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RESEARCH ARTICLE

ASSESSMENT OF OXIDATIVE STRESS IN TYPE 2 DIABETES MELLITUS BY MEASUREMENT OF PLASMA LIPID HYDROPEROXIDES AND TOTAL ANTIOXIDANT CAPACITY.

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Abstract

Context: From all the noncommunicable group of diseases diabetes mellitus type 2 is the fastest growing and responsible for the major morbidity and mortality. The molecular mechanism underlying development of diabetes mellitus type 2 is still poorly understood. Oxidative stress plays important role in the development of DM and its complications.

Aim : The aim of the study is to assess the role of oxidative stress in type 2 diabetes mellitus

Settings and Design : This is a cross sectional study.

Methods and Material : A total number of 101 subjects were included in this study, of which 50 were controls and 51 were cases with Diabetes mellitus. In the diabetics group and control group, Plasma lipid hydroperoxides was measured by Ferrous oxidation in xylenol orange (FOX 2) assay as a marker of oxidative stress induced damage and plasma total antioxidant capacity by Ferric reducing ability of plasma (FRAP) was measured as a marker of antioxidant defense. The data of lipid profile and HbA1c were collected for comparison.

Statistical analysis used: Statistical analysis was performed by Independent t test and Pearson\'s correlation coefficient.

Results: Our results suggest that diabetic patients have a higher level of plasma lipid hydroperoxides (15.49±10.87 µmol/L) than do control subjects (5.42±2.20 µmol/L). We also observed decrease in plasma total antioxidant capacity in diabetic patients (44.69±12.22 µmol/L) in comparison to controls (85.05±26.12 µmol/L). Diabetic patients had higher Total Cholesterol, S.Triglyceride and LDL and lower HDL as compared to control group. There was no correlation found when lipid hydroperoxides and TAC were compared with FBS, HbA1C and lipid profile. Also there is no correlation of different analytes with the duration of DM.

Conclusions: The increased plasma lipid hydroperoxides levels along with the decrease in the antioxidant activity provide direct evidence that diabetes mellitus type 2 is associated with oxidative stress.

INTRODUCTION

The noncommunicable diseases have become one of the major dangers to human health in the 21st century as a result of availability of antibiotics, vaccines and good living conditions. Amongst them prevalence of type 2 DM is increasing very fast. In 2013, International Diabetes Federation (IDF) estimated that 382 million people have

diabetes which is expected to rise to 592 million by 2035. At present 9.1% population have diabetes in India and is expected to be 10.2 (123 million) % in 2035[2] .It significantly increases the risk of cardiovascular disease and stroke thus it increases loss of manpower and economic burden to country as there is no cure.

Type 2 diabetes mellitus (DM) is a group of metabolic disorders characterized by hyperglycemia. Although insulin resistance and beta cell decompensation compose the well accepted basis of type 2 diabetes mellitus, the molecular mechanism underlying their development are incompletely understood[1].Oxidative stress plays an important role in the development of diabetes mellitus. Oxidative stress is an imbalance between the systemic manifestation of reactive oxygen species (ROS) and the biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage[3].Reactive oxygen species(ROS) are generated during oxidative phosphorylation in mitochondria and other metabolic processes[4]. ROS are highly reactive and can damage enzymes, membrane lipids and nucleic acid[5,6]. To prevent oxidative damage by reactive oxygen species, cells have several forms of the antioxidants i.e. superoxide dismutase, catalase, glutathione peroxidase, vitamin C, vitamin E and beta-carotene[7]. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA [5,6]. Under physiological conditions, glucose primarily undergoes glycolytic and oxidative phosphorylation. 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 [3,6,8,9] : Enolisation and alpha-ketoaldehyde formation, Protein Kinase C activation, Dicarbonyl formation and glycation, Sorbitol metabolism, Hexosamine metabolism, Oxidative phosphorylation .Activation of these pathways lead to generation of ROS and heightened oxidative stress(OS). Diabetes mellitus is also associated with decreased levels of antioxidant enzymes like superoxide dismutase(SOD), catalase, glutathione peroxidase[3,8-10].

Increased oxidative stress causes insulin resistance and decreased insulin gene expression which causes decreased insulin secretion [6,8]. Increased oxidative stress is also involved in microvascular and macrovascular complications of diabetes mellitus type 2 [3,6,10-12].

