Original Article

Biochemical scenario behind initiation of diabetic retinopathy in type 2 diabetes mellitus

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Purpose: Despite the disease having similar glycemic status and duration microangiopathy in some patients develop within few years whereas it doesn't appear as a health complication in some diabetics for a considerable period. This study is undertaken to assess the hyperglycemia-induced biochemical background behind the development of diabetic retinopathy (DR) in type 2 diabetes mellitus (DM). **Methods:** Following proper diagnosis, 100 patients of type 2 DM of 10–12 years duration having no DR, and 42 patients of type 2 DM of the same duration and glycemic status as the second group, with mild retinopathy were recruited in the study. Lactic acid, glutamate, malondialdehyde (MDA), nitrate, advanced glycation end-products (AGEs), peripheral blood mononuclear cell reactive oxygen species (ROS), vascular endothelial growth factor (VEGF), and its receptor 2 (VEGFR2) in these two groups were produced in an assay following standard methodology. **Results:** Biochemical markers of anaerobic glycolysis, lipid peroxidation, AGEs, glutamate concentration, oxidative stress, and expression of VEGF and its VEGFR2 with significantly elevated markings were found in them who developed earliest stage of DR rather than them who had not. **Conclusion:** Hyperglycemia-induced anomalous glucose metabolism, lipid peroxidation, advanced glycation, glutamate toxicity, and oxidative stress create a background where apoptosis of retinal capillary endothelial cells invite increased expression of VEGF and VEGFR2, these being the crucial factors behind the development of diabetic microangiopathy.

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Despite remarkable advances in diabetes mellitus' (DM) diagnosis and treatment, based on clinical observations and noninvasive diagnostic optical instrumentations, diabetic retinopathy (DR) remains the leading cause of blindness among the adults, especially counting those in the prime of their productive ages (human resources), both in developed and developing countries.[1,2]

The earliest microvascular dysfunction in retina of patients with diabetes is evoked by hypoxia because of capillary closure and nonperfusion. It has been postulated that hypoxic retina adjacent to acellular capillaries, in turn releases vascular endothelial growth factor (VEGF) in an attempt to increase blood flow in ischemic area. However, it has not yet been clearly established whether hypoxia and its sequel, can develop even without vascular occlusion. It is our hypothesis that gradual onset of DR is due to insidious apoptosis of vascular endothelial cells (ECs) of inner retina, resulting from biochemical changes of persistent hyperglycemia. Thus, increased intracellular glucose in retinal tissue, which is insulin-independent with respect to intracellular transport of glucose, triggers increased anaerobic glycolysis, polyol pathway, increased lipid peroxidation, advanced glycation end-product (AGE) formation, and ultimately increased tissue expression of VEGF which leads to break-down of inner blood-retinal barrier, i.e., all the symptoms appear of DR having been initiated. The hyperpermeability of retinal capillaries resulting from structural damage of capillary wall can be seen. This structural damage of capillary wall occurs due to toxic effects of metabolites, mainly, of anomalous glucose metabolism, lipid peroxidation and endothelial cell injury - secondary to leukostasis owing to resultant response of increased secretion of VEGF. The hike in VEGF follows the consequent appearance of microaneurysm arising as hypercellular saccular outpouching of the capillary wall in solitary or cluster form, spillage of lipid-rich material from the hyper-permeable capillaries accumulating in the inner and outer plexiform layers in the form of hard exudates, and, finally, appearance of intraretinal dot-blot hemorrage as deep red dots and flame-shaped hemorrhage with wispy margins in the nerve fiber layer. Leakage from microaneurysms, or hyperpermeable capillaries, results in localized retinal thickening or diffuse macular edema which may involve the center of macula causing impairment of vision. Thus, microangiopathy in the

