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A study of serum vascular endothelial growth factor and APO-A1 in diabetes mellitus with retinopathy

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Abstract
Background: The present study is aimed to look into the status of serum VEGF and APO A-1 in the patients of type II diabetes with retinopathy without hypertension and its comparisons with the healthy normotensive, non-diabetic controls and also comparing their level among the diabetic retinopathy severity groups.
Materials and Methods: Forty five (45) patients of newly diagnosed type II diabetics with retinopathy and twenty (20) age and sex matched healthy non-diabetic & normotensive individuals of age between 20-65 were selected as the controls. In all cases and controls Fundoscopy was examined by ophthalmologist and severity of retinopathy was graded according to International clinical Diabetic retinopathy Disease severity scale. Complete blood counts (CBC), renal function test (RFT), liver function test (LFT), plasma glucose fasting and postprandial, HbA1c,urine routine/microscopy, 12 lead ECG were done all the cases and controls. Serum VEGF and Apo-A1 level was estimated using ELISA kit. The statistical analysis was done using SPSS for Windows version 16.0 software. For comparing two group of mean Students 't' test and Mann-Whitney U test was used to compare VEGF and APO-A1 in the groups. The p value was considered as statistically significant when it is <0.05.
Results: In our study, males were slightly higher in number in all the groups. In our study, the maximum number of diabetic patients with retinopathy were in the age range of 51-60 years. In the present study FBS, PPBS, HbA1c, VEGF, APO-A1 were significantly elevated in the diabetic patients with retinopathy than in the control group. Significant positive correlation was seen between serum VEGF level and HBA1c.Apo-A 1 was significantly low in moderate NPDR group compared to mild NPDR and significantly low in PDR as compared to moderate NPDR.
Conclusion: In our study we have shown that serum VEGF level represents very well with the severity of retinopathy. It can be a potential tool for diabetic retinopathy early detection and probable severity prediction. Serum Apo-A1 is significantly lower in more severe retinopathy group, suggesting its protective role. In our region data regarding these two marker are scanty and these results should be validated in larger study population.

Keywords: Serum vascular, endothelial, APO-A1, retinopathy

Introduction
Diabetes mellitus (DM) is a chronic metabolic disorder with multifactorial etiopathology. Prevalence of this chronic disease is increasing every year. With urbanization and altered lifestyle and food habit people are more prone to develop diabetes mellitus. According to the International Diabetes Federation (IDF, ninth edition) [1] 463 million adults have diabetes globally and by 2045 this will raise to 700 millions. Chronic course of diabetes mellitus leads to multiple complication, of which diabetic retinopathy is one of the most dreaded. Due to development of retinopathy associated complications patients loose eye sight at an early age. Early diagnosis and strict control of blood glucose is key to prevent retinopathy associated blindness.
Pathophysiology of diabetic retinopathy is multifactorial. Vascular endothelial growth factor (VEGF) is a key 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 [2]. Various pathways are involved in the regulation of VEGF gene expression. Hypoxia is one of the important factors. VEGF production is induced when there is tissue ischemia or hypoxia. Tissue hypoxia leads to the production of hypoxia-inducible factor 1 (HIF-1) which initiates the VEGF mRNA transcription process [3-4]. In diabetes mellitus the chronic hyperglycaemia leads to accumulation of advanced glycation end (AGE) product.

Intravitreal injection of AGEs in experimental animals increased the VEGF mRNA expression in the ganglion, inner nuclear, and RPE cell layers of the retina [5].

A variety of other factors can regulate VEGF mRNA expression. These are insulin, Insulin like growth factor (IGF-I), hormones (TSH, ACTH, and gonadotropin), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), interleukin [IL]-1 and IL-6, and oncogenes (i.e., K-ras, Wnt) [6]. Insulin increased VEGF mRNA expression and VEGF in retinal pigment epithelium (RPE) cells through enhanced transcription of the VEGF gene [7].

