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## Chapter

# Role of Lipid, Protein-Derived Toxic Molecules, and Deficiency of Antioxidants behind the Pathogenesis of Diabetic Retinopathy (DR) in Type 2 Diabetes Mellitus

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## Abstract

To determine the role of NADPH-oxidase mediated formation of different lipid, protein-derived molecules, and depletion of vitamin-C level in vitreous behind the endothelial dysfunction-induced vascular endothelial growth factor secretion and pathogenesis of diabetic retinopathy (DR) in type 2 diabetes mellitus (T2DM). Fourteen T2DM patients with mild non-proliferative diabetic retinopathy (MNPDR), 11 patients without diabetic retinopathy (DNR), 17 T2 DM subjects with high-risk proliferative diabetic retinopathy (HRPDR), and 5 healthy individuals without DM underwent vitreous analysis for estimation NADPH oxidase, lipid peroxide like malondialdehyde (MDA), 4-Hydroxy-noneal (HNE) and advanced lipoxidation end product (ALE) like Hexanoyl-lysine (HLY), protein carbonyl compound (PCC), Vitamin-C and concentration of vascular endothelial growth factor (VEGF) secretion following standard spectrophotometric methods and enzyme-linked immunosorbent assay (ELISA). Vitreous concentration of NADPH-oxidase, different protein and lipid-derived molecule, and VEGF were found to be significantly elevated among DNR and of DR subjects with different grades compared to HC subjects whereasthe vitamin-C level was found to be decreased among different DR subjects and DNR subjects in comparison to healthy individuals. Oxidative stress-mediated lipid and protein-derived biomolecules not only add important mediators in the pathogenesis of DR, but also accelerate the progression and severity of microangiopathy.

**Keywords:** Diabetic retinopathy, NADPH-oxidase, Lipid peroxide, advanced lipoxidation end product, protein carbonyl compound

## 1. Introduction

Despite remarkable advances in diagnosis and treatment, diabetic retinopathy (DR), the most frequently occurring complication causing vision loss in working population, is becoming the burning social problem. Two landmark studies have established that hyperglycemia is the principal contributing factor to the development of the disease, though a reasonable portion of diabetic subjects develops this complication in spite of good control of blood sugar [1–3]. In a large densely populated country like India, where strict control of hyperglycemia is far from reality due to lack of clinic adherence, bad economy and illiteracy. Here, principal diet is carbohydrate since childhood. Enormous intracellular glucose in tissues including retina where glucose transport is insulin independent, overwhelms the glycolysis and citric acid cycle owing to gradual deficiency of oxidized cofactors i.e. nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD), thiamine pyrophosphate (TPP), Coenzyme A and lipoate. The essential vitamins, the precursors of these factors could not be provided with only carbohydrates [4]. Unutilized glucose is diverted to anomalous biochemical pathways like sorbitol pathway, and advanced glycation end products formation [5]. Hexose monophosphate shunt (HMP-shunt) or pentose phosphate pathway (PPP) take-up glucose-6-phosphate for its catabolism and produces specialized products like pentose ribose 5-phosphate to make RNA, DNA, and NADPH for reductive synthesis e.g. reduced glutathione peroxidase and fatty acids, the building blocks of lipid structure.

Beside reductive biosynthesis, NADPH carries life-saving roles to counter the damaging effects of oxygen radicals on erythrocytes, cells of lens, cornea and retina. Retina is a tissue where renewal of outer segment of photoreceptors is continuously going on in one side and other side shows light and oxygen induced death of cells as an inevitable phenomenon caused by oxidative stress.

Poorly controlled glycemia and lack of proper metabolism of glucose mainly result in formation and accumulation of advanced glycation end products (AGEs) and reactive oxygen species (ROS) which in turn cause microvascular endothelial cell dysfunction by oxidative modifications of membrane proteins and lipids [6]. Production of advanced lipoxidation end products (ALEs) during peroxidative damage of lipids may be the important source of protein modification by covalent bonding with catalytic site. Circulating AGEs and ALEs exert their detrimental effects through interactions with their cell surface receptor for AGE (RAGE) leading to post receptor activation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in endothelial cells, mesangial cells and macrophages [7]. Activation and subsequent uncoupling of NADPH oxidase is coupled with increased formation of intracellular reactive oxygen species. Activation of this system also follows the other pathways which suggest increased production of intermediate of faster glycolysis e.g. glycerol 3-phosphate derived inositol triphosphate and diacylglycerol. Inositol triphosphate mediates a transient increase in the level of cytosolic  $\text{Ca}^{++}$  which is essential for activation of NADPH oxidase and induction respiratory burst e.g. generation of large amounts of superoxide anion, hydroxyl anion, and hydrogen peroxide [8].

