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ORIGINAL ARTICLE

Riluzole- and Resveratrol-Induced Delay of Retinal Ganglion Cell Death in an Experimental Model of Glaucoma

Dilara Pirhan¹, Nurşen Yüksel¹, Esra Emre¹, Abdulkadir Cengiz² and Demir Kürtat Yıldız³

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ABSTRACT

Purpose: To evaluate the effects of the neuroprotective agents riluzole and resveratrol on the survival of retinal ganglion cells (RGCs) when administered alone or in combination.

Materials and methods: Experimental glaucoma was induced by injecting hyaluronic acid into the anterior chamber of Wistar albino rats weekly for a six-week period. Intraocular pressure was measured before and immediately after glaucoma induction. The neuroprotective effects of daily intraperitoneal injections of riluzole (8 mg/kg) and resveratrol (10 mg/kg) were evaluated and compared. After the six-week period, dextran tetramethylrhodamine was applied into the optic nerve and the density of surviving RGCs was evaluated by counting the labeled RGCs in whole mount retinas for retrograde labeling of RGCs.

Results: The mean numbers of RGCs were significantly preserved in all treatment groups compared to the vehicle-treated glaucoma group (G). The mean number of RGCs in mm² were 1207 ± 56 in the control group (C), 404 ± 65 in G group, 965 ± 56 in riluzole-treated group in the early phase of glaucoma (E-Ri), 714 ± 25 in riluzole-treated group in the late phase of glaucoma (L-Ri), 735 ± 29 in resveratrol-treated group in the early phase of glaucoma (E-Re), 667 ± 20 in resveratrol-treated group in the late phase of glaucoma (L-Re), and 1071 ± 49 in riluzole and resveratrol combined-treated group in the early phase of glaucoma (E-RiRe group).

Conclusions: When used either alone or in combination, both riluzole and resveratrol, two agents with different mechanisms of action in glaucoma, significantly delayed RGC loss in this study’s experimental glaucoma model.

Keywords: Glaucoma, neuroprotection, resveratrol, retinal ganglion cell, riluzole

INTRODUCTION

Glaucoma is a neurodegenerative disease associated with long-term progressive retinal ganglion cell (RGC) death.¹ The most important risk factor for the onset and progression of the disease is an abnormal elevation of intraocular pressure (IOP), which may lead to RGC loss.²

RGC is highly susceptible to the stress caused by increasing IOP in the retina.³ Currently, approaches for treating the disease to reduce IOP include medical or surgical intervention, sometimes lasers. However, IOP can be conceived as a direct inducer of RGC stress and apoptosis;⁴ RGC failure can occur in the presence of normal IOP,² which means the patients’ visual loss will continue. Therefore, for managing glaucoma, the neuroprotection of RGC has been emphasized as an alternative strategy independent of and complementary to IOP reduction.

Some potential mechanisms of RGC death in glaucoma have been hypothesized, including compromised blood flow in the optic nerve, nitric
oxide-induced injury to the optic nerve, glutamate excitotoxicity and oxidative stress. In addition, several categories of molecules associated with the degenerative processes of glaucoma are currently being investigated, including neurotrophins, glutamate receptor antagonists, calcium channel antagonists, sodium channel blockers, alpha agonists, nitric oxide inhibitors, antioxidants and inhibitors of apoptosis. Neuroprotective compounds approved for neurodegenerative diseases have also been the focus of comprehensive reviews for glaucoma.

Riluzole is a FDA-approved drug treatment due to its life-prolonging effects in amyotrophic lateral sclerosis (ALS). However, the mechanism of neuroprotection has not yet been fully clarified. Voltage-gated sodium channels contribute to the development of axonal degeneration in white matter tracts, including the optic nerve. In vitro and animal model studies have shown that riluzole exerts a neuroprotective activity on axons subjected to injury. Riluzole also blocks sodium channels, inhibits persistent sodium currents and partially protects axons within white matter that have been subjected to anoxia. Riluzole has also been shown to have neuroprotective effects in spinal cord injuries (SCIs).

Resveratrol, a polyphenolic compound found in grapes and wine, has numerous potentially beneficial effects, including cardioprotection, anti-oncogenesis and antioxidant activity, as well as both anti-inflammatory and neuroprotective effects.

