Inhibitor of Lysyl Oxidase in The Optic Nerve Head Complex Imparts Partial Protection Against Injury in Experimental Glaucoma

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Research article

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Abstract

**Background:** Glaucoma is a neurodegenerative disease with the progressive loss of retinal ganglion cells and changes in the optic nerve head (ONH). These changes are exacerbated by an increase in intraocular pressure (IOP).

**Methods:** The effect of scleral and optic nerve softening with beta aminopropionitrile a lysyl oxidase inhibitor (BAPN) and stiffening with genipin, in a model of chronic increase of IOP was evaluated. Changes in optic nerve and retina were evaluated. H&E, Bielschowsky’s silver staining and glial fibrillary acid protein (GFAP) staining was performed on optic nerve, retina and scleral structures. Changes in the expression of the Ywhab, Yhwaz (prosurvival genes), C3 complement (complement C3 inflammatory marker), CPG15 (neurite growth and neural survival gene) GFAP (glial activator marker) genes was carried out in the different groups.

**Results:** Protective effect of BAPN was evident by the preservation of the optic nerve structure, and with the conservation of the retinal structures, while deleterious changes were evident in the stiffening of ONH complex, characterized by the increase in the glia, changes in the optic nerve, and disorganization in the retina. BAPN induced a reduction in the expression of Ywhab, Yhwaz (prosurvival genes), C3 and GFAP (inflammatory and glial marker) and CPG15.

**Conclusions:** These findings support the critical involvement of changes in the ONH stiffness in the progression of glaucoma. The control of this variable as a regulatory mechanism in the progression of neural glaucomatous damage must be considered and would be explored as a possible intervention in glaucoma management.

Background

Glaucoma is the leading cause of irreversible blindness worldwide; in the optic nerve there is a loss of retinal ganglion cells with changes in the optic nerve head, inducing an increase of the cup /disc relation. Increases in IOP induces deformation of the optic nerve head (ONH) and reduction in the retinal ganglion cells by mechanisms such as a reduction in axoplasmic flow [2], a reduction of ocular blood flow in the ONH [3], and changes induced by mechanical strain [4]. Several authors have proposed that cross-linking of the peripapillar sclera decreased the deformation of the ONH, thus reducing the deleterious changes in this area, considering the cross-linking of ONH as a potential therapeutic strategy in the management of glaucoma [5–6]. However, other authors have proposed that the induction of cross-linking could reduce ONH compliance [7], reducing axonal flow, leading to an increase in susceptibility to glaucomatous damage [8] in glaucoma models. To support this theory, noxious effects in the retinal ganglion cells in a rat glaucoma model were exacerbated by cross-linking the ONH [9], such as an increase in collagen and in peripapillary thickness in glaucoma rodent models [10]. In this work, we proposed evaluating the effect of softening the ONH via lysyl oxidase inhibition (LOX inhibition) using beta-aminopropionitrile (BAPN) to inhibit the collagen I and elastin cross-linking, in a glaucoma model.
and to induce stiffening of the ONH with genipin, thus we can confirm the opposite effects in the changes in the optic nerve and in the retina in this model.

Some authors have proposed that stiffening of the ONH may be related to several mechanisms involved with age, such as cross-linking, calcium, lipids and increases in advanced glycation end products (AGEs) affecting the collagenous and non-collagenous components of the extracellular matrix, as well as changes in the phenotype of fibroblasts to myofibroblasts, increasing the fibrosis in the ONH, and leading to a reduction of the ONH compliance [9–11]. It has also been found that glaucoma patients exhibited a higher ocular stiffness than non-glaucoma subjects [12]. In this model, we used genipin, a natural cross-linker that has low toxicity and interesting properties, as a crosslinker agent; this agent induces corneal [13] and scleral stiffening in ex vivo and in vivo models [14], with minimal damage to the corneal endothelial and retinal cells [5–15], and is useful in controlling eye growth in myopia models [16]. In a clinical setting, the uses of scleral strips treated with genipin have been proposed in the control of myopia [17]. Furthermore, the dosage of genipin in the rat eye has been previously characterized [18], showing a good effect as a cross-linker in the optic nerve complex; in the posterior sclera, with only one injection with no toxicity to retinal ganglion cells, there are no changes in gene expression in normal rat eyes [5]. This type of cross-linking is defined as dark cross-linking since there is no need for an external light source in order to achieve the cross-linking effect [6].

