ORIGINAL ARTICLE
J Rehman Med Inst. Vol. 3, No. 1-2, 2017

LEVEL OF SERUM VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN PATIENTS WITH ADVANCED DIABETIC RETINOPATHY

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Submitted: February 14, 2017
Accepted: April 10, 2017

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ABSTRACT

Introduction: Though the pathogenesis of diabetic retinopathy is multifactorial, one of the key events is the growth of new blood vessels in retinal tissues. In some studies, the vaso-proliferative agent, Vascular Endothelial Growth Factor (VEGF), has been implicated as the mediator of neovascularization in diabetic retinopathy. The objective of the present study was to compare the levels of serum VEGF in advanced diabetic retinopathy patients with diabetic non-retinopathy patients and normal healthy controls.

Methods: The comparative study was conducted from June to November 2010 in the outpatient and inpatients department of three major hospitals of Peshawar and one eye hospital from Islamabad, in which 45 diabetic patients with retinopathy were compared with 38 diabetic patients without retinopathy, and 39 healthy individuals. Retinopathy group were further designated as diabetic non-proliferative retinopathy (DNPDR, n=20) and diabetic proliferative retinopathy (DPDR, n=25). Serum VEGF was done by ELISA. Data were analyzed for descriptive statistics by SPSS 14; $p \leq 0.05$ was taken as significant.

Results: Serum VEGF was significantly higher ($p < 0.001$) in DNPDR (202.60 ± 81.75 pg/ml) and DPDR (247.20 ± 85.79 pg/ml) patients than DNR (92.67 ±29.95 pg/ml) patients and normal healthy controls (28.30 ± 6.86 pg/ml).

Conclusion: Diabetic patients with retinopathy have significantly raised serum VEGF levels compared to Diabetic patients without retinopathy and normal healthy individuals; the levels are highest in female patients with proliferative diabetic retinopathy.

Keywords: Vascular Endothelial Growth Factors; Diabetes; Diabetic Retinopathy; Retinal Neovascularization.

The authors declared no conflict of interest. All authors contributed substantially to the planning of research, data collection, data analysis and write-up of the article and agreed to be accountable for all aspects of the work.

Citation: Parveen N, Khan A, Khan Z, Mahmood R, Khan Q, Khan K. Depression among doctors working in public and private tertiary care hospitals in district Peshawar. J Rehman Med Inst. 2017;3(1-2):34-8.

INTRODUCTION

Diabetic retinopathy is one of the leading cause of blindness worldwide, as well as being the principal cause of impaired vision in patients between 25 and 74 years of age.[1] The incidence of blindness is 25 times higher in diabetic patients compared to the general population. Diabetic Retinopathy (DR) is a well-known consequence of long-standing and poorly controlled Diabetes Mellitus (DM), currently affecting approximately 150 million people worldwide. The World Health Organization (WHO) predicts that the number of people affected will double by the year 2025.[2] In patients with DM, the prevalence of any form of diabetic retinopathy has been reported as approximately 24%.[3] The Beijing Eye Study (2009) reported a 37% rate of DR among all diabetic patients and a 54% rate after 10-19 years with the disease.[4]

The pathogenesis of DR is multifactorial but in primarily caused by the metabolic effects of chronic hyperglycemia, which results in vascular changes and subsequent retinal injury and ischemia. Most advanced retinal disease, including proliferative vascular changes and neovascularization in the setting of retinal ischemia, may be medicated by other mechanisms such as the action of vasoactive substances released during the inflammatory process.[5]

Certain growth factors promote the growth of new blood vessels from adjacent vessels in an abortive attempt to revascularize the diseased tissue. Studies in experiment models of retinopathy have shown that neovascularization is mediated in part by the interaction between Insulin Growth Factor 1 (IGF-1) and Vascular Endothelial Growth Factor (VEGF). VEGF is produced by many retinal cells in response to hypoxia. In retinas from diabetic patients, the intensity of immunostaining for VEGF is proportional to the severity of the retinopathy, and vitreous fluid VEGF concentration are increased.[5,6]

VEGF levels in ocular tissues from patients with diabetes are greater than in non-diabetic subjects.[7] VEGF is elevated in patients with diabetic retinopathy and is implicated in the pathogenesis of retinal neovascularization.

Literature from this part of the world appears scarce regarding VEGF in diabetic retinopathy. Thus, the objective of this study was to determine the level of serum VEGF in patients with advanced diabetic retinopathy. The results of the study will provide local statistics on the magnitude of DR; this will be beneficial in early diagnosis and treatment, thereby reducing its morbidity, and also open windows for further research.

