


The Effect of Dietary Antioxidant Supplementation in Patients with Glaucoma

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Purpose: Oxidative stress may be a risk factor for glaucoma, and many previous reports have suggested that antioxidants could be a promising treatment. Here, we investigated the effects of a novel supplement containing three food-derived antioxidants (hesperidin, crocetin, and *Tamarindus indica*) on markers of oxidative stress in patients with glaucoma.

Patients and Methods: This study had a prospective, single arm design. Thirty Japanese glaucoma patients were recruited and given 4 tablets with ample water twice a day for 8 weeks. The treatment was stopped, and the subjects were followed for an additional 8 weeks. We measured biological antioxidant potential (BAP) with a free radical analyzer. We also measured urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG; a marker of oxidative DNA damage). Clinical laboratory data were measured in venous blood samples. Clinical parameters were also recorded. Comparisons used a one-way analysis of variance (ANOVA) followed by Dunnett's test.

Results: The 8-OHdG level was not reduced. We also divided the patients into groups with high or low oxidative stress. In patients with relatively high oxidative stress, the 8-OHdG level was significantly reduced at weeks 4, 8, 12, and 16 ($P < 0.001$, $P < 0.01$, $P < 0.01$, $P < 0.01$), and BAP was significantly elevated at weeks 8 and 12 ($P = 0.03$, $P = 0.04$). In patients with relatively low oxidative stress, the 8-OHdG level was not significantly reduced during supplement intake but was significantly elevated at weeks 12 and 16 ($P = 0.03$, $P = 0.04$), while BAP was not significantly elevated.

Conclusion: An 8-week oral course of antioxidant supplementation was effective in patients with a high oxidative stress level. Dietary supplementation could hold promise in the treatment of systemic oxidative stress-related diseases.

Keywords: oxidative stress, supplement, glaucoma, 8-OHdG

Introduction

Oxidative stress in the body is normally managed by balancing the production of reactive oxygen species (ROS) with the activation of a variety of antioxidative cellular mechanisms. The efficacy of these mechanisms is critical, because if they are impaired, oxidative damage can continue even when overall oxidative stress is within normal levels. Oxidative damage to proteins, DNA, and lipids contributes to many age-related neurodegenerative diseases, as well as age-related macular degeneration, diabetic retinopathy, cataract, and glaucoma.¹⁻⁵ The World Health Organization estimates that among the 37 million people with blindness worldwide, 12% have lost their sight due to glaucoma.⁶

Treatment for glaucoma most commonly involves therapy to reduce intraocular pressure (IOP). However, disease progression persists in some patients even after

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IOP is successfully reduced. This has led to past investigations of possible non-IOP risk factors for glaucoma and its key underlying pathomechanism, retinal ganglion cell (RGC) degeneration. Previously reported risk factors include genetics,⁷ vascular dysregulation,⁸ mitochondrial dysfunction,⁹ and in particular, oxidative stress.² Oxidative stress has three key effects in glaucoma. First, it raises IOP by altering the trabecular meshwork and impairing aqueous humor outflow.^{10,11} Second, it disrupts autoregulation of blood flow to the optic nerve by altering the vessels that feed it.^{12,13} Finally, patients with a low antioxidant level in the eye are also susceptible to systemic oxidative stress, which can induce RGC death, as we have previously reported.^{14,15} Harris et al revealed that one month of oral supplementation with an antioxidant increased biomarkers of blood flow in the eyes of patients with open-angle glaucoma (OAG).¹⁶ Park et al found that a four-week course of daily administration of 160 mg of *Ginkgo biloba* extract led to a significant improvement in retinal blood flow volume and velocity in 15 patients with normal-tension glaucoma (NTG).¹⁷ In vivo experiments using animal models of glaucoma (based on apoptosis signal regulating kinase 1, valproic acid, N-acetylcysteine, spermidine, and coenzyme Q10) have showed that administering antioxidants had a neuroprotective effect.¹⁸ These research suggest that antioxidants are a potential candidate for glaucoma therapy.

