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EVALUATION OF OXIDATIVE STRESS, ANTIOXIDANT AND THYROID HORMONE 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, thyroid hormones status and other biochemical variables. The study population consisted of 100 subjects divided into two groups viz. diabetic (n=50) and healthy controls (n=50). Changes in the levels of lipid peroxidation and antioxidants and thyroid hormones status were determined in diabetic and non-diabetic subjects. The level of thiobarbituric acid reactive substances (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. The level of TSH was significantly decreased whereas the levels of T4 and FT4 were significantly increased in diabetic patients than the control subjects. However, the T3 and FT3 levels did not differ significantly between groups. Our findings indicate that changes in oxidant and antioxidant equilibrium will have biological and possibly pathological role in the development of secondary complications. It also demonstrate that detection of thyroid hormone status in the early stage of the disease will help the patients to improve quality of life and reduce the morbidity rate.

Key Words: *Diabetes mellitus, Oxidative stress, Lipid peroxidation, Antioxidant status, Thyroid hormones.*

Introduction

Diabetes mellitus is the most common endocrine metabolic disorder, affecting about 170 million people worldwide.[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.[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

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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 diseases [8] 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.[9,10]

The influence of other endocrine and non-endocrine organs other than the pancreas on diabetes mellitus is documented. Occasionally other endocrine disorders such as abnormal thyroid hormones levels are found in diabetes [11]. In chemically induced diabetic animals, the alterations in the hypothalamo-pituitary-thyroid axis in diabetic rats are numerous; hypothalamic and plasma TRH, pituitary and plasma TSH, as well as TSH secretion rate are reduced, and the TSH response to TRH is decreased despite normal peripheral TSH metabolism. T_3 and T_4 production and iodide uptake by the thyroid are diminished. There are also important structural changes in the thyroid gland and pituitary that are accompanied by marked alterations in their secretory activity. In addition, T_4 deiodination to T_3 in peripheral tissues is decreased [12,13]. It is found that iodothyronines are insulin antagonist with high levels being diabetogenic while absence of the hormone inhibits the development of diabetes. [14]Hence the present study was undertaken to assess the extent of lipid peroxidation and the status of the antioxidant defense system and thyroid hormone abnormalities 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 Bio Line Research 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.

Each diabetic and non-diabetic subject was physically examined to rule out thyroid disorders. In addition, none of the subjects had a history of previous thyroid disease. Age and sex-matched healthy volunteers without history of diabetes were considered as control subjects. All subjects were informed about the objectives of the study and what roles they were expected to play. The study excluded very ill patients with complications of diabetes mellitus and those with known history of thyroid dysfunction. All samples were specimens taken from subjects who fasted for at least 8 hr before the blood collection.

Classification of the values into raised, low or normal thyroid hormone levels were based on the following criteria. Subjects classified as having raised level of thyroid hormones: had FT_4 values > 1.6ng/l or, TSH < 0.4miu/ or both, those classified as having Low level had FT_4 values < 0.68ng/ml, or TSH values > 5.0miu/ml, or both. Subjects grouped as normal had FT_4 and TSH values within the range > 0.68 – 1.6ng/ml, and 0. 4 – 5.0miu/ml respectively.

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.

Determination of blood glucose, HbA1C, serum lipids and proteins

Biochemical investigation including blood glucose, HbA1C, 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].

The levels of serum thyroid stimulating hormone (TSH), total triiodothyroxine (T_3), free thyroxine (FT_4) and free triiodothyronine (FT_3) were measured by a Microparticle Enzyme Immunoassay (MEIA) on AXSYM System (Abbott Laboratories, Abbott Park, USA) while serum total thyroxine (T_4) was measured by the Fluorescence Polarization Immunoassay (FPIA) method on AXSYM System using the standard laboratory methodologies.

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, Pabst and Jakoby[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 ìM 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, Rosenbrough, Farr and Randall [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 (α-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

Table-I 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.

Table-I

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)

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 ≥120 mg/dl.

Table-II shows the levels of blood glucose, HbA_{1C}, microalbuminurea, urea, creatnine, serum lipids and proteins in control and diabetic subjects.

Table-II

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)

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-III 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.

Table-III

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.

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-IV illustrates the levels of serum thyroid hormone status in diabetic and non-diabetic subjects.

