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ORIGINAL ARTICLE

Thyroid function and oxidative stress indices in type 2 diabetics

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ABSTRACT

Objectives: This study assessed the levels of triiodothyronine (T_3), thyroid stimulating hormone (TSH), total antioxidant capacity (TAC), total plasma peroxides (TPP) and oxidative stress index (OSI) in type 2 diabetics and in different states of metabolic control to determine the possible relationship between oxidative stress, thyroid function and poor metabolic control.

Methods: Ninety consenting subjects aged 40 to 65 years comprising of 50 type 2 diabetics and 40 non-diabetic volunteers in Calabar, Nigeria were recruited into this case control study. Their fasting plasma glucose (FPG), glycated hemoglobin (HbA1c), T_3, TSH, TPP, TAC and OSI were determined using colorimetric methods. Anthropometric indices and socio-demographic information were obtained by standard methods.

Results: The body mass index, FPG, HbA1c, TPP, and OSI were found to be significantly higher and TAC lower in type 2 diabetics compared to the non-diabetic controls. The FPG, T_3, TSH, TPP, TAC and OSI of diabetics with poor glycemic control do not differ from those with good glycemic control. T_3 correlated positively with the duration of diabetes while TAC negatively correlated with TPP and OSI in type 2 diabetics.

Conclusion: Irrespective of the metabolic control state, type 2 diabetes seems to be associated with increased oxidative stress indices which may not be related with changes in T_3 and TSH levels.

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Key Words: Diabetes, glycemic control, thyroid function

INTRODUCTION

The global estimates of the prevalence of diabetes clearly indicate an overall increase of diabetes in the developing countries with type 2 diabetes accounting for about 90% of all diabetes cases [1]. Insulin resistance, reduction in insulin production and chronic hyperglycemia are the major characteristics of type 2 diabetes mellitus [1, 2]. Prolonged exposure to elevated glucose induces both repeated acute changes in intracellular metabolism and cumulative long-term changes in the structure and functions of macromolecules [3]. Hyperglycemia have been associated with increased free radicals and reactive oxygen species (ROS) generation, lipid peroxidation, increased oxidative stress and the associated oxidative damage to cells, organs and tissues [1]. Oxidative stress on the other hand has been implicated in the pathogenesis of insulin resistance by insulin signals disruption and adipocytokines dysregulation [4], thus forming a vicious cycle. Increased ROS production, reduced antioxidant defence mechanisms [4], increased levels of lipid peroxidation products have been demonstrated in diabetic patients, and their presence correlated with the development of complications [5].

Alterations in thyroid hormones metabolism have been reported in type 2 diabetes especially in poor glycemic control states [1]. Thyroid hyper- or hypo-activity has been demonstrated in type 2 diabetes and thyroid hormones have been shown to directly control insulin secretion [6]. Low triiodothyronine (T_3) state characterized by low serum total and free T_3 levels, increase in reverse T_3 (rT_3) but near normal serum tetraiodothyronine (T_4, thyroxine) and thyroid stimulating hormone (TSH) concentrations have been reported in types 1 and 2 diabetes [7]. Thyroid hormones have also been implicated in the production of free radicals and lipid peroxidation products [8, 9]. Increases in free radical production and lipid peroxide levels have been associated with hypermetabolic state in hyperthyroidism [8, 9], while the reverse is the case in hypometabolic state in hypothyroidism [10].

The various interactions between thyroid hormones and oxidative stress indices in type 2 diabetes and poor metabolic states and their relative or absolute contribution to development of thyroid dysfunction in type 2 diabetes in our locality (Calabar, Nigeria) is still uncertain. This study therefore assessed the levels of T_3, TSH, total antioxidant capacity (TAC), total plasma peroxides (TPP) and oxidative stress index (OSI) in type 2 diabetes and in different states of glycemic control to determine the possible relationship between oxidative stress, thyroid function and poor metabolic control.

MATERIALS AND METHODS

Study design

This case control study was carried out at the Diabetic Clinic of the University of Calabar Teaching Hospital (UCTH), Calabar, Cross River State, Nigeria. The study was carried out in accordance with the Ethical Principles for Medical Research involving Human Subjects as

outlined in the Helsinki Declaration in 1975 and subsequent revisions.