Diacylglycerol (DAG) induces the translocation of protein kinase C (PKC) into plasma membrane from cytosol, where it catalyzes the phosphorylation of various proteins including the components of NADPH oxidase, and thus activates this system [9].

Beside structural modifications of retinal membrane, AGEs and ALEs invite up regulation of inflammatory mediators and adhesion molecules in capillary beds which cause apoptosis of endothelial cell and breakdown of tight junctions

of blood-retinal barrier [10]. Active vitamin C is ascorbic acid and acts as a donor of reducing equivalents which is capable of reducing compounds as molecular oxygen, nitrate and cytochromes a and c. This ascorbic acid acts as a water soluble antioxidant reduce oxidized tocopherol in lipid membranes [11]. Such vitamin is also required for hydroxylation of amino acids, proline and lysine in the synthesis of collagen [12]. So, deficiency of vitamin C leading to defective collagen synthesis and accumulation of reactive carbonyl compounds due to oxidative stress results in fragility of retinal capillary membranes and enhancement of break-down of inner blood-retinal barrier. In this study we attempted to determine the accompanying role of different lipid derived toxic molecules and deficiency of antioxidant in the pathogenesis of an inflammatory disease, DR.

## **2. Methodology**

A number of 17 subjects with high-risk proliferative diabetic retinopathy (HRPDR), 14 subjects with mild non-proliferative DR (MNPDR), 11 age and gender-matched diabetic subjects without clinically evident retinopathy (DNR), and 5 healthy controls (HCs), whose clinical condition independently indicated for vitrectomy were enrolled in the present cross-sectional study. Subjects with hypertension (systolic BP > 140 mm Hg and or diastolic BP > 90 mm Hg), cardiovascular diseases, neuropathy (assessed by Michigan Neuropathy Screening Instrument), nephropathy (serum creatinine level > 1.5 mg/dl and or urinary albumin creatinine ratio 300 µg/mg), deficiency of B vitamins, any other ocular diseases (glaucoma, optic neuropathy, cataract, branch retinal vein occlusion, and Eales disease) were excluded from the study. The subjects were chosen consecutively from the 'Outdoor Patient Department' of 'Regional Institute of Ophthalmology, Calcutta Medical College, West Bengal, and Kolkata, India. The institutional ethical committee was approved the study and informed consent was collected from all the study subjects according to the Helsinki guideline.

Subjects with type 2 DM were diagnosed according to the guideline of the American Diabetes Association (2010). The fasting plasma glucose (FPG), postprandial plasma glucose (PPG), and glycated hemoglobin (HbA1c %) levels were used for the assessment of the glycaemic status of each subject. None of the study subjects were taking insulin or lipid-lowering drugs during the study period.

### **2.1 Comprehensive ophthalmological examinations**

The subjects enrolled in the study had undergone different ophthalmological examinations which included slit-lamp biomicroscopy (by  $\pm 90$  diopters and Goldman 3 mirror lens), seven fields of digital fundus photography with fluorescein angiography, and spectral-domain optical coherence tomography (SD-OCT). Visual functions were evaluated by measuring VA. The subjects with different grades of DR were diagnosed according to the modified guideline of 'Early Treatment of Diabetic Retinopathy Study' [13].

### **2.2 Collection of vitreous sample**

Vitreous samples from study subjects were drawn by 3-port pars plana vitrectomy during surgery of vitreous hemorrhage, of idiopathic macular hole or removal of a dropped nucleus which occurred accidentally after blunt trauma. Vitreous was also collected during management of preoperative complication of



phacoemulsification. Undiluted vitreous gel (500  $\mu$ L) was excised from midvitreous by vitreous cutter and carefully aspirated into the hand-held sterile syringe attached to the suction port of the vitrectomy probe. Immediately after collection, the vitreous samples were taken in micro centrifuged tube and centrifuged at 3000 rpm for 5 minutes. The clear solution without any precipitate was then collected in another tube and preserved in - 80° C for farther use.

