Reduction of Conjunctival Fibrosis After Trabeculectomy Using Topical α-Lipoic Acid in Rabbit Eyes

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Purpose: To evaluate the efficacy of α-lipoic acid (ALA) in reducing scarring after trabeculectomy.

Materials and Methods: Eighteen adult New Zealand white rabbits underwent trabeculectomy. During trabeculectomy, thin sponges were placed between the sclera and Tenon’s capsule for 3 minutes, saline solution, mitomycin-C (MMC) and ALA was applied to the control group (CG) (n = 6 eyes), MMC group (MMCG) (n = 6 eyes), and ALA group (ALAG) (n = 6 eyes), respectively. After surgery, topical saline and ALA was applied for 28 days to the control and ALAGs, respectively. Filtrating bleb patency was evaluated by using 0.1% trepan blue. Hematoxylin and eosin and Masson trichrome staining for toxicity, total cellularity, and collagen organization; α-smooth muscle actin immunohistochemistry staining performed for myofibroblast phenotype identification.

Results: Clinical evaluation showed that all 6 blebs (100%) of the CG had failed, whereas there were only 2 failures (33%) in the ALAG and 6 failures in the MMCG on day 28. Histologic evaluation showed significantly lower inflammatory cell infiltration in the ALAGs and CGs than the MMCG. Toxicity change was more significant in the MMCG than the control and ALAGs. Collagen was better organized in the ALAG than control and MMCGs. In immunohistochemistry evaluation, ALA significantly reduced the population of cells expressing α-smooth muscle action.

Conclusions: ALA prevents and/or reduces fibrosis by inhibition of inflammation pathways, revascularization, and accumulation of extracellular matrix. It can be used as an agent for delaying tissue regeneration and for providing a more functional-permanent fistula.

Key Words: α-lipoic acid, mitomycin-C, conjunctival fibrosis, trabeculectomy, scar formation

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Since its introduction by Cairns in 1968, trabeculectomy, with its relatively high success rate, has established itself as the gold-standard technique among the surgical alternatives available for open-angle glaucoma. The main cause of failure with this procedure has been attributed to excessive postoperative conjunctival scarring at the site of the filtering bleb. To reduce the wound-healing process and to inhibit the formation of unwanted scar tissue in challenging cases, antimetabolic agents such as mitomycin-C (MMC) and 5-fluorouracil may be used during trabeculectomy surgery. These agents are believed to improve the success rate of surgery by modulating fibroblast migration and/or proliferation, but their use is associated with a well-known high-complication rate.

α-lipoic acid (ALA) is an organosulfur compound with antioxidant, anti-inflammatory, antifibrotic properties, and has been used successfully in a wide range of nonocular pathologies. It is a key contributor to hepatoprotection by improving liver circulation. Its favorable effects on the treatment of diabetic neuropathy, on the prevention of atherosclerosis, and on cardiovascular diseases have been demonstrated. It has also been shown to reduce tissue inflammation and oxidation processes.

The aim of this study was to evaluate the histologic effects of postoperatively applied ALA on the conjunctival inflammation, scar tissue formation, and filtering bleb patency of rabbit eyes which underwent trabeculectomy, and to compare these results with those obtained with MMC.

MATERIALS AND METHODS

Eighteen eyes from 18 healthy New Zealand white albino male rabbits obtained from the Laboratory Animal Resource Center, Kafkas University (Kars, Turkey); aged 10 to 12 months and weighting between 3 and 3.5 kg were used in the study. The study was carried out in accordance with the Animal Ethical Guidelines for Investigations in Laboratory Animals and was approved by the Medical Experimental Research Ethics Committee of Kafkas University (KAÜ-HADYEK, 2012-002). The rabbits were kept under standard conditions (20 ± 1°C, 12-h light/12-h dark cycles) and received humane care as outlined in the Guide for the Care and Use of Laboratory Animals.

