

Full Length Research Paper

Elevation of oxidative stress markers in Type 1 diabetic children

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Children's diabetes is represented by the Type 1 diabetes mellitus (T1DM). In T1DM, the persistence of hyperglycemia has been reported to cause increased production of oxygen free radicals through glucose autooxidation and nonenzymatic glycation. The aim of this study was to evaluate markers of oxidant/antioxidant status in diabetic children of Western Algeria. This study included 40 children with T1DM with mean age of 7.5 ± 1.7 years and 40 healthy age and sex matched controls. They were subjected to assessment of indicative parameters of lipoperoxidation, protein oxidation, changes in the status of antioxidant defense systems, plasma oxygen radical absorbance capacity (ORAC), glycated hemoglobin (HbA1c), total cholesterol and triglycerides. Malondialdehyde (MDA) and carbonyl proteins levels in plasma were significantly higher (4.03 ± 0.39 versus 2.53 ± 0.4 $\mu\text{mol/L}$, 5.03 ± 0.57 versus 3 ± 0.38 nmol/mg protein, respectively; $P < 0.001$) and a significant reduction in plasma total antioxidant capacity and vitamin C was observed in diabetic children than the controls (1.55 ± 0.28 versus 2.5 ± 0.23 AU, 37.58 ± 5.76 versus 48.8 ± 4.47 $\mu\text{mol/L}$, respectively; $P < 0.001$). Erythrocyte superoxide dismutase (SOD) and catalase (CAT) activities were significantly higher (520 ± 40.42 versus 392.7 ± 42.66 U/g hemoglobin, 71.08 ± 5.18 versus 56.6 ± 2.84 U/g hemoglobin, respectively; $P < 0.001$), whereas erythrocyte glutathione reductase (GSH) reduced significantly (34.98 ± 2.34 versus 42.68 ± 3.03 U/g hemoglobin, respectively; $P < 0.001$) in diabetic children than the control subject. The present finding suggested that young diabetic patients were susceptible to oxidative stress. Appropriate support for enhancing antioxidant supply in these patients may help prevent complications due to oxidative injury.

Key words: Children, oxidative stress, Type 1 diabetes mellitus.

INTRODUCTION

Under normal physiological conditions, there is a critical balance in the generation of oxygen free radicals and

antioxidant defense systems used by organisms to deactivate and protect themselves against free radical

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toxicity (Halliwell and Whiteman, 2004; Sies, 1991). Impairment in the oxidant/antioxidant equilibrium creates a condition known as oxidative stress. Oxidative stress is known to be a component of molecular and cellular tissue damage mechanisms in a wide spectrum of human diseases (Dalle-Donne et al., 2006; Halliwell and Gutteridge, 1999).

Children's diabetes also called Type 1 diabetes mellitus (T1DM) today, was formerly represented by the insulin dependent diabetes. It is a chronic autoimmune disease caused by the specific destruction of pancreatic β cells (Maahs and Rewers, 2006). Diabetes represents one of the most common diseases in school-aged children (Linder, 2014). The increased incidence of the disease in toddlers suggests the influence of the environment of which dietary factors, enterovirus infections, but not vaccines, have been implicated. However, no single factor can be incriminated in the rapid increase of incidence observed in young children (Penno et al., 2013). Extensive studies have focused on the role of the immune system in the development of T1DM, from the initiation of disease to eventual β cells destruction (Beyan et al., 2003; Thomas and Kay, 2000). Some studies have shown that oxidative stress leads to the destruction of pancreatic islets, either by necrosis or apoptosis of β cells (Bonnefont-Rousselot, 2002). Indeed, diabetic patients are exposed to increased oxidative stress due to several mechanisms, including glucose autooxidation and non-enzymatic protein glycation (Sakurai and Tsuchiya, 1988; Wolff, 1993). Nonenzymatic glycation is a spontaneous chemical reaction between glucose and the amino groups of proteins in which reversible Schiff bases and more stable Amadori products are formed (Vlassara, 1994). Advanced glycation end products (AGEs) are then formed through oxidative reactions and cause irreversible chemical modifications of proteins. Chronic hyperglycemia also leads to activation of nicotinamide adenine dinucleotide phosphate-oxidase (NADPH)-dependent aldose reductase (polyol pathway), which diminishes the NADPH available for glutathione reductase (GSH); consequently, the ratio of reduced to oxidized glutathione decreases (Ou et al., 1996).