Here, we gave a daily dietary antioxidant supplement over 8 weeks to patients with glaucoma. The supplement was developed and first reported by Maekawa et al, who identified the components of the supplement through screening and reported that three food-derived compounds, hesperidin, *Tamarindus indica*, and crocetin, had a protective effect in a primary culture of retinal cells under oxidative stress.¹⁹ Moreover, hesperidin reduced apoptosis, oxidative stress and inflammation and had a neuroprotective effect in a mouse glaucoma model. We believe that the findings reported here will shed light on the potential of new treatments for glaucoma based on antioxidant supplementation.

Patient and Methods

Study Design

This study had a prospective, single arm design. Patients with previously diagnosed NTG were recruited at Tohoku University Hospital between March 2018 and

October 2019; all patients were Japanese and all were followed for 16 weeks.

The sample size was based on a preliminary study (unpublished) that used the same method as the present study to measure average BAP before and after supplement intake. The preliminary study showed that BAP increased by 98.6 (standard deviation: 157.9). When we set the α error at 0.05 and the power at 0.8, we found that the number of subjects to obtain statistically significant results in a comparative study was 23. Thus, we set the number of subjects at 30 in anticipation of dropouts.

This study adhered to the Declaration of Helsinki and Clinical Trials Act and was approved by the Clinical Research Review Board of Tohoku University (study 2019-6-068), which is certified by the Japanese Ministry of Health, Labor and Welfare. The trial was registered with the UMIN clinical trial registry, number 000032050. Written informed consent was obtained from all subjects before the start of the study.

Participants

Inclusion criteria for participation were as follows: overall good health, age between 30 and 75 years; NTG confirmed in one or both eyes by a glaucoma specialist; a biological antioxidant potential (BAP) level between 1600 and 2200 nmol/L; and body mass index less than 26 kg/m². Exclusion criteria were as follows: hyperopia (above +3D); high myopia (below -8D); any type of secondary glaucoma; high IOP (> 16 mmHg) despite glaucoma medication; and severe systemic disease, including cancer, hyperthyroidism, and autoimmune disease. No patients used exogenous hormones. Pregnant women, or women planning pregnancy during the study period, were also excluded. Candidates were asked to refrain from the use of vitamin or carotenoid supplements for 2 weeks before the study. If a patient had diabetes, hyperlipidemia, hypertension, or a current smoking habit, it was recorded.

Figure 1 shows an overview of the study design. Participants were examined 5 times, at four-week intervals. At the examination, we measured clinical parameters, confirmed compliance with the study protocol of twice-daily use of the supplement, and recorded any adverse events.

Study Intervention

Maekawa et al previously reported 12 candidate neuroprotective compounds.¹⁹ Based on these results, we developed the novel supplement used in this study, which contained

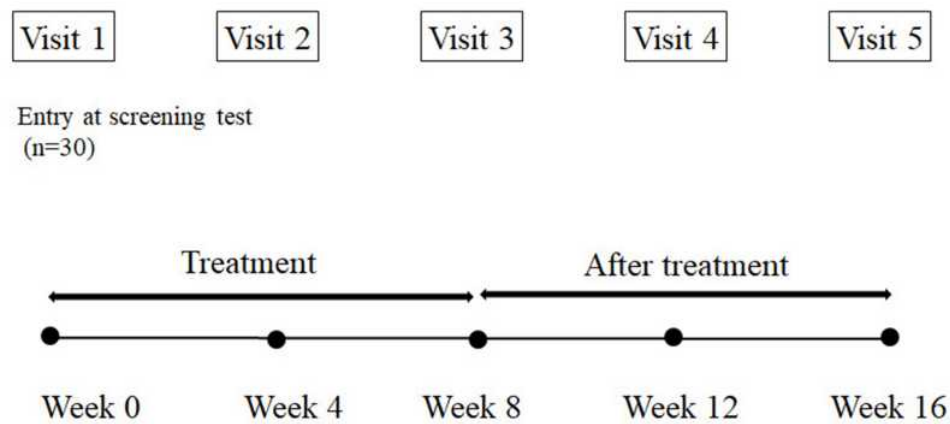


Figure 1 Overview of study design.