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capillary bed of retina of patients of DM is becoming the most frequent single cause of blindness among adults in the age group of 20–75 years.[3,4] Although persistent hyperglycemia is considered to be the most important and decisive factor related to the development of DR, all patients of type-2 DM, with persistent hyperglycemia for a prolonged period, do not develop the symptoms, and their attendant complication, of this disease.[5,6] Some investigators advocate inhibition of glycolysis due to inactivity of glycolytic enzyme as an important cause for the development of DR.[7] Another study hypothesizes that progressively accelerated rate of glycolysis gradually exhausts the supply of oxidized cofactors resulting in pseudohypoxia and anaerobic glycolysis that further exhausts the supply of oxidized cofactor. This is the setting in of nicotinamide adenine dinucleotide positive (NAD^+) leading to different biochemical derangements related to development of DR.[8] Thus, redox state alteration in those tissues, which are not dependent on insulin for intracellular transport of glucose, compels enormous amount of glucose to enter into abnormal biochemical pathways and results in an apoptotic cellular condition, where excessive VEGF secretion is warranted.[9,10]

In this cross-sectional study, we have attempted to determine the differential expression of hyperglycemia-induced anomalous biochemical phenomena, such as AGE formation, lipid peroxidation, oxidative stress, glutamate excitotoxicity and altered secretion of VEGF and VEGF receptor 2 (VEGFR2) in patients of type 2 DM with, and without, onset of DR.

Methods

One hundred and forty-two patients of type-2 DM of 5–10 years duration – who were diagnosed, treated, and referred from the diabetic clinic at the tertiary hospital – were recruited for this study since 2008. All the participants underwent detailed ocular and systemic examinations. Dilated fundus examination was done by direct, indirect ophthalmoscopes, using slit-lamp biomicroscopy with 3 - mirror and + 90D lenses, to detect presence or absence of any form of retinopathy at the beginning of the study.

Exclusion criteria

All participants with inability to give informed consent, ongoing infection, hypertension, cardiovascular (Ischemic and coronary artery disease), connective tissue disorder, and neoplastic diseases were excluded from this study. The approval of the Local Research Ethics Committee was also obtained.

Laboratory investigations

Duly signed by the participants, written informed consent was taken before the following was carried out: First, collection of 10 mL of venous blood by venepuncture from all the participants was needed. In addition, 2 mL plasma and 3 mL serum were separated, and levels of lactic acid, malondialdehyde (MDA), nitrate, glutamate, AGEs, peripheral whole blood (PBMC) reactive oxygen species (ROS), VEGF, and VEGF2 were measured using standard procedures as mentioned elsewhere.[11-17]

Lactic acid was measured by the commercially available Lactate kit (Randox-LC2389, UK), following lactate oxidase and peroxidase enzymatic method, (Burtis, 1998).[11]

The colorimetric method for quantitative analysis of serum MDA free of interference from sialic acids was used as described by Satoh.[12] In this method, 2.5 mL of 20% trichloroacetic acid and 1.0 mL of 0.67% thiobarbituric acid was added to 0.5 mL of serum. Then, the mixture was heated in boiling water bath for 30 min. The resulting chromogen was extracted with 4.0 mL of n-Butyl alcohol, and the absorbance of the organic phase was determined spectrophotometrically at 530 nm.

There was determination of nitrite and nitrate by Griess reaction (Griess reagent, G4410, Sigma USA, sensitivity - <0.64 μmol/L).

This assay indirectly detects NO activity because NO decays in physiological system within seconds. In tissues and blood, NO is largely consumed in the reaction with oxygen species and transition metals, forming nitrite (NO_2^-), and nitrate (NO_3^-). This technique involves enzymatic reduction of nitrate to nitrite, followed by derivatization and spectrophotometric detection of nitrite at 540 nm.[13]

Measurement of glutamate

Plasma glutamate was measured by enzyme-linked spectrofluorimetric assay as described by Wang and Chen.[14] Glutamate present in the sample was oxidized to α-ketoglutarate with the fluorimetric production of NAD phosphate (NADPH) in Krebs–Ringer-Hepes reaction buffer. The fluorescence generated by reduction of $NADP^+$ to NADPH was monitored by spectrofluorimetrically at excitation and emission wavelength of 360 nm and 455 nm, respectively.

