

# Evaluation of Total Oxidant Status, Total Antioxidant Capacity and Oxidative Stress Levels in Humor Aqueous of Diabetic Cataract Patients

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## ABSTRACT

**Purpose:** Diabetic retinopathy (DRP) is one of the most common complications of diabetes mellitus and various studies have demonstrated the role of increased oxidative stress due to hyperglycemia in its pathophysiology. There are few studies in the literature examining total antioxidant capacity (TAC) and total oxidant status (TOS) in humor aqueous, and we were unable to find any, which measured oxidative stress index (OSI). The aim of this study was to investigate whether TAC, TOS, and OSI in the aqueous humor changed according to DRP stage.

**Materials and Methods:** This prospective study included a total of 86 cataract patients: 31 healthy patients with senile cataract (Group 1), 19 diabetic patients without DRP (Group 2), 17 patients with nonproliferative DRP (Group 3), and 19 patients with proliferative DRP (Group 4). Aqueous humor samples were obtained prior to cataract surgery. TAC, TOS, and OSI levels were measured spectrophotometrically and compared between the groups.

**Results:** The analysis of TAC levels revealed that TAC levels were significantly higher in the control group (Group 1) compared to DRP patients (Group 3 and 4,  $p=0.042$  and  $p=0.02$ , respectively), while TOS and OSI levels were lower in Group 1 compared to all diabetic groups (Group 2, 3, and 4) ( $p<0.05$ ).

**Conclusion:** The findings of increased TOS and OSI and decreased TAC levels in diabetic patients support the belief that oxidative stress might be an important etiologic factor in DRP. Increased oxidative stress may be a potential therapeutic target in the prevention and management of DRP.

**Key Words:** Aqueous humour, Diabetic retinopathy, Oxidative stress index, Total antioxidant capacity, Total oxidant status.

## INTRODUCTION

Oxidants are unstable and highly reactive molecules with short half-life. Under normal physiological circumstances, they are required for the maintenance of vital functions such as host defense, cellular proliferation, gene expression, and signal transduction.<sup>1</sup> However, excessive production or inadequate elimination of oxidant molecules gives rise to oxidative stress.<sup>2</sup> Increased oxidative stress causes destruction and senescence in various tissues and cells.<sup>3</sup> Numerous studies have elucidated that oxidative stress contributes to the development and progression of various ocular diseases such as DRP, cataract, glaucoma, age-related macular degeneration, dry eye syndrome, keratoconus, and pseudoexfoliation syndrome.<sup>4,5</sup>

Diabetic retinopathy (DRP) is one of the major microvascular complications of diabetes and is the most common cause of blindness worldwide.<sup>6</sup> Recently, the affiliation between diabetic retinopathy and oxidative stress is being researched intensely and researchers have demonstrated that diabetic hyperglycemia induces oxidative stress by increasing the formation of reactive oxygen species (ROS) and advanced glycation end-products (AGEs) in the blood and other tissues.<sup>7,8</sup> Increased oxidative stress disrupts retinal cell and blood-retina barrier function and induces apoptosis in retinal capillary endothelial cells, retinal neurons, and glia cells.<sup>9,10</sup>

In this study we aimed to investigate the relationship between diabetic retinopathy and oxidative stress in

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terms of humor aqueous total oxidant status (TOS), total antioxidant capacity (TAC) and Oxidative stress index (OSI) levels which were measured with an automated modern method that represents the overall activity of oxidant and antioxidant molecules present in the humor aqueous.

## MATERIALS AND METHODS

The study was conducted after receiving approval from the Selcuk University Medical Faculty Ethics Committee (number 2014/219). Patients who were scheduled to undergo cataract surgery were included and informed consent forms were obtained from all patients.

A total of 86 cataract patients were enrolled in our study. Personal data regarding systemic and ocular diseases, medications used and smoking history were recorded. Ophthalmologic examination included visual acuity assessment, intraocular pressure measurement, and anterior segment and fundus examinations. All patients with diabetes were examined with Spectralis OCT (Spectralis®, Heidelberg Engineering, Heidelberg, Germany) for macular edema. Patients with signs of DRP on fundus examination underwent fundus fluorescein angiography (Carl Zeiss Meditec FF450 Retinal Imaging Fundus Camera) prior to cataract surgery. Patients with DRP were classified according to the Early Treatment Diabetic Retinopathy Study (ETDRS) criteria.

