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Estimation of lipoperoxidative damage and antioxidant status in diabetic children: Relationship with individual antioxidants

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Abstract

Increased oxidative stress has emerged as a potential mechanism implicated in the pathogenesis, progression and cell dysfunction associated with many diseases including diabetes. In routine clinical practice, the estimation of the degree of oxidative damage and antioxidant status, even in paediatric patients, by appropriate techniques appears to be of interest. The aim of this study was to reliably identify patients with increased oxidant stress and/or reduced antioxidant defence mechanisms with a small blood sample and verify the applicability to the study of diabetic children (DC) at clinical onset of the disease. In 1-ml blood samples from 30 DC and 34 controls, techniques for accurately measuring malondialdehyde (MDA) concentrations in plasma and erythrocytes (using HPLC analysis with fluorometric detection), total radical antioxidant potential (TRAP) and blood plasma oxidizability were adapted and validated. Plasma α-tocopherol (HPLC), uric acid and sulfhydryl (SH) groups were also determined. At clinical onset of diabetes a significant reduction in plasma TRAP values ($P < 0.01$) was observed in DC compared with controls. Similarly, a significant fall in individual antioxidant levels (α-tocopherol/total lipids, uric acid and protein SH) was noted in plasma of DC. Highly significant increases were found in both plasma and erythrocyte MDA levels in DC (p-MDA:1.7 ± 0.2 μM; er-MDA: 7.2 ± 0.7 nmol/g Hb) compared with controls (p-MDA:0.86 ± 0.09 μM; $P < 0.0003$; er-MDA:3.8 ± 0.2 nmol/g Hb, $P < 0.0001$). Plasma MDA and triglyceride levels correlated directly only in DC ($P < 0.001$). Whole plasma oxidizability was significantly higher in DC than in controls ($P < 0.0001$) and this parameter correlated significantly with plasma cholesterol and triglyceride concentrations ($P < 0.0001$). The micromethods adapted and applied to the simultaneous detection of lipid peroxidation products and antioxidant status permit accurate and reliable assessment of the oxidative stress process in small plasma samples. Our results clearly show systemic peroxidative damage associated with insufficient defence mechanisms against ROS to be already present at clinical onset of type 1 diabetes mellitus in children and adolescents.

Keywords: *Diabetic children, antioxidants, malondialdehyde, plasma oxidizability, lipid peroxidation*

Introduction

The toxicity provoked by high concentrations of reactive oxygen species (ROS) and radical-derived ROS on cells and tissues and their role in the pathogenesis or progression of diverse human diseases continues to arouse interest, leading to rapid expansion of clinical research in this area [1–3]. In aerobic organisms, ROS are generated constantly during physiological or pathophysiological mitochondrial oxidative metabolism and are efficiently neutralised by cellular antioxidant defences [1]. An excessive and/or sustained increase in free radical production associated with diminished efficacy of the cellular defence systems results in oxidative stress which occurs in many unrelated pathologic processes and may contribute significantly to disease mechanisms; however, increased oxidative stress may also result from a pathologic process [4].

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Diabetic patients are exposed to increased oxidative stress due to several mechanisms, including direct glucose auto-oxidation, non-enzymatic protein glycation, activation of NAD(P)H oxidases, nitric oxide synthase and xanthine oxidase [5–9]. Furthermore, enhanced glucose flux through the polyol pathway leads to depletion of the NADPH available for glutathione reductase, thereby provoking changes in glutathione redox status [10], and hyperglycaemia has been shown to disrupt intracellular antioxidant defence mechanisms [11,12]. Among lipid peroxidation products, plasma MDA concentrations are the most frequently used biomarker for assessing *in vivo* oxidative stress in human subjects [13]. MDA is considered to be the main product of auto-oxidation and degradation of polyunsaturated fatty acids or their esters and studies have shown its concentration to be increased in plasma of diabetic patients [14]. Different spectrophotometric and fluorometric methods have been described for MDA determination in plasma [15–19]. The procedures based on the separation of MDA-TBA adduct by high performance liquid chromatography (HPLC) have been proposed for MDA determination in biologic samples owing to their high analytic sensitivity and specificity, since possible interference by other chromogenous substances is avoided [2,20,21].

In response to greater oxidative stress, living organisms have developed an antioxidant defence network which should prevent the harmful effects of free radical overproduction. Antioxidant systems include small molecules, thiols, antioxidant enzymes and metal-binding proteins. Antioxidant protection in determined pathological processes may be inadequate since essential plasma antioxidant concentrations are often low. The overall antioxidant capacity of an organism depends on the additive and/or synergic relationships which form among the different antioxidants, although these interactions are not well defined. Total plasma antioxidant capacity can be assessed by individually measuring a gamut of antioxidants; however, a series of techniques has also been developed [22–27] permitting total radical antioxidant potential to be determined in a single assay. The advantages of these techniques lie in the fact that they save analysis time, sample volume and, above all, provide an idea of the overall antioxidant capacity of the group of antioxidants present in a biologic sample.

The aim of this study was to develop modifications of techniques which, with a small blood sample, would permit reliable, rapid and accurate determination of both plasma and erythrocyte MDA concentrations, total radical-trapping antioxidant potential (TRAP) and individual antioxidants (α-tocopherol, protein sulfhydryl groups, uric acid) together with plasma susceptibility to oxidation. Individualised analysis for identifying patients with increased oxidative damage integrated with the analysis of antioxidant status would be potentially useful for early prediction of the extent of the ongoing oxidative stress process in children at onset of type 1 diabetes mellitus.

Materials and methods

Subjects

This study was conducted in thirty type 1 diabetic children (DC) (mean age: 7.4 ± 3.5 years; 20 boys, 10 girls) evaluated between 7 and 10 days after the clinical onset of diabetes when hydroelectrolytic disorders and acidosis had returned to normal with therapy. Thirty-six healthy age-matched donors (mean age: 8.4 ± 3.9 years; 21 boys, 15 girls) served as controls. Diabetic (DC) and control children were aged between 2 and 14 years. All patients were diagnosed and followed up at the Paediatric Diabetes Unit of the Vall d'Hebron Children's Hospital. The study was approved by the hospital Ethics Committee for Clinical Research.

