The Effect of Topical and Subconjunctival Coenzyme Q10 on Wound Healing Modulation in an Experimental Trabeculectomy Model

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ABSTRACT

Purpose: To investigate the wound healing effect of topical and subconjunctival Coenzyme Q10 (CoQ) in an experimental trabeculectomy model, and to compare the outcomes with mitomycin C and bevacizumab.

Materials and Methods: Thirty eyes of 27 Wistar Albino rats were included. Group 1 was the control group and the other four groups were mitomycin C (group 2), topical CoQ (group 3), subconjunctival CoQ (group 4), and bevacizumab (group 5). Conjunctival and scleral incisions were made to mimic the trabeculectomy wound. After the rats were sacrificed, histological sections were examined for vasculogenesis, VEGF and TGF-β1 positivity.

Results: The mean vascularization score was lowest in groups 3 and 4 (0.5±0.55). It was followed by group 2 (1.0± 0.89), group 5 (1.5±1.0), and control group (2.5±0.55). The eyes in group 2, 3 and 4 showed significantly less vascularization than the control group ($p = 0.015$, $p < 0.01$, $p < 0.01$). VEGF and TGF-β1 positivity scores were lowest in groups 3 and 4 (1.48±0.45). This was followed by group 2 (2.0±0.34), group 5 (3.5±0.3), and control group (3.94±0.5). TGF-β1 and VEGF positive cells were significantly lower in groups 2, 3 and 4 than group 1 ($p < 0.01$). TGF-β1 and VEGF positivity in groups 3 and 4 were significantly lower than the group 2 ($p = 0.04$).

Conclusion: The application of topical and subconjunctival CoQ resulted in inhibition of vasculogenesis and reduction of TGF-β1 and VEGF-positive cells in trabeculectomy wound healing model. Topical or subconjunctival application of CoQ in trabeculectomy surgery may provide positive effects on wound healing..

Keywords: Wound healing, Coenzyme Q10, Trabeculectomy, Mitomycin C, Bevacizumab.

INTRODUCTION

Glaucoma is a progressive disease that causes a specific optic neuropathy via retinal ganglion cell death. It is the second most frequent reason for blindness in the whole world.[1] Trabeculectomy is still the gold standard for treatment of high intraocular pressure (IOP) when medication and laser surgery are insufficient. Although recently non-penetrating surgeries are becoming more and more popular; the most commonly performed procedure for glaucoma treatment is still trabeculectomy.[2,3] The main reason for failure in trabeculectomy is excessive wound healing of the conjunctiva, tenon capsule and the scleral flap area. For this reason, pharmacological wound healing suppression is critical to the success of trabeculectomy. Antifibrotics like mitomycin-C (MMC) and fluorouracil (5-FU) are commonly used for suppression of wound healing response. Despite the benefits, these agents can cause serious complications like bleb leaking, blebitis, hypotony, corneal endothelial toxicity and endophthalmitis.[2-6]

Because the major cell type in the trabeculectomy wound site is the fibroblast, control of fibroblast activity is crucial. Some growth factors strongly increase the activity of fibroblasts.[2,7] VEGF concentrations in glaucomatous eyes have been found significantly higher than non-glaucomatous eyes in aqueous humor.[8] It has been shown that inflammation is triggered by VEGF in the tissue and

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inflammation itself causes an increase in VEGF expression in turn.[9] Moreover, it is also known that TGF-β1 induces angiogenesis through an increase in VEGF expression.[10-12] TGF-β1 has stimulating effects on fibrosis via angiogenesis and fibroblast activity.[13] Therefore, antifibrotic efficacy of VEGF inhibitors has been examined to improve the success of trabeculectomy.[14] Novel strategies targeting the TGF-β, VEGF and FGF signaling pathways are currently under investigation.

Along with its unique role in the electron transport chain, Coenzyme Q10 (CoQ10, ubiquinone), a lipophilic antioxidant molecule, has some antifibrotic and anti-angiogenic effects.[15-18]

It was shown that CoQ10 inhibits angiogenesis induced by bFGF in a mouse in vivo and ex vivo. In addition, ERK (extracellular-signal-regulated kinase) activation and VEGF expression were decreased by CoQ10 treatment.[19]

This study aims to evaluate the potential anti-scarring efficacy of CoQ10 ophthalmic solution in an experimental scleroconjunctival wound healing model in rats.

MATERIALS AND METHODS

Animals

Adherence to the Declaration of Helsinki with the approval of the Gazi University Ethics committee was provided (Code number: G.U.ET-15.051). Thirty eyes of 27 Wistar Albino Rats were used.

