



# Retinal Ganglion Cell Protection Via Topical and Systemic Alpha-Tocopherol Administration in Optic Nerve Crush Model of Rat

## Rat Optik Sinir Ezme Modelinde Topikal ve Sistemik Alfa Tokoferol Uygulaması Yoluyla Retina Gangliyon Hücre Korunması

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### Summary

**Purpose:** The aim of our study was to investigate the neuroprotective effects of topical  $\alpha$ -tocopherol in optic nerve crush model of rat and to compare its efficacy with that of systemic  $\alpha$ -tocopherol.

**Material and Method:** 50 eyes of 25 Wistar albino rats were included. The eyes were divided into six groups. Optic nerve crush was performed in Groups 1, 3, 5. Additionally, systemic and topical  $\alpha$ -tocopherol therapies were given to Groups 1 and 3, respectively. No treatment was applied in Group 5. Groups 2, 4, and 6 were the fellow eyes of the animals comprising Groups 1, 3, and 5. Eyes were enucleated at day 45 of the study. Retinal ganglion cells (RGCs) were counted with light microscopy.

**Results:** Mean RGC numbers were  $14.5 \pm 3.7$  (10.3-20) and  $27.5 \pm 2.6$  (24-30) in Groups 5 and 6, respectively ( $p: 0.001$ ). They were measured to be  $26.6 \pm 7.8$  (19-45) and  $24.6 \pm 3.9$  (20-32) in Groups 1 and 2 and  $21.1 \pm 7.1$  (11-34) and  $27 \pm 7.5$  (18-42) in Groups 3 and 4 ( $p: 0.659$ ,  $p: 0.094$ , respectively). There was no difference in Groups 2 and 4 compared with Group 6 ( $p: 0.210$ ,  $p: 0.299$ , respectively).

**Discussion:** Topical  $\alpha$ -tocopherol has a significant neuroprotective effects in optic nerve crush model of rat and may be used in the future for the treatment of optic neuropathies such as glaucoma. (*Turk J Ophthalmol* 2013; 43: 161-6)

**Key Words:**  $\alpha$ -tocopherol, retinal ganglion cell, optic nerve crush model

### Özet

**Amaç:** Çalışmanın amacı, topikal  $\alpha$ -tokoferol uygulamasının optik sinir ezme modelinde nöroprotektif etkisinin değerlendirilmesi ve etkinliğinin sistemik  $\alpha$ -tokoferol ile karşılaştırılmasıdır.

**Gereç ve Yöntem:** Yirmi beş Wistar albino ratın 50 gözü çalışmaya dahil edildi. Gözler 6 gruba ayrıldı. Optik sinir hasarı Grup 1,3,5'te uygulandı. Ek olarak, Grup 1 ve 3'te sırasıyla sistemik ve topikal  $\alpha$ -tokoferol tedavileri verildi. Grup 5'te herhangi bir tedavi uygulanmadı. Grup 2,4 ve 6, Grup 1,3 ve 5'teki ratların diğer gözleriydi. Çalışmanın 45. gününde enükleasyon yapıldı. Retina gangliyon hücreleri (RGH) ışık mikroskopisi ile sayıldı.

**Sonuçlar:** Ortalama RGH sayıları sırasıyla Grup 5 ve 6'da  $14,5 \pm 3,7$  (10,3±20) ve  $27,5 \pm 2,6$  (24-30) idi ( $p=0,001$ ). Grup 1 ve 2'de  $26,6 \pm 7,8$  (19-45) ve  $24,6 \pm 3,9$  (20-32); Grup 3 ve 4'te ise  $21,1 \pm 7,1$  (11-34) ve  $27,0 \pm 7,5$  (18-42) olarak saptandı (sırasıyla;  $p=0,659$ ,  $p=0,094$ ). Grup 2 ve 4'te Grup 6'ya göre anlamlı derecede fark mevcut değildi (sırasıyla;  $p=0,210$ ,  $p=0,299$ ).

