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ORIGINAL ARTICLE

Effects of erythropoietin on neuroprotection in an experimental glaucoma model

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Abstract

Purpose: Glaucoma is a progressive, irreversible optic neuropathy that is the leading cause of blindness worldwide. In our study, we aimed to show the neuroprotective effect of erythropoietin (EPO) on glaucoma.

Methods: Twelve male and 12 female albino Wistar rats (6 weeks old; 220±40 grams) from Aydin Adnan Menderes University Experimental Animal Center were used. All animals were housed in a fixed room on a 12/12 h light/dark cycle per day, with food and water provided ad libitum. Rats were divided into four groups as control and glaucoma groups, subconjunctival EPO and topical EPO groups. At the end of the 6th week, the right eyes were enucleated and total retinal thickness, ganglion cell complex (GCC), inner plexiform layer (IPL), and ganglion cell layer (GCL) thickness measurements were determined. Tissue samples stained with HE were examined under a light microscope and photographed. Retinal layer thickness measurements were determined for each eye using the ImageJ program (NIH, USA). The neuroprotective effect of EPO on glaucoma was evaluated by retinal layer thickness measurements.

Results: GCL, IPL, retinal thickness, and GCC thickness were observed the least in the glaucoma group and the most in the control group. There was no significant difference between EPO administration routes ($p>0.05$). Cell layer thicknesses in each group were confirmed by immunohistochemical staining, and apoptotic cells were not detected by bax or bcl-2 staining.

Conclusion: The structural contribution of topical and subconjunctival applications of EPO to retinal layers has been demonstrated, and the study needs to be repeated in larger series.

Keywords: Erythropoietin; glaucoma; neuroprotection; subconjunctival erythropoietin; topical erythropoietin.

Glaucoma is one of the leading causes of irreversible blindness worldwide. It is also a progressive, neurodegenerative eye disorder characterized by degeneration of retinal ganglion cell (RGC) axons and terminal cell loss, primarily resulting in visual field (VF) loss.[1]

Although the exact causes of glaucoma are not known, fac-

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tors associated with glaucoma pathogenesis include elevated intraocular pressure (IOP), increased oxidative stress, aging, glutamate neurotoxicity, and susceptibility genes such as optineurin and myocilin.[2]

Although there are many factors that affect the damage process of glaucoma, high IOP is one of the most important risk factors. Today, the primary goal of current treatment is to reduce IOP, and medical, laser, and surgical treatment methods are used for this purpose. However, despite the effective reduction of pressure with IOP lowering agents, it is known that optic nerve damage due to glaucoma cannot be prevented in some patients, and therefore, it is aimed to develop neuroprotective agents.

Neuroprotective therapy targets the neuron directly.[3] In addition, apoptosis, which is programmed cell death, is the basis of the loss of function in RGCs. For this reason, neuroprotective therapeutic approaches are frequently investigated in the treatment of glaucoma.

Erythropoietin (EPO) is a naturally occurring glycoprotein hormone that is traditionally thought to be responsible for the production of red blood cells in our body.[4] In addition to its hematopoietic effect, neuroprotective and neuroregenerative properties of this cytokine have also been demonstrated in the central nervous system.[4] Pre-clinical studies have been conducted in many ocular diseases such as diabetic retinopathy, retinal detachment, glaucoma, retinopathy of prematurity, age-related macular degeneration, and optic neuritis.[5,6] In many glaucoma models, EPO has been shown to prevent apoptosis of RGCs and preserve visual function, with promising results.[7]

The therapeutic effects of EPO on the retina have been tested with different ocular applications such as systemic routes, intravitreal, subconjunctival, and retrobulbar. While the systemic route causes undesirable secondary effects and an increase in hematopoiesis, ocular applications can lead to ocular complications such as chorioretinitis, retinal detachment, cataract, vitritis, and even endophthalmitis.[7] In the previous studies, it has been shown that in an animal model of glaucoma in rats, EPO reaches the RGC layer in both physiological and glaucoma conditions when administered by the subconjunctival route.[7]

In our study, we aimed to examine the neuroprotective effect of topical and subconjunctival application of EPO molecule on RGCs in an experimental rat glaucoma model, which has not been done before.

