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The Role of Advanced Glycation End Products (Ages) and Oxidative Stress in Diabetic Retinopathy

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Abstract: This work aimed to investigate the role of advanced glycation end products (AGEs) and oxidative stress in the development and progression of diabetic retinopathy (DR). This study included 60 non-insulin dependent diabetic patients (30 diabetics with non-proliferative diabetic retinopathy, NPDR and 30 diabetics with proliferative diabetic retinopathy, PDR) compared to 20 healthy age- matched control subjects. Plasma AGEs levels were assayed by ELISA Oxidative stress levels in terms of malondialdehyde (MDA) along with antioxidant defences as total antioxidant capacity (TAC), glutathione (GSH), glutathione peroxidase (GPx) and superoxide dismutase (SOD) were measured in diabetic and control subjects. Results revealed that the plasma levels of AGEs, MDA and GPx were significantly higher in all diabetic patients compared to controls. Also, AGEs and MDA levels were significantly higher in patients with PDR compared to NPDR patients. On the other hand, there were significant decreases in TAC, GSH and SOD activities in both the NPDR and PDR diabetic patients compared to control subjects. Conclusion: this study clearly demonstrated increased association of AGEs with the severity of DR. Thus; it could be used as a prognostic tool to predict the development and progression of DR. In addition, the study detected increased lipid peroxidation products along with impaired antioxidant status in patients with diabetic retinopathy which may contribute to the progression of DR.

Key words: Advanced Glycation End Products · Oxidative Stress · Diabetic Retinopathy · Antioxidants

INTRODUCTION

Diabetes mellitus, a metabolic disorder characterized by high blood glucose, results from the body's inability to either produce or use insulin [1]. This sustained hyperglycemia leads to the progressive development of long-term complications including both the macrovascular and the microvascular diseases [2].

Diabetic retinopathy (DR) is one of the most severe ocular microvascular complications of diabetes and is a leading cause of acquired blindness in young adults. The cellular components of the retina are susceptible to the high glucose concentration, due to persisting hyperglycemia. The microvasculature of the retina responds to this hyperglycemic milieu through a number of biochemical pathways, including increased of both oxidative stress and polyol pathway, activation of protein kinase C (PKC) and formation of advanced glycation end product (AGEs) [3].

Advanced glycation end products (AGEs) are heterogeneous fluorescent derivatives formed by the Maillard process, a non-enzymatic reaction between the reducing sugars and the free amino groups of proteins, lipids and nucleic acids. Elevated levels of AGEs are believed to play a major pathogenic role in diabetic retinopathy. Therefore, early detection of the biochemical effects of tissue or serum AGEs in patients with diabetic retinopathy could serve as a biomarker, which may be effective in early prediction and treatment of DR [4, 5].

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Oxidative stress is considered as one of the crucial contributors in the pathogenesis of diabetic retinopathy. It appears to be highly interrelated with other biochemical imbalances, e.g. formation of AGEs, augmentation of polyol pathway and activation of PKC and hexosamine pathways, that lead to structural and functional changes and accelerated loss of capillary cells in the retinal microvasculature and, ultimately, pathological evidence of the disease [3].

The aim of this study is to evaluate the change in plasma advanced glycation end products (AGEs) level in type II diabetic subjects as a predictive marker for diabetic retinopathy.

In addition, it also designed to determine the role of oxidative stress in the development and progression of diabetic retinopathy.

Subjects and Methods

Subjects: Sixty patients with non-insulin-dependent diabetes mellitus (NIDDM) were recruited from the outpatient clinic; written consent was obtained from each case in this study, according to the Ethical Committee Approval of the Research Institute of Ophthalmology (RIO), Giza, Egypt.

They Were Classified into 2 Groups:

Group 1: 30 diabetic patients with non-proliferative diabetic retinopathy, NPDR.

Group 2: 30 diabetic patients with proliferative diabetic retinopathy, PDR. They were compared to 20 healthy age-matched control subjects.