A variety of natural antioxidants exist to scavenge oxygen free radicals and prevent oxidative damage to biological membranes. One group of these antioxidants is enzymatic (intracellular), which include superoxide dismutase (SOD), glutathione peroxidase and catalase (CAT). In addition to enzymatic antioxidants, the major natural antioxidants, most of them derived from natural sources by dietary intake are vitamin A, vitamin C, vitamin E and carotenoids. Also, numerous small molecules are synthesized or produced within the body that has antioxidant capacity (Azen et al., 1996; Halliwell and Gutteridge, 1999; Heistad, 2006; Maritim et al., 2003). These non-enzymatic antioxidants act as terminators of free radicals' chain reactions caused by lipid peroxidation (Halliwell and Gutteridge, 1999).

As both free-radical production and antioxidant defenses may be disturbed in diabetes (Lyons, 1991), it has been suggested that oxidative stress may be partly responsible for the development of diabetic complications (Baynes, 1991). Consistent with this, oxidative stress has been implicated in the pathogenesis of T1DM in several studies (Jain, 1989; Sato et al., 1979). Increased levels of lipid peroxidation products and altered antioxidative enzyme activity were also reported in type 2 diabetes mellitus (T2 DM) (Kaji et al., 1985).

To our knowledge, there are a few data regarding the relationship between the diabetes in children and oxidant/antioxidant status. It was, therefore, thought worthwhile to undertake a study in order to evaluate markers of oxidant/antioxidant status in diabetic children in Western Algeria.

METHODOLOGY

Patients

This cross-sectional study was conducted at the Department of Pediatrics of the Hospital of Tlemcen, Algeria, from April 2011 to February 2012. It included 40 patients with T1DM diagnosed in accordance with the criteria for classification and diagnosis of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (1997). They were 22 males and 18 females. Their ages ranged from 5 to 10 years with a mean age of 7.5 ± 1.77 years. Forty age and sex matched healthy individuals were included as the control group. They were 21 males and 19 females. Their age ranged from 6 to 10 years with mean age of 7.7 ± 1.33 years. Most diabetic children in this study present with severe symptoms, very high blood glucose levels, marked glycosuria, and ketonuria. The diagnosis is confirmed without delay by blood glucose measurements, and treatment is initiated immediately, often as a life-saving measure.

Care was taken to ensure the parity of the subjects. The characteristics of both groups are as shown in Table 1. An informed consent has been completed and signed by the parents of all subjects and the study was approved by the Scientific Committee on Research Involving Human Subjects of Tlemcen Hospital.

Blood samples

Fasting venous blood samples were collected in heparinized tubes, centrifuged and plasma was separated for glucose, vitamin C, the oxygen radical absorbance capacity (ORAC), malondialdehyde (MDA) and carbonyl proteins determinations. The remaining erythrocytes were washed three times in isotonic saline, hemolysed by the addition of cold distilled water (1/4) and the cell debris was removed by centrifugation (2000 g, 15 min). The hemolysates were assayed for antioxidant enzyme activities.

Biochemical determination

Plasma glucose was determined by glucose oxidase method using a glucose analyzer (Beckman Instruments, Fullerton, CA, USA). Markers of diabetes control were evaluated on regular medical check-ups of diabetic children; they were represented by the determination of glycated hemoglobin (HbA1c) using isolab column chromatography (Kaplan et al., 1982).

Lipid determination

Plasma triglyceride and total cholesterol contents were determined by enzymatic methods (Kits from Sigma).

