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Original Article

Effects of Intracameral Drugs and Dyes on Corneal Endothelial Cell Apoptosis in a Rat Model: An *In Vivo* and *In Vitro* Analysis

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Abstract

Objectives: To evaluate the effects of intracameral drugs and dyes on rat corneal endothelial apoptosis and cell morphology.
Materials and Methods: The right eyes of 72 rats were injected intracamerally with 1% lidocaine, 0.01% adrenaline, triamcinolone acetonide (TA) 4 mg/mL, 1% trypan blue (TB), 0.5% indocyanine green (ICG), and fortified balanced salt solution as control. Corneal samples were taken 1 day and 1 week post-injection. Corneal endothelial apoptosis was assessed by the TUNEL technique, and the ratio of apoptotic cells in each group was compared with the control. Corneal endothelial cell morphology was evaluated in each specimen by transmission electron microscopy.
Results: The mean apoptotic endothelial cell ratio was significantly higher at 1 day and 1 week after intracameral adrenaline injection when compared to controls (p=0.03 and 0.021, respectively). TB caused a significantly higher apoptotic cell ratio when compared to controls at 1 week after injection (p=0.043). Lidocaine caused a higher apoptotic cell ratio compared to TA and ICG at 1 week, although not statistically significant (p=0.058, 0.09, 0.69, respectively). In all experimental specimens, transmission electron microscopy showed morphological changes associated with apoptosis.
Conclusion: This study showed that intracameral adrenaline, TB, and lidocaine injections may have toxic effects on corneal tissue, as indicated by ultrastructural and histopathological alterations. Therefore, these agents should be used with caution in intraocular surgery.
Keywords: Intracameral injection, corneal endothelium, apoptosis, TUNEL assay, morphology

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Introduction

Intracameral drugs frequently used in ophthalmic practice are useful tools for ocular anesthesia, pupil dilation, safe capsulorhexis, and control of intraocular inflammation. However, the effects and toxicity of these agents on the corneal endothelium are still under investigation.

Apoptosis is a form of cell death that occurs without damaging anatomical structures or disrupting physiological functions.[1,2] It is thought to play a key role in the modulation of corneal tissue through the induction of endothelial and epithelial cells.[3,4] One feature of apoptosis is the fragmentation of DNA, which can be detected in dying cells by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL). Previous studies have addressed various techniques to detect endothelial cell apoptosis induced by intracameral agents. However, the TUNEL technique has been studied in few reports.[5,6,7] It was shown that the TUNEL assay performed on the corneal endothelium allows better identification and quantification of apoptotic cells than other techniques.[8,9]

Previous data on intracameral agents are mostly from isolated reports of *in vivo* and *in vitro* studies. In this study, intracameral agents frequently used in intraocular surgeries were evaluated and compared in a single study using ultrastructural analysis. We aimed to demonstrate the effects of these drugs and dyes on corneal endothelial cell integrity using the TUNEL technique and transmission electron microscopy (TEM) in a rat model.

Materials and Methods

This animal study was performed in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research from the Association for Research in Vision and Ophthalmology, and the protocol was approved by the Institutional Animal Care and Use Committee of Başkent University Hospital in Ankara, Turkey (project no: DA 04/02).

The study was conducted with a total of 72 male Wistar albino rats aged 6 to 9 months and weighing between 301 and 457 g (mean: 357±23.6 g). Intracameral agents used were 0.05 mL of 1% preservative-free lidocaine, 0.01% adrenaline with preservative, triamcinolone acetonide (TA) 4 mg/mL, 1% trypan blue (TB), 0.5% indocyanine green (ICG) (25 mg ICG/0.5 mL aqueous solvent in 4.5 mL balanced salt solution [BSS]), and BSS alone. The rats were randomized and assigned to group 1 (adrenaline), group 2 (lidocaine), group 3 (TA), group 4 (ICG), group 5 (TB), and the control group (BSS). The rats were anesthetized with intramuscular injections of ketamine hydrochloride (Alfamine, Ege-Vet, Turkey) 60 mg/kg and xylazine hydrochloride (Rompun®, Bayer, Germany) 10 mg/kg before the procedure. In the right eye of each rat, the anterior chamber was entered through a long corneal tunnel in the superotemporal quadrant using an MVR knife, and 0.05 mL of aqueous humour was removed using a 30-gauge canula (Figure 1A,B). The same volume of an agent was injected intracamerally with a separate cannula, and the anterior chamber was not irrigated with BSS (Figure 2A-D). One agent was injected in each procedure. Topical ofloxacin 3 mg/mL was administered 3 times a day for 5 days after the injection.