### **2.3 Measurement of NADPH oxidase activity**

NADPH oxidase activity was measured in vitreous using Nitrobluetetrazolium (NBT) as the substrate. Briefly, 100  $\mu$ l plasma/ vitreous was mixed with NBT (4 mg/ml in water) and incubated for 20 minutes at 37°C. 1 M HCl was used to terminate the reaction. Then the samples were centrifuged at 3500 rpm for 5 minutes. 400  $\mu$ l Dimethylsulfoxide (DMSO) was added to form a stable triphenyl-methyl ester whose absorbance was measured at 550 nm using a microplate reader (MerilyzerEiaquant, Meril Diagnostics Pvt. Ltd., Vapi, Gujarat). OD 550 is directly proportional to NADPH oxidase activity [14].

### **2.4 Measurement of vitamin C level**

Vitamin C level was measured by the protocol of Kyaw et al. [15]. Briefly, the colored reagent was prepared using Sodium Tungstate, DisodiumHydrogen Phosphate, H<sub>2</sub>SO<sub>4</sub> and distilled water. Plasma/vitreous sample (1 ml) was thoroughly mixed with 2 ml colored reagent. After 30 minutes incubation at room temperature (RT) the tubes were centrifuged at 3000 pm for 10 minutes. The absorbance was measured at 700 nm from the supernatant, without disturbing the precipitate. Standard curve was prepared using oxalic acid and distilled water is used as substrate blank in the experiment. The vitamin C concentration in samples was expressed in mg/dl.

### **2.5 Measurement of protein carbonylation (PCC)**

Protein carbonylation was measured from vitreous by spectrophotometric method by protein derivatization with 2, 4-dinitrophenyl-hydrazine (DNPH). Briefly, protein lysates from vitreous (50  $\mu$ l) were incubated in dark for 30 minutes with DNPH (10 mM in 2 N HCl, 100  $\mu$ l). After that TCA (20%, 100  $\mu$ l) was used to precipitate proteins and free DNPH was removed by washing with ethanol-ethyl acetate (1:1). The resultant pellet was dissolved in 350  $\mu$ l of sodium dodecyl sulfate (2% SDS) and protein-hydrazone complex's absorbance was measured at 370 nm using spectrophotometer. The carbonyl concentration was calculated using the extinction coefficient of the protein-hydrazone complex (22,000 M<sup>-1</sup> cm<sup>-1</sup>) from the specific absorption (relative to the reagent blank). The final concentration was expressed as nanomoles of carbonyl groups per milligram protein [16–18].

### **2.6 Estimation of malondialdehyde (MDA)**

The MDA level in vitreous was measured by thiobarbituric acid (TBA) assay method. In the assay procedure, the plasma samples were first reacted with trichloroacetic acid (TCA) to remove proteins. Then chromogenic adducts of MDA was precipitated using TBA. Finally the precipitated MDA was extracted using n-butyl alcohol, by vigorous shaking. Then the chromogenic adduct was measured spectrophotometrically at 532 nm; the results were expressed as mM/L [19].

## 2.7 Measurement of HNE

Human vitreous HNE was estimated by competitive inhibition enzyme immunoassay technique (ELISA) using research kit from CUSABIO (cat no: CSB-E16214h). In the assay, an antibody specific for human HNE was coated on the well plate. A series of standards ranging from 39 pg/ml to 2500 pg/ml and samples (vitreous samples were run in 5 fold diluted form respectively) were added into the wells with Horseradish Peroxide (HRP) conjugated HNE. The competitive inhibition reaction was launched between HRP conjugated HNE and HNE in the samples. Then a substrate solution was added to the wells and the color developed is inversely proportional to the amount of HNE in the sample. The color development was stopped using stop solution and the intensity of the color was measured colorimetrically by using 450 nm filter in an ELISA plate reader MerilyzerEiaquant (Meril Diagnostics Pvt. Ltd., Vapi, Gujarat).

