ANAEROBIC GLYCOSIS IS THE CENTRE OF DIFFERENT BIOCHEMICAL DISARRAYS ASSOCIATED WITH THE ONSET OF DIABETIC RETINOPATHY.

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ABSTRACT: BACKGROUND: Despite almost similar hyperglycemic status and duration of the disease, some patients of type-2 diabetes mellitus develop microangiopathy within few years where as some individuals avoid this complication for a considerable period. It is assumed that some subjects of type 2 diabetes mellitus show hyperglycemia induced faster rate of anaerobic glycolytic pathway which liberate excessive intermediates of glycolysis responsible for increased intracellular Ca^{+} and increased extracellular glutamate and lactate, the end product of this metabolic pathway causing apoptosis of pericytes and dysfunction of endothelium of retinal capillaries. This retinal background created by excessive lactate may invite excessive secretion of vascular endothelial growth factor (VEGF) and its receptor-2 (VEGFR2) in an attempt to avoid the resultant irritating and destructive cellular condition. Associated increased activity of matrix metalloproteinases (MMP2 and MMP9) may cause degradation of extracellular matrix (ECM) and disruption of basal membrane of retinal capillaries. Aim : This longitudinal study was undertaken to assess the association of anaerobic glycolysis with lipid peroxidation, NO secretion, VEGF, VEGFR2, MMP2 and MMP9 at the time of development of earliest stage of diabetic retinopathy. **METHODS:** 142 patients of type-2 diabetes mellitus of 5 to 10 years duration having no retinopathy were recruited in this longitudinal study. Molecules like lactate, nitric oxide (NO), malondialdehyde (MDA), VEGF, VEGFR2, MMP2 and MMP9 were measured at the interval of one year for three years following standard methods of estimation. Detailed fundus examination and digital fundus photography were done in each patient to document appearance of microaneurysm. **RESULTS :** Surrogate markers of anaerobic glycolysis, oxidative stress, lipid peroxidation and consequently growth factor VEGF, it's receptor VEGFR2, MMP2 and MMP9 were significantly elevated in 32 patients of this group who developed earliest form of diabetic retinopathy compared to 108 diabetic subjects ($p<0.001$) **CONCLUSION :** Faster rate of glycolysis specially anaerobic glycolysis , lipid peroxidation and nitric oxide production occur in different extent in similar type of type 2 diabetic patients and stimulate differential expression of VEGF, KDR, MMP2 and MMP9. The whole cascade is centred on anaerobic glycolysis which is not beneficial to diabetic patients.

KEY WORDS: Lactate, Malon Di Aaldehyde (MDA), Nitric oxide (NO), VEGF, and KDR.

INTRODUCTION: Microangiopathy in the capillary bed of retina of long standing diabetes mellitus is the most frequent single cause of blindness among adults in the age group of 20 to 75 years.[1,2] Observations of WHO have highlighted that India will lead the world in the prevalence of diabetes mellitus.[3] So the major part of this most common complication of diabetic patients has to be tackled by Indian ophthalmologists. We are trying to reach one of the pathways responsible for the development of this microvascular complication, and for the first time attempting to reveal the role of anaerobic glycolysis which is the principal metabolic mechanism of utilization of huge glucose in retina of type-2 diabetic patients.

Persistent hyperglycemia is considered to be the most important decisive factor related to the development of diabetic retinopathy but all patients of type-2 diabetes mellitus with persistent hyperglycemia for a prolonged period do not develop the complication of this disease .[4,5] Type–2 diabetes mellitus develops diabetic retinopathy in 60 % of patients after 15 years of duration of the disease process where as 40 % of this disease remain asymptomatic. Some investigators have been stressing on continuation of glycolysis to be inhibitor of the development of diabetic retinopathy. We differ from this conception. Our previous studies along with the present study in living diabetic subjects suggest detrimental role of anaerobic glycolysis for excessive up regulation of VEGF and activation of VEGFR2, MMP2 and MMP9.

