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Original Article

Biochemistry Section

Is Long Term Duration of Diabetes a Factor to Cause Endothelial Dysfunction in Patients with Type 2 Diabetes Mellitus?

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ABSTRACT

Introduction: Endothelial Dysfunction (ED) is an earliest pathological process of atherosclerosis. Endothelium regulates vascular tone, platelet activity, leukocyte adhesion and thrombosis. Impaired function of endothelium initiates the development of atherosclerosis. Nitric oxide is one of the most effective endogenous vasodilator and also a marker for ED.

Aim: To assess whether long term duration of diabetes is a factor to cause ED and its complications in patients with Type 2 diabetes mellitus. Hence, the study has been designed to assess the ED in patients with long term duration of Type 2 diabetes for early prediction of vascular complications.

Materials and Methods: The study was conducted on 47 Type 2 diabetic subjects. Among these 27 subjects with <5 years duration of diabetes (Group-I) and remaining 20 subjects with >5 years duration of diabetes (Group-II). Glucose, HbA1c, BMI and lipid profile were estimated by well established methods in auto-analyzer, MDA by Thiobarbituric Acid Reactive Substances (TBARS), total antioxidant capacity as Ferric Reducing Ability of Plasma (FRAP) and NO was estimated by kinetic cadmium reduction method using spectrophotometer. Statistical analysis was performed by "Kruskal-Wallis" test.

Result: Significantly low level of NO was identified in Type 2 diabetic patients with >5 years duration of disease compared to <5 years duration. MDA shows significantly high value in >5 years duration of diabetes and no significant difference in the level of FRAP among the study groups was observed. It has also shown significantly high level of age in >5 years duration of Type 2 diabetes than <5 years. But, no significant differences in the levels of HbA1c, lipid profile were identified between two study groups.

Conclusion: Age and oxidative stress (lipid peroxidation) has been recognized as risk factors for ED and future complications in patients with more than 5 years duration of Type 2 diabetes.

Keywords: Body mass index, Ferric reducing ability of plasma, Glycosylated hemoglobin, Malondialdehyde, Nitric oxide

INTRODUCTION

ED is an earliest pathological process of atherosclerosis [1]. Vascular tone, platelet activity, leukocyte adhesion and thrombosis is regulated by endothelium. Impaired function of endothelium initiates the development of atherosclerosis [2]. The traditional risk factors of premature atherosclerosis such as age, smoking, hypercholesterolemia, hypertension, hyperglycaemia and family history is associated with endothelium dependent vasodilatation in both adults and children. An earlier study had recognized an association of obesity, high C-reactive protein and chronic infection with ED [3].

Oxidative stress is a factor to cause ED by imbalance between oxidants and antioxidants. Increased Reactive Oxygen Species (ROS) production was generally observed in patients with diabetes mellitus. These ROS primarily targets lipid peroxidation and produce MDA. A superoxide ion reduces bioavailability of nitric oxide and causes ED [4].

Nitric oxide is one of the most effective endogenous vasodilator and also a common marker for ED [1]. ED results in either reduced synthesis or decreased bioavailability of nitric oxide. Reduced production of NO is considered to be a primary stage in the development of diabetic vasculopathy and strong interpreter of cardiovascular events in patients with diabetes mellitus [5]. Since, ED is a major factor for vascular complications, the study has been designed to assess ED in patients with long term duration of Type 2 diabetes mellitus.

MATERIALS AND METHODS

This cross-sectional study was carried out in the Department of Clinical Biochemistry in association with Medicine Department OPD of Vinayaka Mission Kirupananda Variyar Medical College and Hospital, Salem, Tamil Nadu, India, between the period of 2012 to 2015. This study was conducted after obtaining ethical clearance from the Institutional Ethical Committee. This study

was part of a thesis work carried out in the Department of Biochemistry. Total 47 Type 2 diabetic subjects were selected for the study in the age group between 30-60 years. Type 2 diabetic subjects with regular anti-diabetic treatment, without documentation of cardiovascular disease were included in the study. Subjects with smoking, alcoholism, hypertension, renal disease, liver disease and thyroid disorders and also subjects taking lipid lowering drugs and antioxidants were excluded from the study.