Scavenging capacity of plasma

Plasma ORAC was measured by a fluorimetric method (Cao et al., 1993). A fluorescent protein, allophycocyanin (APC) was used in this assay (Courderot-Masuyer et al., 2000). ORAC employs the oxidative loss of the intrinsic fluorescence of APC. APC fluorescence decay shows a lag or retardation in the presence of antioxidants, related to the antioxidant capacity of the sample. The reaction mixture (2 ml) contained a final concentration of 37.5 nmol/L APC in 75mmol/L phosphate buffer (pH 7.0) at 37°C in the absence (blank) or presence of 20 µl of trolox (1 µmol/L) or plasma, respectively. The reaction was initiated by the introduction of 9 µmol/L of CuSO₄ and 0.3% H₂O₂ as redox catalysts. This reaction was followed spectrophotometrically by the decrease in fluorescence at 651 nm emission and 598 nm excitation, using a spectrofluorometer SFM25 Kontron. Trolox was used as a reference antioxidant for calculating the ORAC values, with one ORAC unit defined as the net protection area provided by 1 µmol/L final concentration of trolox. ORAC value of the samples was calculated as:

$$\text{ORAC} = ((A \text{ sample} - A \text{ blank}) / (A \text{ trolox} - A \text{ blank})),$$

where A refers to the area under the quenching curve of APC.

Determinations of plasmatic levels of vitamin C

Vitamin C levels were determined in plasma using a biochemical method (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°C for 3 h. Following the addition of 750 µl of 65% (v/v) sulfuric acid, the absorbency was recorded at 520 nm.

Determination of plasma MDA

The MDA level, a marker of lipid peroxidation, was determined in plasma by the reaction of MDA with thiobarbituric acid at 95°C (Ohkawa et al., 1979).

Determination of plasma carbonyl proteins

Plasma carbonyl proteins (marker of protein oxidation) were evaluated following the 2,4-Dinitrophenylhydrazine assay (Levine et al., 1990).

Determinations of erythrocyte antioxidant enzyme activities

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 hemoglobin. CAT (EC 1.11.1.6) activity was measured by spectrophotometric analysis of the rate of hydrogen peroxide decomposition at 240 nm (Aebi et al., 1974). Enzyme activity was expressed as U/g hemoglobin. 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.

Statistical analysis

Values are presented as means ± standard deviation (SD). Statistical analysis of the data was carried out using STATISTICA (version 4.1, Statsoft, Tulsa, OK). The significance of the differences between two groups was determined by Student's t-test. Correlations between parameters were performed by Pearson coefficient. A value of P < 0.05 was considered to be statistically significant.

RESULTS

Clinical and biochemical parameters

The results showed no significant difference in the mean age, body mass index (BMI) between diabetic children with their controls (Table 1).

As expected, fasting glucose and HbA1c were significantly higher in diabetic children compared with controls (Table 1). Diabetic patients demonstrated significantly higher plasma levels of total cholesterol, and triglycerides compared with their controls (Table 1).

Markers of oxidative stress

Plasma ORAC and vitamin C were statistically lower in diabetic children when compared with controls; while MDA and carbonyl proteins were significantly higher in diabetic children when compared with controls (Table 2). The erythrocyte antioxidant enzyme activity of CAT and SOD were significantly higher in diabetic children when compared with controls; however, GSH was significantly lower in diabetic children (Table 3).

Correlations between SOD and oxidative stress parameters

Table 4 showed correlation coefficients between SOD and oxidative stress biomarkers in the diabetic children. In the diabetic children, SOD activity was positively correlated with carbonyl proteins (p < 0.001), ORAC (p < 0.01), vitamin C (p < 0.001), and GSH (p < 0.05). In the control group, correlations between SOD and these parameters were not significant (results not shown).

DISCUSSION

The present study examined the changes in oxidant/antioxidant status in a children suffering from T1DM. Several studies have shown that chronic hyperglycemia induces an increase in oxidative stress in

Table 1. Clinical and biochemical characteristics of diabetic and control groups.

Characteristic	Control	Diabetic	P
Number	40	40	-
Age (years)	7.7 ± 1.33	7.5 ± 1.77	NS
BMI (kg/m ²)	18.61 ± 1	18.81 ± 1.11	NS
Diabetes duration (years)	-	2.84 ± 0.76	-
Fasting glucose (mmol/L)	4.78 ± 0.56	9.83 ± 2.22	<0.001
Glycated hemoglobin (%)	5.7 ± 0.67	10.14 ± 1.6	<0.001
Total cholesterol (mmol/L)	3.66 ± 0.41	4.07 ± 0.59	NS
Triglycerides (mmol/L)	1.18 ± 0.10	1.36 ± 0.17	<0.01

Values are presented as means ± standard deviations (SD). BMI: Body mass index (weight/height²). NS: Not significant.