Vascular leakage as a result of the breakdown of the BRB, is a key pathological feature of DME. By breakdown of the junctional complex of BRB, VEGF increases permeability through cells by forming fenestrae and vesicles [8]. VEGF activates conventional protein kinase C (PKC), which phosphorylates the tight junction protein occludin. This phosphorylation causes the disassembly of the tight junctions and thus increasing vascular permeability [9-10]. Also there is VEGF-induced increased permeability when the adhesion junction protein, VE- cadherin is phosphoralized and internalized [11]. Nitric oxide also have important role in VEGF-induced vascular permeability and also angiogenesis [12].

Activation of PKC-beta 2 isoform is important for VEGF-dependent retinal neovascularization [13]. VEGF induces metalloproteinases, and decreases the tissue inhibitors of metalloproteinases TIMP-1 and TIMP-2.Thus increased destruction of the basement membrane, helps angiogenesis that leads to PDR(14)(15). VEGF can also promote angiogenesis i through the intercellular adhesion molecule 1 (ICAM-1) (16) and vascular adhesion molecule 1 (VCAM-1) [17].

Apolipoprotein A1 produced from liver and intestine, is the principal HDL structural protein. It is essential for reverse transport of cholesterol from peripheral tissue to the liver. It is a cofactor for lecithin cholesterol acyltransferase (LCAT) which catalizes formation of cholesteryl esters. ApoA1 has vasoprotective property due to its reverse cholesterol transport function. LDL-C oxidation products may induce vascular smooth muscle cell cytotoxicity and vascular endothelial cellular dysfunction in the retina. By inhibiting LDL-C oxidation Apo A1 may protect retinal vascular damage [18]. It also has antioxidant and anti-inflammatory effects. In the retina, ApoA-1 is may prevent lipid accumulation [19] and may protect the retina by scavenging the oxygen-reactive species [20].

The data pertaining to the serum level of VEGF and APO A-1 in the type II diabetics with retinopathy are scanty from our country. Present study is aimed to look into the status of serum VEGF and APO A-1 in the patients of type II diabetes with retinopathy without hypertension and its comparisons with the healthy normotensive, non-diabetic controls.

Material and Methods

Our study was conducted from the period of month of June 2016 to July 2017 in the Department of General Medicine, Institute of Medical Sciences, Banaras Hindu University, Varanasi. Biochemical analysis was done in collaboration with Department of Biochemistry, IMS, BHU. For this study forty five [45] patients of newly diagnosed type II diabetics with retinopathy were selected from the department of Medicine, IMS, BHU, Varanashi. Twenty [20] age and gender matched healthy non-diabetic & normotensive subjects were selected for the purpose the controls. Age of both cases and controles were within 20 to 65. Clinical history and systemic examinations was done in all the selected cases and controls. In all cases and controls fundoscopy was examined by ophthalmologist and severity of retinopathy was graded according to International clinical Diabetic Retinopathy Disease severity scale. Then about 5 ml venous blood samples of were collected. Blood was taken 2ml in EDTA vial amd 3 ml in clot vial for analysis. The blood in plain vial was allowed to clot at room temperature. The sera was removed and refrigerated at -20^{0}C in sterile plain glass vials until analyzed in Department of Biochemistry, IMS, BHU. Complete blood counts (CBC), renal function test (RFT), liver function test (LFT), plasma glucose fasting and postprandial, HbA1c, urine routine/microscopy, 12 lead ECG were done in all the cases and controls. Those who were, smoker, hypertensive were excluded. Patient with active infections, who were working in chemical / asbestos / metal factories were not selected. Patient receiving chemotherapy and radiotherapy, were excluded from the study. Patients on multi vitamins and mineral therapy, metformin, linagliptins and glibenclamide were also kept out of the study.

Estimation of Vascular Endothelial Growth Factor (VEGF) and Apo-A1

Serum VEGF concentration of the samples were measured with the help of VEGF ELISA KIT supplied by Ray Biotech and Apo-A1 consentration was measured with the help of human Apo-A1 ELISA kit(Ray Biotech). The minimum detectable dose of human VEGF was determined to be 10pg/ml and that of Apo-A1 was 0.08ng/ml. For both the kits intra assay CV% is <10% and inter assay CV% is <12%.The kits and samples were stored in -80 ºC.

