European Review for Medical and Pharmacological Sciences 2017; 21: 662-668

Vitreous and plasma changes of endothelin-1, adrenomedullin and vascular endothelium growth factor in patients with proliferative diabetic retinopathy

E.M. VINGOLO[1], S. FRAGIOTTA[1], M. MAFRICI[1], A. CUTINI[1], C. MARINELLI[2], A. CONCISTRÈ[2], G. IANNUCCI[2], L. PETRAMALA[2], C. LETIZIA[2]

[1]Department of Medical-Surgical Sciences and Biotechnologies, U.O.S. Ophthalmology, "Sapienza" University of Rome, Terracina, Italy
[2]Department of Internal Medicine and Medical Specialties, UOD Internal Medicine and Secondary Hypertension, "Sapienza" University of Rome, Rome, Italy

Abstract. – OBJECTIVE: To assess vitreous and plasma changes of vascular endothelial growth factor A (VEGF-A), adrenomedullin (ADM) and endothelin-1 (ET-1) in proliferative diabetic retinopathy (PDR).

PATIENTS AND METHODS: 9 patients with PDR in type 2 diabetes (T2DM) and 11 age-matched non-diabetic patients were enrolled. The levels of VEGF-A, ADM and ET-1 were measured using an enzyme (ELISA) and a radioimmunoassay (RIA) both in vitreous and plasma samples.

RESULTS: Vitreous ADM and VEGF-A levels were significantly higher in PDR patients (p=0.04 and p=0.02), whereas no differences were found in ET-1 levels (p=0.29). Plasma ADM levels were significantly higher in the PDR group (p<0.01), whereas no significant differences were found in the plasma ET-1 and VEGF-A levels (p=0.30 and p=0.37). The ADM vitreous/plasma ratio was significantly reduced in PDR group.

CONCLUSIONS: The role of ET-1 in advanced PDR is still controversial; it has been supposed a role limited to induce hypoxic state and promote angiogenesis in the early phases. Once the neo-angiogenic process starts, other mediators are mainly involved as VEGF and ADM. Our findings suggest that ADM is an important marker of advanced PDR as well as VEGF. Conversely, ET-1 is not significantly involved in the advanced stage of PDR.

Key Words:
Neoangiogenic factors, Diabetes, Microvascular complications, Proliferative diabetic retinopathy, Vasoactive peptides.

Introduction

Vascular disease is an important cause of morbidity and mortality in patients with diabetes mellitus, and endothelial dysfunction is closely related to microangiopathy.

Diabetic retinopathy is a common complication of both type 1 (T1DM) and type 2 (T2DM) diabetes mellitus, and an estimated 100 million people are afflicted with vision-threatening worldwide [1].

Proliferative stage of diabetic retinopathy (PDR) is characterized by the formation of new vessels on the retinal surface leading to vitreous hemorrhage, fibrosis, and tractional retinal detachment.

Hyperglycemia causes abnormalities in the blood and vascular permeability, due to imbalance between vasodilators (nitric oxide) and vasoconstrictors [angiotensin II and endothelin-1 (ET-1)], and other permeability factors such as vascular endothelial growth factor (VEGF)[2,3].

Adrenomedullin (ADM) is a potent vasorelaxant peptide of 52 amino acids, firstly isolated from human pheochromocytoma[4]. Previous studies reported that also ADM levels were significantly higher in diabetic patients than control group, both in the plasma and vitreous fluids[5,6].

The purpose of the present study is to determine the changes of vasoactive and angiogenic substances among PDR patients and age-matched nondiabetic controls, measuring the concentrations of ET-1, ADM and VEGF-A both in vitreous fluid and plasma. In addition, the relationship between vitreous and plasma levels of the selected factors was also evaluated.

Patients and Methods

A total of 20 consecutive patients (20 eyes) were enrolled at the Ophthalmology Department,

Corresponding Author: Serena Fragiotta, MD; e-mail: serena.fragiotta@uniroma1.it

University "Sapienza", Rome, Italy. The patients were divided into two groups. In the first group, we included 9 T2DM patients with PDR (age range: 43-84 years). The control group consisted of 11 age-matched non-diabetic patients (age range: 47-78 years), with no evidence of diabetic retinopathy, vitreous hemorrhage and proliferative vitreoretinopathy (PVR).

