ORIGINAL ARTICLES

Oxidative Stress And Apoptosis In Relation To The Progression Of Diabetic Retinopathy In Diabetics

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ABSTRACT

Purpose: to investigate the role of oxidative stress and apoptosis in the progression of diabetic retinopathy.

Experimental: Non-insulin dependent diabetes mellitus patients were recruited from the outpatient clinic of the Research Institute of Ophthalmology (RIO). Patients were divided into 3 groups: (Diabetics without retinopathy; Diabetics with non-proliferative diabetic retinopathy NPDR; Diabetics with proliferative diabetic retinopathy, PDR). A group of healthy age-matched subjects served as control. Lipid profile parameters were measured. Lipid peroxidation end product (MDA) and nitric oxide metabolites (total NO3−) were measured as oxidative markers. SOD and GPx levels were estimated as indices of antioxidant enzymes. Plasma sFas and s-Fas-Ligand were measured as indices for apoptosis. Results: All diabetic patients suffered from dyslipidemia expressed by significant increase in TC, TG and LDL compared to control while HDL level showed significant decrease. MDA, NO3− and sFas significantly increased with progression of retinopathy while SOD and GPx showed significant decrease, levels of sFas ligand were undetectable. Conclusion: The oxidative stress observed in diabetic patients may contribute to the progression of diabetic retinopathy. Also Fas-mediated apoptosis might be involved in the progression of diabetic retinopathy.

Key words: Diabetes, oxidative stress, retinopathy, antioxidant enzymes, apoptosis, nitric oxide, MDA, sFas.

Introduction

Cells generate energy by reducing molecular oxygen to water. During this process, small amounts of partially reduced reactive oxygen forms are produced as an unavoidable by-product of mitochondrial respiration. They are referred to as reactive oxygen species (ROS) that can cause cell injury. The toxic effect of these reactive oxygen species and free radicals can be eliminated by enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx). An imbalance between free radical-generating and radical scavenging systems results in oxidative stress, a condition that has been associated with the cell injury seen in many pathologic conditions (Wakamatsu et al., 2008). Diabetes results in increased oxidative stress which plays an important role in the pathogenesis of diabetic complications. It is postulated to promote the development of retinopathy, neuropathy, nephropathy and myocardial injury (Kowluru and Chan, 2007).

Diabetic retinopathy (DR), a devastating ocular complication of diabetes mellitus, is a leading cause of vision loss and blindness in working-age adults in developed countries and is a serious public health problem throughout the world. Hyperglycemia is known to be the prime-triggering factor for the progression of the disease (Leal et al., 2009; Ali and El-Remessy, 2009). It can be stated that the increase in free radicals by hyperglycemia, lipid peroxidation along with decreased antioxidants are causative agents of the development of retinopathy (Kumar et al., 2001).

Nitric oxide is a free radical that can act as a neurotransmitter and in a paracrine or autocrine manner to produce diverse cellular responses, both beneficial and detrimental. Increased oxidative stress and changes in nitric oxide formation or activity play a major role in the complications of diabetes with decreased antioxidants (Ramakrishna and Jailkhani, 2008). It has been recognized that nitric oxide is involved in the control of basal blood flow in the choroids, optic nerve and retina. Excess NO and / or defective antioxidants cause lipid peroxidation, cellular dysfunction and death (Evereklioglu et al., 2003).

Apoptosis or programmed cell death which is a physiologic process in many organs and cells can also occur in pathological conditions (Granville et al., 1998). Several lines of evidence indicate that hyperglycemia itself (Naruse et al., 2000) might contribute to apoptosis of retinal pericytes. Several mechanisms of apoptosis have been described (Granville et al., 1998), among which, is the Fas system which is composed of Fas ligand (transmembrane glycoprotein) and Fas antigen (transmembrane glycoprotein receptor). Fas (APO-1, CD95) is a type I integral membrane protein, and a member of the tumor necrosis factor (TNF) receptor family. FasL (FasL/APO-1, CD95L) is a type II integral membrane protein and a member of the TNF family, Interaction
between Fas positive cells and FasL positive cells caused intracellular signal transduction in the former cells, which induces apoptosis of the Fas positive cells (Sugita et al., 2000). A dysfunction in the Fas system may be involved in diabetic patients with retinopathy (Guillot et al., 2001).

**Aim of the work:**

The study was planned to investigate the role of oxidative stress in the progression of diabetic retinopathy in diabetic patients. The relationship between plasma oxidants and antioxidants was studied by measuring the plasma MDA-like metabolite and nitric oxide end product (total NO\textsubscript{3}\textsuperscript{−}) as examples of oxidative markers and erythrocyte GPx and SOD as indexes of antioxidants. The relation between these parameters and diabetic retinopathy severity was evaluated. The role of apoptosis in diabetic retinopathy progression was also investigated by examining plasma levels of soluble Fas/APO-1 receptor (sFas), an inhibitor of apoptosis, and soluble Fas ligand (sFas-L), an inducer of apoptosis.