In contrast, beta aminopropionitrile (BAPN) was used for the opposite reason; as an inhibitor of collagen-elastin cross-linking via lysyl oxidase (LOX) inhibitor, it prevents the cross-linking of collagen I and the elastin-induced softening of tissues and reverses myocardial fibrosis in murine models [19]. The aim of this study was to determine whether cross-linking or softening of the ONH is detrimental to retinal cells and the optic nerve in a model of glaucoma in rats. Intraocular pressure changes as well as changes in the retina and ONH morphology and changes in the gene expression of the Ywhab, Yhwaz (prosurvival genes), C3 complement (complement C3 inflammatory marker), CPG15 (neurite growth and neural survival gene) and GFAP genes were evaluated by RT-PCR and compared in different groups (Glaucoma, Glaucoma-genipin, Glaucoma –BAPN, genipin and BAPN).

**Methods**

**Animals**

White albino Wistar rats were used; half were female and half male (n=40). All rats were housed on a 12-hour light-dark cycle and were provided with food and water *ad libitum*. All procedures were approved by the Institutional animal care and use committee from the Facultad de Medicina Universidad Nacional de Colombia, under the approval 010-135-19 and followed the guidelines of ARVO, and Colombian regulation for animals in experimentation. Surgical procedures as well as retrobulbar injections were performed under general anesthesia using ketamine (60 mg/kg) and xylazine (7.5 mg/kg) complemented with topical proparacaine (Alcon Fortworth TX). Retrobulbar injections were performed using a 31 g
microneedle (BD Insulin Syringe Ultra-Fine needle, Becton, Dickinson and Company, Franklin Lakes, NJ) in a volume of 150µl.

**Experimental groups and Glaucoma induction, Cross-linking or softening procedure**

Rats were assigned to one of the five groups as follows. Group 1 (n=8) received a surgical procedure for the induction of glaucoma using the protocol described previously [20]; briefly, a unilateral circumlimbar suture (8.0 nylon) was tied around the equator at 1.5 mm behind the limbus, was anchored by six subconjunctival anchor points, and was tightened firmly inducing the elevation of IOP, demonstrated by tonocare IOP measurement. The contralateral eye was used as a control naïve eye. Group 2 (n=8) received a surgical procedure for the induction of glaucoma and a retrobulbar injection of genipin at a 15mMol dissolved in balanced salt solution (BSS) in a volume of 150µl; the contralateral eye was used as a naïve control. Group 3 (n=8) received the surgical procedure for the induction of glaucoma, and a retrobulbar injection of beta-aminopropionitrile BAPN/Balanced salt solution (10 mMol) at a volume of 150µl; the contralateral eye was used as a naïve control. Group 4 (n=8) Received a retrobulbar injection of genipin/BSS (15 mMol) and Group 5 (n=8) received a retrobulbar injection of beta-aminopropionitrile BAPN/Balanced salt solution (10 mMol).

**Intraocular pressure measurement**

Animals were acclimated to the facility for a week. Then, baseline IOP measurements were performed using a rebound tonometer (Tonolab Icare, Helsinki Finland), and measurements were performed between 11 am and 2 pm in order to avoid diurnal variations. All measurements were performed in awake animals without topical anesthesia, except for the immediate intra-operative and early post-operative measurements. Measurements were taken preoperatively and until 4 weeks postoperative.

**Evaluation of Optic nerve and retina.**

Animals were sacrificed and the whole globe was immersed in 10% buffered formalin as a fixative agent. Eyes were stained with H&E, Bielschowsky’s silver staining, and with anti-GFAP (1/100 DAKO and then stained with DAKO LSAB/HRP). Images from optic nerve were obtained and analyzed with Image software in order to determine the number of cells (NIH).

**Evaluation of Gene expression Changes**

Animals were sacrificed with eutonax at 4 week and the retinas and optic nerve head of the treated eyes were isolated. A total of 4 specimens were isolated per group and immersed in RNA later (ThermoFisher) and stored at -70ºC, after which RNA was extracted using the Monarch total RNA extraction kit (New England Biolabs Boston MA). Extracted RNA was quantified and then cDNA was amplified using the Luna One-Step RT-qPCR universal kit (New England Biolabs Boston MA).