Subjects were selected based on the following criteria: age range between 40 and 65 years at the time of study, known type 2 diabetic patient for the past one year, and non-diabetic according to the World Health Organization diagnostic criteria for diabetes [11]. Subjects who were pregnant, hypertensive or had any form of chronic organ or systemic illness or chronic medication were excluded from the study.

Diabetes in this study was defined based on laboratory findings of fasting plasma glucose levels greater than 7 mmol/l in two or more occasions or 2-hour postprandial plasma glucose levels greater than 11 mmol/l in two or more occasions [11]. Duration of diabetes was defined as time since diagnosis of type 2 diabetes. Socio-demographic characteristics of the study population: family history, social history, past medical history, medication and gynecological history, occupation, physical activity, life habit pattern such as smoking and alcohol consumption were obtained using a semi-structured questionnaire. Anthropometric indices: height, weight, hip and waist circumference were taken to calculate the body mass index (BMI) and waist to hip ratio (WHR) respectively. The blood pressure was also taken to rule out hypertension.

Selection of subjects

Fifty consenting known type 2 diabetic subjects (males and females) and 40 age matched non-diabetic controls from apparently healthy individuals living within Calabar and its environs were consecutively recruited into this case control study. The objectives of the study were explained to the volunteers, informed consent was sought and obtained from each subject before recruitment into the study. All subjects were advised to report to the clinic in the fasting state on the morning of the day for sample collection.

Sample collection

Seven milliliters of fasting venous blood samples were drawn from each subject aseptically by venipuncture. Two milliliters was dispensed into fluoride oxalate container for fasting plasma glucose estimation, 1 ml was transferred into dipotassium ethylenediamine tetraacetic acid (K_2EDTA) container for glycosylated hemoglobin (HbAlc) estimation, the remaining samples were dispensed into plain sample bottles, allowed to clot and retract and then centrifuged at the rate of 3,000 rpm for 5 min to extract the sera for the estimation of T_3, TSH, TAC and TPP within 72 hours.

Blood pressure measurement

The systolic and diastolic blood pressures of subjects were taken at 3 intervals one month prior to sample collection. This is to rule out hypertension. The pressure was also taken on the day of the test in a seated relaxed position. Anthropometric data; body weight and height were measured while subjects wore light indoor clothing but no shoes. This was used to calculate the BMI, which is used as a measure of relative body weight. Normal body weight was defined as BMI between 18-25 kg/m^2 while generalized obesity was defined as BMI > 30 kg/m^2 [12].

Glycemic control

Measurement of glycated hemoglobin was used as index of glycemic or metabolic control. Good glycemic control was defined as HbAlc < 8% while poor glycemic control was defined as HbAlc > 8% [13].

Plasma glucose estimation

The test kit for plasma glucose measurements, depending on the enzymatic glucose oxidase (GOD) method, were obtained from Chem-Index (Hialeah, FL, USA). D-Glucose in the sample is oxidized by the enzyme GOD to gluconic acid, and hydrogen peroxide, in the presence of peroxidase (POD), reacts with p-hydroxybenzene sulfonate (p-HBS) and 4-aminoantipyrine (4-AAP) to form a quinoneimine complex:

$$\text{Glucose} + H_2O + O_2 \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O$$

$$H_2O_2 + \text{4-AAP} + \text{p-HBS} \xrightarrow{\text{POD}} \text{Quinoneimine dye} + H_2O$$

The intensity of the color formed is proportional to the glucose concentration in sample [14].

Glycosylated hemoglobin estimation

The test kit for HbA1c measurements were obtained from Euro diagnostics (Chennai, TN, India). Glycohemoglobin was estimated using column chromatography method with cation exchange resin. A hemolysed preparation of the whole blood is mixed continuously for 5 min with a weak binding cation-exchange resin during this time, HbA1c binds to the resin. After the mixing period, a filter is used to separate the supernatant containing the glycohemoglobin from the resin. The percent glycohemoglobin is determined by measuring the absorbance at 405 nm of the glycohemoglobin fraction and the total hemoglobin fraction. The ratio of the two absorbances gives the percentage HbA1c [15].