**Subjects:**

450 Patients with non-insulin-dependent diabetes mellitus (NIDDM) were recruited from the outpatient clinic of the Research Institute of Ophthalmology (RIO). A history was taken from the patients including age, time of onset or diagnosis of diabetes, first visit to the ophthalmologist, type of antidiabetic medication and the history of any other complaints, systemic or endemic diseases. Accordingly patients who had any ophthalmic complication other than diabetic retinopathy were excluded. Also patients who had any endemic disease (e.g. hepatitis and bilharzias), acute and chronic infections, fever, malignancy, chronic nephritis and coronary heart diseases were all excluded. Thus out of the 450 patients, only 104 (46♂ & 58♀) satisfy the criteria of the study and have been included. Their mean age was 62±7.32 years (age ± SD) with range 42-75 years. Diabetic duration ranged from 3 to 26 years with a mean of 13.87±6.55 years. All patients are treated from diabetes by oral antidiabetic treatment only.

Diabetic patients were examined by an ophthalmologist. According to fundus examination with direct ophthalmoscopy; patients were grouped with respect to the degree of retinopathy to three groups. Diabetic patients without retinopathy D (n = 35; 16♂, 19♀; mean age 58.53±7.91 years, range 45-72), diabetic patients with nonproliferative retinopathy NPDR (n = 29; 12♂, 17♀; mean age 62.13±5.97 years, range 52-73) and diabetics with proliferative retinopathy PDR (n = 40; 18♂, 22♀; mean age 65.33±6.76 years, range 51-75). Age-matched healthy subjects; n=32 (16♂, 16♀) from members of the working staff and relatives served as control with mean age of 55±8.61 years and range 42-70 years. Subjects in the control group were subjected to the same criteria of exclusion as the diabetic patients. All have no history of diabetes, normal fasting plasma glucose and with fundus free from any changes.

**Blood sampling:**

Blood samples were collected from all patients and control following an overnight fast by venopuncture into heparenized tubes. Each blood sample was centrifuged for 10 min. at 4000 rpm. After removal of plasma and buffy coats, erythrocytes were washed three times with two volumes of isotonic saline. Then, erythrocytes were lysed with cold distilled water (1:4), stored in refrigerator at 4°C for 15 min and the cell debris were removed by centrifugation (2000 rpm for 10 min.). Plasma samples and erythrocyte lysates were stored at -70°C until assayed.

**Methods:**

Fasting and postprandial plasma glucose levels were estimated by glucose oxidase method using the commercial glucose kit provided by (Human Gesellschaft für Biochemica und Diagnostica mbH, Germany). Total cholesterol and Triglycerides were measured using the kits provided by (SGM Italia). For the determination of high density lipoprotein (HDL) cholesterol in the plasma samples, (Stanbio Laboratory kit) was used and LDL was calculated from the equation provided with the method. Nitric oxide (NO) release can be determined spectrophotometrically by measuring the Plasma total nitrite using the commercial kit provided by (R&D Systems Inc. Minneapolis, U.S.A). Serum MDA levels were measured as an index of lipid peroxidation using the commercial kit provided by (Biodiagnostic, Egypt),the method was based on that of Satoh, 1978. The activity of Superoxide dismutase (SOD) and Glutathione peroxidase (GPx) were measured in the haemolysate using commercial kit provided by (Randox Laboratories Ltd, U.K.) according to the manufacturer instructions. Results were expressed as U/gHb. sFas was determined using Quantikine sFas Enzyme Linked-Immuno-
Sorbet Assay (ELISA) kit (Research and Diagnostics Systems, Minneapolis, U.S.A.) and results were expressed as ng/ml. sFasL was assayed using s FasL ELISA kit (Dialcone Research, France).

Statistical analysis:

Data were presented as mean ± SD. All experimental data were statistically analyzed by using SPSS for Windows computing program. One way analysis of variance (ANOVA) was applied. When ANOVA gives a significant result, this indicates that at least one group differs from the other groups. Yet, it does not indicate which group differs, so post-hoc comparison of means (least significant difference; LSD) was performed on the means of the biochemical variable data to examine differences among groups. If the difference between two means was greater than the LSD, this expresses significant difference. A value of p< 0.05 was accepted as Statistical significant difference.

Results:

The main characteristics of patients and control subjects are shown in table (1). The duration of diabetes was longer in PDR patients than both NPDR and diabetics without complications. Comparison of means showed that all diabetic patients irrespective to sex, had significantly elevated fasting and post prandial plasma glucose compared to control (p<0.0001). This elevation was more pronounced with progression of retinopathy. It was also noticed that female patients outnumber male patients and comprised 55.76%, 58.62%, 55% in total diabetic patients; NPDR patients and PDR patients respectively.