About 4 mL of blood sample was collected after obtaining informed consent from each subject. Blood samples were transferred into fluoride tube for glucose estimation, EDTA tube for HbA1c, sodium citrate tube for estimation of Nitric Oxide (NO), FRAP and plane tube for lipid profile and MDA. Plasma and serum were separated from blood sample by centrifugation at 3000 rpm for 15 minutes. Glucose, HbA1c and lipid profile were estimated by well established Kit methods (Tulip Diagnostic Ltd) in auto-analyzer.

Blood glucose level was estimated by Glucose Oxidase Peroxidase method (GODPOD). In this method red colour quinonimine complex is formed and measured at 505 nm, which is directly proportional to the glucose concentration [6].

HbA1c was measured by Turbidimetric Immunoassay. Photometric measurement of turbidity, corresponding to antigen-antibody reaction by the end point method at 600 nm to directly determine HbA1c in whole blood [7].

Total cholesterol was estimated by cholesterol esterase and peroxidase. In this method, red colour complex is formed which is directly proportional to the concentration of cholesterol. The absorbance of sample and standard was measured within 60 minutes against reagent blank at 500 nm [8].

Triglyceride was measured by Glycerol phosphate oxidase and peroxidase method. In this method red coloured complex was measured at 510 nm [9].

HDL-cholesterol was measured by Immuno-Inhibition, Two Reagent method. The absorbance of this method was measured at 700/600 nm before and after addition of reagent 2 against reagent blank [10].

Calculation

HDL cholesterol concentration = Difference in absorbance between 700 and 600 nm

LDL and VLDL were calculated by standard Friedwald's equation.

VLDL cholesterol concentration = Triglyceride/5

LDL cholesterol concentration = Total cholesterol – (VLDL+HDL) [11].

Malondialdehyde (MDA), Total antioxidant capacity, Nitric Oxide (NO) were measured by manual methods in spectrophotometer.

MDA was measured by Thiobarbituric Acid Reactive Substances (TBARS) method. Free MDA, as a measure of lipid peroxidation, was measured at 532nm spectrophotometrically as TBA reactive substances after precipitating the proteins with Trichloroacetic Acid (TCA) [12].

Total antioxidant capacity was measured as FRAP. Antioxidant power converts ferric to ferrous ion. Reduction at low pH causes a colored ferrous tripyridyltriazine complex to form. FRAP values are obtained by comparing the absorbance change at 593 nm in mixture (test), with those containing ferrous ion in known concentration (standard) [13].

Nitric Oxide (NO) was estimated by Kinetic cadmium reduction method. In this method, Nitrate the stable product of nitric oxide is reduced to nitrite by cadmium reduction method after deproteinisation of sample by Somogyi reagent. The nitrite produced is determined by diazotization of Sulphanilamide and coupling with Napthylethylene Diamine. Absorbance was measured at 540 nm in spectrophotometer [14].

BMI was calculated by standard formula weight in Kg/Height in m^2.

The study subjects were divided into two groups based on the duration of diabetes. Group-I contains 27 Type 2 diabetic subjects with <5 years duration of disease and Group-II contains 20 Type 2 diabetic subjects with >5 years duration of disease.

STATISTICAL ANALYSIS

Statistical analysis was done by using Microsoft excel sheets and SPSS software version 16.0. Microsoft excel was used for Mean±SD and "Kruskal-Wallis" test was performed by SPSS software to compare variables between two groups. The p-value of <0.05 was considered as statistically significant.

RESULT

In this study Group-II subjects with >5 years duration of type 2 diabetes have significantly high level of age (54.70±8.32) compared to <5 years duration of Type 2 diabetic subjects in Group-I (47.59±10.49), p-value at <0.05. No significant difference in the levels of BMI, Blood sugar and HbA1c were found between two study groups. [Table/Fig-1] shows clinical and biochemical characteristics of Type 2 diabetic subjects with duration of disease.

Parameters	<5 years Duration of T2DM (n-27) – Group-I	>5 years Duration of T2DM (n-20) – Group-II	p-value
Age	47.59±10.49	54.70±8.32	<0.05*
BMI (Kg/m2)	25.67±9.03	24.85±3.05	>0.05
FBS (mg/dl)	174.41±64.73	143.13±50.14	>0.05
PPBS (mg/dl)	290.35±89.24	260.73±75.43	>0.05
HbA1c (%)	9.88±2.72	8.63±2.04	>0.05

[Table/Fig-1]: Clinical and biochemical characteristics of Type 2 diabetic subjects. (Data expressed as mean±SD; statistical significance testing was done by "Kruskal Wallis" test).
*Significant (p<0.05)

