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ROLE OF OXIDATIVE STRESS, INFLAMMATION AND ENDOTHELIAL DYSFUNCTION IN THE PATHOGENESIS OF DIABETIC RETINOPATHY

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ABSTRACT

BACKGROUND: Oxidative stress, inflammation and endothelial dysfunction are commonly found in persons with type II diabetes mellitus (DM), but their role in the pathogenesis of diabetic retinopathy (DR) is not fully elucidated. Therefore, the present study investigates the relationship and the role of these factors in the incidence and progression of different stages of DR. METHODS: This study included 85 subjects divided into four groups. First group consisted of 20 healthy subjects who served as controls. The second group consisted of 23 patients with type II DM without retinopathy, while the third group consisted of 20 patients having non-proliferative diabetic retinopathy (NPDR), and finally the last group consisted of 22 patients having severe proliferative diabetic retinopathy (SPDR). For all subjects in all groups, the levels of glycated hemoglobin (HbA1c %), lipid profiles, malondialdehyde (MDA) and nitric oxide (NO) were measured spectrophotometrically, while tumor necrosis factor-alpha (TNF-α) and soluble Eselectin (sE-selectin) were measured using ELISA technique. RESULTS: All the above measured parameters were significantly elevated in all diabetic patients with or without retinopathy when compared to control subjects, with the most significant increase in case of the SPDR group. There was a significant positive correlation between plasma MDA with both TG & HbA1c%, NO & TNF-α and finally s-Eselectin & HbA1c%. CONCLUSION: Oxidative stress, inflammation and endothelial dysfunction have a fundamental role in the pathogenesis of DR.

Key words: *Endothelial dysfunction; reactive oxygen species; oxidative stress; anti oxidants; nitric oxide; drug toxicity*

[I] NTRODUCTION

Diabetic retinopathy (DR) is among the most common microvascular complications of diabetes [1]. The prevalence of DR is about 4-28% and about 2% of diabetic population is blind as a result of DR [2], thus DR is regarded as one of the leading causes of blindness worldwide [3].

Diabetic retinopathy can be principally classified into non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). In the former type, the earliest clinical signs are microaneurysms, small outpouchings from retinal capillaries, and dot intraretinal hemorrhages [4]. As the NPDR progresses from mild, moderate to severe, patients have an increase in the number and size of intraretinal hemorrhages [5]. This increase may be accompanied by cotton wool spots; both of these signs indicate regional failure of retinal microvascular circulation, which results in ischemia [1, 6]. Proliferative diabetic retinopathy occurs when further retinal ischemia is characterized by the growth of new blood vessels on the surface of the retina or the optic disc [6]. These abnormal vessels may bleed, resulting in vitreous hemorrhage, subsequent

fibrosis, and tractional retinal detachment [1] leading to severe and often irreversible vision loss [7, 8].

The retina has high content of polyunsaturated fatty acids and has the highest oxygen uptake and glucose oxidation relative to any other tissue. This phenomenon renders retina more susceptible to oxidative stress [9] and lipid peroxidation [10]. It has been suggested that the correlation between hyperglycemia, changes in the redox homeostasis and oxidative stress are the key events in the pathogenesis of DR [1]. Oxidative stress, besides creating a vicious cycle of damage to macromolecules by amplifying the production of more reactive oxygen species (ROS), also activates other metabolic pathways that are detrimental to the development of DR [9]. These pathways are mostly dependant on excessive transport of glucose into retinal cells resulting in increased intracellular glucose levels [11]. These pathways include the polyol pathway [9], production of advanced glycation end products (AGEs) [12] and protein kinase C pathway[13].Free radicals are continuously formed in all aerobic cells, and consist of the superoxide radical, hydrogen peroxide and hydroxyl radical. These metabolites are responsible for lipid peroxidation, which is described as a conglomeration reaction of the polyunsaturated fatty acids found in the cell membrane to various products such as peroxides and hydroxy fatty acids[14]. Some lipid peroxidation

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RESEARCH

products such as MDA may bind to proteins and amplify glyco and oxidation generated lesions [15]. Several studies have been made measuring the degree of lipid peroxidation in DR using MDA. Recently, Pan et al. [16] reported a significant increase in the serum MDA levels of type II diabetics with retinopathy when compared to those diabetics without retinopathy and control subjects.

Inflammation is a prominent component of many diseases [17]. Chronic inflammation is characterized by increased vascular permeability, edema, inflammatory cell infiltration, cytokine and chemokine expression, tissue destruction, neovascularization, and attempts at repair [18], and DR exhibits most of these features such as increased blood flow and vascular permeability, tissue macular edema, macrophage infiltration [19], microglial cell activation [20], accelerated cell death [21], acute phase response protein expression [22],increased cytokine expression [23], increased leukocyte adhesion [24], neovascularization, and acute phase response protein expression [22].Several studies have been made on human subjects to postulate the role of TNF-α in the pathogenesis of DR. Doganagy et al. [25] reported that TNF-α levels in the serum of patients with PDR was significantly higher than in patients with NPDR, type II DM and controls.