The protective effects of resveratrol in different tissues have long been studied, and the drug has exhibited several neuroprotective effects in a variety of cerebral ischemia experimental models. Resveratrol provided neuroprotection by showing antioxidant activity and anti-platelet aggregation, as well as vasodilatory, anti-inflammatory and anti-aging effects.

Riluzole and resveratrol are potent anti-apoptotic drugs. Riluzole has shown broad neuroprotective activity in neuronal cell cultures and in animal models of neurodegenerative diseases. By contrast, resveratrol is a naturally occurring polyphenol found in grapes and wine. Its protective effect, which has been shown on ocular tissues, however, is not yet well known.

This combination of riluzole and resveratrol was used also in ALS, which is a neurodegenerative disorder. Since glaucoma exhibits the pathophysiological features of both chronic and neurodegenerative disease, riluzole and resveratrol may be useful in managing glaucoma. In this study, we chose riluzole and resveratrol, which have been approved as safe and efficient in their respective therapeutic categories by using different mechanisms of action. Based on the pharmacological profiles of two drugs, it is likely that a combination of those two drugs may provide more effective therapeutic windows than the monotherapy in reducing glaucomatous injury with reduced side effects. Therefore, this study aimed to evaluate the effects of these neuroprotective agents, riluzole and resveratrol, on the survival of RGCs when administered alone or in combination. It was hypothesized that these two agents might prevent apoptosis and offer neuroprotection in the experimental glaucoma model.

MATERIALS AND METHODS

Animals

Adult male Wistar albino rats were housed in temperature- and light-controlled rooms with a 12-h light/dark cycle as well as food and water ad libitum. All animals experiments were performed in accordance with the guidelines provided by the experimental animal laboratory and approved by the Animal Care and Use Committee of the School of Medicine at Kocaeli University and followed the Association for Research in Vision and Ophthalmology Resolution on the Care and Use of Laboratory Animals. The most common side effect of riluzole and resveratrol is weight loss. Average base weight levels were 372 ± 23 g in the C group, 367 ± 12 g in G group, 370 ± 18 g in E-Ri group, 367 ± 17 g in L-Ri group, 370 ± 17 g in E-Re group, 365 ± 10 g in L-Re group and 369 ± 13 g in E-RiRe group. All rats gained weight, and the average weights at the end of study in groups were 416 ± 22 g for the C group, 404 ± 22 g for G, 412 ± 15 g for E-Ri, 400 ± 18 g for L-Ri, 401 ± 17 g for E-Re, 393 ± 9 g for L-Re and 394 ± 11 g for E-RiRe. There was no evidence of loss of appetite or behavioral disorder in any group.

Treatment Protocols

The rats were randomly divided into the following seven groups based on treatment modalities:

1. Early riluzole-treated group (E-Ri group, n = 12): The group received a single daily dose of 8 mg/kg riluzole via intraperitoneal injection starting with the glaucoma induction for a period of six weeks.
2. Late riluzole-treated group (L-Ri group, n = 13): The group received the same dose of riluzole starting three weeks after glaucoma induction for a period of three weeks.
3. Early resveratrol-treated group (E-Re group, n = 12): The group received a single daily dose of 10 mg/kg resveratrol via intraperitoneal injection starting with glaucoma induction for a period of six weeks.
4. Late resveratrol-treated group (L-Re group, n = 14): The group received the same dose of...
resveratrol starting three weeks after glaucoma induction for a period of three weeks.

(5) Early riluzole and resveratrol combination group (E-RiRe group, n = 14): The group received the same doses of riluzole and resveratrol together starting with the glaucoma induction for a period of six weeks.

(6) Glaucoma group (G group, n = 14): The group received an intraperitoneal injection of physiological saline including 2% ethanol as a vehicle control for all drug groups complying with the drug schedule for six weeks.

(7) Control group (C group, n = 12): The group received no medication, although an equal volume of saline solution was injected into the right eyes.

Riluzole (Sanofi-Aventis, Paris, France) was first dissolved with 0.05% ethanol in 0.9% saline. Resveratrol (Sigma, St. Louis, MO) was dissolved with 2% ethanol in 0.9% saline.