## 2.8 Measurement of HLY

Human vitreous HLY was estimated also by competitive enzyme immunoassay technique using commercial kits (MyBiosource, Catalog no: MBS753480) and utilizing a polyclonal anti-HLY antibody and an HLY-HRP conjugate. At first the assay sample and buffer were incubated together with HLY-HRP conjugate in pre-coated plate for one hour. After the incubation period, the wells were decanted and washed five times. The wells were then incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction forms a blue colored complex. Then, a stop solution was added to stop the reaction, which will then turn the solution yellow. The intensity of color was measured spectrophotometrically at 450 nm in a microplate reader. The intensity of the color was inversely proportional to the HLY concentration present in the sample. A standard curve was also plotted relating the intensity of the color (or O.D.) to the concentration of standards. The HLY concentration in each sample was interpolated from this standard curve.

## 2.9 Measurement of VEGF

Human vitreous VEGF was estimated by sandwich enzyme-linked immune sorbent assay (ELISA) method using Ray Biotech kit (cat no: ELH-VEGF-001, Norcross USA). In the assay, an antibody specific for human VEGF was coated on the well plate. A series of standards ranging from 8.23 pg/ml to 6000 pg/ml and samples (vitreous samples were run in a half diluted form respectively) were added into the wells. VEGF protein present in the sample was bound to the wells by the immobilized antibody. The wells were washed and a biotinylated anti-human VEGF antibody was added. After buffer washing, HRP-conjugated streptavidin was pipette to wash and TMB substrate solution was added into the wells and was placed in incubation at room temperature for 30 minutes. The intensity of the final color product was proportional to the concentration of VEGF protein present in the samples and absorbance of the color product was measured colorimetrically by using 450 nm filter in an ELISA plate reader MerilyzerEiaquant (Meril Diagnostics Pvt. Ltd., Vapi, Gujarat). The concentration of VEGF was determined by a standard curve and the assay detects less than 10 pg/ml of VEGF from the sample.

## 3. Results

As shown in the **Table 1**, different study groups enrolled in the present study showed no statistically significant differences for age, gender distributions, body

Parameters		HC (N = 5)	DNR (N = 11)	MNPDR (N=14)	HRPDR (N = 17)	p value
Age (years)		52.00 ± 7.48	51.29 ± 5.46	52.64 ± 2.853	50.94 ± 7.45	0.905
Gender	M	3(60%)	6(54.54%)	7(50%)	8(47.05%)	0.954
	F	2(40%)	5(45.45%)	7(50%)	9(52.94%)	
BMI (kg/m <sup>2</sup> )		23.30 ± 3.54	25.15 ± 2.183	26.61 ± 4.349	23.15 ± 6.54	0.439
Duration of DM (years)		-----	10.14 ± 5.16	11.19 ± 4.35	11.27 ± 3.80	0.484
Glycaemic Status	FPG (mg/dl)	80.46 ± 8.48	153.5 ± 9.55 <sup>*</sup>	159.3 ± 28.50 <sup>††</sup>	154.9 ± 16.11 <sup>!!!!</sup>	0.0001
	PPG (mg/dl)	118.2 ± 11.19	182.0 ± 33.00 <sup>*</sup>	193.5 ± 62.14 <sup>††</sup>	218.4 ± 32.16 <sup>!!</sup>	0.005
	HbA1C (%)	4.81 ± 0.290	7.61 ± 0.476 <sup>*</sup>	8.43 ± 1.117 <sup>†††</sup>	7.78 ± 1.102 <sup>!</sup>	0.002

HC, healthy control; DNR, diabetic subjects without clinically evident retinopathy, MNPDR, early non-proliferative diabetic retinopathy; HRPDR, high-risk proliferative diabetic retinopathy; BMI, body mass index; FPG, fasting plasma glucose, PPG, postprandial plasma glucose; HbA1C, glycated haemoglobin. The Kruskal Wallis nonparametric ANOVA followed by Dunn's multiple comparisons test was administrated to find out significant differences between the groups. A value of  $p < 0.05$  was considered as statistically significant.

<sup>\*</sup>HC vs DNR,  $p < 0.05$ .  
<sup>†††</sup>HC vs MNPDR,  $p < 0.001$ .  
<sup>††</sup>HC vs MNPDR,  $p < 0.01$ .  
<sup>!!!!</sup>HC vs HRPDR,  $p < 0.0001$ .  
<sup>!!</sup>HC vs HRPDR,  $p < 0.01$ .  
<sup>!</sup>, HC vs HRPDR,  $p < 0.05$ .

