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Correlation of Oxidative Stress Markers, Antioxidants, Vegf and Apoa-I in Patients of Type 2 Diabetes Mellitus without Retinopathy

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Abstract
Introduction: Diabetes mellitus (DM) is one of the most common non-communicable diseases (NCDs) globally. The greatest burden of diabetes is in the low- and middle income countries. Diabetes increases the risk of many different complications that in general can be divided into macrovascular and microvascular disease. The macrovascular complications include coronary heart disease and cerebrovascular disease. The microvascular complications include neuropathy, nephropathy and diabetic retinopathy. Diabetic retinopathy is a major long-term complication of diabetes. Oxidative stress markers, antioxidants and Serum VEGF and ApoA-I play an important role in pathogenesis of diabetic retinopathy in patients of type 2 diabetes. Present study was conducted to look into the status of oxidative stress markers and level of antioxidants and Serum VEGF and ApoA-I in patients of type 2 diabetes without retinopathy and its comparisons with the healthy non-diabetic controls.
Aims & Objectives: To measure the serum level of oxidative stress markers and level of antioxidants, an serum levels of VEGF & ApoA-I in patients of type 2 diabetes without retinopathy and its comparison with the healthy normotensive, non-diabetic controls and find the correlation between them.
Material and Methods: Present clinical study was conducted at the Department of General Medicine and Department of Biochemistry at the Institute of Medical Sciences, Banaras Hindu University, Varanasi. 45 type 2 diabetics without retinopathy, selected from the patients attending the OPD of Department of General Medicine and Endocrinology SSH, BHU or admitted in the indoor Department of General Medicine, SSH, BHU were included in the study. 20 healthy non diabetic, non hypertensive age and sex matched of individuals were selected as controls. With the standard protocol of analysis, we subjected the collected blood for estimation of oxidants like malondialdehyde and protein carbonyl and antioxidants like superoxide dismutase, serum levels of VEGF and ApoA-I with the help of standardized ELISA kits and correlation was calculated.
Results: Present study demonstrated enhanced level of the oxidants (MDA & Protein carbonyl) and a decreased levels of the antioxidants superoxide dismutase (SOD) in the cases i.e. type 2 diabetics without retinopathy whereas the controls showed the lower levels of the oxidants and the higher levels of the antioxidants. VEGF measured was significantly higher in the cases i.e. type 2 diabetics without retinopathy as compared to the healthy subjects. ApoA-I estimation in the diabetic patients was slightly lower as compared to the healthy controls. The difference between both the groups was not statistically significant (P= 0.096).
Conclusion: Antioxidant supplementations and statins may have clinical usefulness in the treatment of this complex disorder and in preventing complications and progression, but the final verdict and consensus can only be obtained after well controlled large randomized studies.

Keywords: Oxidative Stress Markers, Vegf and Apoa-I, Diabetes Mellitus

Introduction
Diabetes mellitus (DM) is one of the most common non-communicable diseases globally. The global prevalence of diabetes mellitus was 366 million in 2001 and it is estimated to increase to 522 million by 2030 [1]. It is a leading cause for the cardiovascular diseases, stroke, blindness, amputations and end stage renal disease in the world (IDF 2005). The most common form of diabetes mellitus is type 2 and constitutes about 90-95% of all the diabetic cases (ADA, 2017).
Diabetes is usually associated with the increased production of free radicals or impaired antioxidant defences [2]. It has been demonstrated that the oxidative stress has an adverse effect on the glucose metabolism. Chronic complication of diabetes mellitus (DM) has also

been attributed to the oxidative stress [3]. Diabetes mellitus (DM) is associated with a wide range of microvascular complications including diabetic retinopathy (DR). One of the main risk factors associated with development of DR is poorly controlled blood sugar as assessed by glycated hemoglobin levels (HbA1c)—the higher the HbA1c, the greater the risk of developing retinopathy [4]. A number of interconnecting biochemical pathways have been proposed as potential links between hyperglycemia and diabetic retinopathy. These include increased polyol pathway flux, activation of diacylglycerol-(DAG) PKC pathway, increased expression of growth factors such as vascular endothelial growth factor.

