ORIGINAL RESEARCH

Relationship between vitreous and serum vascular endothelial growth factor levels, control of diabetes and microalbuminuria in proliferative diabetic retinopathy

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Background: Diabetic retinopathy is a serious microvascular disorder of the retina. Vascular endothelial growth factor (VEGF) expression, induced by high glucose levels and hypoxia, is a main feature in retinopathy. The aim of this study was to evaluate the relationship between vitreous and serum VEGF levels and control of diabetes and microalbuminuria in patients with proliferative diabetic retinopathy.

Methods: Sixty-five patients were enrolled in this case-control study, comprising 30 patients with proliferative diabetic retinopathy (cases) and 35 patients with nonproliferative diabetic retinopathy (controls). The vitreous VEGF level was compared with the serum VEGF level in both groups. Glycosylated hemoglobin (HbA_{1c}), microalbuminuria, serum creatinine, and stage of nephropathy and retinopathy were also measured in patients with proliferative diabetic retinopathy, and the relationship between these parameters and serum and vitreous VEGF levels was investigated.

Results: Mean vitreous and serum VEGF levels were significantly higher in cases compared with controls ($P = 0.001$, $P = 0.011$, respectively). There was also a significant correlation between vitreous and serum VEGF levels ($P = 0.012$, $r = 0.453$). VEGF levels in patients with well controlled blood glucose ($P = 0.039$), on drug treatment ($P = 0.045$) and at an early stage of nephropathy ($P = 0.042$) were significantly lower. There was a significant correlation between VEGF and albumin to creatinine ratio ($P = 0.017$, $r = 0.432$).

Conclusion: Serum and vitreous VEGF levels was significantly lower in patients on oral therapy, in those with well controlled glycemia, and in those with early-stage retinopathy. Administration of anti-VEGF had a good effect in reducing the progression of proliferative diabetic retinopathy.

Keywords: proliferative diabetic retinopathy, vascular endothelial growth factor, vitreous body

Introduction

Diabetic retinopathy is a chronic progressive sight-threatening disease of the retinal microvasculature associated with prolonged hyperglycemia and other conditions linked to diabetes mellitus, such as hypertension, and is one of the main factors leading to blindness, especially in developed countries.[1,2] Diabetic retinopathy is found in 60% of people 20 years after onset of type 2 diabetes; sight-threatening retinopathy is found in 2.0% of people who have had diabetes for more than 5 years and in 15.5% of people who have had diabetes for more than 15 years.[3] In the US, there are six million people with diabetes mellitus, 50% of whom are unaware

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that they have the disease, and only half of these patients receive adequate treatment to reduce complications related to diabetes.[4]

Some adverse consequences of diabetes mellitus in the eye include iris neovascularization, cataract, neuropathy, corneal disorders, and glaucoma, but the most debilitating complication is retinopathy.[5] Another adverse consequence of diabetes mellitus is diabetic nephropathy. Diabetes is a major cause of end-stage renal disease, and the incidence of diabetes is rising rapidly. In this study, we also compared serum and vitreous vascular endothelial growth factor (VEGF) levels and stages of nephropathy, ie, mild (nonspecific light micros-copy changes and electron microscopic-proven glomerular basement membrane thickening/mild mesangial expansion) moderate (severe mesangial expansion, nodular sclerosis, Kimmelstiel–Wilson lesion), and severe (advanced diabetic glomerulosclerosis).[6]

Five major clinical trials provide the scientific basis of standards for clinical management of diabetic retinopathy, ie, the Diabetic Retinopathy Study (DRS, 1971–1975),[7] the Early Treatment Diabetic Retinopathy Study (ETDRS, 1979–1990),[8] the Diabetic Retinopathy Vitrectomy Study (DRVS, 1977–1987),[9] the Diabetes Control and Complica-tions Trial (DCCT, 1983–1993),[10] and the United Kingdom Prospective Diabetes Study (UKPDS, 1977–1999).[11]