Blood sample collection and processing

A small sample of venous blood (1–2 ml) was separated from the blood drawn from subjects after an overnight fast prior to a programmed analysis, collected into tubes containing heparin and processed within 1 h of extraction. Plasma and blood cells were separated by centrifugation at 3000 rpm for 5 min at 4°C and erythrocytes were washed 3 times with phosphate-buffered saline. Plasma aliquots were stored at − 85°C until analysis. An aliquot of 100 μl fresh erythrocytes was haemolyzed by rapid mixing with 100 μl of cold deionized water, followed by deproteinization with 150 μl of cold trichloroacetic acid (20%) and the addition of 10 μl of a butylated hydroxytoluene (BHT) solution. After centrifugation, (15,000 rpm) for 4 min, the supernatant was carefully removed to another tube and either analyzed immediately or frozen at − 80°C and analyzed within one week.

Chemicals

All chemicals were analytical grade. Butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA) and 1,1,3,3-tetraethoxy-propane (TEP), α-Tocopherol, β-phycoerythrin from *Porphirium cruentum* (β-PE), and 5,5′-dithio-bis-(2-nitro-benzoic acid) (DTNB) were purchased from Sigma Chemical Company (St. Louis, MO, USA). High Performance Liquid Chromatography (HPLC) grade acetonitrile from Romil (Cambridge, UK) and n-Butanol, methanol, ethanol and n-Hexane from Merck (Darmstadt, Germany). Highly-purified water (resistivity = 18 MΩ cm) obtained through a Milli-Q water purification system

(Millipore, Bedford, MA) was used for solution preparation and for the mobile phase. Tocopherol acetate and serum calibration standard for vitamin E analysis were obtained from Chromsystems (Munich, Germany). 6-Hydroxy-2,5,7,8-tetramethylchroman 2-carboxylic acid (TROLOX) was obtained from Aldrich Chemical Co. (Milwaukee,WI). 2,2′-Azobis-(2-amidinopropane) hydrochloride (AAPH) was obtained from Polysciences (Warrington,PA). All other general chemicals used were of the highest purity available.

Determination of MDA

HPLC instrumentation and analysis. Plasma MDA concentration was determined after reverse-phase isocratic HPLC separation of the MDA-TBA complex based essentially on the method of Fukunaga et al. [28] with the modifications described below. MDA-TBA adduct was monitored by fluorescence detection (λ excitation, 515 nm; λ emission, 553 nm) on an HPLC system. The chromatographic apparatus was from Waters Associates (Milford, MA) equipped with a Model 510 pump, a Model 470 fluorescence detector and a Model 700 Satellite WISP autoinjector. Separation of MDA-TBA complex was carried out using a Symmetry C18 stainless steel reversed-phase column (4.6 × 150 mm i.d.) packed with 5-μm particles (Waters Associates). The analytical column was protected with a pre-column (Guard-Pak™) filled with a Nova-Pak C_{18} cartridge (Waters Associates). MDA-TBA adduct was eluted using a mobile phase consisting of acetonitrile/water (7:3) at a flow rate of 1 ml/min. Injection volume was 20 μl. Data were processed by peak automatic integration.

Sample preparation. An aliquot of 20 μl plasma was mixed with 200 μl of 0.2% TBA in 0.1 M sodium acetate buffer pH = 3.5 and 10 μl of 5% BHT in 99% ethanol. The sample mixture was incubated at 95°C for 45 min in a dry bath. After cooling, the reaction mixture was centrifuged at 14,000 rpm at room temperature for 5 min. Supernatant (20 μl) was injected into the HPLC system.

Standards. Calibration solutions of 0.5, 1 and 2.5 μmol/l were prepared daily using a stock solution of TEP 10 mM solubilized in 40% ethanol as the calibration standard and determined parallelly with the samples. The stock solution was prepared monthly and stored at 4°C.

Validation of the HPLC method used. Validity of the method was determined using pooled plasma, consisting of equal volumes of EDTA-plasma from 17 different healthy subjects, stored at −85°C in aliquots for no longer than a week. The detection limit, defined as the concentration of 3 times the mean of 4 determinations of the zero calibrator, was 0.05 μmol/l. Within-run imprecision was assessed by measuring 10 pooled plasma samples in the same analytical run; the mean was 1.27 ± 0.05 μmol/l and the coefficient of variation (CV) 3.9%. Between-day imprecision was determined over 6 consecutive days using pooled samples; the mean ± SD was 1.1 ± 0.07 μmol/l and CV 6.3%. Standard curves were linear up to at least 10 μmol/l ($r = 0.9997$). Recovery was 105% and the detection limit was ≥0.001 μmol/l.

Erythrocyte MDA. Erythrocyte MDA content (er-MDA) was quantified on the red blood cell extract by reversed-phase HPLC with fluorescence detection. Briefly, 100 μl of the deproteinized haemolyzed supernatant were mixed with 250 μl of 0.2% TBA in 0.1 M sodium acetate buffer pH = 3.5 and 10 μl of 5% BHT in absolute ethanol, in essentially the same way and under the same instrumentation and reaction conditions as described above for the analysis of plasma MDA. The detection limit was ≥0.01 μmol/l.

Total radical-trapping antioxidant parameter (TRAP) analyses

Total plasma antioxidant activity was measured using a modification of Glazer's fluorometric assay [23]. The water-soluble vitamin E analog Trolox was used as a standard and AAPH as generator of peroxyl radicals. AAPH 12 mM was prepared daily and, once dissolved, immediately embedded in ice. Na-phosphate 75 mM pH = 7 buffer was used for preparation of all dilutions and as the reaction blank. In the fluorometric assay, the final reaction mixture contained AAPH 12 mM (250 μl), sample serum diluted 1/25 (20 μl) and β-PE 0.01113 μM (250 μl). Once the AAPH is added, the reaction mixture is incubated at 37°C and a first reading is taken at 5 min and a second 30 min after incubation. Fluorescence was measured at the emission of 565 nm and excitation of 540 nm. The results were calculated in the following way: %TRAP = 1 − (Δ abs. sample or Trolox/Δ abs. blank) × 100. TRAP levels were expressed as arbitrary units equivalent to Trolox antioxidant activity and calculated from a calibration curve determined daily between the TRAP percentage and the concentration of the three standards. The lineal regression coefficient of the calibration curve was never below 0.999. Intra- and inter-day coefficient of variation were 2.9 and 6.6%, respectively.

Standards. Aliquots of Trolox 1 mM were prepared and stored frozen at −20°C (Stock solution). Twenty microliters of a 50, 75 and 100 μM Trolox stock solution were assayed during each run. Fluorescence

was measured at the emission of 565 nm and excitation of 540 nm, using a F-2000 Hitachi Fluorescence Spectrophotometer.

