Original Article

Circulating Total Glutathione in Normal Tension Glaucoma Patients: Comparison with Normal Control Subjects

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Purpose: Oxidative stress plays a critical role in the pathogenesis of glaucoma. Glutathione is a major antioxidant molecule present in intracellular or extracellular space. Herein, we aimed to examine circulating glutathione level in normal tension glaucoma (NTG), which comprises the largest proportion of glaucoma disease in the Korean population.

Methods: Nineteen NTG patients (NTG group) and 30 age- and gender-matched normal control subjects (control group) were included. Antecubital venous puncture was performed between 8 and 10 o'clock in the morning to obtain a 4 mL venous blood sample. Total glutathione level was measured by the spectrophotometric method at 412 nm. Correlation of total glutathione level with mean deviation and pattern standard deviation from the Humphrey visual field test was analyzed in the NTG group.

Results: Total glutathione level in circulating blood was 524.02 ± 231.09 nmol and 586.06 ± 156.08 nmol in the NTG group and the control group, respectively. The difference between these values was not statistically significant (p = 0.121, F = 2.212). Age had no significant effect on circulating total glutathione level in either the NTG group (p = 0.171, r = -0.328) or the control group (p = 0.380, r = -0.166). In the NTG group, circulating total glutathione level had no significant relationship with mean deviation (p = 0.226, F = 1.636) and pattern standard deviation (p = 0.200, F = 1.766) after correcting for age and gender.

Conclusions: In NTG patients, circulating total glutathione levels were not different compared to those of normal subjects.

Key Words: Glutathione, Low tension glaucoma, Oxidative damage

Glaucoma is the leading cause of irreversible blindness and the second most common cause of blindness after cataract [1]. The main characteristic of glaucoma is a specific optic neuropathy with reduced number of retinal ganglion cells accompanied by characteristic progressive constriction of the visual field [2,3]. Although increased intraocular pressure (IOP) has been considered as a major risk factor for glaucoma [4], there are other concomitant factors affecting the eye such as glutamate excitotoxicity [5], reduced ocular blood flow [6-9], vascular dysregulation [10,11], and reactive oxygen species-related oxidative dam-

age that have been suggested as important etiologic factors in glaucoma pathogenesis [12,13]. The trabecular meshwork, optic nerve, and retinal ganglion cells are known as the major targets of oxidative damage in the pathological development of glaucoma [14-21].

Why is oxidative stress so important in ocular disease? Eyes are continually exposed to light in order to collect data from the outer environment, and thus reactive oxygen species are being produced by ionizing or non-ionizing radiation when the eyes are open [22]. Reactive oxygen species are also produced by aerobic metabolism. Even when the eye is in darkness, the retina consumes more oxygen than when it is in light [23]. Ischemia and reperfusion injury caused by ocular vascular dysregulation also induce oxidative damage [24,25]. Against this vulnerability to oxidative damage, the eye has its own protective mechanism of high levels of both ascorbic acid and glutathione (GSH) [26-32]. Other protective systems including enzymatic

(superoxide dismutase, GSH peroxidase, and catalase) and non-enzymatic antioxidants (cysteines, tocopherols, and retinols) are present in the eye [19]. Under normal physiologic conditions, there is a state of equilibrium between the endogenous production of free radicals and their neutralization by antioxidant defense mechanisms. If these protective systems fail, damage to the ocular tissues can occur, and various diseases can develop such as cataract [33], age-related macular degeneration [34], and glaucoma [35].

GSH is a major non-enzymatic antioxidant present in both intracellular and extracellular spaces [36,37]. The GSH redox system protects ocular tissues from the damage induced by low H_2O_2 concentrations through direct and indirect mechanisms [38,39]. A considerable amount of evidence suggests GSH depletion as an important risk factor for glaucoma. In the aqueous humor of primary open angle glaucoma (POAG) patients, activity of antioxidant enzymes, including glutathione peroxidase, is increased and total antioxidant potential is decreased, suggesting that chronic oxidative damage occurs in outflow pathways [40]. An increase in serum autoantibody against glutathione-S-transferase has been reported in glaucoma patients [41]. Genetic polymorphisms have been detected for GSH transferase isoenzyme, and the GSTM1-null genotype has been found to be significantly common in patients with primary open-angle glaucoma compared to controls [42]. Patients with POAG exhibit low levels of circulating glutathione [43]. In an in vivo study, a mouse retina with systemic GSH reduction showed increased retinal neuronal apoptosis [44] and altered expressions of bcl-2 family mRNA and heat shock protein mRNA [45], suggesting subsequent retinal neuronal protective mechanisms may be changed by systemic GSH depletion.