Measurement of total advanced glycation end-products from serum

The AGE protein adduct content in the sample was determined by comparing it with a standard curve, prepared from AGEs protein adducts present in the sample, and measured by enzyme-linked immunosorbent assay using the kit of Cell Biolabs (catalog no. STA 317, Sandiego, USA). AGEs present in the samples were probed with an ant-AGE polyclonal antibody, followed by HRP conjugated secondary antibody.

AGE-BSA standards range from 0.25 to 5 μL. The absorbance of final color product was read at 450 nm as the primary wave length by Bio Rad multi-plate reader (Model 680) against reduced BSA standard as absorbance blank. The procedure of estimation of AGEs has been described in the previous study by Stitt.[15]

Measurement of reactive oxygen species from mononuclear cells

Mononuclear cells from PBMC were obtained from 10 mL heparinized blood using Histopaque 1077. This process separates media (Sigma Aldrich, St. Louis, MO, USA) density gradient for 40 min at 200 r.p.m and 20°C. Further, PBMCs were subjected to centrifugation at 200 r.p.m for 10 min and washed twice with 1 × PBS (pH 7.2). Thereafter, 5×10^5 cells were pelleted into two different tubes and resuspended in 1 × PBS (pH 7.2) for the estimation of ROS. To measure intracellular ROS generation in mononuclear cells, ROS-sensitive cell permeable dye 2′7′ dihydrodichlorofluorescein diacetate, in the presence of ROS, was oxidized to highly fluorescent 2′7′-dichlorofluorescein in the cell. According to current knowhow, production of intracellular ROS is directly proportional to the oxidation

of 2′7′ dihydrodichlorofluorescein diacetate, elevating the fluorescence level as measured by flow cytometry.[16]

Concentration of VEGF and VEGFR2 in serum of study participants were measured by enzyme-linked immunosorbent assay using commercial ELISA kit (My Biosource, San Diego, CA and Raybiotech, Norcross, USA) according to the manufacturer instructions. Details of the commercially used kits are presented in the study of Paine *et al*.[17]

Statistical analysis

Data obtained from each sample group were presented as mean ± standard deviation). Normally distributed variables (systolic blood pressure [SBP], diastolic blood pressure [DBP]), (fasting blood glucose [FBG], postprandial blood glucose [PBG]), glycosylated hemoglobin (HbA1c), MDA, glutamate, nitrate, lactate, VEGF, and VEGFR2 of two groups were compared by Student's *t*-test. Not normally distributed variables of two groups (age, duration of DM, AGE, and ROS) were compared by Mann–Whitney U-test. Statistical analysis for sex distributions was evaluated by Chi-square test. $P < 0.05$ was considered as minimum level significance. All statistical analysis was performed using GraphPad Prism software (Version-6, Sandiego, CA, USA).

Results

Different study groups enrolled in the present study showed no statistically significant differences for sex distribution, age, duration of DM, SBP, DBP, FBG, PBG, and HbA1c level [Table 1]. Biochemical investigations revealed that generation of MDA ($P < 0.0001$), ROS ($P < 0.0001$), AGE ($P = 0.0005$), glutamate ($P < 0.0001$), lactate ($P < 0.0001$), and nitrate ($P < 0.0001$) was markedly elevated among mild Nonproliferative diabetic retinopathy (NPDR) participants compared to Diabetic with no retinopathy (DNR) participants [Table 2 and Fig. 1a-f]. The expression of VEGF ($P < 0.0001$) and its receptor VEGFR2 ($P = 0.0003$) was also found to be significantly higher among mild NPDR participants compared to DNR participants [Table 2 and Fig. 1g, h].