Estimation of triiodothyronine

For measuring T_3, the ELISA kit from Phoenix Pharmaceuticals (Burlingame, CA, USA) was used. In the T_3 enzyme immunoassay a second antibody (goat antimouse IgG) is coasted on microtiter wells. A measured amount of patient serum, a certain amount of mouse monoclonal anti-T_3 antibody and a constant

amount of T_3 conjugate with horse radish peroxidase are added to the microtiter wells. T_3 and the enzyme conjugated T_3 compete for the unlimited binding sites on the anti-T_3 antibody. After 60 min incubation at room temperature, the wells are washed 5 times by water to remove unbound T_3 conjugate. A solution of 3,3',5,5'-tetramethylbenzidine (TMB) is then added and incubates for 20 min at room temperature, resulting in the development of a blue color. The color development is stopped with the addition of 1 N HCl and the absorbance is measured spectrophometrically at 450 nm. The intensity of the color formed is proportional to the amount of enzyme present and is inversely related to the amount of unlabeled T_3 standards assayed in the same way; the concentration of T_3 in the unknown sample is then calculated. The minimum detectable concentration of T_3 by this assay is estimated to be 0.2 ng/ml [16].

Estimation thyroid stimulating hormone

Again, the needed ELISA kit was obtained from Phoenix Pharmaceuticals. The TSH ELISA test is based on the principle of a solid phase enzyme-linked immunosorbent assay. The assay system utilizes a unique monoclonal antibody directed against a distinct antigenic determinant on the intact TSH molecule. Mouse monoclonal anti-TSH antibody is used for solid phase immobilization (microtiter wells) and goat anti-TSH antibody is in the antibody-enzyme (horseradish peroxidase) conjugate solution. The test sample is allowed to react simultaneously with the two antibodies, resulting in the TSH molecules being sandwiched between the solid phase and enzyme-linked antibodies. After 60 min of overnight incubation **(AQ1: 60 min or overnight?)** at room temperature, the solid phase is washed with water to remove unbound labeled antibodies. A solution of 3,3',5,5'-TMB is added and incubated for 20 min, resulting in the development of blue color. The color development was stopped with the addition of 1 N HCl and the resulting yellow color is measured spectrophotometrically at 480 nm. The concentration of TSH is directly proportional to the color intensity of the test sample. The minimum detectable concentration of TSH by this assay is estimated to be 0.2 μIU/ml [17].

Estimation of total antioxidant capacity

For TAC measurement, the method described by Koracevic *et al* was used [18] A standard solution of Fe-ethylenediaminetetraacetic acid (EDTA) complex reacts with hydrogen peroxide (H_2O_2) by a Fenton type reaction, leading to the formation of hydroxyl radicals (OH•). These ROS degrade benzoate resulting in the release of thiobarbituric acid reactive substances (TBARS). Antioxidant from added sample causes suppression of the production of TBARS. This reaction was measured spectrophotometerically at 532 nm and the inhibition of color development defined as the TAC.

Estimation of total plasma peroxide

The FOX2 method was used for TPP detection [19]. This test is based on oxidation of ferrous ion to ferric ion by various types of peroxides contained within the plasma samples, to produce a colored ferric-xylenol orange complex of which the absorbance can be measured at 560 nm. Total peroxide content of plasma samples was determined as a function of the absorbance difference between test and blank tubes using a solution of H_2O_2 as standard.

Oxidative stress index

The ratio of TPP to TAC is expressed as the OSI; an indicator of the degree of oxidative stress:

$$\text{OSI (\%)} = \text{TPP } \mu\text{mol } (H_2O_2) \text{ x } 100 / \text{TAC } \mu\text{mol/l}$$

Statistical analysis

Data analysis was done using the statistical package for social sciences (SPSS version 20.0). Student's t-test analysis was used to determine mean differences between variables. Analysis of variance (ANOVA) was used to test significance of variations within and among group means and Fisher's least significant difference (LSD) post hoc test was used for comparison of multiple group means. Pearson correlation analysis was employed to determine relationship between variables. The probability value of $P < 0.05$ was considered statistically significant.