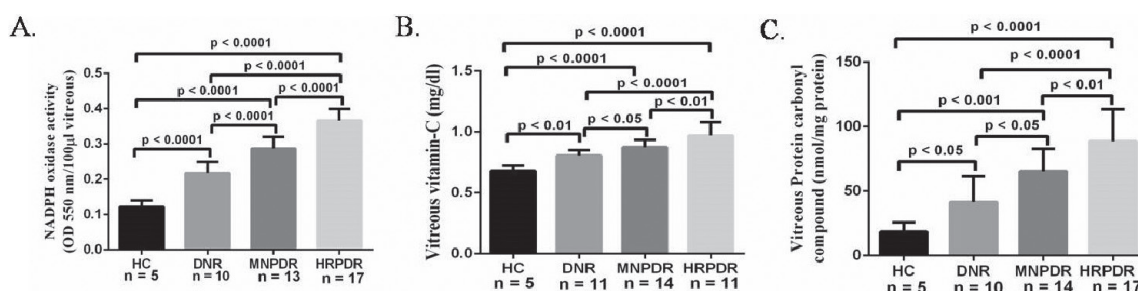
**Table 1.**  
Demographic and clinical characteristics of study subjects.

mass index (BMI), and duration of diabetes, systolic and diastolic blood pressure. Glycaemic parameters like FPG level were found to be increased significantly in the DNR ( $p < 0.05$ ), MNPDR ( $p < 0.01$ ), and HRPDR ( $p < 0.01$ ) subjects compared to the HCs. Similarly, the PPG level were found to be increased significantly in the DNR ( $p < 0.05$ ), MNPDR ( $p < 0.01$ ), and HRPDR ( $p < 0.0001$ ) subjects compared to the HCs. Regarding, HbA1C DNR ( $p < 0.05$ ), MNPDR ( $p < 0.001$ ), and HRPDR ( $p < 0.05$ ) subjects showed significantly higher values of HbA1c compared to HCs. However, statistical analysis showed no significant differences in FPG, PPG, and HbA1C levels between DNR, MNPDR, and HRPDR subjects.

Vitreous NADPH oxidase activity was found to be increased significantly among DNR ( $0.217 \pm 0.031$  OD<sub>550</sub>/100  $\mu$ L, vitreous,  $p < 0.0001$ ), MNPDR ( $0.286 \pm 0.033$  OD<sub>550</sub>/100  $\mu$ L, vitreous,  $p < 0.0001$ ), and HRPDR ( $0.365 \pm 0.032$  OD<sub>550</sub>/100  $\mu$ L, vitreous,  $p < 0.0001$ ) subjects compared to HC ( $0.121 \pm 0.018$  OD<sub>550</sub>/100  $\mu$ L, vitreous) subjects. Again, both the MNPDR ( $p < 0.01$ ) and HRPDR subjects ( $p < 0.0001$ ) showed significantly higher level of NADPH oxidase than DNRs. Further, the HRPDR subjects showed a higher NADPH oxidase level ( $p < 0.0001$ ) than the former (**Figure 1A**).

Regarding vitamin-C concentration in vitreous, the DNR ( $0.807 \pm 0.043$  mg/dl,  $p < 0.01$ ), MNPDR ( $0.874 \pm 0.061$  mg/dl,  $p < 0.0001$ ), and HRPDR ( $0.970 \pm 0.110$  mg/dl,  $p < 0.0001$ ) subjects showed lower vitamin-C level compared to HC ( $0.682 \pm 0.038$  mg/dl) subjects. Again, both the MNPDR ( $p < 0.05$ ) and HRPDR subjects ( $p < 0.0001$ ) showed significantly lower level of vitamin-C than DNRs. HRPDR subjects showed a lower vitamin-C level ( $p < 0.01$ ) than the MNPDR ones (**Figure 1B**).





**Figure 1.** Comparison of vitreous level NADPH oxidase, vitamin-C and protein carbonyl compound among study groups. [A] Comparison of vitreous level NADPH oxidase, [B] Comparison of vitreous level vitamin-C, [C] Comparison of vitreous level protein carbonyl compound. The one way ANOVA followed by Tukey's comparisons test was administrated to find out significant differences between the groups. A value of  $p < 0.05$  was considered as statistically significant.