For practical reasons, neither the rate of oxidant production nor the steady-state levels of ROS are easily measured directly ex vivo in biological systems. Thus, steady-state levels, or the extent of accumulation of oxidation products in tissues and plasma or the changes in antioxidant status are commonly used as measures of increased oxidative stress[13,14,15]. In fact, the measurement of parameters of lipid peroxidation, such as lipid peroxides, conjugated dienes, and malonyldialdehyde (MDA) can be considered the cornerstone of the assessment of oxidative damage in vivo [14].

Therefore, the present study was aimed to undertake a comprehensive and comparative study of oxidative stress measured by lipid peroxidation(lipid hydroperoxides [ROOHs]), which is generated at different stages of the peroxidation cascade and anti-oxidant defense system by measuring total plasma antioxidant capacity (FRAP), a marker of the efficiency of antioxidant defenses in patients of type 2 DM [19]. This would help in better understanding of the relationship between oxidative stress and diabetes. Such studies can form the basis for the development of novel therapeutic approach in the treatment of type 2 diabetes mellitus.

MATERIAL AND METHODOLOGY

The study was carried out at Shree Krishna Hospital and H M Patel Centre for Medical Care and Education from September 2013 to September 2014. Individuals attending 'whole body checkup scheme' at Shree Krishna hospital and patients attending medicine OPD were evaluated, from whom individuals were identified after applying the inclusion and exclusion criteria and recruited in the study. **Group 1** included 50 healthy males & females and **group 2** included 51 DM type 2 patients diagnosed by ADA criteria[16] . Conditions increasing oxidative stress other than Diabetes mellitus like history of ischemic diseases like acute myocardial infarction, pulmonary embolism or peripheral vascular diseases, history of hypertension, renal diseases, liver cirrhosis , cancer , h/o alcohol, tobacco, oral contraceptives, hormone replacement or supplementation therapy e.g. Insulin were excluded from the study.

Clinical laboratory measurements. To assess glycemic control, we measured HbAlc by immunoturbidimetry in COBAS integra 400 plus analyzer. Lipid profile was measured by standard techniques.

Measurement of lipid hydroperoxide

Reagents Ammonium ferrous sulfate, xylenol orange, H2O2, butylate hydroxytoluene (BHT), Triphenylphosphine (TPP) . All general chemicals and reagents were of the highest purity available.

Preparation of plasma. Venous blood was collected after a 12-h overnight fast into sampling vials containing heparin for preparation of plasma. Plasma samples were stored under nitrogen at -70°C and used within 1 month for analysis of FRAP and measurement of ROOHs.

The hydroperoxide content of plasma was determined with the FOX Version II assay for lipid ROOHs (FOX2) [17,18]. This technique relies on the rapid hydroperoxide-mediated oxidation of Fe^{2+} under acidic conditions. Fe^{3+} forms a

chromophore with xylenol orange, which absorbs strongly at 560 run. FOX2 reagent was prepared by dissolving xylenol orange and ammonium ferrous sulfate in 250 mmol/1 H2SO4 to final concentrations of 1 and 2.5 mmol/1, respectively. One volume of this concentrated reagent was added to 9 volume of HPLC-grade methanol containing 4.4 mmol/1 BHT to make the working reagent, which comprised 250 umol/1 ammonium ferrous sulfate, 100 umol/1 xylenol orange, 25 mmol/1 H2SO4, and 4 mmol/1 BHT in 90% (vol/vol) methanol. The working reagent was routinely calibrated against solution of H_2O_2 of known concentration.

Aliquots (90 ul) of plasma lipid extract in HPLC-grade methanol were transferred into 1.5-ml microcentrifuge vials. TPP in methanol (10 ul of 10 mmol/1) was added to the blank samples to selectively reduce ROOHs to hydroxy derivatives having no reactivity with Fe^{2+}. Methanol (10 ul) was added to the test sample. All vials were then vortex-mixed and incubated at room temperature for 30 min before the addition and mixing of 900 ul FOX2 reagent. After incubation at room temperature for a further 30 min, the vials were centrifuged at 12,000g for 10 min. The absorbance of the supernatant was then read at 560nm run. Hydroperoxide content in the plasma samples was determined as a function of the mean absorbance difference of samples with and without elimination of ROOHs by TPP.

PLASMA TOTAL ANTIOXIDANT CAPACITY(TAC)

Method : Ferric reducing ability of plasma(FRAP).