Lipid profile parameters (TC, TG, LDL and HDL) showed that diabetic patients suffer from dyslipidemia (Table 2). TC, TG and LDL significantly increased in the three diabetic groups compared to control (p<0.0001). Also both NPDR and PDR patients had markedly elevated levels of these parameters versus those of diabetics without retinopathy. No significant gender differences were observed in these parameters except for male control TC levels that were significantly higher compared to females (C♂:191.35±10.24 vs. C♀:182.65±11.14; p=0.014) and male control LDL-C levels which were significantly elevated compared with females in the same group (C♂:117.05±10.58 vs. C♀:107.75±11.47; p=0.012). On the other hand, the average values of HDL-C were significantly diminished in diabetics and retinopathy patients compared with control (38.47±5.33; 40.49±5.87; 37.97±3.77 and 48.13±8.87 mg/dl, for D; NPDR; PDR and C respectively; p<0.0001). These values were nearly similar to each other in diabetics with and without retinopathy. Considering gender difference, female control had higher levels of HDL-C than males (C♀:51.15±9.21 vs. C♂:45.11±6.92; p=0.02). In diabetics without retinopathy, the decline in HDL-C levels was more pronounced in males than females (D♂:36.69±4.79 vs. D♀:39.96±5.42; p=0.035) while retinopathy patients showed no significant gender difference (p>0.05).

MDA (Table 3) significantly increased in all diabetic patients compared with control subjects (3.78±0.92; 4.77±0.75, 5.72±0.80 and 2.99±0.64 µmol/L for D, NPDR, PDR and C respectively). Also patients with NPDR had significantly higher levels of MDA compared with diabetics without complications with p<0.0001. Considering the PDR patients, the level of MDA was elevated with statistical difference compared with diabetics (p<0.0001) and NPDR (p<0.05). The values of MDA for males and females show that within the same group there is no significant difference between males and females (p= 0.223, 0.451, 0.166 and 0.441 for C, D, NPDR and PDR respectively). As brief there was significant increase in MDA level which was more pronounced as the disease progress and this increase is not sex - dependant.

Total nitrites results (Table 3) revealed an elevated trend for NO activity in all patients with the highest level in PDR group compared to control (D: 63.60±9.69; NPDR: 77.40±2.50; PDR: 91.20±2.37; C: 51.33±8.91 µmol/L; p<0.0001). High statistical difference between diabetics with no retinopathy and the two retinopathy groups as well as between the NPDR and PDR groups was detected (p value <0.0001). No significant gender difference within the same group was observed (p values 0.315, 0.275, 0.103 and 0.233 for C, D, NPDR and PDR respectively). As an overall observation, total NO3 level increases, irrespective to sex, with the progression of retinopathy.

For antioxidant enzymes, SOD activity (Table 4) was elevated in diabetics without retinopathy (3278.49±442.86u/gHb; p<0.05) but it was diminished in retinopathy patients with statistical significance only in PDR patients compared with control subjects (2128.29±444.60, 2789.61±804.80 u/gHb; p<0.05). GPx activity decreased in both retinopathy groups with a statistical significant difference in case of proliferative retinopathy (C: 61.73±15.79; NPDR: 55.73±6.31, p= 0.16; PDR: 50.67±12.27 u/gHb, p<0.05). No significant gender difference was observed (p>0.05).

The level of sFas (Table 5) was significantly increased with the progression of retinopathy (6.64±1.79, 9.14±1.07 and 9.95±0.83 ng/ml for D, NPDR and PDR respectively) compared with control (5.05±0.75). Also both retinopathy groups demonstrated high significant increase in sFas level in with p<0.0001 versus diabetics...
with no retinopathy. No gender difference was detected. Plasma levels of sFasL were undetectable or very low (<0.1 ng/ml) among patients and control subjects.