All subjects gave written informed consent before enrollment. Institutional Board approval (IRB) was obtained from Ethics Committee of our Department. The research was conducted in conformity to the Declaration of Helsinki.

Patients who had T2DM and advanced PDR, as defined by the Early Treatment Diabetic Retinopathy Study (ETDRS) were included in the study group. The criteria to perform vitrectomy were best-corrected visual acuity less than 20/40, diabetic vitreous hemorrhage at least 2 months of duration with or without tractional retinal detachment or epiretinal membranes.

Exclusion criteria were prior treatment with steroid or non-steroid anti-inflammatory drugs (FANS), history of acute or chronic infections, fever, cancer or organ failure, thrombotic events, other ophthalmic disorders, including intraocular or vitreoretinal surgery within 6 months, and history of intravitreal injection therapies.

The control group consisted of patients who had undergone vitrectomy for other conditions, such as epiretinal membrane (n=5), retinal detachment (n=4) and macular hole (n=2). These patients were free from any systemic diseases.

At the beginning of standard three-port vitrectomy, 0.3 ml of undiluted vitreous fluid was aspirated via pars plana with a vitreous cutter into a syringe, before opening the infusion line. The vitreous fluid samples were collected into sterile tubes and quickly frozen at -80°C. Blood samples were collected into EDTA tubes before start vitrectomy and were subjected to centrifugation for 10 minutes at 3000 g. Then plasma was immediately frozen and stored at -80°C.

The determination of the ET-1, ADM and VEGF-A levels in the plasma and the vitreous fluid was assessed according to the manufacturer's instructions, using specific kits, as previously described[7-9].

ET-1 was determined by specific radioimmunoassay (RIA) using rabbit anti-endothelin antibody (Peninsula Laboratories, Belmont, CA, USA). Inter- and intra-assay variabilities of ET-1 measurements were 13% and 9%, respectively.

ADM was measured by using commercially available RIA (Phoenix Pharmaceuticals, Mountain View, CA, USA). The intra- and inter-assay coefficients of variation were 5.1% and 12%, respectively. VEGF-A was determined by using commercially available enzyme-linked immunosorbent assay (ELISA) (Cusabio Biotech, Carlsbad, CA, USA).

Statistical Analysis

Quantitative data were reported as mean ± standard deviation (SD) and distribution normality verified through Shapiro-Wilk normality test. The significance of differences between groups was assessed using unpaired *t*-test or U-Mann Whitney test, depending on the types of distribution. Chi-squared test was used to compare categorical variables. Pearson or Spearman's rank correlation test (r) was used to assess the relationship between two variables. Statistical significance was set at $p<0.05$. All calculations were carried out using SPSS software (ver.19; SPSS, Inc., Chicago, IL, USA).

Results

Baseline characteristics of the study groups are shown in Table I. The vitreous fluid analysis revealed that ADM and VEGF-A levels were significantly higher in the PDR eyes than controls (+0.30 ng/ml, 95%CI: -0.20,0.80 and +10.50 pg/ml, 95% CI: -2.99, 24.01, respectively). Instead, the levels of ET-1 were not different between groups (0.003 ng/ml, 95% CI: -0.25, 0.03) (Table II). Figure 1 shows the levels of ET-1, ADM and VEGF-A in the vitreous fluid in both groups.

The blood plasma concentration of ADM was significantly higher in the PDR eyes (+7.26 ng/ml, 95% CI: -0.12, 14.64) than control group, whereas ET-1 and VEGF-A did not change significantly between the groups (0.04 ng/ml, 95% CI: -0.22, 0.12 and 17.46 pg/ml, 95% CI: -70.41, 105.34), (Table II). Figure 2 reported the blood plasma concentrations of the studied factors.

A positive correlation was found between vitreous and plasma concentrations of ADM ($r=0.70$, $p<0.03$) as well as VEGF-A ($r=0.77$, $p<0.01$) in the PDR group solely.

The vitreous fluid/plasma ratios for ET-1 and VEGF-A were comparable between groups, whereas the ADM vitreous fluid/plasma ratio was lowest in the PDR group (Table II).

Table I. Main clinical characteristics and biochemical markers of the study groups.