Validation. The linear regression coefficient of the calibration curve was never below 0.999. Intra- and inter-day coefficients of variation were 2.91% ($n = 3$) and 6.6% ($n = 6$), respectively.

Other determinations

Plasma concentrations of α-tocopherol were analysed by reversed-phase HPLC procedure with ultraviolet detection at 280 nm (Waters Model 486 tunable absorbance detector), as detailed previously [11], using 50 μl of plasma. Given the clear metabolic relationship between plasma α-tocopherol and plasma lipid parameters [29], α-tocopherol levels are also expressed here as a ratio of α-tocopherol/total lipids (total cholesterol + triglycerides). Plasma lipid profile and uric acid were measured by quantitative enzymatic assays (Sigma Chemical Co.) according to the manufacturer's protocols, and the results were standardised using calibrator solutions for uric acid, glycerol and cholesterol. Glycated haemoglobin (HbA1c) was tested with a DCA 2000®^{2}+analyser with DCCT and IFCC tractability (Bayer Co.)

Whole plasma oxidation assay was carried out as described by Kontush and Beisiegel [30]. The oxidizability of plasma was measured as an increase in absorbance at 234 nm known to reflect the level of conjugated dienes in the samples. Briefly, heparin plasma (20 μl) was diluted 150-fold with PBS and its oxidation induced by the radical initiator AAPH (330 μM) at 37°C over a period of 20 h. Plasma thiols were measured by the method of Hu [31] and the results expressed as μmol/ml.

Statistical analysis

Statistical analyses were carried out using the Statview 4.5 statistical program (ABACUS Concept, Berkeley, CA). The data (expressed as means ± SEM) were analysed for statistically-significant differences by analysis of variance (ANOVA). Correlations between variables were studied by linear regression. *P* values ≤0.05 were considered significant.

Results

The main biochemical and metabolic control parameters of young diabetic patients at disease onset and the control group are summarised in Table I. Our results show total cholesterol and plasma triglyceride concentrations to be statistically higher in DC than in controls, while uricaemia was statistically lower ($p < 0.0001$). Furthermore, glycated haemoglobin (HbA1c) and fructosamine levels were raised in DC, as expected in diabetes.

Plasma MDA values and their ratio with triglycerides (MDA/TG) in DC and controls are shown in Figure 1A and B, respectively. Plasma MDA concentrations differed significantly between groups (controls:0.86 ± 0.09 μmol/l; DC:1.79 ± 0.24 μmol/l, $p = 0.0003$), being more than double in the diabetic group (108% above control levels). A lower, but significant difference was also found in MDA/TG ratio values between the groups (controls: 1.7 ± 0.2 μM/mM; DC: 2.63 ± 0.44 μM/mM, $p = 0.04$). Erythrocyte MDA content was also significantly higher ($p < 0.0001$) in diabetic children than in controls (Figure 2). Thus, MDA, the most representative secondary end-product of lipid peroxidation, was increased intracellularly and extracellularly in our child diabetic group compared with controls. A significant linear correlation existed between plasma MDA levels and triglyceride levels in the diabetic patient group ($r = 0.56$, $p < 0.005$) (Figure 3), while no correlation was found between these parameters in the control group ($r = 0.16$, $p = 0.33$). However, no significant correlations were observed between cholesterol and MDA concentrations in either group, nor between age of study subjects and triglyceride concentrations ($r = -0.16$, $p = 0.19$), cholesterol ($r = 0.023$, $p = 0.85$) or MDA ($r = -0.24$, $p = 0.052$).

Whole plasma oxidation-induced assay, employed to characterise the oxidizability of plasma lipoproteins in this study, yielded marked differences between DC patients and controls (Figure 4) since plasma oxidizability was found to be significantly higher in diabetic children at onset of the disease ($p < 0.0001$). This measurement correlates positively with the sum of concentrations of the major oxidizable lipids, cholesterol and triglycerides ($r = 0.33$, $p < 0.0001$) and exhibits a negative correlation with urate concentrations ($r = -0.27$, $p < 0.0002$).

Mean values of plasma TRAP and three of its components determined in both groups are presented in Table II. Individual antioxidants studied in plasma were α-tocopherol, protein sulphydryl (SH) groups and uric acid; the table also shows α-tocopherol levels normalised by total plasma lipid levels (cholesterol + triglycerides).

Table I. Plasma biochemical and metabolic control parameters in diabetic patients and in the control group.

	Control subjects ($n = 36$)	Diabetic patients ($n = 30$)
HbA1c %	(4.6–6.6)†	11.9 ± 0.5
Fructosamine (μmol/l)	(180–285)†	499 ± 26
Cholesterol (mmol/l)	4.17 ± 0.13	4.77 ± 0.14**
Triglycerides (mmol/l)	0.59 ± 0.04	0.76 ± 0.05*
Uric acid (μmol/l)	218.1 ± 9.9	153.4 ± 5.4***

Data are given as mean ± SEM. * $p < 0.05$, ** $p < 0.005$ and *** $p < 0.0001$. † Data in parentheses are normal ranges.

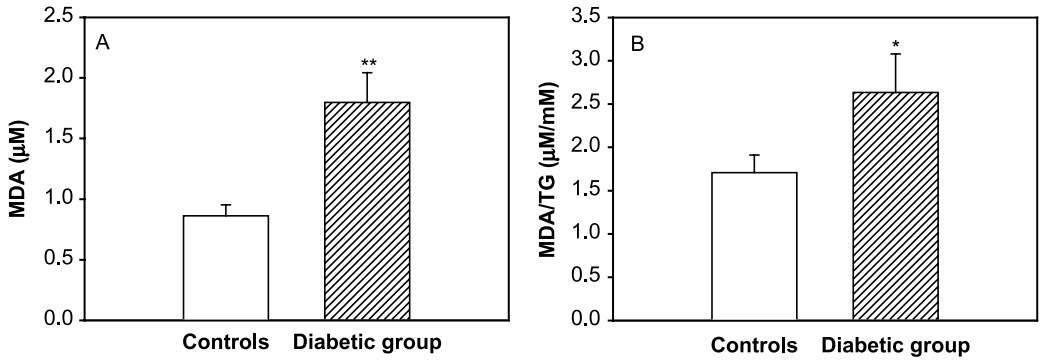

Figure 1. Plasma malondialdehyde (A) and plasma malondialdehyde/triglycerides (B) in diabetic patients at disease onset and in controls. Values are expressed as mean ± SEM. *$p < 0.05$; **$p < 0.0005$ vs. controls.