The DRS, ETDRS, and DRVS have provided guidelines concerning the most opportune time for intervention with laser surgery and vitrectomy for diabetic retinopathy. The DCCT and UKPDS established the benefits of intensive control of blood glucose levels to reduce the risk of pro-gression of diabetic retinopathy and other complications of type 1 and type 2 diabetes, respectively. The modified ETDRS and extended Airlie House classification[12,13] have been devised to assess the severity and extent of the various lesions of diabetic retinopathy.[14] This modification forms the basis of an overall diabetic retinopathy severity scale[12] that ranges from the absence of retinopathy to severe vitreous hemorrhage. Clinical approximations of these levels provide practical guidelines for the diagnosis and management of diabetic retinopathy.[14]

Diabetic retinopathy is classified according to the pres-ence or absence of abnormal new vessels as nonproliferative (background/preproliferative) retinopathy (mild, moderate, severe, very severe), proliferative retinopathy (mild, mod-erate, high-risk), and clinically significant macular edema. Each has a different prognosis for vision.[15]

The main pathogenesis of diabetic retinopathy is unknown, but some assumptions are made in this regard:

- Protein kinase C – kinases transfer the terminal high-energy phosphate group of ATP to a site on a target protein. Diacylglycerol is one of the protein kinase C family activator molecules, intracellular concentra-tions of which are increased during the hyperglycemia of diabetes. Increased protein kinase Cβ levels may be induced by tissue hypoxia, leading to increased VEGF expression, a major factor that increases proliferation of vascular endothelial cells leading to neovascularization and enhancing breakdown of the blood-retinal barrier, and potentially resulting in macular edema. Therefore, protein kinase C inhibitors may prevent the development of diabetic retinopathy.[16]

- Blood viscosity and platelet adhesion – some variable blood disorders are observed in diabetic patients that stimulate production of VEGF, such as increased con-centrations and reduced flexibility of red blood cells, and reduction in accumulation and adhesion of platelets and endothelial injuries, leading to retinal ischemia.[17]

- Aldose reductase and angiogenesis factors – an increase in the level of blood sugar has its own physiological, structural, and anatomical effects on the capillaries. A continuous increase in blood sugar can lead to an aldose reductase shunt in some tissues, the main effect of which is to produce alcohol from sugar. The alcohol produced has an adverse effect on intramural capillary pericytes, leading to incompetence of the vascular wall and the vessel microaneurysms seen as early changes in diabetic retinopathy. These microaneurysms are the main cause of retinal hemorrhage (flame-like or blot and dot).[18]

Damage to the vessel wall changes the structure of the blood-retinal barrier and also makes the retinal blood ves-sels more permeable.[19] Macular edema occurs when the damaged blood vessels leak fluid (exudate) onto the macula, causing blurred vision. Macular edema is the main cause of vision disturbance in patients with nonproliferative diabetic retinopathy, but it can also occur in PDR.[20] Retina ischemia as a result of blood vessel damage is the main stimulus for producing VEGF leading to angiogenesis and increased blood vessel permeability.[21–23]

A clear association has been demonstrated between VEGF and diabetic retinopathy, and the ability to identify biomarkers may be helpful in the diagnosis of severity and progression of PDR, especially in its early stages, to reduce the complications and psychological distress experienced by these patients. The aim of this study was to evaluate the relationship between serum and vitreous VEGF levels and control of diabetes and microalbuminuria in patients with PDR.

Methods and materials

In this descriptive analytical study, vitreous and blood samples were collected from patients who were admitted to Tabriz Nikoukari Educational Hospital and underwent vitrectomy. Before taking samples from patients, the study protocol was approved by the hospital research ethics committee and informed consent was obtained from each patient.

Thirty patients with PDR and 35 patients with nonproliferative diabetic retinopathy were randomly selected and included in this study. The case group included patients with PDR who required vitrectomy for long-term vitreous hemorrhage and/or tractional retinal detachment and patients with active retinopathy leading to vitreous hemorrhage. This group also included patients without active retinopathy. The control group included patients with nondiabetic vitreous hemorrhage that required vitrectomy (due to ischemic vascular disease, such as central retinal or branch vein occlusion). Patients with retinal ischemia due to other pathologies were excluded. The diagnostic criteria for detecting diabetic retinopathy was based on the guidelines of the American Diabetes Association.[24]

Vitreous fluid and whole blood samples were taken from all patients and after separation were maintained at −70°C until analyzed. Patient information, including age, gender, duration of diabetes (less or more than 10 years), glycosylated hemoglobin (HbA_{1c}) level, type of diabetes, type of diabetic treatment, hypertension, nephropathy stage, glycemic control (poor, $HbA_{1c} > 8$; moderate, 7–8; well, <7), albumin to creatinine ratio (ACR), and vitreous VEGF were recorded.