Various studies have suggested vascular endothelial growth factor (VEGF) to be the principal causal agent to the pathogenesis of this disease [6,7] but there is substantial lack of research consecutively on living human subjects about the preceding biochemical derangement leading to altered expression of VEGF and activation of it's receptor-2 in this disorder.

Beside the mitogenic role, VEGF by its physiological function acts as a neuro protectant in ischaemic injury.[8] Pericyte is lost surprisingly before the earliest manifestation of the microangiopathy. It has been suggested by our previous study that some factors other than VEGF, like increased anaerobic glycolysis, accelerated lipid peroxidation, increased extracellular glutamate concentration and increased intracellular influx of Ca^{++} might be responsible for selective apoptosis of pericytes of retinal capillaries.[9]

Second pathomechanism related to the disruption of inner blood-retinal barrier of retina may be caused by degradation of extracellular matrix (ECM) and basal membrane (BM) of retinal capillaries. ECM and BM homeostasis are determined to a great extent by balance between matrix metalloproteinases (MMPs) and tissue inhibitor matrix metalloproteinases (TIMPs). Altered expression and activities of MMPs are considered responsible for increased capillary permeability in initiation of diabetic retinopathy. It is suggested that MMPs, a family of zinc binding, calcium dependent enzymes, are involved in pericellular proteolysis and degradation of ECM in acidic medium created by anaerobic glycolysis.

It is also documented by other study that hyperglycemia is associated with accelerated death of pericytes and endothelial cells.[10]

It may be suggested that lactic acid which is the end product of glycolysis in various tissues including retina and other biochemical events centered on anaerobic glycolysis like increased lipid peroxidation and increased extracellular glutamate due to reduced uptake by muller cells become toxic to microvascular cells resulting in apoptosis of pericytes and endothelial cells.

Death of microvascular cells due to the toxic irritating cellular condition may be responsible for up regulation of VEGF and it's receptor KDR to increase circulation to the

affected areas in an attempt to remove those irritating molecules. Our recent study has also demonstrated hyperglycemia-mediated erythrocyte redox state alteration is associated with the development of diabetic retinopathy.[11]

So this longitudinal study attempted to determine the extent of association of anaerobic glycolysis and related biochemical phenomena with the altered expression of VEGF, VEGFR2, MMP2 and MMP9 at the time of onset of DR.

MATERIALS AND METHODS : One hundred forty patients of type- 2 diabetes mellitus of 5 to 10 years duration diagnosed, treated and referred from the diabetic clinic at the Institute of Post-graduate Medical Education and Research, Kolkata, were recruited for this study since 2008. All subjects underwent detailed ocular and systemic examinations. Dilated fundus examination by direct, indirect ophthalmoscopes, slit-lamp biomicroscopy with 3 – mirror and + 90D lenses excluded any form of retinopathy at the beginning of the study.

Exclusion criteria: all subjects with inability to give informed consent , ongoing infection , hypertension , cardiovascular (ischaemic and coronary artery disease), connective tissue disorder and neoplastic diseases were excluded from this study.
The approval of the Local Research Ethics Committee at the Regional Institute of Ophthalmology, Kolkata was obtained.

Laboratory investigations:
Written informed consent was taken before10 ml of venous blood was collected from all subjects. 2 ml plasma and 3 ml serum were separated and levels of lactic acid , malondialdehyde (MDA), NO, VEGF, VEGF2, MMP2 and MMP9 were measured using standard procedures at the interval of one year for 3 years.

Lactic acid was measured by the commercially available Lactate kit(Randox-LC2389,U.K. s) [(Lactate Oxidase and peroxidase enzymatic method) .[12]

Study subjects were in fasting for at least 4 hours and complete resting state to allow lactate concentration to reach steady state. Venous blood was collected without application of tourniquet and immediately delivered into a pre-measured amount of chilled protein precipitant, perchloric acid. Initially, the lactate is oxidized to pyruvate by lactate dehydrogenase in the presence of NAD^+. The NADH formed in this reaction is measured spectrophotometrically at 550 nm.