Mean values of TRAP, SH groups and uric acid were significantly lower in the DC patient group compared with controls, with the most important statistical difference being in uric acid and SH groups of proteins. No significant differences were observed in α-tocopherol values between the groups, whereas α-tocopherol concentrations standardised by plasma lipid levels were significantly lower in the diabetic children.

The positive, significant correlation between plasma TRAP values and the α-tocopherol/total lipid ratio is shown in Figure 5A and the relationship with uric acid in Figure 5B. The α-tocopherol/total lipid ratio presents a close relationship with total plasma antioxidant capability ($r = 0.515$, $p < 0.0001$). These figures also present the distribution of diabetic children and controls according to their respective TRAP values, α-tocopherol/lipids or uric acid; thus, it can be seen how the diabetic group presents lower uric acid levels than the control group.

The significant inverse correlation between overall plasma antioxidant activity and the lipoperoxidation end product MDA ($r = -0.44$, $p = 0.0005$) is shown in Figure 6A and a similar relationship appeared between TRAP values and the MDA/TG ratio (Figure 6B; $r = -0.43$, $p = 0.0007$). This latter negative relationship remained significant when both the control group ($r = -0.4$, $p = 0.01$) and the diabetic group were considered separately ($r = -0.42$, $p = 0.043$). A significant inverse correlation was found between uric acid and plasma MDA ($r = -0.281$; $p = 0.0243$), thereby indicating the antioxidant potential of uric acid (Figure 6C).

No relationship was observed among metabolic control parameters (fructosamine and glycated haemoglobin) of the diabetic patients with regard to plasma antioxidant activity (TRAP, thiols, α-tocopherol, and uric acid) and neither did they correlate with plasma or erythrocyte MDA levels.

Discussion

In type 1 diabetes, insulin deficiency provokes high blood glucose levels and lipid metabolism alterations and evolution of the disease may be associated with the development of premature vascular complications. Intensified insulin treatment and adequate diet in diabetic patients maintain blood glucose levels in near-normal range and delay the onset of complications long assumed to be related to persistently high

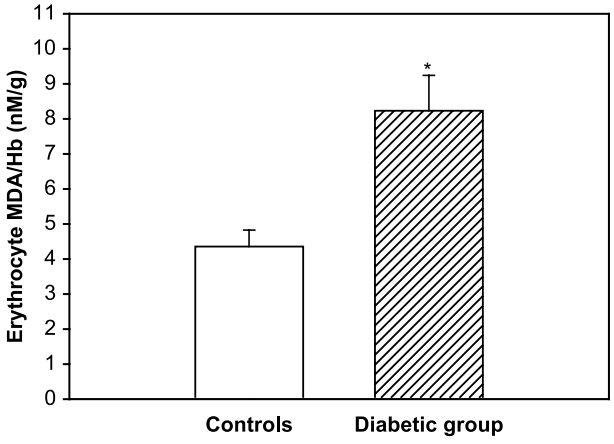

Figure 2. Erythrocyte malondialdehyde levels in diabetic patients at disease onset and in controls. Values represented are mean ± SEM. *$p < 0.001$ vs. controls.

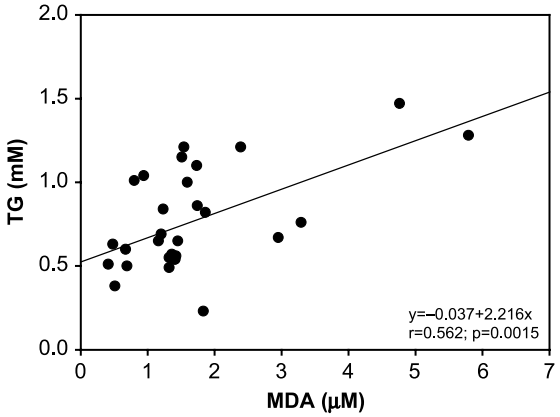

Figure 3. Correlation between plasma triglycerides and malondialdehyde in diabetic patients at disease onset.

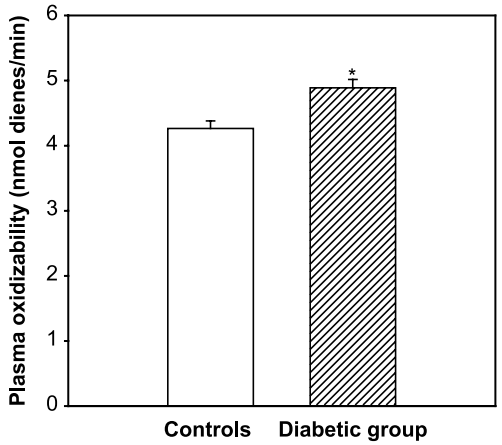

Figure 4. Plasma oxidizability at disease onset and in controls. Values expressed as mean ± SEM. *$p < 0.001$ vs. controls.

glucose levels. However, hyperglycaemia does not appear to be the only factor related to diabetic complications, as established in the conclusions of The Diabetic Control and Complications Trial Research Group [32]. It is generally agreed that lipid peroxidation is elevated in diabetes [33] and that it contributes to the accelerated development of atherosclerosis. Young diabetic patients are also prone to a series of micro- and macrovascular complications, which in adolescence may lead to renal and retinal damage and, in adulthood, to peripheral nervous system and/or cardiovascular system involvement with premature appearance of atherosclerotic vasculopathy. In this study, oxidative modification of lipids and antioxidant capacity deficiency were found in plasma of newly-diagnosed young diabetic patients in whom circulating MDA levels were more than two-fold higher than those of healthy age-matched controls, thereby showing them to be under oxidative stress clearly assessable at clinical level. Elevated levels of lipid-standardised MDA were also found in plasma from diabetic children at clinical disease onset and were not influenced by age, sex or metabolic control parameters. These findings would appear to indicate that the abnormally-high levels of lipid peroxidation end-products are associated with the diabetes itself rather than being a result of tissue injury.

Table II. Total Radical-trapping antioxidant parameter (TRAP) and levels of peroxyl radical scavengers in plasma of diabetic and control children.

	Control subjects ($n = 36$)	Diabetic patients ($n = 30$)
TRAP (μmol/l)	2269.7 ± 33.9	2123.9 ± 41.8**
Sulphydryl groups (μmol/l)	433.9 ± 9.7	355.9 ± 13.1***
α-Tocopherol (μmol/l)	24.41 ± 1.08	23.18 ± 0.86
α-Tocopherol/(TG + C)(μM/mM)	4.91 ± 0.14	4.42 ± 0.16*

Data are given as mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$.