VEGF measured by immunoassay

VEGF in vitreous fluid and serum was measured by enzyme-linked immunosorbent assay using a kit from R&D Systems (Minneapolis, MN). Initially, 100 µL of RDW1 assay solution was put into the microplate, then 100 µL of a standard solution of serum and vitreous fluid were added to the microplate and incubated for 2 hours at room temperature. After three rinses with a special solution, 200 µL of conjugate was added and reincubated at room temperature, and washed three times; 200 µL of substrate was added and centrifuged at 25 rpm, and then incubated. Finally, 50 µL of stop solution was added and the plates were read at a wavelength of 450–570 nm.

Measurement of HbA_{1c}

A 10 mL venous blood sample was taken from patients fasted for 8–12 hours. Measurement of fasting blood sugar was done by a glucose oxidase method using a kit from Zist

Shimi Corporation, Tehran, Iran. High-performance liquid chromatography (HPLC) was used for measurement of HbA_{1c} using a Nycocard system (Oslo, Norway). In summary, this method uses ion-exchange liquid chromatography with gradient washing for separation of HbA_{1c} from hemolytic whole blood. For measurement of HbA_{1c}, 5 µL of whole blood with ethylenediamine was added to 1 mL of hemolytic solution and then optical absorption of hemoglobin fractions, collected by eliminating background material, was read in the optical wavelength of 4.5 nm. Sediment diagnostic methods were used for measurement of serum creatinine (Jaffe method), proteinuria, and albuminuria. Measurement of proteinuria and albuminuria was performed using morning urine samples.

Statistical analysis

The data obtained were expressed as the mean ± standard deviation and also as the frequency and percentage. The data were analyzed by SPSS software (v 15; SPSS Inc, Chicago, IL). Quantitative variables were compared using Student's t-test (independent samples). Comparison of the qualitative variables (categorical) was done using contingency tables and the Chi-square test or Fisher's Exact test depending on conditions. Correlations were tested using Spearman's rank correlation coefficients. Statistical significance was set at $P \leq 0.05$.

Results

Sixty-five patients (30 patients in the case group and 35 patients in the control group) were enrolled in this study. The mean age was 56 ± 10 (range 37–83) years in the cases and 35–80 years in the controls. There was no significant difference with respect to demographic features, including age and gender, between the two groups ($P = 0.891$ and $P = 0.656$, respectively). Nineteen cases were male and 11 were female. Among the cases, 16 (53.3%) were smokers, two (6.7%) had type 1 diabetes, and 28 (93.3%) had type 2 diabetes. The mean vitreous VEGF level in the cases was lower than in controls (383.10 ± 107.48 pg/mL vs 24.81 ± 1.85 pg/mL); this difference was statistically significant ($P = 0.001$; 95% confidence interval [CI]: 159.66–556.91). The mean serum VEGF level in the cases was lower than in the controls (515.12 ± 44.8 vs 343.58 ± 46.41); this difference was statistically significant ($P = 0.01$; 95% CI: 41.46–301.60, Table 1).

Mean HbA_{1c} in the cases was 7.85 ± 60, and there was no statistically significant correlation between HbA_{1c} and serum ($P = 0.403$, $r = 0.158$) or vitreous ($P = 0.837$, $r = 0.039$) VEGF levels (Table 2). Mean serum HbA_{1c} in

Table 1 Mean serum and vitreous fluid concentrations of vascular endothelial growth factor in PDR and NPDR patients

VEGF (pg/mL)	PDR (n = 30)	NPDR (n = 35)
Vitreous fluid		
All patients[a]	383.1 ± 107.48	24.81 ± 1.85
Male[a]	325.81 ± 109.66	24.8 ± 2.24
Female[a]	482.06 ± 228.97	24.3 ± 3.49
Serum		
All patients[a]	515.12 ± 44.8	343.58 ± 46.41
Male[a]	560.8 ± 1	297.25 ± 72.81
Female[b]	488.67 ± 48.27	424.12 ± 37.86

Notes: [a]P values were significant ($P \leq 0.05$); [b]P values were not significant ($P > 0.05$); data are presented as means ± standard deviation.
Abbreviations: VEGF, vascular endothelial growth factor; PDR, proliferative diabetic retinopathy; NPDR, nonproliferative diabetic retinopathy.

diabetic patients on insulin therapy (12 patients, 40%) was 7.87 ± 0.52 and in patients on oral therapy (glibenclamide, metformin, 18 patients, 60%) was 7.84 ± 0.66 ($P = 0.895$; 95% CI: −0.43–0.50). There was no significant difference between these two types of treatment in this regard.