Although there are studies on the use of topical EPO therapy in wound healing in the literature, there is no study on its use in the treatment of glaucoma.[5,6] After the planned study, we aimed to elucidate the effect of EPO on apoptosis and to lay the groundwork for future clinical and experimental studies as well as drug treatments.

Materials and Methods

Study Design

In this study, 12 male and 12 female albino Wistar rats (6 weeks of age, average 220±40 g) from Aydin Adnan Menderes University Experimental Animal Center were used. All animals were housed in a fixed room on a 12/12 h light/dark cycle per day, with food and water provided ad libitum. All rats were treated according to the Association for Research in Vision and Ophthalmology statement and European Union regulations. This study was approved by Aydin Adnan Menderes University Faculty of Medicine Local Ethics Committee for Animal Experiments (date: July 9, 2020; number 64583101/2020/055).

Experimental Groups

The rats were randomly divided into four groups with six subjects (three males and three females) in each group.

Group 1 (control group); no action was taken for six subjects and no additional treatment was given. Six weeks later, the eyes were enucleated with analgesia and anesthesia.

Group 2 (glaucoma group); for glaucoma induction, hyaluronic acid (HA, Viscoat, Alcon) injection was applied to the right anterior chambers of six subjects with a 30 G needle. Six weeks after induction, the eyes were enucleated with analgesia and anesthesia.

Group 3 (subconjunctival EPO treatment group [SUBEPO]); for the induction of glaucoma, HA injection with a 30 G needle was applied and simultaneous subconjunctival 1000 IU human recombinant EPO (rHuEPO, Eporon 4000 IU/0.4 mL pre-filled syringe) was administered to the right anterior chambers of six subjects. Six weeks after induction, the eyes were enucleated with analgesia and anesthesia.

Group 4 (topical EPO treatment group [TEPO]); for the induction of glaucoma, HA injection was applied to the right anterior chambers of six subjects with a 30 G needle, and after the induction, a total of 1000 IU rHuEPO (Eporon 4000 IU/0.4 mL pre-filled syringe) prepared 2×1 drops/day was applied and given as topical drops for 6 weeks. Six weeks after induction, the eyes were enucleated with analgesia and anesthesia.

Anesthesia Technique

In anesthesia and analgesia, a combination of 50 mg/kg ketamine HCl (Ketalar, Eczacibasi, Türkiye) and 5 mg/kg xy-

lazine HCl (Rompun, Bayer, Türkiye) was used intramuscularly. For deep anesthesia, 0.5% proparacaine HCl (Alcaine, Alcon, Türkiye) was instilled into the right eyes of all animals 8 min after the intramuscular injection.

Glaucoma Induction and Subconjunctival-topical EPO Applications

After applying 5% povidone-iodine to the right eyes of the animals, HA injection was applied to the right anterior chambers of rats in Groups 2, 3, and 4 with a 30 G needle. For Group 3, a subconjunctival injection of 1000 IU rHuEPO was administered simultaneously with the glaucoma induction. For Group 4, glaucoma induction was started simultaneously with 1000 IU rHuEPO (Eporon 4000 IU/0.4 mL pre-filled syringe) prepared as a topical drop form, 2×1, for 6 weeks. Topical drops were prepared with artificial tears containing sodium hyaluronate (Refresh tears, Allergan) and treatment was given with the appropriate concentration.

Following anesthesia, HA was injected into the anterior chamber of the rats with a 30 G needle (Fig. 1). HA injection with a 30 G needle under the guidance of a surgical microscope (Leica S8APO) was applied every week during the experiment by making self-sealing entry into the anterior chamber from the corneoscleral limbus, preventing the contact of the needle tip with the iris and lens, and advancing from 12 o'clock to 6 o'clock in the limbus. Injections were repeated at intervals of 1 week for a total of 6 weeks in the glaucoma induction groups. After the operation, the eyes were washed with physiological saline and antibiotic drops were applied. No treatment was applied to the left eyes of the rats.