RT-qPCR was performed (iCycler iQ; Bio-Rad, Hercules, CA) with SYBR green PCR master mix (Bio-Rad). A computer program (version:2020.2 software; Bio-Rad) was used to visualize the data. The standard curve
method was used to determine relative changes in gene expression levels with beta-actin serving as the reference, which did not change significantly in our samples. An ANOVA test was conducted to compare the normalized relative expression values between all groups to determine statistical significance.

Genes evaluated included Ywhab, Ywaz (prosurvival genes), C3 complement, CPG15, GFAP and beta actin, as shown in Table 1.

**Data analysis**

IOP measurements are provided as means ± SEM. Significance was evaluated between two sets of data with Student’s t-test (Paired). Difference between groups were evaluated with Kruskal-Wallis One Way Analysis of Variance on Ranks (ANOVA on ranks) as appropriate, followed by Multiple Comparison Procedures (Dunn's Method). Differences were considered significant if the probability of the null hypothesis (P) was <0.05.

**Results**

**Changes in Intraocular pressure in Glaucoma treated vs treated eyes**

Intraocular pressure showed an increase in the postoperative period with a spike at 1 hour postoperative (60 mmHg) that reduced in the first day. Posteriorly, a stabilization was found between the groups and the values were similar between the glaucoma model groups in the first week; average values ranked in the glaucoma group 21.8±0.6 mmHg, glaucoma-genipin group 25.5±0.5 mmHg and glaucoma-BAPN group 22.5±0.7 mmHg. Then, there was a reduction of IOP in the glaucoma-BPNA group and an increase in the glaucoma-genipin group. In the second week, there was an increase in the glaucoma-genipin group vs. glaucoma and there was a reduction of IOP in the glaucoma-BAPN group. However, those values were not significantly different in the ANOVA analysis. This difference between groups (reduction in glaucoma-BAPN and increase in glaucoma-genipin) remained significantly elevated in weeks 2, 3 and 4; the IOP values at this point were 19.8±1.1 mmHg in group 1 (glaucoma), 22.8±0.58 mmHg in group 2 (glaucoma+genipin), 16.1±0.4 mmHg in group 3 (glaucoma+BAPN), 8.8±0.7 mmHg in group 4 (genipin), 8.3±0.2 mmHg in group 5 (BAPN) and 8.5±0.43 mmHg in untreated eyes. A significant difference was found between glaucoma groups (ANOVA on Ranks p value 0.05) (Figure 1, Table 2).

**Changes in the optic nerve**

The longitudinal optic nerve sections showed a disorganized structure of the optic nerve in the glaucoma (Figure 2A) and glaucoma-genipin treated eyes (Figure 2B) vs glaucoma-BAPN (Figure 2C); in the glaucoma stiffened eyes, the destruction of the structures was evident, as was the loss of bundles in the optic nerve in comparison with the control (Figure 2D). These bundles were well preserved in the control (Figure 2D) and severely altered in the glaucoma (Figure 2A) and glaucoma-genipin groups (Figure 2B) with clearance of the cytoplasm, degenerative changes and microglial cells (asterisks). A Schnabel cavernous optic atrophy like, is observed and characterized with the loss of myelin and axons and
preservation of septa with a spongiform appearance and was evident in the glaucoma and glaucoma-genipin groups. Glaucoma-BAPN showed organization of optic nerve bundles with minimal microglial cells (Figure 2C).

**Changes in retina and nerve fiber evaluation**

At week 4, retinal cell layers were evaluated using the retina cross sections stained with H&E and a comparison between all groups. The normal retina was observed in the control group (Figure 3d), and thinning of the retina was observed in glaucoma (Figure 3a) along with alterations in ONL, OPL, IPL and a reduction in RGC with a loss of inner nuclear cells layer and gliosis-like shape; a loss of the retinal structure was observed in the glaucoma-genipin group (Figure 3b) while a complete evaluation of all layers is observed in the glaucoma-BAPN (Figure 3c), genipin (Figure 3e) and BAPN (Figure 3f).

