.3	27.08±4.6
Diastolic Bp (mmHg)	73.20±5.6	75.00±6.5
Systolic Bp (mmHg)	112.50±14.6	116.00±11.5
CAD*(%)		70.00

*CAD: Coronary artery disease

Table 2: Lipid profile of the study groups

Parameters (mg dL^{-1})	Controls	Patients	p-value
Cholesterol	176.43±44.4	1.87±38.43	NS
Triglyceride	141.53±61.3	179.85±145.5	NS
HDL-C[1]	42.10±22.9	38.15±7.5	NS
LDL-C[2]	96.90±33.56	97.90±24.2	NS

NS: Not significant; [1]High density lipoprotein cholesterol; [2]Low density lipoprotein cholesterol

Table 3: Serum ACE activity, antioxidant and ascorbic acid in CAD patients and controls

Parameters	Controls	Patient	p-value
ACE activity (μmol min L^{-1})	60.7±21.6	71.4±27.2	<0.05
Total antioxidant (μM L^{-1})	540.5±40.4	475.5±18.51	<0.05
Ascorbic acid (mg dL^{-1})	35.3±2.10	25.6±3.80	<0.05
Na^+ (mmol L^{-1})	138.8±2.80	140.4±3.30	NS

NS: Not significant

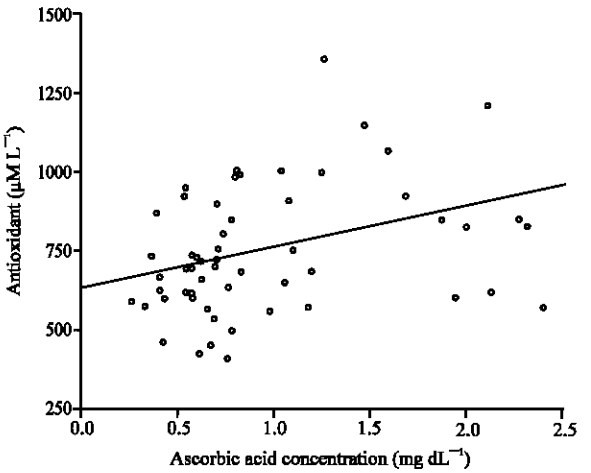

Fig. 1: Scatter plot of antioxidant capacity and ascorbic acid concentration. There is a significant linear correlation between serum antioxidant capacity and ascorbic acid concentration in control group

observed may be a consequence of ACE expression or ACE polymorphism (Martinez *et al*., 2000; Forrester *et al*., 1997; Diet *et al*., 1996). Increased ACE activity will increase AngII. Angiotensin II may play a central role in initiating free radical production, although the mechanism is not fully understood. It is believed that specific binding of Ang II to AT1 receptor subtype is a possible initiating process (Rajagopalan *et al*., 1996; Mulrow, 1999; Griendling and Ushio-Fukai, 2000; Hanna *et al*., 2002). Inactivation and removal of ROS depend on reactions

involving the antioxidative defense system. This may overload antioxidant defense system and result in oxidative stress. It appears that oxidant stress both promotes and is induced by diseases such as hypertension and atherosclerosis. Consistent with our data, a number of studies demonstrated a reduction in total antioxidant status (Serder *et al*., 2006). One of the major metabolic roles of ascorbic acid is its participation as antioxidant agent and free radical scavenger in numerous cellular oxidation processes (Padayatty *et al*., 2003). Results of the present study also displayed a significant correlation between ascorbic acid and antioxidant capacity in control group. Osganion *et al.* (2003), demonstrated an inverse association between intake of ascorbic acid and CAD. Plasma ascorbic acid levels have been inversely correlated with CAD mortality (Diaz *et al*., 1997; Maxwell and Lip, 1997). Present results showed that patients with CAD had lower levels of ascorbic acid than the control group. The reduction in ascorbic acid (antioxidant agent) concentration could be the result of higher ACE activity in patients.

CONCLUSION

The present study indicates that, in patients with CAD, there is significant reduction in both the antioxidant capacity and ascorbic acid with a concomitant increase in the ACE activity. These results suggest, that ACE activity may be associated with oxidative stress in patients with coronary artery disease. As oxidative stress increases, cardiovascular disease develops because the antioxidant defense systems are over loaded.

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