IOP Measurements

A rebound tonometer (Tono-Pen AVIA, REF: 230590, USA) was used to measure IOP of the right eyes 6 times between 4 and 7 min.

When the tip of the Tono-Pen probe came into contact with the cornea, the average value of five consecutive automatic measurements made by the microprocessor inside the device was recorded as the IOP value. IOP was measured just before and after each injection. Injections and measurements were made at the same time of the day (10:00–12:00).

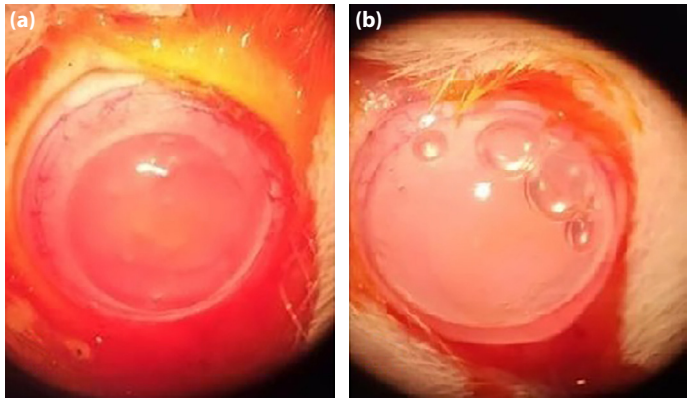

Fig. 1. **(a)** Iris vascularization is clearly observed before glaucoma induction in an albino Wistar rat. **(b)** Pale iris vessels after glaucoma induction (secondary to increased intraocular pressure with HA injection); the appearance of air and viscoelastic material.

Euthanasia and Enucleation

Six weeks after glaucoma induction, pre-euthanasia IOP of the rats was measured and recorded again. Before euthanasia and enucleation, dorsal, ventral, and lateral points were stained with tissue marking dyes. Eyes taken from rats were fixed with 10% formalin for 48 h. Tissues were washed with water to remove formalin and followed using routine follow-up methods on an automatic tissue tracking device (TP1020, Leica, Germany). A 5 µm sections were taken from rat tissues embedded in paraffin with a microtome (RM2235, Leica, Germany) and placed on slides for hematoxylin-eosin (HE) and immunohistochemistry staining (Fig. 2).

Immunohistochemical Examination

BAX and BCL-2 immunohistochemical staining was applied to 5 µm thick sections obtained from paraffin blocks. Sections prepared from the blocks for the immunohistochemical method were placed on (+) loaded (MicroSlides Snowcoat X-tra, Surgipath, Richmond, IL, USA) slides and kept in an oven at 37°C overnight.

Tissue samples stained with immunohistochemistry were examined under an Olympus brand BX51 TF model (Tokyo, Japan) light microscope and photographed with a DP72 camera (Olympus, Tokyo, Japan).

Evaluation of Histology and Retinal Thickness

The 5 µm thick sections taken from the paraffin blocks on the slide were kept in an oven for one night for deparaffinization, and then, the paraffin was removed by applying xylol. Tissues were passed through a decreasing series of alcohol and washed with distilled water. Tissues stained with HE dyes were passed through increasing alcohol series. Tissues dipped in xylol were closed by dripping entellan. Tissue samples stained with HE were examined under a light microscope and photographed.