Nitric Oxide is synthesized from its precursor, L-arginine by the enzyme nitric oxide synthase (NOS). There are three major isoforms of NOS: neuronal NOS(nNOS), endothelial NOS(eNOS), and inducible NOS (iNOS) [26]. The consequences of increased levels of NO in retinas from subjects with diabetes could be twofold: neurotoxicity and angiogenesis. Nitric oxide can be beneficial in its role as a vasodilator, but high concentrations of NO produced by iNOS are neurotoxic [27]. The toxicity of NO has been attributed to multiple mechanisms, including DNA damage, peroxynitrite mediated oxidative damage, and energy failure [28]. Ozden et al. [29] compared the basal serum levels of NO in patient with type II DM without retinopathy and different stages of DR with the levels in non-diabetic control subjects and the results showed a significant increase in serum NO levels in patients with different stages of DR than in type II diabetics which were both significantly higher than control subjects.

Recently, leukocyte activation and adhesion to the endothelium have been considered as a cause of capillary occlusion in DR [30]. Leukocytes of individuals with DR show decreased deformability and increased adhesion to retinal capillaries leading to leukostasis, which appears to play a role in retinopathy, in diabetic patients [31].

Leukocytes are present within microaneurisms and may play roles in the development of these abnormal vessels. E-selectin is an adhesion molecule which is selectively synthesized by activated endothelial cells [32].Increased levels of serum sE-selectin were reported to be associated with endothelial cell dysfunction in previous studies [33],however, the relationship of sE-selectin to DR is currently not fully explained. Olsen et al. [30] measured the serum concentrations of soluble E-Selectin molecules in serum of diabetic patients with different stages of DR and compared it with healthy control subjects. Results have shown a significant increase in the level of this marker being the highest in case of severe NPDR.

The aim of this study is to determine the role of these inflammatory and oxidative stress markers as well as adhesion molecules in the pathogenesis of DR.

[II] MATERIALS AND METHODS

2.1. Study Subjects

The study compromised 85 subjects divided into 46 males and 39 postmenopausal females aged 45-69 years. All of the study subjects were non-smokers. Twenty of them were healthy volunteers serving as the control group. Twenty three patients suffering from type II DM without retinopathy were recruited from the Department of Endocrinology of El Mattariah Hospital, Cairo, Egypt and these were representing the second group. Forty four patients were recruited from the Research Institute of Ophthalmology, Giza, Egypt. Those patients were divided into 20 patients suffering from NPDR representing third group and 22 patients suffering from SPDR representing the fourth group as shown in **Table–1**. All diabetic patients with or without retinopathy were under treatment of oral hypoglycemics or insulin and the level of DR was determined by fundus findings where all the diabetic patients underwent a complete ocular examination including visual field testing, slit lamp biomicroscopy and indirect opthalmoscopy. Exclusion criteria included: age over 70 years, ischemic cardiovascular disorders, hepatic disorders, history of malignancy, presence of hematological diseases and renal disorders. The study protocol was approved by the local university committee and informed consent was obtained from all subjects in accordance with the principles of the Helsinki Declaration.

2.2. Sample collection

For all subjects, blood samples (5-10 ml) were collected in the morning after overnight fasting. Samples were divided into three portions: First portion of blood was collected on vacutainer tubes containing Na_2 EDTA for assay of HbA1c. The second portion was collected on vacutainer tubes containing Na_2 EDTA for assay of MDA in plasma. Plasma samples were separated after 20 minutes by centrifugation at 2500 rpm for 15 minutes. The last portion was collected on plain vacutainer tubes for serum preparation used for the assay of the lipid profiles, NO, TNF-α and sE-selectin. Sera were separated from clotted blood after 30 minutes by centrifugation at 4000 rpm for 15 minutes. Plasma and sera samples were kept frozen in aliquots at -80 °C until assayed.

2.3. Assays

Fresh blood aliquots were used for the measurement of HbA1c, while sera aliquots were used for the measurement of total cholesterol (TC) and triglycerides (TG) by standard enzymatic techniques using commercially available kits [34, 35]. High density lipoprotein-cholesterol (HDL-C) was determined after the precipitation of apoliporotein B-containing lipoproteins [36]. Low density lipoprotein-cholesterol (LDL-C) was calculated according to Friedewald equation [37]. All spectrophotometric measurements were done by UV/Visible spectrophotometer, Jenway, model no. 6305.

2.3.1. MDA Measurement

Levels of MDA were determined as thiobarbituric acid-reactive substances (TRABS) following a protocol described previously by Uchiyama and Mihara [38].