the following ingredients in each daily dose of 4 tablets: hesperidin (50 mg/4 tablets), crocetin (7.5 mg/4 tablets), and *Tamarindus indica* (25 mg/4 tablets). The supplement was manufactured and supplied by Wakamoto Pharmaceutical Co. Ltd (Tokyo, Japan). The candidates took 2 tablets with ample water twice a day (ie, 4 tablets/day) for 8 weeks, and no supplementation for 8 weeks. Any unused supplements were returned for a pill count at the 8-week visit.

Measurements

A complete ophthalmic examination of all patients was performed by a glaucoma specialist. This comprised measurement of best-corrected visual acuity (recorded as the logarithm of the minimum angle of resolution) examinations with slit-lamp biomicroscopy and funduscopy, and evaluation of the optic disc with a 90-diopter lens. The patients with glaucoma also underwent measurement of mean deviation (MD) with the Humphrey field analyzer (HFA). If both eyes had glaucoma, the analysis included the worse-MD eye.

Clinical Laboratory Measurements

Samples of blood were stored in containers with EDTA at weeks 0 and 8. We confirmed supplement safety based on clinical laboratory measurements. Plasma samples were stored at -80°C for later biochemical analysis. Serum samples from the screening visit were sent to LSI Medience Co. (Tokyo, Japan), analyzed for AST, ALT, γ -GTP, total bilirubin, total protein, albumin, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, total cholesterol, triglycerides, creatinine, blood urea nitrogen, uric acid, glucose, HbA_{1c}, white and red blood cell count, platelet count, hematocrit, and hemoglobin.

Blood Sampling and Oxidative Stress Measurement

Serum samples were evaluated for oxidative stress levels and anti-oxidative potential with a free radical analyzer system (Free Carpe Diem, Wismerll Company Ltd., Tokyo, Japan). Blood samples were collected more than 3 hours after a subject ate. Total plasma antioxidant capacity in the samples was measured with the BAP test, which is based on the ability of a sample to reduce iron from its ferric (Fe^{3+}) to its ferrous (Fe^{2+}) state. The analyses were performed as previously reported.^{14,15}

Urinary Sampling

Urinary levels of 8-oxo-2'-deoxyguanosine (8-OHdG), an oxidative stress marker, were measured with the commercially available ELISA kit (LSI Medience Co., Tokyo, Japan). Creatinine levels were also measured in urine samples. Urine samples were stored at -4°C .

Statistical Analysis

The significance of differences between groups was determined with the Mann–Whitney *U*-test and Fisher's exact test. Comparisons of groups used one-way analysis of variance (ANOVA) and Dunnett's test. All numerical findings are mean \pm SD. Statistical significance was set at $P < 0.05$. SPSS version 23.0 (SPSS Inc., Chicago, IL, USA) was used for all analyses.

Results Subjects

This study enrolled 30 patients with NTG. Table 1 shows the demographic characteristics of the subjects. We also