Discussion

Type 2 DM develops DR after 15 years of duration of the disease process in 60% of patients observed, whereas 40% patients of this disease remain asymptomatic. Chronic uncontrolled hyperglycemia has been suggested as a major influencing factor for the development of microangiopathy in type 2 DM. Among the different biochemical pathways implicated in the pathogenesis of DR, the process of accumulation of AGEs, increased extracellular glutamate concentration, nitric oxide secretion, lipid peroxidation, and excessive generation of oxidative stress are considered to converge to increased tissue expression of VEGF and its receptor VEGFR2. Different studies have already demonstrated VEGF as the principal causal agent to the pathogenesis of DR; however, there is lack of substantial research-based evidence concerning living human subjects on how the preceding biochemical derangements lead to altered expression of VEGF, and its related disorder, that is the activation of its receptor-2.[18,19]

Besides the mitogenic role, it has been found that VEGF, by its physiological function, acts as a neuroprotectant in ischemic injury.[20] Surprisingly, pericytes are lost before the earliest manifestation of the microangiopathy. It has been postulated by previous inquiries into the matter that factors

Table 1: Clinical characteristics of study groups

Parameters	DNR (*n*=100)	Mild NPDR (*n*=42)	*P*
Sex (%)			
Male	55 (55)	45 (20)	0.42
Female	45 (45)	(52.38)	
Duration of DM (years)	8.1±1.72	8±2.13	0.89
Blood pressure (mmHg)			
Systolic	132±2.66	133±3.43	0.47
Diastolic	82.44±2.40	83.11±4.13	0.68
Blood glucose (mg/dl)			
Fasting	134.41±7.26	137.33±11.33	0.46
Postprandial	154.58±9.10	159.5±18.05	0.40
Glycosylated hemoglobin (%)	7.1±0.61	7.1±0.52	0.97

Comparison between two groups enrolled in the present study showed no statistically significant differences for sex distribution, age, and duration of disease, blood pressure, blood glucose level and glycosylated hemoglobin level. Data were represented as mean±SD and $P<0.05$ was considered as statistically significant. *n*: Sample size, SD: Standard deviation, DM: Diabetes mellitus, DNR: Diabetic with no retinopathy, NPDR: Nonproliferative diabetic retinopathy

Table 2: Serum, plasma or peripheral blood mononuclear cell level of malondialdehyde, reactive oxygen species, advanced glycation end-product, glutamate, lactate, nitrate, vascular endothelial growth factor, and vascular endothelial growth factor receptor among study groups

Parameters	DNR (*n*=100)	Mild NPDR (*n*=42)	*P*
Serum MDA level (nmol/ml)	2.61±1.14	3.95±1.4	<0.0001
PBMC ROS (geomean of DCF/5×10^5 cells)	98.6±21.35	135.34±38.8	<0.0001
Serum AGE level (μg/ml)	2.2±1.3	3.2±1.85	0.0005
Plasma glutamate level (μmol/L)	55.13±20.8	65.94±20.29	<0.0001
Plasma lactate level (mmol/L)	1.9±0.76	3.14±1.14	<0.0001
Serum nitrate level (μmol/L)	20.65±8.76	39.42±11.39	<0.0001
Serum VEGF level (pg/ml)	96.66±37.35	182.61±49.36	<0.0001
Serum VEGFR2 level (ng/ml)	51.37±21.36	64.78±22.72	0.0003

Data were represented as mean±SD and $P<0.05$ was considered as statistically significant. All (as stated above) biochemical parameters were found to be increased significantly among mild NPDR subjects compared to DNR subjects. MDA: Malondialdehyde, PBMC: Peripheral blood mononuclear cell, ROS: Reactive oxygen species, AGE: Advanced glycation end-product, VEGF: Vascular endothelial growth factor; VEGFR2: Vascular endothelial growth factor receptor-2, SD: Standard deviation, *n*: Sample size, DNR: Diabetic with no retinopathy, NPDR: Non poliferative diabetic retinopathy

