·Basic Research·

Effect of pan-retinal laser photocoagulation on plasma VEGF, endothelin-1 and nitric oxide in PDR

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Abstract

• AIM: To study plasma levels of vascular endothelial growth factor (VEGF), endothelin-1 (ET-1) and nitric oxide (NO) in patients with proliferative diabetic retinopathy (PDR) before and after pan-retinal photocoagulation (PRP).

• METHODS: Forty patients (23 females and 17 males, mean age 48.5 ± 12.2 years) with PDR without previous PRP therapy were studied. Blood samples were obtained before and 3 months after the last PRP session. Baseline (prelaser) plasma levels of VEGF, ET-1 and NO were compared with their levels in 30 healthy age- and sex- matched controls and also with plasma levels 3 months post-PRP.

• RESULTS: Patients with PDR had significantly raised plasma VEGF (375 ± 89ng/L), ET-1 (20 ± 5ng/L) and NO (135± 53μ mol/L) when compared with healthy control group (P<0.01). After PRP, there was a significant reduction in plasma VEGF (179± 66ng/L), ET-1 (11± 5ng/L) and NO (91± 49μ mol/L) levels at 3 months' follow-up but still significantly higher than healthy controls.

• CONCLUSION:Patients with PDR demonstrate elevated VEGF, ET-1 and NO, which decrease after successful laser treatment.

• KEYWORDS: diabetic retinopathy; microangiopathy; pan-retinal photocoagulation; vascular endothelial growth factor; endothelin-1; nitric oxide
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INTRODUCTION

Diabetic retinopathy (DR), a devastating microvascular complication in the eye, is one of the leading causes of blindness. Most diabetic patients, especially those with poor glycemic control, develop DR, which remains the major cause of onset of blindness among diabetic adults [1]. The Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) showed that 28.8% of diabetic patients develop retinopathy early, whereas 22.2% with the history of diabetes irrespective of glycemic exposure do not develop retinopathy [2]. DR is characterized by vascular permeability, increased tissue ischemia, and angiogenesis. Vascular endothelial growth factor (VEGF) is a potent angiogenic growth factor [3]. In the eye, numerous types of retinal cells are recognized to produce VEGF, including retinal pigment epithelial cells, pericytes, endothelial cells, Müller cells, and astrocytes[4]. Intraocular VEGF levels have also been studied in animal models and human vitreous fluid, where the levels are found to be high in patients with active intraocular neovascularization, such as proliferative diabetic retinopathy, ischaemic central retinal vein occlusion, rubeosis iridis, and retinopathy of prematurity [4]. Another possible factor for diabetes is nitric oxide (NO). NO causes increased oxidative stress due to free radicals and could play an important role in the pathogenesis of microvascular complications in humans [5]. NO production has been reported to be either increased or decreased in the presence of high glucose concentrations [6]. Endothelin-1 (ET-1) is a potent and prolonged vasoconstrictor and mitogenic endothelium derived peptide, and has been considered as a marker for endothelial damage and potential contributor to the development of the atherogenic process. Increased circulating ET-1 levels were found in patients with atherosclerosis, as well as in patients with type 2 diabetes mellitus suggesting a role in the pathogenesis of these disorders [7]. Vasoactive factors like endothelins, by virtue of the microvascular regulation as well as by other effects, possibly play important parts in the pathogenesis of diabetic retinal microangiopathy[7]. Pan-retinal laser photocoagulation is still the standard management for proliferative diabetic retinopathy, although the possibility of using other modalities of treatment is available [8]. An alternative new therapeutic strategy for the treatment of severe florid PDR complicated or not by vitreous hemorrhages is to inhibit the

high level of vascular endothelial growth factor (VEGF), responsible for multiple retinal and papillary neovascularizations, by intravitreal injection of bevacizumab, a humanized monoclonal antibody that binds all isoforms of VEGF[9].