	PDR group (n=9)	Control group (n=11)	*p*-value
Age (years)	63.33 ± 12.52	67.18 ± 9.66	0.22[a]
Gender (M/F)	4/5	6/3	0.42[b]
Disease duration (years)	13.42 ± 6.72	-	-
Pan Retinal photocoagulation	7/9	-	-
Insulin therapy	6/9	-	-
Metformin therapy	5/9	-	-
Fasting blood glucose (mg/dl)	140.55 ± 39.97	106 ± 25.70	0.01
HbA1c (%)	8.5 ± 3.21	4.6 ± 1.13	<0.001[a]
Creatinine (mg/dl)	2.14 ± 3.48	0.84 ± 0.16	0.11

Data are expressed as the mean ± standard deviation; [a]unpaired student *t*-test; b χ square test;
PDR, proliferative diabetic retinopathy, HbA_{1c}, glycosylated hemoglobin;

No statistically significant correlations were found between vitreous or plasma factors and age, disease duration or glycemia. Instead, a positive correlation between serum creatinine and plasmatic ET-1 levels was detected (r=0.80, p<0.01).

Discussion

Diabetic retinopathy is a common microvascular complication, involving more than 60% of patients with T2DM after 20 years. The most

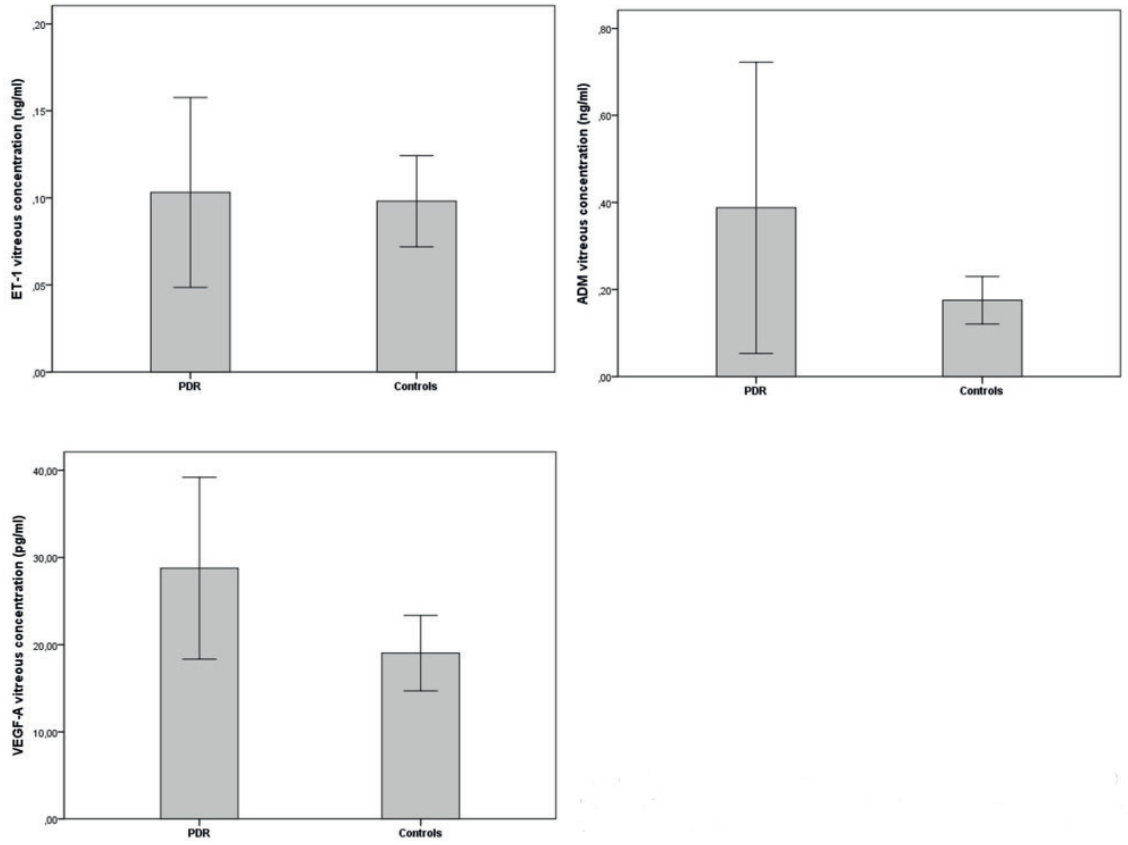

Figure 1. Vitreous concentrations of endothelin-1 (ET1), adrenomedullin (ADM) and anti-vascular endothelial growth factor A(VEGF-A) in patients with proliferative diabetic retinopathy (PDR) and controls.

