Antioxidant Status and Levels of Different Vitamins Determined by High Performance Liquid Chromatography in Diabetic Subjects with Multiple Complications

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Abbreviations: CAD, coronary artery disease; CAT, catalase; DWC, diabetes without complications; GSH-Px, glutathione peroxidase; HDL-C, HDL-cholesterol; HPLC, high performance liquid chromatography; HTA, hypertension; LDL-C, LDL-cholesterol; RF, renal failure; SOD, superoxide dismutase.

Abstract. Plasma vitamin A, C and E levels and erythrocyte antioxidant enzyme activities were investigated in type I and type II diabetic subjects with and without complications, i.e., hypertension, coronary artery disease and renal failure. Reverse phase HPLC was used to quantify vitamin A and E levels. We observed that the vitamin C levels were not significantly different between control and diabetic subjects. However, vitamin A and E levels were significantly lower in type I and type II diabetic subjects compared to controls. Superoxide dismutase (SOD) activity was significantly lower in type II, but not in type I, diabetic patients compared

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to controls. Interestingly, glutathione reductase and peroxidase activities were diminished in type I, but not in type II, diabetic subjects as compared to controls. Catalase activity was lower in both types of diabetic patients in comparison with their respective controls. Altogether these results suggest that diabetes mellitus may be associated with altered antioxidant status regardless to various complications.

Introduction

Diabetes mellitus is a chronic disease characterized by an absolute or relative deficiency of insulin and/or by insulin resistance. All forms of diabetes, both inherited and acquired, are characterized by hyperglycaemia, development of specific microvascular pathology and accelerated atherosclerosis responsible for coronary artery disease (Ruderman et al. 1992). There has been currently great interest in the potential contribution of increased oxidative stress to the development of complications in diabetes mellitus (Sinclair 1993; Baynes and Thorpe 1999). An increase in oxidative stress may occur due to an increase in the production of free radicals. These reactive oxygen species are capable of chemically altering all major classes of biomolecules (e.g., lipids, proteins and nucleic acids) by changing their structure and function (Therond et al. 2000), thus leading to cell damage in diabetes (Sinclair 1993). The biological effects of free radicals are normally controlled *in vivo* by a wide range of antioxidants such as vitamin A, C and E, glutathione and antioxidant enzymes. Vitamin E, the main liposoluble antioxidant in human beings, scavenges peroxyl-radicals, produced during lipid peroxidation (Therond et al. 2000). Reduced glutathione and vitamin C regenerate vitamin E (Therond et al. 2000). Vitamin A and C have also the ability to react directly with reactive oxygen species. Among antioxidant enzymes, superoxide dismutase (SOD) catalyzes dismutation of the superoxide anion (O^{2-}) into H_2O_2, and glutathione peroxidase (GSH-Px) and catalase (CAT) both detoxify H_2O_2 and convert lipid hydroperoxides to nontoxic alcohols (Guemouri et al. 1991; Therond et al. 2000). Since oxidative stress seems to play a key role in the pathology of diabetes mellitus, several methods have been developed to assess the antioxidant status such as the determination of circulating vitamins and antioxidant enzymes (Guemouri et al. 1991; Maxwell et al. 1997a; Courderot-Masuyer et al. 2000; Therond et al. 2000; Aydin et al. 2001). However, the investigators have not come to a consensus due to contradictory results reported in this pathology (Jones et al. 1988; Courderot-Masuyer et al. 2000; Aydin et al. 2001; Pieri et al. 2001). In addition, the implication of oxidative status in diabetes mellitus with its major complications such as hypertension (HTA), renal failure (RF) and coronary artery disease (CAD) is not well known. It was, therefore, thought worthwhile to undertake a study in order to evaluate erythrocyte antioxidant enzyme activities (SOD, GSH-Px, CAT) and the levels of vitamin A, C and E in diabetes mellitus associated or not with these complications.

