Journal of Applied Sciences Research 5(7): 770-775, 2009

Evaluation of Oxidative Stress and Antioxidant Status in Patients with Diabetes Mellitus

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Abstract: Oxidative stress is currently suggested as mechanism underlying diabetes and diabetic complications. The aim of the study was to evaluate the magnitude of oxidative stress in patients with diabetes by measuring the lipid peroxidation as well as the status of the antioxidant defense system. The study population consisted of 100 subjects (age-matched male only) divided into two groups viz. diabetic (n=50) and healthy controls (n=50). Changes in the levels of thiobarbituric acid reactive substances (TBARS) and antioxidants were determined in diabetic and non-diabetic subjects. The level of plasma TBARS was found to be increased significantly in diabetic patients compared to healthy controls. On the other hand, the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-*S*-transferase (GST), reduced glutathione (GSH), vitamin A, vitamin E and vitamin C were found to be decreased significantly in diabetics when compared to control subjects. We also noticed a marked increase in serum total cholesterol, triglyceride, low-density lipoprotein cholesterol (LDL-C), very low-density lipoprotein cholesterol (VLDL-C), and decrease in high-density lipoprotein cholesterol (HDL-C), total protein and albumin in diabetic patients. Our findings indicate that changes in oxidant and antioxidant equilibrium will have biological and possibly pathological role in the development of secondary complications.

Key words: Diabetes mellitus- Oxidative stress- Lipid peroxidation- Antioxidant status

INTRODUCTION

Diabetes mellitus is the most common endocrine metabolic disorder, affecting about 170 million people worldwide. The fundamental defect in diabetes mellitus is the relative lack of biologically active insulin, which results in the impairment of uptake and storage of glucose and reduced glucose utilization for energy purposes [1]. Defects in glucose metabolizing machinery and consistent efforts of the physiological system to correct the imbalance in glucose metabolism place an over exertion on the endocrine system. Continuing deterioration of endocrine control exacerbates the metabolic disturbances and leads primarily to hyperglycemia [2]. Prolonged exposure to elevated glucose induces both repeated acute changes in intracellular metabolism and cumulative long-term changes in the structure and function of macromolecules [3]. The injurious effects of hyperglycemia are characteristically observed in tissues that are not dependent on insulin for glucose entry into the cell and hence they are not capable of down-regulating glucose transport along with the increase of extracellular glucose concentrations [4].

Free radicals are very reactive chemical species, can cause oxidative injury to the living beings by attacking the macromolecules like lipids, carbohydrates, proteins and nucleic acids. 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 toxicity [5]. 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 [6,7].

Formation of lipid peroxides by the action of free radicals on unsaturated fatty acids has been implicated in the pathogenesis of atherosclerosis and vascular

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diseases [8]. Diabetic patients have an increased incidence of vascular disease, and it has been suggested that free radical activity is increased in diabetes [9,10]. Mechanisms that contribute to increased oxidative stress in diabetes may include not only increased non-enzymatic glycosylation and autoxidative glycosylation but also metabolic stress resulting from changes in energy metabolism, alterations in sorbitol pathway, changes in the level of inflammatory mediators, the status of antioxidant defense systems and localized tissue damage results from hypoxia and ischemic reperfusion injury [11]. Increased levels of the products of oxidative damage to lipids have been detected in serum of diabetic patients, and their presence correlates with the development of complications [12,13]. Hence the present study was undertaken to assess the extent of lipid peroxidation and the status of the antioxidant defense system in patients with diabetes.

MATERIALS AND METHODS

Study Population: The study population consisted of 100 subjects (age-matched male subjects) divided in to two groups viz., diabetic patients (type 2 diabetic subjects; n=50) and healthy controls (n=50). The prospective study was carried out at KG Hospital and Post Graduate Medical Institute, Coimbatore, Tamil Nadu, India, during June 2006 to December 2007. General health characteristics such as age, sex, smoking status, menopausal status, alcohol consumption, and dietary habits, particularly as related to preference were investigated by a self-administered questionnaire.