The colorimetric method for quantitative analysis of serum MDA free of interference from sialic acids was used as described by Satoh K (Satoh, 1978).[13] In this method 2.5 ml of 20 % Trichloroacetic acid and 1.0 ml of 0.67 % Thiobarbituric acid (TBA) was added to 0.5 ml of serum. Then the mixture was heated in boiling water bath for 30 minutes. The resulting chromogen was extracted with 4.0 ml of n-Butyl alcohol and absorbance of the organic phase was determined spectrophotometrically at 530 nm.

Determination of nitrite and nitrate by Griess reaction [14][Griess reagent, G4410 ,Sigma USA, Sensitivity - < 0.64 µmol/L] indirectly detects NO activity because NO decays in physiological system within seconds. In tissues and blood, NO is largely consumed in reaction with oxygen species and transition metals, forming nitrite ($NO2^-$) and nitrate ($NO3^-$). This technique involves enzymatic reduction of nitrate to nitrite followed by derivatization and spectrophotometric detection of nitrite at 540nm (Guevara et al, 1998).

Total concentration of VEGF and KDR in serum of study subjects were measured by Enzyme-linked immunosorbent assay using commercial ELISA kit (Ray Biotech) according to the manufacturer instruction.

Total concentration of MMP2 and MMP9 and their natural inhibitors TIMP1 and TIMP3 in serum of study subjects were measured using commercial ELISA kit (R & D systems.

Statistical analysis: Statistical analysis were performed by using daniel soper online statistical calculator. All the data are expressed as Mean ± Standard deviation. Continuous variables were compared by one way ANOVA followed by evaluation of Fisher's F value. The independent variables (sex, age, duration of diabetes, blood pressure, glycemic and nutritional status) were also compared by Student's t test.

RESULTS: Age, sex blood pressure, and nutritional status were matched among the study patients. Our yearly clinical and biochemical investigations revealed that the serum lactate was elevated markedly in those patients who developed micro aneurysms in this longitudinal study, VEGF along with its receptor-2 (KDR) were significantly elevated in all diabetic subjects but not in similar fashion though the HbA1c level were almost same (table– 1 & table-2). In progression of time molecules like NO and MDA were also elevated significantly in those diabetic patients who showed very mild microangiopathy detected yearly for 3 years (table-3).

During follow-up period 7, 9 and 16 patients of the study population consequently manifest very mild microangiopathy. Production of lactate, MDA, NO, VEGF and VEGFR2 in these 32 patients was statistically higher than the remaining asymptomatic 108 patients (P = 0.0001; p =0.0001; p = 0.005; p = 0.0001; p = 0.003 respectively).

The serum level of MMP2 and MMP9 were found to be significantly elevated in mild NPDR (MMP2 : 167.36 ± 42..4 ng \ ml, MMP9 : 732.62 ± 256.51 ng \ ml compared to diabetic subjects without DR (MMP2 : 99.44 ± 27 ± 27.58 ng \ ml, MMP9 : 611.11 ± 60.26 ng \ ml ; p< 0.001 for MMP2 and p< 0.001 for MMP9.

DISCUSSION: Although many investigators have suggested different biochemical mediators and growth factors to be involved in the already developed diabetic retinopathy, the exact pathomechanism just before the earliest manifestation of this complication remains elusive.[15,16] Hyperglycemia induced retinal metabolic abnormalities are considered to contribute to it's earliest pathogenesis. Recently some basic experimental studies on animal models focus on the role of inhibition of specific glycolytic enzyme on the development of diabetic retinopathy.[17]

We first time follow up longitudinally the effects of anaerobic glycolysis, the principal metabolic events in retina and other tissues leading to the up regulation of VEGF and it's receptor VEGFR2 in human subjects having type-2 DM.

In our study out of 140 patients of type-2 diabetes mellitus of 5 to 10 years duration 32 patients developed microangiopathy within 3 years follow-up period despite having almost similar glycemic status. Those 32 patients showed gradually increased anaerobic glycolysis resulting in gradually increasing production of lactate. Consequently accelerated lipid peroxidation and nitric oxide secretion which occurred in extended manner in those patients were detected.