Table II. Vitreous and bood serum concentrations of ET-1, ADM and VEGF-A among study groups and their ratios.

	PDR group (N=9)	Control group (N=11)	*p*-value
Vitreous concentration			
ET-1 (ng/ml)	0.10	0.09	0.29[a]
95%CI	0.08, 0.12	0.08, 0.10	
ADM (ng/ml)	0.47	0.17	0.04[b]
95%CI	0.001, 0.77	0.11, 0.23	
VEGF-A (pg/ml)	29.51	19.03	
95%CI	16.75, 40.78	14.21, 23.86	0.02[a]
Blood plasma concentrations			
ET-1 (ng/ml)	0.38	0.43	0.30[a]
95%CI	0.29, 0.64	0.35, 0.50	
ADM (ng/ml)	10.79	3.53	<0.01[a]
95%CI	2.72, 14.92	1.68, 5.38	
VEGF-A (pg/ml)	162.15	144.69	0.37[b]
95%CI	124.06, 194.62	60.13, 229.24	
Vitreous/serum ratio			
ET-1	0.25	0.23	0.34[b]
95%CI	0.19, 0.32	0.20, 0.27	
ADM	0.04	0.07	0.05[b]
95%CI	0.02,0.06	0.05, 0.11	
VEGF-A	0.17	0.16	0.42[b]
95%CI	0.12, 0.22	0.13, 0.20	

PDR, proliferative diabetic retinopathy; 95% CI 95% confidence interval; [a]unpaired student *t*-test; [b]U Mann-Whitney test;

serious and vision-threating stage is proliferative diabetic retinopathy[10].

In T2DM both metabolic and vasoactive factors contribute to the development of diabetic microvascular complications[11] and endothelial dysfunction is an early feature of the disease[12].

The vascular endothelium can maintain vascular tone, performing also an important hemostatic function. The endothelial dysfunction plays an important role in the pathogenesis of diabetic angiopathy[13].

An imbalance between some vasoactive substances such as ET-1 (potent vasoconstrictor and pro-inflammatory peptide)[14] and ADM (potent vasodilator)[15] represents a prominent feature of the endothelial dysfunction.

Although our results showed that ADM and VEGF-A concentrations were highest in the vitreous samples of the proliferative diabetic eyes, the ET-1 levels did not significant change between groups. Moreover, the plasma analysis demonstrated that only ADM levels were significantly higher in proliferative diabetic eyes than controls.

In our opinion, there are several possible explanations for the behavior of ET-1. Ocular tissues, vascular and extravascular sites in the retina are a rich source for ET-1 expression, and ET-1 itself contributes to abnormal retinal hemodynamics in diabetic retinopathy[16,17]. The ET-1 expression is associated with several factors, such as clinical course of disease, insulin, sulfonylureas therapy, and serum creatinine levels[18]. Moreover, panretinal laser photocoagulation can also reduce plasma ET-1 levels[19].

Indeed, vitreous and/or plasma modifications of the ET-1 are still controversial in proliferative diabetic retinopathy. Adamiec-Mroczek et al[20] reported no significant differences in plasmatic ET-1 concentration between PDR patients and control group, but a significant increase in the vitreous fluid. However, Ogata et al[21] reported lower ET-1 levels in the vitreous fluid of PDR patients compared with controls.

Our observations suggest that ET-1 levels in plasma and vitreous are strongly influenced by various factors, resulting in high variability of its expression. In fact, a strong positive correlation