For the euthanasia of the experimental rats, a high dose of intramuscular anesthetics or an intracardiac injection of potassium chloride was administered 1 day or 1 week after intracameral injection. In each group, 6 rats were sacrificed on day 1 and 6 rats at 1 week before their corneal samples were taken. Corneal transparency was clinically evaluated using a spotlight just before euthanasia. Immediately following death, the corneas were prepared for TUNEL staining and TEM analysis. The corneas were removed with a knife and scissors, leaving a 1 mm scleral rim, and the iris diaphragm was stripped from the corneal endothelium.

Preparation of the Corneal Samples

The corneas were divided into two parts, and one part was fixed in a glutaraldehyde fixative and the other part in a 4% formaldehyde solution for electron microscopic analysis. Formalin-fixed and paraffin-embedded 5 µm thick tissue sections were stained using the hematoxylin-eosin technique. DNA fragmentation was detected in situ by 3' end labelling using the ApopTag® Plus Peroxidase *In Situ* Apoptosis Kit (Oncor, Gaithersburg, MD, USA). TUNEL-stained apoptotic cells in the corneal samples were counted by the same person (B.B.) under a microscope using the 40x objective lens. Labelled cells were proportioned to the total number of cells and this was expressed as the rate of apoptosis. The percentage of apoptotic cells were scored as follows; grade 0: 0%, grade 1: 1-5%, grade 2: 5-25%, grade 3: 25-50%, and grade 4: ≥50%.

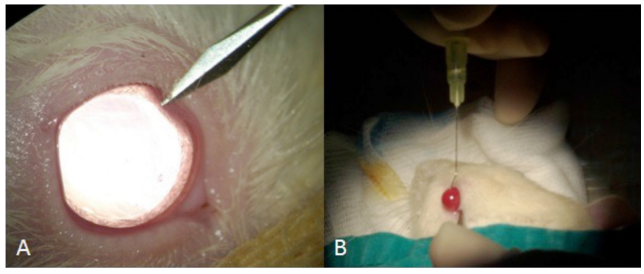

Figure 1. The anterior chamber was entered via the superotemporal corneal quadrant with an MVR knife (A) and 0.05 mL of aqueous humour was drained with a 30-gauge cannula (B) before injection of an intracameral agent

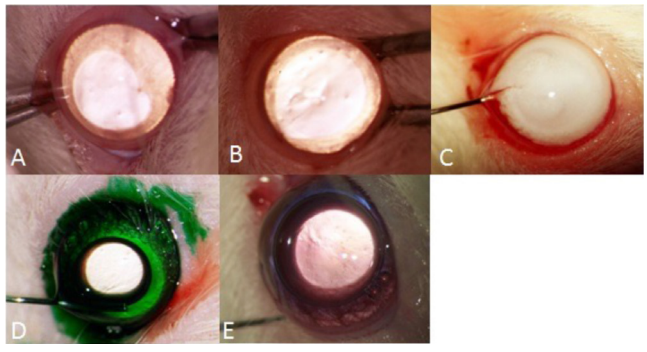

Figure 2. Intracameral injection of 1% preservative-free lidocaine (A), 0.01% adrenaline (B), triamcinolone acetonide 4 mg/mL (C), 0.5% indocyanine green (25 mg/0.5 mL aqueous solvent in 4.5 mL BSS) (D), and 1% trypan blue (E) into the rat anterior chamber

TEM Analysis

For the evaluation of corneal samples under TEM, the corneas were fixed for 24 hours in 2.5% glutaraldehyde solution in a phosphate buffer, post-fixed in 1% osmium tetroxide and 0.5% uranyl acetate, dehydrated through a graded sequence of acetone soaks, embedded in resin, sectioned and contrasted in 1% borax solution with 1% methylene blue and 1% azure II. After the ultrathin sections were cut, the material was counterstained with uranyl acetate and lead citrate. The specimens were initially embedded in dodecenyl succinic anhydride, Araldite CY212 (1:1, vol/vol), and benzyldimethylamine. The blocks were sectioned at 1 µm (thick section) and 0.05 µm (thin section) with an ultramicrotome. The thin sections were stained with uranyl acetate and lead citrate for examination with Carl Zeiss 906E (Oberkochen, Germany) TEM.