**Experimental Glaucoma Model**

Rats were anesthetized by intraperitoneal injection with 1.5 mL/kg solution of 5 mL ketamine (100 mg/mL) and 2.5 mL xylazine (20 mg/mL). A drop of 0.5% proparacaine hydrochloride was applied to each eye. IOP was measured immediately after anesthesia induction and then hyaluronic acid was injected and IOP was measured again. Chronic elevation of IOP was induced in one eye of each animal by intracameral injection of hyaluronic acid (Sigma catalog no. H1751; Sigma Chemical Co., St. Louis, MO) weekly (Figure 1a) by using a syringe with a 30-gauge needle, as previously described by Moreno et al.26 An equal volume of saline solution was also injected into the right eyes of the C group.

**IOP Measurements**

IOP measurements were taken at the same time under using the same anesthesia. A calibrated Tono-Pen® XL (Reichert, Inc., Depew, NY) tonometer was used to determine IOP. IOP was measured weekly before and immediately after every glaucoma induction. All measurements were always taken by the same examiner in the morning to minimize both variations and IOP fluctuations due to either circadian rhythm27 or the elevation of IOP itself.28 With gentle manual restraint, IOP readings were obtained in each eye by using firm contact with the cornea. Readings acquired were omitted as the instrument was removed from the eye. Instrument-generated averages were ignored, because they have proven uncertain when compared with actual IOP. The average of 15 successive measurements was recorded as IOP, as suggested by Moore et al.29,30

**Retrograde Labeling of RGCs**

The fluorescent tracer dextran tetramethylrhodamine (DTMR; 3000 MW; Molecular Probes, Inc., Eugene, OR) was injected into the optic nerve 48 h prior to sacrifice, as previously described.31–33 DTMR diffuses passively through the axon toward the cell soma, which produces an intense labeling in normal31,32,34,35 and ocular hypertension eyes.35

**Tissue Sampling**

Rats were heavily anesthetized and perfused transcardially through the ascending aorta with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), as previously described by Salinas-Navarro et al.31,32 Rats with corneal opacity, lens damage and/or retinal injury were excluded from this study. Six rats, two from G group (one lens damage and one corneal opacity) one from E-Ri group (corneal opacity), one from L-Ri group (corneal opacity), one from E-Re group (corneal opacity) and two from E-RiRe group (two retinal injury) were excluded from this study. As a consequence, final group numbers were 12 for all.

Globes were marked for orientation from the superior limbus to the fixed eye, and enucleation was performed immediately after the sacrifice of animals. The lens and vitreous were extracted by cutting the anterior chamber at the ora serrata. The eyecups were postfixed in the same fixative for 2 h, after which the retinas were removed and flat-mounted with the RGC layer uppermost using PBS/glycerol (1/1). The superior retina was then marked for orientation.

**RGC Counting Procedure**

For quantitative analysis, the retinas in which DTMR had been applied were photographed shortly after mounting. Retinal whole mounts were examined with an Olympus BX51 (Olympus, Tokyo, Japan) fluorescence microscope coupled with a digital camera and equipped with an appropriate ultraviolet filter to observe DTMR-labeled cells. Counts were taken from comparable areas of four quadrants of each retina along two radii in four directions (i.e. superior, temporal, inferior and nasal) centered on the position of the optic nerve head at a distance of 2 mm from the optic disc. Three fields 1 mm from each other were counted with a 40 super wide-field objective along each radius, which yielded 24 fields per retina.
Fluorescent ganglion cells were clearly visible throughout the retina (Figure 1c). RGCs were counted in 24 areas of 0.134 mm² and each area per retinal quadrant. From these, the average number per area were calculated (Figure 1d). An experienced observer who was masked to both the procedure and the treatments implemented performed the RGC-counting process. Images were counted by Image Analysis Software Image Pro Plus (IPP 5.1 for Windows; Media Cybernetics, Silver Spring, MD).

**RESULTS**

**Intraocular Pressure**

All eyes that inducted to glaucoma (n = 72) developed elevated IOP within 0 d of induction. In all groups, all animals’ IOP increased rapidly, iris vessels faded in color. Corneal edema appeared and adequate IOP values were reached during the six-week study period. The mean IOP of the glaucomatous eyes was significantly greater than the controls for each treatment group (p < 0.01) (Supplementary Figure). The mean IOP of the glaucoma group and the five different treatment groups showed no significant difference, which suggests that any difference in RGC survival could be attributed to the treatment (Figure 2).