Table 2. Serum oxidative stress markers in diabetic and control group.

Makers	Control	Diabetic	P
ORAC (Arbitrary units)	2.50 ± 0.23	1.55 ± 0.28	<0.001
Vitamin C (µmol/L)	48.8 ± 4.47	37.58 ± 5.76	<0.001
MDA (µmol/l)	2.53 ± 0.40	4.03 ± 0.39	<0.001
Carbonyl proteins (nmol/mg protein)	3.00 ± 0.38	5.03 ± 0.57	<0.001

Values are presented as means ± standard deviations (SD). ORAC: Plasma oxygen radical absorbance capacity; MDA: malondialdehyde

Table 3. Erythrocyte antioxidant enzyme activities in diabetic and control group.

Enzyme activity	Group 1	Group 2	P
SOD (U/g hemoglobin)	392.7 ± 42.66	520 ± 40.42	<0.001
CAT (U/g hemoglobin)	56.60 ± 2.84	71.08 ± 5.18	<0.001
GSH (U/g hemoglobin)	42.68 ± 3.03	34.98 ± 2.34	<0.001

Values are presented as means ± standard deviations (SD). SOD: Superoxide dismutase; CAT: catalase; GSH: reduced glutathione.

Table 4. Correlation coefficients (Pearson's) between SOD and oxidative stress parameters in diabetic children.

Parameter	r
MDA (µmol/L)	-.016
Carbonyl proteins (nmol/mg protein)	0.42***
ORAC (Arbitrary units)	0.40**
Vitamin C (µmol/L)	0.47***
GSH (U/g hemoglobin)	0.34*
CAT (U/g hemoglobin)	0.22

Values represent correlation coefficients (r). MDA: Malondialdehyde; ORAC: plasma oxygen radical absorbance capacity; GSH: reduced glutathione; CAT: catalase. Statistically significant: *P < 0.05; **P < 0.01; ***P < 0.001.

diabetic children (Dominguez et al., 1998; Lin et al., 2014; Mishra and Singh, 2013). Our results are in accordance with those of previous findings which show that increased

glucose level induces overproduction of oxygen free radicals and consequently increases the protein oxidation and lipid oxidation.

Indeed, the plasma concentration of MDA which is a final product of the peroxidation of polyunsaturated fatty acids was increased in patients compared with controls. These results are in agreement with previous studies (Lin et al., 2014; Mishra and Singh, 2013; Varvarovská et al., 2003). Another study demonstrated the elevated concentrations of plasma MDA, 8 days after clinical onset of diabetes when metabolic control had returned to normal; this suggests that oxygen free radicals may already have exerted their cytotoxic effects in the early clinical stage of the disease (Dominguez et al., 1998). Furthermore, in children and adolescents with T1DM, MDA levels continued to rise over the course of the disease, indicating overproduction of free radicals and leading to lipid peroxidation and cell oxidative injury, which is considered by some authors to be related to the development of diabetic complications (Velazquez et al., 1991; Wolff, 1994).

It is important to note that glycemic control plays an important role in peroxidation of fatty acids (Wierusz-Wysocka et al., 1995) and the well-controlled diabetic patients (HbA1c<6.5%) demonstrate a lower level of lipid peroxidation markers (Griesmacher et al., 1995; Jain and McVie, 1999; Vantuyghem et al., 2000). Since the mean HbA1c in the diabetic children was >8%, therefore this can explain the increase in MDA in this study. However, we did not find a statistically significant correlation between any parameter of oxidative stress and diabetes control; similar findings were presented by Varvarovská et al. (2003).