**Table 1:** Characteristics of patients and control subjects

<table>
<thead>
<tr>
<th>Item</th>
<th>Group 1 control</th>
<th>Group 2 D</th>
<th>Group 3 NPDR</th>
<th>Group 4 PDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number(n)</td>
<td>32 (16/16)</td>
<td>35 (16/19)</td>
<td>29 (12/17)</td>
<td>40 (18/22)</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>42±10</td>
<td>45±72</td>
<td>52±73</td>
<td>51±75</td>
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<tr>
<td>Mean ±SD</td>
<td>55±8.61</td>
<td>58.53±7.91</td>
<td>62.13±5.97</td>
<td>65.33±6.76</td>
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<tr>
<td>Duration (years)</td>
<td>---</td>
<td>(3-10)</td>
<td>(8-20)</td>
<td>(10-26)</td>
</tr>
<tr>
<td>Mean ±SD</td>
<td>---</td>
<td>6.87±2.26</td>
<td>14.2±3.47</td>
<td>20.53±4.19</td>
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<tr>
<td>Fasting glucose (mg/dl)</td>
<td>All</td>
<td>84.07±7.05</td>
<td>176.07±38.54</td>
<td>203.33±38.16</td>
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<td>♂</td>
<td>82.27±6.17</td>
<td>185.07±37.62</td>
<td>199.78±41.7</td>
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<td></td>
<td>♀</td>
<td>85.87±7.14</td>
<td>168.48±38.66</td>
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<td>&lt;0.004</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>2 h pp. glucose (mg/dl)</td>
<td>All</td>
<td>106.87±10.66</td>
<td>204±44.61</td>
<td>248.93±41.08</td>
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<tr>
<td></td>
<td>♂</td>
<td>109.00±11.93</td>
<td>206.62±47.48</td>
<td>241.06±45.22</td>
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<td></td>
<td>♀</td>
<td>104.73±8.30</td>
<td>203.02±43.29</td>
<td>254.49±38.32</td>
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<td></td>
<td>(p value)</td>
<td>&lt;0.008</td>
<td>&lt;0.004</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>All</td>
<td>48.13±8.87</td>
<td>38.47±5.33</td>
<td>40.49±5.87</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>45.11±6.92</td>
<td>36.69±4.79</td>
<td>40.04±7.50</td>
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<td></td>
<td>♀</td>
<td>51.15±9.21</td>
<td>39.96±5.42</td>
<td>40.81±4.61</td>
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<tr>
<td></td>
<td>(p value)</td>
<td>&lt;0.004</td>
<td>&lt;0.004</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>All</td>
<td>112.4±11.96</td>
<td>146.4±21.60</td>
<td>175.07±21.18</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>117.05±10.58</td>
<td>149.18±24.51</td>
<td>173.15±24.24</td>
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<tr>
<td></td>
<td>♀</td>
<td>107.75±11.47</td>
<td>144.06±11.71</td>
<td>176.42±23.05</td>
</tr>
<tr>
<td></td>
<td>(p value)</td>
<td>&lt;0.004</td>
<td>&lt;0.004</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Values are mean ±SD; D (Diabetes without retinopathy); NPDR (non-proliferative diabetic retinopathy); PDR (proliferative diabetic retinopathy); pp (post prandial).

**Table 2:** Lipid profile parameters in the study groups

<table>
<thead>
<tr>
<th>Item</th>
<th>Group 1 control</th>
<th>Group 2 D</th>
<th>Group 3 NPDR</th>
<th>Group 4 PDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mg/dl)</td>
<td>187±11.78</td>
<td>221.53±16.10</td>
<td>246±15.75</td>
<td>257±16.78</td>
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<tr>
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<td>♂</td>
<td>191.35±10.24</td>
<td>223.66±12.83</td>
<td>248.74±15.50</td>
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<td>♀</td>
<td>182.65±11.14</td>
<td>219.74±18.57</td>
<td>244.06±16.10</td>
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<tr>
<td></td>
<td>(p value)</td>
<td>&lt;0.004</td>
<td>&lt;0.004</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>128±31.88</td>
<td>159±14.43</td>
<td>176.73±16.38</td>
<td>183±18.11</td>
</tr>
<tr>
<td></td>
<td>♂</td>
<td>136.72±22.04</td>
<td>156.73±15.47</td>
<td>177.24±16.41</td>
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<td></td>
<td>♀</td>
<td>119.28±36.3</td>
<td>161.15±13.58</td>
<td>176.37±16.86</td>
</tr>
<tr>
<td></td>
<td>(p value)</td>
<td>&lt;0.004</td>
<td>&lt;0.004</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>48.13±8.87</td>
<td>38.47±5.33</td>
<td>40.49±5.87</td>
<td>37.97±3.77</td>
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<tr>
<td></td>
<td>♂</td>
<td>45.11±6.92</td>
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<td>LDL-C (mg/dl)</td>
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<td></td>
<td>(p value)</td>
<td>&lt;0.004</td>
<td>&lt;0.004</td>
<td>&lt;0.001</td>
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</tbody>
</table>

Data are presented as mean ± SD. TC: Total cholesterol; TG: triglycerides; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol.