**RGC Density**

The area of each field in our microscope camera is 0.134 mm², which yields a total counted area of 3.21 mm² or a 6.45% sample of the average 50.1 mm² Wistar rat retina.36
The mean density of DTMR-labeled RGC density (mean cell number/mm² ± standard deviation) was 1207 ± 56 in the C group, 404 ± 65 in G, 714 ± 25 in E-Ri, 965 ± 56 in E-Re, 735 ± 29 in L-Ri, 714 ± 25 in L-Re and 1071 ± 49 in E-RiRe (Figure 3; Table 1). Six weeks after glaucoma induction, the mean RGC loss as compared with the C group was 66 ± 5% in G, 19 ± 5% in E-Ri, 40 ± 3% in L-Ri, 38 ± 3% in E-Re, 44 ± 5% in L-Re and 11 ± 3% in E-RiRe.

A multiple comparison of the groups is listed in Table 1. The RGC density was significantly greater in the C group than in any other group (p < 0.001 for all) and significantly less in the G group compared to those of all treatment groups (p < 0.001 for all). RGC density in the E-RiRe group was found to be the highest of all groups (p < 0.001 for all), while RGC density in the E-Ri group was also significantly higher than those in the L-Ri, E-Re and L-Re groups (p < 0.001 for all). RGC density in the L-Ri group was not significantly different from those in the E-Re and L-Re groups (p = 0.923 and p = 0.179, respectively). RGC density in the E-Ri group was significantly higher than that in the L-Re group (p = 0.010). The effect of riluzole on RGC survival was more pronounced in early treatment compared to late treatment. Resveratrol therapy initiated in the early period was more effective than late treatment; these approaches simply slowed the progression of the disease.

We also examined RGC density in all quadrants (Table 2). Although there was certain variability in the location of highest RGC density areas within each of the retinas, there was a tendency for higher densities to be located in the temporal retina. DTMR-traced RGCs are densest in the temporal region in control group although in all region. The control group was significantly densest in comparison with others in terms of mean RGC counts. Except L-Ri group did not significantly differ from E-Re groups, there is statistically clear variation in the regional numbers of RGC between the groups as in their total retinal RGC count. Thus, the loss of RGC within each quadrant in response to six weeks of glaucoma was delayed with riluzole and resveratrol treatments similar to total retinal count.

**DISCUSSION**

In this study’s model of experimental glaucoma with both riluzole and resveratrol, agents with different mechanisms of action in glaucoma were found to have a significant neuroprotective effect on RGC survival. Furthermore, the combined use of these drugs was associated with a significantly higher survival rate of RGCs than either the riluzole or resveratrol mono-therapy groups.

A number of mechanisms that may initiate RGC apoptosis in glaucoma have been proposed. These include endothelial dysfunction, neurotrophic factor deprivation, hypoperfusion/ischemia, glial cell activation, glutamate excitotoxicity and abnormal immune response. Red wine polyphenolic flavonoids are naturally available and have several proposed biological actions, which make this drink a potentially important agent in the treatment of glaucoma. Resveratrol exert vasoprotective effects by inhibiting the synthesis of endothelin-1, a vasoactive peptide that plays a crucial role in the pathogenesis of glaucoma. In addition, it has been shown to inhibit the production of the extracellular levels of vascular endothelial growth factor and has free radical scavenging properties.

Ischemia is one of the main causes of RGC death in various ocular diseases. It has also been suggested...
that chronic ischemia of the retina or optic nerve head may be partly involved in the development of glaucomatous damage. Ion channels such as glutamate N-methyl-d-aspartate (NMDA) or the voltage gated Ca\(^{2+}\) or Na\(^{+}\) channels are considered to be involved in these processes. Riluzole has multiple molecular actions. It inhibits both synaptic glutamate release and voltage gated-sodium channel activity, and it is neuroprotective against a variety of excitotoxic challenges. Voltage-gated sodium channels, which are normally involved in the initiation and propagation of action potentials, are also implicated in the pathogenesis of glaucoma.