The extent of lipid peroxidation depends on at least three factors: ROS generation, presence of oxidisable substrates and antioxidant activity. Hyperglycaemia may cause ROS overproduction and activation of oxidative stress through auto-oxidative glycosylation, non-enzymatic glycation of proteins, increased polyol pathway, activation of NAD(P)H oxidases, nitric oxide synthase and xanthine oxidase [5–10]. However, increased free radical generation may merely reflect homeostatic mechanisms or changes that are not toxic to the cell. This does not appear to occur in recently-diagnosed diabetic children, in whom hyperglycaemia-induced metabolic disorders seem to trigger very early on the sequence of oxidative reactions leading to excessive intra- and extracellular MDA formation, which reflects systemic lipid peroxidation and supports other results previously reported by us [11]. Increased MDA concentrations in serum and erythrocytes, being a marker of oxidative degradation of lipids, represent a reliable index of free radical attack with potentially cytotoxic consequences since oxidised lipids have wide-ranging effects on membrane function and cell metabolism. Erythrocytes, the cell type characterised by the highest oxidative stress sensitivity, are continuously exposed to high oxygen concentrations and pro-oxidative action of iron from haemoglobin. As mature human erythrocytes have no nucleus or other organelles, the plasma membrane, containing a high proportion of polyunsaturated fatty acids, is the critical target for oxidative injury. In this study, erythrocytic MDA, showed an evident increase in diabetic children. This finding had not been previously reported in these patients, but is wholly consistent with the above results of raised plasma MDA since, by separate analysis of extracellular and erythrocyte MDA, our data demonstrate that, in recently-diagnosed diabetic patients, red blood cells and surrounding blood plasma have already suffered peroxidative damage, thereby indicating that oxygen radical activity has been produced and may cause several cell alterations through direct oxidisation of cellular macromolecules. The increase in erythrocyte lipid peroxide content could be related to our finding of a 30% decline in plasma uric acid values in this group of newly-diagnosed diabetic children and to the significant protective action of urate against oxidative damage of plasma lipids and erythrocyte membrane ghosts [22,34,35]. This association could also be supported by the inverse relationship found between uric acid and MDA, thereby confirming the salient role of urate in antioxidant defence [36] and the possible pathophysiologic consequences of hypouricaemia in type 1 diabetic children.

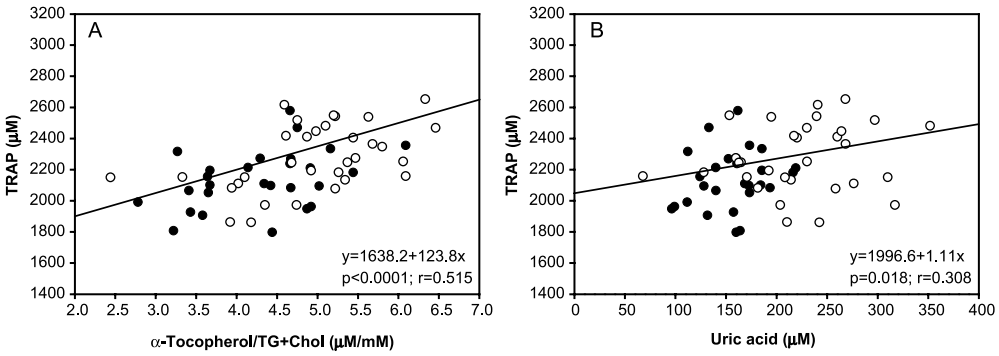

Figure 5. Correlations between plasma TRAP and α-tocopherol/total lipids (A), plasma TRAP and uric acid (B), in diabetic patients at disease onset (●) and in controls (○).

Dyslipidaemia is common in diabetes and, via oxidation of susceptible lipids, may contribute to increased rates of lipid peroxidation reactions that give rise to a great diversity of aldehydes, some of which are highly reactive and may be considered as toxic second messengers which disseminate and augment initial free radical events [37]. The findings of this study permit a direct relationship, hitherto undescribed, to be established between circulating peroxidable lipids and plasma lipid peroxidation products only in the diabetic patient group, while no correlation was found between these parameters in healthy controls. Lipids, the substrate under greatest attack by free radicals, have been the most extensively studied and in diabetic patients are of special interest since their hyperlipidaemia (excess of substrate) is considered to be a significant risk factor for the later development of microvascular complications [7,38].

Lipid abnormalities, oxidative modification of low-density lipoproteins (LDLs) and "response to injury" are the fundamental mechanisms proposed as being involved in the pathogenesis of atherosclerosis [39–42], the development and progression of which are accelerated in diabetes. In this study, lipid susceptibility to oxidation of unfractionated serum samples from diabetic children was measured *in vitro* to identify possible oxidative mechanisms whereby plasma lipoproteins become atherogenic. Plasma oxidizability, the assay employed to characterise the oxidation of all major plasma lipoproteins [30], was found to be significantly higher in our diabetic children at onset of the disease and this measurement correlated positively with the sum of concentrations of the major oxidisable lipids, cholesterol and triglycerides, thereby supporting the potential role of increased circulating lipid levels in producing oxidants. These results strongly point to diabetes-related hyperlipidaemia as a major source of oxidised lipids in the blood of young diabetic patients which would potentiate the progression of atherogenesis and endothelial dysfunction from the early stages of diabetes and thereafter. However, disturbed blood lipid levels in diabetes may not be the only aetiological factor involved in oxidative injury since, as has been demonstrated, lipotoxicity requires hyperglycaemia to exert harmful effects, whereas glucose toxicity can occur in the absence of hyperlipidaemia [43]. A recent study by our group [44] also supports the concept of glucose toxicity and lipotoxicity being interrelated, since we found a significant rise in plasma peroxidative markers in gestational diabetes compared with those obtained in pregnant women with abnormal blood lipid levels, which would suggest that oxidative damage, to the extent it can be attributable to hyperlipidaemia, occurs to a greater degree in the context of hyperglycaemia.