*significant difference compared to control. †significant difference compared to diabetics without retinopathy, ‡significant difference compared to NPDR. n.s. No significant difference between males and females; s.d. significant difference between males and females.
Diabetes mellitus is a metabolic disorder characterized by hyperglycemia. The oxidative stress in diabetes was greatly increased due to prolonged exposure to hyperglycemia and impairment of oxidant / antioxidant equilibrium (Ramakrishna and Jailkhani, 2008). Diabetic retinopathy is a common and potentially devastating microvascular complication in diabetes and is a leading cause of acquired blindness among the people of occupational age. Studies suggested that oxidative stress is increased in diabetic patients and is more severe in diabetic retinopathy (Yamagishi et al., 2008; Pan et al., 2008). It was observed from patient's characteristics that PDR appears in patient with longer diabetic duration. This pattern might reflect that diabetic retinopathy progression occur with prolonged exposure to high levels of circulating blood glucose and that diabetic duration could be a risk factor for initiation and progression of retinopathy. This is in accordance with Doganay, et al. (2002) who mentioned that degradation and loss of retinal pericytes that are seen in proliferative diabetic retinopathy, results as a consequence of systemic metabolic abnormalities associated with prolonged hyperglycemia. Mohamed et al. (2007); Yam and Kwok (2007) and Manaviat et al. (2008) supported present results in that duration of diabetes and severity of hyperglycemia are major factors for retinopathy in type II diabetic patients. The number of female patients was more than that of males despite the fact that, demographic structure of the Egyptian population indicates that males outnumbered females in ages below 64 years old (CAPMAS, 2008). This might suggest that females are more susceptible to diabetes and its complications than males. Ding, et al. (2006) suggested that endogenous sex
hormones may differentially modulate glycemic status and risk of type II diabetes in men and women. High testosterone levels are associated with lower diabetic risk in men but higher risk in women.

Lipids present in plasma, mitochondrial, and endoplasmic reticulum membranes are major targets of ROS attack and peroxidation. End products of lipid peroxidation; known as lipid peroxides, can be toxic to cells and require removal by antioxidant defence system (Vincent et al., 2004). In the present study, the levels of TC, TG and LDL-C of the three diabetics groups, despite sex, were significantly higher than the control group. Also, both retinopathy groups (NPDR, PDR) showed higher levels for the same parameters compared with diabetics without retinopathy. This increase was not statistically significant between the two retinopathy stages which might point to a relation between dyslipidemia in diabetic patients and the development of retinopathy, but not the progression of the disease. Concerning sex differences, results showed that in control group, males recorded significantly higher levels of total cholesterol (TC) and LDL-C than females. This might be related to factors as smoking, stress, sex hormones, body mass index, metabolic disturbance and others. Soliman (2008) found no gender effect on the TC, LDL-C and HDL-C levels of the control groups.

TC and TG and LDL-C levels in the diabetics with and without retinopathy did not show significant gender difference; suggesting that once diabetes is developed, both males and females become exposed to dyslipidemia. This coincides with Mahboob, et al. (2005); Soliman, (2008) and Nayak, et al. (2012) in TC levels; but, Soliman, (2008) found that male Egyptian diabetic patients had higher TG levels and lower LDL-C compared to female diabetics while Nayak, et al. (2012) found that female diabetics had higher TG levels than males attributing this to different circulating sex hormones in women compared to men. Pérez- López, et al.(2010) stated that in women, endogenous female sex hormones, especially estrogens has a vital role in decreasing levels of low-density lipoprotein (LDL). Diabetic retinopathy patients (NPDR and PDR) had significantly higher levels of TC, TG and LDL compared to control healthy subjects. The levels in PDR were higher than NPDR but with no statistical significance. Different literature support the hypothesis that dyslipidemia in diabetic patients is associated with the initiation and progression of diabetic retinopathy (Cai et al., 1998; Leiter, 2005; Bloomgarden, 2007). Elevated serum lipid levels are positively associated with retinal hard exudates in DR. Hard exudates, in turn, are associated with visual impairment and subretinal fibrosis from macular oedema (Yam and Kwok, 2007). Results of Wu, et al. (2008) that examined the association of dyslipidemia with the initiation and progression of diabetic retinopathy, showed that intraocular oxidized LDL was absent in non-diabetics but present in all diabetic groups and it increased with the severity of retinopathy.

HDL-C in control subjects was higher in females than males which coincide with studies of Erem et al., 2008 and Goh et al., 2007. Pérez- López, et al. (2010) attributed this to cardio-protective role of endogenous female sex hormones, especially estrogens. For all diabetics, HDL-C declined significantly irrespective to sex or stage of retinopathy that is in accordance with Meisinger, et al. (2002). Also the decline in male diabetics without complications was more than females which agree with Schneider, et al. (2009) and Perreault et al., (2008).