**FIGURE 3** Intraperitoneal injections of riluzole 8 mg/kg and 10 mg/kg per day had a significant neuroprotective effect on survival of retinal ganglion cells six weeks after the induction of glaucoma. (a–g) representative whole-mounted retinas from eyes labeled with dextran tetramethylrhodamine (DTMR) after treatment with riluzole, resveratrol or vehicle. (a) Riluzole-treated glaucomatous eye in the early phase of glaucoma (n = 12). (b) Resveratrol-treated glaucomatous eye in the early phase of glaucoma (n = 12). (c) Control/saline eye (n = 12). (d) Vehicle-treated glaucomatous eye (n = 12). (e) Combined-treated glaucomatous eye in the early phase of glaucoma (n = 12). (f) Riluzole-treated glaucomatous eye in the late phase of glaucoma (n = 12); and (g) Resveratrol treated glaucomatous eye in the late phase of glaucoma (n = 12). There were more labeled cells in the combined-treated retina than in the vehicle-treated one and one drug treated eyes. Note the increased number of surviving retinal ganglion cells, which appear as bright dots, in eyes treated with riluzole and resveratrol (e) compared with control eyes (c). Bar = 100 \(\mu m\).

**TABLE 1** Multiple comparisons of RGC density of groups.

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<tr>
<th></th>
<th>RGC density</th>
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<th>p Value</th>
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<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Median</td>
<td>Min–Max</td>
<td>Control</td>
<td>G</td>
<td>E-Ri</td>
<td>L-Ri</td>
<td>E-Re</td>
<td>L-Re</td>
</tr>
<tr>
<td>Control</td>
<td>1207 ± 56</td>
<td>1218</td>
<td>1115–1291</td>
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<tr>
<td>G</td>
<td>404 ± 65</td>
<td>383</td>
<td>316–574</td>
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<tr>
<td>E-Ri</td>
<td>965 ± 56</td>
<td>983</td>
<td>857–1040</td>
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<td>0.000*</td>
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<tr>
<td>L-Ri</td>
<td>714 ± 25</td>
<td>715</td>
<td>667–757</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
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<tr>
<td>E-Re</td>
<td>735 ± 29</td>
<td>746</td>
<td>692–773</td>
<td>0.000*</td>
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<td>0.000*</td>
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<tr>
<td>L-Re</td>
<td>667 ± 20</td>
<td>668</td>
<td>636–706</td>
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<td>0.000*</td>
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<tr>
<td>E-RiRe</td>
<td>1071 ± 49</td>
<td>1065</td>
<td>1003–1128</td>
<td>0.000*</td>
<td>0.000*</td>
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*The mean difference is significant at 0.05 level.
potentials, are proposed to axonally degenerate in CNS myelinated tracts including the optic nerve by facilitating excessive depolarization, intracellular sodium overload and presynaptic release of glutamate; \(^{50,51}\) Na\(^+\) influx through voltage gated Na\(^+\) channels is a critical step in the CNS white matter injury cascade. Na\(^+\) accumulation results in Ca\(^{2+}\) release via reversal of the Na\(^+\)–Ca\(^{2+}\) exchanger.\(^{50,51}\) Glutamate can be released by the reverse operation of Na\(^+\)-dependent transporters, aggravating the overload of cellular Ca\(^{2+}\). This Ca\(^{2+}\) overload leads to neuronal injury. Therefore, expecting sodium channel blockers to reduce the harmful effects of ischemia and have protective effect on injured white matter axons should not be surprising.

Phenytoin and lamotrigine are well known Na channel blockers and have been used in experimental glaucoma models.\(^{8,52}\) Our findings of RGC counts are in agreement with the phenytoin study. On the other hand, treatment with lamotrigine was shown to have no neuroprotective effect on RGC in chronic hypertension model. But disparities could be due to differences in capacity of these drugs to block persistent sodium currents a feature not attributed to lamotrigine. Thus one proposed mechanism of the neuroprotective effects of riluzole may result from a blockade persistent sodium channels whose activation following injury has been associated with degeneration of neural tissue, and the prevention of a reversal of the Na\(^+\)/calcium (Ca\(^{2+}\)) exchanger. A second proposed mechanism is anti-glutamatergic activity via the inhibition of glutamate release, modulation of glutamate receptors (NMDA and \(\alpha\)-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate [AMPA]) and the increase of glutamate uptake,\(^{53,54}\) which are known to be involved in the mechanism of RGC death in glaucoma. The literature provides only limited information focused on the effect of riluzole and resveratrol in ocular tissue, hence this was our rationale for considering the use of riluzole and resveratrol in glaucoma.