Patients with inflammatory and rheumatologic systemic diseases (rheumatoid arthritis, sarcoidosis, etc.), smoking habit, complicated cataract, previous or active ocular disease other than cataract, antioxidant vitamin and mineral supplementation, or malignant tumors were excluded from the study.

The cataract patients were divided into 4 groups:

Group 1: Control group without any systemic disease (n:31),

Group 2: Diabetic patients without DRP (n:19),

Group 3: Diabetic patients with non-proliferative DRP (n:17) and

Group 4: Diabetic patients with proliferative DRP (n:19)

On the day of surgery, the pupils were dilated by instilling one drop each of cyclopentolate, tropicamide, and phenylephrine drops prior to the surgery.

**Sample collection:** At the beginning of the operation, a side port was created using a 20-gauge MVR blade and a 27-gauge cannula was used to obtain 0.10-0.15 mL anterior chamber samples which were immediately frozen

at -80 °C. If this volume of sample could not be obtained, the patients were not included in the study.

**Total Antioxidant Capacity (TAC) Measurement:** The TAC assays were performed with the commercial diagnostic kit (RL0017 coded kit, Rel Assay Diagnostics, Gaziantep, Turkey) according to the colorimetric method described by Erel.<sup>13</sup> The antioxidants in the sample reduce the dark blue-green ABTS radical to the reduced, colorless ABTS form. The absorbance change reflects the total antioxidant level of the sample. The results are expressed in terms of mmol Trolox equivalent per liter (Trolox equiv./L).

**Total Oxidant Status (TOS) Measurement:** The TOS assays were performed by the Erel's method using the commercial diagnostic kit (RL0024 coded kit, Rel Assay Diagnostics, Gaziantep, Turkey). Oxidant molecules in the sample oxidize the ferrous ion complex to ferric ion and the colorimetric change occurring after this reaction is measured spectrophotometrically and is related to the level of the oxidant molecules in the sample. The results are expressed in terms of micromolar hydrogen peroxide equivalent per liter ( $\mu\text{mol H}_2\text{O}_2$  Equiv./L).<sup>14</sup>

Those methods are based on the spectrophotometrically measurement of the colorimetric changes occurring after the oxidation or reduction reaction in the sample. The color changes correlate with the total oxidant and antioxidant molecules level in the sample. The aqueous humor samples stored at -80 °C were analyzed using the above mentioned colorimetric assays with an Abbott Architect C16000 (Japan) fully automated biochemistry autoanalyzer in the Selcuk University Faculty of Medicine Biochemistry Department.

**Oxidative Stress Index (OSI) Measurement (Arbitrary Unit):** OSI was obtained by dividing TOS by TAC using the following equation:

$$[\text{TOS } (\mu\text{mol H}_2\text{O}_2 \text{ equiv./L}) \div \text{TAC } (\mu\text{mol Trolox Equiv./L}) \times 100]$$

## Statistical Analysis

Statistical Package for the Social Sciences (SPSS) software version 18.0 for Windows was used for statistical analyses. To compare multiple means, ANOVA test and Bonferroni test were used for variables showing normal distribution and the non-parametric Kruskal-Wallis test was used for variables with non-normal distribution. Comparisons between two independent groups were made using Mann-Whitney U test. Spearman correlation analysis was used to evaluate correlations between the parameters. The results were assessed within a 95% confidence interval and significance was accepted at  $p < 0.05$ .

## RESULTS

The participants ranged in age between 44 and 107 years (mean age  $66.48 \pm 11.31$  years); 41 (47.7%) were male and 45 (52.3%) were female. The mean ages of male and female patients were  $64.4 \pm 9.4$  years and  $68.4 \pm 12.6$  years, respectively. There were no significant differences in age or gender between the groups ( $p > 0.05$ ) (Table 1).

We observed an incremental decrease in mean TAC values from Group 1 to Group 4. Although the difference between Groups 1 and 2 was not significant ( $p > 0.05$ ), there were significant differences between Group 1 and the DRP groups (Groups 3 and 4) ( $p = 0.042$  and  $p = 0.002$ , respectively) (Table 2).