Vascular endothelial growth factor (VEGF) is a well-known pathogenic factor for the disruption of the blood retinal barrier (BRB) and neovascularization, which are the primary pathogenic events of diabetic macular edema (DME) and proliferative diabetic retinopathy (PDR), respectively. Apolipoprotein AI is the major protein component of HDL particles in plasma**.** In the retina, ApoA-I is proposed as a key factor for preventing lipid accumulation and a potent scavenger of oxygen-reactive species for protecting the retina from the oxidative stress caused by diabetes [5].

The data pertaining to the oxidative stress and Antioxidants and serum level of VEGF and ApoA-I in the type 2 diabetics without retinopathy are scanty from our country and especially from this region. Present study is aimed to look into the status of oxidative stress markers and level of antioxidants and Serum VEGF and ApoA-I in patients of type 2 diabetes without retinopathy and its comparisons with the healthy non-diabetic controls.

Materials and Methods

Study population: Present study was conducted in the Department of General Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi in collaboration with Department of Biochemistry in the period of month of June 2016 to July 2017. 45 patients of newly diagnosed type 2 diabetics without retinopathy of age between 20 to 65 years were selected from the Department of Medicine and endocrinology IMS, BHU, Varanasi. 20 age and sex matched healthy non-diabetic & Normotensive individuals were selected as the controls, whose blood samples were drawn with their consent for comparison with the blood samples of the cases.

Estimation of Oxidants (Malon Dialdehyde, Protein carbonyl), Anti-oxidants [Superoxide dismutase (SOD)] and Serum levels of VEGF and ApoA-I: Detailed history and clinical examination (including fundoscopy by ophthalmologist) was done in all the selected cases and controls. Then the venous blood samples of about 5 ml were collected. 3 ml was taken in a clean and dry plain vials without any anticoagulant. The blood was allowed to clot at room temperature. The sera was removed and stored at -20 ^{0}C in a sterile plain glass vial until analyzed in Department of Biochemistry, IMS, BHU. 2ml of blood was also taken in EDTA vial for analysis. Estimation of oxidants (Malon Dialdehyde, protein carbonyl), anti-oxidants [(Superoxide dismutase (SOD)] and serum levels of VEGF and ApoA-I was done with the help of standardized methods. Calculation of results for VEGF and ApoA-I is done by mean absorbance for each set of duplicate standards, controls and sample was calculated and average zero standard optical density was subtracted. The standard curve on log-log graph paper (using Sigma plot software) was plotted, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points was drawn.

Sensitivity

The minimum detectable dose of Human VEGF was determined to be 10pg/ml and for Human ApoA was determined to be 0.08 ng/ml. Minimum detectable dose for both is defined as the analyte concentration resulting in an absorbance that is 2 standard deviations higher than that of the blank.

Statistical Analysis

The statistical analysis was done using SPSS for Windows version 16.0 software. Descriptive statistics like mean, frequency and percentages of various parameters were calculated. For categorical variable Chi-Square test and Fischer's Exact test was used. For comparing two group of mean Students 't' test and for paired samples Paired 't' test was used. The p value <0.05 was considered as statistically significant

Results

Clinical Characterstics

The study consisted of 45 patients of type 2 Diabetics without retinopathy and 20 healthy non diabetic and non-hypertensive age and sex matched controls. In our study, males were slightly higher in number in both groups (Table 1).

Table 1: Clinical Characteristics of Study Population

Parameter	Cases	Control
Age (in years)	50.13±9.132	52.35±5.779
Male / Female	28 / 17	11 / 9
HbA1C	9.66000±1.775003	5.40000±0.502625
Fasting blood sugar (mg/dl)	222.93±74.567	87.55±11.038
Post Prandial blood sugar (mg/dl)	313.31±92.567	126.55±8.507

Oxidants Malondialdehyde (MDA) and Protein Carbonyl levels

In the present study, median serum MDA level in the age & sex matched healthy controls was 0.002 (0.001-0.003) µmol/L, while in type 2 Diabetes mellitus without retinopathy group it was 0.015 (0.008-0.034) µmol/L. The median serum MDA level in type 2 Diabetes mellitus without retinopathy group was elevated as compared to the median MDA levels in control group. The difference between both the groups was statistically highly significant (P<0.001). Mean serum protein carbonyl level in the age & sex matched healthy controls was 7.60050±0.920535 ηmol/ml, while in type 2 Diabetes mellitus without retinopathy group it was 1.55713±1.756019 ηmol/ml. The mean serum protein carbonyl level in the type 2 Diabetes mellitus without retinopathy group was elevated as compared to the mean Protein carbonyl level in control group. The difference between the both groups was statistically significant (P<0.001).