2.3.2. NO determination

Levels of NO were determined based on the conversion of nitrate to nitrite by Vanadium (III) chloride according to the method of Cox [39]. The reaction is followed by a colorimetric detection of nitrite as an azo dye product of the Griess reaction according to the method of Griess [40].

2.3.3. TNF-a and sE-selectin determinations:

These two markers were assayed by a validated ELISA technique using commercial kits provided by R&D Systems, Inc., USA. All ELISA procedures were done by Hyprep ® automated ELISA system, USA, according to the instructions of the manufacturer.

2.3.4. Statistical Analysis

All statistical analyses were performed using Statistical Package for Social Science (SPSS) version 9 software. Data were represented as mean ± SEM. Differences between groups were compared using a one-way analysis of variance (ANOVA) followed by LSD post-hoc analysis. A P value < 0.05 was considered statistically significant. Pearson correlation coefficient was used to determine correlation between different parameters.

[III] RESULTS

With regard to the levels of HbA1c % and the lipid profiles (TC, TG, HDL-C and LDL-C), all the diabetic groups with or without retinopathy showed a significant increase when compared to the control group with the exception of HDL-C level which was significantly lower as shown in **Table 2**. The levels of MDA, NO, TNF-α and sE-selectin were significantly higher in all diabetic groups when compared with the healthy controls as shown in table 3. In case of MDA, the levels were 5.28 ± 0.34, 5.57 ± 0.43 and 6.81 ± 0.49 nmole/ml, respectively, for type II DM, NPDR and SPDR groups whereas, the normal control was 2.15 ± 0.19 nmole/ml. As for NO, the levels were 41.5 ± 2.29, 40.43 ± 3.51 and 49.76 ± 3.0 mmole/l for the former three groups, respectively while the normal control was 16.1 ± 0.68 mmole/l. The levels of TNF-α in type II DM, NPDR and SPDR groups were 21.7 ± 0.63, 22 ± 0.51 and 25.8 ± 1.23 pg/ml, respectively while the control group was 16.1 ± 0.68 pg/ml. As for the sE-selectin levels in these diabetic groups, they were 30.28 ± 1.96, 37.74 ± 2.67 and 63.7 ± 4.65 ng/ml, respectively while the control group was 10.86 ± 0.74 ng/ml.

3.1. Correlation data

Evaluation of the correlation coefficient of the biomarkers in all diabetic patients, comprising type II DM, NPDR and SPDR groups, revealed a positive and significant association of MDA with HbA1c (fig1a), MDA with TG **[Figure–1 b]**, NO with TNF-α **[Figure– 2]** and sE-selectin with HbA1c % **[Figure–3]**.

Table: 1. Clinical characterization of the study subjects. Data are expressed as mean ±SEM

Groups	Control (n=20)	Type II DM (n=23)	NPDR (n=20)	SPDR (n=22)
Sex (M / F)	12 / 8	11 / 12	9 / 11	14 / 8
Age (year)	M: 51.59 ± 0.70	M: 54.65 ± 2.24	M: 54.11 ± 2.23	M: 59.65 ± 1.22
	F: 55.63 ± 1.11	F: 57.92 ± 1.11	F: 58.82 ± 1.05	F: 59.13 ± 1.30
Duration of Diabetes(years)	—	6.87 ± 0.39	7.08 ± 0.38	8.98 ± 0.51
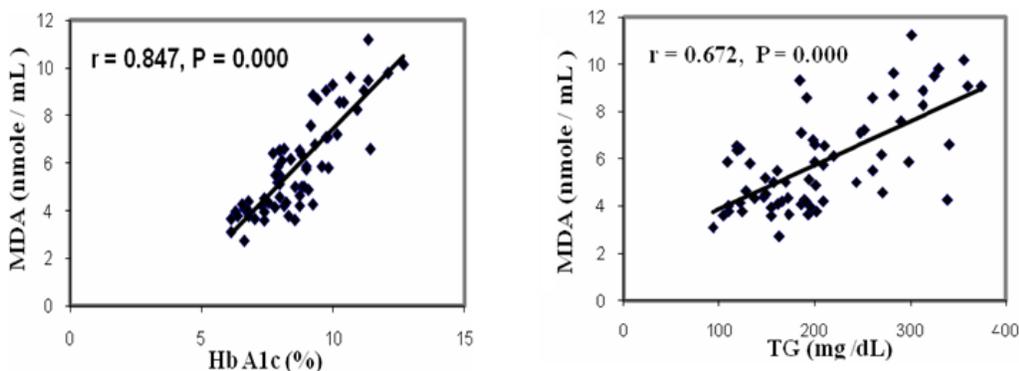
Fig: 1. Correlation between (a) MDA and HbA1c, (b) MDA and TG in all diabetic patients