Full Ophthalmological Examinations Included:

- Intraocular pressure measured by Goldman applanation tonometry.
- Slit lamp examination to determine anterior chamber depth and the presence of iris neovascularization.
- Indirect ophthalmoscopy and biomicroscopy to evaluate the grade of vitreous proliferation and to determine the presence and nature of macular oedema.
- Retinopathy was diagnosed on the basis of fundoscopy to differentiate between NPDR and PDR.
- Fundus fluorescein angiography was done using Topcon fundus camera TRC 50 Ex on image net, 5 ml of 10% sodium fluorescein was injected in the antecubital vein and photography was carried out.

Medical History Was Taken for Each Subject Examination Including:

- Routine laboratory investigations were performed to all cases and controls (Fasting and 2 hours blood glucose, liver function tests, kidney function tests and lipid profiles).
- Exclusion criteria: only those patients who did not have hepatic or renal diseases were selected. Any patient with serum creatinine >1.2 mg / dl or urinary albumin excretion > 150 mg / 24 hrs was not included in this study. Also any patient with local eye disease such as cataract, glaucoma or uveitis was excluded from the study.

Blood Sampling: Approximately 8 ml of venous blood sample was drawn from the antecubital vein on an anticoagulant agent following 12 h overnight fasting from each subject. For erythrocyte lysate preparation, red blood cells were mixed four times its volume with cold saline solution and then centrifuged at 4°C at 3000 rpm for 15 min. The erythrocyte lysate was collected and stored at-80°C. For plasma preparation, samples were centrifuged at 4°C at 3000 rpm for 15 min. Plasma was collected and stored at-80°C.

MATERIALS AND METHODS

Fasting glucose levels in plasma were estimated by enzymatic colorimetric method using a commercial kit supplied by (BioMe´rieux, CA 61-269; France). Blood hemoglobin concentration was measured by cyanomet hemoglobin method according to Betke *and* Savelsberg [6]. For the determination of glycosylated hemoglobin ($HbA1_c$) in the blood samples (Non diabetic reference 5.5 - 7.7%), ion Exchange Resin method was perfrmed using a kit provided by (NS Biotec, Egypt).

Plasma MDA levels (As an index of oxidative stress), plasma total antioxidant capacity (TAC) and erythrocyte lysate superoxide dismutase (SOD) activities were measured by a colorimetric method using a kit supplied by (Biodiagnostic, Egypt). Erythrocyte reduced glutathione (GSH) activity was determined according to the method of Beutler *et al.* [7]. Glutathione peroxidase (GPx) activity in erythrocyte lysate was measured using a kit provided by (Enzo Life Sciences, ADI-900-158, USA).

Eventually, enzyme linked immunosorbent assay (ELISA) procedure was used for quantitative determination of plasma AGEs levels using a commercial kit supplied by (Cell Biolabs, Inc, CA 92126, San Diego, USA).

Statistical Analysis: Data were presented as mean ± standard error (SE). All values were statistically analyzed using Microsoft excel (Version 10) and statistical package for social sciences (SPSS) software (Version 20) [8]. One-way analysis of variance (ANOVA; with post-hoc LSD analysis) was used to compare the groups on continuous variables. The degree of association between the variables was assessed using Pearson's correlation coefficient (r). A receiver-operating characteristic (ROC) curve was generated by plotting sensitivity versus 1-specificity and by selecting cutoffs that provide the best combination of sensitivity and specificity. For all statistical tests, $P > 0.05$ was considered as the level of significance.

RESULTS

The levels of fasting blood glucose (FBG) and glycosylated hemoglobin (HbA1c) were significantly higher (*p*-values<0.001) in both NPDR and PDR groups compared to control group.

Meanwhile, as shown in Table 1, there was a highly significant increase in plasma malondialdehyde (MDA) in both NPDR and PDR groups compared to control group (*p*-values<0.001). Furthermore, there was a statistical significant increase in plasma MDA level in the PDR group compared to NPDR group (*p*-values<0.05).