Groups

All rats were randomly divided into five groups. There were one surgical control group and four experimental groups (Group 1-5). Group 1, in which six eyes of three rats were included, was determined as the control group. Six eyes of six rats were included in every other group. Group 2 (MMC) comprised eyes that were applied a sponge soaked in MMC during the surgery. Group 3 (T CoQ) included eyes which topical CoQ10 was instilled postoperatively. In group 4 (SC CoQ), CoQ10 was injected in subconjunctival space at the last stage of the surgery. In group 5 (SC B), bevacizumab was injected in subconjunctival space at the end of the surgery.

Administration of Ophthalmic Solutions

Trabeculectomy was performed in all eyes as described below. No ophthalmic solution was applied to the eyes in group 1 during the surgery. Topical balanced salt solution (BSS) was instilled 4 times a day in the 21-day postoperative period. After the fornix-based conjunctival flap was created in the eyes in Group 2, a surgical sponge soaked in 0.2 mg/mL MMC was applied to the scleral wound area for 3 minutes. Then the entire MMC application area was irrigated with 10 mL of BSS. No ophthalmic solution was applied to the eyes in Group 3 during surgery. Coqun® eye drop (CoQ10 0.1% (w/v) and Vitamin E TPGS (d-alpha-tocopheryl polyethylene glycol 1000 succinate) 0.5% (w/v), Visufarma SpA, Rome, Italy) was applied topically for a period of 21 days following the surgery. Eyes in group 4 (SC CoQ) and group 5 (SC B) were injected 0.1 ml of CoQ10 (Coqun®) solution and 2.5 mg/0.1 mL of bevacizumab subconjunctivally at the end of the surgery, respectively.

Surgical Procedure

Surgeries were performed under anesthesia with intramuscular ketamine (Ketalar; Eczacıbaşı, Istanbul, Turkey) and xylazine (Rompun; Bayer, Leverkusen, Germany) and with topical 0.5% proparacaine hydrochloride ophthalmic solution (Alcaine; Alcon, Hünenberg, Switzerland). After placing a lid speculum, a corneal traction suture was applied with an 8-0 silk suture. After a peritomy at 5 mm was performed, a fornix-based conjunctival flap was made. A 2 mm long half-thickness scleral incision was performed 2 mm posterior to the limbus and temporal to the superior rectus muscle to mimic the trabeculectomy wound. For the rats in the MMC group, 0.2 mg/mL MMC was applied for 3 minutes with a cotton applicator. The conjunctival wound was closed by suturing with 8-0 vycril sutures. All eyes received tobramycin 3mg/mL and dexamethasone 1mg/mL eye drops 4 times daily for 21 days. No infection was detected in any eyes. Enucleation was performed under general anesthesia for histological analysis.

Histologic Examinations and Immunohistochemistry

Eye samples were put in 10% neutral formalin for fixation. Antigen retrieval were applied to 4 µm cross sections with Citrate Buffer (pH: 6. 0) (Lot: MK161004, Thermo). 3% hydrogen peroxide (Fisher Scientific, Melrose Park, IL, USA) diluted with phosphate buffered saline (PBS) for 15 min was used for blocking. After application of serum blocking solution (Lot: 1779354A, LifeTech) for 15 minutes, the slides were incubated with primary antibodies of VEGF (sc-7269, Lot #K1816, Santa Cruz), TGF-β1 (sc-146, Lot #G2114, Santa Cruz) (diluted with PBS at a rate of 1/100) at overnight in 4^{0}C. The biotinylated secondary antibody (Lot: 1779354A, LifeTech) was used for 10 minutes. Streptavidin peroxidase (Lot: 1779354A, LifeTech) was applied to the slides for 10 minutes, DAB (Lot: 38703, DAB Chromogen/Substrate Kit, ScyTek) was used as a chromogen. Afterwards, all slides were counterstained with Harris's hematoxylin. Slides were examined with Photo-light microscope (DM4000B Image

Analyze System, Leica, Germany) and Leica DFC280 plus camera.

The number of immune positive cells was measured by using LAS software program in consecutive areas for serial cutaways taken from. The following semi-quantitative scoring system was used to assess the immunolabeling intensity: (0) no staining, (1) weak, (2) moderate to weak, (3) moderate, (4) moderate to strong and (5) strong labeling. Degenerative Criteria Table was also formed and assessed as (0) no, (1) weak, (2) moderate and (3) strong. Two independent observers who were blind to the treatment protocol performed the immunolabeling score evaluations independently.