RESULTS

The mean age, BMI, WHR, blood pressure (systolic and diastolic), fasting plasma glucose (FPG), HbA1c, T_3, TSH, TPP, TAC and OSI in type 2 diabetics and non-diabetics were summarized in Table 1. In brief, type 2 diabetics had higher BMI, FPG, HbA1c, TPP and OSI, but lower TAC compared to the non-diabetic controls ($P < 0.05$). No significant differences were observed among the levels of other indices in the two groups studied ($P > 0.05$).

The effect of glycemic control and duration of diabetes on the FPG, T_3, TSH, TPP, TAC and OSI in type 2 diabetics are to see in Table 2. The FPG, T_3, TSH, TPP, TAC and OSI of diabetics with poor glycemic control do not differ from those with good glycemic control ($P > 0.05$). T_3 levels increased with increasing duration of diabetes ($P < 0.05$). Duration of diabetes did not exert any significant effect on the levels of other indices studied ($P > 0.05$).

Figure 1 shows the correlation plot of duration of diabetes against T_3 in type 2 diabetics studied; significant positive correlation was observed between T_3 and duration of diabetes ($r = 0.366$, $P = 0.009$). Figure 2 presents the correlation plot of TAC against TPP in type 2 diabetics studied; TAC correlated negatively with TPP ($r = -0.577$, $P < 0.001$). Figure 3 exhibits the correlation plot of OSI against TAC in type 2 diabetics studied; TAC correlated negatively with OSI ($r = -0.850$, $P < 0.001$).

Table 1. Mean age, body mass index (BMI), waist/hip ratio (WHR), blood pressure (BP; systolic, diastolic) fasting plasma glucose (FPG), glycated hemoglobin (HbA1c), triiodothyronine (T3), thyroid stimulating hormone (TSH), total plasma peroxides (TPP), total antioxidant capacity (TAC) and oxidative stress index (OSI) in type 2 diabetics and non-diabetics

Index	Diabetics (n = 50)	Non-diabetics (n = 40)	P value
Age (years)	44.97 ± 1.65	43.67 ± 1.46	0.566
BMI (kg/m²)	27.17 ± 0.58	23.8 ± 0.87	**0.002**
WHR	0.83 ± 0.05	0.81 ± 0.05	0.092
Sys BP (mmHg)	128.6 ± 8.57	127.8 ± 9.1	0.652
Dias BP (mmHg)	82.8 ± 10.5	82.2 ± 11.11	0.782
FPG (mmol/l)	12.61 ± 1.75	4.6 ± 0.23	**< 0.001**
HbA1c (%)	8.85 ± 0.25	6.04 ± 0.28	**< 0.001**
T_3 (mg/dl)	1.95 ± 0.17	1.8 ± 0.10	0.464
TSH (IU/l)	2.77 ± 0.24	2.26 ± 0.29	0.182
TPP (µmol/l)	2.68 ± 0.05	1.28 ± 0.04	**< 0.001**
TAC (mmol/l)	150.38 ± 9.23	209.92 ± 11.91	**< 0.001**
OSI (%)	2.39 ± 0.26	0.7 ± 0.05	**< 0.001**

Table 2. Effect of glycemic control and duration of diabetes on biochemical indices in type 2 diabetics

	FPG (mmol/l)	T_3 (mg/dl)	TSH (IU/l)	TPP (µ µmol/l)	TAC (mmol/l)	OSI (%)
Glycemic State						
Good control (HbA1c < 8%, n = 14)	8.23 ± 1.03	1.79 ± 0.14	2.53 ± 0.49	2.28 ± 0.21	142.45 ± 24.27	6.85 ± 5.12
Poor control (HbA1c > 8%, n = 36)	10.06 ± 0.9	2.01 ± 0.23	2.86 ± 0.27	3.92 ± 1.04	145.41 ± 10.37	3.97 ± 1.06
P value	0.377	0.419	0.565	0.131	0.912	0.594
Duration						
≤ 4 yrs (n = 16)	8.35 ± 1.17	1.52 ± 0.18	3.12 ± 0.34	2.4 ± 0.19	158.78 ± 16.39	2.16 ± 0.43
5-10 yrs (n = 23)	9.19 ± 0.86	2.01 ± 0.26	2.99 ± 0.41	2.97 ± 0.24	146.87 ± 12.90	2.76 ± 0.41
≥ 10 yrs (n = 11)	9.83 ± 1.16	2.68 ± 0.45	2.44 ± 0.47	2.45 ± 0.43	142.87 ± 23.44	1.87 ± 0.44
F ratio	0.419	3.221	0.557	1.640	0.253	0.92
P value	0.66	**0.049**	0.557	0.205	0.778	0.405