Normal tension glaucoma (NTG) is the most common type of glaucoma in Korea and Japan [46-48]. This is unique in comparison with Western populations. The prevalence of NTG is about 3.5-fold higher than that of POAG with increased IOP in Korea (The Namil Study, the Korean Glaucoma Society) [48]. However, the pathophysiologic mechanism of NTG is little known. Vascular dysregulation in the optic nerve head and some influence of high normal intraocular pressure are known as mild risk factors [10,11,49]. Although IOP is still the major target of treatment for NTG in current practice [50], attention is shifting to other modalities such as neuroprotection, improvement of ocular blood flow, and antioxidant supplementation [51]. Moreover, some patients with POAG are also facing these new forms of therapy due to the progression of optic neuropathy despite well-controlled IOP [52] due to the multifactorial etiology of glaucoma.

Therefore, information about the status of systemic GSH level in NTG will help to elucidate the role of oxidative stress in the disease progression of NTG. The purpose of this study was to determine the status of circulating total GSH levels in patients with normal tension glaucoma and to compare levels with those of people without glaucoma.

Materials and Methods

Study sample

Patients with NTG attending the glaucoma clinic of the Catholic Eye Center at St. Mary's Hospital (Seoul, Korea) between June 2008 and October 2008 were considered for inclusion in this prospective study. Ethical approval was obtained from the committee of St. Mary's Hospital institutional review board, and written informed consent was received from all subjects before entry into the study.

Patients were diagnosed as having NTG if IOP measurements were less than 22 mmHg by Goldmann applanation tonometry, characteristic glaucomatous cupping of the optic disc was observed on funduscopic examination, normal open anterior chamber angles was seen on gonioscopy, and repeatable visual field defects consistent with the diagnosis of glaucoma were observed in the results obtained with program 24-2 of the Humphrey field analyzer (Carl Zeiss Meditec, Dublin, CA, USA). The study's ocular exclusion criteria included any IOP measurement greater than 21 mmHg, narrow iridocorneal angles, evidence of secondary open-angle glaucoma, history of previous intraocular surgery, and other nonglaucomatous ocular diseases including cataract, diabetic retinopathy (DR) and age-related macular degeneration (ARMD). The control group was recruited from a group of age- and gender-matched volunteers and was composed of subjects who had never had glaucoma or other ocular diseases such as cataract, DR, or ARMD.

Other exclusion criteria for both groups were smoking and a history of any chronic systemic disease with presumed low GSH level, including autoimmune diseases, alcoholic liver disease, cancer, and diabetes mellitus. After this selection, the experimental group was narrowed to 19 patients with NTG, and that control group contained 30 subjects.

Blood sampling

Subjects were instructed to fast from midnight to 8 a.m. on the morning of the test. All blood samples were obtained by a qualified registered nurse in the morning, between 8 and 10 a.m. Four milliliters of blood were collected in EDTA-treated tubes (to prevent oxidation) by venipuncture to the antecubital vein. One thousand microliters of blood was then transferred into a centrifuge tube for initial processing. Red blood cells (RBC) were separated from plasma by centrifugation at 600 $\times g$ for 10 minutes. The pellet RBCs were then transferred into another microcentrifuge tube and washed twice with 3 volumes of

phosphate buffered saline (pH = 7.4). A 200 microliter aliquot of RBC pellets was placed in another microcentrifuge tube, to which 200 microliters of 5% 5-sulfosalicylic acid (SSA) were added and then incubated for 10 minutes at 4°C. After centrifugation at 10,000 ×g for 10 minutes, 100 microliters of the supernatant were immediately cooled at -70°C. All of these procedures were performed within 2 hours from the time of blood collection. The next steps of the GSH analysis were performed within 10 days.