In diabetic subjects nerve tissues including retina, lens epithelium, nephrons and erythrocytes which are not insulin dependent for intracellular transport of glucose show higher rise of glucose concentration resulting in higher activity of glycolysis and polyol pathway.[18] In the

sorbitol pathway glucose undergoes reduction by NADPH to sorbitol followed by oxidation of sorbitol to fructose in the presence of NAD^+ and sorbitol dehydrogenase. This enzyme catalyses the transfer of one hydride ion (H^-) from sorbitol to cofactor NAD^+ that is reduced to cytosolic NADH and removes second hydrogen atom as a proton in cell cytosol.[19] The cytosolic reductive stress generated in the cells in which glucose transport is insulin insensitive may manifest altered ratio of lactate\ pyruvate due to altered ratio of NAD^+ \ NADH.[20]

As glycolysis occurs faster in those tissues, the intermediate of glycolytic system e.g. glycerol 3-phosphate is produced excessively which ultimately form inositol triphosphate and diacylglycerol. Those molecules increase intracellular Ca^{++}which in turn activates phospholipase enzyme causing degradation of membrane phospholipids and liberation of eicosanoids leading to vasoconstriction of retinal capillaries.

SO the ischaemia of retina and increased intracellular Ca^{++} stimulate excessive release of glutamate which acts as a neurotransmitter at more than 90 % of the synapses in the retina.[21] Further metabolism of eicosanoids produce oxygen derived free radicals which cause peroxidative damage of lipid of retina which is rich in polyunsaturated fatty acid. Malondialdehyde (MDA) the byproduct of accelerated lipid peroxidation becomes accumulated excessively in retinal tissue. This lipid peroxide which is also the biochemical marker of free radical production owing to lipid peroxidation, is toxic to cell.[22]

Muller cells have high affinity L glutamate\ L aspartate transporter (GLAST) expression and plays an important role in transport of extracellular glutamate through GLAST transporter from the synaptic spaces of the retina.[23] Glutamate is the major excitatory amino acid and toxic to retinal neurons when present in high concentration.[24] Transport of glutamate from extracellular space is PH dependent and lowering of PH slows or even reverses glutamate uptake. [25]

Increased generation of lactate in anaerobic glycolysis makes the medium surrounding the muller cells and the microvasculature of the retina acidic and therefore may inhibit glutamate uptake. Beside the effect of lactic acidosis of severely damaging the viability of central nervous system cells, lactate influences increased expression of vascular endothelial growth factor from retinal neurons in a concentration-dependent way.[26, 27]

In the present study 32 patients who developed microangiopathy, also demonstrated higher lactate production.

It has been documented that increased activation of NMDA receptor causes an increase in neuronal and endothelial nitric oxide synthase activity, which further promote increased formation of nitric oxide and peroxy nitrite (NO \ $ONOO^-$).[28] Peroxy nitrite is a potent cytotoxin that attacks vascular endothelium.[29]

So the increased generation of molecules like lactate and consequently increased intracellular Ca^{++}, extracellular glutamate, MDA, NO and peroxy nitrite 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.

Acidic medium and increased intracellular Ca^{++} favor the proteolysis of ECM and BM of the capillary bed of retina.

Elevated serum levels of MMP2 and MMP9 at the time of onset of DR indicate their pathogenic potentiality for the initiation of this complication.

Our previous study demonstrated that increased blood lactate level which is the end product of anaerobic glycolysis, is associated with decreased visual function in some long

standing type-2 diabetic patients even without retinopathy.[30] So this anaerobic glycolysis related cascade may also be related to retinal dysfunction before manifestation of diabetic retinopathy.

We have been stressing on the role of increased anaerobic glycolysis as an important risk factor related to visual dysfunction and development of diabetic retinopathy since 2004.[31] Now different studies demonstrated that NAD^+ \ NADH ratio mediated redox potential in vascular smooth muscle cells is predominantly determined by the cytosolic concentration of major end products of glycolysis e.g lactate and pyruvate which are oxidant and reductant metabolites respectively.[32,33]

Our recent study also demonstrated that lactate dehydrogenase in presence of excessive cytosolic NADH causes reduction of pyruvate to lactate which diffuses out of retinal microvascular cells resulting impairment of extra cellular glutamate uptake.[11]

In our present longitudinal study we have observed that some diabetic patients who developed microangiopathy, showed increased production of lactate, MDA and NO and consequently increased secretion of VEGF and activation of VEGFR2, MMP2 and MMP9 compared to other patients of same glycemic status who did not develop any sign of diabetic retinopathy.