The animals were randomly divided into 3 groups: a control group (CG), an α-lipoic acid group (ALAG), and a mitomycin-C group (MMCG). Each group included the same number of eyes (n = 6). Limbus-based conjunctival incision was performed, and Tenon’s capsule was dissected. After Tenon’s capsule dissection; the MMCG received an antimetabolism drug administered with small (2 × 4 mm) lint impregnated with 0.25 mg/mL mitomycin (Mitomycin-C, Kyowa Hakko Kogyo, Japan), ALAG and CG received ALA and saline solution on the sclera and the Tenon’s capsule.
capsule for 3 minutes, respectively. The sponges were then removed and the surgical field was irrigated with copious amounts of balanced salt solution. Trapezoidal scleral flap was created and 3 × 1 mm trabeculectomy and peripheral iridectomy was performed. The ALAG received 1% ALA (Thiocticacid 600t; MEDA, Hamburg, Germany) and CG received saline eye drops 5 times/d for 28 days. Twenty-eight days after trabeculectomy, the animals were killed, the trabecular region was removed, and the histopathologic and immunohistochecmical analyses were performed by scoring the tissue changes and then comparing them among the 3 groups.

Samples were analyzed on the 28th day, because Miller and colleagues showed that in rabbits, 94% of blebs were closed on the 17th day, whereas in a study by Zhong and colleagues, their control group, all blebs were closed on the 51.3rd day in the MMC group. Corderio and colleagues showed that in rabbits, 94% of blebs were closed on the 16th day in the control group and on the 19.0 ± 2.0. In similar studies, Tina and colleagues reported that blebs were closed on the 16th day in the control group and on the 51.3rd day in the MMC group. Corderio and colleagues showed that in their control group, all blebs were closed by the 14th day. Tissues were taken from the rabbits on 28th day to account for the “bleb closure time” mentioned in the literature.15–18

Anesthesia Procedure

Food was withheld from the animals for 12 hours before the operation. General anesthesia was performed intramuscularly with a combination of xylazine HCl (Rampun, 2%, Bayer; 5 mg/kg body weight) and ketamine HCl (Ketalar, 50 mg/mL, Pfizer; 50 mg/kg body weight). Before the surgical procedure, proparacaine HCl 0.5% (Alcaine, Alcon) was also applied as a topical anesthetic eye drop to produce a more analgesic effect. At the 28th post-operative day, the rabbits were euthanized using an intra-cardiac injection of 1 mL Pentobarbital Sodium (Nembutil, Abbott, Turkey).

Surgical Procedure

Trabeculectomies were performed based on a standard study protocol: A 6 mm limbus-based conjunctival incision was produced 5 mm from the limbus. The underlying Tenon’s capsule was opened and dissection was continued to the limbal area. Each rabbit underwent a limbus-based trabeculectomy. A half thickness trapezoidal scleral flap (3 × 2 mm) was created, starting 2 mm behind the limbus and continuing until the blade was just visible in the anterior corneal stroma. The anterior chamber was entered using a 45-degree blade. A piece of tissue containing the inner sclera, trabeculum, and peripheral cornea measuring approximately 3 × 1 mm was excised. Peripheral iridectomy was performed. The anterior chamber was filled and formed with ophthalmic viscousurgical device (1.8% Na Hyaluronate, Protectalon; VSY, Turkey). The scleral flap was closed with 10/0 nylon sutures (Ethicon; Johnson & Johnson; New Jersey, NJ). At the end of surgery, the conjunctiva was closed with 8/0 Vicryl sutures (Ethicon, Johnson & Johnson). All glaucoma filtration surgeries were performed by a single surgeon with experience in using the rabbit model (ME). Topical antibiotic drops [Tobramycin (Tobrared; Bilim İlaç, İstanbul, Turkey)] and antibiotic ointment (Tobrared; Bilim İlaç) was applied to all eyes for 14 days postoperatively. We did not prefer to use steroid and/or nonsteroid anti-inflammatory agents in order to assess the primary effectiveness of ALA.

Clinical Evaluation

After the surgery, macroscopic assessment and bleb function were controlled. Intraocular pressure was not collected as an endpoint as our specific metric of interest involved bleb function and histologic differences between groups. Macroscopic assessment was performed by Pentlight to evaluate the absence of infection. To evaluate the filtrating bleb patency, we injected 0.1 mL 0.1% trepan blue dye into the anterior chamber and observed its passage into the subconjunctival space. The dye applied into the anterior chamber by the same surgeon. Injections were applied without allowing excessive deepening of the anterior chamber and sudden increase in intraocular pressure.