Both the kit come with 96 wells coated with anti-human VEGF antibody and anti-human Apo-A1 antibody respectively. The standards and samples are pipetted into the wells and the specific immobilizing antibody coating in it captures the antigen (VEGF/Apo-A1) present in the sample. The wells are washed and biotinylated antihuman VEGF/Apo-A1 antibody added. Now HRP conjugated streptavidin is pippeted into the wells after the unbound biotinylated antibody are washed. Now reactants become antibody-antigen (VEGF/Apo-A1)-antibody -enzyme complex. The wells are washed again and a TMB substrate solution is added in to the wells. Now colour develops in proportion of VEGF/Apo-A1present in sample. The stop solution is added which changes the colour from blue to yellow and intensity of colour is measured at 450nm.

The 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. In the graph standard concentration was plotted on x –axis and absorbance on the Y –axis. The best fit straight line through the standard points drawn.

Statistical analysis

Mean, frequency and percentages of different groups were calculated. The SPSS for Windows version 16.0 software was used for statistical analysis. For categorical variable Chi-Square test and Fischer's Exact test were used. Students 't' test was used for comparing mean of two group. Mann-Whitney U test was used to compare VEGF and APO-A1 in the groups. The p value was considered as statistically significant when it is <0.05.

Observations and Results

In our study, males were slightly higher in number in all the groups. There were 30 male among 45 diabetic patients with retinopathy (66.7%) while non-diabetic non-hypertensive controls had 11 out of 20 (55%). Female diabetic patients with retinopathy were 15 (33.3%) whereas 9 (45%) were in the non-diabetic non-hypertensive control group. (Table no.1) In our study, the maximum number of diabetic patients with retinopathy were 17 (37.78%) in the age range of 51-60 years. In the present study fasting blood sugar was significantly elevated in the diabetic patients with retinopathy (mean 238.38mg/dl) than in the control group (87.1mg/dl) $p< 0.001$ (Table No.3). In our study post prandial blood sugar was significantly high in the diabetic patients with retinopathy (mean 336.60mg/dl) than in the control group (127.35mg/dl) $p< 0.001$(Table No.3). HBA1c was significantly high in the diabetic patients with retinopathy (mean10.009) than in the non-diabetic non-hypertensive controls (5.45) $p< 0.001$(Table No.3). In the present study, mean serum VEGF level in the controls was 20.32 (6.36-38.26) pg/ml while in the Normotensive diabetic with retinopathy group it was 354.57 (155.52-556.83) pg/ml (Table No.4). The mean serum VEGF level in the Normotensive diabetic with retinopathy group was significantly increased as compared to the mean VEGF levels in control group. The difference between the groups was statistically highly significant ($P< 0.001$). Among the Diabetics mean VEGF are 163.84 (99.34-192.96) pg/ml in mild non-proliferative diabetic retinopathy (mild NPDR) group, 488.63 (232.28-576.69) pg/ml in moderate non-proliferative diabetic retinopathy (moderate NPDR) group and 1744 (1688-1800) pg/ml in Proliferative retinopathy group (PDR) (Table No.5). In our study, mean serum APO-A1 level in the controls was 12.92 (7.64-15.68) g/L while in the normotensive diabetic with retinopathy group it was 0.89 (0.20-2.24) g/L (Table No.4). The mean serum APO-A1 level in the normotensive diabetic with retinopathy group was significantly decreased as compared to the mean APO-A1 levels in control group. The difference between both the groups was statistically highly significant ($P< 0.001$). Among the patients of Diabetes mean APO-A1 were 2.69 (2.34-5.54) g/L in mild non-proliferative diabetic retinopathy (mild NPDR) group, 0.42 (0.077-1.35)g/L in moderate non-proliferative diabetic retinopathy (moderate NPDR) group and 0.018 (0.015-0.021)g/L in Proliferative retinopathy group (PDR), (Table No.5).

Serum VEGF level showed significant positive correlation with HbA1c level of Diabetic Patients with retinopathy. (Fig.1) (Pearson Correlation = 0.471, p = 0.001). Serum APO-A1 level showed negative correlation with HbA1c level of Diabetic Patients with retinopathy though the study was not statistically significant. (Fig.2) (Pearson Correlation = - 0.180, p = 0.236).