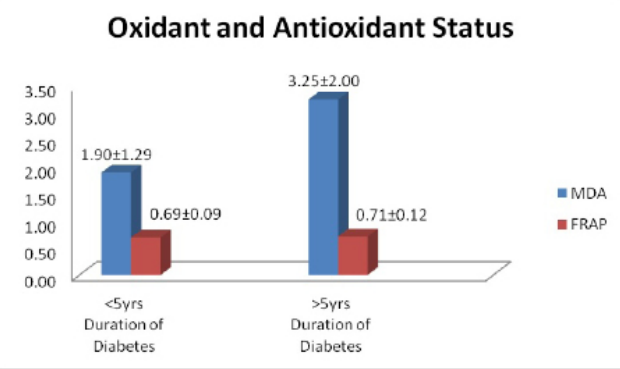
[Table/Fig-2] shows no significant difference in the level of lipid profile between patients with <5 years and >5 years duration of Type 2 diabetes mellitus.

Significantly high level of MDA was found in Group-II (3.25±2.00) compared to Group-I (1.90±1.29) and there is no significant difference in the level of FRAP between the groups [Table/Fig-3].

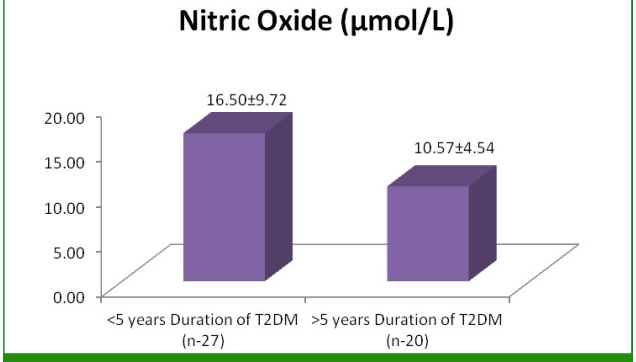
Significantly low level of NO was identified in Group-II subjects (10.57±4.54) compared to Group-I subjects (16.50±9.72), p-value <0.05 [Table/Fig-4].

Parameters	<5 years Duration of T2DM (n-27) – Group-I	>5 years Duration of T2DM (n-20) – Group-II	p-value
Total Cholesterol (mg/dl)	195.92±29.66	190.55±43.93	>0.05
Triglyceride (mg/dl)	163.56±72.20	159.75±60.95	>0.05
HDL (mg/dl)	40.41±8.77	41.55±6.95	>0.05
LDL (mg/dl)	188.58±34.93	180.85±47.65	>0.05
VLDL (mg/dl)	32.67±14.47	32.00±12.24	>0.05

[Table/Fig-2]: Lipid profile in <5 years and >5 years duration of Type 2 diabetic patients. (Data expressed as mean±SD; statistical significance testing was done by "Kruskal Wallis" test).
*Significant (p<0.05)



[Table/Fig-3]: Comparison of oxidant and antioxidant status in between the groups.
*Significant (p<0.05); Non-significant (p>0.05)



[Table/Fig-4]: Status of nitric oxide levels in between the study groups.
*Significant (p<0.05); Non-Significant (p>0.05)

DISCUSSION

The present study found significantly reduced function of endothelium in association with duration of disease in type 2 diabetic subjects. Here, age and oxidative stress might be the reason to cause ED by increasing the duration of disease. This is further involved in the progression of atherosclerosis and cardiovascular disease in diabetes. So, we find a relationship between duration of disease and ED, which can be helpful to predict vascular complication in diabetic subjects.

An earlier, Diabetes Control and Complications Trial (DCCT) has reported 41% reduction in the risk of CVD with intensive control. Nine years post-DCCT follow-up cohort study has shown 42% reduction in CVD risk and 57% reduction in the risk of myocardial infarction, stroke or CVD death compared to standard treatment [15]. UKPDS has observed 16% reduction of cardiovascular complications in intensive glycaemic control. However, no significant reduction in cardiovascular events with intensive glycaemic control has demonstrated by other two trials such as Action in Diabetes and Vascular Disease-Preterax and Diamicron Modified Release Controlled Evaluation [ADVANCE] and Veterans Affairs Diabetes Trial (VADT) [16].