In studies performed with in vitro human Tenon’s capsular fibroblasts, it has been shown that trepan blue dye does not affect the antimetabolic effect of MMC. It has also been reported that during cataract surgery, trepan blue dye can be useful to evaluate filtering bleb patency and Baerveldt tube function by observing trepan blue passage into the subconjunctival space after injection of the dye into the anterior chamber.19–22

Histologic Assessment

A large amount of tissue including the trabeculectomy site was removed using a lancet. The architecture of the superior fornix and conjunctival tissues around the drain- age site was preserved. The tissues were fixed in 10% buffered formal saline for 24 hours, stored in 70% alcohol, and fixed in paraffin wax. Sequential 5-μm sections of the operative wound site were prepared, and histologic staining was performed to demonstrate cellularity and extracellular matrix (ECM) deposition including: hematoxylin and eosin (for total cellularity), Masson trichrome frozen sections of 4- to 5-μm thickness (for collagen organization) α-smooth muscle actin (α-SMA) immunohistochemistry (monoclonal mouse anti-human smooth muscle actin antibody, Clone 1A4; Dako, High Wycombe, UK) (for myofibroblast phenotype identification). The stained samples were observed under a Leica DM6000B photomicroscope and the parameters were graded randomly by 2 masked observers.

For evaluating cellularity, the polymorphonuclear leukocyte, plasmocyte, lymphocyte, and lymphoid aggregate and vascular structures were calculated in 1 magnification area (40 × : magnifications).

For evaluating toxicity, analysis of the conjunctiva and the ciliary body were made and the findings were evaluated by scoring according to the grading systems published by Polak et al23 and Apfel et al,24 with slight modifications. The scale used for conjunctival changes was: 0 = no histologic change: no inflammation; 1 = minimal histologic change/conjunctiva epithelium preserved, thickening of conjunctiva; 2 = mild histologic change/conjunctiva epithelium preserved, thickening of conjunctiva, and mild inflammatory cell infiltration; 3 = moderate histologic change/score 2 with loss of collagen fibril organization; 4 = severe histologic change: loss of conjunctival epithelium, total disorganization, and necrosis of the underlying scleral stroma. The scale used for ciliary body changes was: 0 = no histologic change; 1 = minimal histologic change/ciliary epithelium height normal, demonstrating minimal fibroblast proliferation, congestion, and edema, no fibrin; 2 = mild histologic change/ciliary epithelium height decreased, demonstrating moderate fibroblast proliferation with fibrin, moderate congestion and edema; 3 = moderate histologic change/score 2 with
inflammatory cell infiltration; 4 = severe histologic change/desquamation of ciliary epithelium, total disorganization, and necrosis of ciliary body.

To evaluate the collagen organization we used a modified scoring system (which was based on that described by Janin-Manificat et al25). The scale used for collagen organization was: 0 = no collagen disorganization/parallel mature collagen fibers; 1 = weak collagen disorganization/long, discrete collagen fibers; 2 = moderate collagen disorganization/shortened, focal perpendicular sequence of collagen fibers; and 3 = strong collagen disorganization/collagen fibers shortened and pointing in all directions.

For evaluating immunohistochemistry, the percentage of stained cells and the grade of staining were employed as criteria and a semiquantitative method was used: 0 = no staining; 1 = weak staining; 2 = moderate staining; and 3 = strong staining. For sections, an immunohistochemistry score was calculated using “H-SCORE” (H-SCORE = I x PC; I, the grade of staining; and PC, the percentage of stained cells).26

**Statistical Analysis**

All data were expressed as mean grade ± SD (mean ± SD). The histologic data were compared using Kruskal-Wallis nonparametric tests. Post hoc tests were performed with the Mann-Whitney U test. Values of P < 0.05 were regarded as significant. Calculations were performed using the Statistical Package for the Social Sciences (SPSS) version 18.0 system for personal computers (SPSS Inc., Chicago, IL).
RESULTS

Experimental Details and Clinical Findings

Of the 18 rabbits observed for the duration of the 28-day experimental period, all completed the experimental protocol. No intraocular infection or cataract was observed. The tolerability of ALA drops was found very well and no side effect (conjunctival redness, chemosis, or discharge) was detected. When the filtrating bleb patency was evaluated using 0.1% trepan blue dye, it was observed that all 6 blebs (100%) of control group had failed, whereas only 2 (33%) from the ALAG and none from the MMCG had failed on the 28th postsurgical day (Fig. 1). There was a statistically significant difference between CG and MMCG ($P = 0.002$), whereas the difference between CG and ALAG did not reach statistical significance ($P > 0.05$).