Table 1 Characteristics of Normal Tension Glaucoma Patients

Demographics	All	8-OHdG < 6.86 (ng/mg Creatinine)	8-OHdG > 6.86 (ng/mg Creatinine)	P-value
Number of patients	30	19	11	–
Age (years)	59.1 ± 9.0	60.7 ± 8.5	56.3 ± 9.6	0.23
Sex (male: female)	15: 15	10: 9	5: 6	1.00*
Visual acuity (logMAR)	0.11 ± 0.37	0.01 ± 0.30	0.08 ± 0.34	0.28
IOP (mmHg)	11.03 ± 2.25	11.48 ± 1.87	11.58 ± 1.97	0.94
Mean deviation (dB)	−14.24 ± 6.58	−15.20 ± 6.36	−12.89 ± 6.99	0.49
BMI (kg/m ²)	22.34 ± 2.15	23.14 ± 1.59	20.95 ± 2.35	0.02
8-OHdG (ng/mg creatinine)	6.86 ± 3.52	4.69 ± 1.41	10.59 ± 2.88	< 0.01
BAP (μmol/L)	2022.37 ± 139.94	2039.56 ± 110.53	1992.64 ± 182.40	0.64
Diabetes (%)	1 (3.33)	0 (0.00)	1 (9.09)	0.37*
Hypertension (%)	4 (13.33)	4 (21.05)	0 (0.00)	0.27*
Current smoker (%)	6 (20.00)	3 (15.79)	3 (27.27)	0.64*
Hyperlipidemia (%)	6 (20.00)	5 (26.32)	1 (9.09)	0.37*

Notes: Unmarked *P* values: Mann–Whitney *U*-test; *Fisher exact test.

Abbreviations: 8-OHdG, 8-hydroxy-2'-deoxyguanosine; logMAR, logarithm of minimum angle of resolution; IOP, intraocular pressure; BMI, body mass index; BAP, biological antioxidant potential.

divided the patients into groups with high or low oxidative stress, defined by an 8-OHdG level higher or lower than 6.86 ng/mg of creatinine, respectively, and performed separate analyses of these groups. There was a significant difference in body mass index and 8-OHdG between the two groups ($P = 0.02$, $P < 0.01$). There were no supplement-related adverse events in any patient, and all blood tests were normal (data not shown).

Oxidative Stress

BAP was significantly elevated at weeks 8 and 16 ($P = 0.01$, $P = 0.02$, respectively; Figure 2B). There were reductions in the 8-OHdG level, but these did not reach statistical significance at any of the five time points (Figure 2A). In the high-oxidative stress group, the 8-OHdG level was significantly reduced at weeks 4, 8, 12, and 16 ($P < 0.001$, $P < 0.01$, $P < 0.01$, $P < 0.01$, Figure 3B) and BAP was significantly elevated at weeks 8 and 12 ($P = 0.03$, $P = 0.04$, Figure 3D). In the group with relatively lower oxidative stress, the 8-OHdG level was not significantly reduced during supplement intake or after intake was stopped, but was significantly elevated at weeks 12 and 16 ($P = 0.03$, $P = 0.04$, Figure 3A); BAP was not significantly elevated (Figure 3C).

Discussion

The results of this study are of particular interest for the treatment of patients with a low antioxidant level; we found that antioxidant supplementation may be an effective option for such patients. This is especially promising for the treatment of glaucoma, an age-related chronic neurodegenerative disease in which oxidative stress has been shown to play an important role.

The glaucoma patients in this study who had a relatively higher level of oxidative stress and took a daily antioxidant supplement showed an increase in BAP and a decrease in 8-OHdG, while the glaucoma patients with a relatively lower level of oxidative stress who took the supplement did not show an increase in BAP and showed a decrease in 8-OHdG. This suggests that our supplement was only effective in patients with a high oxidative stress level. Previously, Maekawa et al reported that three food-derived compounds, hesperidin, *Tamarindus indica*, and crocetin, had a protective effect in a primary culture of retinal cells under oxidative stress, and that hesperidin reduced apoptosis, oxidative stress and inflammation. Maekawa confirmed that hesperidin had these effects in vivo in mice, reducing oxidative stress and preventing RGC death caused by N-methyl-