Antioxidant Superoxide Dismutase (SOD) levels

The median serum SOD level in the age & sex matched healthy controls was 3.500 (2.800-4.175) U/L, while in patients of type 2 Diabetes mellitus without retinopathy group it was 1.500 (1.000-2.000) U/L. The median serum SOD level in the type 2 Diabetes mellitus without retinopathy group was decreased as compared to the median SOD level in the control group. The difference between the both groups was statistically highly significant (P<0.001).

VEGF levels and ApoA-I levels

In the present study, median serum VEGF level in the age & sex matched healthy controls was 20.328 (6.368-38.261) pg/ml, while in patients of type 2 diabetes mellitus without retinopathy group it was 60.680 (38.590-87.050) pg/ml. The median serum VEGF level in the in patients of type 2 diabetes mellitus without retinopathy group was elevated as compared to the median serum VEGF level in control group. The difference between the both groups was statistically significant (P<0.001). Median serum ApoA-I level in the age & sex matched healthy controls was 12.920(7.648-15.680) pg/ml, while in patients of type 2 diabetes mellitus without retinopathy group it was 10.733 (1.210-14.646) pg/ml. The median serum level of ApoA-I in the type 2 Diabetes mellitus without retinopathy group was slightly reduced as compared to the median ApoA-I level in control group. The difference between the both groups was not statistically significant (P =0.096).

Correlation between Oxidants, Antioxidants, VEGF and ApoA-I

Protein carbonyl shows positive correlation with MDA (r=0.607, p<0.001) (Figure 1). VEGF shows positive correlation with MDA (r=0.688, p<0.001) (Figure 2). Apo-A1 shows no correlation with MDA (r=0.052, p=0.735) (Figure 3). ApoA-I shows no correlation with VEGF (r= -0.021, p= 0.889) (Figure 4). SOD shows negative correlation with MDA (r= -0.441, p<0.002) (Figure 5)