Total retinal thickness, ganglion cell complex (GCC), inner plexiform layer (IPL), and ganglion cell layer (GCL) thickness measurements were determined for each eye using

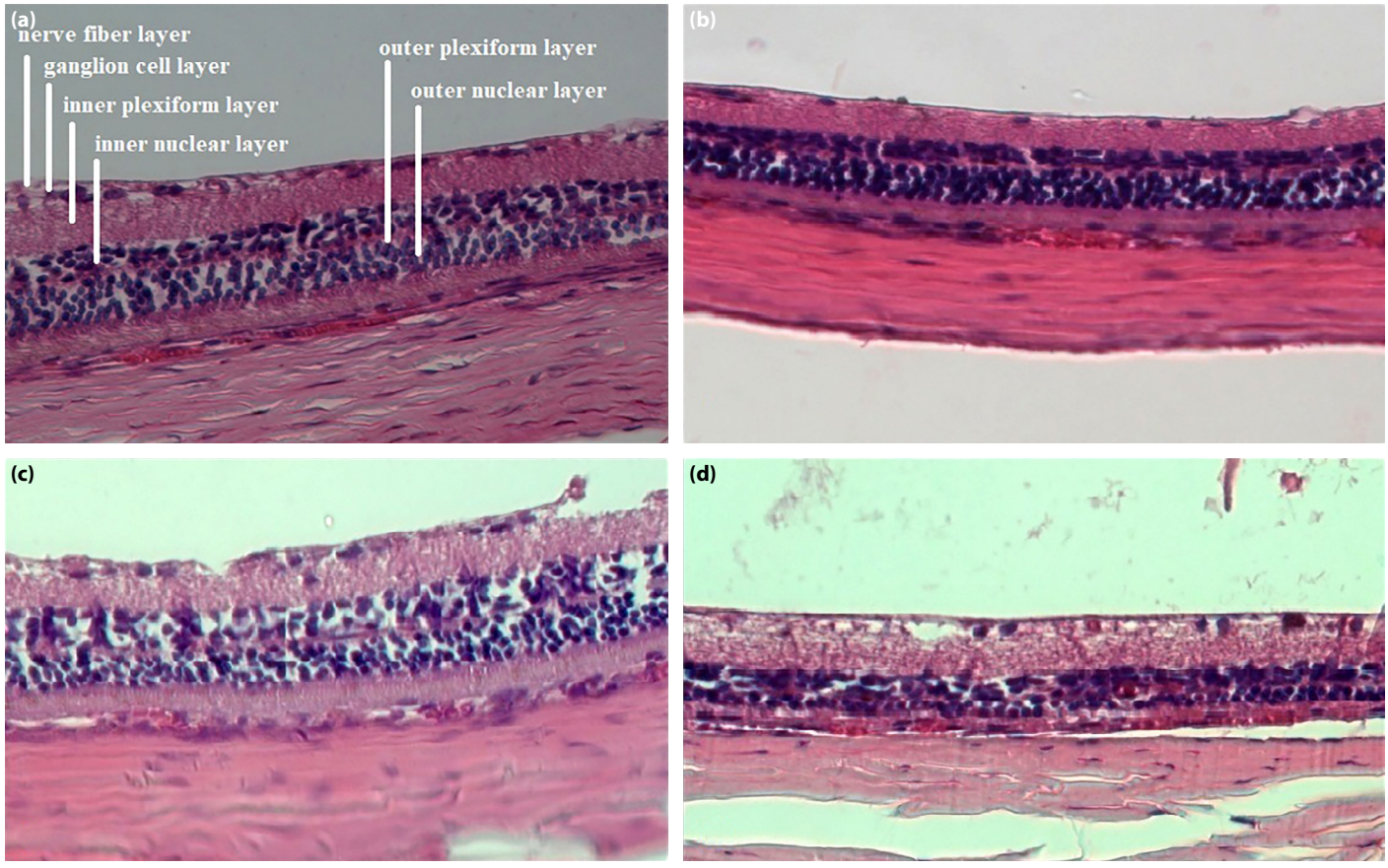

Fig. 2. Hematoxylin-eosin staining (40×) after 5 µm sections was taken from rat tissues embedded in paraffin with a microtome. **(a)** Control group, **(b)** glaucoma group, **(c)** SUBEPO group, and **(d)** TEPO group.

the ImageJ program (NIH, USA). The neuroprotective effect of EPO on glaucoma was evaluated by measuring retinal thickness, IPL thickness, GCL thickness, and GCC thickness measurements.