**Tartışma:** Topikal  $\alpha$ -tokoferol rat optik sinir ezme modelinde anlamlı nöroprotektif etkinliğe sahiptir ve gelecekte glokom gibi çeşitli optik nöropatilerin tedavisinde kullanılabilir. (*Turk J Ophthalmol* 2013; 43: 161-6)

**Anahtar Kelimeler:**  $\alpha$ -tokoferol, retina gangliyon hücresi, optik sinir ezme modeli

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## Introduction

Optic neuropathies usually cause death of retinal ganglion cells (RGCs) in the early stages of the disease. Glaucoma, as the most common optic neuropathy, is also characterized by progressive loss of RGCs leading to irreversible loss of vision. Generally, reducing intraocular pressure (IOP) is effective for the treatment of glaucoma. However, in some cases reducing IOP is not enough to prevent the progression of the disease and loss of vision. In these kind of cases, neuroprotection and neuroprotective treatment strategies have been of considerable interest. Over the past years, various experimental procedures causing death of RGCs have been used to test the efficacy of such neuroprotective treatments.<sup>1,2</sup>

Optic nerve crush or transection is one of the most common experimental procedures simulating some ocular diseases such as glaucoma, in which slow RGC loss is a leading cause of visual decrease.<sup>2</sup> On the other hand, major limitation of this method is the reproducibility of the result. This problem was tried to be overcome by using some special clips or application of predetermined amount of force during crushing.<sup>3,4</sup>

Up to date, neuroprotective effects of various groups of agents were investigated in some experimental studies. Natural antioxidants constitutes one of these groups that have gained popularity also in ophthalmology literature. Vitamin E is a well-known natural agent that is a family of essential micronutrients comprising lipid soluble tocopherols and tocotrienol with a strong antioxidant and neuroprotective activities.  $\alpha$ -tocopherol was found to reduce degeneration of hippocampal cells after cerebral ischemia.<sup>5</sup> Engin et al.<sup>6</sup> also described the neuroprotective effects of orally administered tocopherol in glaucoma patients as determined by the average differences between pulsatility indexes and resistivity indexes of both ophthalmic arteries and posterior ciliary arteries and also mean deviations.

In the current study, we aimed to investigate the neuroprotective effects of topically applied  $\alpha$ -tocopherol and to compare its efficacy with that of systemic  $\alpha$ -tocopherol in optic nerve crush model of rat.

## Material and Method

### Preparation of Nanosuspension

$\alpha$ -tocopherol (Sigma-Aldrich, Inc., St. Louis, USA) and Eudragit RS 100 (Röhm GmbH were co-dissolved at room temperature in ethanol. The solution was slowly injected (0.5 mL min<sup>-1</sup>) with a syringe into water containing Tween 80 (0.02%) and benzalkonium chloride (0.1%) and was kept in an iced-water bath. During the injection, the mixture was highly mixed by an Ultra-Turrax. Ethanol residues were left to evaporate off under a slow magnetic stirring of the nanosuspension at room temperature for 12 hours. Finally, the prepared nanosuspension had mean size around 6 nm which makes it suitable for ophthalmic applications. In vivo efficacy was assessed on the rat eye.

### Animals

50 eyes of 25 Wistar albino rats were included in the study. All procedures were performed in accordance with the guide for the care and use of laboratory animals, and the study was approved by the institutional review board. Rats were anesthetized with intramuscular ketamine hydrochloride, 40 mg/kg, and xylazine hydrochloride, 5 mg/kg.

### Experimental Procedure and Groups

Surgical steps for creating optic nerve crush model was as follows: After the completion of anesthesia, conjunctiva of animals was dissected superiorly, then retrobulbar optic nerve was exposed. Optic nerve crush was performed in right eyes of each animal in Groups 1, 3, and 5. 30-sec constant pressure was applied with forceps by same author (ZA). Conjunctival tissue was then closed by using 8.0 vicryl suture. In Groups 1 and 3, optic nerve crush was performed together with systemic (intramuscular) and topical  $\alpha$ -tocopherol application, respectively. Systemic  $\alpha$ -tocopherol was administered as a single dose (0.5 cc, 50 IU) in Group 1, whereas topical  $\alpha$ -tocopherol (0.05 cc, 5 IU) was given two times a day in Group 3.