The degree of oxidative stress and severity of subsequent endothelial damage might depend on the imbalance between an excess in ROS production and the individual antioxidant defence systems. This study showed plasma thiol groups to be susceptible to oxidative damage since levels in children with recent-onset type 1 diabetes were low. It is worth noting that plasma thiol compounds have already suffered a partial loss in early stages of the disease. These compounds, among other redox-sensitive molecules, play a significant role in the cell by minimising the deleterious consequences of oxygen activation processes. Essentially, all plasma SH groups are protein-associated and as albumin is the most abundant plasma protein, it would lose in part its extracellular antioxidant power; therefore, decreases found in thiols could also be considered very early products of protein oxidation [31].

Increased lipid peroxidation raises the need for lipid-soluble antioxidants, such as tocopherols and carotenoids. Vitamin E, mainly α-tocopherol, is the major peroxyl radical scavenger in biological lipid phases such as membranes or LDLs. Its antioxidant action has been ascribed to its ability to chemically act as a lipid-based free radical chain-breaking molecule, thereby inhibiting lipid peroxidation and oxidised LDL formation and protecting the organism against oxidative damage. Plasma α-tocopherol levels in our young patients with type 1 diabetes did not differ from those of the controls; nevertheless, when the values were normalised by total lipids, the ratio was significantly decreased in the diabetic children. Close correlations exist between plasma vitamin E and lipid concentrations owing to

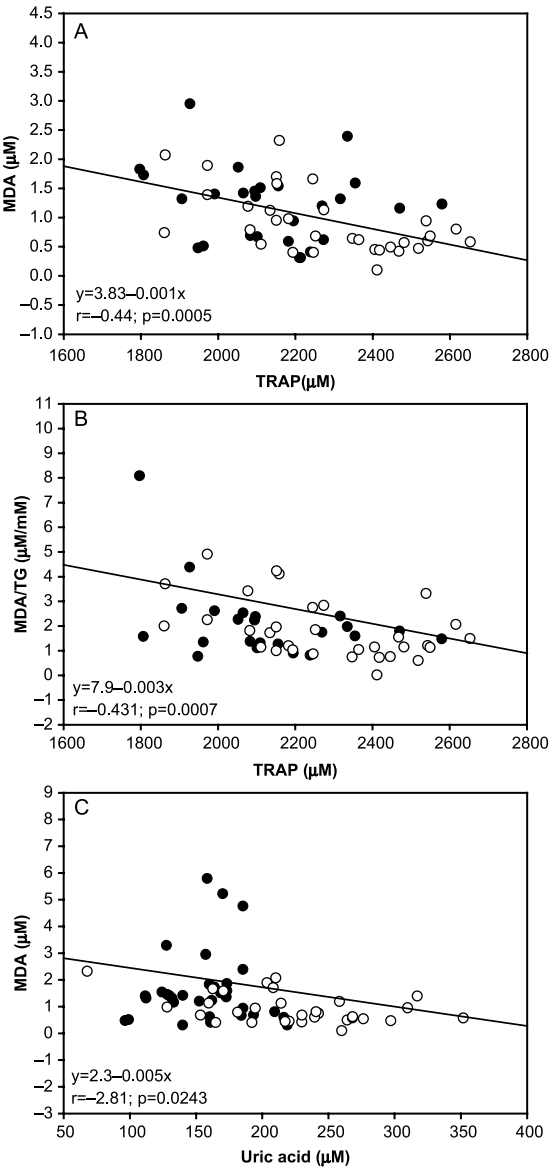

Figure 6. Correlations between plasma malondialdehyde (MDA) and TRAP (A), plasma malondialdehyde/total lipids and TRAP (B) and plasma malondialdehyde and uric acid (C) in diabetic patients at disease onset (●) and in controls (○).

the lipophilic properties of vitamin E and since metabolic disorders in diabetic patients are known to imply a certain degree of hyperlipidaemia, α-tocopherol values must be standardised by plasma lipid concentrations to ascertain whether differences exist between diabetic and nondiabetic groups [45]. This relative decrease in plasma α-tocopherol may therefore be attributed to its consumption while scavenging free radicals in biomembranes or lipoproteins. The role of vitamin E in diseases involving lipid oxidation and endothelial damage, such as diabetes-induced atherosclerosis, has been widely studied although contradictory results concerning plasma vitamin E in diabetes have been reported. Thus, vitamin E levels in plasma of diabetic patients have been reported to be significantly increased in both types of diabetes and statistically correlated with blood cholesterol, apoprotein B and triglycerides [46,47], and significantly lowered [48,49], or even tending to be lower in insulin- and non-insulin-dependent diabetic patients [11,50]. Intervention studies have shown that vitamin E treatment could reduce protein glycation in diabetic patients [51] or afford cardiovascular protection in diabetes [52]. It has recently been reported that vitamin E supplementation increases cellular glutathione, lowers HbA1c and restores MDA to normal concentrations in erythrocytes of type 1 diabetic children [53].

Human plasma protection against free radical injury is offered by a wide spectrum of antioxidants with synergic action so that individual measurements of antioxidant concentrations in blood do not always reflect the level of antioxidant status. Extracellular fluids have different protective mechanisms from the intracellular environment and, though poorly protected by antioxidant enzymes, contain various antioxidants which, by their very presence at a much lower concentration than an oxidisable substrate, delay or inhibit peroxidation processes [54], thus protecting the vascular endothelium against oxidative injury. An assay that detects the total peroxyl radical-trapping antioxidant activity (TRAP) of serum was used to measure chain-breaking antioxidants as a whole. The directly-measured TRAP value was decreased in sera of diabetic children probably due to the low levels of antioxidants such as uric acid, sulphydryl compounds and to insufficient α-tocopherol content for the hyperlipidaemia of diabetic patients, which points to an inadequate capacity of plasma to protect its environment from free radical aggression in newly-diagnosed diabetic children and might account for the lack of maintenance of the overall redox network in plasma of these patients. The disappearance of plasma endogenous antioxidants in our newly-diagnosed diabetic patients concurs with the rate of changes reported elsewhere in plasma antioxidant concentrations during induced peroxyl radical attack, which showed the first line of defence to be provided by the plasma sulphydryl groups, followed in turn by complete consumption of ascorbate and the partial depletion of bilirubin, alpha-tocopherol and urate, which is spared during the initial stages of the reaction [55]. At this point, uric acid merits a special mention since it has been proposed as a powerful antioxidant molecule and, being low in plasma of this group of diabetic children at levels 30% lower than in plasma of healthy controls, would appear to not confer the physiologic protection expected against free radical activity. Persistent hypouricaemia is a usual finding attributed to glomerular hyperfiltration produced in the first years of type 1 diabetes that would contribute to lowering serum uric acid by increasing renal urate clearance [56–58]. Though few reports exist, hypouricaemia and hyperuricosuria

have been described in childhood diabetes [59] which concurs with the findings of this study in which serum uric acid levels, despite acceptable maintenance of metabolic control, were already found to be low in the early stages of diabetes due, at least in part, to an osmotic diuretic effect of hyperglycaemia leading to excessive uric acid excretion. Given the potential protective physiologic function of uric acid in free radical-scavenging processes *in vivo* together with the hypouricaemia produced in diabetic children, the possible consequences of this pathophysiologic situation in childhood diabetes should not be overlooked.