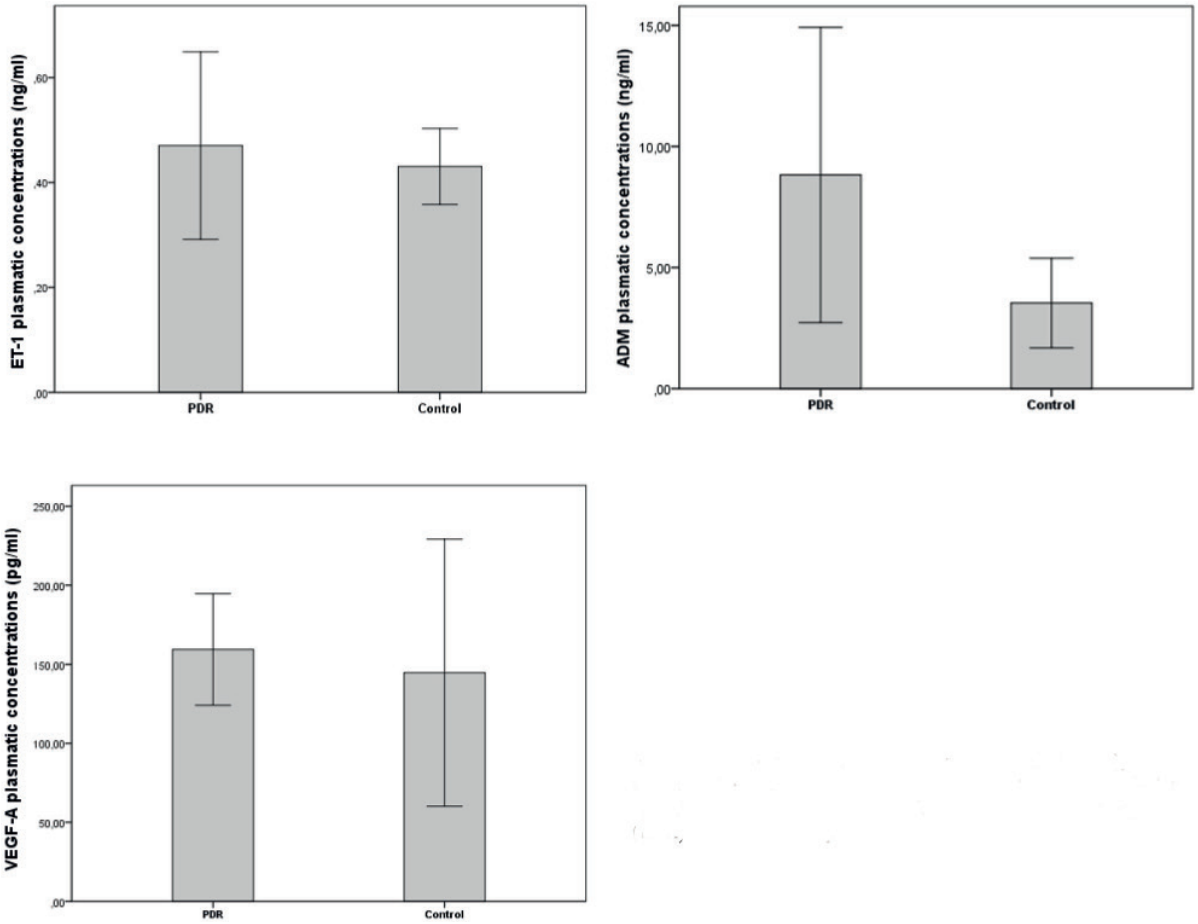

Figure 2. Blood plasma concentrations of endothelin-1 (ET1), adrenomedullin (ADM) and anti-vascular endothelial growth factor A(VEGF-A) in patients with proliferative diabetic retinopathy (PDR) and controls.

was found between serum creatinine and plasma ET-1 levels, in agreement with previous studies reporting also an increased kidney glomerular filtration rate or mesangial expansion[18,22-23].

In the current study, the VEFG-A was elevated in the vitreous fluid of PDR patients, as previously reported[24-25]. Otherwise, the plasma levels of VEGF-A were higher than controls but not significantly, even if directly related to its vitreous concentration. The VEGF is a well-known factor in the development of proliferative diabetic retinopathy. VEGF acts as proangiogenetic factor, involved in both vascular proliferation and increased vascular permeability[26-27].

Interestingly, vitreous and plasma levels of ADM were highly elevated in the PDR eyes and directly correlated each other.

ADM is a potent vasorelaxant peptide found not only in normal adrenal medulla but also in various tissues organs including vascular smooth muscle cells, endothelial cells, and human retinal cells[17]. In detail, during inflammatory process, various types of cells in the eye may produce ADM, including RPE cells, vascular endothelial and smooth muscle cells and fibroblasts[28].