Bielschowsky's silver staining shows preservation of the inter-neuronal unions and axonal staining in the normal retina (Figure 4D), genipin alone (Figure 4E) and BAPN alone (Figure 4F), as well as in the glaucoma-BAPN group (Figure 4C); in the glaucoma group (Figure 4A) and glaucoma-genipin group (Figure 4B), severe fiber distortion and a loss of the axonal union was observed in the central area of the retina. Staining with GFAP antibody in the glaucoma-genipin (Figure 5B) and glaucoma (Figure 5A) groups was evident in the nerve fiber layer (NFL), ganglion cell layer (GCL), and projections to inner plexiform layer (IPL), while there was minimal staining in the NFL in the control group (Figure 5D) and glaucoma-BAPN group (Figure 5C).

**Changes in Gene Expression and effects of BAPN and genipin in glaucoma eyes**

Gene expression of C3 complement was up-regulated in glaucoma +6.1±0.4 in comparison with the control group; a significative down-regulation observed in the BAPN/glaucoma group and in the genipin/glaucoma group. No differences were noted in the BAPN and genipin group (Figure 6A).

The GFAP gene was up-regulated in glaucoma by +1.4±0.02 in comparison to the control group, as well as in BAPN (+1.3±0.9) and was down-regulated in the glaucoma-genipin group (-19±0.9), BAPN/glaucoma group (-33±2.0) and in the genipin group (-16±1.4) (Figure 6B).

Cpg15 gene was down regulated in glaucoma by -1.8±0.7 in comparison with the control group, as well as in the BAPN-glaucoma group by -20±2.0 and in the genipin-glaucoma group by -14±0.9; in the genipin group, this was reduced by -2.7±1.4 and was increased in the BAPN group by +3.7±1.0 (Figure 6C).

Ywhab gene was up-regulated in the glaucoma group by 1.8±0.27 and down-regulated in the glaucoma-genipin and glaucoma-BAPN group, as well as severely up-regulated in the genipin group (77±1.42) (Figure 7A).

The Ywhaz gene was down-regulated in glaucoma by -5.3±0.4 in comparison to the control group, and was also down-regulated in the BAPN/glaucoma group -21±2.1; in BAPN alone, a significant reduction
was also observed by -30±1.2, while an increase in expression was observed in the genipin/glaucoma group by +3.7±0.95 and in the genipin group by +10.3±1.4 (Figure 7B).

**Discussion**

Scleral collagen cross-linking has been proposed as a possible treatment for disorders that involve changes in the ocular diameter as myopia, in rodent models [16], and is proposed in the management in the reduction of biomechanical stress on the optic nerve head [5–6], thus reducing the stress of RGC and protecting the loss of those cells [1]. Several authors have demonstrated that scleral cross-linking with genipin has minimal adverse effects in the normal eye, and in their retinal function [5]; however, there is some controversy around whether axonal damage is exacerbated, and retinal axon ganglion loss is induced by scleral cross-linking with glyceraldehyde [9]. The aim of this work is to evaluate the changes with a less toxic cross-linking agent (genipin) in a glaucoma model and to compare with a BAPN, a known substance that induces the opposite effect, producing a softening effect in several tissues [20], by the inhibition of lysyl oxidase (LOX) in models of thoracic aortic aneurysm and improving the outflow facility (OF) in the trabecular meshwork [21].

Our findings have several implications: the increased stiffness with cross-linking simulating the effect of aging, changing the tissue to be less elastic, mechanically weaker and more rigid than younger tissues, as happen in ocular tissues in glaucoma eyes [8]. This could be similar in the ONH and an increase in neuronal damage, as observed in this study. Some of the effects observed could be related to changes in the outflow facility (OF) due to the reduction observed with genipin in previous studies [21], or by the increase in the OF observed with BAPN. Despite this effect which could be affecting IOP, the observed effects are more closely related to the direct effect in the ONH complex area, unlocking the importance of the softening or stiffening of ONH. Some authors have demonstrated that an increase in strain in the peripapillar scleral fibroblasts induce myofibroblast differentiation; this type of change is required for tissue injury repair response [22] and could be related to an induction of pro-fibrotic and pro-inflammatory response, increasing collagen deposition and then reducing the compliance of the ONH complex.