MATERIALS AND METHODS

Subjects This study was conducted over 2007-2008 in ophthalmology department of Assiut University Hospitals with the approval of the Ethics Committee of our institute, within which the work was undertaken, and it conformed to the provisions of the Declaration of Helsinki in 1995. Informed consent was obtained from all subjects and patient anonymity has been preserved. We recruited patients with proliferative retinopathy secondary to diabetes with no previous pan-retinal photocoagulation (PRP) therapy from the retina clinic. All were subjected to a clinical evaluation which involved fundus examination by a binocular indirect ophthalmoscope and a slit lamp biomicroscopic examination with a 90D lens. Patients' details such as age, sex, duration of diabetes, other systemic illness and treatment details were also documented. Forty patients with proliferative diabetic retinopathy (PDR) (23 females and 17 males) underwent PRP therapy in view of obvious neovascularization compared with 30 age- and sex- matched healthy controls (mean age 48.5±12.2 years).

Methods Blood samples were obtained before and 3 months after the last PRP session, for plasma VEGF, NO and ET-1, as well as random blood glucose level. Baseline (prelaser) plasma VEGF, NO and ET-1 levels in patients with PDR were compared with levels in 30 healthy, age- and sex- matched "healthy controls" recruited from those attending hospital for non-acute surgical conditions, such as cataract surgery and from members of the hospital staff. None of the patients or healthy controls had a history of renal or liver disease, uncontrolled hypertension, malignancy, connective tissue disease or deep vein thrombosis. Three months after the last PRP session plasma VEGF, NO and ET-1 levels were compared with those from baseline and healthy controls.

After obtaining informed consent, about 5mL blood samples were withdrawn from the antecubital vein in all subjects of the study group under complete aseptic condition into the tubes containing EDTA. Each blood sample was immediately centrifuged at 500r/min for 15 minutes. The separated plasma samples were stored at -20℃ until assayed. VEGF levels were measured by ELISA kit for quantitative determination of VEGF (Cat. No. DVE00, R&D systems, Inc., Minneapolis, USA). The measurement was performed according to method described by Kondo *et al* [10]. ET-1 was determined by an ELISA kit for quantitative determination of ET-1 (Cat. No. BBE5, R&D systems, Inc., Minneapolis, USA) .The measurement was performed according to method described by Takakuwa *et al*[11].NO levels were measured by chemical methods using Griess reagent. The measurement was performed according to the method described by van Bezooijen *et al* [12].

Table 1 Plasma VEGF, ET-1 and NO levels in diabetic patients before and 3 months after last PRP session

	Healthy controls	Pre-PRP	3 month post PRP
VEGF(ng/L)	65.7± 23.6	375.4±89.0	178.0±66.5
ET-1 (ng/L)	2.3± 1.0	20.3± 5.3	10.8±5.0
NO (μmol/L)	49.7 ± 14.6	135.3± 53.5	90.5±49.1

Statistical Analysis Differences between groups (i.e. healthy, diabetic patients at baseline and follow up) were analyzed by ANOVA and subsequently pairwise *t*test and regression analysis were performed. Non-parametric analysis (Kruskal-Wallis for more than 2 groups) was added, due to the relatively small sample size. All results were confirmed by the non-parametric analysis. The statistical analysis was made with SAS version 8.1.

RESULTS

The mean random blood sugar level was 171±17.99mg/dL (mean±SD). Plasma VEGF was elevated in the 40 patients with PDR compared with 30 healthy subjects (375±89 *vs* 66±24ng/L, $P<0.01$). Plasma ET-1 levels were found to be 20.3±5.3ng/L in the PDR group, whereas 2.3±1.0ng/L in the control group, which was statistically significant ($P<0.01$). The plasma NO levels were 135±54μmol/L in patients with PDR and 50±15μmol/L in healthy control group and the difference was also statistically significant ($P<0.01$). After PRP, there was a significant reduction in plasma VEGF levels at 3 months' follow-up ($P<0.01$), ET-1 ($P<0.01$) and NO ($P<0.01$) in the 40 patients compared to the prelaser baseline values (Table 1). Post-PRP plasma VEGF, ET-1 and NO were still significantly higher than those levels in healthy controls with $P<0.01$, $P<0.01$ and $P=0.002$ respectively. At 3 months' follow-up the mean random blood sugar in the diabetic patient group was 175.0±22.3mg/dL which was not statistically significant when compared with the initial level.