Statistical Analysis

Statistical Package for the Social Sciences (SPSS® 15.0 for Windows; SPSS Inc., Chicago, USA) program was used for statistical analysis of the data. Categorical data were given as numbers and percentages, and numerical data were given as mean and minimum-maximum. Kruskal–Wallis test was used for comparison of groups and Kruskal–Wallis one-way ANOVA test was used for pairwise comparisons. $P<0.05$ was accepted for statistical significance.

Results

A total of 24 rats were used in the study, six of which were randomized in each group. During the experiment, three rats were excluded from the study due to complications such as cataracts and three during anesthesia complications. Two more rats were excluded from the study to have an equal number of rats in the groups. The study was terminated with four rats in each group. Measurements were made at 6 weeks.

Mean IOP values in the control, glaucoma, SUBEPO, and TEPO groups are shown in Figure 3. There was a significant difference between the glaucoma group, SUBEPO treatment group, TEPO treatment group, and control group in terms of mean IOP values ($p<0.05$). At the end of the 6th week, adequate IOP values were achieved in the glaucoma

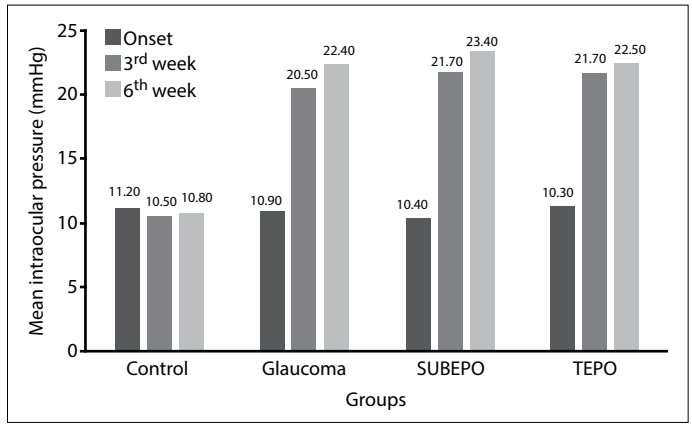

Fig. 3. Comparison of intraocular pressures of the groups at baseline, 3rd and 6th weeks.

Table 1. Pairwise comparison of retinal thickness between groups

Groups	Test statistics	p-value*
Glaucoma group-TEPO group	–1.188	1.000
Glaucoma group-SUBEPO group	–2.376	0.105
Glaucoma group-control group	3.565	0.002
TEPO group-SUBEPO group	1.188	1.000
TEPO group-control group	2.376	0.105
SUBEPO group-control group	1.188	1.000

*Kruskal–Wallis one-way ANOVA. SUBEPO: Subconjunctival erythropoietin; TEPO: Topical erythropoietin.

Table 2. Pairwise comparisons of inner plexiform layer thickness between groups

Groups	Test statistics	p-value*
Glaucoma group-TEPO group	–1.337	1.000
Glaucoma group-SUBEPO group	–2.748	0.036
Glaucoma group-control group	3.045	0.014
TEPO group-SUBEPO group	1.411	0.950
TEPO group-control group	1.708	0.526
SUBEPO group-control group	0.297	1.000

*Kruskal–Wallis one-way ANOVA. SUBEPO: Subconjunctival erythropoietin; TEPO: Topical erythropoietin.

Table 3. Pairwise comparisons of ganglion cell layer thickness between groups

Groups	Test statistics	p-value*
Glaucoma group-TEPO group	–1.485	0.825
Glaucoma group-SUBEPO group	–2.302	0.128
Glaucoma group-control group	3.045	0.014
TEPO group-SUBEPO group	0.817	1.000
TEPO group-control group	1.559	0.713
SUBEPO group-control group	0.743	1.000

*Kruskal–Wallis one-way ANOVA. SUBEPO: Subconjunctival erythropoietin; TEPO: Topical erythropoietin.

