Antioxidant Status and Lipid Peroxidation End Products in Patients of Type 1 Diabetes Mellitus

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Background and Objective: *In Type 1 diabetes mellitus (DM), hyperglycemia is considered a primary cause of diabetic vascular complications and is associated with oxidative stress. The role of antioxidants, particularly α tocopherol, in Type 1 DM and its contribution in the development of vascular complications is not clear. Therefore, the present study aims to investigate the relationship between antioxidant status (α tocopherol) and lipid peroxidation end products (malondialdehyde; MDA) in the plasma of 20 Type 1 DM and 20 nondiabetic healthy control subjects.*

Material and Method: *Lipid levels in all subjects were analyzed spectrophotometrically by enzymatic reagent kits. Plasma MDA was assessed by spectrofluorometry, whereas plasma α tocopherol was estimated by high performance liquid chromatography in Type 1 DM as well as in the control subjects of matched sex and ages. The results of Type 1 DM were compared with a control group using unpaired Student's t-test. The correlations between fasting plasma glucose and other laboratory parameters were assessed by Pearson rank correlation coefficient.*

Results: *The plasma MDA concentration was significantly higher in Type 1 diabetic patients as compared to controls, (p < 0.01). A significantly reduced plasma antioxidant status of Type 1 DM patients was found only in α tocopherol / total lipid as compared to controls (p < 0.05). However, no significant difference was observed in plasma α tocopherol and α tocopherol / total cholesterol (p > 0.05) as compared to the control subjects. The positive correlation between MDA and FPG was demonstrated in Type 1 diabetic compared with normal subjects.*

Conclusion: *We conclude that antioxidant supplementation may be necessary for treatment to reduce oxidative stress for diabetic complication protection in Type 1 DM.*

Keywords: *Lipid peroxidation, Antioxidant, Atherosclerosis, Oxidative stress, Type 1 diabetes*

Type 1 diabetes mellitus (DM) usually begins in childhood with a lifelong tendency for hyperglycemia and a high risk of developing serious complications, including retinopathy, nephropathy, neuropathy, and accelerated coronary artery disease. Both diabetes and its associated complications have become public health problems of considerable magnitude. Oxidative stress in the individual with a genetic predisposition for Type 1 DM plays an important role in the pathogenesis of this disease. Hyperglycemia results in oxidative stress by generating free radicals and causes different lesions including DNA, lipids, proteins, and carbohydrate damage. Superoxide converted to hydrogen peroxide penetrates through cell membrane and can cause damage to beta cell[1].

Oxidative stress in diabetes leads to further unfavorable processes: increased production of cholesterol due to activation of hydroxymethylglutamyl CoA reductase, insufficient removal of acetyl CoA; higher flows of calcium ions into cells with activation of intracellular proteases due to inactivation of mem-

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brane ATPase; increased platelet aggregation; increased cell proliferation; increased lipid peroxide causing crosslink formation between single molecules of proteins; increased oxidation of LDL particles leading to early occurrence of atherosclerotic changes; and free radical overproduction causing consumption of antioxidants (vitamin C, E, intracellular glutathione, etc.). Free radicals participate both in the origin of Type 1 DM and in the development of its late complications.

A number of studies have evaluated the role of oxidative stress in Type 1 diabetes and its complications but with inconsistent results. Oxidative stress was implicated in the pathogenesis of DM, in particular lipid peroxidation (measured as levels of malondialdehyde or MDA). MDA is formed as an end product of lipid peroxidation. MDA level was found significantly higher in diabetic patients[2-6]. Traditionally, patients with Type 1 DM have been considered at risk for marginal or deficient in nutritional status of several micronutrients (vitamin A, E, C and carotenoids)[7,8]. In addition, a greater oxidative stress and lower concentrations of antioxidant have been reported in these patients, which may contribute to accelerated aging and atherosclerosis in diabetes and microangiopathic complications of the disease[9,10]. However, others have reported no change in indices of oxidative stress[11] and antioxidant status[7,12,13]. For this reason, the current study was undertaken to evaluate the levels of α tocopherol and lipid peroxidation in plasma of type 1 DM and then compared with non-diabetic controls.