The mean serum VEGF level in patients on insulin therapy was 623.94 ± 139.51 and in those on oral therapy was 442.57 ± 276.28, ie, the serum VEGF level on oral therapy was significantly lower compared with patients on insulin ($P = 0.045$; 95% CI: 3.97–358.74). However, there was no significant difference in the mean vitreous VEGF level between patients on insulin therapy (264.98 ± 167.94) and those on oral agents (461.85 ± 746.00, $P = 0.379$; 95% CI: −647.93–254.09).

The PDR patients were then divided into three groups with respect to glycemia, ie, poorly controlled (n = 15), moderately controlled (n = 13), and well controlled (n = 2).

A significantly lower serum VEGF level was observed in the group with good glycemic control ($P = 0.039$), but there was no statistically significant difference in the vitreous VEGF level between the three groups (Table 3).

We also divided the PDR patients into three groups according to their stage of nephropathy, ie, mild (n = 2, 6.7%), moderate (n = 17, 56.7%), or severe (n = 11, 36.7%). Serum VEGF levels were significantly lower in the patients with mild nephropathy ($P = 0.042$). However, there was no

significant difference in the vitreous VEGF level between the three groups ($P = 0.319$, Table 4). Further, with respect to stage of retinopathy, patients with PDR were divided into three groups, ie, mild (n = 8, 26.7%), moderate (n = 14, 46.7%), or severe (n = 8, 26.7%). As shown in Table 5, there was no significant difference in serum and vitreous VEGF levels ($P = 0.601$ and $P = 0.063$, respectively).

Mean levels of albuminuria, serum creatinine, and urinary ACR in the cases were 249.43 ± 97.91 mg/dL, 1.33 ± 0.50 mg/dL, and 191.53 ± 69.70 mg/dL, respectively. There was a significant correlation between serum VEGF levels and ACR ($P = 0.017$, $r = 0.432$), but not between serum VEGF levels and albuminuria ($P = 0.414$, $r = 0.155$). There was also no significant correlation between vitreous VEGF levels and albuminuria ($P = 0.917$, $r = −0.020$) or ACR ($P = 0.844$, $r = 0.038$). We also compared serum and vitreous VEGF levels with duration of diabetes in the cases (Table 6). There was no significant correlation between serum VEGF levels in patients with diabetes for less than 10 years and those with diabetes for more than 10 years ($P = 0.908$; 95% CI: −220.24–246.93).

Discussion

In this study, we compared serum and vitreous VEGF levels in patients with PDR and nondiabetic retinopathy. Mean serum and vitreous VEGF levels in patients with PDR were 515.12 ± 245.55 pg/mL and 383.10 ± 588.69 pg/mL, respectively, and in patients with nonproliferative diabetic retinopathy were 343.58 ± 274.55 pg/mL and 24.81 ± 10.97 pg/mL, respectively. Both serum and vitreous VEGF levels were significantly higher than normal in diabetic patients ($P = 0.011$ and $P = 0.001$, respectively).

Funatsu et al compared 57 patients with PDR and 16 controls and found mean vitreous VEGF levels of 1135.2 pg/mL and 19.3 pg/mL, respectively. This difference was statistically significant.[25] Hernandez et al compared 20 patients with PDR and 20 controls, and reported significantly higher vitreous VEGF levels in patients with PDR than in controls (134 pg/mL vs 9 pg/mL, respectively), but there was no significant difference in serum VEGF levels

Table 2 Correlation coefficient between vascular endothelial growth factor with HbA$_{1c}$, Alb/Cr, and Alb in patients with proliferative diabetic retinopathy