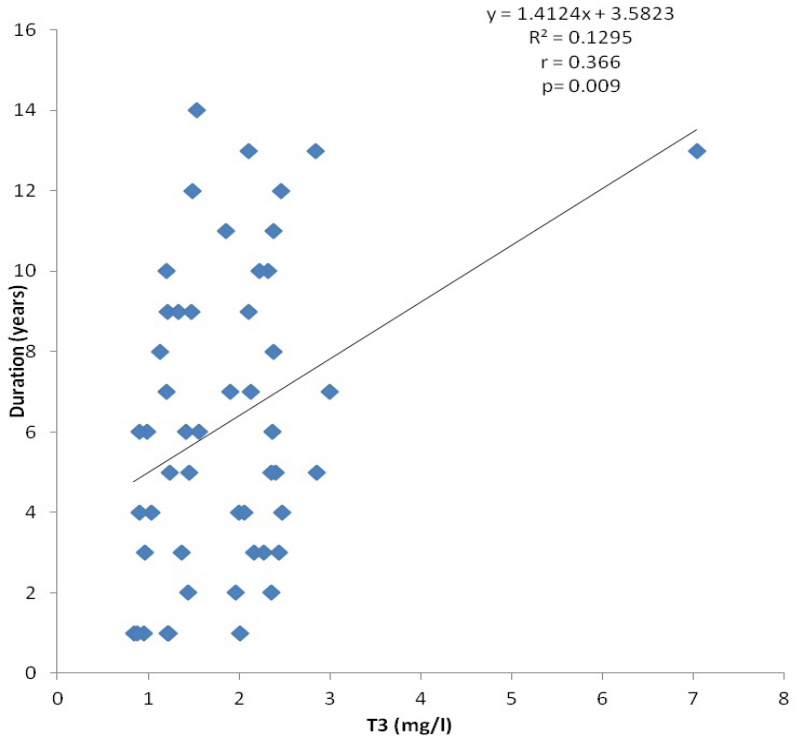

Figure 1. Correlation plot of duration of diabetes against triiodothyronine (T_3) in type 2 diabetics

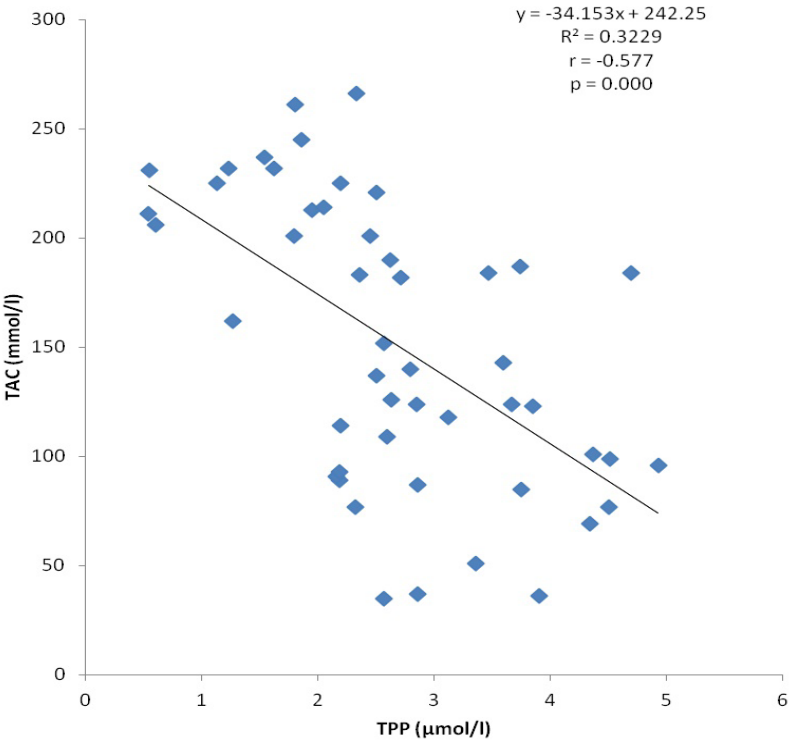

Figure 2. Correlation plot of total antioxidant capacity (TAC) against total plasma peroxides (TPP) in type 2 diabetics