Materials and Methods

Subjects

A total of 40 type I and 42 type II diabetic patients were recruited from the Department of Diabetics, University Hospital of Tlemcen (Algeria). Medical records were screened by specialist physicians. The diabetic type I or type II patients were divided into four groups: group I consisted of diabetic patients without complications (DWC); group II consisted of patients with coronary artery disease (CAD), diagnosed by clinical symptoms of angina pectoris, electrocardiogram examination or documented myocardial infarction; group III consisted of patients with hypertension (HTA), defined as a blood pressure $\geq$140/90 mm Hg; group IV consisted of patients with renal failure (RF), evaluated by significant renal impairment such as abnormal creatinine or macroalbuminuria.

All diabetic patients received insulin (for type I diabetes), or oral hypoglycemic agents like sulphonylureas or metformin (for type II diabetes). In addition, diabetic patients with HTA or with RF were treated by angiotensin converting enzyme inhibitors. Diabetic patients with CAD were treated by calcium antagonists. No patients received lipid lowering medications.

Two groups of control subjects were selected. Controls and diabetic subjects were matched with respect to age and body mass index (BMI) as determined by the weight and height of patients. All individuals were non smokers. None had taken vitamin supplements. The characteristics of patients are given in Table 1. The study was approved by the ethical committee of the Tlemcen-University Hospital and informed written consent was obtained by all the subjects.

Blood samples

Fasting venous blood samples were collected in heparinized tubes. Blood samples were centrifuged to obtain the plasma for immediate analysis of glucose, glycosylated hemoglobin (Hb A1C) and lipids. Aliquots of plasma were frozen at $-20\,℃$ for further determinations of vitamins and total antioxidant capacity. After removal of plasma, erythrocytes were washed three times with two volumes of isotonic saline. Erythrocytes were lysed with cold distilled water (1/4), stored in refrigerator at $4\,℃$ for 15 min and the cell debris was removed by centrifugation ($2000 \times g$ for 15 min). Erythrocyte lysates were assayed for antioxidant enzyme activities.

Lipoprotein isolation

Plasma lipoprotein fractions (LDL, $d < 1.063\ \mathrm{g \cdot ml^{-1}}$; HDL, $d < 1.21\ \mathrm{g \cdot ml^{-1}}$) were separated by sequential ultracentrifugation in a beckman ultracentrifuge (Model L5-65, 65 Ti rotor), using sodium bromide for density adjustment, according to Havel et al. (1955). LDL and HDL fractions were dialyzed against 0.15 mol/l NaCl and 1 mmol/l disodium EDTA, pH 7.4, at $4\,℃$ in spectra/por 2 dialysis tubing (spectrum Medical Industries, Los Angeles, CA).