Sample Collection and Hemolysate Preparation: Blood samples were collected by venous puncture in heparinized tubes and the plasma was separated by centrifugation at 1000 g for 15 min. After the collection of plasma, the buffy coat was removed and the packed cells were washed thrice with cold physiological saline. A known volume of the erythrocytes was lysed with hypotonic phosphate buffer (pH 7.4). The hemolysate was separated by centrifugation at 2,500 g for 10 min at 2°C. Biochemical estimations were carried out immediately.

Biochemical Analysis: Biochemical investigation including blood glucose, $HbA1_C$, urea, creatnine, total protein, albumin, total cholesterol, triglyceride, HDL-C and LDL-C were determined using fully automated clinical chemistry analyzer (Hitachi 912, Boehringer Mannheim, Germany). Serum VLDL-C was calculated according to Friedewald *et al* [15].

Estimation of Lipid Peroxidation: Lipid peroxides were estimated by measurement of thiobarbituric acid reactive substances in plasma by the method of Yagi [16]. The pink chromogen produced by the reaction of thiobarbituric acid with malondialdehyde, a secondary product of lipid peroxidation was estimated. The absorbance of clear supernatant was measured against reference blank at 535 nm.

Assay of Superoxide Dismutase (SOD) and Catalase (CAT): SOD was assayed utilizing the technique of Kakkar et al. [17] based on inhibition of the formation of nicotine amide adenine dinucleotide, phenazine methosulfate and amino blue tetrazolium formazan. A single unit of enzyme was expressed as 50% inhibition of NBT (nitroblue tetrazolium) reduction/min/mg protein.

CAT was assayed colorimetrically at 620 nm and expressed as µmoles of H_2O_2 consumed/min/mg protein as described by Sinha [18]. The reaction mixture (1.5 ml, vol) contained 1.0 ml of 0.01 M phosphate buffer (pH 7.0), 0.1 ml of erythrocyte lysate and 0.4 ml of 2 M H_2O_2. The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3).

Assay of Glutathione Peroxidase (GPx) and Reduced Glutathione (GSH): GPx activity was measured by the method described by Rotruck et al [19]. Briefly, reaction mixture contained 0.2 ml of 0.4 M Tris-HCl buffer pH 7.0, 0.1 ml of 10 mM sodium azide, 0.2 ml of homogenate (homogenized in 0.4 M, Tris-HCl buffer, pH 7.0), 0.2 ml glutathione, 0.1 ml of 0.2 mM H_2O_2. The contents were incubated at 37°C for 10 min. The reaction was arrested by 0.4 ml of 10% TCA, and centrifuged. Supernatant was assayed for glutathione content by using Ellmans reagent (19.8 mg of 5,5'-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate). GSH was determined by the method of Ellman [20]. 1.0 ml of supernatant was treated with 0.5 ml of Ellmans reagent and 3.0 ml of phosphate buffer (0.2 M, pH 8.0). The absorbance was read at 412 nm.

Assay of Glutathione-S-transferase (GST): The GST activity was determined spectrophotometrically by the method of Habig, *et al*[21]. The reaction mixture contained 1.0 ml of 0.3 mM phosphate buffer (pH 6.5), 0.1 ml of 30 mM 1-chloro-2, 4-dinitrobenzene (CDNB) and 1.7 ml of double distilled water. After preincubating the reaction mixture at 37°C for 5 min, the reaction was started by the addition of 0.1 ml of homogenate and 0.1 ml of glutathione as substrate. The absorbance was followed for 5 min at 340 nm. Reaction mixture without the enzyme was used as blank. The activity of GST is expressed as µM of GSH-CDNB conjugate formed/min/mg protein using an extinction coefficient of 9.6 mM^{-1} cm^{-1}. Protein was determined by the method of Lowry, *et al*[22] using Bovine Serum Albumin (BSA) as standard, at 660 nm.