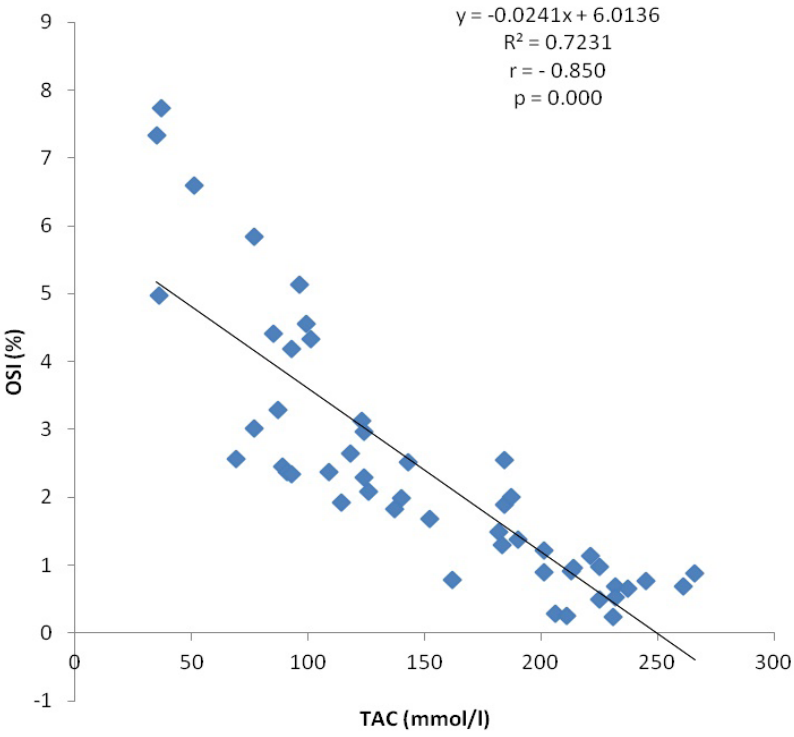

Figure 3. Correlation plot of oxidative stress index (OSI) against total antioxidant capacity (TAC) in type 2 diabetics

DISCUSSION

Metabolic disturbances and alterations in thyroid hormone homeostasis have been described in type 2 diabetes. The relationship between thyroid dysfunction and oxidative stress indices in type 2 diabetes is still uncertain. In this study, the FPG and glycated hemoglobin levels were significantly higher in type 2 diabetics compared to non-diabetic controls. Higher FPG and HbA1c in diabetics is the result of insulin deficiency, insulin resistance or both and increase protein glycation associated with diabetes mellitus [20, 21].

Lower TAC and higher TPP and OSI were seen in type 2 diabetics compared to the non-diabetic controls studied. These observations may be attributed to increased production of free radicals, reactive oxygen species and lipid peroxidation products associated with hyperglycemia [1, 3]. Under pathological conditions of hyperglycemia, excessive glucose levels can swamp the glycolytic process and inhibit glyceraldehydes catabolism which cause glucose, fructose 1,6-bisphosphate and glyceraldehyde-3-phosphate to be shunted to other pathways like enolisation and alpha-ketoaldehyde formation, protein