Table 4. Pairwise comparisons of ganglion cell complex thickness between groups

Groups	Test statistics	p-value*
Glaucoma group-TEPO group	–1.337	1.000
Glaucoma group-SUBEPO group	–2.451	0.086
Glaucoma group-control group	3.342	0.005
TEPO group-SUBEPO group	1.114	1.000
TEPO group-control group	2.005	0.270
SUBEPO group-control group	0.891	1.000

*Kruskal–Wallis one-way ANOVA. SUBEPO: Subconjunctival erythropoietin; TEPO: Topical erythropoietin.

and treatment groups (IOP >20 mmHg) (Fig. 3). Elevated IOP was observed in all subjects who underwent glaucoma induction.

The retinal thicknesses in the control, glaucoma, SUBEPO, and TEPO groups were 152.3 (148.9–155.1), 101.2 (92.8–108.4), 138.1 (134.3–143.0), and 117.8 (114.1–124.2) μm, respectively (p=0.003). According to the pairwise comparisons of the groups, the difference between the control group and the glaucoma group was statistically significant (Table 1, p=0.002). Although the mean retinal thickness values of the SUBEPO and TEPO groups were higher than the glaucoma group, the difference was not statistically significant (p>0.05).

The IPL thicknesses in the control, glaucoma, SUBEPO, and TEPO groups were 29.9 (28.1–31.5), 21.0 (19.9–23.0), 31.1 (27.5–32.9), and 24.0 (23.5–28.4) μm, respectively (p=0.008). According to the pairwise comparisons of the groups, the difference between the control group and the glaucoma group, and between the glaucoma group and the SUBEPO group was statistically significant (Table 2, p=0.014 and 0.036, respectively). Although the mean values of the TEPO group were higher than the glaucoma group, the difference was not statistically significant (p>0.05).

The GCL thicknesses in the control, glaucoma, SUBEPO, and TEPO groups were 16.3 (11.7–20.6), 11.1 (8.8–12.5), 13.2 (11.8–13.7), and 12.0 (10.2–13.1) μm, respectively (p=0.017). According to the pairwise comparisons of the groups, the difference between the control group and the glaucoma group was found to be statistically significant (Table 3, p=0.014). Although the mean values of the SUBEPO group and TEPO group were higher than the glaucoma group, the difference was not statistically significant (p>0.05).

The GCC thicknesses in the control, glaucoma, SUBEPO, and TEPO groups were 44.9 (42.3–52.0), 30.9 (29.0–31.9), 41.5 (40.3–44.6), and 36.7 (34.9–41.0) μm, respectively (p=0.006). According to the pairwise comparisons of the groups, the difference between the control group and the glaucoma group was found to be statistically significant (Table 4, p=0.005). Although the mean values of the SUBEPO group and TEPO group were higher than the glaucoma group, the difference was not statistically significant (p>0.05).

Although cell layer thicknesses in each group were confirmed by immunohistochemical staining, apoptotic cells could not be detected by bax or bcl-2 staining.

Discussion

In our study, retinal GCL thickness was the lowest in the glaucoma group and the highest in the control group, as expected. Retinal thickness was higher in the EPO group

than in the glaucoma group, regardless of the route of administration. Although the retinal thickness was greater in the SUBEPO group, no significant difference was found between the administration routes.

EPO is a hematopoietic growth factor of approximately 30.4 kDa.[8] It is accepted as a neuroprotective drug in clinical practice and its effectiveness is being investigated in studies.[9] EPO is mostly produced in the adult kidneys and to a lesser extent in the central nervous system.[10] A wide distribution of EPO and EPO receptors has been reported in adult humans, suggesting autocrine or paracrine actions in the brain and retina.[7,10]

Various models of ophthalmic and non-ophthalmic neurodegenerative diseases suggest that EPO has important neuroprotective and neuroregenerative properties due to its ability to reduce apoptosis, inflammation, oxidation, and excitotoxicity.[11,12] Therefore, EPO seems to be a valuable drug for preventing apoptosis of RGCs and can be applied to the eye by systemic, intravitreal, and retrobulbar routes.[13]

Subconjunctival administration of EPO is an easy and safe procedure because of its minimal side effects. The main ocular barriers to subconjunctival and topical applications are barriers to flow (blood flow and lymphatic flow), which make penetration into tissue difficult.[14] Here, scleral tissue is the most important barrier to consider. However, transscleral delivery of immunoglobulins and other major compounds to the choroid and retina has proven possible.[15] Because glaucoma is a chronic disease, multiple treatments are required to maintain RGCs. Therefore, it seems more appropriate to use topical and subconjunctival routes with less risk.