Ettaiche et al.\(^{49}\) investigated the neuroprotective effects of riluzole in a retinal ischemia model and were the first to report the drug’s potent

### TABLE 2. Comparisons of regional RGC density of groups.

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<tr>
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<th>Temporal</th>
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<td>Mean ± SD</td>
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<tr>
<td>Control</td>
<td>307 ± 26</td>
<td>303</td>
<td>271–371</td>
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<tr>
<td>G</td>
<td>113 ± 34</td>
<td>110</td>
<td>77–199</td>
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<tr>
<td>E-Ri</td>
<td>244 ± 24</td>
<td>257</td>
<td>201–265</td>
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<tr>
<td>L-Ri</td>
<td>185 ± 16</td>
<td>182</td>
<td>164–222</td>
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<tr>
<td>E-Re</td>
<td>202 ± 14</td>
<td>202</td>
<td>179–227</td>
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<tr>
<td>L-Re</td>
<td>196 ± 21</td>
<td>193</td>
<td>172–228</td>
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<tr>
<td>E-RiRe</td>
<td>262 ± 33</td>
<td>250</td>
<td>222–333</td>
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<td></td>
<td>Nasal</td>
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<tr>
<td>Control</td>
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<td>86 ± 12</td>
<td>83</td>
<td>70–109</td>
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<td>E-Ri</td>
<td>242 ± 10</td>
<td>244</td>
<td>222–258</td>
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<tr>
<td>L-Ri</td>
<td>183 ± 10</td>
<td>182</td>
<td>159–195</td>
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<tr>
<td>E-Re</td>
<td>182 ± 14</td>
<td>183</td>
<td>158–211</td>
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<tr>
<td>L-Re</td>
<td>162 ± 13</td>
<td>158</td>
<td>147–186</td>
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<tr>
<td>E-RiRe</td>
<td>253 ± 13</td>
<td>256</td>
<td>236–284</td>
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<tr>
<td>E-Ri</td>
<td>215 ± 27</td>
<td>211</td>
<td>180–252</td>
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<tr>
<td>L-Ri</td>
<td>158 ± 13</td>
<td>158</td>
<td>128–179</td>
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<tr>
<td>E-Re</td>
<td>189 ± 12</td>
<td>192</td>
<td>172–207</td>
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<tr>
<td>L-Re</td>
<td>167 ± 11</td>
<td>169</td>
<td>144–182</td>
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<tr>
<td>E-RiRe</td>
<td>255 ± 11</td>
<td>258</td>
<td>236–276</td>
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<td>Inferior</td>
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<tr>
<td>Control</td>
<td>285 ± 19</td>
<td>279</td>
<td>264–324</td>
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<tr>
<td>G</td>
<td>95 ± 12</td>
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<td>77–120</td>
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<tr>
<td>E-Ri</td>
<td>225 ± 19</td>
<td>230</td>
<td>189–245</td>
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<tr>
<td>L-Ri</td>
<td>161 ± 17</td>
<td>159</td>
<td>129–183</td>
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<tr>
<td>E-Re</td>
<td>133 ± 21</td>
<td>129</td>
<td>99–175</td>
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<tr>
<td>L-Re</td>
<td>116 ± 21</td>
<td>124</td>
<td>78–138</td>
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<tr>
<td>E-RiRe</td>
<td>254 ± 13</td>
<td>253</td>
<td>238–279</td>
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ANOVA (Tukey Test)

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neuroprotective effect against retinal ischemia by reducing reported changes in the retinal cytoskeleton, necrotic cell damage and DNA fragmentation. In addition, Vorwerk et al.\textsuperscript{48} investigated the effect of intraperitoneal therapy with nimodipine, memantine or riluzole on glutamate level after optic nerve crush and they showed that memantine and riluzole block the increase of intravitreal glutamate induced by optic nerve damage thus they have a neuroprotective effect. Since riluzole treatment prevented RGC loss; the drug showed neuroprotective effects in both monotherapy and in combination with resveratrol.

Resveratrol treatment also protected against retinal ischemia/reperfusion injury.\textsuperscript{55} Luna et al.\textsuperscript{56} investigated the effect of resveratrol in trabecular cell culture by creating a chronic oxidative stress model. They demonstrated that resveratrol inhibited production of oxidative stress markers, which suggested that the drug could prevent or halt the damage in trabecular meshwork in patients with primary open angle glaucoma. Our results were in accordance with previous studies and also suggest the neuroprotective effects of resveratrol in an experimental glaucoma model. As resveratrol is widespread as a dietary supplement, it can easily be given to newly diagnosed glaucoma patients in order to prevent an irreversible loss of RGCs.