The fellow eyes of animals in Groups 1 and 3 constituted Group 2 and 4, respectively. In Group 5, only optic nerve crush was performed, and this group was taken as sham control. Group 6 was composed of fellow eyes of animals in Group 5.

### Detection of topically applied $\alpha$ -tocopherol in vitreous Reagents and Chemicals

$\alpha$ -tocopherol (Sigma-Aldrich, Inc., St. Louis, USA), disodium sulphate (99%), HPCL grade ethyl alcohol, ethyl acetate, n-butanol, acetonitrile, and methanol were obtained from Sigma-Aldrich (Steinheim, Germany).

### Equipments

Supelco<sup>TM</sup> LC18DB (150x4.0 mm ID, 3  $\mu$ m) analytical column, Supelguard<sup>TM</sup> LC18DB (2 cm) guard column (Supelco Inc., PA, USA), Hewlett Packard model 1050 HPLC pump and UV detector, HP 3396 integrator (Avondale, PA, USA) were used for  $\alpha$ -tocopherol analysis.

### Preparation of Standard

Stock standard of  $\alpha$ -tocopherol was prepared in acetonitrile. Various concentrations of working standard (range in 1-20  $\mu$ g/mL) were prepared in n-butanol:ethyl acetate (1:1,v/v), protected from light and kept at -20°C when not in use.

### Preparation and Measurement of Sample

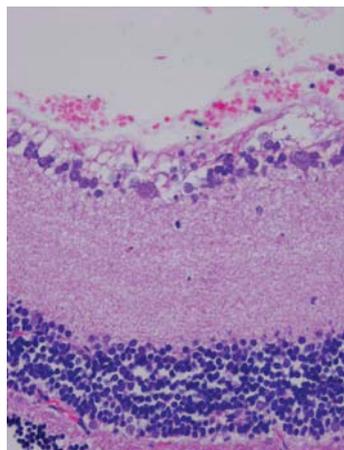
6 eyes were used to test whether topically applied  $\alpha$ -tocopherol penetrates into the posterior segment of the eye, firstly.  $\alpha$ -tocopherol levels were determined with isocratic HPLC method described by Lee et al.<sup>7</sup> 50  $\mu$ L ethyl alcohol was added to vitreous sample. After vortex-mixing, specimen was extracted with 50  $\mu$ L of n-butanol:ethyl acetate (1:1, v/v) and further mixed for 1 min. 5 mg sodium sulphate was added. After vortex-mixing for another 1 min, the sample was stand at -20 °C for 20 min before centrifugation at 15 000xg for 5 min. The organic upper layer was transferred into an Eppendorf tube and stored at -70 °C until analysis. The mobile phase, pumped at 1 mL/min, consisted of methanol:n-butanol:water (75:20:5, v/v).  $\alpha$ -tocopherol was measured at 290 nm.



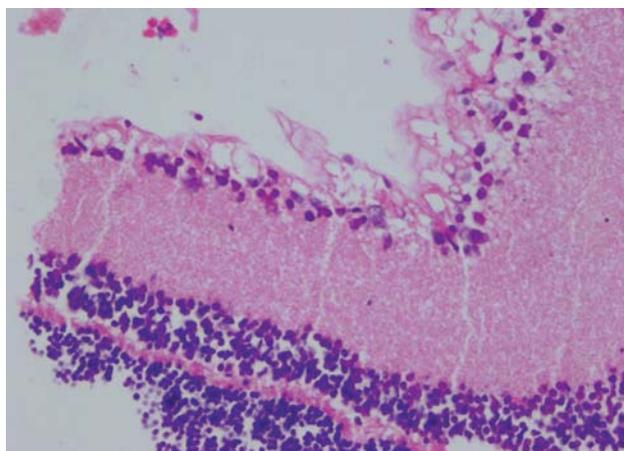
21.1±7.1 (11-34) and 27±7.5 (18-42) in Group 3 (topically treated eyes) and Group 4, respectively ( $p=0.659$ ;  $p=0.094$ , respectively). When a comparison regarding RGC numbers was performed between Groups 1 and 3, the difference was not statistically significant ( $p=0.216$ ). On the other hand, RGC numbers were not significantly different in Group 2 and Group 4 (control eyes of treatment groups) compared with Group 6 (control eyes of sham group) ( $p=0.210$ ;  $p=0.299$ , respectively).

