Changes of total antioxidant capacity and total oxidant status of aqueous humor in diabetes patients and correlations with diabetic retinopathy

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Abstract

• AIM: To measure changes of total oxidant status (TOS) and total antioxidant capacity (TAC) of aqueous humor (AH) in diabetic retinopathy (DR) patients, and to determine if there were any differences in TOS and TAC of AH in diabetic patients without retinopathy compared with non–diabetic patients.

• METHODS: One hundred and three eyes of 103 patients who were enrolled for cataract surgery were included in this study. Patients were grouped according to presence of diabetes and stage of DR. Prior to cataract surgery, 0.1mL to 0.2mL of AH was aspirated and analyzed for TAC and TOS level using a colorimetric method.

• RESULTS: TOS levels were highest among proliferative diabetic retinopathy (PDR) patients and lowest in patients with only cataracts. Results were statistically significant between all groups (P<0.05). Whereas result between diabetic without retinopathy patients and non – proliferative diabetic retinopathy (NPDR) patients was not statistically significant (P =0.757). TAC levels were highest in patients with only cataract and lowest among PDR patients and results were statistically significant between all groups (P<0.05).

• CONCLUSION: Aqueous humor TAC levels are low in diabetic patients and reduced further in DR patients, TOS levels are increased in diabetic patients and this is exacerbated in DR patients.

• KEYWORDS: aqueous humor; diabetic retinopathy; total antioxidant capacity; total oxidant status

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INTRODUCTION

Diabetic retinopathy (DR) is the leading cause of preventable blindness in working-aged people[1,2]. Major factors for DR progression are hyperglycemia, diabetes duration, hypertension, pregnancy and puberty [3]. Although there are clearly defined risk factors for DR progression, it is currently not possible to predict who will develop retinopathy or progress to advanced stages of the disease. Recognizing the potential role of oxidative stress in the progression of DR has changed the scope for predictive oxidative biomarkers and its ability to detect DR risk factors earlier in the disease process. There are many studies investigating the relationships between DR and oxidative stress. Despite these studies, there are still no sensitive markers that can predict or quantify total oxidant status (TOS) and total antioxidant capacity (TAC) in DR patients. This lack of reliable biomarkers prevents early, cost-effective measures to target those who might benefit from intensive diabetic therapy. It is important to note that, besides these classical antioxidants such as glutathione peroxidase, glutathione reductase and some metals (such as zinc, manganese and selenium) may also play an important role in the treatment of diabetes[4-7]. We believe that it is not relevant to investigate one by one analysis of antioxidants or metals, because their effects are
additive and may produce synergistic or antagonistic effects and may not be the most effective strategy when trying to predict a patients risk of developing DR. Therefore investigation of TOS and total TAC of the samples would be more relevant than the investigation of individual markers (such as glutathione peroxidase, glutathione reductase, zinc, manganese etc).

To our knowledge this is the first study, which investigates TOS and TAC levels of aqueous humor (AH) in DR patients and the relationship between these levels and retinopathy stages. Hence, the current study was undertaken to evaluate the role of oxidative stress status and antioxidant capacity of AH in DR progression.

SUBJECTS AND METHODS

Subjects The current study was performed at the Turkish Ministry of Health Ankara Ulucanlar Eye Education and Research Hospital Retina Clinic between July 2010 and March 2011. The eyes of 103 patients who were enrolled for cataract surgery were included in this study. All patients were Turkish Caucasians. All procedures were conducted in accordance with the Declaration of Helsinki and informed consent was obtained from all patients. The local medical ethics committee approved the study. All subjects received ophthalmologic examinations including: medical history, preoperative best-corrected visual acuity (BCVA), slit-lamp biomicroscopy with and without pupil dilatation, and applanation tonometry for intraocular pressure (IOP). Systemic diseases, duration of diabetes and fasting blood glucose level of patients were recorded. BCVA was checked with a Snellen chart and then converted to logMAR values. Fundoscopic examination was completed using Volk® +78D lens and if required fundus fluorescein angiography (FFA) was performed to determine the stage of retinopathy. If retinopathy was found, retinopathy stage was determined according to Early Treatment Diabetic Retinopathy Study (ETDRS) as no retinopathy, non-proliferative diabetic retinopathy (NPDR) or proliferative diabetic retinopathy (PDR).

Methods

Patient grouping Patients were grouped according to presence of diabetes and the stage of DR. Retinopathy was graded by single retina specialist prior to cataract surgery. For all patients nine fundus photographs were taken of each eye after maximum dilation in nine fields of gaze. If required FFA was performed. Every retinal lesion was assessed and retinopathy grading was performed according to ETDRS scoring system. All diabetic patients were type 2. Group 1 (control) was composed of 33 non-diabetic patients with cataracts. Group 2 was composed of 19 diabetic patients without retinopathy. Group 3 was composed of 25 NPDR patients and group 4 composed of 26 PDR patients. Inclusion criteria allowed for diabetic and non-diabetic patients diagnosed with senile cataracts. Exclusion criteria included: patients who with dense cataracts that hindered retinopathy staging, presence of macular edema, previous ocular surgery or laser photoocoagulation, type 1 diabetes, history of glaucoma, retinal vein occlusion, uveitis, and any other posterior segment pathologies.