Lipid peroxidation in the present study was evidenced by a significant increase in plasma MDA in diabetic patients, regardless to sex, compared to control subjects. Different studies found significant increase in MDA in diabetic patients compared with control (Vanizor et al., 2001; Memisogullari et al., 2003; Atli et al., 2004; Vericel et al., 2004; Mahboob et al., 2005; Song et al., 2007; Ramakrishna and Jaikhani, 2008; Soliman, 2008). A significant correlation between MDA levels and duration of diabetes was detected by Nakhjavani, et al. (2010). Simbaljević, et al. (2007) stated that the observed abnormality in lipid metabolism in NIDDM patients is closely associated with increased lipid peroxidation, changes in antioxidative defense and erythrocyte morphology. The findings introduced by Peerapatdit, et al. (2006) and Likidlilid, et al. (2010) strongly confirmed the evidence that diabetic patients were susceptible to oxidative stress and higher blood glucose level had an association with free radical-mediated lipid peroxidation. The data of Goodarzi, et al. (2008) showed an increase in lipid peroxidation and oxidative stress in diabetes and also indicated a positive correlation between the degree of hyperglycemia and oxidative stress. All aforementioned findings are in accordance with the results of the present study. Contrary other studies could not detect any significant difference in lipid peroxidation products between diabetics and controls (Maejima et al., 2001; Polak and Zagorski, 2004) attributing this to the relatively high age of all tested subjects (diabetics and control). Retinopathy patients (NPDR and PDR), regardless their sex, had significantly higher levels of MDA compared with diabetics without retinopathy or control healthy group. This elevation was more pronounced with the progression of retinopathy. The increase 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. The present findings are in accordance with those of Mancino, et al. (2011) which supports the hypothesis that oxidative stress is associated with the progression of diabetic retinopathy to its proliferative form. The present results also coincide with Gurler, et al. (2000), Kurtul, et al (2005) and Pan, et al (2008). They attributed this elevation to increased reactive oxygen products as a result of auto-oxidation of glucose and glycosylated proteins, polyl pathways and decreased non-enzymatic antioxidants. The ROS induced peroxidation of the polyunsaturated fatty acids that lead to the formation of MDA as an end product of lipid peroxidation. Gupta and Chari (2005) findings suggested that
lipid peroxidation increases with the increase in duration of diabetes and severity of retinopathy, which is in harmony with the present results. Also, Turk, et al. (2011) found correlation between malondialdehyde (MDA) and the development of diabetic retinopathy in patients with diabetes mellitus. Gender had no effect on the level of MDA in controls or any of the three diabetic groups. Both male and female diabetics and diabetic retinopathy patients had elevated MDA levels. The work of Mahboob, et al. (2005) coincides with the present results.

The present study showed a significant increase in the total NO$_3^-$ (end product of nitric oxide) in diabetic patients of both sexes compared to control healthy subjects. This result is in accordance with (Aydin, et al., 2001; Abou-seif and Youssef, 2004; Ramakrishna and Jaiiklni, 2008). Also, the results of Pacheco, et al. (2006) suggested that high glucose increases inducible nitric oxide synthase induction and subsequent increase in NO production by activating the protein kinase C II. The significant increase in NO metabolite levels in diabetic patients compared with healthy control is possibly due to the NO generating cells such as endothelial cells and macrophages found in inflammation, since these cells are also known to be involved in the pathogenesis of diabetes (Dogany, et al., 2002). To the contrary of the present study; Vanizor, et al. (2001) recorded significant decrease in the level of total NO$_3^-$ in poor glycemic control diabetic patients. They introduced two possibilities for this decline; the first is that the elevated glycosylated end-products in diabetic patients react with nitric oxide, resulting in NO inactivation. The second possible explanation may be that the effects of elevated glucose levels are exacerbated by increased aldose reductase activity leading to depletion of the NADPH required for the generation of nitric oxide from L-arginine by nitric oxide synthase.

Results pointed to a relation between the high level of total nitrates in all diabetics and the progression of diabetic retinopathy. This finding is supported by the findings of Doganay, et al. (2002) which showed that NO level was correlated with the stage of DR attributing this to the increased vascular permeability in the retina, and therefore the progression of the disease. Results of Maejima, et al. (2001) and Cicik, et al. (2003) concur with present results indicating the role of NO in the progression of DR. Concerning gender differences in total nitrates, no significant differences were observed in this study within each group that despite sex, all diabetics and diabetic retinopathy patients had high levels of total nitrates.

One of the most investigated mechanisms of apoptosis is the Fas system pathway which is composed of Fas Ligand (FasL), a type II transmembrane glycoprotein and Fas antigen (Fas/ APO-1/CD95), a type I transmembrane glycoprotein receptor. Cross-linking of Fas by FasL triggers apoptosis in various target cells (Guillot et al., 2001). The interaction of Fas with its ligand promotes the deletion of potentially harmful, damaged or unnecessary cells during the immune response. This interaction also regulates tissue remodeling and homeostasis. Impaired Fas-induced apoptosis results in abnormal cell proliferation and accumulation, whereas inappropriate expression or excessive Fas activity causes tissue damage (Stassi et al., 1997). Malfunction of Fas / FasL system can result in either survival of cells that should be eliminated (e.g., cancer) or inappropriate death of functioning cells (e.g., AIDS) and also affect critical cells such as the retinal pigment epithelium which would be vulnerable to elimination by apoptosis (Jiang et al., 2008). In the present study, sFas and sFasL were measured in plasma samples of diabetic patients with and without retinopathy and compared to those of control healthy subjects to investigate the involvement of Fas system in the progression of diabetic retinopathy.

