Oxidative DNA Damage in the Human Trabecular Meshwork

Clinical Correlation in Patients With Primary Open-Angle Glaucoma

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Objective: To evaluate the intensity of oxidative molecular damage and its clinical correlations: visual field damage, intraocular pressure, age, and disease duration.

Methods: DNA was extracted from human trabecular meshwork specimens collected from 17 glaucoma-affected patients using standard filtration surgery. Twenty-one specimens from healthy eyes collected for cornea transplants serve as controls. Oxidative DNA damage was evaluated by determining 8-hydroxy-2'-'deoxyguanosine levels. All patients underwent a Humphrey 30-2 visual field examination and diurnal tonometry before surgery.

Results: The mean±SD DNA oxidative damage was 8.51±5.44 and 1.75±1.80 8-hydroxy-2'-'deoxyguanosine molecules/10^5 normal nucleotides in patients with glaucoma and controls, respectively. A statistically significant correlation was found among human trabecular meshwork DNA oxidative damage, visual field damage, and intraocular pressure. No other statistically significant correlations were found.

Conclusions: Oxidative stress may represent an important pathogenetic step in primary open-angle glaucoma because it could induce human trabecular meshwork degeneration, favoring an intraocular pressure increase, thus priming the glaucoma pathogenetic cascade.


The trabecular meshwork represents the "key" region in glaucoma pathogenesis, its alterations resulting in increased resistance of the outflow system. In the primate eye, approximately 75% of the resistance to aqueous humor outflow resides in the tissues between the anterior chamber and the lumen of the Schlemm canal. In particular, the human trabecular meshwork (HTM) is a specialized eye tissue that regulates the aqueous humor outflow and controls intraocular pressure (IOP). The HTM has been interpreted as a biological filter to be transited by the aqueous humor to reach the Schlemm canal. More recently, it has been established that HTM cells are essential for maintenance of the whole outflow system homeostasis, thus recognizing the possible importance of HTM cell damage in the pathogenesis of glaucoma. Although many researchers suggest that glaucoma is the consequence of an ischemic injury, it is recognized that IOP has a pathogenetic role toward glaucomatous neuropathy, possibly by inducing the formation of oxidative free radicals. A previous study in patients with open-angle glaucoma provided evidence of the increased oxidative DNA damage in patients with glaucoma and its relationship to the lack of the GSTM1 gene, which catalyzes the neutralization of free radicals by their conjugation with glutathione.

In the present study, we examine the levels of 8-hydroxy-2'-'deoxyguanosine (8-OH-dG), a fundamental molecular biomarker of oxidative damage that can be detected in the DNA of HTM specimens from patients with primary open-angle glaucoma (POAG). We also evaluated the relationship between 8-OH-dG and the following clinical data: IOP, visual field (VF) damage, and disease duration. The aim of the present study is to explore whether oxidative stress is involved in the pathogenesis of glaucoma, thus supporting the hypothesis that HTM DNA oxidative damage affects the depletion of the trabecular cell population.

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METHODS

The ethics board of the Department of Neurosciences, Ophthalmology, and Genetics, Clinica
Oculistica, Genoa University, approved this study. Seventeen patients (9 women and 8 men) with POAG aged 50 to 82 years (mean ± SD age, 69.87 ± 11.08 years) are included. All enrolled patients provided informed written voluntary consent. Disease duration was 6 to 19 years. The inclusion criteria required the presence of POAG with no tonometric compensation and the absence of the pseudoxefoliation syndrome, diabetes mellitus, uveitis, systemic collagenopathy, and objective neurologic signs. In all patients undergoing filtration surgery, the IOP values were greater than 25 mm Hg, with maximal pharmacologic therapy at 2 or more measurements. The presence of typical perimetric glaucomatous defects, at least in the fellow eye, were examined using reliable computerized VF testing by determining the occurrence of 1 of the following situations: (1) 2 contiguous points with a 10-dB loss or greater in the superior or inferior Bjerrum area compared with perimeter-defined age-matched controls, (2) 3 contiguous points with a 5-dB loss or greater in the superior or inferior Bjerrum area, or (3) a 10-dB difference across the nasal horizontal midline in 2 or more adjacent locations. All patients underwent a Humphrey 30-2 VF examination 12 to 27 days before surgery.