	Vitreous and serum VEGF	Vitreous VEGF and HbA$_{1c}$	Serum VEGF and HbA$_{1c}$	Serum VEGF and Alb	Serum VEGF and Alb/Cr	Vitreous VEGF and Alb	Vitreous VEGF and Alb/Cr
P	0.012[a]	0.403[b]	0.837[b]	0.414[b]	0.017[a]	0.917[b]	0.842[b]
r	0.453	0.158	0.039	0.155	0.432	0.020	0.038

Notes: [a]P values were significant ($P \leq 0.05$); [b]P values were not significant ($P > 0.05$); data are presented as the mean ± standard deviation.
Abbreviations: VEGF, vascular endothelial growth factor; Alb, albumin; Cr, creatinine.

Table 3 Comparison of serum and vitreous vascular endothelial growth factor levels in different groups based on intensity of glycemic control

Diabetic control	Poor	Moderate	Well
Serum VEGF (pg/mL)	524.65 ± 247.43	567.22 ± 207.73	105.00 ± 23.75
Vitreous VEGF (pg/mL)	250.53 ± 412.217	441.70 ± 624.21	383.10 ± 588.69

Note: Data are presented as the mean ± standard deviation.
Abbreviation: VEGF, vascular endothelial growth factor.

Table 5 Comparison of serum and vitreous vascular endothelial growth factor levels in different groups based on stage of retinopathy

Retinopathy stage	Mild	Moderate	Severe
Serum VEGF (pg/mL)	439.83 ± 272.91	533.77 ± 232.32	557.77 ± 256.57
Vitreous VEGF (pg/mL)	125.42 ± 124.78	648.53 ± 775.67	176.28 ± 192.62

Note: Data are presented as the mean ± standard deviation.
Abbreviation: VEGF, vascular endothelial growth factor.

(177 pg/mL vs 170 pg/mL).[26] In another similar study by Malik et al in 11 patients with PDR and 23 controls, vitreous VEGF levels were higher in cases (720 pg/mL vs 180 pg/mL, respectively).[27] In a study by Hernandez et al of 23 PDR patients and 17 controls, vitreous VEGF levels in the cases were higher than in controls (142 pg/mL vs 9 pg/mL) but there was no significant difference in serum VEGF levels (150 pg/mL vs 120 pg/mL).[28] In a study by Simo et al in 37 PDR patients and 21 controls, the vitreous VEGF level in the cases was higher than in controls (138 pg/mL vs 9 pg/mL) but there was no significant difference in the serum VEGF level (130 pg/mL vs 160 pg/mL).[29] Watanabe et al, in their study of 41 PDR patients and 18 nondiabetic patients, showed that vitreous VEGF levels in PDR patients was higher than in nondiabetics (812 pg/mL vs 17 pg/mL).[30] In a similar study by Sydorova and Lee, the serum and vitreous VEGF levels in PDR patients were higher than in controls.[31]

Hogeboom Van Buggenum et al compared 39 PDR patients with 11 controls, and reported mean vitreous VEGF levels of 1134 pg/mL and 59 pg/mL, respectively. This difference was statistically significant.[32] In a similar study by Aiello et al in 70 PDR patients and 25 controls, vitreous VEGF levels in cases were higher than in controls (360 pg/mL vs 100 pg/mL).[33] Deng et al compared 27 PDR patients with 14 control patients, and found mean vitreous VEGF levels of 410 pg/mL and 17 pg/mL, respectively. This difference was statistically significant.[34] In a study by Ambati et al

in 22 PDR patients and 28 controls, the vitreous VEGF levels in cases were higher than in controls (1759 pg/mL vs 27 pg/mL, respectively).[35] In a study by Zhou and Zhang in 19 PDR patients and seven controls, vitreous VEGF levels in cases were higher than in controls (566 pg/mL vs 350 pg/mL, respectively).[36] Adamis et al also reported similar results.[37] In a study by Burgos et al in 20 PDR patients and 13 control patients, the vitreous VEGF level in cases was higher than in controls (175 pg/mL vs 9 pg/mL) but there was no significant difference in serum VEGF level.[38]