Results showed significant increase in sFas in diabetic patients of both sexes compared with control subjects. This concurs with the results of Cosson, et al. (2005) which pointed to a dysregulation of Fas-mediated apoptosis in patients with type II diabetes. They hypothesized that the shear stress induced by diabetes stimulates apoptosis which may result in a dynamic process of tissue remodeling. Also, Guillot, et al. (2001) found that serum sFas levels in diabetics without complications were significantly increased when compared with normal controls offering two possible mechanisms to explain the increase in sFas. The first is a stimulation of the Fas gene yielding to sFas by alternative Fas mRNA splicing and the second mechanism is an over expression of the Fas receptor and consequently an increase in sFas. Gender had no effect on the activity of Fas in control or diabetic patients with and without retinopathy as shown by comparison of means within each group. This coincide with results of Choi (2006) suggesting that subject’s age or gender does not significantly influence serum Fas concentrations, at least in healthy individuals. Endothelial cell death is a hallmark of diabetic retinopathy that underlies the formation of acellular capillaries, lesions that produce irreversible retinal ischemia. Chronic low-grade endothelial cell death of early diabetes is repairable, but as diabetes progresses, the vascular endothelium reach its Hay flick number and can no longer repair its damaged endothelial lining. At this point, a cellular capillary formation ensues leading to transition to the proliferative stage of retinopathy (Joussen et al., 2003).

The observations introduced by Abu El-Asrar, et al. (2004) suggested that diabetes induces an apoptogenic environment in the retina and that the Fas death system and glial cells may be involved in the induction of cell death by apoptosis of ganglion cells. Few data about the role of Fas system in type II diabetic patients suffering from diabetic retinopathy were available in the reviewed literature.

In the present study, plasma sFas level of males and females in both retinopathy groups (NPDR and PDR) was significantly higher than that of the diabetics without complications as well as that of the control healthy group. Similar results were obtained by Moemen and El-Malt (2002) and Abdel Hamid and Helal (2003). Their
data suggested that apoptosis is involved in the initiation of diabetic retinopathy, and that plasma sFas level might be a predicting factor for diagnosis. Results of Gulliot, et al. (2001) proposed that the sFas serum level increase might appear before clinical symptoms in diabetic patients who will present diabetic complications later on. They also suggested that the increase in sFas may be a result of continuous activity of peripheral blood mononuclear cells remaining after insulin or inflammations that can overexpress Fas and secrete cytokines involved in cell injury or death. Other data suggested mechanistic role of Fas-FasL in the development of diabetic retinopathy and demonstrated that diabetes increases the susceptibility of the retinal vasculature to Fas-mediated apoptosis and death of retinal vascular endothelial cells resulting in blood-retinal barrier breakdown (Joussen et al., 2003).

Present results revealed no significant difference in plasma sFas among the two retinopathy groups (NPDR and PDR) proposing that sFas is not involved in the progression of retinopathy. Thus, although sFas could be involved in the onset of retinopathy as results indicate, yet present study did not reflect its participation in the progression of retinopathy if present. The mechanistic explanation offered by Joussen, et al. (2003) demonstrated that retinal vasculature are more susceptible to Fas-mediated apoptosis in diabetes and targeting the Fas/FasL pathway may prove beneficial in preventing the sight-threatening complications of diabetic retinopathy. In contrast, sFas serum level in the study of Gulliot, et al. (2001) was found to be normal in diabetics with predominant retinopathy which were attributed to the inability of the antibodies used in the study to recognize the sFas isoform released by pericytes or endothelial cells. Cosson, et al. (2005) did not find correlation between sFas and diabetes duration or retinopathy.

Concerning sFasL in the present study, the levels of plasma sFasL were either undetectable or very low in the control healthy group and this was the case for the rest of the tested groups. This might be due to cross linking of FasL with sFas and consequently its free soluble form not available in the sample or that the used antibodies are not appropriate for the detection of this protein, thus could not recognize the sFasL epitop present in the samples from patients and controls (Gulliot et al., 2001). Although in the study conducted by Cosson, et al. (2005) FasL was found to be undetectable in more than half the patients with diabetes versus none of the controls, yet their data detected lower sFasL in diabetics than in non-diabetics suggesting that sFasL but not sFas may be influenced by insulin resistance. In brief, the present study suggested that Fas-mediated apoptosis may be involved in type II diabetes and its complications represented here by diabetic retinopathy.