Histopathologic Features

Histologic analysis of the specimens was performed at the center of the sclerotomy site, as indicated by the location of the iridectomy.

Inflammatory cell infiltration was obviously lower in the ALA and control groups compared with the MMCG (Fig. 2). With respect to lymphocyte scores, there was a statistically significant difference between the MMC and ALA groups and between the MMC and control groups ($P < 0.05$). There was no statistically significant difference between ALA and control group scores ($P > 0.05$). There were no statistically significant differences among all groups when plasmocyte and lymphoid aggregate scores were evaluated. With respect to polymorphonuclear leukocyte scores, there was a statistically significant difference between the ALA and MMC groups and the ALA and control group scores ($P < 0.05$); there was no statistically significant difference between the MMC and control group scores ($P > 0.05$). With respect to vascular structure scores, there was statistically significant difference between the control and ALA groups, and the control and MMC groups ($P < 0.05$); there was no statistically significant difference between the ALA and MMC group scores ($P > 0.05$) (Table 1).

In the control group, mild histologic changes due to surgical trauma were observed in the conjunctiva and in the ciliary body. The conjunctival epithelium was preserved with mild inflammatory reaction, cell infiltration, and minimal thickening. The height of the epithelium of the ciliary body was normal. There was minimal fibroblast proliferation, congestion, and edema in the ciliary body. In the ALA group, minimal histologic changes were observed in the conjunctiva. The height of the conjunctival epithelium was normal, with minimal fibroblast proliferation, congestion, and edema. In the ciliary body, the height of the epithelium was normal, with minimal fibroblast proliferation, congestion, and edema. In the MMC group, the histologic changes observed in the conjunctiva were a decrease in the collagen density of the conjunctival epithelium, the loss of organization in the collagen fibrils, the slowing of mature collagen formation, epithelial cell loss, and increased inflammatory cell infiltration. The histologic changes observed in the ciliary body were a decrease in the height of pigmented and nonpigmented epithelium, the change of the cuboidal epithelium into a columnar epithelium, the accumulation of fibrin, and the disorganization of collagen fibers, with congestion and edema. When conjunctival and ciliary body toxicity scores were evaluated,
there was statistically significant difference among all the groups (ALA < control < MMC) (Table 1).

Masson trichrome staining showed a shortened, focal perpendicular sequence of collagen fibers in the CG, organized collagen fibers in the ALA group, and collagen fibers that were much looser and pointed in all directions, hypocellular connective tissue, and rare blue collagen deposition in the MMC group (Fig. 3). When Masson trichrome collagen organization scores were evaluated, there were statistically significant differences among all the groups (ALA < MMC < control) (Table 1).

When α-SMA staining scores (H-scores) were evaluated, there were statistically significant differences among all groups (MMC < ALA < control). ALA significantly reduced the population of cells expressing α-SMA, indicating less fibroblast differentiation into the myofibroblast phenotype ($P < 0.05$) when compared with the CG (Fig. 4) (Table 1).

FIGURE 3. Tissues stained with Masson trichrome on day 28. Tissues shown in (A and B) are from the control group, (C and D) from the ALA group, and (E and F) from the MMC group. In the control group: moderate collagen disorganization/shortened, focal perpendicular sequence of collagen fibers. In the ALA group: weak collagen disorganization/long, discrete collagen fibers, and in the MMC group: strong collagen disorganization/shortened and pointing in all directions; collagen fibers can be seen. ALA indicates α-lipoic acid; C, cornea; CB, ciliary body; CJ, conjunctiva; MMC, mitomycin-C; SS, sclerostomy site: moderate disorganization of collagen fibers. ★: strong disorganization of collagen fibers.