Almost all studies performed in this field until now are mentioned above. As can be seen, in all studies, the vitreous VEGF level in PDR patients was significantly higher than in controls. Serum VEGF levels are inconsistent, in the summarized results of other studies, the mean vitreous VEGF levels in PDR patients are 623 (138–1759) pg/mL and mean serum VEGF levels in PDR patients are 234 (120–509) pg/mL. The results of our study are also in the above range, but it should be noted that current results are calculated based on the level of vitreous proteins. Selecting vitreous proteins for measurement eliminates confounding factors and increases the accuracy of the results. This is one of the strong points of the current study. Burgos et al in their study showed that there was no significant correlation between serum and vitreous VEGF levels. They believe that all vitreous VEGF is made inside the eye and the serum emission is absent or negligible.[38]

In our study, we observed a high and significant correlation between serum and vitreous VEGF levels ($P = 0.012$

Table 4 Comparison of serum and vitreous VEGF levels in different groups based on the stage of nephropathy

Nephropathy stage	Mild	Moderate	Severe
Serum VEGF (pg/mL)	105.00 ± 23.75	556.35 ± 232.85	525.97 ± 226.70
Vitreous VEGF (pg/mL)	996.35 ± 1391.09	324.22 ± 536.69	383.10 ± 588.69

Note: Data are presented as means ± SD.
Abbreviation: VEGF, vascular endothelial growth factor.

Table 6 Serum and vitreous level of vascular endothelial growth factor based on duration of diabetes

Duration of diabetes	Level of vitreous VEGF	Level of serum VEGF
More than 10 years	457.68 ± 634.66	512.45 ± 252.39
Less than 10 years	84.78 ± 153.76	525.80 ± 237.74

Note: Data are presented as the mean ± standard deviation.
Abbreviation: VEGF, vascular endothelial growth factor.

and $r = 0.453$), and serum VEGF levels were higher in PDR patients than control patients. This is in contrast with results reported by other investigators. However, to reach firm conclusions in this regard, we suggest further studies. The current study showed that the vitreous VEGF level has no significant correlation with treatment of diabetes, glycemic control, severity of nephropathy, duration of diabetes, ACR ratio, severity of retinopathy, or HbA_{1c} level ($P > 0.05$). As far as we have identified, few studies have been performed in this field. Malik et al in their study indicated that there was no significant correlation between HbA_{1c} levels and the vitreous level of VEGF.[27] Shinoda et al in another study showed that there was no significant correlation between the vitreous VEGF level and type and duration of diabetes, type of treatment, HbA_{1c} level, and renal dysfunction.[39]

With regard to the correlation between the vitreous VEGF level and the severity of retinopathy, existing studies have indicated that the level of this substance in cases of active disease is more than in inactive disease, but there is not any study similar to our study.[25,26,29] On the other hand, in our study, the serum level of VEGF was low in patients on oral therapy, those with good glycemic control, and the first stage of diabetic nephropathy. Another significant and positive correlation was observed between the level of this factor and serum ACR. There is no similar study in this field. To confirm these cases, we need to perform more research. In summary, the mean level of VEGF in serum and vitreous in patients with retinopathy was significantly higher than in patients with PDR. A significant and positive correlation was found between serum and vitreous VEGF levels.

The mean ratio of urinary albumin to serum creatinine and serum VEGF in patients with oral agent therapy with good glycemic control, in the mild stages of diabetic nephropathy were low. There was no correlation between serum and vitreous VEGF levels with severity of retinopathy and diabetes duration. A significant and positive correlation was seen between serum VEGF level and level of albuminuria/creatinine. However, there was no significant correlation between serum or vitreous VEGF levels and the level of HbA_{1c}.

The serum VEGF levels in comparison with the vitreous VEGF levels is a good factor to determine the status of patients with proliferative diabetic retinopathy and as it is easy to measure, it is recommended that serum VEGF is quantified when patients are evaluated. Given the positive effect of good glycemic control on serum VEGF level, and VEGF association with eye lesions (in diabetic patients), the importance of appropriate blood sugar control is more apparent; further studies in this field with a larger sample size are recommended.

Conclusion

Serum and vitreous VEGF levels were significantly lower in patients on oral therapy, in patients with well controlled diabetes, and in those with early-stage retinopathy. Administration of anti-VEGF had a good effect in reducing the progression of PDR. In contrast with previous studies, we have demonstrated the ACR to be a factor predictive of retinopathy.

Disclosure

The authors report no conflicts of interest in this work.

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