Mean TOS values in Group 1 were significantly lower than in the diabetic groups (Groups 2, 3, and 4) ( $p = 0.04$  and  $p < 0.001$  and  $p < 0.001$ , respectively). However, despite an increase in TOS with disease severity, the differences between Groups 2, 3, and 4 were not statistically significant ( $p > 0.05$ ) (Table 3).

Mean OSI values in Group 1 were also significantly lower than those in the diabetic groups (Groups 2, 3, and 4) ( $p = 0.04$ ,  $p = 0.004$ , and  $p < 0.001$ , respectively). The difference in mean OSI between Groups 2 and 3 was not significant ( $p > 0.05$ ) (Table 4).

There were no significant differences between Groups 2, 3, and 4 in TAC, TOS, or OSI values ( $p > 0.05$ ).

**Table 1.** Gender, age, HbA1c level, and diabetes duration of the study groups.

	Group 1 (n=31)	Group 2 (n=19)	Group 3 (n=17)	Group 4 (n=19)	p value
Gender (M/F)	14/17	11/8	7/10	9/10	$p > 0.05$
Age (years) Mean $\pm$ SD	$65.1 \pm 11.3$	$68.4 \pm 16.7$	$65.6 \pm 8.9$	$67.4 \pm 6.5$	$p > 0.05$
HbA1c (%) Mean $\pm$ SD		$7.57 \pm 1.98$	$9.39 \pm 2.6^*$	$8.69 \pm 1.15$	
Duration of Diabetes (years), Mean $\pm$ SD		$8.69 \pm 1.15$	$12.88 \pm 5.4^*$	$14.84 \pm 6.2^*$	

\* Significantly higher than Group 2 ( $p < 0.05$ ).

**Table 2.** Mean TAC levels (TAC unit: mmol/L) of the study groups and comparison between groups (Kruskal-Wallis test).

TAC	N	Minimum	Maximum	Mean	Std. Deviation	Comparison
Group 1	31	1.1	3.2	2.23	0.481	Group 1-2: $p = 0.113$ Group 1-3: $p = 0.042$ Group 1-4: $p = 0.002$ Group 2-3: $p = 0.661$ Group 2-4: $p = 0.223$ Group 3-4: $p = 0.639$
Group 2	19	0.9	3.4	1.984	0.596	
Group 3	17	0.6	2.7	1.852	0.572	
Group 4	19	1.2	2.7	1.784	0.407	

**Table 3.** Mean TOS levels (TOS unit:  $\mu\text{mol/L}$ ) of the study groups and comparison between groups (Kruskal-Wallis test).

TOS	N	Minimum	Maximum	Mean	Std. Deviation	Comparison
Group 1	31	7.98	21.98	10.756	3.801	Group 1-2: $p = 0.041$ Group 1-3: $p < 0.001$ Group 1-4: $p < 0.001$ Group 2-3: $p = 1.00$ Group 2-4: $p = 0.981$ Group 3-4: $p = 1.00$
Group 2	19	8.28	28.11	15.477	6.661	
Group 3	17	8.5	31.89	17.572	7.215	
Group 4	19	8.41	33.41	18.043	6.252	

**Table 4.** Mean OSI levels of the study groups and comparison between groups (Kruskal-Wallis test).

OSI	N	Minimum	Maximum	Mean	Std. Deviation	Comparison
Group 1	31	0.26	1.1	0.504	0.185	Group 1-2: $p = 0.041$ Group 1-3: $p = 0.004$ Group 1-4: $p < 0.001$ Group 2-3: $p = 0.244$ Group 2-4: $p = 0.981$ Group 3-4: $p = 0.754$
Group 2	19	0.25	3.12	0.913	0.692	
Group 3	17	0.34	4.59	1.21	1.071	
Group 4	19	0.38	2.57	1.085	0.529	

Age, duration of diabetes, and HbA1c level were not significantly correlated with TAC, TOS, or OSI values ( $p>0.05$ ).

There was no significant difference between the TAC, TOS, and OSI values of diabetes patients using insulin and those using oral antidiabetic drugs ( $p>0.05$ ).