Table 1. Different groups of diabetic and control subjects

. .	Control subjects		Diabetic subjects							
	C1	C2	Type I				Type II			
			DWC	CAD	HTA	RF	DWC	CAD	HTA	RF
Number	10	10	12	8	10	10	10	10	12	10
M/F ratio	5/5	5/5	6/6	5/3	5/5	6/4	5/5	5/5	6/6	5/5
Age (years)	28.0 ± 1.0	56.0 ± 1.2	30.0 ± 1.0	28.0 ± 2.0	29.0 ± 1.0	30.0 ± 1.0	54.0 ± 2.0	55.0 ± 1.0	56.0 ± 1.3	56.0 ± 1.2
BMI (kg/m^2)	21.2 ± 1.8	25.3 ± 1.3	22.2 ± 1.5	22.6 ± 1.8	22.5 ± 1.6	20.1 ± 1.7	25.8 ± 1.9	24.4 ± 2.0	24.8 ± 2.4	22.9 ± 2.0
Duration of disease (years)	–	–	8.2 ± 0.5	10.0 ± 1.0	9.1 ± 1.0	12.0 ± 1.0	5.2 ± 0.8	8.5 ± 0.9	12.0 ± 1.4	12.7 ± 1.2
Fasting glucose (mmol/l)	4.9 ± 0.1	5.2 ± 0.2	9.0 ± 0.8*	12.7 ± 1*	10 ± 0.8*	10.8 ± 1*	9.6 ± 0.6*	10.8 ± 0.5*	9.0 ± 0.8*	9.4 ± 0.7*
Hb A1C (%)	5.0 ± 0.2	5.4 ± 0.3	7.4 ± 0.5*	9.9 ± 0.3*	9.1 ± 0.4*	10.2 ± 0.3*	8.0 ± 0.6*	10.7 ± 0.6*	10.0 ± 0.5*	9.7 ± 0.7*
Total cholesterol (mmol/l)	5.7 ± 0.4	5.4 ± 0.5	5.4 ± 0.6	5.5 ± 0.4	6.0 ± 0.6	7.2 ± 0.3*	5.8 ± 0.5	6.1 ± 0.6	6.1 ± 0.4	6.69 ± 0.3*
Triglycerides (mmol/l)	1.3 ± 0.2	1.0 ± 0.3	1.3 ± 0.2	1.4 ± 0.2	2.0 ± 0.4*	2.3 ± 0.3*	1.9 ± 0.3*	2.6 ± 0.4*	2.8 ± 0.4*	2.4 ± 0.3*
LDL–C (mmol/l)	3.6 ± 0.2	3.8 ± 0.5	3.5 ± 0.3	3.6 ± 0.3	4.1 ± 0.5	5.2 ± 0.3*	3.8 ± 0.3	4.3 ± 0.2	4.4 ± 0.5	5.3 ± 0.4*
HDL–C (mmol/l)	1.7 ± 0.1	1.5 ± 0.2	1.5 ± 0.5	1.1 ± 0.2*	1.5 ± 0.3	1.5 ± 0.4	1.6 ± 0.4	1.1 ± 0.1*	1.4 ± 0.4	1.3 ± 0.4
SBP (mm Hg)	127 ± 3	132 ± 4	128 ± 3	130 ± 3	176 ± 4*	128 ± 2	130 ± 3	132 ± 3	180 ± 5*	136 ± 2
DBP (mm Hg)	81 ± 2	87 ± 3	84 ± 2	86 ± 2	98 ± 3*	84 ± 2	88 ± 1	85 ± 3	96 ± 3*	88 ± 2

BMI, body mass index; CAD, coronary artery disease; DBP, diastolic blood pressure; DWC, diabetes without complications; HDL-C, high density lipoprotein-cholesterol; HTA, hypertension; LDL-C, low density lipoprotein-cholesterol; RF, renal failure; SBP, systolic blood pressure; * $p < 0.05$, diabetic subjects *versus* controls. C1 and C2 represent, respectively, the control groups belonging to type I and type II diabetic groups.

Determinations of hemogloblin and plasmatic levels of lipoprotein

Glycosylated hemoglobin levels (Hb A1C) were determined by isolab column chromatography (Kaplan et al. 1982). Plasma glucose was determined by glucose oxidase method using a glucose analyzer (Beckman Instruments, Fullerton, CA, USA). Plasma triglyceride and total cholesterol, LDL- and HDL-cholesterol (LDL-C and HDL-C) contents were determined by using enzymatic methods, according to the instructions furnished with the kit (Boehringer, Mannheim, Germany).

Determination of plasmatic levels of vitamin A and E by high performance liquid chromatography

Plasma α-tocopherol (vitamin E) and retinol (vitamin A) were determined by reverse phase HPLC (Zaman et al. 1993). This method was used to quantify in a single chromatographic run with an internal standard, Tocol (Lara Spiral, Couternon, France), added for estimation of recovery. The stationary phase was constituted by greffed silica (C18 column, HP ODS Hypersil C18; 200 mm $\times$ 4.6 mm; Lara Spiral, maintenance temperature of analytical column, 35 ℃). The mobile phase was a mixture of methanol/water (98/2, v/v) at a flow rate of 1 ml/min. Vitamins were extracted by hexane, dried under nitrogen and resuspended in methanol. The HPLC peaks were detected by an UV detector at 292 nm for vitamin E and 325 nm for vitamin A. Representative chromatograms were obtained by injecting standard solutions. In order to evaluate the daily performance of the HPLC system, the external standard was injected every day in the beginning, middle and at the end of the chromatographic system.