MATERIALS & METHODS

The present comparative study was carried out from June to November 2010 in the outpatients and inpatients department of three major hospitals of Peshawar (Khyber Teaching Hospital, Hayatabad Medical Complex, Lady Reading Hospital) and Al-Shifa Eye Trust Hospital located in Rawalpindi, Pakistan. Informed consent was obtained from patients and hospital authorities. Convenience sampling was used to select 45 confirmed diabetic patients with retinopathy without consideration of ethnic and regional background. They were further designated as Diabetic Non- Proliferative Retinopathy (DNPR) and Diabetic Proliferative Retinopathy (DPR) groups. Inclusion criteria were marked diabetes, adult onset of disease and visual symptoms. Exclusion criteria were systemic diseases, kidney and heart disease, liver malfunction, and respiratory and gastrointestinal disorders. For comparison purposes, two control groups were 38 Diabetic Non-Retinopathy (DNR) and 39 Normal Heathy Subjects (NS). Written informed consents were taken from all subjects. Detailed history was followed by standard physical examination including blood pressure, testing of visual equity and fundus examination.

Estimation of Vascular Endothelial Growth Factor (VEGF)

VEGF concentration in serum was estimated through a standard solid phase sandwich enzyme linked-immunosorbent assay (ELISA) using a commercial kit obtained from AssayPro (Belgium).

Principle

A polyclonal antibody specific for human VEGF coated onto the wells of the microtiter strips is used. Samples, including standards of known human VEGF content, control specimens, and unknowns, are pipetted into these wells. During the first incubation, the Hu VEGF antigen binds to the immobilized (capture) antibody on one site. After washing, a biotinylated monoclonal antibody specific for Hu VEGF is then added. During the second incubation, this antibody binds to the immobilized Hu VEGF captured during the first incubation. After removal of excess secondary antibody,

streptavidin-peroxidase is added which binds to the biotinylated antibody to complete the four-member sandwich. After a third incubation and washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of Hu VEGF present in the original specimen. To prepare the standard curve, for human VEGF, dilutions ranging from 0-1500 pg/ml, while for streptavidin-HRP dilutions ranging from 2-12 ml were made.

Assay method: Procedure and Calculation

All reagents were brought to room temperature approximately 30 min before use. Standard curve was prepared simultaneously with the measurement of test samples. Wells for reagent blank were determined and 100 µl each of 'tube-4' EIA buffer was put into it. Wells for test sample blank, test sample and diluted standard were determined. 100 µl was each of the test sample blank (tube 8), test sample and dilutions of standard (tube-1-7) were added to the appropriate wells. Pre-coated plate was incubated for 60 min at 37^0C or overnight at 4^0C. Each well of the pre-coated plate was washed vigorously with wash buffer. Wash buffer was removed completely from the pre-coated plate by snapping them onto paper towel. Labeled antibody solution of 100 µl was pipetted into the wells of test samples, diluted standard and test samples blank. The pre-coated plate was incubated for 30 min at 4 ^{0}C after covering it with plate lid, washed the plate nine times as above. Required quantity of 100 µl "6, chromogen" was taken and pipetted into the wells. The pre-coated plate was incubated for 30 min at room temperature in the dark. The liquid was turned blue by the addition of "6, chromogen". Stop solution (1N H_2SO_4) of 100 µl was pipetted into the wells and mixed by tapping the side of pre-coated plate. The liquid was turned yellow by the addition of stop solution. Samples were run on the plate reader and absorbance was recorded at 450 nm. The measurements were done within 30 min after addition of the stop solution. Absorbance of standards was plotted against the standard concentration. Hu VEGF concentration was read for unknown samples and controls from the standard curve. Same assay procedure was used for both the vitreous and serum. No background problem or cross reactivity was seen.

Limitations of the Procedure

Standard curve did not extrapolate beyond the 1500 pg/ml and the minimum detectable dose of VEGF is <5 pg/ml. Intra and inter-assay coefficients were 4.7 and 8.1 respectively.

Specificity

The following substances were tested and found to have no cross reactivity: human IL-1b, IL-2, IL-6. IL-8, IL-10, IL-13, IL-15, EGF, FGF basic, FGF acidic, G-CSF, GM-CSF, IFN-g, RANTES, SCF, TGF-a, TNF-a; mouse IL-1b, IL-6, IL-10, G-CSF, GM-CSF, IFN-g, TNF-a; rat IL-1b,IL-6, IL-10,GM-CSF,IFN-g, TNF-a. Mouse and rat VEGF-165 showed 0.25 % and 0.11% cross-reactivity, respectively. Human VEGF-121 showed 100 % cross-reactivity and complete parallelism with huVEGF-165.