Collagen cross-linking happens naturally, mainly by two pathways: enzymatic cross-linking via LOX activity and non-enzymatic cross-linking via reducing sugars forming advanced glycation end-products (AGEs). LOX is an extracellular enzyme that catalyzes the conversion of lysine to allysine to their aldehyde, which condenses to induce covalent cross-linking [7–8]. This enzyme is required for the cross-linking of collagen and elastin. In the eye, LOX is involved in changes in the trabecular meshwork by cross-linking via the gremlin pathway and thus increasing aqueous humor outflow resistance [23]. LOX deficiency could be induced with the irreversible inhibitor β-aminopropionitrile (BAPN) [24].

An increase of the pro-inflammatory C3 was found in the glaucoma group. It is well known that glaucoma induces the expression of C3 in human tissue and in glaucoma models, and it has been proposed that C3 targeting reduces the optic nerve degeneration in DBA glaucoma mice [25]. An unexpected result is the reduction in the expression in the glaucoma-genipin and in the glaucoma-BAPN group; these findings
could be explained by the anti-inflammatory effect of genipin by itself [26], and by the preservation of the ONH anatomy by BAPN, reducing the damage in the RGC and in the ONH complex, thus reducing inflammation.

Changes in the Ywhaz gene expression with a function as a mitochondrial import stimulation factor, was also observed in this study. A down-regulation in glaucoma (as previously demonstrated) (27) and in the glaucoma-BAPN group was found in this work, and their increase in the glaucoma-genipin group and in the genipin group is observed; this gene is considered an inductor of pro-survival pathways in RGC. Despite this, BAPN induced a reduction of this gene and genipin increased the expression of this gene. This effect could be related to the neuroprotective effects of genipin itself, as has been demonstrated in models of Alzheimer degeneration (26); despite the up-regulation, the net effect was neuronal damage, demonstrating that the effect of stiffening by itself is more injurious than the possible neuroprotective effect of genipin. However, more studies are required to elucidate this change. Ywhab was also down-regulated in the treated glaucoma-BAPN and glaucoma-genipin group but was overexpressed in genipin control. This effect could also be explained by the neuroprotective effect of genipin mediated by the reduction of ROS species and RNS species [8–9]; as previously stated, stiffening induced more deleterious changes than the effect of genipin as a neuroprotective agent.

GFAP gene expression was observed in the glaucoma group and a reduction in the glaucoma-BAPN group, glaucoma-genipin group and genipin group. GFAP is a complex system that is up-regulated in neurodegenerative diseases; its role could be related to a reduction of the neuronal damage, and an increase in the affected area to form a physical barrier to isolate the damaged tissue [10]. A protective effect in the treatment may explain the reduction in the expression of GFAP as well as the expression in the Ganglion cell layer in glaucoma and in glaucoma-genipin retina.

Cpg15, a candidate plasticity gene that promotes neurite outgrowth, is down-regulated in glaucoma models; this finding was also present in our study, as well as an important down-regulation in treated groups. This down-regulation may be related to the reduction of ischemia in the ONH, as has been proposed in models of transient global cerebral ischemia, where ischemia is required to induce the overexpression of Cpg15 [11]; however, more studies are required to clarify this point.

Conclusions

In conclusion, our model confirms the effects of changing the stiffness of the ONH complex in a glaucoma model, showing that this factor is crucial for the progression of glaucoma. A proposed mechanism is presented in Figure 8. Stiffening the ONH complex induces a reduction in scleral compliance and induces axon damage in the ONH and in the retinal layers with changes in the optic nerve. There is also local degeneration, cavernous atrophy and the induction of pro-inflammatory molecules, as well as a reduction of prosurvival genes. In contrast, softening of the ONH induces the preservation of axons and optic nerve structure as well as preservation of the retinal layers and a
reduction in the induction of inflammatory genes. ONH softening may represent a new target in the management of glaucoma and deserves more studies.

**Abbreviations**

ONH Optic Nerve Head

IOP Intraocular Pressure

BAPN Beta aminopropionitrile

GFAP Glial fibrillary acid protein

LOX Lysyl oxidase

OF Outflow facility

AGEs Advanced glycation end-products

RGC Retinal ganglion cells

**Declarations**

Ethical Approval and Consent to participate: Ethical approval was obtained by the IRB 010-135-19 Facultad de Medicina Universidad Nacional de Colombia.