Determinations of plasmatic levels of vitamin C

Vitamin C levels were determined in plasma using the method of Roe and Kuether (1943). After protein precipitation with 10 % trichloroacetic acid and centrifugation, the supernatant (500 μl) was mixed with 100 μl sulfuric acid (9 N) containing 30 mg/ml dinitrophenylhydrazine, 4 mg/ml thiourea and 0.5 mg/ml copper sulfate and incubated at 37 ℃ for 3 h. Following the addition of 750 μl of 65 % (v/v) sulfuric acid, the absorbency was recorded at 520 nm.

Determinations of erythrocyte antioxidant enzyme activities

Catalase (CAT, EC 1.11.1.6) activity was measured by spectrophotometric analysis of the rate of hydrogen peroxide decomposition at 240 nm (Aebi 1974). Enzyme activity was expressed as U/g Hb. Glutathione peroxidase (GSH-Px, EC 1.11.1.9) was assessed by the method of Paglia and Valentine (1967), using cumene hydroperoxide as substrate. One unit of glutathione peroxidase activity is defined as the amount of enzyme which gives a 90 % decrease in glutathione concentration *per* min at a 1 mmol/l starting glutathione concentration. Glutathione reductase (GSSG-Red, EC 1.6.4.2) activity was determined by measuring the rate of NADPH oxidation in the presence of oxidized glutathione (Goldberg and Spooner 1992). The unit of enzyme activity was defined as the amount of enzyme which oxidized 1 mmol of

NADPH/min. Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured by the NADPH oxidation procedure (Elstner et al. 1983) and expressed as units of SOD *per* g Hb.

Statistical analysis

Values are means ± S.E.M. Statistical analysis of the data was carried out using *Statistica* (version 4.1, Statsoft, Tulsa, OK). Data were evaluated by analysis of variance. Mann-Whitney U-test was employed for the comparison between patients and control groups.

Results

Hb A1C, plasma glucose, cholesterol and triglyceride concentrations, and LDL-C and HDL-C levels

Significant differences were found between diabetic and control subjects for plasma glucose and Hb A1C levels which were high in all diabetic patients regardless to complications (Table 1). However, the highest glucose concentrations were apparent in diabetes mellitus (type I and II) associated with CAD. Plasma total and LDL-C concentrations were not significantly altered in diabetic patients having CAD and HTA, except those associated with RF who exhibited higher levels than controls. HDL-C amounts were lower only in type I, and II diabetes mellitus with CAD compared to control and other diabetic patients. Plasma triglyceride levels were significantly increased in all type II and type I diabetic patients, having HTA and RF.