As shown in Table 1, highly statistical significant decreases in the plasma total antioxidant capacity (TAC) level, erythrocyte reduced glutathione (GSH) concentration and erythrocyte superoxide dismutase (SOD) activity were observed in both NPDR and PDR groups compared to control group (*p*-values<0.001). Moreover, there was a significant decrease in erythrocyte SOD activity in PDR group compared to NPDR group (*p*-value<0.05).

Furthermore, there was a statistically significant increase in erythrocyte glutathione peroxidase (GPx) activity in both NPDR and PDR groups compared to control group (*p*-values<0.05). On the other hand, there was no statistical significant increase in GPx activity in PDR group compared to NPDR group (*p*-value>0.05).

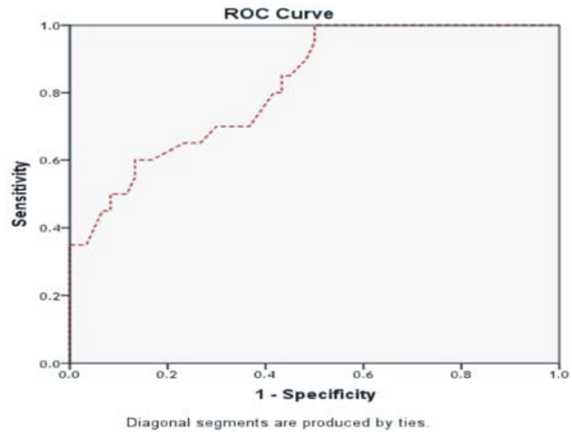

Fig 1: ROC curve of AGEs in NPDR and PDR groups compared to control group.

As shown in Table 1, the mean plasma levels of AGEs were highly significantly elevated in both NPDR and PDR groups compared to control group (*p*-values<0.001). Also, plasma levels of AGEs were significantly elevated in PDR group compared to control group (*p*-values<0.05).

Plasma levels of AGEs were significantly positively correlated with plasma FBG levels (r=0.397, *p*<0.01), plasma MDA levels (r=0.495, *p*<0.01) and erythrocyte GPx activities (r=0.430, *p*<0.01) in NPDR and PDR groups (Fig. 1, 2, 3). However, plasma AGEs levels were significantly inversely correlated with plasma TAC levels (r=-0.233, *p*<0.05) and erythrocyte SOD activities (r=-0.229, *p*<0.05) in NPDR and PDR groups.

The receiver operating characteristic (ROC) curve analysis revealed that at a value of 6.90 µg/mL (The best cut-off), the sensitivity and the specificity of AGEs marker in both NPDR and PDR groups compared to control group were 35 % and 96.7 %, respectively. The area under curve (AUC) = 0.820 (Fig. 1).

Table 1: Characteristic features and biochemical parameters studies of the studied groups

Group	Sex (M/F)	Age (year)	Fasting blood glucose (mg/dL)	Blood hemoglobin (g/dL)	$HbA1_c$ (%)	(MDA) (nmol/mL)	TAC (mmol/L)	GSH (mg/dL)	Gpx (U/g Hb)	SOD (U/g Hb)	(AGEs) (µg/mL)
Control (n=20)	8/20	58.36 ± 2.69 a	101.52 ± 2.88 a	13.14 ± 0.53 a	7.25 ± 0.60 a	1.73 ± 0.06 a	4.59 ± 0.32 a	82.69 ± 5.97 a	52.76 ± 2.32 a	1695.05 ± 53.69 a	8.52 ± 0.78 a
NPDR (n=30)	14/30	63.25 ± 2.36 a	165.86 ± 6.37 b	15.12 ± 0.52 b	10.55 ± 0.53 b	3.12 ± 0.10 b	1.82± 0.11 b	61.27 ± 3.63 b	63.12 ± 2.12 b	1208.33 ± 33.71 b	11.90 ± 0.59 b
PDR (n=30)	17/30	64.72 ± 2.56 a	188.40 ± 9.02 b	15.45 ± 0.50 b	11.58 ± 0.46 b	3.64 ± 0.19c	1.49 ± 0.12 b	51.56 ± 3.45 b	66.30 ± 2.35b	1086.16 ± 26.68 c	14.61 ± 0.57 c
F ratio		1.468	32.890	4.827	15.83	41.266	81.431	12.808	8.201	67.298	20.913
P value		NS 0.237	** 0.000	* 0.011	** 0.000	** 0.000	** 0.000	** 0.000	* 0.001	* 0.000	** 0.000