Table: 2. **Levels of glycated hemoglobin (HbA1c), triglycerides (TG), total cholesterol (TC), high density-lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) in all diabetic patients and in healthy controls. Results are expressed as mean ±SEM**

Groups	Control	Type II DM	NPDR	SPDR
HbA1$_c$ (%)	5.178 ± 0.22	8.37 ± 0.28 [a]	7.86 ± 0.32 [a]	9.59 ± 0.29 [a,b,c]
TG (mg/dL)	78.63± 4.92	188.6 ±16.85 [a]	199.6 ± 14 [a]	233.49 ± 15.57 [a,b]
TC (mg/dL)	142.32 ± 4.79	225.24 ± 9.73 [a]	242 ±13 [a]	258.73 ± 11.54 [a,b]
HDL-C (mg/dL)	50.75 ±1.65	41.63 ± 0.97 [a]	41.26 ± 0.85 [a]	39 ± 0.75 [a]
LDL-C (mg/dL)	75.85 ± 4.57	145.9 ± 9.43 [a]	160.88 ± 12.1 [a]	173 ± 10.96 [a,b]

a: Significantly different from the healthy controls at P ≤ 0.001.
b: Significantly different from the type II DM group at P ≤ 0.05.
c: Significantly different from the NPDR group at P ≤ 0.001.

Table: 3. **Levels of malondialdehyde (MDA), nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), soluble E-selectin (sE-selectin) in all diabetic patients with or without retinopathy and in healthy controls. Results are expressed as mean ±SEM**

Groups	Control	Type II DM	NPDR	SPDR
MDA (nmole/mL)	2.15 ± 0.19	5.28 ± 0.34 [a]	5.57 ± 0.43 [a]	6.81 ± 0.49 [a,b,c]
Nitric Oxide (mmole/L)	20.77 ± 1.24	41.5 ± 2.29 [a]	40.34 ± 3.51 [a]	49.76 ± 3.00 [a,b,c]
TNF- α (pg / mL)	16.1 ± 0.68	21.7 ± 0.63 [a]	22 ± 0.51 [a]	25.8 ± 1.23 [a,b,c]
sE-Selectin (ng /mL)	10.86 ± 0.74	30.28 ± 1.96 [a]	37.74 ± 2.67 [a]	63.7 ± 4.65 [a,b,c]

a: Significantly different from the healthy controls at P ≤ 0.001.
b: Significantly different from the   type II DM group at P ≤ 0.03.
c: Significantly different from the NPDR group at P ≤ 0.03.

[IV] DISCUSSION

Complications of DM, which are the cause of major morbidity and mortality, are related mainly to chronic level of glycemia [41]. The risk of DR is increased with poor glycemic control [42]. Early epidemiologic studies have shown a consistent relationship between HbA1c% levels and the incidence of DR. This important observation has been confirmed in large randomized clinical trials demonstrating that tight glycemic control reduces both the incidence and progression of DR [6]. Early in the course of diabetes, hyperglycemia is responsible for many of the functional retinal vascular changes, including impairment of retinal blood flow, increased leukocyte and monocyte adhesion in the retinal micro vessels, and capillary closure resulting in localized hypoxia [43].In addition, retinal neuronal function may also exhibit abnormalities early in the course of the disease. One of the earliest and most specific retinal changes induced by hyperglycemia is the death of pericytes[44]. The death of pericytes and the loss of vascular intercellular contacts may predispose to endothelial cell proliferation, facilitating the development of microaneurysms [45].Alterations in hemodynamics and vascular autoregulation that are characteristic of the diabetic state [46] can produce venous dilation and beading as well as intraretinal microvascular abnormalities that represent dilated small

vessels [47]. Impairments of vascular cell to-cell contacts and altered barrier permeability function can lead to small intraretinal hemorrhages and fluid leakage. When water is reabsorbed, the plasma lipids and proteins precipitate as hard exudates [43].
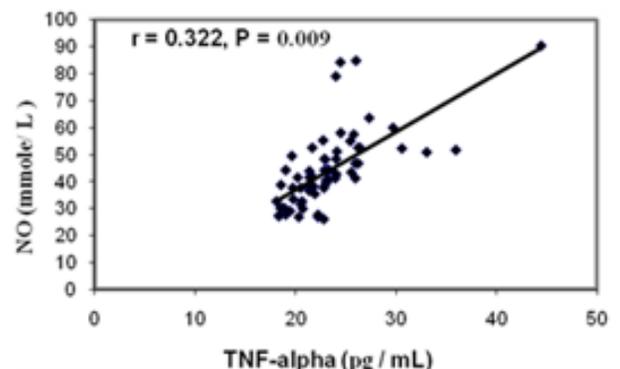
Fig: 2. Correlation between NO and TNF-α in all diabetic patients