#### Histopathological Analysis

Light microscopic examination of Group 5 (sham controls) revealed decreased total cell number of RGC layer with significant morphologic alterations due to the traumatic effect of optic nerve crush that was performed (Figure 5A). On the other hand, in Group 6 (control group), retinal anatomy was completely normal (Figure 5B). In systemically and topically treated Groups 1 and 3, retinal anatomy was disrupted but found to be relatively well preserved compared with that of Group 5 (Figure 3A and 4A). Histopathologic sections in eyes of Groups 2 and 4 (fellow eyes of the animals treated in Groups 1 and 3) seemed to be completely normal (Figure 3B and 4B).



**Figure 4A.** Transverse section of the retina in Group 3 treated with topically administered  $\alpha$ -tocopherol. Retinal architecture in RGC layer (arrow) also seems relatively to be well-preserved (HE $\theta$ x40)

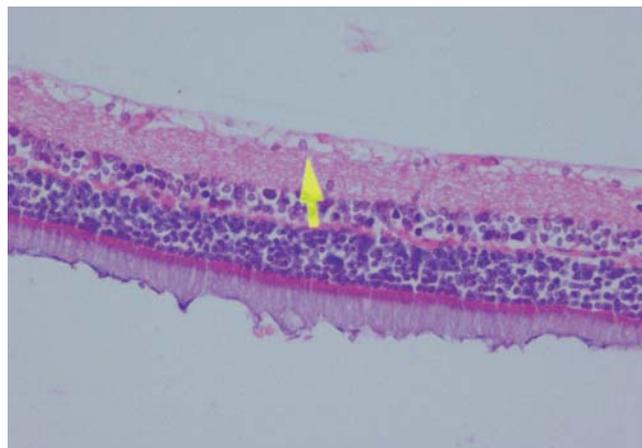


**Figure 4B.** Transverse section of the retina in Group 4 (control of Group 3) showing normal RGC\* anatomy and number

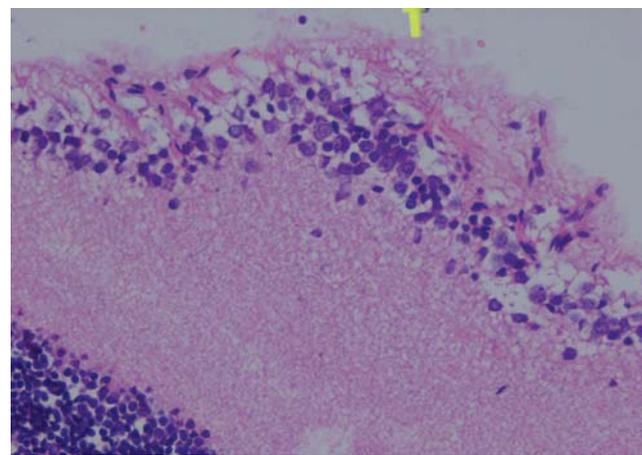
## Discussion

Vitamin E and its antioxidative properties have been known for years. Various studies including all tissues of the body demonstrated that vitamin E, especially  $\alpha$ -tocopherol is an efficient chain-breaking antioxidant during lipid peroxidation.<sup>8,9</sup> It has also antioxidant properties in the eye. It has been reported that vitamin E deficiency might lead to retinal degeneration.<sup>10</sup> Antioxidant effects of alpha-, gamma-, and succinate tocopherols were also found to have antioxidant effects in guinea pig retina during ischemia-reperfusion injury. Therapeutic effects of antioxidants and nutritional support in ocular diseases have been examined in various studies. Glaucoma is one of these ocular diseases that has been found to be treated by some natural agents to some extent. It is a progressive optic neuropathy presenting with optic nerve excavation and visual field damage clinically. Progressive visual field damage is known to result from progressive RGC apoptosis and death.

