Endothelin-1 and Nitric Oxide Levels in Patients With Mitral Annulus Calcification

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SUMMARY

Mitral annulus calcification (MAC) is a chronic degenerative noninflammatory process. The goal of this study was to determine endothelin-1 (ET-1) and nitric oxide (NOx) levels in patients with MAC and compare them with those in normal subjects. The study group included 39 patients [26 females (66%), age, 63 ± 8 years] with MAC and 20 [11 females (55%), age, 61 ± 7 years] healthy subjects. The patients were divided into two subgroups, group A with severe MAC and group B with mild MAC, according to the severity of the MAC. Plasma ET-1 levels were higher and NOx levels were lower in patients than controls [(6.5 ± 5.6 pg/mL vs 3.7 ± 2.9 pg/mL for ET-1 and 35.0 ± 10.6 µmol/L vs 42.3 ± 9.9 µmol/L for NOx; P < 0.05 for both)]. In the subgroups, ET-1 levels were higher in group A than group B (8.65 ± 6.84 pg/mL vs 4.74 ± 3.45 pg/mL, P < 0.05) and the control group (8.65 ± 6.84 pg/mL vs 3.70 ± 2.88 pg/mL, P < 0.05). There was no difference between group B and the control group. Plasma NOx levels were significantly decreased in group A compared to controls (32.22 ± 11.88 µmol/L vs 42.25 ± 9.99 µmol/L, P < 0.05). However, no significant difference was observed between group B (37.38 ± 9.06 µmol/L) and the other groups. Diabetes mellitus, coronary artery disease, and dyslipidemia were significantly associated with ET-1 levels. However, this association was not observed for NOx. In conclusion, patients with MAC have increased ET-1 and decreased NOx levels. This seems to be more prominent in patients with severe MAC. (Jpn Heart J 2004; 45: 487-495)

Key words: Mitral annulus calcification, Nitric oxide, Endothelin-1

MITRAL annulus calcification (MAC) is a chronic degenerative noninflammatory process in the fibrous base of the mitral valve.1-3 On transthoracic echocardiography (TTE), which is the best method to demonstrate MAC, it appears as opacity in various shapes and thicknesses.4 The incidence of MAC is greater with advanced age, and is more common in females.1,2,5,6
Previous pathological studies have claimed that MAC in the elderly is a form of atherosclerosis and suggested that coronary atherosclerosis, MAC, and aortic valve calcification in the elderly have a similar etiology. It was previously demonstrated that there was a highly significant association between the presence of MAC and aortic atheroma.7-9) Demopoulos, et al also found a very strong association between the presence of aortic atheroma and carotid artery atherosclerotic disease.10)

The endothelium acts as a dynamic organ and secretes some substances in response to various physical and humoral stimulants. These biologically active substances are vasoconstrictors and vasodilators, which control these processes. Several endothelium-dependent agonists have been identified, each of which acts through a membrane receptor. Today, endothelin-1 (ET-1) is the strongest known vasoconstrictor peptide and has significant effects on the cardiovascular system. It increases coronary vascular resistance, has positive inotropic effects on cardiomyocytes and is mitogenic for smooth muscle cells.11,12) Because of these properties, it contributes to the progression of atherosclerosis, and is an important component in the pathogenesis of heart failure and hypertension.13) Previous studies also showed a significant increase in ET-1 levels after tachycardia and during PTCA in ischemic heart disease patients.14,15) Nitric oxide (NOx) is also an endothelium-derived key molecule in normal autoregulatory mechanisms like the vasodilatation response to tachycardia and exercise.16) It plays a role in cell proliferation, and modulates myocardial functions and metabolism.17)

The aim of this study was to assess the levels of ET-1 and NOx in patients with MAC and their differences in relation to normal subjects because, to the best of our knowledge, no previous study has evaluated the levels of these endothelium-derived molecules in this type of patient.