The effect of hyperlipidemia in the progression of DR is mainly due to the changes that occur in the fibrinolytic system resulting in the formation of hard exudates which is one of the

major reasons for vision loss in DR, in addition to elevation in the blood viscosity[48]. Moreover, the incorporation of TGs into the cell membrane leads to changes in membrane fluidity and leakage of plasma constituents into the retina resulting in haemorrhage and edema in the retina [49].
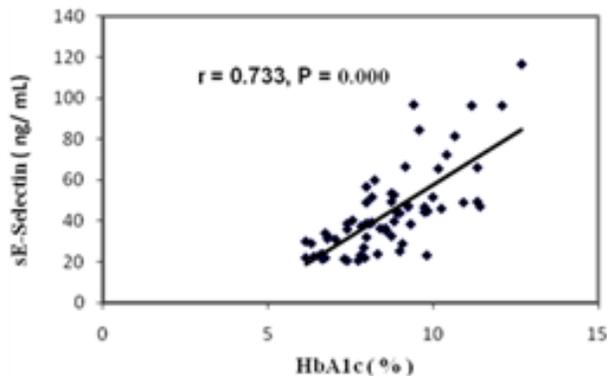
Fig: 3. Correlation between HbA1c% and sE-selectin in all diabetic patients

Oxidative stress is believed to play a significant role in the development of DR [50]. Increased superoxide ion production increases the oxidative load with greater reactive oxidative intermediates and AGEs, which also lead to increased release of vascular endothelium growth factor by the retinal pigment epithelial cells [51], that in turn increases the risk of neovascularization and ischemia in diabetic retina [52]. Both ischemia and increased oxidation can lead to an increased production of lipid peroxidation products such as MDA, which are themselves angiogenic [42].The increased plasma levels of MDA in all diabetic patients may be due to oxidative stress in diabetic patients as increased free fatty acid oxidation in mitochondria produces superoxide free radical [53, 54], in addition to increased reactive O2 products as a result of auto-oxidation of glucose and glycosylated proteins, polyol pathways, and decreased non-enzymatic antioxidants. Finally, hypertriglyceridemia, hypercholesterolemia and AGEs are associated with oxidative modification of LDL, thus leading to excess production of MDA.

The significant correlation found between MDA and HbA1c% may be attributed to the fact that poor glycemic control causes a hypoxia like imbalance by increasing the NADH-to-NAD ratio. This altered ratio has been hypothesized to be a mechanism for ischemic retinopathy and a cause of increased production of the superoxide ion [42] that in turn leads to increased production of lipid peroxidation products. Also, the positive correlation between MDA and TG explains that this increase in MDA, may be due to an increase in the lipid substrate available and increased oxidation of LDL which in turn damages the retinal endothelial cells and the pericytes [55].

On the other hand, iNOS in particular is known to release a great deal of NO continuously compared with nNOS and eNOS, especially in patients without active neovascularization [56].The two fundamental abnormalities in DR are increased retinal vascular permeability and progressive retinal vessel closure , which leads to tissue hypoxia and ischemia which in turn induce iNOS. This induction leads to microenviromental changes in diabetic retinas resulting in sustained and high NO production [57].This increased NO release can cause oxidation and overproduction of peroxynitrite, a ROS mediated by NO, that has been reported to cause vascular endothelial cell dysfunction and breakdown of the blood-retinal barrier, which are important components of the development of DR [25].

Several studies have addressed the recent hypothesis that the angiogenesis of PDR is due to the release of growth factors and interleukins from the ischaemic retina [58]. Abnormal production of cytokines such as TNF-α [25] may also be important in the progression of DR. The mechanism of TNF-α contribution to DR is not fully elucidated. It has been suggested that hyperglycemia may lead to the activation of proinflammatory cytokines that are crucial for micro- and macroangiopathy developments [59]. In diabetic patients, an increased synthesis of the macrophage's RAGE receptors, which bind final glycation products, has been noted [60]. The RAGE receptors signalize the proinflammatory cytokines' cascade induction, including TNF-α, interleukin-6, and interleukin-12 [59, 60].These cytokines may mediate the synthesis of acute phase proteins which are able to initiate and support inflammatory process in the vascular wall. In our study, the significant correlation between NO and TNF-α may be explained by the fact that NO mediates the angiogenic activity of platelet-activating factor and TNF-α [61]. Moreover, diabetes causes microangiopathy in retina and causes hypoxia. Transcription factor kappa (NF-KB) is activated by hypoxia and controls the expression of many gens, some involved with angiogenic factors [62].