Statistical Analysis

The histologic data were compared using Mann-Whitney U nonparametric tests. Values of $P < 0.05$ were regarded as significant. Calculations were performing using the Statistical Package for the Social Sciences (SPSS) version 22.0 system for personal computers (SPSS Inc., Chicago, IL).

RESULTS

There were no complications and intraocular infections during the 21 days follow-up. Enucleated eyes were analyzed histologically and immunohistochemically (Figure 1-5).

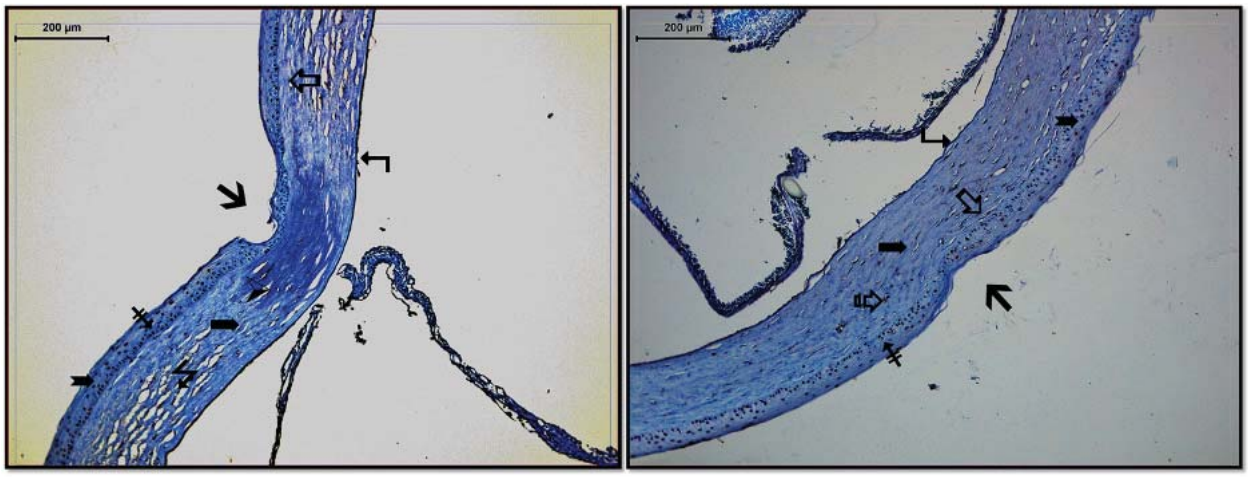

Figure 1: Group 1 *scleral incision area.*
(➔), conjunctiva ephitel (↕), episclera (⇨), stroma (➡), collagen fibers (➢), vessel (↯), subarachnoid layer (↳), fibroblasts (⇪)immunoreactive cells (➡) (Immunoperoxidase-Hematoxylin x100) (Left: VEGF, Right: TGF-β1).