Material and Method
1. Subjects

Twenty normal healthy subjects with fasting plasma glucose (FPG) < 110 mg/dl were a control group. The ages ranged from 18 to 48 years. Twenty Type 1 diabetic subjects whose FPG > 140 mg/dl were selected from medical outpatient department and diabetic clinics in Siriraj hospital. The ages ranged from 14 to 55 years. Patients with any renal dysfunction, (i.e. raised blood urea and serum creatinine levels), with coexistent illness (i.e. infections), congestive heart failure, acute myocardial infarction, proliferative retinopathy, with diabetic macroangiopathic complications (i.e. coronary artery disease, peripheral vascular disease and stroke: diagnosed by clinical history and examination) were excluded from the study. Control group and patients who supplemented with antioxidants were also excluded. All patients were on insulin treatment more than 6 months.

Informed consent was obtained from all participants according to the ethical guidelines of the Helsinki declaration. The work was carried out with the approval of the ethical clearance committee of the Faculty of Medicine Siriraj Hospital, Mahidol University.

2. MDA assay in plasma

MDA in plasma was performed as described by Satoh[14]. In brief, plasma was mixed with 20% TCA and allowed to stand for 10 min. Then, 0.05 M H_2SO_4 and TBA were added. The mixture was mixed and place in boiling water bath for 30 min. The resulting chromogen was extracted with n-butanol and centrifuged at 1871 x g for 10 min and measured against butanol blank at 532 nm excitation and 553 nm emission by spectrofluorometer.

3. Plasma α tocopherol assay

α tocopherol was assayed by HPLC[15]. In brief, plasma, αtocopheryl acetate as an internal standard and ethanol was mixed for 15 sec. Then hexane was added and mixed vigorously for 2 min. The tube was centrifuged at 5198 x g, 4ºC for 5 min. The hexane layer was transferred and evaporated under a stream of nitrogen gas. The lipid residue was dissolved in ethanol and injected into the Sphere clone 5 μ ODS, 250 x 4.60 mm. of HPLC. The mobile phase was methanol: acetonitrile: chloroform (25:60:15) at a flow rate 1.5 ml/min. α tocopherol in plasma and tocopheryl acetate were detected at 290 nm. Plasma α tocopherol was expressed as plasma αtocopherol concentration (μg/ml), α tocopherol: cholesterol ratio (mg/g) and α tocopherol: sum of total cholesterol and triglyceride (total lipid) concentration (mg/g).

4. Plasma glucose and lipid profile

Plasma glucose, total cholesterol, HDL-cholesterol, and triglycerides were measured using commercially available test.

5. Statistical analysis

Results are expressed as mean ± SEM. Different between the two groups were tested by unpaired Student's t test. The correlation of each parameter was performed by Pearson correlation, and $p < 0.05$ values were considered as significant.

Results
Characteristic of the patients with type 1 DM

Table 1 showed the data of age, gender, FPG,

Table 1. Database of age, gender, FPG, hematological data and lipid profiles in normal healthy subjects and type 1 diabetic patients (Values are mean $\pm$ SEM)

Patient characteristics	Normal subjects	Type 1 diabetes	p-value
Age (yrs)	30.45 ± 2.60	34.25 ± 3.40	0.381
Gender (M/F)	13/7	9/11	
FPG (mg/dl)	96.71 ± 3.52	210.58 ± 21.35	<0.0001
Hb (g/dl)	16.07 ± 0.45	13.80 ± 0.60	0.005
Hct (%)	45.79 ± 0.86	45.69 ± 1.30	0.952
Total cholesterol (mg/dl)	189.81 ± 9.61	214.48 ± 13.28	0.140
LDL cholesterol (mg/dl)	96.80 ± 8.51	131.81 ± 11.65	0.020
VLDL cholesterol (mg/dl)	21.15 ± 2.74	29.37 ± 4.63	0.136
Triglyceride (mg/dl)	105.73 ± 13.65	146.89 ± 23.14	0.140
HDL cholesterol (mg/dl)	71.86 ± 4.09	53.30 ± 3.57	0.002

Table 2. Plasma malondialdehyde (p-MDA) (μmol/L) in healthy normal subjects and type 1 diabetic patients (Values are mean $\pm$ SEM)

Subjects	p-MDA (mol/L)	p-value
Normal (n = 20)	2.20 ± 0.08	
Type 1 DM (n = 20)	3.00 ± 0.15	<0.001

Table 3. Plasma α tocopherol in healthy normal subjects and type 1 diabetic patients (Values are mean $\pm$ SEM)