DISCUSSION

In diabetic patients, some of the earliest pathophysiological retinovascular changes include selective loss of capillary pericytes, impairment of retinal vascular autoregulation, and the failure of the capillary circulation. These changes subsequently lead to increased retinal vascular permeability, chronic retinal hypoxia and extensive retinal ischaemia, eventually resulting in retinal neovascularization. These processes are thought to be mediated by various growth

factors, such as vascular endothelial growth factor (VEGF)[13]. This study has demonstrated high plasma VEGF levels in patients with untreated PDR. This work has confirmed 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 [6]. High VEGF mRNA in retinal sections has previously been noted in diabetics with proliferative retinopathy and ischaemic central retinal vein occlusion [9]. Lip *et al* [14] in their pilot study have demonstrated high plasma VEGF levels in patients with untreated proliferative retinopathy. In another study Lip *et al*[15] have confirmed the same result. Two studies failed to find significant relationships between serum VEGF levels and stage of retinopathy, although aqueous levels were significantly different [16,17] nevertheless, the use of serum to measure systemic VEGF levels has to be interpreted with caution, because activated platelets (during blood clotting) release VEGF into serum, and thus, results based on serum samples may be inaccurate in view of the possible artifact relating to the source of VEGF levels [18]. The present study avoids this problem by measurement of VEGF levels from citrated plasma.

There is a marked reduction in VEGF levels after PRP. The same result was observed by Lip *et al* [14] in their pilot study and they suggested that the angiogenic stimuli responsible for increased VEGF appeared to have decreased. In contrast with this present study, Lip *et al* [15] have demonstrated no significant reduction in VEGF levels after panretinal photocoagulation in another cross sectional study. They explain this controversy by the fact that their first pilot study included patients with proliferative retinopathy secondary to both diabetes and retinal vein occlusion, while the successive one was confined to diabetic patients only and significant cardiovascular disease or hypertension were exclusion criteria and many patients were also treated with ACE inhibitors at baseline, and pre-intervention VEGF levels were also much lower than that reported in their pilot study, making a substantial change secondary to intervention more difficult to show. Whereas, in our study, pre-PRP VEGF levels were markedly elevated in those poorly controlled diabetics making any change due to PRP treatment more easy to show. Vasoactive factors like endothelins, by virtue of the micro-vascular regulation as well as by other effects, possibly play important parts in the pathogenesis of diabetic retinal microangiopathy. Short-term vasoactive properties of ET-1 up-regulation could lead to vasoconstriction and alterations in blood flow in the retina and peripheral nerve in diabetes [19]. In this study, we found significantly elevated ET-1 levels in diabetic patients with untreated PDR compared to healthy controls. This finding is confirmed by Zhu *et al* [20] who reported a higher plasma ET-1 concentration in the diabetic patients with retinopathy than in those without retinopathy. Kawamura *et al*[21] compared ET-1 levels of diabetic patient with and without retinopathy and healthy subjects and found significantly elevated ET-1 levels in diabetic patients with retinopathy. Laurenti *et al* [22] reported a significant correlation between ET-1 levels and grade of retinal vascular damage. Chakravarthy *et al* [23] studied ET-1 levels in ocular and retinal vascular bed and veins and found elevated ET-1 in streptozotocin-treated rats when compared with normal rats. There was a significant reduction in ET-1 levels after pan-retinal photocoagulation but still significantly higher than healthy controls. To our knowledge there is not any current research studying the effect of PRP treatment on plasma ET-1 levels in diabetic patients. However, Kakizawa *et al* [24] had reported that improved glycemic control did not affect plasma ET-1 concentration. Endothelial dysfunction is considered an intrinsic element in the pathogenesis of diabetic angiopathies. The free radical NO is derived from endothelium. NO is a non-stable radical and converted to nitrite/nitrate anion (NO_2^-/NO_3^-) in a very short time which is more stable. However, excess NO can exert cytotoxic and cytostatic effects[25]. In this study, we found high plasma NO levels in diabetic patients suffering from PDR before PRP compared to healthy controls. There is a significant reduction in NO levels after PRP but still significantly higher than healthy controls.

There are only a few reports on the relationship between plasma NO levels and microvascular complications, especially DR. In accordance with our results, Izumi *et al*[26] reported significantly higher plasma NO levels in patients with type 2 diabetes than those in the control group. Aydn *et al* [27] found high plasma NO_2^-/NO_3^- levels in diabetic patients before and after glycemic control compared to controls. However, according to our knowledge there is no current report about the effect of PRP on NO plasma levels in diabetic patients with PDR.

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