Plasma vitamin A, C and E concentrations

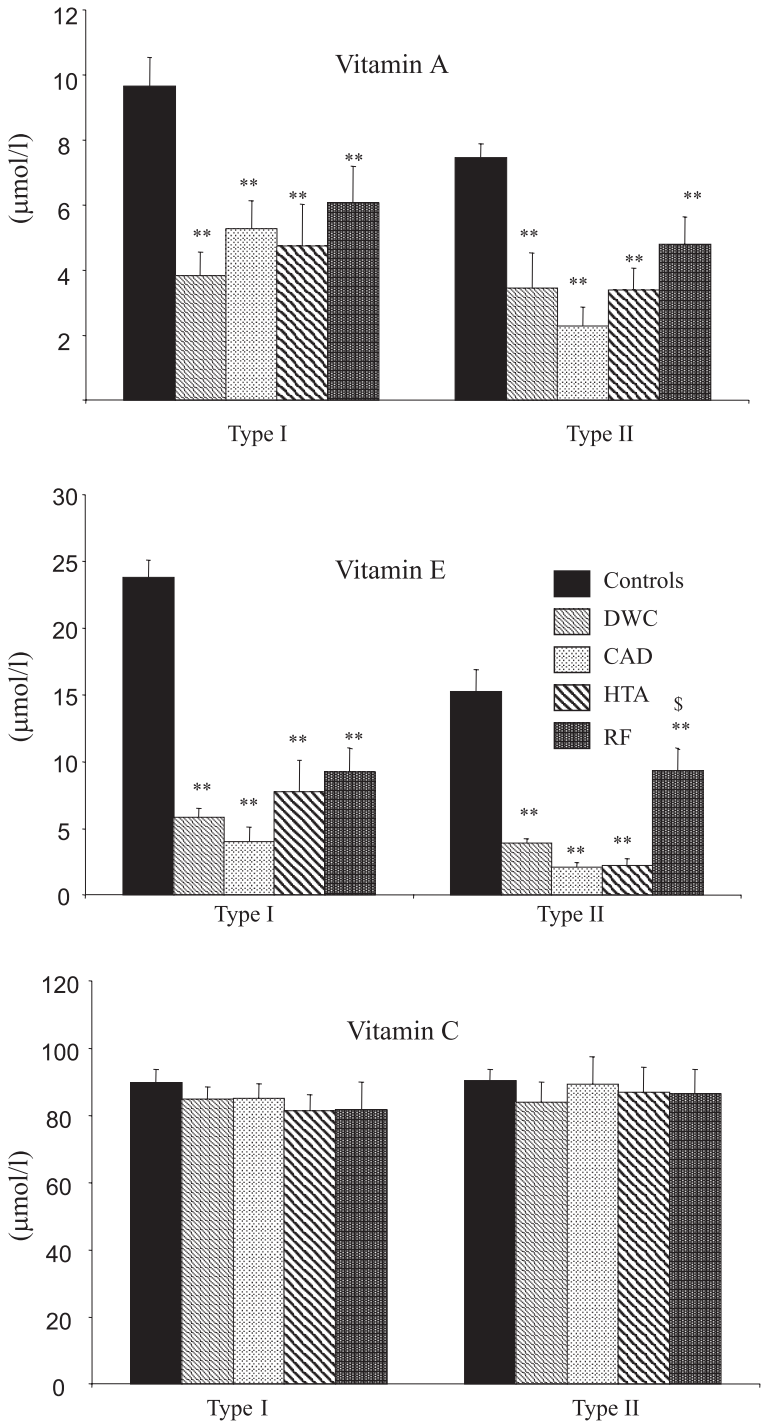
While vitamin C levels did not differ between the diabetic and control groups, vitamin A and E levels were significantly lower in all diabetic groups compared to controls (Figure 1).

Erythrocyte antioxidant enzyme activities

SOD activity was significantly lower in all groups of type II diabetes mellitus when compared with controls. However, no significant difference was observed between type I diabetic groups and their controls (Figure 2).

Catalase activity was lower in both types of diabetic patients compared with their respective controls (Figure 2). On the contrary, glutathione reductase and peroxidase activities were diminished in all type I, but not in type II, diabetic patients as compared to controls (Figure 3).

Figure 1. Plasma vitamin A, C and E concentrations in diabetic and control subjects. The levels of different vitamins were determined as described in Materials and Methods. Values are means ± S.E.M. Statistical analysis was performed by employing the Mann-Whitney U-test. ** $p < 0.001$, diabetic *vs.* control subjects; \$ $p < 0.05$ significant compared to DWC, CAD, HTA and RF patients.