Statistical Analysis

The mean percentage of endothelial apoptotic cells in each group 1 day and 1 week after intracameral injection was compared with the control group. Data were analyzed using SPSS 11.0 for windows (SPSS Inc., Chicago, IL, USA). The Kruskal-Wallis test was used to evaluate the differences between all groups, and differences between two groups were evaluated with the Mann-Whitney U test. P values less than 0.05 were considered statistically significant.

Table 1. The mean TUNEL-positive endothelial cell (apoptotic cell) ratio in each group at 1 day and 1 week after intracameral injections

	Post-injection day 1		Post-injection week 1	
Agent	**Mean ± SD**	**P value**	**Mean ± SD**	**P value**
Adrenaline	0.403±0.036	0.03	0.626±0.081	0.021
Lidocaine	0.188±0.015	0.42	0.361±0.026	0.058
TA	0.248±0.021	0.24	0.295±0.024	0.09
TB	0.233±0.03	0.18	0.428±0.032	0.043
ICG	0.210±0.04	0.76	0.318±0.027	0.69
BSS (control)	0.220±0.034	Ref.	0.316±0.03	Ref.

TA: Triamcinolone acetonide, TB: Trypan blue, ICG: Indocyanine green, BSS: Balanced salt solution, SD: Standard deviation

Results

The study examined 72 rats, and each group comprised 12 eyes. The mean ratio of TUNEL-positive apoptotic cells for each group 1 day and 1 week after intracameral injection is shown in Table 1. On postoperative day 1, the adrenaline group had a statistically significantly higher mean apoptotic cell ratio than the control group (p=0.03). The mean apoptotic cell ratios in the lidocaine, TA, TB, and ICG groups were not significantly different from the control group (p>0.05). At postoperative 1 week, the adrenaline and TB groups had statistically significantly higher mean apoptotic cell ratios than the control group and other agent groups (p=0.021 and 0.043, respectively). When apoptotic cell ratios were scored, apoptosis varying between grade 1 and grade 4 was detected in all agent groups at 1 day and 1 week after injection (Figure 3A-D). Grade 4 endothelial apoptosis was observed at 1 day and 1 week after injection only in the group given adrenaline (Table 2).

During the injection, minimal iris prolapse occurred in two rats. However, the iris was repositioned with proper

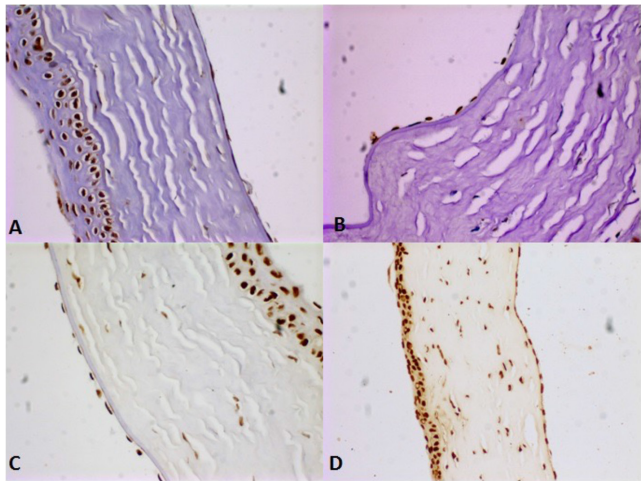

Figure 3. The effect of intracameral agents on endothelial cell apoptosis along the corneal folds was demonstrated by TUNEL technique. Cells with chromatin condensation were labelled and proportioned to the total number of cells. The rate of apoptosis was scored as Grade 0 (0%), Grade 1 (1-5%) (A), Grade 2 (5-25%) (B), Grade 3 (25-50%) (C), and Grade 4 (<50%) (D)

Table 2. The apoptotic scores in each rat corneal endothelium at 1 day and 1 week after the injection of intracameral agents