Estimation of Non-enzymatic Antioxidants: Plasma vitamin A (β-carotene) was estimated by the method of Bradle and Hombeck [23]. Proteins were precipitated with ethanol and the carotenes were extracted into light petroleum. The intensity of the yellow color due to carotene was read directly at 450 nm using a violet filter. Vitamin E was measured by the method of Baker et al [24] on the basis of the reduction of ferric ions to ferrous ions by vitamin E (a-tocopherol) and the formation of a red colored complex with 2.2'-dipyridyl at 520 nm. Vitamin C (ascorbic acid) was estimated by the method of Roe and Kuether [25]. This involves oxidation of ascorbic acid by copper followed by treatment with 2,4-dinitrophenylhydrazine that undergoes rearrangement to form a product with absorption maximum at 520 nm.

Statistical Analysis: All data were expressed as mean ± SD. The statistical significance was evaluated by Student's t test using Statistical Package for the Social Sciences (SPSS Cary, NC, USA) version 10.0.

RESULTS AND DISCUSSION

Results: Table 1 shows the information about the investigated characteristics of study population. The mean age limit was 40 ± 15 years in diabetic patients and 42 ± 12 years in control subjects. A significant increase in body mass index (BMI) was observed in diabetic patients (35 ± 6.2 kg/m^2) when compared to control subjects (30 ± 6.2 kg/m^2). Diabetic participants were defined as those with a fasting blood glucose concentration minimum $\geq$120 mg/dl.

Table 1: Demographic characteristics of control and diabetic patients

Parameter	Control subjects	Diabetic patients
Age (year)	42 ± 12	40 ± 15^{NS}
Smokers (%)	10 %	25%
Alcohols (%)	15%	28%
Hypertension (%)	2 %	18 %
Diabetes mellitus (%)	-	100 %
Body mass index (kg/m^2)	30 ± 6.2	35 ± 6.2***

Values are given as mean ± S.D from 50 subjects in each group. Diabetic patients compared with control subjects. (***p<0.001, NS-Not significant)

Table 2 shows the levels of blood glucose, HbA1$_C$, microalbuminurea, urea, creatnine, serum lipids and proteins in control and diabetic subjects. The level of blood glucose, HbA1$_C$, microalbuminurea, urea, creatnine and serum lipids was significantly increased in diabetics than non-diabetic subjects. On the other hand, the levels of serum total protein; albumin and HDL-C were significantly decreased in diabetic patients when compared to healthy control subjects.

Table 3 illustrates the level of circulatory lipid peroxidation and antioxidant status in control and diabetic subjects. The extent of lipid peroxidation was significantly increased in diabetic patients when compared to healthy controls. For studying the deleterious consequence of diabetes on antioxidant status, the activities of enzymatic antioxidants SOD, CAT, GPx, GST and non-enzymatic antioxidants GSH, vitamin A, vitamin C, vitamin E were measured. The activities of enzymatic and the levels of non-enzymatic antioxidant were significantly decreased in diabetic patients when compared to healthy control subjects.

Table 2: Comparison of biochemical changes in control and diabetic subjects

Parameter	Control subjects	Diabetic patients
Blood glucose (mg/dl)		
Fasting	92 ± 13	215 ± 37***
Postprandial	125 ± 25	373 ± 30***
HbA$_{1C}$ (%)	3.2 ± 1.7	13.3 ± 2.01***
Microalbuminurea (mg/l)	9 ± 7	35 ± 20***
Urea (mg/dl)	21 ± 15	30 ± 20**
Creatnine (mg/dl)	0.5 ± 0.3	1.1 ± 0.9**
Total protein (g/dl)	7.5 ± 0.6	5.9 ± 1.0***
Albumin (g/dl)	4.2 ± 1.2	3.3 ± 0.6***
Total cholesterol	160 ± 15	190 ± 22**
Triglyceride (mg/dl)	120 ± 30	165 ± 40**
HDL-C (mg/dl)	45 ± 7	37 ± 12*
LDL-C (mg/dl)	60 ± 13	85 ± 15**
VLDL-C (mg/dl)	32 ± 10	42 ± 17*

Values are given as mean ± S.D from 50 subjects in each group. Diabetic patients compared with control subjects. (*p<0.05, **p<0.01, ***p<0.001)