Vitamin A
(µmol/l)
12
10
8
6
4
2
**
Type I
Type II
Vitamin E
30
25
20
15
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Controls
DWC
CAD
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120
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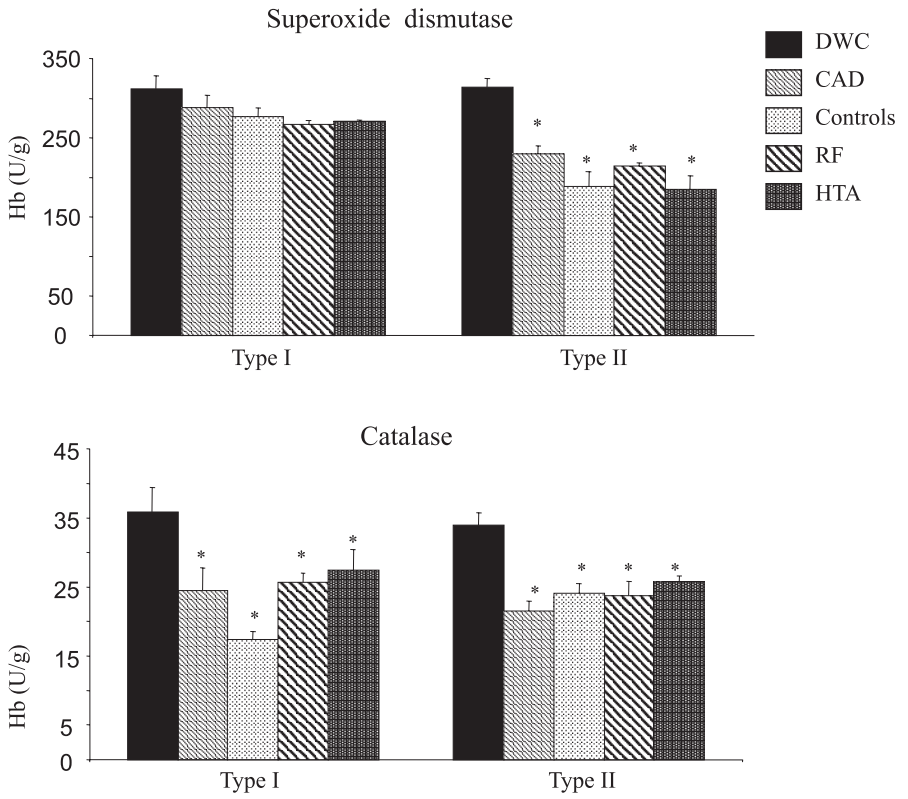

Figure 2. Superoxide dismutase and catalase enzyme activities in diabetic and control subjects. The enzyme activities were determined as described in Materials and Methods section. Values are means ± S.E.M. Statistical analysis was performed by employing the Mann-Whitney U-test. * $p < 0.05$, diabetic *vs.* control subjects.

Discussion

Since there are contradictory results on the antioxidant status in diabetic patients, we conducted the present study on type I and type II diabetes mellitus with or without complications like hypertension (HTA), coronary artery diseases (CAD) and renal failure (RF). We observed that abnormalities in lipid and lipoprotein in the diabetic population were associated with high fasting glucose and Hb A1C levels. Hypertriglyceridemia was observed in all type II diabetic and only in type I diabetic subjects with HTA and RF. Total and LDL-C concentrations were increased in diabetes mellitus with RF. HDL-C levels were decreased only in diabetic patients with CAD. Hyperglyceridemia is the most common feature in type II diabetes (Elstner et al. 1983). In fact, overproduction of VLDL with increased secretion of triglycerides or decreased lipoprotein lipase-mediated hydrolysis of VLDL triglyc-