Agents	**Grade at 1 day post-injection (n=6)**						**Grade at 1 week post-injection (n=6)**					
Adrenaline	1	1	2	2	2	4	2	3	2	2	4	1
Lidocaine	1	1	2	1	1	1	3	3	2	1	2	1
TA	0	1	0	1	1	1	0	1	1	1	2	0
TB	0	2	1	0	1	1	1	3	2	2	3	1
ICG	2	1	1	1	2	0	3	1	2	0	2	1
BSS (control)	0	1	1	0	2	0	0	1	2	1	1	0

TA: Triamcinolone acetonide, TB: Trypan blue, ICG: Indocyanine green, BSS: Balanced salt solution

manipulation. Corneal edema was observed at 1 week in two rats with grade 4 apoptosis in the adrenaline group and one rat in the TB group. Minimal hemorrhage was observed in the anterior chamber in three rats on day 1. There was no sign of infection or endophthalmitis.

In TEM analysis, the control group displayed normal endothelium with intact cell junctions and organelles at 1 day and 1 week after BSS injection (Figure 4A). In the mid phase of apoptosis, the corneas showed chromatin clusters and mitochondrial swelling with vacuolization (Figure 4B). In the late apoptotic phase, chromatin condensation with mitochondrial swelling and shrinkage of the nucleus was observed (Figure 4C).

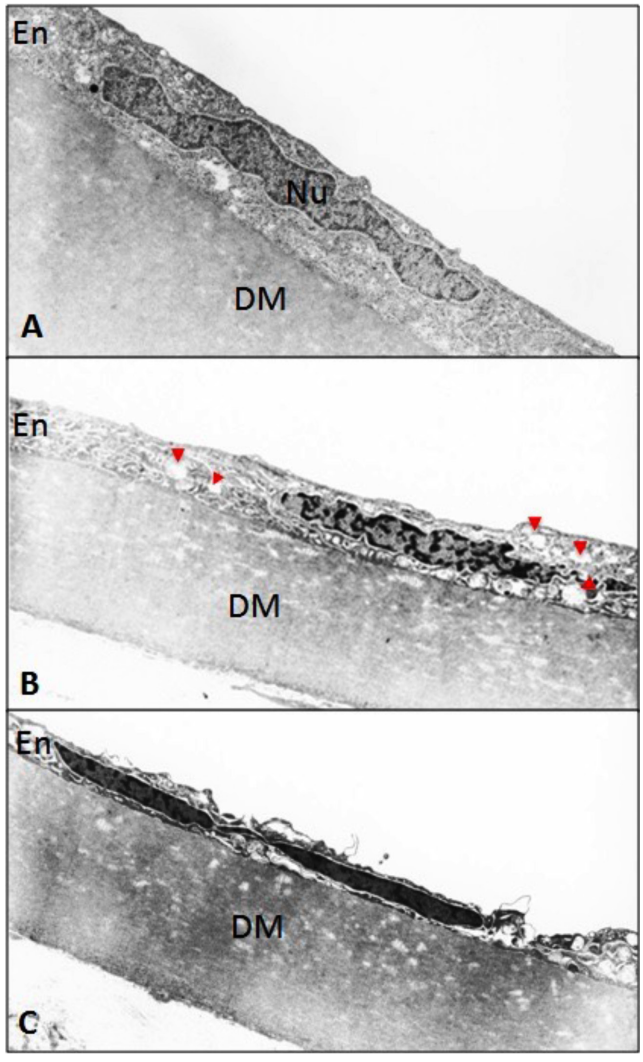

Figure 4. High-magnification transmission electron micrograph of rat corneal endothelium. The normal endothelium (En) is adherent to Descemet's membrane and the nucleus (Nu) appears long and undulated within the cell (A). The cells in the mid-phase of apoptosis show mitochondrial swelling, cytoplasmic vacuolization (arrows), and chromatin clustering (B). In the late apoptotic phase, the surface cell membrane is disrupted and chromatin condensation, mitochondrial swelling, and nucleus shrinkage are observed

Discussion

Our results showed that intracameral administration of adrenaline and TB induced significantly a higher rate of apoptotic response in the corneal endothelium. Lidocaine also caused more pronounced apoptotic changes in the first week, but there was no significant difference compared to the control group. Comparing day 1 and week 1 analyses, the proportion of apoptotic cells was higher after a week than after a day, suggesting that longer exposure may increase the apoptotic effect over time.

In the present study, TEM demonstrated the characteristic morphological features considered the hallmarks of apoptosis. These features include chromatin condensation, nuclear fragmentation, cytoplasmic vacuolization, and mitochondrial swelling, which were observed in the corneal endothelium 1 day and 1 week after drug administration.