Subjects	Plasma α tocopherol (μg/ml)	α tocopherol/total cholesterol (mg/g)	α tocopherol/total lipid (mg/g)
Normals (n = 20)	13.42 ± 1.26	6.93 ± 0.38	4.57 ± 0.29
Type 1 DM (n = 20)	12.57 ± 1.24	5.91 ± 0.45	3.63 ± 0.29
p-value	0.630	0.091	0.027

hematological data and lipid profile between normal healthy group and type 1 DM patients. This table showed that FPG in type 1 DM patients was significantly higher than that in normal group. The study of plasma lipid profile also showed that total cholesterol, VLDL-cholesterol and triglycerides in type 1 DM patients were not significantly different from the normal healthy group. This was due to the treatment of cholesterol-lowering drug in diabetic patients. However, the significant increase of LDL-cholesterol and significant decrease of HDL-cholesterol were also found in type 1 DM subjects. The increasing of LDL-choles-

terol and decreasing of HDL-cholesterol may exacerbate the cardiovascular complication in this group of diabetes. In addition, Hb concentration was also significantly lower in type 1 DM as compared to normal control subjects whereas hematocrit (Hct) was not different.

Plasma malondialdehyde level

Plasma malondialdehyde (p-MDA) level, the index of lipid peroxidation, in type 1 DM was significantly higher when compared to healthy normal group (p < 0.01) as indicated in Table 2. The increasing level

of p-MDA suggested the PUFA in phospholipids of lipoproteins, especially LDL, was mainly oxidized by ROS leading to the atherogenic formation in this diabetics.

Plasma α tocopherol level

The mean comparisons of these plasma α tocopherol in type 1 DM and normal healthy subjects were expressed as plasma α tocopherol concentration, α tocopherol/total cholesterol ratio, and α tocopherol/total lipid ratio as shown in Table 3. The result showed that plasma α tocopherol concentration (plasma vitamin E), and α tocopherol/total cholesterol ratio were not significantly different whereas α tocopherol/total lipid ratio showed significantly different ($p < 0.05$).

Discussion

Type 1 diabetic patients have been generally described as having high levels of oxidative stress[16]. Insulin therapy has also been shown to be effective in the improvement and long-term maintenance of nearly normal glycemic control, a key factor for reducing metabolic and clinical complications and reducing the risk for developing long-term micro-and macroangiopathic complication of the disease from oxidative stress[17,18]. Increased levels of MDA provide evidence for increased lipid peroxidtion and possibly increased tissue damage by free radicals. In the present study, we found significantly elevated plasma MDA in insulin-treated type 1 diabetic patients ($p < 0.05$), reflecting increased oxidative stress. This is in agreement with observations from other investigators, who reported that systemic oxidative stress was increased during insulin treatment in young diabetic patients[19]. In general, extracellular fluids contain several antioxidants that interfere with the oxidative process. One of the most important antioxidants is α tocopherol, which was measured in this study. Several studies have reported different effects of insulin treatment on serum levels of ·tocopherol in type 1 diabetes. With regard to α tocopherol plasma levels, both lower and equal plasma concentrations have been reported in Type 1 diabetic patients, a finding that has been related to the inhomogeneity of inclusion criteria, presence of hyperlipemic, or poor metabolic control of the disease[8]. Adjustment of α tocopherol plasma concentrations for cholesterol levels (α tocopherol: total cholesterol ratio) has been reported to reflect more reliably vitamin E nutritional status[20], and on correcting for lipid concentration, α tocopherol levels in diabetic patients are not different from those observed in normolipemic subjects[8,12,15,21].

In this regard, the lack of significant changes in relation to metabolic control of the disease may need a more reliable marker of vitamin E status (α tocopherol: total plasma lipid ratio) as suggested earlier[22,23].

Our results found that no significant differences were observed in plasma α tocopherol concentration and α tocopherol: total cholesterol ratio corresponding to the previously reported in insulin-treated type 1 DM[12]. When plasma α tocopherol was expressed as α tocopherol: total lipid ratio, it showed significantly lower. This implied that the measurement of antioxidants in terms of plasma α tocopherol concentration or α tocopherol: total cholesterol ratio may be less specific because it reflects indirectly to the whole organism to increase oxidative stress. In the other hands, we think that α tocopherol: total lipid ratio reflects oxidative stress better than plasma α tocopherol level or α tocopherol: total cholesterol ratio. α tocopherol: total lipid ratio could potentially provide a new tool in clinical practice because the decrease of this parameter can demonstrate an increase in oxidative stress in type 1 DM that was not related to blood glucose concentration ($r = -1.56$, $p = 0.38$), whereas the increase of plasma MDA level was related to the blood glucose concentration ($r = 0.57$, $p = 0.01$).

A test for these parameters, therefore, potentially identifies diabetic patients at increased risk for these complications who might benefit from intensive treatment with antioxidants and other prophylactic drugs.