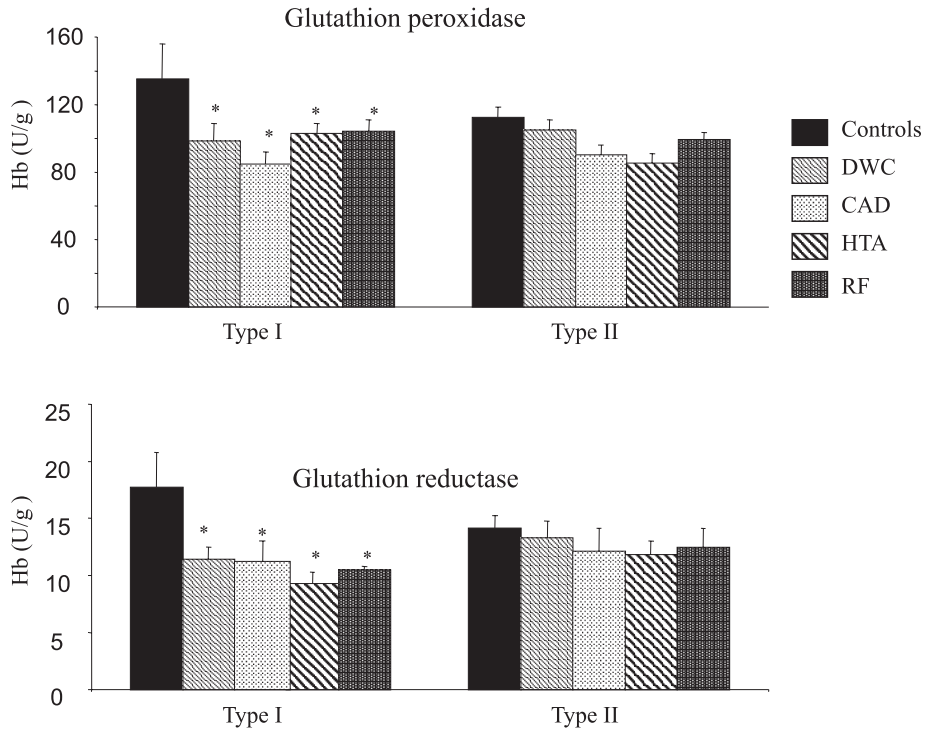

Figure 3. Glutathione reductase and peroxidase enzyme activities in diabetic and control subjects. The enzyme activities were determined as described in Materials and Methods section. Values are means ± S.E.M. Statistical analysis was performed by employing the Mann-Whitney U-test. * $p < 0.05$, diabetic *vs.* control subjects.

erides might contribute to elevated triglyceride levels in type II diabetes (Taskinen 1997). In addition, patients with high blood pressure tend to be hypertriglyceridemic (MacMahon et al. 1985). Thus, it is not surprising that all our type II and only type I diabetic subjects with HTA possessed high triglyceride levels. On the other hand, diabetes mellitus with renal complications is known to be associated with elevated plasma triglyceride, cholesterol and LDL-C levels (Jensen et al. 1988; Mattock et al. 1988). In the nephrotic syndrome, hyperlipidemia is assumed to be caused by increased hepatic lipoprotein synthesis in response to hypoalbuminaemia, low plasma oncotic pressure and renal protein leakage (Short and Durrington 1990). In our study, lipoprotein changes in type I and type II diabetics with RF are in agreement with several reports that have demonstrated that abnormalities in lipids are associated with diabetic nephropathy (Jensen et al. 1988; Mattock et al. 1988). Our data showed that type I and type II diabetic subjects with CAD had low HDL-C levels while LDL-C concentrations were normal. In general, consideration of metabolic risk factors for CAD has focused on the role of LDL-C. However,

it is becoming increasingly apparent that CAD can occur in the absence of hypercholesterolemia (Laws and Reaven 1990). In addition, low HDL-C is identified as the increasing risk factor of CAD in diabetes mellitus (Ginsberg 1991; Reaven and Laws 1994). In our study, plasma cholesterol, triglyceride, LDL-C and HDL-C levels were not different in type I diabetes, without complications, compared to control subjects, and this observation is in agreement to previous study (Dullaart 1995).