Groups with different letters have a statistically significant difference. *p**= significant at *p*-value<0.05, *p***= highly significant at *p*-value<0.001 and NS= non-significant at *p*-value>0.05.

DISCUSSION

Diabetic retinopathy (DR) is one of the most serious microvascular complications of diabetes mellitus; it is rarely detected in the first few years of diabetes [10]. The incidence of DR increases to 50% by 10 years and to 90% by 25 years from the disease diagnosis. It progresses to its advanced form, proliferative diabetic retinopathy (PDR), which affects over 60%, of diabetic patients [11]. In Egypt, the prevalence of DR among the Egyptian population is about 20.5% [12].

The Diabetes Control and Complications Trial (DCCT) and United Kingdom Prospective Diabetes Study (UKPDS) clinical trials confirmed a strong relationship between chronic hyperglycemia and the development and progression of diabetic retinopathy [13, 14]. A number of interconnecting biochemical pathways have been proposed as potential links between hyperglycemia and diabetic retinopathy. These pathogenic mechanisms include increased polyol pathway flux, activation of diacylglycerol-protein kinase C (DAG-PKC) pathway, accelerated formation of advanced glycation end products (AGEs), stimulation of oxidative stress, increased expression of growth factors, activation of the renin angiotensin system (RAS) and subclinical inflammation [15].

In this study, a highly statistical significant increase in fasting blood glucose (FBG), blood hemoglobin (Hb) and glycosylated hemoglobin ($HbA1_c$) levels was detected in both the NPDR and PDR groups compared to control group. These results were agreed with the results obtained by Gupta *and* Chari. [16] and Aldebasi *et al*. [17] who reported that patients with poor glycemic control were found to be two fold more susceptible to retinopathy than normoglycemic subjects. Moreover, Van Leiden *et al.* [18] studies in diabetic patients had shown that tight glucose control reduces the risk of retinopathy.

In diabetes, increased oxidative stress may result from over production of precursors to reactive oxygen radicals and/or decreased efficiency of inhibitory and scavenger systems [19]. This impairment in the oxidant/antioxidant equilibrium induces lipid peroxidation of the cellular structures, which is thought to play an important role in pathogenesis of diabetic retinopathy [20].

Thus, oxidative stress is proposed to be one of the most important risk factors in the development of diabetic retinopathy [21]. Malondialdehyde level is considered as a sensitive marker of lipid peroxidation, which is a useful measure of oxidative stress status [22].

In this study, a highly statistical significant increase in plasma MDA level was detected in both the NPDR and PDR groups compared to control group. This result was in agreement with the findings of Pan, *et al.* [22], Gurler *et al.* [23] and Kurtul *et al.* [24] who attributed such elevation in MDA levels to increased reactive oxygen products as a result of autoxidation of glucose and glycosylated proteins, polyol pathways and decreased non-enzymatic antioxidants.