Glutathione analysis

Total GSH levels were assessed by the 5, 5'-dithiobis-(2-nitrobenzoic acid) recycling procedure, as described in a previous study [52]. The glutathione assay kit from Sigma (Glutathione assay kit, product code CS0260; St Louis, MO, USA) was used.

A standard curve from 3.125 to 50 nanomoles in double-fold increments using a GSH standard solution was prepared for each 96-well plate. The standards contained the same final concentrations of SSA as used for the samples. To each well of a 96-well plate, 150 microliters of working buffer and 10 microliters of standard or sample were added in duplicate, and the plate was incubated at room temperature for 5 minutes. Finally, 50 microliters of 0.16 mg/mL nicotinamide adenine dinucleotide phosphate (NADPH) was added, and the plate was read at 412 nm using a 96-well plate reader. A standard curve was then generated by linear regression.

Statistical analysis

The statistical analysis was performed on a computer equipped with SPSS ver. 12 (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation. Differences between groups at baseline for age, mean deviation (MD) and pattern standard deviation (PSD) were calculated using Student's t-test. Difference between groups at baseline for gender was calculated using the χ^2-test (Fisher's exact test). Differences between the two study groups in blood GSH level were computed by analysis of covariance using age as a cofactor. Differences between males and females in blood GSH level were computed by

Student's t-test for each study group. A correlation was performed to determine the association of age and blood GSH level. A multiple regression was performed to test the influences of age, gender, and blood GSH level on MD and PSD from the 24-2 test pattern of the Humphrey field analyzer. Results with $p = 0.05$ were considered statistically significant.

The minimum sample size needed was calculated from the formula $[(u + v)(\sigma_1^2 + \sigma_2^2) / (\mu1 - \mu2)^2]$ with 90% power and a 5% level of significance. The sample size needed was determined to be 18 for each group $[(1.28 + 1.96)^2(100^2 + 100^2) / (300 - 200)^2]$ using the reported data [44].

Results

Nineteen patients with NTG and 30 normal control subjects were included in this study. The demographics of the study groups are described in Table 1. There was no significant difference in age ($p = 0.056$) or gender ($p = 0.337$, $\chi^2 = 0.921$) distribution between the NTG group and control group. There were significant differences in MD ($p = 0.000$) and PSD ($p = 0.000$) between the two groups.

Total glutathione level in circulating blood was 524.02 ±
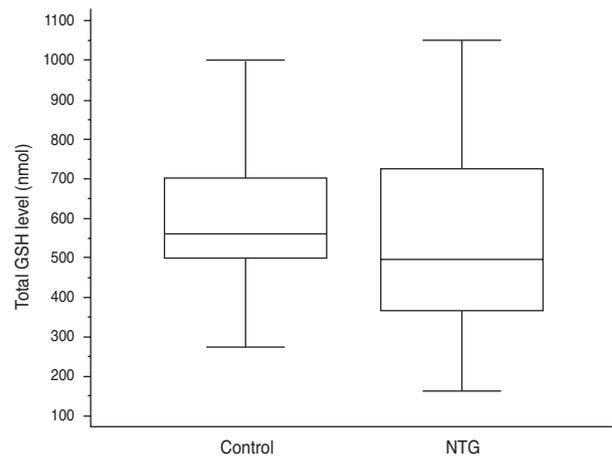
Fig. 1. Median and interquartile range values of total circulating glutathione (GSH) level in the control group and normal tension glaucoma (NTG) group. Total GSH levels were not statistically different between the two groups ($p = 0.121$, F = 2.212).

Table 1. Patient demographics (mean ± SD)

|	Control	NTG	p-value
No. of patients	30	19	
Age (yr)	52.70 ± 6.70	56.26 ± 8.48	0.056
Sex (male : female)	20 : 10	10 : 9	0.377
MD	-0.75 ± 1.24	-5.66 ± 4.32	0.000
PSD	1.58 ± 0.94	5.06 ± 4.03	0.000

Statistical analysis was performed with Student's t-test (age, MD, and PSD) and Fisher's exact test (sex).
NTG = normal tension glaucoma; MD = mean deviation; PSD = pattern standard deviation.