Drawbacks of this study are that we had no scope to measure other markers of anaerobic glycolysis like LDH 5, PDK 1 or GLUT 1.

Despite the drawbacks this study probably in the first time, draws our attention towards the role of increased anaerobic glycolysis on differential expression of VEGF and VEGFR2 occurring in same type of diabetic patients. The initiating pathomechanism of diabetic retinopathy is associated with the anomaly of anaerobic glycolysis and assessment of end product of this metabolic pathway may provide information regarding prediction of onset of retinal microangiopathy in type-2 diabetic subjects.

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Table 1. Demographic characteristics of study groups.

Sample Group	Age (years) Mean ± SD	Female n (%)	Male n (%)	Total
Diabetic without DR	46 ± 12.0	66 (47. 14%)	74 (52.85%)	140
P Value	P> 0.05	P> 0.05	P> 0.05	

Table 2: Nutrition and glycaemic status of the study groups.

Parameters	Female n (%)	Male n (%)	P value
	47. 14%	52.85%	
Serum Total Protein Protein	7.1 ± 0.88	7.1 ± 1.44	(P>0.05)
Duration of Diabetes	8 ± 3 (years)	8 ± 3 (years)	
B.P	132 ± 6 mmHg 82 ± 5 mmHg	129 ± 7 mmHg 83 ± 3 mmHg	(P>0.05)

Table 3: Level of different biochemical parameters in serum and plasma of study groups.

Parameters	DC (n = 108)	NPDR (n = 32)	P value
Results obtained at the time of ascertainment of study			
Serum VEGF level (pg/ml)	98.36 ± 34.5	109.78 ± 36.7	0.1073
Serum KDR level (ng/ml)	52.6 ± 21.34	55.7 ± 22.43	0.4768
Serum MDA level (nmol/ml)	2.78 ± 1.05	3.06 ± 1.34	0.217
Plasma Lactate Level (nmol/L)	1.72 ± 0.49	1.88 ± 0.6	0.1262
Serum NO Level (umol/L)	22.37 ± 8.93	20.67 ± 7.63	0.3308
Results obtained after one year follow-up			
Serum VEGF level (pg/ml)	106.72 ± 33.02	130.69 ±41.25	0.0009
Serum KDR level (ng/ml)	50.6 ± 18.93	55.23 ±20.41	0.2347
Serum MDA Level (nmol/ml)	2.63±1.14	3.16±1.38	0.0296
Plasma Lactated Level (mmol/L)	1.89±1.2	2.08±1.1	0.4244
Serum NO Level (µmol/L)	19.63±7.12	23.61±8.83	0.0098
Results obtained after two years follow up			
Serum VEGF Level (pg/ml)	102.76±31.3	169.93±39.44	0.0001
Serum KDR Level (ng/ml)	53.73±18.62	59.37±20.25	0.1425
Serum MDA Level (nmol/ml)	2.48±1.07	3.26±1.47	0.0012
Plasma Lactate Level (mmol/L)	1.98±0.92	2.7±1.08	0.0003
Serum NO Level (µmol/L)	23.7±9.32	32.24±10.42	0.0001
Results obtained after three years follow up			
Serum VEGF Level (pg/ml)	96.66±37.35	182.61±49.36	0.0001
Serum KDR Level (ng/ml)	51.37±21.63	64.78±22.72	0.002
Serum MDA Level (nmol/ml)	2.61±1.14	3.95±1.4	0.0001
Plasma Lactate Level (mmol/L)	1.9±0.76	3.14±1.14	0.0001
Serum NO Level (µmol/L)	20.65±8.76	39.42±11.39	0.0001