The results of this study demonstrate that, already at clinical onset, diabetes is associated with a state of oxidative damage demonstrated by the systemic presence of increased lipid peroxidation markers and by decreased antioxidant activity. The concentration of antioxidants in human blood plasma appears to be central to the investigation and understanding of the relationship between oxidative stress and human disease. Modifications in techniques developed in this study permitted, with a small blood sample, sensitive and accurate assessment of a wide panel of antioxidants that include TRAP and determinations of the antioxidants α-tocopherol, protein sulphydryl groups and uric acid; furthermore, the more representative markers of oxidative damage to biomolecules can be reliably measured. Some of the molecular damage products, which are markers of oxidative stress, are reported for the first time in this initial stage of childhood diabetes. This work clearly enabled us to demonstrate increased systemic lipid peroxidation and reduced antioxidant activity, evaluated both globally and through individual antioxidants, in our cohort of young diabetic patients, which fuels the idea that in its early phases the pathogenic mechanisms of type 1 diabetes involve higher oxidative status.

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References

[1] Sies H. Oxidative stress: Oxidants and antioxidants. Exp Physiol 1997;82:291–295.
[2] Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine. 3rd ed. Oxford: Clarendon Press; 1999.
[3] Halliwell B, Gutteridge JM, Cross CE. Free radicals, antioxidants, and human disease: Where are we now? J Lab Clin Med 1992;119:598–620.
[4] Halliwell B. Free radicals, antioxidants, and human disease, cause, or consequence? Lancet 1994;344:721–724.
[5] Wolff SP, Dean RT. Glucose autooxidation and protein modification. The potential role of "autoxidative glycosylation" in diabetes. Biochem J 1987;245:243–250.
[6] Brownlee M, Cerami A, Vlassara H. Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. N Engl J Med 1988;318:1315–1321.
[7] Inoguchi T, Li P, Umeda F, Yu HY, Kakimoto M, Imamura M, Aoki T, Etoh T, Hashimoto T, Naruse M, Sano H, Utsumi H, Nawata H. High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C-dependent activation of NAD(P)H oxidase in cultured vascular cells. Diabetes 2000;49:1939–1945.
[8] Cosentino F, Hishikawa K, Katusic ZS, Luscher TF. High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. Circulation 1997;96:25–28.
[9] Desco MC, Asensi M, Márquez R, Martínez-Valls J, Vento M, Pallardó F, Sastre J, Viña J. Xanthine oxidase is involved in free radical production in type 1 diabetes: Protection by allopurinol. Diabetes 2002;51:1118–1124.
[10] Williamson JR, Chang K, Frangos M, Hasan KS, Ido Y, Kawamura T, Nyengaard JR, van denEnden M, Kilo C, Tilton RG. Hyperglycemic pseudohypoxia and diabetic complications. Diabetes 1993;42:801–813.
[11] Domínguez C, Ruíz E, Gussinyé M, Carrascosa A. Oxidative stress at onset and in early stages of Type I Diabetes in children and adolescents. Diabetes Care 1998;21:736–742.
[12] Ceriello A, Morocutti A, Mercuri F, Quagliaro L, Moro M, Damante G, Viberti GC. Defective intracellular antioxidant enzyme production in type 1 diabetic patients with nephropathy. Diabetes 2000;12:2170–2177.
[13] Nielsen F, Mikkelsen BB, Nielsen JB, Anderson HR. Plasma malondialdehyde as biomarker for oxidative stress: Reference interval and effects of life-style factors. Clin Chem 1997; 43:1209–1214.
[14] Slatter DA, Bolton CH, Bailey AJ. The importance of lipid-derived malondialdehyde in diabetes mellitus. Diabetologia 2000;43:550–557.
[15] Pryor WA, Stanley JP, Blair E. Autoxidation of polyunsaturated fatty acids: II. A suggested mechanism for the formation of TBA-reactive materials from prostaglandin-like endoperoxides. Lipids 1976;11:370–379.
[16] Yagi K. Assay for blood plasma or serum. Methods Enzymol 1984;105:328–331.
[17] Conti M, Morand PC, Levillain P, Lemonnier A. Improved fluorometric determination of malondialdehyde. Clin Chem 1991;37:1273–1275.
[18] Janero DR. Malondialdehyde and thiobarbituric acid-reacti vity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radic Biol Med 1990;9:515–540.
[19] Knight JA, Pieper RK, McClellan L. Specificity of the thiobarbituric acid reaction: Its use in studies of lipid peroxidation. Clin Chem 1988;34:2433–2438.
[20] Esterbauer H, Lang J, Zadravec S, Slater T. Detection of malonaldehyde by high-performance liquid chromatography. Methods Enzymol 1984;105:319–328.
[21] Draper HH, Squires EJ, Mahmoodi H, Wu J, Agarwal S, Hadley M. A comparative evaluation of thiobarbituric acid methods for the determination of malondialdehyde in biological materials. Free Radic Biol Med 1993;15:353–363.
[22] Wayner DDM, Burton GW, Ingold KU, Locke S. Quantitative measurement of the total peroxyl radical-trapping antioxidant capacity of human blood plasma by controlled peroxidation. FEBS Lett 1985;187:33–37.
[23] Glazer AN. Phycoerythrin fluorescence-based assay for reactive oxygen species. Methods Enzymol 1990;186: 161–168.
[24] Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, Milner A. A novel method for measuring antioxidant capacity and its