**METHODS**

**Study population:** The study group included 39 patients [26 females (66%), age, 63 ± 8 years] with MAC and 20 [11 females (55%), age, 61 ± 7 years] healthy subjects as the control group. Patients who had cardiac diseases other than coronary artery disease, renal failure (patients who have creatinine ≥ 1.4 mg/dL or creatinine clearance < 90 mL/min), or hepatic failure were excluded. All patients and controls were analysed for atherosclerosis risk factors. The echocardiographic examinations and ET-1 and NOx levels of all patients and controls were evaluated. Due to the severity of the MAC, the values of the subgroups were also evaluated (group A, severe MAC; group B, mild MAC).

**Echocardiography:** Two-dimensional and Doppler color flow examinations were performed in all patients and controls using a Vingmed System Five
echocardiography system (GE Vingmed Ultrasound A/S in Horten, Norway) and a 2.5 MHz electronic probe by an experienced cardiologist. The 2-dimensional echocardiographic criteria for MAC included an intense echo-producing structure located at the junction of the atrioventricular groove and posterior mitral valve leaflet on the parasternal long axis and apical 4-chamber views or an intense echo-dense structure located posterior to the posterior mitral valve leaflet on the parasternal short-axis view. MAC was considered severe when the thickness of the intense echo-producing structure was ≥ 5 mm measured by 2-dimensional echocardiography in the 4-chamber view.3)

Analysis of endothelin-1: Plasma samples were drawn into chilled EDTA tubes (1 mg/mL blood) containing aprotinin (500 KIU/mL of blood). The whole blood samples were centrifuged at 1600 x g for 15 minutes at 0°C. The plasma fractions were transferred to a plastic tube and stored at -70°C for long-term storage. After a short incubation the excess sample was washed out and a polyclonal antibody to endothelin-1 labeled with the enzyme horseradish peroxidase was added. This labeled antibody bound to the endothelin-1 captured on the plate. After a short incubation the excess labeled antibody was washed out and substrate was added. The substrate reacted with the labeled antibody bound to the endothelin-1 captured on the plate. The color generated with the substrate was read at 450 nm, and was directly proportional to the concentration of endothelin-1 in the sample (Human Endothelin-1, catalog no EIA-3111, DRG International Inc., USA).

Analysis of serum nitrite and nitrate: The levels of nitrite and nitrate were determined using a procedure based on the Griess reaction. Blood samples were centrifuged at 4000 rpm for 10 minutes. Serum samples were then separated and stored at -70°C until used for assay. Equal volumes of serum and potassium phosphate buffer were placed in an ultrafilter and centrifuged at 4000 rpm for 45 minutes. The ultrafiltrate was collected and used in the test. Nitrates were quantitatively converted to nitrites for analysis. Enzymatic reduction of nitrate to nitrite was carried out using coenzymes (NADPH, FAD) in the presence of nitrate reductase in the incubation assay step. N-1-(naphthyl) ethylenediamine dihydrochloride, sulfanilamide and incubation solutions were mixed at a ratio of 1:1:2 (v/v). These mixtures were incubated for 5 minutes at room temperature and measured at 540 nm. Sodium nitrite (1.00 mM) was used as the standard for determination of nitrite and 80 mM potassium nitrate was used as the standard for determination of nitrate (Nitric oxide colorimetric assay, 1-756-281, Roche Diagnostics GmbH, Mannheim, Germany).

Statistical analysis: Statistical analysis was performed using an SPSS software package (version 10.0, SPSS Inc, Chicago, Illinois, USA). Categoric variables are expressed as counts and percentages and continuous variables as the mean ± SD. Comparisons of categoric variables were performed using the Pearson chi-
square test or Fisher's exact test. Continuous variables are presented as the mean ± SD. ET-1, NOx, and other continuous variables in the patient and control groups were compared with a one-way ANOVA test. The post-hoc analysis was performed using Bonferroni's test. Univariate analysis of variance was used to evaluate the association among ET-1, NOx, and coronary artery disease risk factors. All hypothesis testing was 2-tailed. P values < 0.05 were considered to be significant.