These findings demonstrate that type 1 DM with advanced oxidative stress has lower α tocopherol: total lipid ratio and higher plasma MDA level. It correspond to the finding that low plasma vitamin E and high plasma MDA concentrations bring greater risk to the development or progression of atherosclerosis[24,25]. Supplementation by vitamin E has been shown to have no effect on oxidative damage[26] but improved endothelial function[27] in type 1 diabetic patients. The antioxidative effect of α tocopherol may be dose-dependent. Whilst a low dose has a protective effect, a high dose may worsen the endothelial vasodilator function[28]. However, vitamin E form only a part of the antioxidative mechanisms taking part in the protection of vascular changes.

In summary, our findings support that oxidative stress is increased in type 1 DM, as reflected by elevated MDA levels and decreased α tocopherol: total lipid ratio as compared with healthy persons. These parameters may merit further study as candidate biomarkers of antioxidant status for the risk of compli-

cations in diabetes mellitus.

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สภาวะแอนติออกซิแดนท์และผลผลิตไลปิดเปอร์ออกซิเดชั่นในพลาสม่าของผู้ป่วยเบาหวานประเภทที่ 1

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ภูมิหลังและวัตถุประสงค์: *น้ำตาลที่เพิ่มสูงขึ้นในเลือดของผู้ป่วยเบาหวานประเภทที่ 1 เป็นสาเหตุสำคัญในการทำให้เกิดโรคแทรกซ้อนเกี่ยวกับระบบหลอดเลือดและสัมพันธ์กับภาวะออกซิเดทีฟ สเตรส บทบาทของแอนติออกซิแดนท์ โดยเฉพาะไวตามิน อี (α-tocopherol) ในส่วนที่เกี่ยวข้องกับการเกิดโรคแทรกซ้อนในระบบหลอดเลือดยังไม่เป็นที่เข้าใจมากนัก ดังนั้นจุดประสงค์ของการศึกษานี้คือการศึกษาความสัมพันธ์ระหว่างสภาวะแอนติออกซิแดนท์ (α-tocopherol) และผลผลิตไลปิดเปอร์ออกซิเดชั่น (มาลอนไดอัลดีไฮด์ ; MDA) ในพลาสม่าของผู้ป่วยเบาหวานประเภทที่ 1 จำนวน 20 ราย เปรียบเทียบกับคนปกติจำนวน 20 ราย*

วัสดุและวีธีการ: *ระดับไขมันในเลือด จะถูกวิเคราะห์ด้วยเครื่องเทียบสีโดยการใช้เอนไซม์ในน้ำยาสำเร็จรูป ระดับของ MDA ในพลาสม่าจะวัดการเรืองแสงโดยใช้เครื่อง spectrofluorometer และระดับของไวตามิน อี จะถูกวัดโดยเครื่อง High performance liquid chromatography ทั้งในผู้ป่วยเบาหวานประเภทที่ 1 และคนปกติที่มีเพศและอายุสมนัยกัน ผลการทดลองของทั้ง 2 กลุ่มจะถูกเปรียบเทียบโดยใช้ unpaired Student's t-test และสหสมพันธ์ระหว่างระดับ น้ำตาล ในเลือดและค่าตัวแปรต่างๆจะถูกทดสอบโดยใช้ Pearson rank correlation coefficient test*

ผลการศึกษา: *จากการศึกษาพบว่าความเข้มข้นของ MDA ในพลาสม่าของผู้ป่วยเบาหวานประเภทที่ 1 จะสูงกว่าคนปกติอย่างมีนัยสำคัญ (p< 0.01) ส่วนสภาวะของแอนติออกซิแดนท์ในผู้ป่วยเบาหวานประเภทที่ 1 จะลดลงอย่างมีนัยสำคัญ (p< 0.05) เมื่อเทียบกับคนปกติเฉพาะในส่วนที่เป็น α-tocopherol/total lipid สำหรับในส่วนของ plasma α-tocopherol และ α-tocopherol/total cholesterol พบว่าไม่มีความแตกต่างจากคนปกติ (p> 0.05) สหสัมพันธ์ระหว่าง MDA และ FPG สามารถพบได้ในผู้ป่วยเบาหวานประเภทที่ 1 เมื่อเทียบกับคนปกติ*

สรุป: *การเสริมแอนติออกซิแดนท์ในผู้ป่วยเบาหวานประเภทที่ 1 อาจจะมีความจำเป็นในการรักษาผู้ป่วยเพื่อป้องกันโรคแทรกซ้อนที่เกิดจากสภาวะออกซิเดทีฟสเตรส*