231.09 nmol and 586.06 ± 156.08 nmol in the NTG group and the control group, respectively. Circulating total GSH levels were not statistically different between the NTG group and control group ($p = 0.121$, F = 2.212) (Fig. 1). In the NTG group, the mean glutathione level was 589.41 ± 239.64 nmol in males and 451.35 ± 210.39 nmol in females. In the control group, the mean glutathione level was 618.98 ±208.16 nmol in males and 569.61 ± 125.70 nmol in females. There was no statistically significant difference in total GSH level between males and females in the NTG group ($p = 0.424$) (Fig. 2) or in the control group ($p = 0.202$) (Fig. 3). There was no significant assotciation be-

tween age and total GSH level in the NTG group ($p = 0.171$, r = -0.328) (Fig. 4) or in the normal group ($p = 0.380$, r = -0.166) (Fig. 5). In the NTG group, after correcting for age and gender influences on systemic total GSH level, there was no significant association between GSH level and visual field parameter (MD: $p = 0.226$, $R^2 = 0.260$; PSD: $p = 0.200$, $R^2 = 0.275$) (Figs. 6 and 7).

Discussion

Glutathione is normally present in the cornea, lens, trabecular meshwork, ciliary body, and retina. Changes of
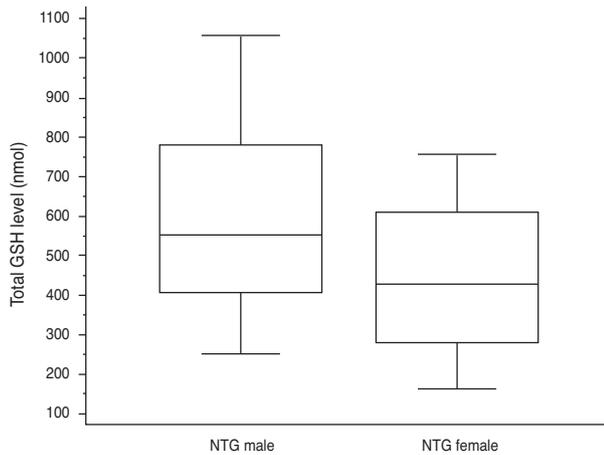
Fig. 2. Median and interquartilic range values of total glutathione (GSH) level in the normal tension glaucoma (NTG) group; comparison between male and female patients. There was no significant difference between males and females ($p = 0.424$) in total circulating GSH level.
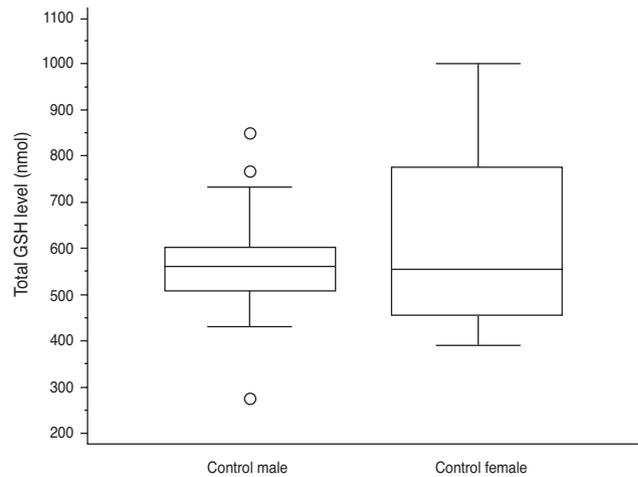