Adhesion molecules such as E-selectin have been implicated in the pathogenesis of DR [63].Neovascularization , a process involved in the pathogenesis of DR, is a result of microvascular thrombi leading to retinal ischaemia. E-selectin and cell adhesion molecules, being expressed on retinal vascular endothelium, may take part in this process [64].sE-selectin is expressed on activated endothelial cells and initiates rolling and tethering of leucocytes on the endothelium [65]. This leukocyte recruitment may be the first step in the ensuing endothelial dysfunction resulting in increased permeability of the vessel wall, capillary occlusion, retinal ischemia and ultimately new vessel formation, all characteristics of various stages of DR [66]. The positive correlation between sE-selectin and HbA1c may be explained by the fact that massive hyperglycemia and subclinical tissue injury as well as increased fat mass seen in DM elevate blood levels of inflammatory cytokines, especially TNF-α, which in turn stimulate an acute phase response marked by elevated levels of C-reactive protein [67]. Localization of this inflammatory cascade by vascular endothelial cells is mediated by cellular adhesion molecules including sE-selectin whose surface

expression is a common endothelial response to a variety of toxic stimuli.

[V] CONCLUSION

From our results we conclude that dyslipidemia, oxidative stress and inflammation as well as endothelial dysfunction are all involved in the pathogenesis of DR.

[VI] REFERENCES

[1] Fowler MJ. [2008] Microvascular and macrovascular complications of diabetes. *Clinical Diabetes* 26: 77–82

[2] Malla OK. [2006] The retina in diabetes. *Kathmandu University Medical Journal* 4: 2–3.

[3] Resnikoff S, Pascolini D, Etya'ale D, et al. [2004] Global data on visual impairment in the year 2002. *ulletin of the World Health Organization* 82: 844–851.

[4] Bloomgarden ZT. [2007] Diabetic retinopathy and diabetic neuropathy. *Diabetes Care* 30: 761–765.

[5] Khan ZA, Chakrabarti S. [2007] cellular signaling and potential new treatment targets in diabetic retinopathy. *Exp Diabetes Res* 1–12.

[6] Mohamed Q, Gillies MC, Wong TY. [2007] Management of diabetic retinopathy. *JAMA* 298: 902 – 916.

[7] Fong DS, Aiello L, Gardner TM, Klein R. [2004] Retinopathy in diabetes. *Diabetes Care* 27: 84-87.

[8] Erickson KK, Sundstrom JM, Antonetti DA. [2007] Vascular permeability in ocular disease and the role of tight junctions. *Angiogenesis* 10: 103-117.

[9] Kowluru RA, Chan PS. [2007] Oxidative stress and diabetic retinopathy. *Exp Diabetes Res 2007: 43603.*

[10] Evereklioglu C, Er H, Doganay S, et al. [2003] Nitic oxide and lipid peroxidation are increased and associated with decreased antioxidant enzyme activities in patients with age-related macular degeneration. *Documenta Opthalmologica* 106: 129–136.

[11] Cai J, Boulton M. [2002] The pathogenesis of diabetic retinopathy: old concepts and new questions. *Eye* 16: 242–260.

[12] Beisswenger PJ, Drummond KS, Nelson RG, Howell SK, Szwergold BS, Mauer M. [2005] Susceptibility to diabetic nephropathy is related to dicarbonyl and oxidative stress. *Diabetes* 54: 3274–3281.

[13] Adamis AP, Berman AJ. [2008] Immunological mechanisms in the pathogenesis of diabetic retinopathy. *Semin Immunopath* 30: 65–84.

[14] Gurler B, Vural H, Yilmaz N, Oguz H, Satici A, Aksoy N. [2000] The role of oxidative stress in diabetic retinopathy. *Eye* 14: 730–735.

[15] Gillery P. [2006] Oxidative stress and protein glycation in diabetes mellitus. *Ann Biol Clin* 64: 309.

[16] Pan HZ, Zhang H, Chang D, Li H, Sui H. [2008] The change of oxidative stress products in diabetes mellitus and diabetic retinopathy. *British Journal of Ophthalmology* 92: 548–551.

[17] Wellen KE, Hotamisligil GS. [2005] Inflammation, stress, and diabetes. *J Clin Invest* 115: 1111.

[18] Antonetti DA, Barber AJ, Sarah KB, et al. [2006] Diabetic retinopathy: Seeing beyond glucose- induced microvascular disease. *Diabetes* 55: 2401–2411.

[19] Cusick M, Chew EY, Chan CC, Kruth H, Murphy RP, Ferris FL. [2003] Histopathology and regression of retinal hard exudates in diabetic retinopathy after reduction of elevated serum lipid levels. *Ophthalmology* 110: 2126–2133.

[20] Zeng XX, Ng YK, Ling EA. [2000] Neuronal and microglial response in the retina of streptozotocin-induced diabetic rats. *Visual Neurosci* 17: 463–471.

[21] Mizutani M, Kern TS, Lorenzi M. [1996] Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. *Journal of Clinical Investigation* 97: 2883–2890.

[22] Gerhardinger C, Costa MB, Coulombe MC, Toth I, Hoehn T, Grosu P. [2005] Expression of acute-phase response proteins in retinal muller cells in diabetes. *Invest Ophthalmol Vis Sci* 46: 349 –357.