Major preventable risk factor for the glaucoma progression is elevated IOP. Besides reducing the IOP, neuroprotection is other



**Figure 5A.** Transverse section of the retina in Group 5 (sham control). Remarkable disruption of retinal architecture and cell loss in RGC\* layer (arrow) are shown (HE $\theta$  x40)



**Figure 5B.** Transverse section of the retina in Group 6 (control of Group 5) showing normal RGC\* anatomy and number

treatment of choice that aims to prevent progressive RGC death. In a large number of patients, glaucomatous neuropathy progresses in spite of the decreased IOP. Thus, neuroprotection and neuroprotective treatments have gained popularity over the last years. Up to date, various animal models have been used to investigate some neuroprotective treatment strategies. There are various natural compounds available, with neuroprotective properties.<sup>12</sup>  $\alpha$ -tocopherol is one of these natural compounds that shows neuroprotective effects in some experimental models.

Besides its neuroprotective properties,  $\alpha$ -tocopherol was reported to have some antiproliferative effects and to inhibit human Tenon's capsule fibroblast proliferation.<sup>13</sup> It was found to have a positive effect on the success of filtering surgery in rabbit.<sup>14</sup> It was also reported to be protective against cataract formation in animals and also in humans.<sup>15,16</sup>

Glaucoma, as one of the leading causes of blindness worldwide, usually presented with characteristic appearance of the optic nerve secondary to underlying apoptotic death of RGCs. Although reducing IOP is one of the most important therapeutic targets for the prevention of disease progression, it may not be enough in some cases. Underlying mechanisms of disease progression other than increased IOP have been popular field of research over years. Thus, number of studies investigating therapeutic agents to prevent RGC death have been performed in current literature. To investigate the neuroprotective properties of some therapeutic agents, various animal models simulating slow RGC loss similar to that seen in glaucoma have been designed. Optic nerve crush model is one of these animal models that have been used to provoke slow RGC death. It has been shown that optic nerve crush induces optic nerve injury and causes retrograde degeneration of RGC of the rat.<sup>17</sup> Optic nerve crush model is easy to perform and it causes slow RGC loss that is much more similar to the nature of glaucoma than that of optic nerve transection model. Optic nerve damage may be induced by crushing optic nerve with clamps, forceps or other mechanic devices.<sup>4</sup> On the other hand, it is often difficult to compare the results of studies obtained by different investigators owing to different methods for measuring IOP and noncomparable pressure levels.<sup>4</sup> In the current study, this problem was tried to be overcome to some extent by IOP measurements done by the same author (ZA).

Increasing RGC survival is one of the important steps for neuroprotection. Neuroprotection implies prevention of neuronal death secondary to any injury or disease and is also of great importance in the treatment of glaucoma and some neurodegenerative diseases. In the present study, we aimed to investigate the neuroprotective effect of topical  $\alpha$ -tocopherol and compare its efficacy with that of systemic  $\alpha$ -tocopherol in optic nerve crush model.

Once an agent said to be neuroprotective in eye, it must initially penetrate into vitreous, keep its concentration for a given period of time, and must have receptors on its target tissues that are expected to be retina and the optic nerve. Since the  $\alpha$ -tocopherol is a liposoluble molecule, its penetrance to the

vitreous in topical form might have been difficult. In our study,  $\alpha$ -tocopherol was prepared as a nanosuspension with a mean size around 6 nm. Following the preparation of nanosuspension, it was administered topically twice a day. Its presence in the vitreous was demonstrated by HPLC method. High peaks were detected in vitreous samples that was supporting the presence of topically applied  $\alpha$ -tocopherol in vitreous. Microparticulate structure of the formulation might have increased the penetration of the  $\alpha$ -tocopherol into the vitreous.