As it is known that ED is an initiator for the development and progression of vascular complications such as hypertension, atherosclerosis and coronary artery disease. ED provides an important link with other diseases such as chronic renal failure and diabetes [1]. Several risk factors associated with ED such as classical factors like diabetes mellitus, dyslipidemia, hypertension, aging and novel factors like infection, physical inactivity, post-prandial state, homocysteine and obesity [3]. It is closely associated with obesity or insulin resistance or Type 2 diabetes mellitus [1]. Hence, ED is considered as primary stage for the development of atherosclerosis and vascular complications.

Body Mass Index (BMI): It is broadly accepted being overweight having >25 kg/m^2. Elevated BMI is a well established contributor to the etiology of cardiovascular disease, Type 2 diabetes and certain site-specific cancers including colorectal and breast cancer [17]. The present study has found no significant difference in the level of BMI between two groups of Type 2 diabetic patients [Table/Fig-1]. Similarly an earlier study has reported no significant association between BMI and duration of diabetes (4-17 years). It also demonstrated high risk of CVD in Type 2 diabetic subjects irrespective of BMI [18]. The factors influence BMI level such as lifestyle, intake of calorie, physical activity, poor glycaemic control and medication. Effect on weight gain, weight loss and weight balance may vary between the medications. In anti-diabetic treatment, metformin therapy can cause weight loss, sulfonyl urea drugs and insulin therapy may results in weight gain [19]. In our study, diabetic patients were under medication, but groups were divided with irrespective of the treatment. This might be the reason for insignificant difference in the level of BMI between the groups.

Glycosylated Hemoglobin (HbA1c): HbA1c is widely used in diabetic patients to monitor long-term glycaemic control. It is recommended to be measured at least two times in a year to predict diabetic complications [20]. The cardiovascular risk was 2-4 times higher in diabetic than non-diabetic. Among diabetic population glycemic control may be an important mediating factor in preventing CVD [21]. Recent study noted elevated level of glycemia was associated with cardiovascular disease among general population without diabetes [22]. The present study has shown no significant difference in the levels of fasting and post prandial blood sugar and HbA1c in between two groups [Table/Fig-1]. An earlier study has found that poor glycaemic control in Type 2 diabetic patients with 6-10 years duration of disease and good glycaemic control in patients with 3-7 years duration of diabetes. It also showed significant correlation between glycaemic control and HbA1c in diabetic patients [23].

Tan KC et al., has reported that high level of HbA1c in diabetic patients with duration of 3-16 years compared to healthy control [24]. Elevated level of HbA1c was associated with poor glycaemic control [25]. In the present study, both study group subjects were under poor glycaemic control. This might be the reason for insignificant difference in the level of HbA1c between two diabetic duration groups.

Lipid Profile: Type 2 diabetic patients typically have an atherogenic lipid profile that characterized by hypertriglyceridemia, low HDL and small dense LDL particles. Diabetic dyslipidemia has been associated with increased risk of CVD and lead to death in Type 2 diabetes [26]. Patients with diabetes have shown ED, this is due to frequent association with other cardiovascular risk factors including dyslipidemia, hypertension and obesity [27]. The present study shows no significant difference in the level of lipid profile (Cholesterol, Tgl, HDL, LDL and VLDL) between two groups [Table/Fig-2]. On comparison with healthy control level of lipid profile was found to be high in patients with 4-10 years duration of diabetes [28]. Mullugeta Y et al., observed that Type 2 diabetes lipidus directly affected by hyperglycaemia. Regulation of lipid profile was associated with glycaemic control in Type 2 diabetic patients [29]. In our study, both groups of diabetic subjects were under poor glycaemic control. This might be the reason for insignificant difference in the level of lipid profile between two diabetic groups.

Malondialdehyde (MDA): Oxidative stress is defined as imbalance between oxidants and antioxidants status in the body. Increased free radicals damage cellular organelles and enzymes, lipid peroxidation and develop complications in diabetes mellitus. Hence, it is also considered to be causative factor for the development of complications in diabetes [30]. The present study shows significantly high level of MDA in patients with >5 years duration of diabetes compared to <5 years duration of diabetes [Table/Fig-3]. An earlier study reported an increased MDA level in >5 years duration diabetes compared to <5 years duration of diabetes. As duration of diabetes increases, chronic hyperglycemia increases production of free radical and alters antioxidant defense [30]. So, here glycaemic control might be a critical factor for lipid peroxidation in duration of diabetes. Oxidative stress also increases in elderly subjects by uncontrolled production of free radicals from aging mitochondria and then modifies lipids, proteins and DNA [31]. In our study, ageing process might be the reason for high level of MDA in >5 years duration of diabetes. However it is still controversial on oxidative stress in aging process.