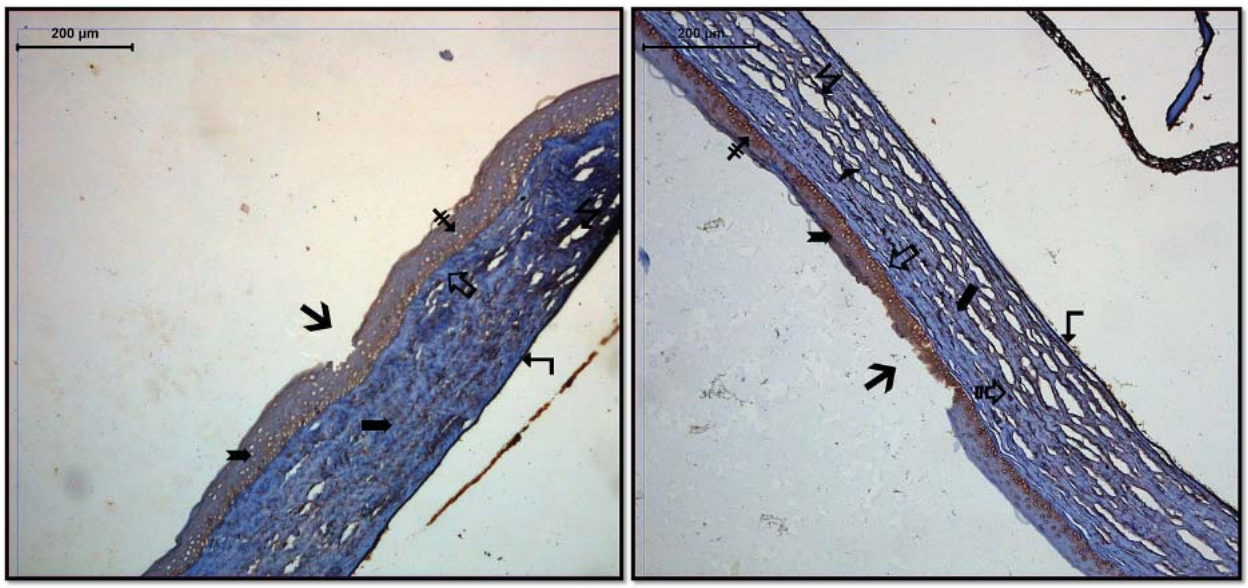

Figure 2: Group 2 *scleral incision area.*
(➔), conjunctiva ephitel (↕), episclera (⇨), stroma (➡), collagen fibers (➢), vessel (↯), subarachnoid layer (↳), fibroblasts (⇪)immunoreactive cells (➡) (Immunoperoxidase-Hematoxylin x100) (Left: VEGF, Right: TGF-β1).

Figure 3: Group 3 *scleral incision area.*
(➔), conjunctiva ephitel (↕), episclera (⇨), stroma (➡), collegen fibers (➢), vessel (↯), subarachnoid layer (↳), fibroblasts (⇪)immunoreactive cells (➡) (Immunoperoxidase-Hematoxylin x100) (Left: VEGF, Right: TGF-β1).

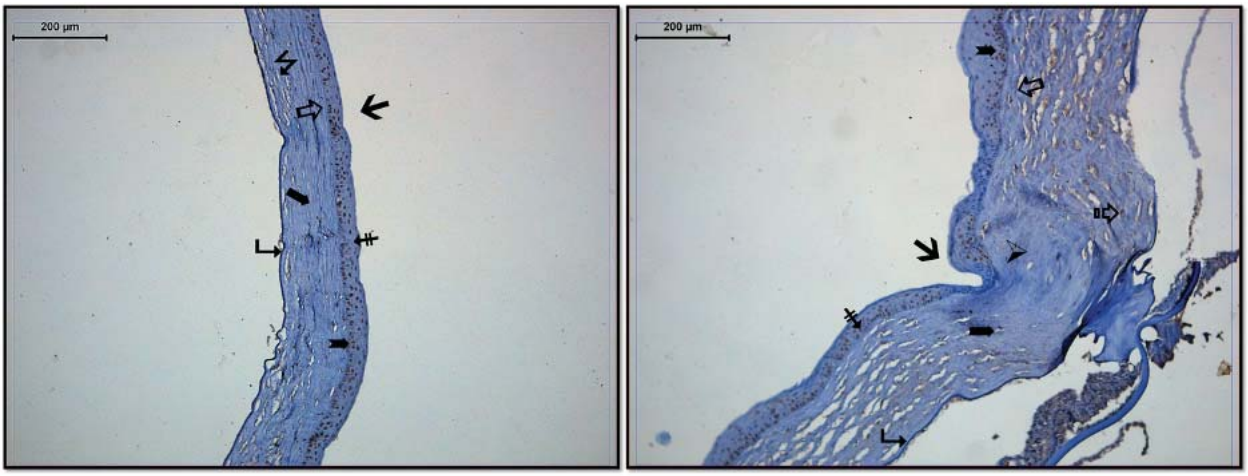

Figure 4: Group 4 *scleral incision area.*
(➔), conjunctiva ephitel (↕), episclera (⇨), stroma (➡), collegen fibers (➢), vessel (↯), subarachnoid layer (↳), fibroblasts (⇪)immunoreactive cells (➡) (Immunoperoxidase-Hematoxylin x100) (Left: VEGF, Right: TGF-β1).