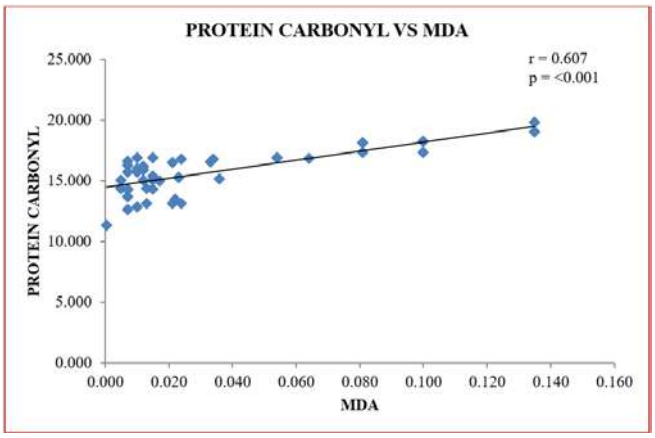

Fig 1: Correlation between Protein carbonyl and MDA

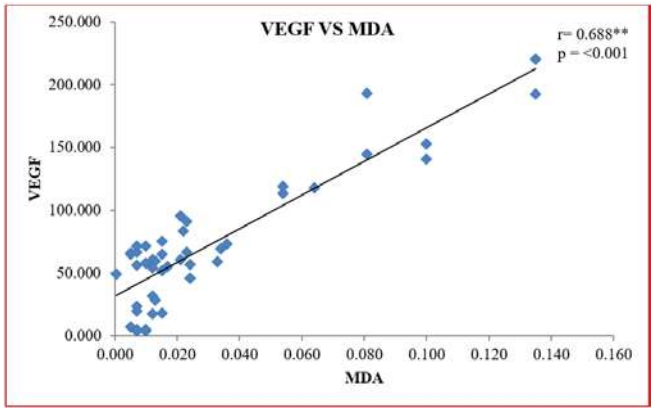

Fig 2: Correlation between VEGF and MDA

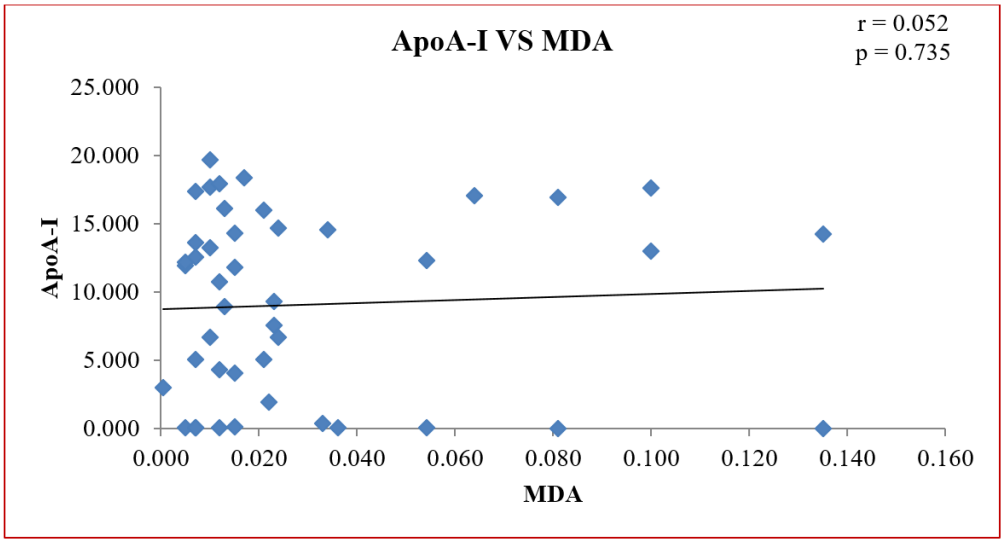

Fig 3: Correlation between ApoA-I and MDA

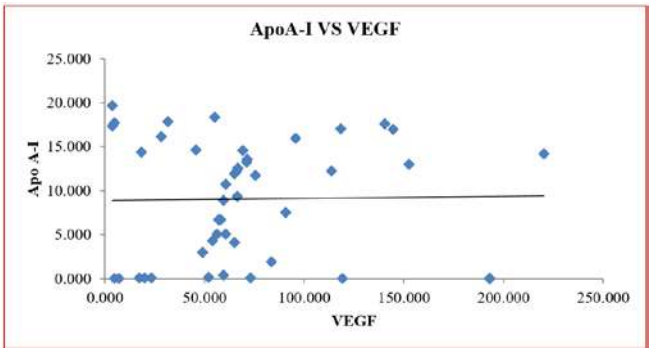

Fig 4: Correlation between VEGF and ApoA-I

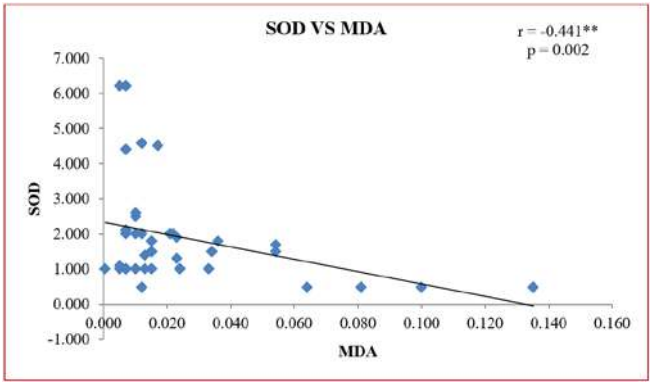

Fig 5: Correlation between SOD and MDA

Discussion

Diabetes mellitus is a metabolic disorder that is characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency [6]. Diabetes is associated with macrovascular (coronary artery, cerebrovascular and peripheral vascular disease) and microvascular (retinopathy, nephropathy, and neuropathy) complications. Diabetes is associated with the increased production of the free radicals [7] and impaired antioxidant defense. In the present study, Oxidants were measured by malondialdehyde and protein carbonyl estimation in the serum. Anti-oxidants were measured by the estimation of Superoxide Dismutase. Further by ELISA kits, levels of VEGF and ApoA-I were measured in the serum of both groups.