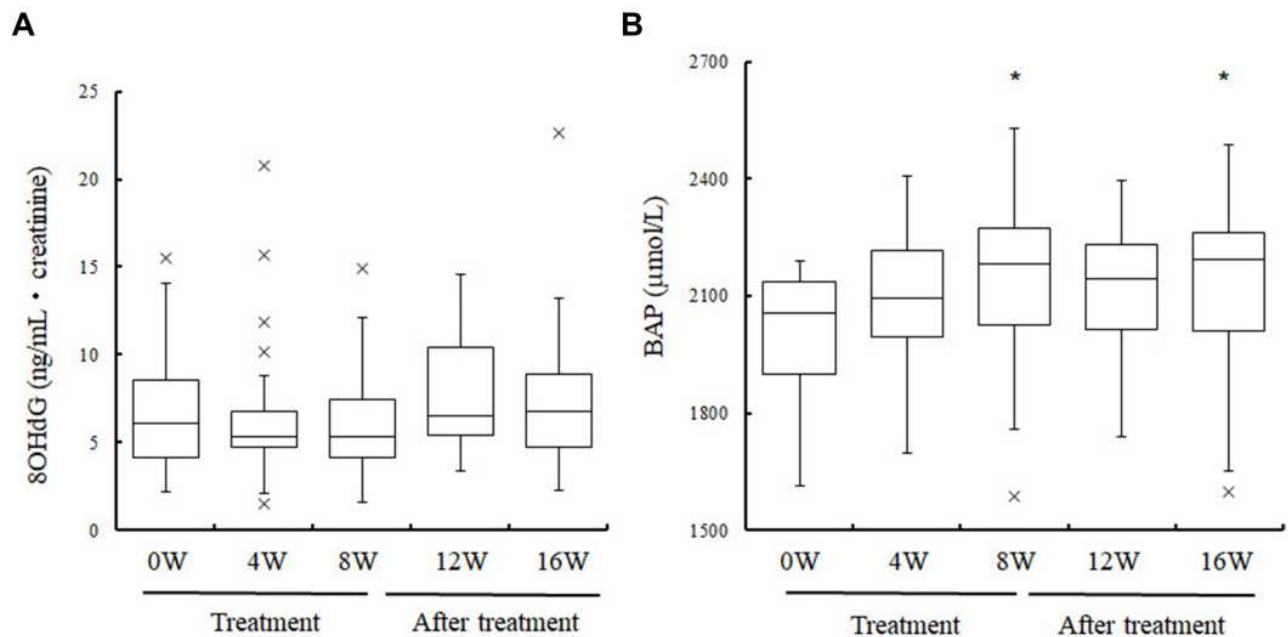


Figure 2 Comparison of findings before and after antioxidant treatment in patients with glaucoma. (A) There was no significant increase in 8-OHdG level. (B) BAP level significantly increased at weeks 8 and 16. * $p < 0.05$.

D-aspartate (NMDA)-induced excitotoxicity.¹⁹ *Tamarindus indica* is indigenous to tropical Africa. It is high in tartaric acid, B vitamins, and minerals. It has antioxidant,^{20,21} anti-inflammatory,²² anti-diabetic,²³ and anti-atherosclerotic effects.²⁴ Crocetin is a natural apocarotenoid dicarboxylic acid that is found in the crocus flower and in *Gardenia jasminoides*. Crocetin has various effects, acting as an antioxidant²⁵ and anti-inflammatory,²⁶ and can inhibit the caspase pathway, preventing retinal damage induced by NMDA.²⁷ These results suggest that these three food-derived compounds, used as a dietary supplement, might help reduce RGC degeneration in retinal disease.

A variety of antioxidant molecules can be found in human plasma, with the following compounds being most common: α -tocopherol, β -carotene, albumin, ascorbic acid, bilirubin, catalase, ceruloplasmin, ferritin, glutathione peroxidase, lycopene, reduced glutathione, SOD, and uric acid.²⁸ All these compounds can catalytically remove reactive species such as free radicals. After taking our novel supplement, the subjects in this study showed decreased oxidative stress and an increased antioxidative level. Interestingly, we found that patients showing a higher level of antioxidative activity also showed a lower level of oxidative stress. This is especially interesting for glaucoma, which can sometimes progress even with successful IOP-lowering treatment.