Resende et al.[16,17] showed that the subconjunctival administration of EPO has positive effects on the RGCs of the retina and also has neuroprotective effects. This route of administration avoids potential side effects associated with stimulation of hematopoiesis (systemic administration) and complications such as endophthalmitis, retinal detachment, uveitis, or cataracts (intravitreal and retrobulbar administration).[9,18,19]

In addition, in a study on topical EPO, it was shown that topical application of EPO β ex vivo can penetrate the porcine conjunctiva, cornea, and sclera.[20] Topical ocular administration of EPO over subconjunctival administration has the advantages of being a non-invasive route that does not require sedation or local anesthesia. Despite these attractive features, significant ocular hurdles to overcome must be taken into account.

In another recent study by Silva et al.,[21] when pig eye tissues were evaluated immunohistochemically with a nanoformulation of topical EPO (CS/HA6-EPO) using chitosan and hyaluronic acid molecules, they found that the fastest penetration was from the conjunctiva, followed by the sclera, and third from the cornea. In our study, we compared these two routes of administration in rats with in vivo glaucoma, referring to these ex vivo studies.

In an experimental mouse model of glaucoma, a single dose of intravitreal administration of 200 ng EPO was found to be effective in preventing at least some of the high-pressure loss of RGCs.[13] Exogenous EPO also shows an antiapoptotic mechanism of action by significantly reducing terminal apoptotic events in ischemic retina.[22]

Carvalho et al.[23] found that the number of RGC in the group given EPO in the glaucoma model occurring in the ischemic background was higher than in the rats that were not administered EPO.

When Loeliger et al.[24] examined the effect of EPO on retinal damage caused by endotoxins on fetal sheep, they found that it caused a significant increase in the number of RGCs.

Although not fully elucidated, studies suggest that EPO may have neuroprotective, antiapoptotic, anti-inflammatory, antioxidative, antipermeability, and angiogenic functions in the retina.[7,25]

The presence of many EPO receptors in retinal tissue suggests that EPO has important physiological roles in the eye.[26]

Rodent models of glaucoma have greatly improved our understanding of glaucoma pathophysiology and continue to serve as a useful tool for investigating neuroprotective agents. In addition, rodents show some similarities with humans in terms of their ocular anatomical structures.[27]

Hyaluronic acid is one of the main components of the extracellular matrix. HA can be injected into the anterior chamber through the corneascleral limbus, avoiding iris or lens contact. This gradually increases the anterior chamber depth and increases IOP through repeated injections.[28]

Studies have reported an 80% (approximately 9.4 mmHg) IOP increase in rats after 7–8 days with a single injection (25 µl HA [10 mg/ml saline]).[29,30] In addition, 40% RGC loss was detected with repeated injections (up to 10 weeks). However, in these studies, it was reported that 6–12.5% of rats developed corneal or lenticular complications after injection. In our study, in subjects with glaucoma due to repetitive injections, cataract, endophthalmitis, and per-

foration complications were observed at a rate of 12.5%, and death due to repetitive anesthesia inductions was observed at a rate of 12.5%. However, in our study, repeated injections were preferred to trigger glaucoma because the daily change in IOP was significantly higher and the IOP rise time was shorter. The disadvantage of repeated injections is that phthisis bulbi, cataracts, and transient corneal edema may be seen in animal eyes as a result of maintaining high IOP.[31]