To evaluate VF damage, 3 different staging systems were used. The VF map of every patient was evaluated by 2 of us (S.C.S. and P. Caprio) so that each judgment would be given in the most independent way. The VF defect score was assigned according to the Aulhorn “frequency distribution of glaucomatous visual field defects” criteria,10 the Brussini Glaucoma Staging System,11 the Hodapp VF staging criteria,11 and the mean deviation VF index.12

The mean defect VF index allows precise evaluation of the total loss of light sensitivity independent of the shape of defects. To give more consistent weight to the morphologic features of VF damage, the Glaucoma Staging System was applied to take into account the correct pattern standard deviation index, which is the marker of the heterogeneity of VF sensitivity, more suggestive of glaucomatous damage. The Hodapp staging system is more sensitive for the topography of VF damage and particularly for defects near the fixation point. The Aulhorn criteria, originally created for kinetic perimetry, were transferred on the automatic static perimetry to specifically evaluate the shape of the defects.

All patients underwent daily tonometry before surgery. The same physician performed all the measurements using Goldmann tonometry. The IOP was measured every 2 hours between 8 AM and 8 PM, and the results were plotted on daily tonometric curves. Tonometry was performed using maximal topical therapy (β-blockers, 100%; topical carbonic anhydrase inhibitors, 33%; pilocarpine nitrate, 66%; prostaglandin F2α analogs, 100%; and α2-agonists, 20%) and systemic therapy with carbonic anhydrase inhibitors in 60% of cases, without the influence of any other systemic pharmacologic therapy. The experimental protocol required removal of the HTM specimens during surgery. The HTM specimens were obtained according to standard surgical procedures: to removal all specimens, a 30° knife was used to cut a 1.8- to 2.0-mm button of corneoscleral tissue at the limbus back to the blue-white transition zone. This area contains the complete meshwork, from the Schwalbe line to the scleral spur, including the Schlemm canal and the pigmented band of the trabecular meshwork. To be sure that the removed specimens contained HTM cells, the cut of the scleral flap was executed under microscopic control, ensuring that the posterior excision contained the pigmented band of the trabecular meshwork. No patient underwent laser trabeculoplasty. The presence of HTM cells in the specimens was determined by transmission electron microscopic examination (Siemens Elmiskop 101; Siemens AG, Berlin, Germany) of randomly selected samples from 3 controls and 2 patients with glaucoma. Immediately after collection, the samples were immersed in an antioxidant that contains chondroitin sulfate/dextran cornel storage medium (Optisol; Chiron Vision, Irvine, Calif) at 4°C and sent to the Department of Health Sciences, where tissues were frozen at –80°C until DNA extraction, which was performed according to the method of Izziotti et al,13 obtaining similar performances for DNA quality and recovery.

Twenty-four HTM specimens destined for cornea transplantation from unaffected individuals (13 women and 11 men) were provided by the Melvin Jones Eye Bank in Genoa. The mean ± SD age of the donors was 67.92 ± 9.21 years, which is not significantly different (P = .35) from that of the POAG group, as evaluated using the χ2 test, dividing participants into groups based on 5-year age intervals. Similarly, the sex distribution was not significantly different (P = .38) between patients with glaucoma and controls, as evaluated using the χ2 test. Amounts of 8-OH-dG detected in specimens from patients with POAG were compared with those obtained from the control group.

**Determination of 8-OH-dG Levels**

DNA was depolymerized by nuclease and normal nucleotides selectively removed by trifluoracetic acid, as previously reported.14-16 8-OH-dG was 32P labeled by kinase reaction in the presence of ATP-[γ-32P],15,17 resolved using thin-layer chromatography in unbuffered formic acid,15,16 and quantified using a γ imager (InstantImager; Packard, Meriden, Conn). The 8-OH-dG–positive reference samples were obtained by incubating DNA with hydrogen peroxide, as previously reported.18 A DNA-free negative control was included in each analysis. The appearance of the thin-layer chromatograms obtained was similar to that of chromatograms published in previous articles.8,11 Amounts of 8-OH-dG are reported as 8-OH-dG molecules per 107 normal nucleotides.15,17,18

The stability of 8-OH-dG under the storage conditions used, that is, 1, 3, and 6 hours before analysis, was tested by analyzing 8-OH-dG levels in 3 separate aliquots of the same control sample. The experiment was performed in duplicate on 3 different samples.