Consent for publication: Non Applicable

Availability of supporting data: Supporting data is available upon request to the Corresponding author as a PDF format

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Authors’ contributions : CMC.; Designed and performed experiments, analysed data and co-wrote the paper. MB Performed experiments, SP Performed experiments and Performed bioinformatic analyses, PGF Performed pathologic analyses, JAA Performed experiments, ZD Supervised the research and designed experiments, MYA Designed experiments, analyzed data and co-wrote the paper and supervised the research. All authors read and approved the final manuscript.

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Tables

Table 1 Primers of genes evaluated in the Glaucoma model RT-qPCR
| Gene Name                                                                 | Primers                                                                 | Amplicon |
|--------------------------------------------------------------------------|-------------------------------------------------------------------------|----------|
| C3: complement component 3                                               | F: CTC TGG GGA GAA AAG CCC AAT ACC                                       | 97       |
|                                                                         | R: AAA CCA CCA TTG TTT CTG TGA ATG CCC                                   |          |
| Ywhab: tyrosine 3-monoxygenase/tryptophan 5-                            | F: TCC TGA AAA GGC CTG TAG CCT GG                                         | 184      |
| monoxygenase activation protein, beta polypeptide                        | R: TTC TCT CCC TCT CCA GCA TCT CC                                       |          |
| Ywhaz: tyrosine 3-monoxygenase/tryptophan 5-                            | F: ATC AGA CTG GGT CTG GCC CTC AA                                       | 216      |
| monoxygenase activation protein, zeta polypeptide                       | R: TGC TTC GTC TCC TTG GGT ATC CG                                       |          |
| Cpg15: candidate plasticity gene 15                                       | F: TAT CCA AGG CAG CTT ATT CG                                             | 200      |
|                                                                         | R: ATG GCT CTT CTC GAT TT                                                |          |
| Gfap: Glial fibrillar acid protein                                       | F: TGGACACCAAATCTGTGTCAGAAGG                                             | 200      |
|                                                                         | R: ATCACATCCTTGCTCCCTGCTTG                                              |          |
| Beta actin                                                              | F: GCTACAGCTTCACCACCA                                                    | 117      |
|                                                                         | R: TCTCCAGGGAGGAGAGGAT                                                   |          |

Table 2 is not available with this version.

Figures
Figure 1

Changes in Intraocular pressure in the groups. Glaucoma, Glaucoma-Genipin, Glaucoma-BAPN increases IOP in the follow up a reduction of IOP was observed in the Glaucoma-BAPN at week 3 and 4. Control groups include Genipin, BAPN and untreated eyes with similar IOP.
Figure 2

Longitudinal optic nerve changes. A glaucoma, B Glaucoma-Genipin, C BAPN-Glaucoma, D Control. Cavernous optic changes are observed in glaucoma as well as loss of nerve bundles (A), glaucoma – genipin (B) and minimally in glaucoma-BAPN (C) vs control (D). line 50 microns
Figure 3

Retinal changes in treated vs non treated eyes. A Normal retina, B Glaucoma, C Glaucoma-Genipin, D Glaucoma-BAPN, E Genipin, F BAPN. (HE). GCL ganglional cell layer, IPL internal plexiform layer, INL Internal nuclear layer, OPL outer plexiform layer, ONL Outer nuclear layer. H&E line 50um

Figure 4

Bielschowsky's silver Staining retina. A Glaucoma, B Glaucoma-Genipin, C Glaucoma-BAPN, D Control, E Genipin, F BAPN.
**Figure 5**

Glial Fibrilar Acid Protein in retina. A Glaucoma, B Glaucoma –Genipin, C Glaucoma-BAPN, D Control. A positive staining was observed in Glaucoma and in Glaucoma-Genipin in the ganglionar cell layer, and in the inner plexiform layer.
Figure 6

Gene expression. Complement C3, Glial Fibrilar Acid Protein and CPG15.
Figure 7

Gene expression. Ywhab and Ywhaz.

Figure 8

Schematic of effect Softening ONH-Complex and Stiffening ONH. A reduction of the compliance by glaucoma and stiffening the ONH complex induces optic nerve damage in this model. B Softening by BAPN induces preservation of optic nerve and retinal structures precluding the deleterious damage of glaucoma.