kinase C activation, dicarbonyl formation and glycation, sorbitol metabolism, hexosamine metabolism and oxidative phosphorylation. Activation of these pathways leads to generation of ROS and heightened oxidative stress [22]. Generated ROS oxidatively attack membrane lipids (cholesterol, polyunsaturated fatty acids (PUFA)) leading to the formation and the accumulation of lipid peroxidation (LPO) products seen in type 2 diabetes [23, 24]. Lower TAC levels seen may be attributed to increased utilization of antioxidants to buffer the deleterious effects of ROS, low dietary intake of such antioxidants as vitamin C, E, beta carotene and essential amino acid methionine due to poor economic status and insufficient synthesis of antioxidant enzymes as superoxide dismutase (SOD), glutathione peroxidase (GPx) which may in turn result from decreased micronutrient availability as zinc (Zn), copper (Cu), selenium (Se) and manganese (Mn) [25]. The findings of the present work are consistent with similar observations made by other studies [22, 23, 26]. Significantly elevated values of LPO and 8-hydroxy-2'-deoxyguanosine (8-OHdG) were found in diabetic subjects compared with the non diabetic controls [27]. Contrary to our findings, higher TAC levels were reported in type 2 diabetes compared to controls by Kopprasch *et al* [28]. Negative correlations were observed between TAC and TPP and OSI in type 2 diabetics of the present study; this is expected since type 2 diabetes is associated with increased generation of ROS and LPO products. Increased ROS and LPO products entails increased OSI and hence decreased TAC as a result of increased utilization to buffer deleterious effects of oxidative stress.

Glycemic control does not seem to have any effect on TAC, TPP and OSI in diabetics studied. Similar observations have been documented [22, 29, 30]. Lipid peroxidation, antioxidant depression and oxidative stress in diabetes is a gradual chronic process, so evaluation of effects of glycemic control on the levels of theses indices using glycated hemoglobin concentration as an index may be a poor reflection of the actual event since it can only account for metabolic control for a duration of three months which is the life span of hemoglobin. However, a proportional decrease in the antioxidant enzyme activity with declining glycemic control [31], and a significant positive correlation between FPG and plasma lipid hydroperoxides [22, 28] have been described in diabetes.

The T_3 and TSH levels of type 2 diabetics of the current study were not significantly different from those of non-diabetic controls and in different states of glycemic control. A similar observation has been documented by Pasupathi *et al* [3] who also reported no significant differences in T_3 and FT_3 levels between diabetics and healthy controls. Significantly increased T_3 levels were however observed with increasing duration of diabetes, with a corresponding decrease in TSH levels though not statistically significant. Higher levels of circulating insulin associated with insulin resistance in type 2 diabetes have been shown to have a proliferative effect on thyroid tissues resulting in larger thyroid size and increased formation of nodules [32]; effects which may be aggravated with increasing duration of diabetes. Contrary to our findings, lower and higher levels of thyroid hormones have been independently reported in diabetes by others [1, 3, 6]. Higher serum levels of T_3, T_4, free T_3 (FT_3) and lower TSH and FT_4 have also been reported in diabetic subjects compared to the non-diabetic subjects [33]. Significant positive correlations of HbA1c with rT_3 and to rT_3/T_3 ratio have also been reported in type 1 and in type 2 diabetics [1]. The thyroid hormones, T_3 and T_4 have been described as insulin antagonists that also potentiate the action of insulin indirectly as low levels of these hormones have been shown to inhibit the development of diabetes [3]. Thyroid hormone concentrations have been correlated with insulin receptor number, insulin responsiveness and insulin sensitivity. The insulin receptor number in isolated adipocytes were shown to be increased by 70% in hypothyroidism and decreased by 40% in hyperthyroidism. The sensitivities of the effects of insulin on lipolysis and glucose oxidation were increased 4-fold in hypothyroidism and decreased 5-fold in hyperthyroidism [34]. Thus thyroid hormones modify insulin action in a dose dependent manner, antagonizing their action at higher concentration and potentiating it at lower levels. Insulin has also been shown to enhance the levels of FT_4 while it suppresses the levels of T_3 by inhibiting hepatic conversion of T_4 to T_3 [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 [35]. These situations may explain the findings of low or raised thyroid hormones status in diabetic subjects reported by these studies. Moreover, thyrotropin-releasing hormone (TRH) synthesis has been shown to be decreased in diabetes and this could also be responsible for the occurrences of low thyroid hormone levels in diabetics as reported by some studies.

In conclusion, the present findings have shown that type 2 diabetes may be associated with depressed antioxidant status, increased lipid peroxidation products and oxidative stress index with elevated T_3 associated with increasing duration of diabetes. On the other hand, thyroid dysfunction in type 2 diabetes seems not be associated with the state of glycemic control.

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