Vitreous PCC concentration was found to be increased significantly among DNR ( $41.57 \pm 19.96$  nmol/mg protein,  $p < 0.05$ ), MNPDR ( $65.43 \pm 17.31$  nmol/mg protein,  $p < 0.001$ ), and HRPDR ( $88.65 \pm 24.93$  nmol/mg protein,  $p < 0.0001$ ) subjects compared to HC ( $18.46 \pm 7.18$  nmol/mg protein) subjects. Again, both the MNPDR ( $p < 0.05$ ) and HRPDR subjects ( $p < 0.0001$ ) showed significantly higher level of PCC than DNRs. Further, the HRPDR subjects showed a higher PCC level ( $p < 0.01$ ) than the former (**Figure 1C**).

Vitreous MDA level was found to be increased significantly among DNR ( $2.814 \pm 0.482$   $\mu$ M/L,  $p < 0.01$ ), MNPDR ( $4.58 \pm 0.655$   $\mu$ M/L,  $p < 0.0001$ ), and HRPDR ( $8.51 \pm 1.23$   $\mu$ M/L,  $p < 0.0001$ ) subjects compared to HC ( $1.129 \pm 0.579$   $\mu$ M/L) subjects. Again, both the MNPDR ( $p < 0.0001$ ) and HRPDR subjects ( $p < 0.0001$ ) showed significantly higher level of MDA than DNRs. Further, the HRPDR subjects showed a higher MDA level ( $p < 0.0001$ ) than MNPDR subjects (**Figure 2A**).

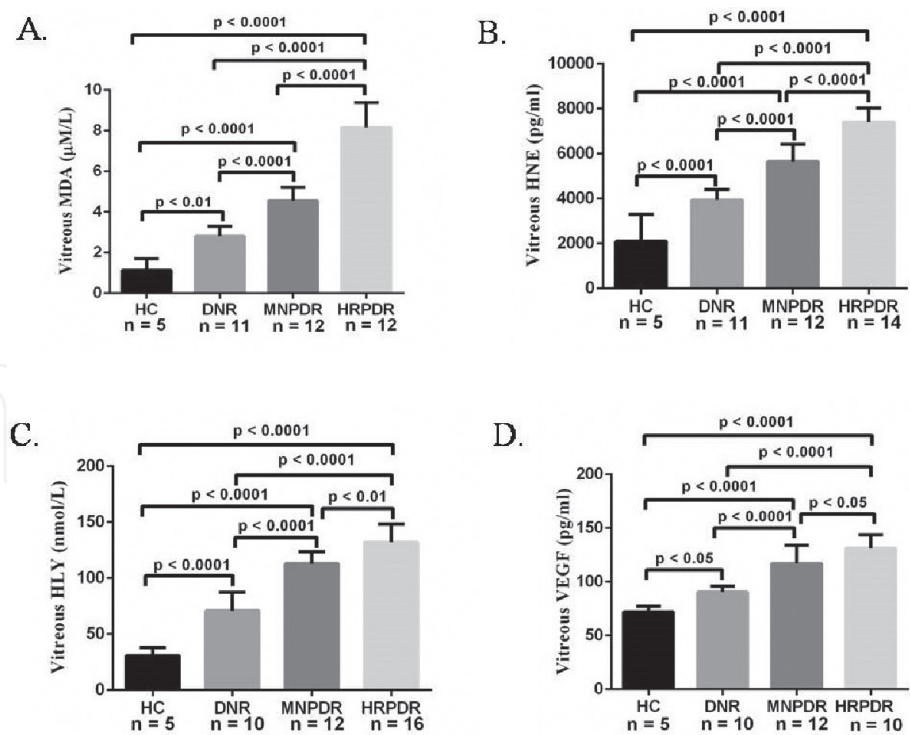
Regarding HNE concentration in vitreous, the DNR ( $3936 \pm 457.2$  pg/ml,  $p < 0.0001$ ), MNPDR ( $8643 \pm 771.8$  pg/ml,  $p < 0.0001$ ), and HRPDR ( $7407 \pm 622.3$  pg/ml,  $p < 0.0001$ ) subjects showed higher HNE level compared to HC ( $2092 \pm 1201$  pg/ml) subjects. Again, both the MNPDR ( $p < 0.0001$ ) and HRPDR subjects ( $p < 0.0001$ ) showed significantly higher level of HNE than DNRs. HRPDR subjects showed a higher HNE level ( $p < 0.0001$ ) than the MNPDR ones (**Figure 2B**).