Based on the severity of the disease, high level of plasma MDA was observed in patients with PDR compared to patients with NPDR. These findings were in agreement with those of Mancino *et al.* [9] which supported that oxidative stress is associated with the progression of diabetic retinopathy severity to its proliferative form. It was also agreed with Gupta *and* Chari. [16] findings who suggested that lipid peroxidation increases with the increase of both diabetes duration and severity of retinopathy. This rise in MDA levels in patients with diabetic retinopathy could be due to the oxidant impact produced by hyperglycemia that enhances the generation of ROS, which in turn can oxidize other major biomolecules including membrane lipids [25]. In addition, the increase in the blood free fatty acid levels in diabetic patients, as a function of the degree of lipolysis, could result in excessive production of MDA [21].

There are several scavenger systems that protect the cells and tissues from oxidative stress damage [26]. Antioxidants may act at different levels, depending on the susceptibility of various tissues to oxidative stress. They inhibit the formation of ROS, scavenge free radicals, or increase the antioxidants defense enzyme capabilities [15, 33]. The level of these antioxidants is critically associated with the development of diabetes complications. This revealed the importance of determining the antioxidant status (Indirect evaluation of oxidative stress) in diabetics [21].

The present study showed a highly statistical significant decrease in plasma total antioxidant capacity (TAC) level in both the NPDR and PDR groups compared to control group. These results were in accordance to those performed by Abdel Hamid *et al.* [27]. The decreased levels of TAC in diabetic retinopathy patients might be due to the destructive effects of oxidative stress caused by increased vascular inflammation and gene expression of growth factors and cytokines, such as vascular endothelial growth factor (VEGF) [28, 29]. As a consequence, this decrease in the antioxidant levels could further potentiates the deleterious effects of AGE on diabetic retinopathy through the overproduction of VEGF [30]. Also, the overconsumption of TAC during the scavenging mechanism without adequate compensatory production further results in TAC depletion.

Moreover, the present study showed a highly statistical significant decrease in erythrocyte GSH concentration in both the NPDR and PDR groups compared to control group. This finding was in agreement with that declared by Kumar *et al.* [31] and Yildirim *et al.* [32]. It also agreed with Samuel *et al.* [21] that correlated the depletion in GSH concentration might represent its susceptibility to oxidative injury.

The decrease in GSH concentration observed in patients with diabetic retinopathy might be due to the preferentially consumption of the reduced nicotinamide dinucleotide phosphate (NADPH), a necessary co-factor for GSH regeneration, in polyol pathway and thus leading to GSH depletion [33]. Moreover, GSH also participates in a variety of oxidation-reduction reactions. Intracellularly, it is converted to its oxidized form (Glutathione disulphide, GSSG) by selenium containing glutathione peroxidase, which catalyses the reduction of hydrogen peroxide (H_2O_2). Glutathione-S-transferase also catalyzes such reaction. This leads to increased utilization of GSH by glutathione peroxidase and transferase, resulting in GSH depletion [38].

A statistical significant increase in erythrocyte GPx activity was detected in both the NPDR and PDR groups compared to control group. This result was in accordance with those obtained by Gupta *and* Chari [16],Rema *et al.* [34] and Sundaram *et al.* [35].

The possible explanation for this paradoxical rise in GPx activity associated with diabetic retinopathy patients could be due to insulin deficiency, which promotes β-oxidation of fatty acids resulting in increase in hydrogen peroxide (H_2O_2) formation. Thus, with the increase in the lipid peroxide levels, the paradoxical increase in the GPx levels could be a compensatory mechanism adopted by the body to prevent tissue damage [16].

However, a highly statistical significant decrease in erythrocyte SOD activity was found in both the NPDR and PDR groups compared to control group. This result was agreed with the findings of Gupta and Chari [16], Kumar *et al.* [31] and Rema *et al.* [34]. Furthermore, this study demonstrated a significant lower activity of erythrocyte SOD in patients with PDR compared to patients with NPDR (p-value<0.05). A similar result had previously been confirmed by Aldebasi *et al.* [17].