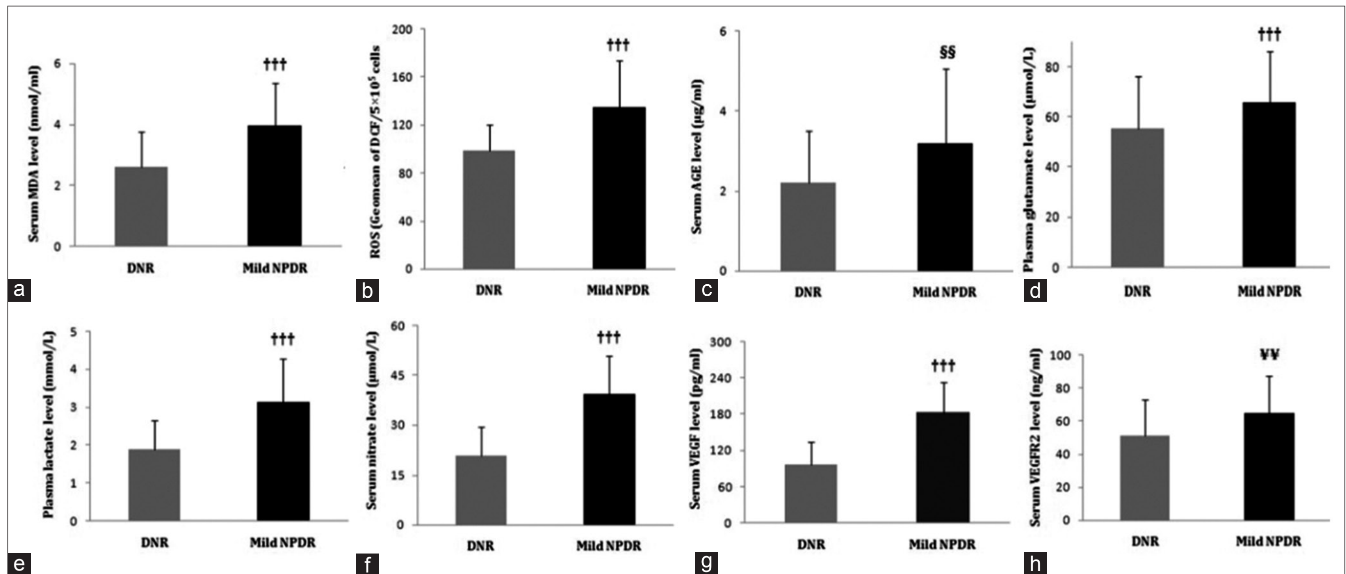

Figure 1: Blood level of different biochemical parameters among study groups. Bar diagrams represent mean ± standard deviation of malondialdehyde (a), reactive oxygen species (b), AGE (c), glutamate (d), lactate (e), nitrate (f), vascular endothelial growth factor (g) and vascular endothelial growth factor receptor 2 (h) among DNR and mild NPDR groups. $^{†††}P < 0.0001$, $^{¥¥}P = 0.0005$ and $^{§§}P = 0.0003$, respectively

other than VEGF might be responsible for selective apoptosis of pericytes of retinal capillaries.[9] There is now ample evidence that hyperglycemia is associated with accelerated death of vascular and neural cells of retina.[21] Enormous quantities of intracellular glucose in retinal tissues are bound to follow the faster rate of glycolysis and polyol pathways resulting in a deficiency of NAD^+, while sorbitol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase enzymes consume this essential oxidized cofactor excessively to remain active.[22] Hence, the condition of pseudohypoxia and, therefore, anaerobic glycolysis produce excess of lactate causing the ratio of lactate to pyruvate to elevate, while the ratio of NAD^+/NADH falls.[10] In this study, we found that plasma lactate level was increased remarkably in patients with diabetic with mild NPDR as compared to DNR, and this finding conclusively indicates the increased occurrence of anaerobic glycolysis in type 2 diabetic subjects who developed mild retinopathy.