Vitreous HLY level was found to be increased significantly among DNR ( $70.93 \pm 16.29$  nmol/L,  $p < 0.0001$ ), MNPDR ( $113.0 \pm 10.56$  nmol/L,  $p < 0.0001$ ), and HRPDR ( $132.1 \pm 16.22$  nmol/L,  $p < 0.0001$ ) subjects compared to HC ( $30.68 \pm 7.29$  nmol/L) subjects. Again, both the MNPDR ( $p < 0.01$ ) and HRPDR subjects ( $p < 0.0001$ ) showed significantly higher level of HLY than DNRs. Further, the HRPDR subjects showed a higher HLY level ( $p < 0.01$ ) than MNPDR subjects (**Figure 2C**).

Vitreous VEGF level was found to be increased significantly among DNR ( $90.53 \pm 5.611$  pg/ml,  $p < 0.05$ ), MNPDR ( $117.0 \pm 17.09$  pg/ml,  $p < 0.0001$ ), and HRPDR ( $131.3 \pm 12.21$  pg/ml,  $p < 0.0001$ ) subjects compared to HC ( $72.06 \pm 5.109$  pg/ml) subjects. Again, both the MNPDR ( $p < 0.0001$ ) and HRPDR subjects ( $p < 0.0001$ ) showed significantly higher level of VEGF than DNRs. Further, the HRPDR subjects showed a higher VEGF level ( $p < 0.05$ ) than MNPDR subjects (**Figure 2D**).

In the DNR and DR group, vitreous NADPH activity showed significant positive correlations with PCC, MDA, HNE, and HLY respectively. However, the study showed no significant correlation with the same in the HC group (**Table 2**).





**Figure 2.** Comparison of vitreous level MDA, HNE, HLY and VEGF among study groups. [A] Comparison of vitreous level MDA, [B] Comparison of vitreous level HNE, [C] Comparison of vitreous level HLY, [D] Comparison of vitreous level VEGF. The one way ANOVA followed by Tukey's comparisons test was administrated to find out significant differences between the groups. A value of  $p < 0.05$  was considered as statistically significant.

Study groups	PCC	MDA	HNE	HLY
HC	$r = 0.300, p = 0.491$	$r = 0.205, p = 0.741$	$r = 0.200, p = 0.825$	$r = 0.100, p = 0.100$
DNR	$r = 0.833, p = 0.005$	$r = 0.673, p = 0.033$	$r = 0.632, p = 0.040$	$r = 0.733, p = 0.040$
DR	$r = 0.667, p < 0.0001$	$r = 0.768, p < 0.0001$	$r = 0.891, p < 0.0001$	$r = 0.726, p < 0.0001$

A Pearson or spearman correlation coefficient ( $r$ ) was used and  $p < 0.05$  was considered statistically significant.

**Table 2.** Correlation of vitreous NADPH oxidase activity with PCC, MDA, HNE and HLY in HC, DNR and DR (MNPDR+HRPDR) group.

The VEGF level of vitreous showed a significant negative correlation with vitamin-C and positive correlations with PCC, MDA, HNE, and HLY levels respectively in both DNR and DR groups. However, the study showed no significant correlation with the same in the HC group (Table 3).

Parameters	HC group	DNR group	DR group
Vitamin-C	$r = 0.16, p = 0.722$	$r = -0.755, p = .007$	$r = -.451, p = 0.035$
PCC	$r = 0.235, p = 0.684$	$r = 0.774, p = 0.009$	$r = .748, p < 0.0001$
MDA	$r = 0.285, p = 0.691$	$r = 0.810, p = 0.003$	$r = .660, p = 0.003$
HNE	$r = 0.085, p = 0.875$	$r = 0.871, p < 0.0001$	$r = .807, p < 0.0001$
HLY	$r = 0.145, p = 0.802$	$r = 0.783, p = 0.007$	$r = .655, p = 0.002$

A Pearson or Spearman correlation coefficient ( $r$ ) was used and  $p < 0.05$  was considered statistically significant.