There is extensive clinical evidence that systemic oxidative stress contributes to glaucomatous optic neuropathy. Moreover, antioxidant therapy has shown promising results in animal- and human-based research. Antioxidant treatment can decrease the activation of NF- κ B and decrease the production of cytokines in the optic nerve and retina.²⁹ In a rat glaucoma model, overexpression of thioredoxins can protect the RGCs.³⁰ Higher green leafy vegetable intake is associated with a lower primary OAG risk.³¹ Lee et al found that *Ginkgo biloba* extract protected against the effects of glaucoma in some patients.³² These results suggest that insufficient serum antioxidant proteins underlie the contribution of oxidative stress to glaucoma, indicating that antioxidants should have a neuroprotective effect. Our past research also showed that visual field damage was strongly correlated with systemic antioxidant levels in young, male patients with OAG.¹⁴ Antioxidant treatments might therefore be a useful therapeutic option to delay or prevent disease progression.

Limitations of this study included, first, that it lacked a placebo arm, which is needed to prove the efficacy of any supplement. Thus, we plan to conduct further studies with a randomized, doubled-blind, placebo-controlled design. Second, we observed a significant elevation in BAP after supplementation for eight weeks in the overall group of NTG patients (Figure 2B), and for four weeks in

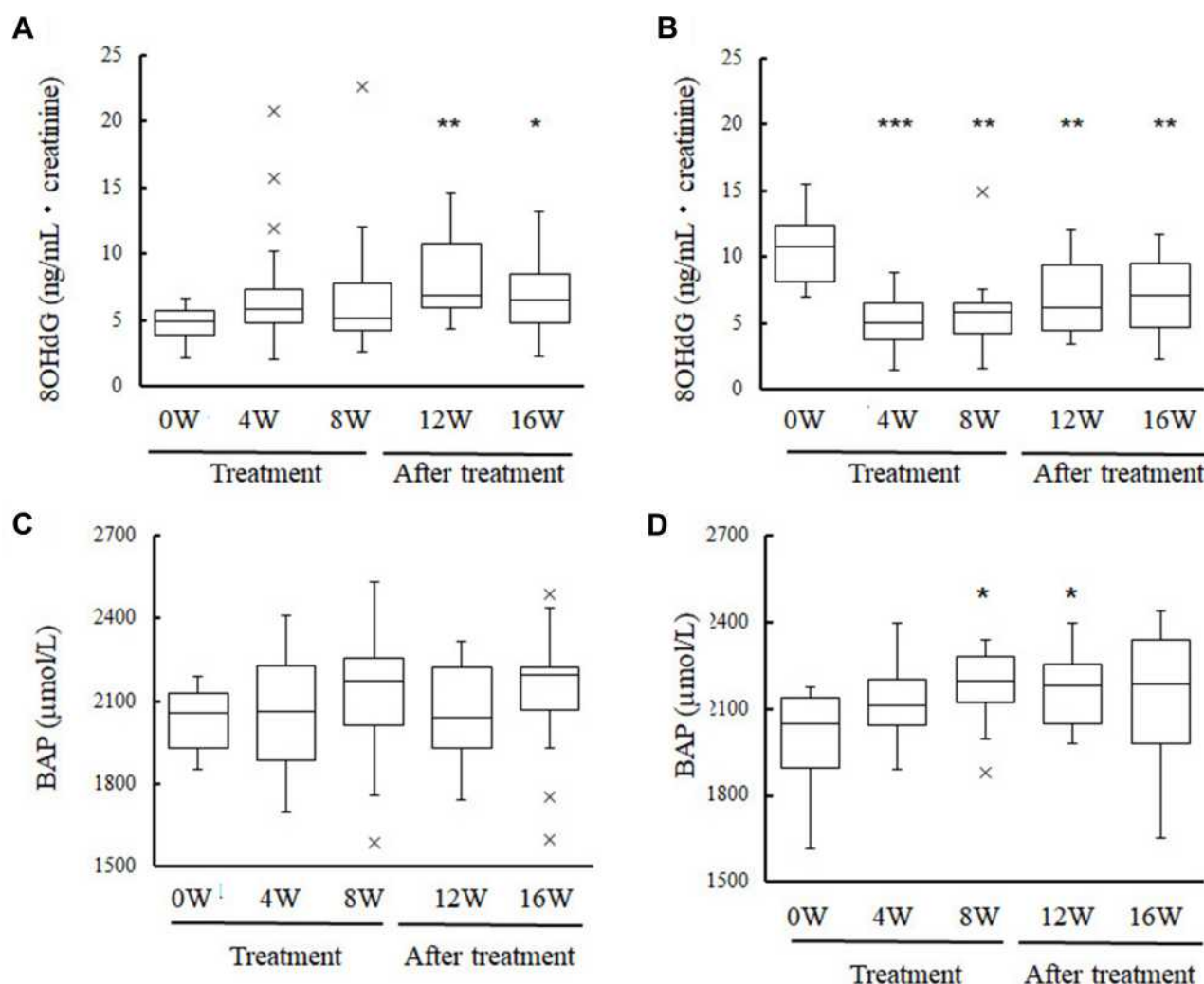


Figure 3 Comparison of findings before and after antioxidant treatment in patients with relatively high or low oxidative stress. **(A)** There was a significant increase in the 8-OHdG level at weeks 12 and 16 in patients with relatively low oxidative stress. **(B)** There was a significant decrease in 8-OHdG level at weeks 4, 8, 12 and 16 in patients with relatively high oxidative stress. **(C)** BAP level did not significantly change before and after treatment in patients with relatively low oxidative stress. **(D)** BAP level significantly increased at weeks 8 and 12 in patients with relatively high oxidative stress. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

the group of NTG patients with relatively higher oxidative stress (Figure 3D). This might have been due to the participants improving their lifestyle after starting this study, potentially affecting our finding of a change in antioxidative stress levels after supplementation was stopped. Nevertheless, when we analyzed a group of patients with a higher BAP level than the overall average for our participants (2022.37 $\mu\text{mol/L}$), we found that their BAP level was not significantly elevated during supplement intake (data not shown). The BAP change we observed in the other groups was interesting. We speculate that the increased BAP level might return to its baseline with longer term observation. There is therefore a need for a longer follow-up study. Third, we observed a significant elevation in 8-OHdG after supplementation