Table 3: Circulatory lipid peroxide and antioxidant status in control and diabetic subjects

Parameter	Control subjects	Diabetic patients
TBARS (nmole/ml)	2.96 ± 0.21	7.12 ± 0.25***
SOD (Unit^a mg/Hb)	3.21 ± 0.36	2.54 ± 0.17***
CAT (Unit^b mg/Hb)	67.5 ± 7.60	50.7 ± 6.70***
GPx (Unit^c mg/Hb)	8.15 ± 1.69	6.53 ± 0.85***
GST (Unit^d mg/Hb)	2.10 ± 0.30	1.12 ± 0.35***
GSH (mg/dl)	36.18 ±1.67	28.09 ± 3.14**
Vitamin A (mg/dl)	0.87 ± 0.08	0 .54 ± 0.061***
Vitamin C (mg/dl)	1.31 ± 0.21	0.74 ± 0.13***
Vitamin E (mg/dl)	1.32 ± 0.28	0.53 ± 0.13***

Values are given as mean ± S.D from 50 subjects in each group. Diabetic patients compared with control subjects (**p<0.01, ***p<0.001)

a- One unit of activity was taken as the enzyme reaction, which gave 50% inhibition of NBT reduction in one minute.

b - µmole of H_2O_2 consumed/minute.

c - µg of GSH consumed/min.

d - µmole of CDNB–GSH conjugate formed/min.

Discussion: Diabetes mellitus is a complex and multifactorial disease indulging severe insulin dysfunction in conjunction with gross abnormalities in glucose homeostasis, lipid and protein metabolism. The metabolic dysregulation associated with diabetes causes secondary pathophysiologic changes in multiple organ systems that impose a heavy burden of morbidity and mortality from macrovascular and microvascular complications [26]. Oxidative stress plays an important role in chronic complications of diabetes and is postulated to be associated with increased lipid peroxidation. The present study was examined the changes in both extra and intracellular antioxidants and oxidant status in diabetic patients.

In uncontrolled or poorly controlled diabetes there is an increased glycosylation of a number of proteins including hemoglobin and α-crystalline of lens. $HbA1_C$ was found to increase in patients with diabetes to approximately 16% and the amount of increase is directly proportional to the fasting blood glucose level. During diabetes the excess glucose present in blood reacts with hemoglobin [27,28]. In the present study, we noticed a marked increase in $HbA1_C$ level in diabetic patients, which could be due to excessive glycosylation of hemoglobin. Diabetes is also grossly reflected by profound changes in protein metabolism and by a negative nitrogen balance and loss of nitrogen from most organs. Increased blood urea production in diabetes may be accounted for by enhanced catabolism of both liver and plasma proteins [29]. The diabetic hyperglycemia induces elevation of the plasma levels of urea and creatinine, which are considered as significant markers of renal dysfunction. The decrease in total protein and albumin may be due to microproteinuria and albuminuria, which are important clinical markers of diabetic nephropathy, and/or may be due to increased protein catabolism.

Diabetes has been shown to be associated with numerous thrombotic, atherosclerotic, and cardiovascular diseases. Cholesterol has been singled out as the cause of atherosclerosis. However, other lipids, such as triglycerides and phospholipids, also show similar correlations [30]. In our study, the levels of serum lipids were found to be elevated in diabetic patients. The abnormally high concentration of serum lipids in diabetes is mainly a result of the increase in mobilization of free fatty acids from peripheral depots, because insulin inhibits the hormone-sensitive lipase. On the other hand, glucagons, catecholamines, and other hormones enhance lipolysis. The marked hyperlipemia that characterizes the diabetic state may therefore be regarded as a consequence of the uninhibited actions of lipolytic hormones on fat depots. The increase and fall in the individual lipoprotein levels is a reflection of the total serum cholesterol levels; that is, the levels of VLDL-C, LDL-C, and HDL-C increase or decrease with the level of total serum cholesterol, and it is their ratio that determines the pathophysiology of lipoprotein metabolism [30,31].