application to monitoring the antioxidant status in premature neonates. Clin Sci 1993;84:407–412.
[25] Cao G, Alessio HM, Cutler RG. Oxygen-radical absorbance capacity assay for antioxidants. Free Radic Biol Med 1993;14:303–311.
[26] Ghiselli A, Serafini M, Maiani G, Azzini E, Ferro-Luzzi A. A fluorescence-based method for measuring total plasma antioxidant capability. Free Radic Biol Med 1995; 18:29–36.
[27] Chapple IL, Mason GI, Garner I, Matthews JB, Thorpe G, Maxwell SR. Enhanced chemiluminescent assay for measuring the total antioxidant capacity of serum, saliva and crevicular fluid. Ann Clin Biochem 1997;34:412–421.
[28] Fukunaga K, Suzuki T, Takama K. Highly sensitive high-performance liquid chromatography for the measurement of malondialdehyde in biological samples. J Chromatogr 1993;621:77–81.
[29] Jordan P, Brubacher D, Moser U, Stähelin B, Gey KF. Vitamin E and vitamin A concentrations in plasma adjusted for cholesterol and triglycerides by multiple regression. Clin Chem 1995;41:924–927.
[30] Kontush A, Beisiegel U. Measurement of oxidizability of blood plasma. Methods Enzymol 1999;299:35–49.
[31] Hu M-L. Measurement of protein thiol groups and glutathione in plasma. Methods Enzymol 1994;233:380–385.
[32] Diabetes Control and Complications Trial Research Group The effect of intensive treatment of diabetes on the development of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med 1993;329:977–986.
[33] Packer L, Rösen P, Tritschler HJ, King GL, Azzi A. Antioxidants in Diabetes Management. New York, USA: Marcel Dekker, Inc; 2000.
[34] Frei B, Stocker R, Ames BN. Antioxidant defenses and lipid peroxidation in human blood plasma. Proc Natl Acad Sci USA 1988;85:9748–9752.
[35] Ames BN, Cathcart R, Schwiers E, Hochstein P. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: A hypothesis. Proc Natl Acad Sci USA 1981;78:6858–6862.
[36] Becker BF. Towards the physiological function of uric acid. Free Radic Biol Med 1993;14:615–631.
[37] Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med 1991;11:81–128.
[38] Baynes JW, Thorpe SR. Glycoxidation and lipoxidation in atherogenesis. Free Radic Biol Med 2000;28:1708–1716.
[39] Goldstein JL, Ho YK, Basu SK, Brown MS. Binding site on macrophages that mediates uptake and cholesterol deposition. Proc Natl Acad Sci USA 1979;76:333–337.
[40] Solberg LA, Strong JP. Risk factors and atherosclerotic lesions. A review of autopsy studies. Arteriosclerosis 1983; 3:187–198.
[41] Parthasarathy S, Steinberg D, Witztum JL. The role of oxidized low-density lipoproteins in the pathogenesis of atherosclerosis. Ann Rev Med 1992;43:219–225.
[42] Kutuk O, Basaga H. Inflammation meets oxidation: NF-kappaB as a mediator of initial lesion development in atherosclerosis. Trends Mol Med 2003;9:549–557.
[43] Robertson R, Harmon J, Tran PO, Poitout V. Beta-cell glucose toxicity, lipotoxicity, and chronic oxidative stress in type 2 diabetes. Diabetes 2004;53:S119–S124.
[44] Llurba E, Gratacós E, Martin-Gallán P, Cabero Ll, Domínguez C. A comprehensive study of oxidative stress and antioxidant status in pre-eclampsia and normal pregnancy. Free Radic Biol Med 2004;37:557–570.
[45] Gross M, Yu X, Hannan P, Prouty C, Jacobs Jr, DR. Lipid standardization of serum fat-soluble antioxidant concentrations: The YALTA study. Am J Clin Nutr 2003;77: 458–466.
[46] Krempf M, Ranganathan S, Ritz P, Morin M, Charbonnel B. Plasma vitamin A and E in type 1 (insulin-dependent) and type 2 (non-insulin-dependent) adult diabetic patients. Int J Vitam Nutr Res 1991;61:38–42.
[47] Asayama K, Uchida N, Nakane T, Hayashibe H, Dobashi K, Amemiya S, Kato K, Nakazawa S. Antioxidants in the serum of children with insulin-dependent diabetes mellitus. Free Radic Biol Med 1993;15:597–602.
[48] Sundaram RK, Bhaskar A, Vijayalingam S, Viswanathan M, Mohan R, Shanmugasundaram KR. Antioxidant status and lipid peroxidation in type II diabetes mellitus with and without complications. Clin Sci 1996;90:255–260.
[49] Maxwell SR, Thomason H, Sandler D, Leguen C, Baxter MA, Thorpe GH, Jones AF, Barnett AH. Antioxidant status in patients with uncomplicated insulin-dependent and non-insulin-dependent diabetes mellitus. Eur J Clin Investig 1997;27:484–490.
[50] Nourooz-Zadeh J, Tajaddini-Sarmadi J, McCarthy S, Betteridge DJ, Wolff SP. Elevated levels of authentic plasma hydroperoxides in NIDDM. Diabetes 1995;44:1054–1058.
[51] Ceriello A, Giugliano D, Quatraro A, Donzella C, Dipalo G, Lefebvre PJ. Vitamin E reduction of protein glycosylation in diabetes. Diabetes Care 1991;14:68–72.
[52] Gazis A, Page S, Cocjcroft J. Vitamin E and cardiovascular protection in diabetes. Br Med J 1997;314:1845–1846.
[53] Jain SK, McVie R, Smith T. Vitamin E supplementation restores glutathione and malondialdehyde to normal concentrations in erythrocytes of type 1 diabetic children. Diabetes Care 2000;23:1389–1394.
[54] Halliwell B, Gutteridge JM. The antioxidants of human extracellular fluids. Arch Biochem Biophys 1990;280:1–8.
[55] Frei B, Stocker R, Ames BN. Antioxidant defenses and lipid peroxidation in human blood plasma. Proc Natl Acad Sci USA 1988;85:9748–9752.
[56] Herman JB, Goldbourt U. Uric acid and diabetes: Observations in a population study. Lancet 1982;2(8292):240–243.
[57] Shichiri M, Iwamoto H, Marumo F. Diabetic hypouricemia as an indicator of clinical nephropathy. Am J Nephrol 1990;10:115–122.
[58] Marra G, Cotroneo P, Pitocco D, Manto A, Di Leo MA, Ruotolo V, Caputo S, Giardina B, Ghirlanda G, Santini SA. Early increase of oxidative stress and reduced antioxidant defenses in patients with uncomplicated type 1 diabetes: A case for gender difference. Diabetes Care 2002;25:370–375.
[59] Dura Trave T, Moya Benavent M, Casero Ariza J. Renal hypouricemia in juvenile diabetes mellitus. An Esp Pediatr 1996;44:425–428.