RESULTS

The age, gender, hypertension, diabetes mellitus, smoking, and family history for coronary artery disease parameters were similar between the patient and control groups. Coronary artery disease and dyslipidemia were more frequent in the patient group. The clinical characteristics of the patients and controls are summarized in Table I.

Patients were divided into two groups, a severe MAC group who had ≥ 5 mm calcification (group A; n = 18, 65 ± 8 years) and a mild MAC group (group B, n = 21, 62 ± 7 years). Group A patients had higher incidences of more frequent diabetes mellitus, hypertension, and documented coronary artery disease than group B patients (P < 0.05 for all). The clinical characteristics of group A and group B patients are summarized in Table II.

Plasma ET-1 levels were higher and NOx levels were lower in patients than in controls [(6.5 ± 5.6 pg/mL vs 3.7 ± 2.9 pg/mL for ET-1 and 35.0 ± 10.6 µmol/L vs 42.3 ± 9.9 µmol/L for NOx; P < 0.05 for both)]. When we assessed these parameters in the subgroups; ET-1 levels were higher in group A than group B (8.65 ± 6.84 pg/mL vs 4.74 ± 3.45 pg/mL, P < 0.05) and the control group (8.65 ± 6.84 pg/mL vs 3.70 ± 2.88 pg/mL, P < 0.05). There was no statistically significant difference between group B and the control group (Figure 1).

<table>
<thead>
<tr>
<th>Table I. Clinical Characteristics of the Patient and Control Groups</th>
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<tr>
<td>MAC (n = 39)</td>
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<tr>
<td>Age (years)</td>
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<tr>
<td>Female, n (%)</td>
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<tr>
<td>Diabetes mellitus, n (%)</td>
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<td>Hypertension, n (%)</td>
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<td>Dyslipidemia, n (%)</td>
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<td>CAD* n (%)</td>
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<td>Family history of CAD, n (%)</td>
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*CAD = Coronary artery disease.
Plasma NOx levels were significantly decreased in group A compared to controls (32.22 ± 11.88 μmol/L vs 42.25 ± 9.99 μmol/L, $P < 0.05$). However, no significant difference was observed between group B (37.38 ± 9.06 μmol/L) and the other groups (Figure 2).

Univariate analysis of variance was used to determine the associations between coronary artery disease risk factors and ET-1 and NOx in the patient and control groups and revealed that diabetes mellitus ($P < 0.002$, observed power = 0.908), coronary artery disease ($P < 0.023$, observed power = 0.635) and dyslipidemia ($P < 0.001$, observed power = 0.918) were significantly associated with ET-1 levels. However, this association was not observed for NOx levels.
Most of the patients had (only 4 did not) aortic calcification, but there was no relationship between the degree of MAC and aortic calcification.

Groups A and B and the controls did not differ with respect to creatinine levels ($1.03 \pm 0.26$, $1.05 \pm 0.15$, and $0.97 \pm 0.19$, respectively, $P = 0.45$) or creatinine clearance ($96.81 \pm 4.64$, $94.9 \pm 4.14$, and $93.85 \pm 4.60$, respectively, $P = 0.75$). Creatinine levels were not associated with ET-1 ($r = -0.008$, $P = 0.95$) and NOx ($r = -0.06$, $P = 0.96$) levels. Creatinine clearance values were also not associated with ET-1 ($r = -0.066$, $P = 0.62$) and NOx ($r = 0.033$, $P = 0.80$) levels.

**DISCUSSION**

The results of this study demonstrate that plasma ET-1 levels were higher and NOx levels were lower in patients with MAC (especially when severe) than in the age- and sex-matched group of control subjects. Furthermore, patients who had severe MAC had higher ET-1 levels than patients with mild MAC.