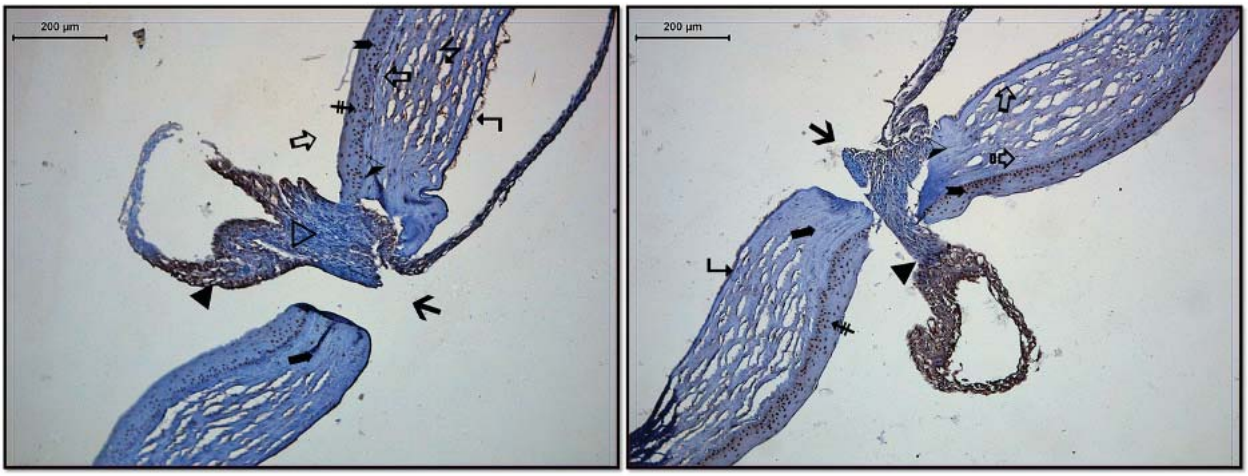

Figure 5: Group 5 *scleral incision area.*
(➔), conjunctiva ephitel (↕), episclera (⇨), stroma (➡), collegen fibers (➢), vessel (↯), subarachnoid layer (↳), fibroblasts (⇪)immunoreactive cells (➡) (Immunoperoxidase-Hematoxylin x100) (Left: VEGF, Right: TGF-β1).

Vascularization scores

The means, standard deviations and the statistical comparison of the vascularization scores of the groups were summarized in table 1. The mean vascularization score was lowest in T CoQ and SC CoQ groups (0.5±0.55). It was followed by MMC (1.0± 0.89), SC B (1.5±1.0) and control groups (2.5±0.55), respectively. Eyes of MMC, T CoQ and SC CoQ groups showed statistically significant less vascularization than control group (p = 0.015, p <0.001, p <0.001). When the vascularization scores of T CoQ or SC CoQ groups were compared with MMC and SC B groups individually, no statistically significant difference was found (p= 0.4 and 0.09, respectively).

VEGF and TGF-β1 positivity scores

VEGF and TGF-β1 positive cells were detected in tissues by immunohistochemical examination by using LAS software program and graded with the semi-quantitative scoring system. The means, standard deviations and the statistical comparison of the immunohistochemical positivity scores of the groups were summarized in tables 2 and 3. Immunohistochemical positivity scores of VEGF and TGF-β1 were equal and lowest in groups 3 and 4 (1.48±0.45). This was followed by group 2 (2.0±0.34) and 5 (3.5±0.3) and was highest in the control group (3.94±0.5). There was a statistically significant difference in VEGF and TGF-β1 positivity score between the control group and group 2, group 3 or group 4 (p <0.01). There was no statistically significant difference between group 5 and the control group (p = 0.1). There was also a statistically significant difference between the comparison of groups 3 and 4 with group 2 (p = 0.04).

Table 1: *Mean vascularization scores.*

Groups	Mean±SD	p value*
Control	2.5±0.55	
MMC	1.0± 0.89	0.015
T CoQ	0.5±0.55	<0.01
SC CoQ	0.5±0.55	<0.01
SC B	1.5±1.0	0.09

SD: Standard deviation, MMC: Mitomycin-C,
T CoQ: Topical CoQ10, SC CoQ: Subconjunctival CoQ10,
SC B: Subconjunctival Bevacizumab.
*Statistical comparison of control group with other groups

Table 2: *Mean VEGF positivity scores.*

Groups	Mean+SD	p value*
Control	3.94±0.5	
MMC	2.0±0.34	<0.01
T CoQ	1.48±0.45	<0.01
SC CoQ	1.48±0.45	<0.01
SC B	3.5±0.3	0.1

SD: Standard deviation, MMC: Mitomycin-C,
T CoQ: Topical CoQ10, SC CoQ: Subconjunctival CoQ10,
SC B: Subconjunctival Bevacizumab.
*Statistical comparison of control group with other groups

Table 3: *Mean TGF-β1 positivity scores.*

Groups	Mean+SD	p value
Control	3.94±0.5	
MMC	2.0±0.34	<0.01
T CoQ	1.48±0.45	<0.01
SC CoQ	1.48±0.45	<0.01
SC B	3.5±0.3	0.1