Total antioxidant capacity was measured by FRAP assay according to the method of Benzie.F.F. and J.J.Strain(1999). At low pH, a ferric tripyridyltriazine (Fe+3 TPTZ) complex is reduced to the ferrous (Fe+2) form and an intense blue colour develops which is measured at 593 nm [19].

REAGENTS

1)Acetate buffer,(200 mmol/L, pH 3.6)

2) TPTZ (2.4.6-tripyridyl-striazine) (10 mmol/L)- 40 mmol/L HCl - dissolve 0.2 ml HCl in 20 ml D.W. Then add 31.3 mg TPTZ in 10 ml 40mM HCL.

3) $FeCl_3$. 6H2O(20 mmol/L)- Weigh 0.05406 gm $FeCl_3$. 6H2O in glassware and add it immediately to 10 ml D.W before preparing working reagent.

4) Trolox.

FRAP WORKING SOLUTION:

25 ml acetate buffer, 2.5 ml TPTZ solution and 2.5 ml $FeCl_3$ solution. It is necessary to prepare freshly the working solution. The method was calibrated with Trolox standard. The Trolox is a water soluble form of vitamin E.

Procedure of FRAP measurement

The reaction was performed by adding of 20 µl plasma to 900 µl FRAP working reagent and the mixture was incubated for 10 min at 37 º C. The absorbance was measured in spectrophotometer at 593nm which was compared to a blank mixture where 20 µl water was added to the working FRAP reagent instead of plasma. The results were expressed in mmol Trolox equivalent/L of plasma (mM/L) .

STATISTICAL ANALYSIS

Statistical analysis was performed using the commercially available statistical software SPSS – 14.0 version, and Microsoft excel. Values are given as means ± SD. Comparisons between diabetic and control groups were made using the Student's *t* test. Linear regression analysis was used for detecting relationships between FRAP values and lipid hydroperoxides levels. The p value of less than 0.0001 was considered statistically significant.

RESULTS

Our data showed that type2 DM patients had significantly lower levels of total plasma antioxidant defenses and increased levels of lipid peroxidation products, compared with control subjects. As shown in Table 2, FRAP values were 44.69±12.22µmol/L vs. 85.05±26.12 µmol/L *(P* < 0.0001), and lipid hydroperoxides were 15.49±10.87µmol/L vs. 5.42±2.20µmol/L *(P* < 0.0001).

FBS ,GlyHbA1c and lipid profile are statistically significantly higher in diabetic group than control group as shown in the table 1.

We found no significant correlation between plasma lipid hydroperoxides levels and total antioxidant status of normal subjects (r = -0.123 & p = 0.39) and diabetic subjects (r = -0.22& p = 0.88).We also did not find significant correlation between oxidative parameters(lipid hydroperoxide and plasma TAC) with lipid profile.

The mean values of HbAlc were 7.5 ± 1.2% in type 2 DM patients. No correlation was found between metabolic control and the levels of the markers of oxidative stress, such as ROOHs and FRAP. Furthermore, these parameters of oxidative stress did not correlate with age, sex, and diabetes duration.

Table 1 : Clinical characteristics of control and Type 2 DM subjects

	Control	Type 2 DM	P value
n	50	51	NS
Sex(M/F)	26/25	27/23	NS
Age(years)	55.5	59	NS
Duration (years)	-	6.3	<0.0001
FBS(mg/dl)(Mean ±SD)	98.62± 8.27	165.03±59.08	<0.0001
HbAlc (4.5-6.5%)	5.46± 0.43	9.42 ± 1.92	<0.0001
Triglycerides (mg/dl)	119.0± 59.94	182.21± 58.44	<0.0001
Cholesterol(mg/dl)	174.32± 33.82	224.21±64.09	<0.0001
HDL cholesterol(mg/dl)	46.9± 10.21	36.47± 8.67	<0.0001
LDL cholesterol(mg/dl)	106.24± 24.63	151.29± 58.0	<0.0001

Table 2 : Comparison of plasma lipid hydroperoxides and total antioxidant capacity (TAC) in diabetic and control group. (Independent t test)

Parameters	Diabetic	Control	P value
Plasma lipid hydroperoxide(µmol/L) (Mean ±SD)	15.49±10.87	5.42± 2.20	<0.0001
Total antioxidant capacity(TAC)(µmol/L) (Mean ±SD)	44.69± 12.22	85.05± 26.12	<0.0001

DISCUSSION

Oxidative stress occurs when there is an imbalance between free radical generation and scavenging capacity of anti oxidative defense mechanism. In our study, we found significantly elevated plasma lipid hydroperoxides levels in diabetic patients.