[23] Gariano RF, Gardner TW. [2005] Retinal angiogenesis in development and disease. *Nature* 438: 960–966.

[24] Chibber R, Ben-Mahmud BM, Coppini D, Christ E, Kohner EM. [2000] Activity of the glycosylating enzyme, core 2 GlcNAc (beta1,6) transferase, is higher in polymorphonuclear leukocytes from diabetic patients compared with age-matched control subjects: relevance to capillary occlusion in diabetic retinopathy. *Diabetes* 49: 1724–1730.

[25] Doganay S, Evereklioglu C, Er H, et al. [2002] Comparison of serum NO, TNF-alpha, IL-1beta, sIL-2R, IL-6 and IL-8 levels with grades of retinopathy in patients with diabetes mellitus. *Eye* 16: 163–170.

[26] Dawson TM, Snyder SH. [1994] Gases as biological messengers: nitric oxide and carbon monoxide in the brain. *J Neurosci* 14: 5147–5159.

[27] Dawson VL, Brahmbhatt HP, Mong JA, Dawson TM. [1994] Expression of inducible nitric oxide synthase causes delayed neurotoxicity in primary mixed neuronal-glial cortical cultures. *Neuropharmacology* 33: 1425–1430.

[28] Dawson VL, Dawson TM. [1996] Nitric oxide neurotoxicity. *J Chem Neuroanat* 10: 179–190.

[29] Ozden S, Tatlipina rS, Biçer N, et al. [2003] Basal serum nitric oxide levels in patients with type 2 diabetes mellitus and different stages of retinopathy. *Can J Ophthalmol* 38: 393-396.

[30] Olson JA, Whitelaw CM, McHardy KC, Pearson DWM, Forrester JV. [1997] Soluble leucocyte adhesion molecules in diabetic retinopathy stimulate retinal capillary endothelial cell migration. *Diabetologia* 40: 1166–1171.

[31] Bloomgarden ZT. [2008] Diabetic retinopathy. *Diabetes Care* 31: 1080-1083.

[32] Ersanli D, Top C, Oncul O, Aydin A, Terekeci H. [2007] Relationship between serum soluble E-selectin levels and development of diabetic retinopathy in patients with type 2 diabetes. *Scand J Clin Lab Invest* 1-6.

[33] Knudsen ST, Foss CH, Poulsen PL, Bek T, Ledet T, Mogensen CE. [2003] E-selectin-inducing activity in plasma from type 2 diabetic patients with maculopathy. *Am J Physiol Endocrinol Metab* 284: E1-6.

[34] Richmond W. [1973] Preparation and properties of a cholesterol oxidase from Nocardia sp. and its application to the enzymatic assay of total cholesterol in serum. *Clin. Chem* 19: 1350–1356.

[35] Wahlefeld AW, [1974] Triglycerides Determination after enzymatic hydrolysis in *Method of enzymatic analysis*, H.V. Bergmeyer, Editor. Verlag Chemie, *Academic Press*, New York. p. 1831–835.

[36] Finley PR, Schifman RB, Williams RJ, Lichti DA. [1978] Cholesterol in high-density lipoprotein: use of Mg^{2+}/dextran sulfate in its enzymic measurement. *Clin Chem* 24: 931–933.

[37] Friedwald WT, Levy RI, Fredrickson DS. [1972] Estimation of the concentration of low density lipoprotein cholesterol in

plasma without use of preparative ultracentrifuge. *Clin. Chem* 18: 499–501.

[38] Uchiyama M,Mihara M. [1978] Determination of malonaldehyde precursor in tissues by Thiobarbituric acid test. *Analytical Biochemistry* 86: 271–278.

[39] Cox RD. [1980] Determination of nitrate and nitrite at the parts per billion level of chemiluminescence. *Anal Chem* 52: 331–333

[40] Griess JP. [1879] Bemerkugen zu der Abhandlungder HH: Wesely and Benedict "Uber eingie Azoverbindungen." *Ver Deutsch Chem Ges* 12: 425–427.

[41] Monnier L, Mas E, Ginet C, et al. [2006] Activation of oxidative stress by acute glucose fluctuations compared with sustained chronic hyperglycemia in patients with type 2 diabetes *JAMA* 295: 1681–1687.

[42] Hartnett ME, Stratton RD, Browne RW, Rosner BA, Lanham RJ,Armstrong D. Serum markers of oxidative stress and severity of diabetic retinopathy. *Diabetes Care* 23:234–240.

[43] Sheetz MJ,King GL. [2002] Molecular understanding of hyperglycemia's adverse effects for diabetic complications. *JAMA* 288: 2579–2588.

[44] Masson E, Wiernsperger N, Lagarde M, El Bawab S. [2005] Involvement of gangliosides in glucosamine-induced proliferation decrease of retinal pericytes *Glycobiology* 15: 585–591.