SD: Standard deviation, MMC: Mitomycin-C,
T CoQ: Topical CoQ10, SC CoQ: Subconjunctival CoQ10,
SC B: Subconjunctival Bevacizumab.
*Statistical comparison of control group with other groups scores

DISCUSSION

During the wound healing process, scar tissue formation increases along with the amount of VEGF in the medium. This is explained by the enhancing effect of VEGF on the number of fibroblasts and myofibroblasts. In addition, less scar tissue has been shown to be formed when injecting Anti-VEGF antibody into wound tissue. However, this relationship with inflammation is controversial.[20,21] Coenzyme Q10 is a fat-soluble, organic, electron-transporting molecule with antioxidant properties.[22] Coenzyme Q10 has also been shown to have anti-angiogenic effects by decreasing VEGF expression in endothelial cells.[18] In addition, there are also positive effects on wound healing.[23,24]

The long-term success of the glaucoma filtration surgery depends on the wound healing response in the surgical field. Fibrotic tissue caused by complex cellular and extracellular events such as fibroblast proliferation and abnormal collagen synthesis is the main cause of bleb failure which leads to unsuccessful surgical results.[25] With the use of antifibrotic agents such as MMC and 5-fluorouracil, which are used to prevent bleb failure, an increase in surgical success rates have been achieved in trabeculectomy.[2] Antifibrotic agents achieve this inhibition by immune modulation effects in the wound site.[26] On the contrary, when TGF-β is applied to conjunctiva after MMC treatment, it reverses the effect of MMC on

the wound healing process.[27] Similar to these results, it is seen that TGF-β expression was significantly reduced by MMC in our study compared to the control group. Besides the positive effects of antifibrotic agents on the success of trabeculectomy, they can lead to complications such as hypotony, corneal decompensation or cataract development.[6,28]

High complication rates of antifibrotic agents have led clinicians to investigate more selective agents to reduce scar formation. Successful and unsuccessful results have been reported by testing different molecules, especially with anti-VEGF agents.[14, 29-32] It has been shown that VEGF increases myofibroblast transformation by inducing TGF-β1 and may increase the subconjunctival fibrosis in the trabeculectomy wound site.[33] Based on this mechanism, anti-VEGF agents such as Bevacizumab were applied to the trabeculectomy procedure alone or to enhance the effect of antifibrotic agents. Despite the positive results of Anti-VEGF agents, success rates were still lower than antifibrotic agents.[34] In our study, it is seen that MMC leads to lower VEGF and TGF-β1 positivity and vascularization compared to bevacizumab. This finding is probably one of the reasons why MMC is more effective than bevacizumab.

In this study, CoQ10 was expected to prevent bleb failure because of better wound healing in the scleral incision site in glaucoma filtration surgery, possibly with the help of anti-angiogenic and wound healing modulation effects of VEGF inhibition. For this purpose, CoQ10 was injected into the bleb during surgery in one of the groups, and the other was administered topically 4 times daily for 21 days after surgery. Vascularization scores of both topical and subconjunctival CoQ groups were significantly lower than the control group but there was no difference when CoQ groups compared to each other. Furthermore, when CoQ groups were compared with MMC and SC B groups, no statistically significant difference was found. Immunohistochemical analysis for VEGF and TGF-β1 supported this result. The mean VEGF and TGF-β1 positivity scores in both CoQ groups were lower than the other three groups. They were also statistically significant difference from all groups. These surprising results suggest that administration of SC or topical CoQ in filtration surgery can provide better wound healing modulation with anti-angiogenesis effect and predict increasing the success of the surgery and reducing the complications.

The limitations of this study are the use of the rat eye for the experiment and the inability to apply all the steps of trabeculectomy for the reason that the rat sclera is too thin to perform a complete trabeculectomy surgery. For this reason, decreasing in IOP's, evaluating the bleb functions and complications such as hypotony cannot be investigated. After these positive results, we recommend that the CoQ should be investigated in studies with more eyes and re-planned in subjects on which all steps of trabeculectomy can be performed.

In summary, when CoQ is administered topical or subconjunctival, inhibits vascularization similarly to MMC or bevacizumab and inhibits VEGF and TGF-β1 expression better than MMC or bevacizumab. For this reason, with the use of CoQ, glaucoma surgeons can achieve better wound site modulation, which has more success and low complication rates than current trabeculectomy techniques.

Compliance with ethical standards

Funding: This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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