MAC is a chronic degenerative process. In most patients it is asymptomatic and does not affect mitral valve function. However, when severe, it can contribute to mitral regurgitation and/or stenosis. In our study, none of the patients had mitral regurgitation or stenosis severe enough to affect cardiac function or warrant exclusion from the study.

ET-1 is the strongest known vasoconstrictor peptide and has significant effects on the cardiovascular system. It increases coronary vascular resistance and has positive inotropic effects on cardiomyocytes and is mitogenic towards

![Figure 2. Nitric oxide levels in study groups. (Group A, patients with severe MAC; Group B, patients with mild MAC).](image)
smooth muscle cells.\textsuperscript{11,12} Because of these properties, it contributes to the progression of atherosclerosis, and is an important component in the pathogenesis of heart failure and hypertension.\textsuperscript{13} NOx is also an endothelium-derived molecule in normal autoregulatory mechanisms like the vasodilatory response to tachycardia and exercise.\textsuperscript{16} It plays various roles in the cardiovascular system and systemic metabolism.\textsuperscript{17} Endothelial dysfunction is associated with the loss of NO bioavailability due to either reduced formation or accelerated degradation of NO.\textsuperscript{19} Increased plasma levels of ET-1 and decreased levels of NOx are associated with endothelial function impairment in pathological conditions.\textsuperscript{14-16,19-21}

Previous pathophysiological studies have reported the presence of foam cells, which are the early signs of atherosclerosis, in the endothelium of epicardial coronary arteries, on the ventricular face of the posterior mitral valve, and on the aortic faces of the aortic valves, in patients aged between 13-39.\textsuperscript{6,7} This would suggest similar etiologies for coronary atherosclerosis, MAC, and degenerative aortic calcification in the elderly. The incidence of coronary artery disease, new coronary events, carotid artery disease, cerebrovascular accidents, and peripheral vascular disease have also been found to be more frequent in patients with MAC.\textsuperscript{22-28} Additionally, it was suggested that MAC and aortic atherosclerotic plaques are associated with increased cardiovascular mortality.\textsuperscript{20} In support of these theories, some studies have suggested a correlation between MAC and atherosclerotic risk factors such as hypertension, diabetes mellitus, and dyslipidemia.\textsuperscript{2,30-35} It is also known that all these diseases, including atherosclerosis and heart failure, are associated with endothelial dysfunction and abnormalities in the plasma levels of these substances.\textsuperscript{15,20,21,36,37} Therefore, it was not surprising for us to find increased ET-1 and decreased NOx levels in patients with MAC. In addition, in our study diabetes mellitus, coronary artery disease, and dyslipidemia were significantly associated with ET-1 levels. However, this association was not observed for NOx levels.

In the present study, the patients with MAC had higher ET-1 and lower NOx levels than controls. However, this difference primarily seems to have originated from the values of the patients with severe MAC (MAC \( \geq 5 \) mm of thickness) because when we assessed the subgroups, the patients with severe MAC had higher ET-1 levels than controls and patients with mild MAC, and lower NOx levels than controls. Furthermore, there were no statistically significant differences in the ET-1 and NOx levels between patients with mild MAC and controls. This suggests that the significant impairment in the levels of NOx and ET-1 (and probably endothelial dysfunction) is only seen in patients with severe MAC.

It has been reported there is a close correlation between endothelial function in the human coronary and peripheral circulations.\textsuperscript{38} It is also known that endothelial dysfunction has prognostic value for cardiovascular patients.\textsuperscript{39-41} Thus, it
is clear that more studies that assess directly the endothelial function in this group of patients are needed.

**Conclusions:** In conclusion, patients with MAC have increased ET-1 and decreased NOx plasma levels. This seems to be more prominent in patients with severe MAC. Further controlled studies using a larger patient population are needed to determine the association of the plasma levels of these substances with endothelial function and other risk factors in these patients and identify new therapeutic options.

**REFERENCES**