The involvement of free radicals in diabetes and the role of these toxic species in lipid peroxidation and the antioxidant defense system have been studied. Lipid peroxide-mediated damage has been observed in the development of type 1 and type 2 diabetes mellitus. Insulin secretion is also closely associated with lipoxygenase-derived peroxides [32]. Low levels of lipoxygenase peroxides stimulate the secretion of insulin, but when the concentration of endogenous peroxides increases, it may initiate uncontrolled lipid peroxidation leading to cellular infiltration and islet cell damage. The observed increase in the level of plasma TBARS in diabetic patients is generally thought to be a consequence of increased production and liberation into the circulation of tissue lipid peroxides due to pathological changes.

Antioxidants constitute the foremost defense system that limit the toxicity associated with free radicals. The levels of these defense mechanisms are altered in diabetes and, therefore, the ineffective scavenging of free radicals plays a crucial role in determining the extent of tissue injury [33]. SOD and CAT are considered primary enzymes since they are involved in the direct elimination of ROS. SOD scavenges the superoxide radical by converting it to H_2O_2 and hence reduces the toxic effects due to this radical or other free radicals derived from secondary reactions. The activity of SOD was found to be lower in diabetic subjects. The observed decrease in SOD activity could result from inactivation by H_2O_2 or by glycation of the enzyme, which have been reported to occur in diabetes [34].

CAT, which is present virtually in all mammalian cells, is responsible for the removal of H_2O_2. The decrease in CAT activity could also result from inactivation by glycation of the enzyme. Moreover, an increase in the SOD activity may protect CAT against enzyme inactivation by superoxide radical as these radicals have been shown to inactivate CAT. Therefore, the increase in SOD activity may indirectly play an important protective role in conserving the activity of CAT. The reduced activities of SOD and CAT in the erythrocytes have been observed during diabetes and this may result in a number of deleterious effects due to the accumulation of superoxide radicals and hydrogen peroxides [30,34].

Under *in vivo* conditions, GSH acts as an antioxidant and its decrease was reported in diabetes. We have observed a significant decrease in GSH content in diabetic erythrocytes. The decrease in GSH content represents increased utilization due to oxidative stress. The depletion of GSH level may also lower the GST activity as GSH is required as a substrate for GST activity [35]. Depression in GPx activity was also observed in erythrocytes during diabetes. GPx has been shown to be an important adaptive response to condition of increased peroxidative stress.

Earlier research has shown that diabetics have low levels of vitamin-C and vitamin E and that vitamin-E supplementation can help prevent the development of glucose intolerance and diabetes [36]. Vitamin E is a well-known physiological antioxidant and membrane stabilizer. It interrupts the chain reaction of lipid peroxidation by reacting with lipid peroxy radicals, thus protecting the cell structures against damage. The decreased level of vitamin E observed in the diabetic patients is compatible with the hypothesis that plasma vitamin E excess plays a protective role against increased peroxidation in diabetes. Vitamin C is a hydrophilic antioxidant in plasma, because it disappears faster than other antioxidants when plasma is exposed to reactive oxygen species. The observed significant decrease in the level of plasma vitamin C could be caused by increased utilization of vitamin C as an antioxidant defense against reactive oxygen species or by a decrease in GSH, which is required for the recycling of vitamin C. The present study showed that people with diabetes had lower level of β-carotene than people without diabetes. Recent studies have also shown that plasma concentrations of vitamin A and its carrier proteins, retinol-binding protein, and transthyretin are decreased in diabetic patients [37]. The underlying cause for decreased availability of this vitamin in diabetes is not clearly understood. It appears that the increased hepatic store of vitamin A is attributed to a decreased availability of its carrier proteins.

Increasing evidence in both experimental and clinical studies suggest that oxidative stress plays a major role in the pathogenesis of diabetes mellitus. The present study revealed the importance of determining the antioxidant status in diabetes, in addition to the markers of oxidative stress and lipid profiles to enable the formulation of specific therapies for an early intervention and better management of the disease.

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