for four and eight weeks, while we observed significant differences in 8-OHdG before and after supplementation in patients with relatively lower oxidative stress (Figure 3A). This discrepancy might have been due to an increase in oxidative stress after supplementation, causing increased endogenous antioxidant activity. Finally, we could not include a control group, because we could not find potential candidates without cataracts, due to the advanced age of our patients. We were also limited in our ability to detect relatively modest associations, making longer follow-up of the subjects necessary in a future study.

Our study indicates that patients with a low systemic antioxidant level may have increased susceptibility to oxidative stress, which might accelerate the progression of glaucoma. This raises the possibility that antioxidant

supplementation may be a viable option to delay or prevent age-related glaucoma progression.

Conclusion

Oxidative stress may be a key non-IOP risk factor for age-related cataracts and glaucoma. Our study revealed that antioxidant supplementation was effective in patients with high oxidative stress level, suggesting that such supplementation may be a novel way of combating diseases induced by systemic oxidative stress, and could contribute to individualized treatment for these diseases.

Abbreviations

ROS, reactive oxygen species; IOP, intraocular pressure; RGC, retinal ganglion cell; OAG, open-angle glaucoma; NTG, normal-tension glaucoma; BAP, biological antioxidant potential; MD, mean deviation; HFA, Humphrey field analyzer; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; NMDA, N-methyl-D-aspartate.

Data Sharing Statement

The authors do not intend to share participant level data.

Ethics Approval and Informed Consent

This study adhered to the Declaration of Helsinki and Clinical Trials Act was approved by the Clinical Research Review Board of Tohoku University (study 2019-6-068), which is certified by the Japanese Ministry of Health, Labor and Welfare. The trial was registered with the UMIN clinical trial registry, number 000032050. Written informed consent was obtained from all subjects before the start of the study.

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the

article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects or the work.

Disclosure

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