Table 1: Gender distribution

Sex	Diabetic with retinopathy (n = 45)		Non-diabetic non-hypertensive (n = 20)	
	No.	%	No.	%
Male	30	66.7	11	55.0
Female	15	33.3	9	45.0
Total	45	100	20	100

Table 2: Age distribution in study population

Age (in years)	Diabetics with retinopathy		Non-diabetic non-hypertensive	
	No.	%	No.	%
30-40	6	13.33%	0	0
41-50	13	28.89%	5	25%
51-60	17	37.78%	11	55%
≤65	9	20.00%	4	20%

Table 3: Fasting blood sugar, post prandial blood sugar, HBA1cin study population

	Diabetics with retinopathy	Non-diabetic non-hypertensive	t -value	p -value
FBS (mg/dl)	238.38 ± 73.860	87.10 ± 13.69	7.25	<0.001
PPBS (mg/dl)	336.60 ± 86.957	127.35 ± 8.45	8.64	<0.001
HbA1c	10.009 ± 1.8863	5.45 ± 0.51	8.271	<0.001

Table 4: VEGF and APO-A1 in study population

Group	Diabetic with retinopathy (n = 45)	Non-diabetic non-hypertensive (n = 20)	Mann-Whitney U test	p -value
VEGF (pg/ml) (Median, IQR)	354.57 (155.52-556.83)	20.32 (6.36- 38.26)	4.00	<0.001
APO-A1(g/l) (Median, IQR)	0.89 (0.20-2.24)	12.92 (7.64-15.68)	25.00	<0.001

Table 5: VEGF and APO-A1 distribution among diabetic retinopathy groups

Diabetic Retinopathy Severity (No of patients)	Mild NPDR(13)	Moderate NPDR(26)	PDR(6)
VEGF(pg/ml)	163.84 (99.34-192.96)	488.63 (232.28-576.69)	1744 (1688-1800)
APO-A1(g/l)	2.69 (2.34-5.54)	0.42 (0.077-1.35)	0.018 (0.015-0.021)

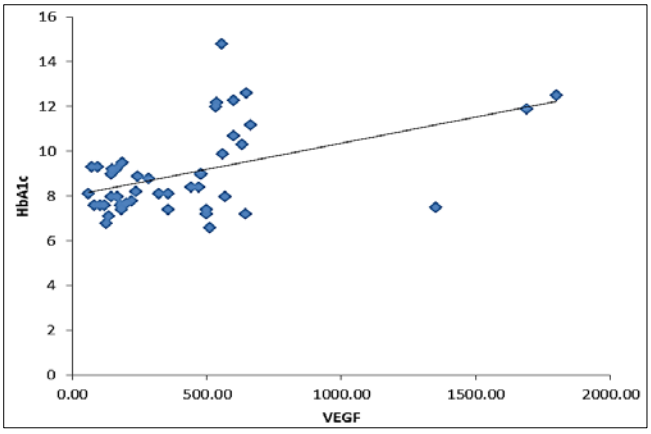

Fig 1: Correlation between HBA1c and VEGF

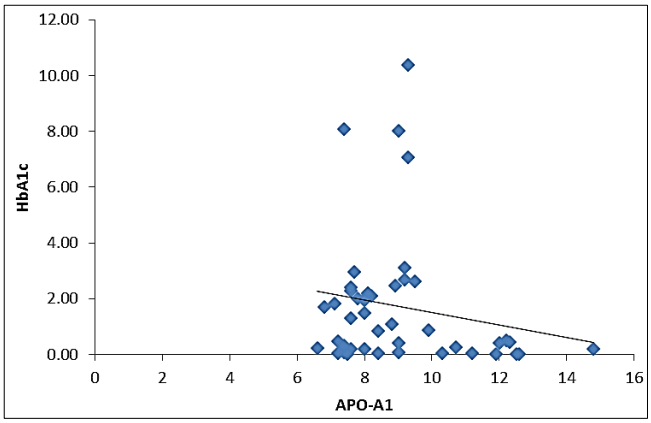

Fig 2: Correlation between HBA1c and APO-A1

Discussions

Diabetes Retinopathy is one of the dreaded microvascular complication. In the present study we compared the vascular endothelial growth factor (VEGF) and APO-A1 levels between 45 diabetic retinopathy group and 20 healthy controls.