Table-IV

Serum thyroid hormone status in diabetic and non-diabetic subjects

Parameter	Control subjects	Diabetic patients
TSH (mIU/ml)	2.35 ± 1.25	1.92 ± 1.03*
T4 (μg/dl)	8.51 ± 2.20	10.86 ± 4.83**
T3 (ng/ml)	0.91 ± 0.37	1.02 ± 0.65^{NS}
FT4 (ng/ml)	1.35 ± 0.74	1.73 ± 0.83*
FT3 (pg/ml)	2.80 ± 0.83	3.09 ± 0.77^{NS}

Values are given as mean ± S.D from 50 subjects in each group. Diabetic patients compared with non-diabetic subjects (* p<0.05, ** p<0.01, NS - Not significant)

The level of TSH was significantly decreased whereas the levels of T_4 and FT_4 were significantly increased in diabetic patients compared to control subjects. However, the levels of T_3 and FT_3 in diabetic and non-diabetic subjects did not differ significantly.

Discussion

Diabetes mellitus is a complex and multifactorial disease. 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].

HbA_{1C} 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. Increased blood urea production in diabetes may be accounted for by enhanced catabolism of both liver and plasma proteins [29].

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 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].

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.

Antioxidants constitute the foremost defense system that limit the toxicity associated with free radicals. The levels of these defense mechanisms are altered in diabetes .[33] SOD and CAT are considered primary enzymes since they are involved in the direct elimination of ROS. The observed decrease in SOD and CAT activity could result from inactivation by H_2O_2 or by glycation of the enzyme, which have been reported to occur in diabetes [34].

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]. The present study showed that people with diabetes had lower level of â-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.

The thyroid hormones, triiodothyronine and tetraiodothynine are insulin antagonists that also potentiate the action insulin indirectly [38]. TRH synthesis decreases in diabetes and these facts could be responsible for the occurrences of low thyroid hormone levels in diabetics. In our study, the TSH was significantly lower in diabetics than in non-diabetics. The abnormal thyroid hormones levels may be the outcome of the various medications the diabetics were receiving. For example, it is known that insulin; an anabolic hormone enhances the levels of FT_4 while it suppresses the levels of T_3 by inhibiting hepatic conversion of T_4 to T_3. On the other hand, some of the oral hypoglycemic agents such as the phenylthioureas are known to suppress the levels of FT_4 and T_4, while causing raised levels of TSH [39].

These situations may explain the finding of low or raised thyroid hormone status in diabetic subjects. Never the less the situation in these diabetics does not seem to follow the pattern previously recorded in other non-thyroidal diseases such as liver diseases and Cushing syndrome where low thyroid hormone levels were recorded. The presence of both raised and low levels of thyroid hormones levels in diabetics in this study may also be due to modified TRH synthesis and release and may depend on the glycemic status of the diabetics studied. Glycemic status is influenced by insulin, which is known to modulate TRH and TSH levels [40].

The TSH level in diabetic males was significantly lower than the level in females. The incidence of hyperthyroidism was lower in females than in males, but the number of subjects in hypothyroid state was higher in females than in males. This finding is probably associated with the higher prevalence of obesity recorded in female diabetics. Insulin, which is used in treating diabetes and is produced in normal quantities or in excess, has been associated with increased anabolic activity [41].

Suzuki et al. [39] attributed the abnormal thyroid hormone levels found in diabetes to the presence of thyroid hormone binding inhibitor (THBI), an inhibitor of extra thyroidal conversion enzyme of T_4 to T_3, and dysfunction of the hypothalamo-pituitary-thyroid axis. These situations may prevail in diabetics and would be aggravated in poorly controlled diabetics. Stress, which is associated with diabetes, may also cause changes in the hypothalamus anterior-pituitary axis in these diabetics. It appears that the presence of sub-clinical hypothyroidism and hyperthyroidism may result from hypothalamus-hypophyseal-thyroid-axis disorders as suggested by Celani et al. [42]. Failure to recognize the presence of these abnormal thyroid hormone levels in diabetics may be a primary cause of poor management often encountered in some treated diabetics. There is therefore need for routine assay of thyroid hormone on diabetics, particularly those whose conditions are difficult to manage.

In conclusion, our findings demonstrate that detection of thyroid hormone status in addition to other biochemical variables in the early stage of the disease will help the patient to improve and reduce the morbidity rate.

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