[45] Aiello LP, Cavallerano J, Bursell SE. [1996] Diabetic eye disease. *Endocrinol Metab Clin North Am* 25: 271–291.

[46] Gilmore ED, Hudson C, Nrusimhadevara RK, et al. [2007] Retinal arteriolar hemodynamic response to an acute hyperglycemic provocation in early and sight-threatening diabetic retinopathy. *Microvascular Research* 73: 191–197.

[47] Garhofer G, Kopf A, Polska E. [2004] Influence of exercise induced hyperlactatemia on retinal blood flow during normo- and hyperglycemia. *Curr Eye Res* 28: 351–358.

[48] Freyberger H, Schifferdecker E, Schatz H. [1994] Ruckbildung harter exsudate bei diabetischer hintergrundretinopathie unter therapie mit dem lipidsenker etofibrat. *Medizinische Klinik* 89: 594–597.

[49] Su DH, Yeo KT. [2000] Diabetic retinopathy and serum lipids. *Singapore Med J*41: 295–297.

[50] Hong-Zhi P, Hong Z, Dong C, Hui L,Hong S. [2008] The change of oxidative stress products in diabetes mellitus and diabetic retinopathy. *British Journal of Ophthalmology* 92: 548–551.

[51] Strauss O. [2005] The retinal pigment epithelium in visual function *Physiol Rev* 85: 845–881.

[52] Churchill AJ, Carter JG, Ramsden C, et al. [2008] VEGF polymorphisms are associated with severity of diabetic retinopathy. *Investigative Ophthalmology and Visual Science 49*: 3611–3616.

[53] Brownlee M. [2005] The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* 54: 1615–1625.

[54] Brownlee M. [2001] Biochemistry and molecular cell biology of diabetic complications. *Nature* 414: 813–820.

[55] Chowdhury TA, Hopkins D, Dodson PM,Vafidis GC. [2002] The role of serum lipids in exudative diabetic maculopathy: is there a place for lipid lowering therapy? *Eye*16: 689–693.

[56] Tsai DC, Chiou SH, Lee FL, et al. [2003] Possible involvement of nitric oxide in the progression of diabetic retinopathy. *Ophthalmologica* 217: 342–346.

[57] Santilli F, Cipllone F, Mezzatti A,Chiarelli F. [2004] The role of nitric oxide in the development of diabetic angiopathy. *Horm Metab Res* 36: 319–335.

[58] Ideta R, Yamashita H, Tanaka Y, Kato S, Kitano S, Hori S. [1999] Roles of cytokines in diabetic retinopathy. *Arch Ophthalmol* 117: 700–701.

[59] McCarter RJ, Hempe JM, Gomez R, Chalew SA. [2004] Biological variation in HbA1c predicts risk of retinopathy and nephropathy in type 1 diabetes. *Diabetes Care* 27: 1259–1264.

[60] Yokoi M, Yamagishi SI, Takeuchi M. [2005] Elevations of AGE and vascular endothelial growth factor with decreased total antioxidant status in the vitreous fluid of diabetic patients with retinopathy. *British Journal of Ophthalmology* 89: 673–675.

[61] Abu El-Asrar AM, Desmet S, Meersschaert A, Dralands L, Missotten L,Geboes K. [2001] Expression of the Inducible Isoform of Nitric Oxide Synthase in the Retinas of Human Subjects With Diabetes Mellitus. *Am J Ophthalmol* 132:551–556.

[62] Cicik E, Tekin H, Akar S, et al. [2003]Interleukin-8, nitric oxide and glutathione status in proliferative vitreoretinopathy and proliferative diabetic retinopathy. *Ophthalmic Res* 35: 251–255.

[63] Meigs JB, Hu FB, Rifai N, Manson JAE. [2004]Biomarkers of endothelial dysfunction and risk of type 2 diabetes mellitus. *JAMA* 291: 1978–1986.

[64] Siemianowicz K, Francuz T, Gminski J, Telega A,Syzdol M. [2005] Endothelium dysfunction markers in patients with diabetic retinopathy. *Int J Mol Med* 15: 459 – 462.

[65] Krieglstein CF,Granger DN. [2001]Adhesion molecules and their role in vascular disease. *Am J Hypertens* 14: 44–45.

[66] Spijkerman AM, Gall MA, Tarnow L, et al. [2007] Endothelial dysfunction and low-grade inflammation and the progression of retinopathy in type 2 diabetes. *Diabetic Medicine* 24: 969–976.

[67] Yudkin JS, Stehouwer CDA, Emis JJ, Coppack SW. [1999] C-reactive protein in healthy subjects: associations with obesity, insulin resistance and endothelial dysfunction: a potential role for cytokines originating from adipose tissue? *Arterioscler Thromb Vasc Bio* l19: 972–978.