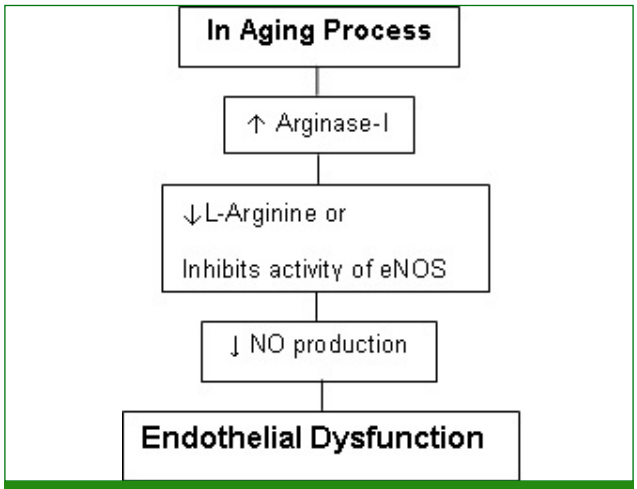
FRAP (Ferric Reducing Ability of Plasma): No significant difference in the level of FRAP was observed between two different duration of diabetic groups [Table/Fig-3]. Earlier studies have identified lower levels of antioxidants in diabetic patients with different durations of diabetes (5-10 years and 6-10 years) than healthy control [32,33]. In hyperglycemic condition, glucose is diverted to sorbitol pathway that consumes NADPH. It is an important cofactor for regeneration of GSH by glutathione reductase. Hence, hyperglycemia indirectly reduces GSH, which leads to increase oxidative stress [32]. In our study, both groups have same glycaemic status, this might be the reason for insignificant difference in the level of FRAP between two study groups.

Nitric Oxide (NO): ED refers to wide alterations in endothelial functions that may contribute to the development of clinical phase of atherosclerosis. The assessment of endothelial-dependent vasodilation has emerged as an indicator of the function of endothelium. Predominantly, increased production of endothelium-derived NO has been proved as assessing endothelial-dependent vasodilation in humans [3]. Since, ED has considered being a promoter of the process of atherosclerosis and cardiovascular morbidity and mortality. Schiekofer S et al., has reported ED as an earlier event in Type 1 and Type 2 diabetes mellitus and its relation to the development and progression of vascular complications in diabetes [34].

Significantly lower level of NO was found in patients with >5 years duration of diabetes compared to <5 years duration of diabetic patients [Table/Fig-4]. There are several factors involved in ED other than diabetes mellitus/hyperglycaemia such as age, obesity, physical inactivity, inflammation, dyslipidaemia, smoking, hypertension and homocysteine [3].

In this study, average age of Group-II was found to be significantly high when compared to Group-I [Table/Fig-1]. So, the aging process can be assumed as the major reason for observed low level of NO in >5 years duration of diabetic subjects. Age alone can cause ED even in the absence of other risk factors [35]. Activity of arginase-I has been found to be increased during aging process. This enzyme impairs the production of NO either by reducing the level of L-Arginine or by inhibiting the action of eNOS and causes ED [Table/Fig-5] [36].

Impaired NO bioavailability by different mechanisms may determine the progressive impairment of endothelial function. In this study, increased duration of diabetes along with aging



[Table/Fig-5]: Aging process cause endothelial dysfunction.

process might reduce the endothelial function and it can leads to vascular complications. Earlier studies also reported that increased age was associated with reduction in NO production. Hence, in recent years ED has been characterized a major promoter of atherosclerosis and thrombosis and an independent predictor of the risk of CVD in humans [21].

LIMITATION

The sample size was less due to exclusion of subjects treated with lipid lowering drugs, anti-oxidants and diabetic complications. Hence, further studies are needed to get better result with more number of samples or follow-up study.

CONCLUSION

Reduced NO level was identified in patients with >5 years duration of Type 2 diabetes along with aging process. Here ageing process was identified as a risk factor for both oxidative stress and endothelial dysfunction. So, estimation of NO along with regular parameters might be helpful to predict earlier risk of ED and its complications in Type 2 diabetic subjects. We need further studies on the effect of duration of diabetes and aging process on ED in association with good and poor glycaemic control.

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