The identification of oxidation proteins was made by assay of carbonyl proteins, whose concentration increased in plasma of diabetic children when compared with control; these results are in agreement with previous studies (Dominguez et al., 1998; Hernández-Marco et al., 2009). Carbonyl group formation is considered an early and stable marker for protein oxidation. Oxidized proteins constitute a substantial fraction of the catalytically inactive or less active forms of enzymes, which may have direct metabolic consequences (Jain and McVie, 1999; Velazquez et al., 1991).

Elevation in pro-oxidant species and peroxidation of lipids and proteins observed in diabetic patients was associated with increase of antioxidant erythrocyte of SOD and CAT activity; we can presume that this increase could be interpreted as a positive feedback mechanism that reflects a favorable response of the organism to oxidative stress. These results are in agreement with previous studies (Dominguez et al., 1998; Zivić, et al., 2008).

The increase in erythrocyte SOD activity found in patients could protect against the elevation of the superoxide anion. In fact, SOD, catalyzing the decomposition of the superoxide anion to hydrogen peroxide (H_2O_2), prevents against eventual generation of free radicals (Yu, 1994). Superoxide radicals are converted by SOD in H_2O_2 which are destroyed by CAT. This process may

result in lipid peroxidation if H_2O_2 is not decomposed immediately (Gumsulu et al., 2002).

The SOD level correlated positively with the carbonyl proteins which suggest that this enzyme is susceptible to glycation and may have its activity inhibited through blockage of the active site itself or by structural alteration which in turn affects the active site. The SOD from the plasma has been shown to be glycated *in vivo*; the proportion of glycated SOD being considerably higher in diabetic patients (Adachi et al., 1991).

GSH is a ubiquitous tripeptide that presents in red cells and participates in glutathione peroxidase (GPx) reaction. When H_2O_2 is detoxified by GPx; the GSH is simultaneously converted to oxidized glutathione (GSSG).

In some studies (Likidilid et al., 2007; Mishra and Singh, 2013; Varvarovská et al., 2003), the authors found that GSH levels in T1DM patients were significantly lower than that in the same age-matched control subjects. These results are in good agreement with results found in this study.

As already mentioned, GSH serves as an essential cofactor for the enzyme GPx and GSSG during the enzyme processes. Thus, increases in GPx activities imply higher consumption of GSH. Other mechanisms that may explain the GSH reductions in diabetes are that the GSH is regenerated by the enzyme GSH, using reducing equivalents from NADPH. The NADPH is generated in red blood cells through the pentose phosphate pathway, which is stimulated by insulin (Wierusz-Wysocka et al., 1995), and in T1DM, NADPH production may be sluggish, probably resulting in lowered GSH activity and reduced GSH recycle.

It was found out that there was a positive correlation between carbonyl proteins and GSH, suggesting that enhanced oxidative stress in diabetes may result in increased protein glutathionylation, having an adverse effect on cellular glutathione levels (Livingstone and Davis, 2007).

The present study has also demonstrated a significantly lower plasma ascorbate in diabetic children compared with their controls; these results are in agreement with previous studies (Ramakrishna and Jaiikhani, 2007).

Because of the relative difficulty in measuring each antioxidant separately, some assays have been designed to measure the plasma ORAC (Cao et al., 1993). ORAC has been found to be a good index of oxidative stress in diabetes mellitus (Merzouk et al., 2004). Our data revealed that the total antioxidant activity (ORAC) decreased in the plasma of children with diabetes in favor of an oxidative stress in such patients. These results are in agreement with previous studies (El Samahy et al., 2013; Varvarovská et al., 2003).

There is a positive correlation between SOD and ORAC. This suggests that the increase in enzymatic activity of SOD in return can induce increase in ORAC. Indeed several studies have considered that improvement

in SOD activity may explain the higher plasma ORAC in diabetic patients. Our results corroborate this hypothesis (Ginty and Conklin, 2012; Lluís et al., 2013).

Conclusively, our results showed an imbalance in the oxidant/antioxidant status in diabetic children. It may be appropriate to evaluate markers of oxidative stress in addition to routine laboratory assessments in evaluation of T1DM pediatric patients. Antioxidant supplementation may be required to reduce oxidative stress and prevent complications of diabetes.

Conflict of interest

The authors have no relevant conflict of interest to disclose.

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