Aqueous humor sampling Cataract patients received topical cyclopentolate HCl 1.5% (Sikloplejin®, Abdi Ibrahim, Turkey) and tropicamide 1.5% (Tropamid forte®, Bilim Drug, Turkey) prior to surgery. Nonsteroidal anti-inflammatory agents were not administered before all surgical procedures. In all cases, this was the patients' first intraocular surgical procedure. At the beginning of cataract surgery, 0.1mL-0.2mL of AH was aspirated with a 26 gauge insulin syringe followed by cataract surgery was carried out using standard techniques. No complication occurred in any step of AH sampling. Samples were stored immediately at -80°C until biochemical analysis.

Biochemical determinations of TOS and TAC Measurement of TAC and TOS levels of the AH was performed using a colorimetric method, which was first described by Erel [8]. Briefly, in this method, when a standard hydrogen peroxide solution is oxidized with free radicals, a yellow-brown color is produced. Therefore, antioxidants within the sample suppress the oxidation and color formation. This reaction is monitored by spectrophotometry and thus TAC can be indirectly measured. Similar to TAC measurement, oxidation of ferrous iron by oxidants in the sample causes a quantifiable color change allowing TOS measurement. Results were expressed as millimolar trolox equivalent per liter (mmolEqv./L) for TAC and micromolar hydrogen peroxide equivalent per liter (µmolL H₂O₂ Eqv./L) for TOS.

Statistical Analysis SPSS Version 15.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Statistical analysis included the difference in each parameter studied between groups. Spearman correlation test and analysis of variance ANOVA test were used for statistical analysis in the study. A two-tailed probability of 0.05 was considered statistically significant.

RESULTS

The eyes of 103 patients were included in this study. Forty-nine samples were from the right eye of patients and 54 from the left eye of patients. Forty-five patients were male and 58 were female. Gender distribution and eye lateralization were similar between the groups, and there was no significant difference in age and gender across groups (P>
Additionally, there were no significant differences in BCVA and IOP levels between groups (\( P > 0.05 \)). However, the duration of diabetes in patients was significantly different between groups (\( P < 0.05 \)) (Table 1).

TOS and TAC levels from the AH were compared between the four groups of patients. There was a linear relationship between TOS levels and the severity of RD in patients, with the lowest TOS levels recorded from group 4 (PDR patients) and highest TOS levels found at group 1 (control patients) (Figure 1A and Table 2). There were significant differences in TOS levels from the AH between all groups except between group 2 (diabetes, no retinopathy patients) and 3 (NPDR patients) (\( P < 0.05 \), between group 2-3 \( P = 0.757 \)). There was also a linear relationship in TAC levels as well, with the highest TAC levels observed in group 1 (control patients) and lowest levels observed at group 4 (PDR patients) (Figure 1B and Table 2). When mean TAC levels of groups were compared, they were significantly different between all groups (\( P < 0.05 \)). Mean TAC and TOS levels of groups were shown in Table 2.

Mean duration of diabetes at group 2 (diabetes, no retinopathy) was 7.74±7.30 (0.1-25) years, at group 3 (NPDR), diabetes duration was 8.20±6.13 (4-30) years and at group 4 (PDR) it was 16.26±6.87 (6-30) years. When diabetes duration was compared, there was a significant difference between groups (\( P < 0.001 \). We also went on to determine if there was a correlation between the duration of diabetes and TOS/TAC levels in the AH. Indeed, there was positive correlation between duration of diabetes and TOS levels (\( P < 0.001 \), \( R^2 = 0.400 \)) and negative correlation between duration of diabetes and TAC levels (\( P < 0.05 \), \( R^2 = 0.526 \)).