The decrease in SOD activity in patients with diabetic retinopathy could be due to diminished synthesis and/or deactivation of the enzyme activity by progressive glycation. This results in impairment of the effective scavenging and intracellular defense system of SOD against superoxide radical-mediated toxicity [24]. Moreover, the products of membrane lipid peroxidation and other oxidants, like hydrogen peroxide, may react with SOD resulting in oxidative modification, thereby causing loss of the enzyme activity [36].

Advanced glycation end products (AGEs) are a heterogeneous group of molecules formed by a non-enzymatic reaction of reducing sugars with free amino groups of proteins, lipids and nucleic acids [37]. This reaction proceeds via the formation of the Schiff bases, then the Amadori products which undergo slow and complex rearrangements leading to formation of irreversible AGEs [6, 38]. A number of clinical studies have reported that the formation and accumulation of AGEs detected in retinal blood vessels, serum as well as vitreous of diabetic patients, were found to correlate with the progression and development of diabetic retinopathy [30, 39, 40].

The results obtained from the present study revealed a highly statistical significant increase in plasma AGEs level in both the NPDR and PDR groups compared to control group. This result was agreed with that performed by Anitha *et al.* [8] who correlated increased levels of AGEs to the development and progression of DR. Also [41] found that by diminishing AGEs levels could prevent the initiation and progression of DR. Moreover, the current study demonstrated a significant increase in plasma AGEs level in the PDR group compared to NPDR. This was agreed with that reported by Kerkeni *et al.* [41] and Choudhuri *et al.* [42].

This elevated rate of formation and accumulation of AGEs in diabetic retinopathy patients might be due to increased availability of glucose. [43] via autoxidation and protein glycosylation [44].

The increase in AGEs is observed within retinal capillary cells can cause loss of pericytes [6], Stitt *et al.* [45] and basement membrane thickening, which are the characteristic features of DR [46]. Moreover, AGEs can disturb the microvascular homeostasis through interaction with advanced glycation end product receptor (RAGE). This interaction can induce intracellular signaling, oxidative stress and plays a crucial role in the inflammation, neurodegeneration and microvascular dysfunction in DR [47]. Thus, the elevated levels of AGEs are believed to play a causative role in pathogenesis and progression of DR.

In the current study, statistically significant positive correlations were found between AGEs and fasting blood glucose and $HbA1_c$ in both proliferative and non-proliferative diabetic retinopathy patients. These results were agreed with those reported by Sampathkumar *et al.* [48].

Moreover, AGEs were found to be significantly and positively correlated with MDA and GPx. Meanwhile, inversely correlated with TAC and SOD in diabetics with both proliferative and non-proliferative retinopathy. These findings were in agreement with Bansal *et al.* [49] who found a significant positive correlation between AGEs and MDA and stated that AGEs may mediate increased ROS generation leading to enhanced oxidative stress. This demonstrated the possible involvement of enhanced formation of AGEs in inducing oxidative stress.

In this study, the receiver operating characteristic (ROC) curve was used to reach the value of the best sensitivity and specificity of AGEs and to evaluate its diagnostic performance to predict the initiation and progression of DR. It was found that at a value of 6.90 µg/mL (the best cut-off), the sensitivity was 35 % and the specificity was 96.7 %. This was confirmed by Bansal *et al.* [49] who found two fold increases of AGEs levels from normal value that leads to development and progression of vascular complications in diabetics. Therefore AGEs could be considered a novel progressive risk marker and its measurement appears to be of value in predicting diabetic retinopathy.

CONCLUSION

This study clearly demonstrated accumulation of AGEs and lipid peroxidation products along with impaired antioxidant status in patients with diabetic retinopathy. These observations suggest that AGEs formation and oxidative stress generation are correlated to form a positive feedback loop, having an important role in the initiation and progression of diabetic retinopathy. Thus, inhibition of AGE formation or oxidative stress generation could be a potential target for therapeutic intervention in sight threatening diabetic retinopathy. Moreover, this study suggested that AGEs measurement could be used as a diagnostic tool in primary screening programs to predict the development and progression of DR.

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