ADM production in the eye is stimulated by hypoxic state, which is a typical pathological feature of diabetic microangiopathy leading to angiogenesis[5,29,30]. ADM seems to be implicated in the advanced PDR in which fibrovascular membranes are formed. Not surprisingly, the same cells forming the fibrovascular membranes are also involved in the ADM production[28,29].

Conclusions

In summary, ET-1 may be considered as an early mediator that acts, as demonstrated previously, inducing hypoxic state and promoting angiogene-

sis[31], but it is not remarkable in advanced PDR, in which seems to be involved mainly other mediators especially VEGF and ADM.

To support these hypotheses, Masuzawa et al[32] in a diabetic rat model reported that treatment with an ET_A receptor antagonist, in the early stage of the diseases, might be useful in preventing the progression of diabetic retinopathy.

This is the first study that simultaneously measures concentrations of ET-1, ADM, and VEGF-A, the peptides involved in the PDR pathogenesis. Our results suggest that ET-1 may be a role as target molecule in the early stages of diabetic retinopathy, whereas ADM seems to be involved in the advanced stages as well as VEGF. Also, the high levels of ADM detected in both vitreous and plasma may represent an important marker of proliferative diseases. These findings are important to clarify the pathogenic mechanisms and to assess the potential target molecules at different stages of diabetic retinopathy.

Further studies with a large number of patients are encouraged both to confirm and extend these findings and especially to evaluate the potential role of ADM as a new target molecule.

Conflict of interest

The authors declare no conflicts of interest.

References

1) Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. Diabetes Res Clin Pract 2010; 87: 4-14.
2) Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature 2001; 414: 813-820.
3) Shao Y, Xu TT, Zhang CG, Pei CG, Zhou Q. The use of optical coherence tomography (OCT) to evaluate the efficacy of different photo-coagulations in diabetic macular edema treatment. Eur Rev Med Pharmacol Sci 2016; 20: 2993-2998.
4) Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, Eto T. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun 1993; 192: 553-560.
5) Lu Y, Xu Y, Tang C. Changes in adrenomedullin in patients with proliferative diabetic retinopathy. Curr Eye Res 2011; 36: 1047-1052.
6) Ito S, Fujisawa K, Sakamoto T, Ishibashi T. Elevated adrenomedullin in the vitreous of patients with diabetic retinopathy. Ophthalmologica 2003; 217: 53-57.
7) Arca M, Montali A, Pigna G, Antonini R, Antonini TM, Luigi P, Fraioli A, Mastrantoni M, Maddaloni M, Letizia C. Comparison of atorvastatin versus fenofibrate in reaching lipid targets and influencing biomarkers of endothelial damage in patients with familial combined hyperlipidemia. Metabolism 2007; 56: 1534-1541.
8) Vizza CD, Letizia C, Badagliacca R, Sciomer S, Poscia R, Della Rocca G, Iacoboni C, Leonardo de L, Quattrucci S, Dario C, Luigi P, Fedele F. Plasma adrenomedullin and endothelin-1 concentration during low-dose dobutamine infusion: Relationship between pulmonary uptake and pulmonary vascular pressure/flow characteristics. Regul Pept 2006; 136: 85-91.
9) Aversa A, Letizia C, Francomano D, Bruzziches R, Natali M, Lenzi A. A spontaneous, double-blind, double-dummy cross-over study on the effects of daily vardenafil on arterial stiffness in patients with vasculogenic erectile dysfunction. Int J Cardio 2012; 160: 187-191.
10) Wilkinson CP, Ferris FL 3rd, Klein RE, Lee PP, Agardh CD, Davis M, Dills D, Kampik A, Pararajasegaram R, Verdaguer JT. Global Diabetic Retinopathy Project Group: Proposed international clinical diabetic retinopathy and diabetic macular edema disease severity scales. Ophthalmology 2003; 110: 1677-1682.
11) Groop PH, Forsblom C, Thomas MC. Mechanisms of disease: Pathway-selective insulin resistance and microvascular complications of diabetes. Nat Clin Pract Endocrinol Metab 2005; 1: 100-110.
12) Sena CM, Pereira AM, Seiça R. Endothelial dysfunction--a major mediator of diabetic vascular disease. Biochim Biophys Acta 2013; 1832: 2216-2231.
13) Rask-Madsen C, King GL. Mechanisms of Disease: endothelial dysfunction in insulin resistance and diabetes. Nat Clin Pract Endocrinol Metab 2007; 3: 46-56.
14) Barton M, Yanagisawa M. Endothelin: 20 years from discovery to therapy. Can J Physiol Pharmacol 2008; 86: 485-498.
15) Kato J, Tsuruda T, Kita T, Kitamura K, Eto T. Adrenomedullin: a protective factor for blood vessels. Arterioscler Thromb Vasc Biol 2005; 25: 2480-2487.
16) Kohner EM, Patel V, Rassam SM. Role of blood flow and impaired autoregulation in the pathogenesis of diabetic retinopathy. Diabetes 1995; 44: 603-607.
17) Pang IH, Yorio T. Ocular actions of endothelins. Proc Soc Exp Biol Med 1997; 215: 21-34.
18) Strzalka-Mrozik B, Nowak A, Gola J, Kowalczyk M, Kapral M, Mazurek U. Factors associated with changes in endothelin-1 gene expression in patients with diabetic retinopathy in type 2 diabetes mellitus. Mol Vis 2010; 16: 1272-1279.
19) Mohamed TA, Mohamed Sel-D. Effect of pan-retinal laser photocoagulation on plasma VEGF, endothelin-1 and nitric oxide in PDR. Int J Ophthalmol 2010; 3: 19-22.