**DISCUSSION**

In the current study, we have shown that AH TOS was increased in diabetic patients when compared to patients with only cataracts. In addition, the increase in TOS was even more marked in diabetic patients with retinopathy. Aqueous humor TAC was decreased in diabetic patients, which was also exacerbated in diabetic patients with retinopathy, when compared to patients with cataracts alone. Yokoi et al.\(^{9}\) have showed that increased oxidative stress and decreased TAC vitreous levels in diabetic patients may play role in diabetic retinopathy pathogenesis via induction of vascular endothelial growth factor (VEGF). It was shown that oxidative stress increases advanced glycation end-products formation and these products increase level of VEGF, which is an oxidative stress marker and has a role in

![Figure 1 TOS and TAC levels of groups](image-url)

**Table 1  Demographic data and clinical properties of participants**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (a)</td>
<td>67.7±11.2</td>
<td>66.1±11.8</td>
<td>64.8±9.5</td>
<td>62.4±8.6</td>
</tr>
<tr>
<td>Gender, n (%)</td>
<td>M 15 (45.5)</td>
<td>7 (36.8)</td>
<td>11 (44)</td>
<td>12 (48)</td>
</tr>
<tr>
<td></td>
<td>F 18 (54.5)</td>
<td>12 (63.2)</td>
<td>14 (56)</td>
<td>13 (52)</td>
</tr>
<tr>
<td>¹BCVA (logMAR²)</td>
<td>1.072±0.57</td>
<td>0.39±0.72</td>
<td>1.41±0.66</td>
<td>1.84±1.02</td>
</tr>
<tr>
<td>³IOP (mmHg)</td>
<td>14±3.42</td>
<td>19.4±3.89</td>
<td>16.80±3.76</td>
<td>16.19±3.21</td>
</tr>
<tr>
<td>Duration of diabetes (year)</td>
<td>-</td>
<td>7.74±7.30</td>
<td>8.20±6.13</td>
<td>16.26±6.87</td>
</tr>
</tbody>
</table>

¹Best corrected visual acuity; ²Logarithm of the minimum angle of resolution unit; ³Intraocular pressure.

**Table 2  Mean TAC and TOS levels**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>33</td>
<td>19</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td>¹TOS (μmol H₂O₂ Eqv./L )</td>
<td>31.26±20.64</td>
<td>75.91±37.70</td>
<td>79.56±33.82</td>
<td>95.05±25.78</td>
</tr>
<tr>
<td>²TAC (mmol Eqv./L)</td>
<td>2.54±0.71</td>
<td>2.02±0.65</td>
<td>1.46±0.55</td>
<td>0.88±0.65</td>
</tr>
</tbody>
</table>