**Statistical Analyses**

Statistical analyses were performed using Spearman correlations and statistical analysis software (SPSS version 6.0; SPSS Inc, Chicago, Ill). The data were analyzed using parametric descriptive and probabilistic statistics to define all study variables. To analyze the effects of different variables on the course of glaucoma, the Spearman correlation coefficient between selected items was calculated. Differences in experimental variables between cases and controls were evaluated using 1-way analysis of variance at a threshold of P = .05. The percentage of daily IOP variation (z score) was determined using the following formula:

\[ z = \frac{(A - B)}{B} \]

where A is each single IOP value and B is their daily mean.

**Results**

The stability of 8-OH-dG under the storage conditions used was high. On average, 8-OH-dG variations were negligible, falling inside the interexperimental variability. In fact, an 8.5% increase was observed during the first 3 hours, and a 1% decrease was observed during the following 3 hours. Therefore, 8-OH-dG was demonstrated to be stable under the experimental conditions applied.

Transmission electron microscopic examination confirmed that collected samples included HTM cells. Be-
cause the whole amounts of the 2 samples from patients with POAG were used for this analysis, they cannot be further processed for 8-OH-dG determination. Therefore, results referring to 8-OH-dG and IOP analyses for 15 patients with POAG are reported in Table 1. The results show that oxidative DNA damage of the HTM, reported as 8-OH-dG levels, is significantly (5.01-fold) higher in POAG specimens than in controls (Table 2) (P<.001 by 1-way analysis of variance). Oxidative stress in the HTM did not show any statistically significant correlations with either the age of the patients or the duration of disease.

The VF damage was classified according to the 4 variables given in Table 1. The assessment of VF damage by 2 examiners matched. The 3 VF staging systems used had good correlation among themselves and with the mean deviation VF index (Table 3). This correlation is the expression of the good agreement between the global loss of sensitivity of VF and the morphologic features, shape, and location of the damage.

Spearman rs testing showed good correlations among 8-OH-dG levels, mean deviation VF index (rs =0.77; P =.001), the Aulhorn criteria (rs =0.72; P =.002), the Glaucoma Staging System (rs =0.77; P =.002), and the Hodapp VF staging criteria (rs =0.67; P =.002) in 13 glaucoma cases (87%) (Table 1 and Figure 1). Examples of increasing VF defects in patients with POAG and increasing levels of HTM oxidative damage are shown in Figure 2. In the remaining 2 cases (13%), no significant correlations among the considered variables were revealed. In 1 case (patient G7 in Table 1), the VF damage was enhanced because of the presence of high myopia; in the second case (patient G22 in Table 1), the papillary morphologic features strikingly differed from his VF damage. Therefore, these 2 samples were not included in the analysis.

Table 1. Results of 8-OH-dG, Visual Field Damage, and Intraocular Pressure Analyses in 15 Patients With Primary Open-Angle Glaucoma

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Patient Age, y</th>
<th>Oxidative DNA Damage, 8-OH-dG Molecules/105 Nucleotides</th>
<th>Visual Field Damage</th>
<th>Intraocular Pressure</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MD</td>
<td>AUL</td>
</tr>
<tr>
<td>G14</td>
<td>51</td>
<td>1.80</td>
<td>3.90</td>
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<td>G95</td>
<td>76</td>
<td>14.70</td>
<td>8.30</td>
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</tr>
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<td>70</td>
<td>9.79</td>
<td>25.25</td>
<td>4</td>
</tr>
<tr>
<td>G15</td>
<td>83</td>
<td>3.60</td>
<td>1.90</td>
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<td>74</td>
<td>15.90</td>
<td>32.00</td>
<td>4</td>
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<td>G63</td>
<td>79</td>
<td>11.40</td>
<td>29.00</td>
<td>4</td>
</tr>
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<td>G103</td>
<td>52</td>
<td>2.92</td>
<td>5.87</td>
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<tr>
<td>G22*</td>
<td>63</td>
<td>10.00</td>
<td>2.26</td>
<td>1</td>
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</table>

Abbreviations: 8-OH-dG, 8-hydroxy-2-deoxyguanosine; AUL, Aulhorn “frequency distribution of glaucomatous visual field defect” criteria; EGS, Hodapp visual field staging criteria; GSS, Glaucoma Staging System; MD, mean deviation visual field index.

*Cases with no clinical correlations (see the “Results” section of the text).