Our results are similar to some other studies as lipid hydroperoxides are concerned [20,21].Many studies have evaluated the role of oxidative stress in the development of type 2 DM and also its complications. Different researchers have applied different methods for detection of ROS induced damage and antioxidant defense. Most researchers have taken lipid peroxide as a measure of ROS induced damage. Plasma lipid hydroperoxides is one of the lipid peroxidation products frequently used to determine the oxidant/antioxidant balance in diabetic patients .Many researchers have measured Malondialdehyde (MDA) activity in type 2 DM patients as an index of lipid peroxidation [22,23].All have found significant increase in MDA in type 2 DM patients as compared to normal participants. As per the standard texts, a major disadvantage of the MDA measurement (TBARS) test is that it is nonspecific and measures many parameters in addition to lipid peroxidation[24].Moreover Malondialdehyde is a secondary degradation product of lipid peroxidation whereas in our study we measured primary product i.e. lipid hydroperoxide so it gives more specific result[24]. Sridevi Devaraj et al.[25] studied urinary Isoprostanes F2α and oxidized LDL as measures of oxidative stress .They found significant increase in urinary PGF2α in type 2 DM patients as compared to controls. However they did not find difference in oxidized LDL in type 2 DM and controls. Although isoprostane PGF2α is more reliable marker its measurement requires specialized instrument and it is more costlier and time consuming[7].

In our study we measured total antioxidant capacity (TAC) by FRAP (ferric reducing ability of plasma) assay . We found significant reduction in plasma TAC in patients of DM as compared to normal healthy individuals. Our rests are similar to other studies.[26-29]

There are different methods for TAC measurement. The results obtained by these methods are hardly comparable because of the different mechanisms, redox potentials, pH and solvent dependencies, etc. of various assays. Because it is difficult to separate, detect, and quantify individual antioxidants from a complex biological matrix due to chemical diversity of natural antioxidants, the total antioxidant power is often more meaningful to evaluate health beneficial effects because of the cooperative action of individual antioxidant species[7].

In the present study , we found significant increase in S. cholesterol , S.TG , S.LDL and significant reduction in HDL in diabetics in comparison to controls. Nada Zorika et al.[30] also found significant increase in

S.TGs in diabetics as compared to controls. Widad et al.[31] also found increase in S.Cholesterol, S.TGs and S.LDL in diabetics as compared to controls.

The oxidative parameters studied did not appear to be correlated with metabolic control (HbAlc levels) and lipid profile. J.Nourooz-Zadeh et al.[20] also did not find correlation between fasting plasma glucose and plasma lipid hydroperoxides in control and diabetic group ($r = 0.10$; $P = 0.32$). Elizabeth et al.[21] found significant positive correlation between fasting plasma glucose and plasma lipid hydroperoxides in control and diabetic group.

The reason for finding no correlation of plasma lipid hydroperoxides and total antioxidant activity with various parameters like FBS, HbA1c, lipid profile in our study could be heterogeneous group of population. Patients could be taking extra care in the form of exercise, diet restrictions, taking herbal preparation on diagnosing DM. Small Sample size could also affect the correlation.

Conclusion

The present study was undertaken to analyze the oxidative stress levels in patients with diabetes mellitus type 2 and compare it with controls.

We have observed that the diabetic patients have a higher level of plasma lipid hydroperoxides than do control subjects. We also observed decrease in plasma total antioxidant capacity in diabetic patients in comparison to controls. Diabetic patients had higher Total Cholesterol, S.Triglyceride and LDL and lower HDL as compared to control group.

There was no correlation found when lipid hydroperoxides were compared with FBS, HbA1C and lipid profile. There was also no correlation found between TAC on one hand and FBS, HbA1c and lipid profile on the other. Although there was decrease in TAC and increase in plasma lipid hydroperoxides in diabetics, there was no correlation found between plasma lipid hydroperoxides and TAC.

Also levels of plasma lipid hydroperoxides and total antioxidant capacity did not differ with duration of diabetes mellitus.

Thus, the increased lipid hydroperoxides levels along with the decrease in the antioxidant activity provides direct evidence that diabetes mellitus type 2 is associated with oxidative stress.

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