The present study showed increase in the SOD activity in diabetics compared with healthy controls which could be a body response to eliminate the load of oxidative stress as a consequence of hyperglycemia and scavenge the superoxides to reduce the oxidative damage. Several studies were in accordance with the present results (Palanduz et al., 2001; Seghrouchni et al., 2002; Turk et al., 2002; Cimbaljević et al., 2007). Contrarily, others found decrease in the SOD activity in diabetic patients (Pan et al., 2010; Abou -Seif and Youssef, 2004; Song et al., 2007; Goodarzi et al., 2008). Ramakrishna and Jailkhani (2008) concluded that the high level of glucose hamper the activity of enzymatic antioxidants. No significant gender difference was observed.

Regarding the GPx activity in male and female diabetic patients, present results did not show any significant difference when compared with healthy controls of both sex. This finding was in agreement with those of Walter, et al. (1991), Aydin, et al. (2001), Seghrouchni, et al. (2002) and Atli, et al. (2004). On the other hand, other reports detected significant decrease in GPx activity in diabetic patients (Palanduz et al., 2001; Memisogullari et al., 2003; Vericel et al., 2004; Goodarzi et al., 2008). The low GPx activity in diabetic patients detected by Ramakrishna and Jailkhani (2008) was explained by either low GSH content or enzyme inactivation under severe oxidative stress. To the contrary, Likidlilid, et al. (2010) detected significant increase in GPx activity in type II diabetic patients that were probably due to adaptive response to pro-oxidant in diabetic state. Gender did not affect activity of GPx in diabetic patients. Regarding diabetic retinopathy patients, the activity of both enzymes (SOD and GPx) in males and females was clearly diminished with a higher magnitude in PDR patients. The results of the present work are in consistent with those of other studies on antioxidant status in diabetic patients with retinopathy concerning SOD and/or GPx (Rema et al., 1995; Fahmy et al., 1996; Cai et al., 1998).

Although the results of Hartnett, et al. (2000) coincide with the present results, yet they found no association between the decrease in the enzymes activity and the severity of diabetic retinopathy. Gupta and Chari (2005) found that SOD decreases with the progression of the diabetic retinopathy, while GPx tends to increase in the later part of the disease. Other reports found significant elevation in the SOD activity in diabetics with retinopathy compared with diabetics without retinopathy (Turk et al., 2002; Siemianowicz et al., 2004) as well as in GPx activity (Rema et al., 1995). Some results did not reflect any significant difference among the enzymes level in diabetics, diabetic retinopathy and control subjects as those of Walter, et al. (1991) and Gurler, et al. (2000).

In the present study, the observed decline in the activity of both SOD and GPx with the progression of retinopathy, especially the SOD, could be attributed to their inability to cope with the burden of oxidative stress and excessive production of reactive species due to persistent hyperglycemia as well as the development of retinopathy. In other words, overproduction of reactive species was beyond their capability to overwhelm or that
the two enzymes are over-consumed during scavenging without adequate compensatory production, thus their activities decrease. Abou-Seif and Youssef (2004) attributed the decrease in SOD activity to possible three reasons; 1) overproduction of oxidants due to hyperglycemia, 2) increase in glycosylated SOD that lead to enzyme inactivation, and 3) loss of the two factors (Cu²⁺ and Zn²⁺) that are essential for the synthesis of the enzyme. Another reasonable explanation was given by Song, et al. (2007) that the observed decrease in SOD activity in diabetics was possibly associated with progressive glycation of this enzymatic protein by high level of glycated hemoglobin (HbA1c) resulting in the decreased activity of the enzyme.

The decrease of GPx activity with advanced diabetes and retinopathy might be related to the effect of high glucose level on the glutathione redox cycle. It is known that GPx catalyses the degradation of H₂O₂ to H₂O and O₂ in presence of glutathione as a cofactor and the regeneration of glutathione by glutathione reductase requires NADPH (Valko et al., 2007).

General Conclusion:

An overall conclusion drawn from the results of the present study and reviewed literature suggests that oxidative stress and impaired antioxidant defence is a feature in type II diabetes that is present early in the disease and there is growing evidence emphasizing the role of oxidative stress in the onset and progression of diabetes-related complications that is presented here by retinopathy, one of the most devastating complications of diabetes. The imbalance between oxidative and antioxidative system may trigger the cell and tissue damage occurring in the eyes of diabetic retinopathy patients as well as triggering lipid hydroperoxide production. In addition, oxidative stress might contribute in apoptosis (death) of retinal cells observed in retinopathy and a dysfunction in the Fas system might be involved in the process.

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