Table 2. Intensity of Oxidative DNA Damage in the Human Trabecular Meshwork of 15 Patients With POAG and 18 Controls

<table>
<thead>
<tr>
<th></th>
<th>Arithmetic Mean ± SD (Range)</th>
<th>Geometric Mean</th>
<th>Square Mean</th>
<th>F Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>1.70 ± 1.65 (0.19-6.70)</td>
<td>1.14</td>
<td>14.47</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Patients with POAG</td>
<td>8.51 ± 5.44 (1.80-19.20)</td>
<td>6.68</td>
<td>388.35</td>
<td>26.836</td>
<td>&lt;.001</td>
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</table>

Abbreviations: 8-OH-dG, 8-hydroxy-2-deoxyguanosine; NA, not applicable; POAG, primary open-angle glaucoma.

Table 3. Statistical Correlations Among Visual Field Damage Staging Systems as Evaluated by Calculating the Spearman rs Coefficient*

<table>
<thead>
<tr>
<th></th>
<th>AUL</th>
<th>GSS</th>
<th>EGS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD</td>
<td>0.95</td>
<td>0.96</td>
<td>0.88</td>
</tr>
<tr>
<td>AUL</td>
<td>1.00</td>
<td>0.97</td>
<td>0.89</td>
</tr>
<tr>
<td>GSS</td>
<td>1.00</td>
<td>0.92</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: AUL, Aulhorn “frequency distribution of glaucomatous visual field defect” criteria; EGS, Hodapp visual field staging criteria; GSS, Glaucoma Staging System; MD, mean deviation visual field index.

*Reciprocal correlations are highly significant (P<.001).
mum daily IOP peaks ($r_s = 0.66; P = .01$) and the minimum IOP values ($r_s = 0.60; P = .03$) (Table 1). The mean IOP value correlated with the oxidative DNA damage in the HTM ($r_s = 0.79; P = .001$).

Aerobic organisms possess antioxidant defense systems that deal with reactive oxygen species produced as a consequence of aerobic respiration. Low concentrations of reactive oxygen intermediates may be beneficial or even indispensable in processes such as intracellular messaging and defense against microorganisms, but higher amounts of active oxygen may be harmful to cells and organisms. Oxidative damage to DNA can occur by many routes, including the oxidative modification of nucleotidic bases and sugars or the formation of molecular cross-links. Such modifications can lead to DNA mutations, pathologic conditions, cellular aging, and death. Oxidation of proteins seems to play a causative role in many chronic diseases, including cataractogenesis, rheumatoid arthritis, and various neurodegenerative diseases, including Alzheimer disease.19

Human trabecular meshwork oxidative stress manifests itself as the occurrence of DNA damage inside HTM cells, as evaluated in this study by determining 8-OH-dG levels. This nucleotide alteration is a pivotal indicator of the burden of oxidative DNA damage, being the most abundant oxidative DNA lesion inside the cell.7 It derives from the interaction of reactive oxygen species and DNA at the level of the nucleophilic site C8 of guanine, with those arising at C8 ketonic guanine tautomerizing the enolic isomer to 8-OH-dG.20 This DNA alteration can induce point mutations, such as G-C transversion, but also the formation of apurinic/apyrimidinic sites.7 Although specific repair pathways are recognized for these lesions,21 whenever these mechanisms are overwhelmed by an excessive amount of 8-OH-dG, stable DNA alterations can occur. These alterations can represent a step forward in the development of a variety of chronic degenerative diseases, including atherosclerosis and aging.16,22

Figure 1. Scatterplots with regression lines of the relationship between the level of oxidative damage (8-hydroxy-2'-deoxyguanosine [8-OH-dG]) in the trabecular meshwork of patients with primary open-angle glaucoma and their visual field defects as evaluated by 4 different staging systems: the mean deviation visual field index (MD), the Authorh "frequency distribution of glaucomatous visual field defect" criteria (AUL), the Glaucoma Staging System (GSS), and the Hodapp visual field staging criteria (EGS).
The present results confirm previous findings indicating that the HTM undergoes oxidative DNA damage in a more extensive manner in patients with POAG than in controls (Table 2). The occurrence of such a situation has been hypothesized in the past by several researchers and was indicated as a possible pathogenic factor for glaucoma development.

However, usually, the toxic potential of the reactive oxygen species is neutralized by a complex network of antioxidant factors, enzymatic (eg, superoxide dismutase, catalase, and glutathione peroxidase) and nonenzymatic (reduced glutathione, ascorbic acid, vitamin E, etc). Under pathologic conditions, this balance may be altered in favor of pro-oxidant factors, and, therefore, oxidative stress may be produced. Alvarado et al found that the decline of HTM cellularity is linearly related to age, with the loss of approximately 0.58% of total HTM cells each year. This phenomenon specifically affects the filtering area. Furthermore, the specific activity of superoxide dismutase demonstrates an age-dependent decline in normal HTM collected from cadavers. This HTM cell loss or the altered functionality of HTM cells has been suggested to be the result of an increase in oxidative stress. Kahn et al, studying the role of the oxide-reducing system in glaucoma, demonstrated that outflow resistance increases in the presence of high levels of hydrogen peroxide.