Fig. 3. Median and interquartilic range values of total glutathione (GSH) level in the control group; comparison between male and female patients. There was no significant difference between males and females ($p = 0.202$) in total circulating GSH level.
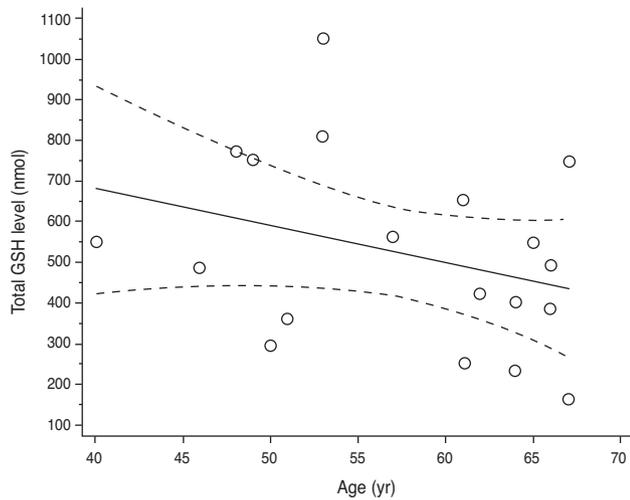
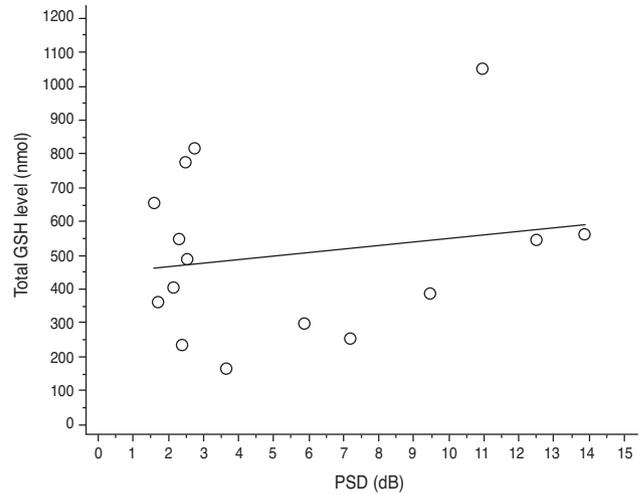**Fig. 4.** Correlation between total glutathione (GSH) level and age in year in the normal tension glaucoma group. There was no significant association between total GSH level and age (r = -0.328, $p = 0.171$).
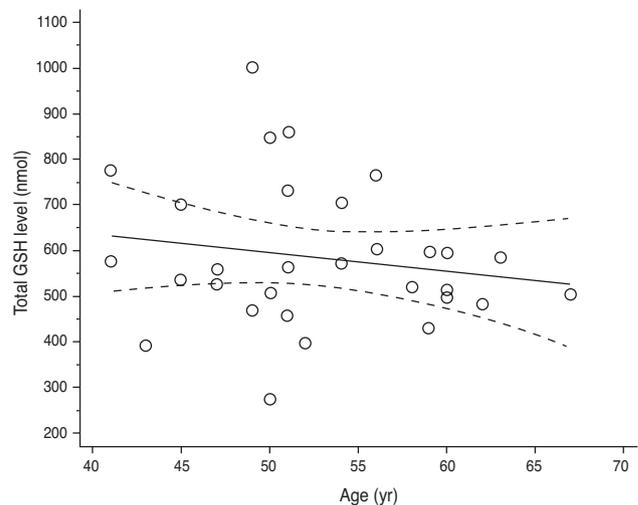
Fig. 5. Correlation between total glutathione (GSH) level and age in the control group. There was no significant association between total GSH level and age (r = -0.166, $p = 0.380$).
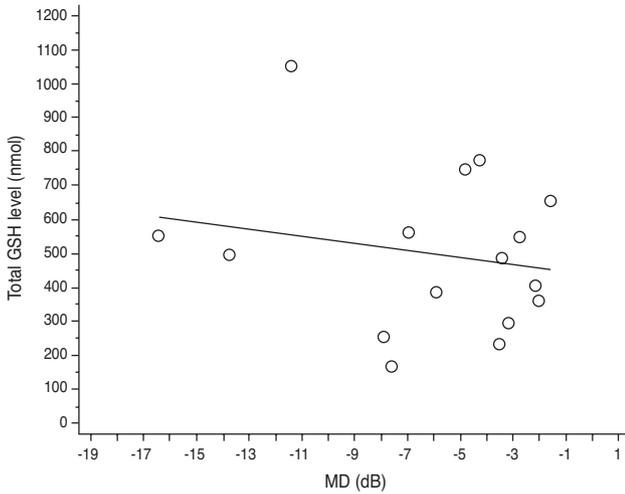
Fig. 6. Correlation between total glutathione (GSH) level and mean deviation (MD) in the normal tension glaucoma group. After correcting for age and gender influence, there was no significant association between GSH level and MD ($p = 0.226$, $R^2 = 0.260$).