**Table 3.** Correlation of vitamin-C, PCC, MDA, HNE and ALE with VEGF in different study groups.

## 4. Discussion

Our study showed that a gradual increment of NADPH Oxidase activity with the pathogenesis and severity of DR (HC < DNR < MNPDR < HRPDR). However there is no direct evidence regarding gradual increment and association of NADPH Oxidase activity with different graded of DR. An in-Vitro study, Meng et al. [20] demonstrated that NADPH Oxidase augment insulin-induced VEGF expression and angiogenesis. In another study by Ushio-Fukai, [21] also showed that VEGF expression is augmented through ROS, produced by NADPH Oxidase.

Vitreous level of vitamin C was also found to be declined with pathogenesis and severity of DR (HC > DNR > MNPDR > HRPDR). However there is lack of evidence between vitreous level of Vitamin C and different stages of DR. Vitamin C suppress the VEGF gene expression through HIF-1 $\alpha$  pathway [22].

Vitreous PCC level was found to be increase gradually with progression of DR (HC < DNR < MNPDR < HRPDR). Nevertheless there is no such evidence of vitreous level of PCC and different stages of DR. Loukovaara et al. [23] shown that amount of protein carbonylation and HIF-1 $\alpha$  elevated in vitreous of PDR subjects.

The present study showed a gradual increment of LPO products like MDA and HNE and ALE like HLY both in plasma and vitreous sample towards the development and progression of DR. The studies by Chatziralli et al. [24], Mondal et al., [4] also showed plasma MDA level increases towards the DR pathogenesis and progression. Another study by Mancino et al. [25] showed that vitreous MDA level increases among NPDR and PDR subjects compared to nondiabetic HC subjects. Researchers have demonstrated that the MDA compound is associated with protein modification in a pH-dependent fashion. At the physiological pH, it rapidly forms enolates, which are of lower reactivity and do not react as avidly with nucleophilic species as other aldehydes [26]. However, at a lower pH, MDA exists as  $\beta$ -hydroxyacrolein form, exhibiting a higher reactivity, readily reacting with Lys residues of proteins to form the enamine type MDA adduct, N $\epsilon$ -(2-propenal) lysine, and the fluorescent product, dihydropyridine (DHP) lysine and thereby alters proteins structure and functions [27]. On the other hand, the role of HNE in diabetes and its complications is not well understood [28]. Clinical studies have reported elevated levels of HNE in the blood of diabetic patients with retinopathy compared to those without retinopathy and healthy controls [29]. There is also evidence showing that HNE and HNE-derived ALEs increase in the retinas of rats rendered diabetic for 4-6 weeks [30]. Another study confirmed these findings and showed that HNE may contribute to the pathogenesis of DR by activating the WNT signaling pathway through stabilization of the WNT co-receptor LRP6 [31]. Other animal studies have linked HNE to retinal hemodynamics changes during DR. Retinal perfusion deficits during early diabetes are thought to be mediated, at least in part, through the reduced activity of large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> (BK) channels on the retinal vascular smooth muscle cells, causing vasoconstriction [32, 33]. HNE impairs BK channel function in rat retinal arterioles, as demonstrated by reduced vasodilatory responses to the BK channel opener, BMS-191011 [34]. HNE exposure is reported to result in endoplasmic reticulum stress, mitochondrial dysfunction, and apoptosis in cultures of human retinal capillary pericytes and Müller glia [35].

The ALE component like HLY also found to be increased with the pathogenesis and severity of DR in the present study. A previous study by [36] reported a significant elevation of ALE levels among DNR and MNPDR subjects compared to HCs. Moreover, significant elevation of HLY in the vitreous and serum of patients with PDR was also observed by Izuta et al. [37], which is following our findings.

The study showed a significant negative correlation of VEGF with Vitamin-C level and positive correlations with PCC, MDA, HNE, and HLY. Decrease vitamin C level with increased NADPH oxidase activity may turn oxidative stress, which further damages protein and lipids subsequently causes endothelial dysfunction induced VEGF secretion.

## 5. Conclusion

Oxidative stress-mediated lipid and protein-derived biomolecules not only add important mediators in the pathogenesis of DR, but also accelerate the progression and severity of microangiopathy.

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