Reference Values: Serum: 33-86 pg/ml

Statistical Analyses

Analyses were done using SPSS version 14 (Chicago, Illinois, USA). Data are presented as Mean ± SD (standard deviation). Students T-test was used to compare the differences in mean values of groups; p≤0.05 was considered significant.

RESULTS

Median age of diabetic and diabetic retinopathic patients was 50 years ranging from 37-65 years, while of normal subjects was 53 ranging from 35-61 years. No difference in age was found between each of these groups compared to normal subjects. Females of NS group and males of CDNR and NPDR groups had significantly older age as compared to their counterparts (p=0.041, *P*<0.001, and p=0.002 respectively).

Out of the 45 diabetic patients with retinopathy, 23 were male and 22 were female; 20 patients were in DNPR group and 25 were in DPR group. Serum VEGF levels were significantly greater (p<0.001) in DNPR (202.60 ± 81.75 pg/ml) and DPR (247.20 ± 85.79 pg/ml) than DNR (92.67 ± 29.95 pg/ml) and NS (28.30 ± 6.86 pg/ml).

Table 1: Serum levels of VEGF in different groups of subjects.

#	Groups	VEGF level (mean ± SD: pg/ml)		Overall pg/ml	Range pg/ml	p value
		Male	Female			
1	Normal (39)	28.05 ± 7.43	28.50 ± 6.55	28.30 ± 6.86	20.0-43.0	NS
2	DNR (38)	76.03 ± 24.29	103.52 ± 28.66	92.67 ± 29.95	42.5-145.0	
3	DNPR (20)	185.16 ± 70.25	228.75 ± 95.30	202.60 ± 81.75	92.0- 390.0	<0.001
4	DPR (25)	421.75 ± 167.05	515.62 ± 239.29	247.20 ± 85.79	110.0- 430.0	

DISCUSSION

The present study showed that serum VEGF increased in patients with advancement of retinopathy. The study was in conformity with previous observations of increased plasma VEGF levels in diabetic patients, as well as previous observations of higher plasma VEGF levels in patients with more severe retinopathy.[8] Despite advances in ophthalmological care, DR remains a major cause of preventable blindness.[9]

VEGF is the most attractive candidate for stimulating new vessel formation and vascular hyperpermeability in DR. It is a mitogen for endothelial cells, and its expression both in vivo and in vitro can be induced by hypoxia.[10,11] Retinal hypoxia-induced inflammation and increased expression of VEGF has been implicated in the pathogenesis of DM. The pivotal role of VEGF in DM is further supported with the studies that showed regression of DM after the intravitreal injection of anti-VEGF drugs.[12]

In current study, all the patients had disease duration of 5-20 years and all were on antidiabetic medications, such as metformin, thiazolidines, glyburide and others, in combination or alone, according to disease status. Level of VEGF was found elevated; as regards PDR, the level of VEGF was more markedly raised in females, which is also supported by other studies.[13,14] Besides genetic factors, females seem to be victims of retinopathy and other diabetic complications due to ignorance, gender preference, etc., which can play a major role in the alarming rise of blindness in females, especially in Khyber Pakhtunkhwa.

The results of current study are consistent with the study of Zakareia et al,[15] who state that there was significant increase in plasma levels of VEGF in type II diabetics with retinopathy compared to controls and diabetics without complications. This could help to support a causative role of VEGF in diabetic proliferative retinopathy. VEGF plays a role in the neovascularization and in the breakdown of the blood-retinal barrier which is characterized by hyperpermeability of retinal vessels.[16] VEGF production is stimulated by hyperglycemia, Advanced Glycation End Products (AGEP), angiotensin II, and hypoxia, all of which are present in the retinal microvascular bed. Hypoxia is generally considered to represent a fundamental stimulus for angiogenesis through VEGF production in diabetic retinopathy.

However, VEGF overexpression can result in retinal neovascularization, and increased retinal vascular permeability, macular edema, bleeding, fibrosis, and loss of vision may follow. Local and systemic VEGF antagonists have been proposed as potential therapeutic interventions for the treatment of diabetic macular edema and proliferative retinopathy.[17]

CONCLUSION

VEGF level in diabetic patients with retinopathy were raised as compared to relevant controls; the finding was more prominent in females with Proliferative Diabetic Retinopathy.

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