Fig. 7. Correlation between total glutathione (GSH) level and pattern standard deviation (PSD) in the normal tension glaucoma group. After correcting for age and gender influence, there was no significant association between GSH level and PSD ($p = 0.200$, $R^2 = 0.275$).

glutathione and related enzyme activity in the lens, cornea, retina, and other eye tissues occur with aging, cataract, diabetes, irradiation, and administration of some drugs. Many ocular diseases including keratoconus, cataract, glaucoma, diabetic retinopathy, and age-related macular degeneration may occur by oxidative damage induced by an abnormal glutathione redox state.

In terms of glaucoma, the major interest lies on two specific structures, the trabecular meshwork and retina, because the trabecular meshwork is a rate-limiting structure of aqueous outflow pathway controlling intraocular pressure, and the retina is one of the target structures of glaucomatous optic neuropathy.

Studies on the calf trabecular meshwork (TM) have shown the presence of glutathione, glutathione reductase, and glucose-6-phosphate dehydrogenase, indicating that the TM is well supplied with the capacity to generate and use NADPH to maintain GSH. It has been supposed that GSH is able to protect the TM against H_2O_2-induced oxidative damage, which would decrease the aqueous humor outflow [19]. Altered GSH and GSH activities have also been reported in the trabecular meshwork and aqueous humor of patients with glaucoma [40,41,53-56]. All of these results imply that reduced activity of GSH in the TM may influence aqueous outflow facility and thus elevate IOP.

Additional studies of the role of glutathione in glaucoma pathogenesis are related to studies of the trabecular meshwork. However, the retina is a major target organ in glaucoma pathogenesis, and glutathione also exists in the retina. Therefore, abnormal glutathione function within the retina may play an important role in glaucoma. Although the function of glutathione in the retina is not fully understood, there is some evidence that it may have a role in neuroprotection under harmful conditions such as ischemia and oxidative damage. In the retina, glutathione is predominately localized to Muller cells and horizontal cells. According to a study by Schutte and Werner [57], using immunocytochemical methods for glutathione, postmortem ischemic injury longer than 10 minutes resulted in strong labeling of neurons, particularly of retinal ganglion cells, whereas Muller cells were essentially devoid of immunoreactivity. This means that glutathione molecules were transferred from Muller cells to retinal ganglion cells to protect ganglion cells from ischemic injury. In a study by Carter-Dawson et al. [58], Muller cells in glaucomatous monkey retinas showed significantly greater immunoreactivity for glutathione than control retinas, increasing with the duration of elevated intraocular pressure.

According to our previous study, a 15% reduction of total retinal GSH level by intraperitoneal injection of buthionine sulfoximine caused retinal neuronal apoptosis in mice [44]. To reach this level of GSH in the retina, circulating total GSH might need to be lowered even further. In the same mouse, total GSH level in the heart was reduced to 60% of that of the control mouse (unpublished data). We hypothesized that systemic total glutathione level might be changed in NTG patients in whom there may be additional factors other than increased IOP for glaucomatous damage. If there is little influence of IOP on glaucomatous optic neuropathy in NTG pathogenesis, and if oxidative damage is one of the major risk factors for NTG, the antioxidant capacity of GSH might have been changed in NTG patients. However, as the results of this study show, there was no difference in circulating GSH level between the

NTG group and control group. This difference in results between human and animal subjects is possibly due to the difference in background factors such as genetic homogeneity and diet.