Although the neuroprotective effects of both riluzole and resveratrol have been reported in animal models of brain ischemia,\textsuperscript{57,58} SCI,\textsuperscript{11,59,60} traumatic brain injury,\textsuperscript{58,61} Parkinson’s disease,\textsuperscript{62} post-traumatic peripheral neuropathy\textsuperscript{63} and acute noise-induced hearing loss,\textsuperscript{64} no study has yet evaluated their neuroprotective effects on RGCs in glaucoma. Hence, the precise mechanism by which resveratrol and riluzole produce neuroprotection in glaucoma is not clear. Our study showed for the first time that a combination therapy of riluzole and resveratrol showed significantly better neuroprotective effects in a rat model of glaucoma. The neuroprotective ability of combined use of riluzole and resveratrol may be the result of distinct mechanisms of action, the capacity of acting at different stages of glaucomatous injury or the synergistic effect of the two agents upon one another on excitotoxicity and neuromodulation.

The difference in efficacy between early and late treatment is not surprising, since glaucoma is a multifactorial and progressive disease. In glaucoma, numerous proteins may be upregulated during the progression of the disease.\textsuperscript{65} Different downstream signaling pathways may be disturbed in different times. For these reasons, early and late treatments showed different results. We showed beneficial synergistic effects of concurrent treatment with same doses of riluzole and resveratrol against glaucomatous damage in rats. Because RGC die at different times, we conclude that early treatment stop or slow the progression of the glaucomatous damage.

Stimulating the survival signal at the early stage of injury may play a critical role control of the death or survival decision phase of RGCs. A late triggering of survival causes death of many RGCs that lose the capacity to stand out against extrinsic injury caused by chronic ocular hypertension.

Only the L-Ri group did not differ significantly from E-Re and L-Re group. This might be attributed to late treatment with riluzole that is as effective as early resveratrol treatment. Furthermore, the finding that the antioxidant resveratrol, unlike riluzole, was unable to prevent significantly glaucoma-induced RGC death can be explained by the fact that this compound has a poor efficacy on RGC survival compared to riluzole in experimental glaucoma model.

Since the mean IOP in the glaucomatous eyes was significantly greater than that in the controls for each treatment group ($p<0.01$) and since the mean IOP of the glaucoma group and of the five different treatment groups revealed no significant difference, any difference in RGC survival could be attributed to the treatment.

This study aimed at short-term results. It may be understood from this study that systemic treatment with these drugs proposed to prevent its progression with the survival of RGCs in experimental glaucoma at six weeks. Delay of RGC loss should be evaluated by monitoring the progression, and we suggest loss delayed usually by pharmacological means. Thus additional studies should be done to identify the specific mechanism of action of these drugs and duration of protection.

This prospective experimental study has some serious limitations. First, the efficacy of riluzole and resveratrol treatment was not demonstrated on axonal counts, which act as a significant correlate for RGC counts, due to some technical difficulties. Second, this study did not contain observations of functional changes in photoreceptors induced by glaucoma, such as electroretinogram. Finally, biochemical evaluations may be investigated in a time and dose-dependent manner. Since axonal degeneration is associated with loss of RGCs, future studies evaluating the axonal density and functional measurements will provide additional data about the potential use of the two agents in prevention of this neurodegenerative disease.

The significant neuroprotective effect of riluzole in experimental glaucoma suggests that clinical studies with this FDA-approved drug should continue to evaluate its clinical therapeutic potential in glaucoma patients. These multimodal drugs have diverse pharmacologic properties that act on multiple targets and thus can be potentially better treatments for glaucoma.

Altogether, both riluzole and resveratrol, which demonstrate different mechanisms of action in glaucoma, significantly protect the RGC against the
deleterious effects of a hypertensive insult, when administered either alone or in combination. Given the available data, in vitro and animal model studies that compare riluzole and resveratrol with other potent biologic agents determine the optimal treatment for neuroprotection in glaucoma. When our data are confirmed by future studies, the combination of resveratrol and riluzole in glaucoma may become a promising treatment option in the future.

**DECLARATION OF INTEREST**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

**REFERENCES**


SUPPLEMENTAL MATERIAL
Supplemental data for this article can be accessed at www.tandfonline.com/icey.