## DISCUSSION

In diabetic retinopathy patients, oxidative stress has been reported to affect almost all ocular layers including retinal pigment epithelium, retinal cells, vitreous and lens. In our study, it was shown that oxidative stress index is significantly higher even in the humour aqueous. According to our results, TOS and OSI were significantly higher in diabetic patients than those in healthy controls, regardless of the presence of DRP, and TAC was significantly lower in both proliferative and non-proliferative DRP patients compared to the otherwise healthy cataract patients in the control group. We were unable to find any studies in the literature that measured OSI in the aqueous humor of diabetic patients.

Mitochondrial ROS production has been shown to increase in hyperglycemia. The mitochondrial abnormalities are irreversible, and mitochondrial ROS production continues even after normoglycemia is restored. This phenomenon is referred to as the 'metabolic memory' or 'legacy effect'.<sup>11</sup> In vitro experiments have shown that retinal cells and retinal pericytes subjected to hyperglycemia continue to overproduce ROS even in the normoglycemic environment.<sup>1</sup> These findings indicate that in diabetic patients,

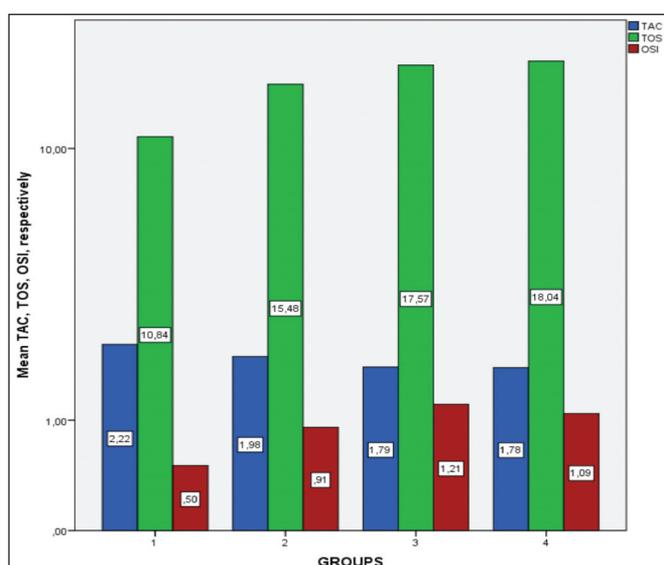
irreversible changes in tissues due to hyperglycemia lead to increased oxidative stress and trigger the development and progression of various complications of diabetes.

Oxidative stress is well known to be closely associated with diabetic complications, however it is not yet fully understood whether oxidative stress is a cause or a consequence of diabetes.<sup>12</sup> Oxidative stress has been shown to induce diabetes by inducing apoptosis in pancreatic  $\beta$  cells and play a role in the formation of insulin resistance in tissues. Hyperglycemia has also been shown to induce oxidative stress in diabetic patients. The resulting vicious cycle accelerates the emergence of diabetic complications.<sup>3</sup> However, the precise mechanism by which oxidative stress may contribute to the development of diabetic complications has not been identified.<sup>7,9</sup>

In the literature there are some conflicting findings regarding oxidant and antioxidant substance levels in the eyes of the diabetic retinopathy patients, some studies put forward that oxidative stress markers are elevated in the ocular structures while other studies claim the opposite. Our study indicates that oxidative stress is closely related with the diabetic retinopathy, and the antioxidant levels were found to be lower in the diabetic patients. The antioxidant molecules rapidly deplete in the face of increased oxidative stress.

A variety of enzymatic and non-enzymatic antioxidant defense mechanisms are available in the eye. In several studies, levels of many antioxidant molecules such as ascorbic acid, glutathione, uric acid, glutathione peroxidase, catalase etc. were evaluated and some contradictory results were obtained.<sup>13</sup> However, the retina and other ocular structures has complex antioxidant defense mechanisms, some of which can not be measured quantitatively. Mancino et al. stated that measuring each antioxidant separately is difficult and that the level of each antioxidants will vary in different patients depending on their disease stage and the chemical structure of the antioxidant. Therefore, they claimed that TAC, which measures the total capacity of all antioxidants, is more reliable.<sup>14</sup>