Systemic glutathione level can change within a day and is affected by diet and medications. We obtained blood samples at a fixed time (8 a.m.) after fasting, as in the previous report [43], in order to reduce the sampling bias.

Gherghel et al. [43] demonstrated that patients with POAG exhibit low levels of circulating glutathione, suggesting a general compromise of the antioxidative defense. In Gherghel's study, total GSH level in the POAG group was about 75% (225.27 ± 83.03) of that of the control group (332.23 ± 98.50). In addition to the IOP range, there is one more thing we should consider about the difference in results between Gherghel's study and ours. In contrast to Caucasians, Korean and Japanese people have a higher prevalence of NTG than POAG with a 3.5-fold elevation in pressure. In Choe and Hong [46]'s study of Koreans, the estimated prevalence of POAG in the population over 20 years of age was 2.04%, and the prevalence of NTG was 1.71% (83.8% of POAG). In Iwase et al. [47]'s study of Japanese individuals, the estimated prevalence of POAG in the population older than 40 years of age was 3.9%, and the prevalence of cases of POAG with IOP levels of 21 mmHg or less was 3.6% (92.3% of POAG). Korean and Japanese people might have different risk factors compared to Caucasian populations. Different racial characteristics may lead to different results. These issues illustrate the importance of further studies of POAG with elevated IOP in Korea because of the absence of data on systemic glutathione levels in Korean POAG patients.

Although there were no statistically significant differences in total GSH level between the two groups in this study, the range of total GSH level in the NTG group showed a wider distribution compared to that of the control group. The proportions of patients with very low total GSH level and with very high total GSH level were larger in the NTG group. Considering the smaller sample size of the NTG group, this characteristic might be more meaningful. From this point of view, we can also hypothesize that the homeostasis of systemic glutathione-related antioxidant capacity may be inadequately controlled in the NTG patient. Both a low level and a high level of total GSH may be related to insufficient anti-oxidant activity against reactive oxygen species.

It is known that blood GSH level is influenced by gender, with men demonstrating higher levels than women. Our results found that mean total GSH level was slightly higher in men then in women (NTG group, 589.41 ± 239.64 nmol in males and 451.35 ± 210.39 nmol in females; control group, 618.98 ± 208.16 nmol in males and 569.61 ± 125.70 nmol in females), but there were no significant differences in blood GSH level between men and women in either the NTG group ($p = 0.424$) or the control group ($p = 0.202$). This might be due to either the small sample size or racial characteristics. There are few data on normal circulating GSH levels in the Korean population [59,60]. Further population-based studies are needed to fully explain the results of this study.

Age is known as one of the influencing factors on systemic GSH level. It has been reported that about half of healthy elderly people show low blood GSH level. A negative correlation between age and blood GSH level has also been reported. Our results demonstrated a slightly decreasing trend of GSH level as age increases but found no significant correlation between age and total circulating GSH level in either the NTG group or control group. This might be also due to the small sample size.

We also expected that if the systemic total GSH level is related to the range of glaucomatous optic neuropathy, then the extent of visual field damage in NTG patients might be proportional to the decrement of total GSH level. But the results of this study also showed no significant relation between the MD and PSD of the visual field test and total GSH level. We think this might be due to the finding of no significant difference in total GSH level. Since there was no difference of total GSH level between the NTG group and normal subject group, the extent of visual function may not have been influenced by GSH level. Another possibility is that there might be a change in circulating GSH only in patients with fast progression of optic nerve damage. If we had had a greater number of patients, we could have divided patients into two groups with progressing and non-progressing optic nerve damage and compared them. However, because normal tension glaucoma is a slowly progressing disease in most patients, this would require enormous time and effort to recruit a sufficient number of volunteers with progressive disease.

A major concern of this study is that the study results came from only one measurement of serum glutathione level. Therefore, these results might not be able to represent the long-term status of patients. However, because there are few reports about the relationship between normal tension glaucoma and glutathione, this study represents a meaningful step toward understanding the pathophysiology of normal tension glaucoma.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgements

of the authors and do not necessarily represent those of Merck & Co., Inc.

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