20) Adamiec-Mroczek J, Oficjalska-Młynczak J, Misiuk-Hojło M. Roles of endothelin-1 and selected proinflammatory cytokines in the pathogenesis of proliferative diabetic retinopathy: Analysis of vitreous samples. Cytokine 2010; 49: 269-274.

21) Ogata M, Naruse M, Iwasaki N, Katoh S, Ohta Y, Hori S, Demura H, Iwamoto Y. Immunoreactive endothelin levels in the vitreous fluid are decreased in diabetic patients with proliferative retinopathy. J Cardiovasc Pharmacol 1998; 31 Suppl 1: S378-S379.

22) De Mattia G, Cassone-Faldetta M, Bellini C, Bravi MC, Laurenti O, Baldoncini R, Santucci A, Ferri C. Role of plasma and urinary endothelin-1 in early diabetic and hypertensive nephropathy. Am J Hypertens 1998; 11: 983-988.

23) Zanatta CM, Gerchman F, Burttet L, Nabinger G, Jacques-Silva MC, Canani LH, Gross JL. Endothelin-1 levels and albuminuria in patients with type 2 diabetes mellitus. Diabetes Res Clin Pract 2008; 80: 299-304.

24) McAuley AK, Sanfilippo PG, Hewitt AW, Liang H, Lamoureux E, Wang JJ, Connell PP. Vitreous biomarkers in diabetic retinopathy: A systematic review and meta-analysis. J Diabetes Complications 2013; 28: 419-425.

25) Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, Park JE, Nguyen HV, Aiello LM, Ferrara N, King GL. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N Engl J Med 1994; 331: 1480-1487.

26) Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. Am J Pathol 1995; 146: 1029-1039.

27) Adamis AP, Miller JW, Bernal MT, D'Amico DJ, Folkman J, Yeo TK, Yeo KT. Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. Am J Ophthalmol 1994; 118: 445-450.

28) Udono-Fujimori R, Udono T, Totsune K, Tamai M, Shibahara S, Takahashi K. Adrenomedullin in the eye. Regul Peptides 2003; 112: 95-101.

29) Udono T, Takahashi K, Nakayama M, Yoshinoya A, Totsune K, Murakami O, Durlu YK, Tamai M, Shibahara S. Induction of adrenomedullin by hypoxia in cultured retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 2001; 42: 1080-1086.

30) Hayashi M, Shimosawa T, Isaka M, Yamada S, Fujita R, Fujita T. Plasma adrenomedullin in diabetes. Lancet 1997; 350: 1449-1450

31) Ergul A. Endothelin-1 and diabetic complications: focus on the vasculature. Pharmacol Res 2011; 63: 477-482.

32) Masuzawa K, Goto K, Jesmin S, Maeda S, Miyauchi T, Kaji Y, Oshika T, Hori S. An endothelin type A receptor antagonist reverses upregulated VEGF and ICAM-1 levels in streptozotocin-induced diabetic rat retina. Curr Eye Res 2006; 31: 79-89.