A study in which lipid peroxidation (LPO) levels and antioxidant capacity levels in humour aqueous of diabetic cataract patients were evaluated, LPO levels were found to be higher and antioxidant capacity levels to be lower in aqueous humoral samples of diabetic cataract patients than in the control group, but the difference was not significant.<sup>14</sup> While that study only assessed lipid peroxidation levels as oxidative stress markers, in our study we examined TOS, which represents the total level of oxidative molecules. It has been reported that measuring only one or more oxidant molecules separately does not accurately indicate



**Figure 1.** Total antioxidant capacity (TAC), total oxidant status (TOS), and oxidative stress index (OSI) mean levels of the study groups (TAC: mmol Trolox equiv./L,  $\mu\text{mol H}_2\text{O}_2$  Equiv./L).

a patient's total oxidant levels. Oxidant molecules can have synergistic interactions and, depending on this synergism the total oxidative effect of a sample might be higher than the sum of the individual effects of oxidant molecules, that's why measurement of TOS is easier and more reliable.<sup>15</sup>

Chronic hyperglycaemia induces overproduction of AGEs and leads to structural and functional disorders by causing glycation in proteins. AGEs are associated with fibrosis, deterioration of cell signaling mechanism, vascular dysfunction and increase of inflammation, oxidative stress, VEGF expression and platelet aggregation.<sup>16, 17</sup> Yokoi et al. found that TAC in vitreous samples obtained from DRP patients were significantly lower and Advanced Glycation End Products (AGEs) levels were significantly higher than those in the control group. There was a significant positive correlation between the vitreous AGEs and VEGF levels, while both AGE and VEGF levels were inversely correlated with the total antioxidant status.<sup>16</sup>

In a study by Hashimoto et al., Vitreous TAC levels were found to be significantly lower in patients with proliferative diabetic retinopathy and retinal venous occlusion than epiretinal membrane and macular hole patients.<sup>18</sup> Mancino et al. reported that patients with proliferative DRP exhibited lower TAC in the vitreous and aqueous humor compared to the nonproliferative DRP and control groups.<sup>14</sup> In another study, there was no significant difference in aqueous humor TAC in the proliferative DRP group compared to the control group. However, the patients with proliferative DRP had significantly higher levels of the oxidant molecule nitric oxide (NO) in humor aqueous.<sup>19</sup> NO is a pro-inflammatory and pro-oxidant molecule. In animal studies, reactive NO end-products (Nitrite and Nitrate) levels were found to be higher in diabetic rat retinas. NO derivatives elevate vascular leukocyte adhesion, trigger the breakdown of the blood-retina barrier and the progression of DRP.<sup>20</sup>

In a study by Yoshida et al. Pigment epithelium-derived factor (PEDF, a molecule found in the retinal pigment

epithelial cells with anti-inflammatory, anti-oxidant and anti-angiogenic functions) levels and TAC levels in humor aqueous were found to be significantly lower in patients with proliferative DRP (n=9) compared to uveitis patients (n=34).<sup>21, 22</sup> However, the number of diabetic patients in that study was low and uveitis cases were used as a control group instead of healthy individuals.

Kirboga et al. reported no significant difference between proliferative DRP patients and a healthy control group in terms of aqueous humoral TAC, TOS, and total thiol levels.<sup>22</sup> However, in this study, diabetic retinopathy was not evaluated according to the stages as in our study. In contrast to our findings, Cunha et al. found that antioxidant power in aqueous humoral samples of patients with DRP were higher than in diabetic patients without retinopathy and healthy controls.<sup>23</sup> In this study, classical methods were not used as an antioxidant capacity measurement method. In their method ROS production in phorbol dibutyrate (PDB, a protein kinase C activator)-stimulated granulocytes from diabetic patients with or without retinopathy was inhibited by autologous aqueous humor. This inhibition was measured spectrophotometrically and estimated as an antioxidant capacity marker.

Our study revealed higher TOS and OSI in diabetic patients and decreased TAC in DRP patients, supporting the theory that oxidative stress might be an important factor in the progression of DRP. However, the limited number of the study population is the weakness of our study. Mechanisms for breaking the vicious cycle of oxidative stress in diabetic patients may lead to significant reductions in the development and progression of DRP and other diabetic complications. More advanced and extensive studies are needed to identify such mechanisms.

#### **Disclosure**

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