The effect of the H2O2 on the adhesion of HTM cells to extracellular matrix proteins results in a rearrangement of cytoskeletal structures that may result in reduced trabecular meshwork cell adhesion, cell loss, compromised HTM integrity, and pathologic consequences. Previous data on cellular cultures suggest that oxidative stress can affect the biological reactions of the HTM cells themselves. Therefore, oxidative stress is likely to represent a reliable mechanism for the development of glaucoma damage, which manifests its occurrence in the HTM as an IOP increase.

We demonstrated the existence of a statistically significant correlation between oxidative DNA damage and daily mean, minimum, and maximum IOP values. On the other hand, we did not find correlations with tonometric fluctuations, probably because the daily pressure peak has a pathogenetic meaning, whereas the daily tonometric fluctuation reflects properly circadian rhythms.

The correlation between IOP and 8-OH-dG level in the HTM was demonstrated in a pool of patients affected by various glaucoma types (angle recession, neovascular, juvenile, pseudoxefoliative syndrome, and POAG) for IOP mean and fluctuation, not for IOP minimum and maximum values. Differences in these findings could be related to a possible different pathogenic role of IOP in the various types of glaucoma. The present data show that glaucoma VF damage and 8-OH-dG levels are correlated (Figure 1). The present results not only indicate the presence of HTM oxidative damage in POAG but, demonstrating a fair relationship with clinical data, also support the possible pathogenic role of this phenomenon.

The amount of visual field damage is correlated with the rate of oxidative DNA damage in the human trabecular meshwork. A, In patient G44, the 8-hydroxy-2′-deoxyguanosine (8-OH-dG) level was 1.90 molecules/10^5 nucleotides. B, In patient G103, the 8-OH-dG level was 2.92 molecules/10^5 nucleotides. C, In patient G108, the 8-OH-dG level was 5.79 molecules/10^5 nucleotides. GH indicates glaucoma hemifield test; CPSD, corrected pattern standard deviation; MD, mean deviation; PSD, pattern standard deviation; and SF, short fluctuation.
There are 2 main theories that explain glaucoma VF damages consequent to the death of the ganglion cells: the vascular theory, based on the hypothesis that ocular blood flow is reduced, and the pressure-mechanic theory. According to the second theory, the pressure on the axons develops a compression and loss of the neurotrophic support of the ganglion cells. Neither theory explains exactly the whole pathogenesis of the glaucoma neuropathy because the first does not justify the relationship between the reduction of blood flow and the increase of IOP and the other does not explain the alterations in circulation that occur in the optic nerve. However, both theories use oxidative stress as a common pathogenic way. In the vascular theory, ischemia produces free radicals responsible for the oxidative damage to the axons. In the pressure-mechanic theory, the oxidative DNA damage would be the primum movens of HTM alterations.

Recently, it has been demonstrated that an oxidative-induced molecular defense mechanism, that is, the endothelial leukocyte adhesion molecule-1/interleukin induced molecular defense mechanism, that is, the endothelial leukocyte adhesion molecule-1/interleukin-1/nuclear factor κB pathway, is activated in HTM cells collected from patients with glaucoma. Although it has been established that antioxidants have protective effects on rescuing retinal ganglion cells from death in eyes with elevated IOP, we do not know whether the oxidative HTM DNA damage we reported is a common pathogenetic mechanism that also affects optic nerve head degeneration. In fact, other pathogenetic factors could also be involved, such as ischemia or excitotoxicity, thereby ascribing to POAG a possible multifactorial origin.

A previous study demonstrated that in patients with POAG there exists a predisposition toward oxidative DNA damage related to the absence of the GSTM1 gene. The perturbation of the pro-oxidant/antioxidant balance caused by this genetic deficit can lead to increased oxidative damage, especially when the first line of antioxidant defense weakens with age. In conclusion, our results support the belief that glaucomatous damage is the pathologic consequence of oxidative stress. This stress could be a common, fundamental, pathogenetic element that could be acting in HTM and the optic nerve head. Therefore, further studies are needed to elucidate the role of oxidative stress in the pathogenesis of glaucoma.

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