Adrenaline is an agent used to provide rapid pupil dilation during intraocular surgery and minimize iris damage in patients with floppy iris syndrome.[10,11] Intracameral adrenaline use has been shown in numerous studies to be safe and effective.[12,13,14,15] However, there is still controversy regarding the possible endothelial toxicity. Liou et al.[16] observed that there were no significant changes in cell density or corneal thickness between rabbits that received intracameral injections of adrenaline and saline and that electron microscopic analysis showed healthy endothelial cells in all groups. Hong et al.[17] also showed that intracameral injection of adrenaline (up to 1%) did not affect the viability or morphology of endothelial cells in the rabbit cornea. In contrary, Hull et al.[18] reported that the endothelial damage caused by adrenaline was caused by the 0.1% bisulfite it contains, which is used to enhance the stability of the drug. Some studies also indicated that adrenaline has a high concentration of free radicals, which may contribute to endothelial toxicity.[19,20]

In our study, we observed corneal edema with grade 4 apoptosis in the adrenaline group at 1 week. Recently, toxic anterior segment syndrome was identified after an intracameral injection of 2.5% adrenaline and longer exposure was thought to be the cause.[21]

Lidocaine is an effective local anesthetic agent that acts on all nerve fibers in the anterior chamber. While some studies have indicated that low lidocaine concentrations do not affect corneal endothelial cells,[22,23] other studies have discussed the possibility of adverse effects to intraocular tissues at higher concentrations.[24,25,26,27] Cytotoxic effects have been demonstrated in relation to the concentration or duration of application of intracameral anesthetic agents.[22,23,25,26] It has been reported that 2% lidocaine with or without preservative induces a significant amount of apoptosis in rabbit corneal endothelium.[6,28] In terms of duration, Chang et al.[26] reported that a 1-minute exposure to 1% or 2% lidocaine appears to be safe for rabbit endothelial cells, but longer exposure may cause cytotoxicity. Atilla et al.[27] found that even a short exposure to intracameral lidocaine may result in histologic changes and functional defects in ocular tissues. Kim et al.[25] did not observe apoptosis in the rabbit endothelial cells 1 day after the administration of 1% lidocaine. However,

another study demonstrated apoptotic endothelial cell loss and morphologic changes which were temporary and resolved by 1 week.[6] According to our study, the risk of corneal endothelial cell apoptosis was increased by lidocaine relative to the risk presented by BSS exposure at 1-week analysis. This can be attributed to the longer time the agent remains in the anterior chamber.

TA is used to visualize and manage vitreous loss in the anterior chamber during complicated cataract surgery.[29,30,31] Furthermore, it has been shown to decrease postoperative inflammation and cystoid macular edema.[30] In a study by Oh et al.,[32] TA was administered into the anterior chamber of rabbit eyes, and their analysis showed no significant change in endothelial cell count after 2 hours. However, they observed a decreased amount of microvilli when TA was administered without resuspension. Another study showed cytotoxic effects on cultured rabbit endothelium, which was attributed to the preservative in the vehicle.[33] Histopathological studies conducted on retinal pigment epithelium cells also support the idea that the toxic effects of TA may be caused by 0.025% benzyl alcohol used as preservative.[34,35] In our study, no cytotoxic effect was observed due to TA at 1 day or 1 week after injection.

TB is used for capsulorhexis during cataract surgery. It is also used in staining and stripping the endothelium from the donor lenticule in deep anterior lamellar keratoplasty. Several clinical studies have tested TB toxicity on different structures of the anterior segment, and all have shown good biocompatibility with 0.1% TB.[36,37,38] Chung et al.[39] also evaluated the safety of 1% TB to improve visualization of the anterior capsule of a mature white cataract and found it to be safe. Although TB was shown to be feasible, there have been reports of toxicity related to dose and duration. *In vivo* and *in vitro* studies have demonstrated TB toxicity for corneal endothelium and corneal fibroblasts at higher concentrations and longer exposure periods.[37,40,41,42] Increasing the clinically used concentration resulted in a 38% to 55% decrease in the viability of endothelial cells. One study showed that intracameral TB injection may damage corneal tissue, as shown by oxidative stress parameters and histopathological assessment.[43] Teratogenic and carcinogenic potency has also been shown in animal studies.[44,45] Given these results, there is uncertainty as to whether TB is safe for corneal tissue. Briefly, TB is harmless to corneal cells at widely used concentrations, both in cataract surgery and in corneal tissue banks. However, extreme caution is advised at higher concentrations or longer exposures.