As far as the vitamins are concerned, we found no alteration in the levels of vitamin C, whereas the levels of vitamin A and E were lower in type I and type II diabetes mellitus with or without complications than control subjects. There have been conflicting reports regarding the plasma vitamin concentrations in diabetes mellitus. Makimattila et al. (1999) reported that plasma vitamin C and E in type II diabetic patients were not significantly different from those in control groups, while vitamin A levels were significantly decreased. On the other hand, Sundarm et al. (1996) reported low levels of vitamin E and C in diabetic patients. In our study, low levels of vitamin A and E could reflect their high utilisation rate, suggesting that these vitamins may be used to reduce oxidative stress in diabetic patients. It is well reported that oxidative stress is induced by both the increases in free radicals and disturbance of the free radical scavenging system in diabetes mellitus (Sinclair 1993; Therond et al. 2000). Alternatively, it is also possible that reduced vitamin A and E concentrations reflect low intake, which resulted in decreased antioxidant defence system in diabetic subjects. On the other hand, high levels of vitamin E were observed in type II diabetic subjects with RF. Increased levels of vitamin E may be related to the well known high serum lipid concentrations in these patients (Bonnefont-Rousselot et al. 1997). Taken together, our data provide convincing evidence for altered plasma antioxidants concentrations in diabetic patients, irrespective to their complications. The inverse correlation between Hb A1C and vitamin A and E levels was consistent with the idea that chronic hyperglycemia may increase oxidative stress (Hunt et al. 1990). Vitamin A and E supplementation would provide good metabolic control and benefits in the reduction of diabetes-induced complications. Gokkusu et al. (2001) indicated that vitamin E administration significantly reduced fasting glucose and increased C-peptide and insulin levels in type I diabetic subjects. In addition, vitamin E treatment lowers urinary albumin excretion rate (AER) in type II diabetic patients with micro/macroalbuminuria (Gaede et al. 2001). On the other hand, Zobali et al. (2002) showed that vasomotor activity depends on the prevention and/or inhibition of peroxidative stress which is achieved by combined treatment with vitamin A plus insulin in streptozotocin induced-diabetic rats.

In our study, diabetic subjects showed altered erythrocyte antioxidant enzyme activities. SOD activity was reduced in all the type II, but not type I, diabetic subjects regardless their complications. In contrast, glutathione reductase and peroxidase activities were diminished in type I diabetes but unchanged in type II diabetes, whatever complications were prevalent. Moreover, catalase activity was lower in all diabetic patients compared to controls. Our findings agree with the ob-

servations of Sekeroglu et al. (2000) who have reported decreased erythrocyte SOD activity in type II diabetic patients. Uzel et al. (1987) and Kedziora-Kornatowska et al. (1998) have also shown low SOD and catalase activities in type II diabetics compared to controls. In contrast, Aydin et al. (2001) noticed that SOD activity is elevated while glutathione peroxidase and catalase activities are normal in erythrocytes of type II diabetics. Ruiz et al. (1999) indicated that whereas erythrocyte glutathione peroxidase activity in type I diabetic subjects was decreased, there was no difference in SOD activity compared to control values. The reason why erythrocyte antioxidant enzyme activities were reported so differently in previous studies might be related to the treatment of diabetics related complications and the duration of disease.

Nonetheless, as observed in our study, the poor glycemic control in diabetic subjects is associated with the diminution of protective antioxidant enzyme activities (Maxwell et al. 1997b). Low anti-oxidant enzyme activities render cells vulnerable to oxygen radical attack, and thus increased diabetic complications. These alterations could not be ascribed to associated complications, but seemed to be related to the glycemic control in these diabetic patients. However, the diminution in antioxidant defences may contribute to the development of complications in diabetes mellitus. Therefore, antioxidant status might be used as marker in the management of glycemic control and the development of diabetic complications.

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