Excessive generation of lactic acid owing to increased anaerobic glycolysis lowers the pH of the microenvironment of retina which impairs the activity of L-glutamate/L-aspartate transporter (GLAST). Muller cells express GLAST, which plays an important role in the transport of extracellular glutamate, where it moves away from the synaptic spaces of retina to keep its extracellular concentration below neurotoxic level.[23,24] A previous study reported that under high glucose concentration, glutamate uptake, and GLAST expression decreased significantly, along with increased generation of ROS.[25] Glutamate excitotoxicity disrupts retinal neurons which has been proposed as a mechanism of neural apoptosis. In our present study, plasma glutamate showed significantly elevated level in MNPDR participants pointing to its role in the pathogenesis of earliest stage of DR. The other mechanism played by glutamate may be increased activation of N-methyl-D-aspartate (NMDA) receptor that stimulates nitric oxide synthase resulting in increased production of nitric oxide. It would be reasonable to conclude that the excessive nitric oxide combines with superoxide free radicals to form peroxynitrate that attack pericytes and ECs as powerful cytotoxin. In the present study, the nitrate level – which reflects the production of nitric oxide – showed significantly elevated margin in MNPDR group compared to DNR. Glutamate cascade also has an important role in lipid peroxidation. To recount this briefly, the pathway is glutamate-induced activation of NMDA receptor which results in increased intracellular Ca^{++} that activates endogenous phospholipase enzyme. Next, phospholipase degrades membrane phospholipids and produces arachidonic acid, eicosanoids and oxygen-derived free radicals which cause peroxidative damage of lipid-rich retinal membrane. This, without doubt, generates lipid peroxide such as MDA. As a result, this lipid peroxide - which is the biochemical marker in free radical production owing to lipid peroxidation - is toxic to retinal cell.[26] In this study, the estimation of MDA demonstrated significantly higher levels in MNPDR participants as compared to diabetics with no retinopathy.

Besides the effect of lactic acidosis of severely influencing glutamate cascade, the lactate influences increased expression of VEGF from retinal neurons in a concentration-dependent way.[27]

Without NAD^+, glycolysis cannot continue, and anaerobic glycolysis ultimately slows down owing to diminished activity of glycolytic enzymes under lactate-induced low cellular pH. Hence, the excess unutilized glucose stimulates nonenzymatic glycation of proteins, along with the formation of reactive AGEs which are believed to have a role in long-term diabetic microvascular complications.[28] To define it, AGEs are a class of complex glycoxidized products, produced during the reaction between carbonyl group of reducing sugars and free ε-amino groups of protein to form amadori product. It is well known that circulating AGEs exert their deleterious effects through the interactions with their cell surface receptor for AGE, resulting in postreceptor activation of reduced NADPH oxidase in pericyte, ECs, mesangial cells and macrophases. Activation,

and subsequent uncoupling, of NADPH oxidase, is coupled with increased production of intracellular ROS. This ultimately causes cellular oxidative stress.[29] It is common knowledge that ROS perturb cellular function causing pericyte dysfunction and is considered to be one of the earliest histopathological sign of DR. AGEs are known not only to result in pericyte loss but also are considered responsible for other key features, like basement membrane thickening, blood-retinal barrier dysfunction, formation of microaneurysms and capillary dropout in early stages of DR.[30] In the present study, compared to that in diabetics with no retinopathy, the serum levels of total AGEs and PBMC ROS increased significantly in MNPDR subjects, which highlights their roles in the pathogenesis DR.

Hence, the increased generation of molecules such as lactate and consequently increased intracellular Ca^{++}, extracellular glutamate, nitrate, MDA, AGEs, and ROS are considered to cause death of pericytes of vascular endothelium of retinal capillaries. VEGF and VEGFR2 are then expressed increasingly in an attempt to harvest the ischemic and apoptotic vascular bed of retina leading to breakdown of inner blood-retinal barrier.

In summary, we have observed that some diabetic patients, who developed microangiopathy, demonstrate increased production of molecules such as lactate, glutamate, AGEs, MDA, NO, and ROS. Consequently, there is increased secretion of VEGF and VEGFR2 in these diabetic patients compared to that in other patients of same glycemic status who did not develop any sign of DR within similar duration.

Conclusion

Despite the drawbacks of a cross-sectional study, this study probably draws our attention toward the role of increased anaerobic glycolysis on differential expression of VEGF and VEGFR2 occurring in same type of diabetic patients. That, the initiating pathomechanism of DR is associated with the anomaly of glycolysis and assessment of biomarkers of the associated biochemical derangements, may provide information regarding prediction of the onset of retinal microangiopathy in type-2 diabetic subjects.

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Conflicts of interest

There are no conflicts of interest.

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