Tanito et al<sup>18</sup> also demonstrated increased concentration of  $\alpha$ -tocopherol and  $\alpha$ -tocopherol-3 in ocular tissues including neural retina after a 4-day application of 5 $\mu$ L pure  $\alpha$ -tocopherol and  $\alpha$ -tocopherol-3 (2.23 $\pm$ 0.14 mg). However, topical  $\alpha$ -tocopherol-3 was reported to reach much more drug concentration in ocular tissues than those detected with topical  $\alpha$ -tocopherol. We used topical  $\alpha$ -tocopherol at a lower dose but for 45 days. Another study by Nagata et al<sup>19</sup> demonstrated that  $\alpha$ -tocopherol acetate concentration was increased in aqueous humor and lens after 3 weeks of topical application. In the current study, we demonstrated peaks in chromatogram after the topical administration of  $\alpha$ -tocopherol. Mean vitreous concentration of topically applied  $\alpha$ -tocopherol was 2.27 $\pm$ 0.61  $\mu$ g/mL. We also showed that  $\alpha$ -tocopherol protected RGCs at much more lower concentration than those reported in literature. In our study,  $\alpha$ -tocopherol, given in both systemic (Group 1) and topical (Group 3) forms was found to increase RGC survival as determined by RGC count. Furthermore, RGC counts were found to be comparable in Groups 1 and 3 ( $p=0.216$ ).

$\alpha$ -tocopherol is a well-known antioxidant substance. Neuroprotective properties of some natural substances including  $\alpha$ -tocopherol have been studied in various studies.<sup>12</sup> It was found to be neuroprotective in ischemia-reperfusion models.<sup>11,19</sup> On the other hand, in these studies,  $\alpha$ -tocopherol was usually administered intraperitoneally or subcutaneously. Engin et al<sup>6</sup> investigated the neuroprotective effect of  $\alpha$ -tocopherol against glaucomatous damage. This was a clinical study in which orally administered  $\alpha$ -tocopherol was given to the patients. The authors reported that resistivity indexes and pulsatility indexes were significantly lower in patient groups in which  $\alpha$ -tocopherol supplementation was given. This study clinically demonstrated the therapeutic impact of  $\alpha$ -tocopherol in the treatment of glaucoma patients. On the other hand, there is no data showing the therapeutic effect of topically applied  $\alpha$ -tocopherol in the current literature. Thus, in our experimental study,  $\alpha$ -tocopherol was given both systemically and topically, and the neuroprotective effects of  $\alpha$ -tocopherol in both forms were investigated and compared. We found that  $\alpha$ -tocopherol was neuroprotective in both systemic and topical forms. There is no experimental study demonstrating the neuroprotective effects of topically applied  $\alpha$ -tocopherol on RGCs. It is important to know that it's also neuroprotective in topical form. There is also no other study investigating the neuroprotective effects of  $\alpha$ -tocopherol in optic nerve crush model of rat. Since it is easier to apply  $\alpha$ -tocopherol topically than systemically in ophthalmic

practice, this might be advantageous for the patients who need RGC protection.

To summarize, neuroprotective agents have been one of the most important components of the treatment of optic nerve diseases characterized by progressive loss of the RGCs such as glaucoma. There are various natural compounds including  $\alpha$ -tocopherol which were found to be promising in the treatment of glaucoma.<sup>12</sup> To the best of our knowledge, this is the first study demonstrating the neuroprotective effects of topically applied  $\alpha$ -tocopherol in comparison with systemic  $\alpha$ -tocopherol. The results of our study clearly indicate that topically applied  $\alpha$ -tocopherol increases the survival of the RGCs as much as systemic  $\alpha$ -tocopherol application. As a strong antioxidant agent,  $\alpha$ -tocopherol, may also be used topically for the treatment of glaucoma in the future. Further clinical and prospective studies are needed to demonstrate the neuroprotective effects of topically applied  $\alpha$ -tocopherol.

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