In the present study, serum malondialdehyde (MDA) was found to be significantly elevated in the diabetic patients 0.015(0.008-0.034) µmol/L as compared to healthy controls (0.002(0.001-0.003) µmol/L) (p <0.001). Similar results were observed in other studies by Dulal HP *et al.* [7] which, the diabetic patients (with or without complications) had significantly elevated MDA as compared to the healthy nondiabetic controls. Protein carbonyl was found to be significantly elevated in the diabetic patients (1.55713±1.756019 ηmole/ml) when the compared to healthy controls (7.60050±0.920535 ηmole/ml) (p < 0.001). The study of Odetti and his co-workers (1999) reported the slight but non- significant result with respect to the protein carbonyl content in the controls compared to type 2 diabetics. However in the present study the results were consistent and promising as a stable marker for oxidative stress. Similar results were observed in other studies by Dayanand *et al.* [8] in which, the diabetic patients (with or without complications) had significantly elevated Serum Protein Carbonyl as compared to the healthy non diabetic controls.

The Superoxide Dismutase (SOD) was found to be significantly reduced in the diabetic patients 1.500 (1.000-2.000) U/L) when compared to the healthy controls 3.500 (2.800-4.175) U/L) (p <0.0001). Similar results were observed in the other studies by Dayanand *et al.* [9] and in which, the diabetic patients (with or without complications) had significantly reduced SOD as compared to the healthy controls.

VEGF were significantly higher in the diabetic patients without retinopathy (60.680 (38.590-87.050) pg/ml) as compared to the healthy controls (20.328 (6.368-38.261) pg/ml) with p<0.001. VEGF shows positive correlation with oxidant (MDA) (r=0.688, p=<0.001) in current study. ApoA-I is proposed as a key factor for preventing lipid accumulation and a potent scavenger of oxygen-reactive species for protecting the retina from the oxidative stress caused by diabetes. Sasongko *et al.* [5] found that there were significant associations between the decreased ApoA1 and low ApoA-I/ApoB ratio in serum with PDR. In our study the levels of ApoA-I in the diabetic patients was slightly lower (10.733 (1.210-14.646) pg/ml) as compared to healthy controls 12.920 (7.648-15.680) pg/ml). The difference between the both groups was not statistically significant (*P* =0.096). The result have shown the protective role of ApoA-I in preventing the development of DR as ApoA-I is proposed as a key factor for preventing lipid accumulation and a potent scavenger of oxygen-reactive species for protecting the retina from the oxidative stress caused by diabetes.

Conclusion

The present study demonstrated enhanced level of the oxidants (MDA & Protein carbonyl) and a decreased levels of the antioxidants (SOD) in the cases i.e. type 2 diabetics without retinopathy. Whereas the controls, showed the lower levels of the oxidants and the higher levels of the antioxidants. VEGF measured was significantly higher in the cases i.e. type 2 diabetics without retinopathy as compared to the healthy subjects. ApoA-I estimation in the diabetic patients was slightly lower as compared to the healthy controls. The difference between both the groups was not statistically significant (*P*= 0.096). Studies regarding the levels of VEGF and ApoA-I in diabetes with complications (Microvascular and Macrovascular) will support their further role in the progression and severity of Diabetes. Yet it is difficult to come on a definite conclusion whether the altered levels of the oxidative stress are cause or the effect of diabetes mellitus. The extent of role of growth factor (VEGF) and the lipoprotein (ApoA-I) in the pathogenesis of ocular complication of diabetes mellitus (Diabetic retinopathy) will need further larger cohort studies. However till date studies have shown their significant role in the diabetic retinopathy. Antioxidant supplementations and statins may have clinical usefulness in the treatment of this complex disorder and in preventing complications and progression, but the final verdict and consensus can only be obtained after well controlled large randomized studies.

Conflict of interest: There is no conflict of interest to this study.

Funding: Cost to this study is nil.

Ethical consideration: Patients were not subjected to any additional procedure for the purpose of study. The study was conducted on ethical guidelines for biomedical research on human subject as given in "Declaration of Helsinki" and by Central Ethics Committee on Human Research (CECHR) of ICMR, New Delhi.

Patient consent: Patients were informed beforehand that biopsy is a routinely established procedure with no additional risk. Confidentiality will be maintained.

Data availability statement: The supporting data is not shared

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