Glaucoma is expected to affect approximately 112 million people in 2040.[11] While 1% of people over the age of 40 are affected by this pathology, this rate rises to 5% in people over 70 years old and up to 10% in people over 80 years old. Primary open-angle glaucoma (POAG) is the most common form of glaucoma.[32] POAG is characterized by degeneration of the trabecular meshwork, which is responsible for the drainage of aqueous humor from the anterior chamber of the eye, which increases IOP. The resulting ocular hypertension causes disruption of the axons of the RGCs forming the optic nerve and then the concentric loss of the RGCs. However, additional mechanisms other than increased IOP play a role in the development and progression of this degenerative disease. Current treatments are mainly aimed at lowering IOP. Indeed, despite appropriate IOP control, 15–25% of patients may experience glaucoma progression.[33,34]

High IOP is a risk factor for optic nerve damage and RGC loss, but lowering IOP alone is not sufficient to preserve visual function.[35] While lowering IOP is the basis of glaucoma treatment, treatment models targeting neuroprotection are also required.[36]

The death order of normal retinal tissue cells starts from the RGC layer and progresses from the inside out.[37,38] Retinal damage caused by glaucoma results in apoptosis of RGC as a result of axonal degeneration, activation of microglial tissue, release of TNF-α, and cytokines followed by activation of the complement pathway.[4,39]

In our study, while retinal GCL thickness was the least in the glaucoma group, it was the most in the control group. Retinal thickness was greater in the groups receiving EPO treatment than in the glaucoma group, regardless of the route of administration. Although the retinal thickness was greater in the SUBEPO group, no significant difference was found between the administration routes. This may be due to the small number of subjects in the experimental groups. If the study is repeated with more subjects, the difference between the EPO administration routes may also result in statistically significant results.

Cheng et al.[38] reported that the damage in the IPL regressed after EPO application in rats with retinal damage. Loss of the IPL is highly correlated with overall VF loss and is, therefore, a potential biomarker for assessing glaucoma progression in patients.[38] Neuroprotection can be used to prevent IOP-independent RGC loss.[39] In our study, IPL thickness was found to be higher in subconjunctival application than topical drop application.

GCC is affected early in glaucoma and is, therefore, an early indicator of glaucoma-related damage.[39] In our study, when the GCC thicknesses were examined between the groups, it was observed that the thickness was the least in the glaucoma group and the most in the control group. Although the SUBEPO group had thicker GCC than the TEPO group, no significant difference was found between them.

One of the limitations of our study is the use of single injections of EPO. Repeated subconjunctival injections of EPO should be administered to assess both local and systemic potential side effects. The glaucoma model used is also seen as another limitation. Although this experimental model of glaucoma has been used before and has been shown to be reliable and reproducible, glaucoma is a multifactorial and very complex disease. Therefore, combining data from studies using several different models of glaucoma are important to understand the full picture of EPO ocular neuroprotection used in glaucoma disease. Despite the limitations noted, these studies open new perspectives on the application of EPO for future studies targeting ocular neuroprotection in both preclinical and clinical scenarios.

Conclusion

In our study, the beneficial effects of subconjunctival and topically applied rHuEPO on RGCs, GCC layer, IPL, and total retinal layer after glaucoma induction were demonstrated. However, further studies are required to evaluate subconjunctival and topical EPO kinetics in glaucoma conditions.

Ethics Committee Approval: This study was approved by the Aydin Adnan Menderes Universty Animal Experiment Ethics Committee (date: 09.07.2020, number: 64583101/2020/055).

Peer-review: Externally peer-reviewed.

Authorship Contributions: Concept: Y.O., T.K., K.E.; Design: Y.O., TK; Supervision: Y.O., T.K., S.C.; Resource: Y.O., T.K., S.C.; Materials: Y.O., S.C.; Data Collection and/or Processing: Y.O.; Analysis and/or Interpretation: Y.O., T.K., S.B.; Literature Search: Y.O., S.B., S.C.; Writing: Y.O., T.K., S.B., S.C., K.E.; Critical Reviews: T.K., K.E.

Conflict of Interest: None declared.

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