Vascular Endothelial Growth Factor

In the present study, the mean serum VEGF level in the age & sex matched healthy controls was 20.32 (6.36-38.26) while in the Normotensive diabetic with retinopathy group it was 354.57 (155.52-556.83). The mean serum VEGF level in the Normotensive diabetic with retinopathy group was significantly increased as compared to the mean VEGF levels in control group. The difference between both the groups was statistically highly significant ($P< 0.001$). Also serum VEGF level showed significant positive correlation with HbA1c level of Diabetic Patients with retinopathy. (Fig.1)

Among the patients of Diabetics mean VEGF are 163.84 (99.34-192.96) in mild NPDR group, 488.63 (232.28-576.69) in moderate NPDR group and 1744 (1688-180) in Proliferative retinopathy group. VEGF value is highest in the Proliferative diabetic retinopathy group and is lowest in mild NPDR. Serum VEGF was significantly raised in PDR group as compared to moderate NPDR(p-value 0.004) and its level in moderate retinopathy group was significantly raised than in mild diabetic retinopathy group.(p-value 0.001).

The present study result is similar to previous study by Cavusoglu *et al.*, (2007) [22] where serum VEGF level was increased significantly between NPDR and PDR (P = 0.018) and also between diabetic with NPDR and Healthy controls ($p< 0.000$) [21]. Jain *et al.*, (2013) [23] in a study of serum VEGF level in the Diabetic retinopathy showed similar result [22]. Study done by Ozturk *et al.*, 2009 [24] showed higher VEGF level in those with NPDR compared to controls (p = 0.01) and in PDR compared to controls (p = 0.02). No significant difference between NPDR and PDR (p = 0.87) [23]. Thereby, VEGF is very well correlated with severity of retinopathy as per most of the above studies.

APO-A1

In the present study, the mean serum APO-A1 level in the normotensive diabetic with retinopathy group was significantly decreased as compared to the mean APO-A1 levels in control group. The difference between both the groups was statistically highly significant ($P< 0.001$).

Among the patients of Diabetes with retinopathy APO-A1 value is lowest in the Proliferative diabetic retinopathy group and is highest in mild NPDR. Serum APO-A1 was significantly lower in PDR group as compared to moderate NPDR (p-value = 0.004) and also APO-A1 level in moderate NPDR group was significantly lower than mild NPDR. (p-value <0.001).

Our study result was similar to the previous studies of apolipoproteins in type II diabetic patients with retinopathy. In a Chinese population, Hu *et al.*, [25] collected serum samples from 25 type 2 diabetic patients with very mild NPDR and 25 type 2 diabetic patients with PDR1 [19]. They found that there were significant associations between the decreased ApoA1 and low ApoA1/ApoB ratio in serum and PDR [24]. Their findings were consistent with the results obtained by Sasongko *et al.*, [26]. The findings from these two study groups are very encouraging. The beneficial associations of ApoA1 and deteriorating associations of ApoB/A1 with microvascular function seen in this study may be similar to findings in larger vessels. However, the sample size in these two studies was small and their findings need to be reproduced in larger longitudinal studies.

Recently, Sasongko *et al.*, [26] conducted a cross-sectional study of 224 diabetic patients to compare the associations of serum Apo lipoproteins with DR [25]. In this study, ApoA1 levels were inversely associated with DR, whereas increased ApoB and the ApoB-to-ApoA1 ratio were positively associated with diabetic retinopathy. ApoA1 and ApoB and the ApoB-to-ApoA1 ratio were significantly and independently associated with DR and DR severity and improved the ability to discriminate DR by 8% [25].

Conclusion

Our study shows significantly increased VEGF and decreased APO- A1 in diabetic patients with retinopathy as compared to nondiabetic normotensive healthy control. Anti VEGF treatment for PDR is approved and our study in Indian population further supports it and also strengthen its pathological role in the diabetic retinopathy. A decreased APO –A1 in patients of diabetic retinopathy indicates its possible protective role in this condition and should be confirmed by further studies.HbA1c showed significant positive correlation with serum VEGF level of diabetic retinopathy patients and thus further reinforcing the importance of long term blood sugar control to prevent the progression of diabetic retinopathy.

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