¹Total oxidant status; ²Total antioxidant capacity.
neovascularization in advanced diabetic retinopathy rats [9]. Sone et al. [10] studied VEGF in the ocular tissues of diabetic rats and they showed increased level in the AH. Nitric oxide is also an oxidant molecule that contributes to TOS of a sample by producing unstable toxic chemicals, such as superoxide [11]. Chiou et al. [12] showed higher levels of nitric oxide in the AH of diabetic patients, and emphasized that control of nitric oxide levels in ocular tissues of diabetes may decrease angiogenesis and neovascularization. Chakrabarti et al. [13] studied on diabetic rats and their findings suggest the potential role of endothelin-1, a pro-inflammatory and oxidative vasoconstrictor mediator in DR. They concluded that vasoactive factors such as endothelin-1, VEGF and nitric oxide, on account of their multi-functional capabilities, may play significant roles as effector molecules in mediating the pathogenesis at early changes in DR [13]. These studies do not represent the TOS of diabetic patients since they focused on one or two mediator of oxidation and thus, show changes only in a few oxidative markers. At this point, it is unknown how much endothelin-1, VEGF and nitric oxide contribute to the TOS of a sample. To date, there are no studies that show a correlation between these markers and TOS, but should be studied in the future. Turk et al. [14] studied serum levels of superoxide dismutase, catalase activities and thiobarbituric acid reactive substance levels in type 2 diabetic patients and found increased superoxide dismutase and thiobarbituric acid reactive substance levels and decreased serum catalase levels. They elucidated this difference as compensation mechanism of the body. Many studies have worked on markers of oxidative stress in diabetic patients. However, most of these studies have measured only one marker of oxidative stress, generally from the serum of diabetic patients. It is well known that markers of oxidative stress often work together and their effects may be synergistic [15]. Therefore, we suggest that showing total amount of oxidative markers will ultimately be more relevant for the treatment of diabetic patients. Also, showing the oxidative status of AH rather than serum may be more relevant. In the current study, we analyzed both TOS and TAC levels of AH in diabetic patients and found significant differences in both when compared to non-diabetic controls. TAC levels were decreased, while TOS levels were increased in DR patients. Most striking was the finding that as DR progressed, TOS increased and TAC decreased. The highest levels of TOS in AH were found in PDR patients while the lowest TOS levels were in non-diabetic controls. In contrast, the lowest TAC levels were found in PDR patients and the highest TAC levels were in non-diabetic controls. In a study by Memisogullari et al. [16], serum glutathione and glutathione peroxidase levels were shown as significantly decreased in diabetic patients. Lamont et al. [17] have shown a positive correlation between TAC levels and antioxidants including glutathione, albumin, uric acid, bilirubin and creatinine. Importantly, TAC is a measure of the antioxidant capacity of all antioxidants in a biological sample and not of a single compound. While, correlation of TAC with individual antioxidants should also be elucidated. The relationship between TAC and individual antioxidants was studied by Nemec et al. [18], and they reported a trend for a positive correlation between TAC and albumin, vitamin E and lipid standardized vitamin E in serum from beagles. Additionally, they found a trend toward a negative correlation between TAC levels and bilirubin, vitamin A and β-carotene. Erel's group has previously reported a correlation between serum TAC levels and different antioxidant molecules including: vitamin C, vitamin E, bilirubin and proteins, which are main components of serum by the Erel method. They found that proteins account for almost half (48.89%) of the total antioxidant response in serum [19]. Additionally, they measured the estimated contribution of each of these molecules to the total antioxidant response of serum. Thus measuring TAC can give a sample's overall antioxidant status, which may include known antioxidant molecules effects or those of antioxidants not yet determined. In the current study, we measured TOS and TAC levels from AH by the Erel method. There are different methods to measure TOS and TAC, and the majority of these methods have analytical problems and technical restrictions. The Erel method is a sensitive and reliable method that is highly reproducible with strong linearity, and utilizes newly developed ferrous ion-o-dianisidine complex [20,19]. There are a large number of studies measuring TAC and TOS by this method [20-25]. However, there are no studies which have used this method to show TAC and TOS of AH. This method also has some disadvantages. ABTS [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical used to analyze antioxidant molecule has high extinction coefficient that limits the measurement of too little antioxidant molecules. We do not know if this method capable of measurement of AH accurately. Further studies on healthy subjects or with large groups should be initiated to show whether this method accurately tests TAC and TOS of AH or not. Correlations of TOS and TAC with some individual oxidant/antioxidant markers (such as VEGF, nitric oxide and endothelin-1) should also be clarified. To our knowledge this is the first study to demonstrate AH TOS and TAC levels according to retinopathy stages. AH TOS levels increased as DR progressed while TAC levels
decreased. Yoshida et al.\(^{[26]}\) compared AH TAC between patients with PDR and uveitis. They demonstrated that AH antioxidant capacity was lower in PDR patients than uveitis patients. In our current study, we used a control group (non-diabetics with cataracts) and patients presenting at various stages of DR, which allowed us to analyze patients according to grade of retinopathy. We showed that as DR progressed, TAC level decreased. Moreover, a recent study of type 1 diabetics suggested that antioxidant therapy with vitamin E might normalize diabetic retinal hemodynamics\(^{[27]}\). Therefore, it may be important to further investigate TAC levels of AH in diabetics in future antioxidant treatment studies. Hartnett et al.\(^{[28]}\) also studied serum levels of TOS and antioxidant levels; superoxide dismutase and glutathione peroxidase in DR patients. They showed a positive correlation between oxidative stress in DR and negative correlation between glutathione peroxidase and superoxide dismutase levels in DR. These results are in agreement with the results from our study. However, they did not find any statistically significant difference between duration of diabetes and DR. There are many studies in the literature demonstrating cataract formation increases AH oxidant status and decreases AH antioxidant capacity \(^{[28-30]}\). All patients in our study had cataracts. Thus, we aimed to compare the TOS and TAC levels of AH in diabetic and non-diabetic otherwise healthy patients with cataracts. Our results demonstrate that diabetes increases TOS and decreases TAC levels in cataract patients. Thus, the differences in TOS and TAC levels are related to diabetes status rather than the cataracts themselves. Hashim et al.\(^{[31]}\) studied serum oxidant and antioxidant markers in senile and diabetic cataract patients. Similar to our findings, they showed increased oxidative stress markers and decreased antioxidant markers in diabetic cataracts, which is concordant with our study. In the current study it was clear that diabetic cataract patients had lower AH antioxidant status than non-diabetic cataract patients. Our findings demonstrate that diabetic patients have an increased AH oxidant level and a decreased antioxidant capacity with retinopathy progression. These results suggest an imbalance between free radical generation and antioxidant defense which may play a role in the progression of DR. Correlation analysis reveals that the extent of oxidative stress is an imbalance in the levels of oxidants and antioxidants, which is related to the severity and duration of diabetes. Based on our findings, it is evident that diabetic patients have higher oxidative stress in AH than non-diabetic patients. In addition, diabetic patients in later stages of retinopathy progression, TOS levels are concurrently increasing. It is noteworthy that besides the stage of retinopathy, the duration of diabetes was also related to the oxidative status in these patients. AH oxidative stress was positively correlated with longer duration of diabetes. In conclusion, free radical formation along with antioxidant deficiency in diabetes mellitus increases over time and may play an important role in the development of DR, which is an important complication of the disease. Further work is needed to elucidate whether there exists an association between antioxidant nutrient intake and reduction in the development of diabetic complications particularly retinopathy.

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