ICG is used as an intraocular stain in cataract and vitreoretinal surgery to improve the visualization of tissues. Intracameral administration is used for anterior capsular staining for safe capsulorhexis. Previous posterior segment studies have revealed that ICG may be toxic to the retina.[46,47] Clinical data showed that retinal glial cells, the nerve fiber layer, retinal ganglion cells, and the optic nerve can be damaged as a result of unknown mechanisms. The use of intracameral ICG tends to be well tolerated by the corneal endothelium during ophthalmic surgery. McEnerney and Peyman[48] demonstrated that ICG selectively stains dead corneal endothelial cells, and does not seem to be harmful to living cells. Holley et al.[49] indicated that the human corneal ultrastructure showed no harmful effects after ICG exposure in their TEM study. Our results based on an animal model also suggest that no toxic effects can be attributed to the dye. However, further research in the clinical setting is needed to document the effects of this stain.

The rats have a flat anterior chamber and thin iris stroma, which may lead to iris prolapse during injection. Among our subjects, only two rats had iris prolapse, and repositioning did not result in any endothelial contact or lens damage that could induce apoptosis. Events that cause inflammation in the anterior chamber may induce apoptosis. However, as all injections were performed by the same person using the same technique, we believe that all rats were subject to the same conditions.

Study Limitations

The present study has several limitations. Our objective was to investigate whether the doses of intracameral agents that are frequently used in clinical practice and determined to be safe in previous studies had an effect on apoptosis. Thus, we did not assess long-term effects or the effects of different doses of intracameral agents on endothelial cell function. Our study's primary objective was to explore the effects of anesthetic, mydriatic, and capsule staining agents in ocular surgery. Cefuroxime, on the other hand, has also been found to trigger apoptosis when administered intracamerally at the end of surgery.[50,51] This subject, however, was beyond the scope of our study.

Although rats are a common experimental model for studying the human cornea, human corneas may have different structural components and levels of endothelial stress than rat corneal endothelium. The rat cornea is thicker in the center and thinner in the periphery when compared to the human cornea.[52] Vasoactive intestinal peptide-positive parasympathetic nerve fibers identified in rat corneas but not in human corneas were reported to minimize corneal endothelium loss and improve corneal allograft survival following transplantation.[53] Human corneas, on the other hand, are protected from toxic injury by proteins and ion concentration in the aqueous humour, as well as a thicker endothelium mucin layer.[54,55] Furthermore, the use of viscoelastic and continuous irrigation during phacoemulsification can minimize these toxic effects. Additionally, the stromal component accounts for 90% of the human cornea, whereas it accounts for 70% in rats.[52] Thus, the disparity between the human eye and rat model could present difficulties in translating the findings. Future clinical trials are needed to support these results in humans. Also, it is known that DNA damage is not a unique feature of apoptosis and can also occur in necrosis. Therefore, using another independent method in conjunction with the TUNEL test may be essential to confirm and characterize apoptosis.

Conclusion

Intracameral injections of 1% lidocaine, 4 mg/mL TA, and 0.5% ICG did not cause damage to rat corneal endothelial cells. However, intracameral injection of 0.01% adrenaline or 1% TB

can induce microstructural changes in the corneal tissue. This should be considered when planning cataract or other ocular surgeries.

Ethics

Ethics Committee Approval: This animal study was performed in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research from the Association for Research in Vision and Ophthalmology, and the protocol was approved by the Institutional Animal Care and Use Committee of Başkent University Hospital in Ankara, Turkey (project no: DA 04/02).

Peer-review: Externally and internally peer reviewed.

Authorship Contributions

Surgical and Medical Practices: S.A.B., Concept: S.A.B., Y.A.A., Design: S.A.B., Data Collection or Processing: S.A.B., B.B., G.K., Analysis or Interpretation: S.A.B., Z.K.Ö., Literature Search: S.A.B., Z.K.Ö., Writing: S.A.B., Z.K.Ö.

Conflict of Interest: No conflict of interest was declared by the authors.

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