FIGURE 4. Immunohistochemical staining of filtration blebs on day 28. Tissues shown in (A and B) are from the control group, (C and D) is the ALA group and (E and F) is the MMC group. There was a reduction in the number of α-SMA-expressing cells in ALA(C-D) and MMC (E and F) groups compared with the control (A and B) group. The brown color stained the myofibroblasts (activated collagen secreting α-SMA+ fibroblast). ALA indicates α-lipoic acid; MMC, mitomycin-C; SMA, smooth muscle actin.
DISCUSSION

Trabeculectomy is a filtrating procedure. Its long-term efficiency is entirely dependent on the permanency of the surgically created fistula. It is unanimously agreed that fistula obstruction arises from an abnormal subconjunctival tissue regeneration process. Many procedures have been developed to inhibit unwanted fibrotic activity in the trabeculectomy area, using different pharmacological agents with variable success. Among these, the most commonly used has been MMC perioperatively applied. However, its use has been reported to be associated with a high complication rate.

In this study we investigated inhibitory effect of topically applied ALA on inflammation and proliferation; and we observed that it can be used as an agent to reduce scar tissue formation in trabeculectomy surgery and can provide filtering bleb patency, which increases the success of the surgery.

To the best of our knowledge, this is the first experimental study in which topical ALA has been used in rabbit eyes undergoing trabeculectomy to prevent scar tissue formation at the trabeculectomy area, and in which trepan blue dye has been used to evaluate filtering bleb patency.

Tissue healing is a very complex biological mechanism. It involves inflammation, inducing fibroblast proliferation, and revascularization as 2 main components. Angiogenesis is a key element of the proliferative phase of healing, supplying oxygen and nutrients to support the rapid growth of the cells and mediating repair. Fibroblasts play the most important role in scar formation. When activated, they transform into myofibroblasts (activated collagen secreting α-SMA+ fibroblast). Chronic inflammation and repair can trigger excessive accumulation of ECM components, which leads to the formation of fibrotic scarring. During this period, profibrotic cytokines and growth factors, such as transforming growth factor-β (TGF-β), IL-13 and platelet-derived growth factor play a critical role. Some chemokines/cytokines and their functions on tissue healing are shown in Table 2.

MMC is an antibiotic agent with antiproliferative properties and is classified as a cell cycle nonspecific alkyating agent. Thus, it inhibits both DNA replication and protein synthesis. MMC causes DNA damage directly by inhibiting both DNA replication and also decreases IL-1 and TNF-α concentration in the aqueous humor. Studies have shown that ALA decreases TGF-β concentration in the aqueous humor increases. Studies have shown that ALA decreases TGF-β, IL-6, and TNF-α levels. Topically applied ALA, which is used as a drop, penetrates to the surgical area of topically applied ALA. In our study, less inflammatory cells infiltration and lower collagen fiber density were observed; the collagen fibers were better organized and the number of α-SMA-expressing cells was lower than for the control group. When the filtering bleb

| Chemokines/Cytokines | Function in Tissue Healing | Effect of ALA |
|----------------------|---------------------------|--------------|
| Transforming growth factor-β (TGF-β) | Inhibition of matrix protein removal<sup>31</sup> Stimulation of the expression of protease inhibitors including plasminogen activator inhibitor-1 (PAI-1)<sup>32</sup> | Inhibition of TGF-β |
| Plasminogen activator inhibitor-1 | Accumulation of ECM | Decreases TGF-β-induced PAI-1 expression<sup>36</sup> |
| Reactive oxygen species (ROS) | Inhibition of fibrinolysis | Inhibition of ROS-induced endothelial injury<sup>37</sup> |
| Interleukin-6 and interleukin-17 | Inhibition of the deposition of ECM<sup>34</sup> Regulation of the expression of other important cytokines such as interleukin-1 and TNF-α<sup>35</sup> | Decreases serum Interleukin-6 and interleukin-17 levels. Prevents upregulation of ICAM-1 and VCAM-1<sup>38</sup> |
| Adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule (VCAM-1) | Key roles on angiogenesis | |

ALA indicates α-lipoic acid; ECM, extracellular matrix.
patency was evaluated using 0.1% trepan blue dye, it was observed that 2 (33%) blebs had failed in the ALAG on 28th day. This result did not reach statistically significance when compared with the control group, but in our opinion, and as Whitley et al.\(^{47}\) and Petrie et al.\(^{48}\) defined, a result can be of clinical significance but not statistical significance because of a small number of subjects.

All the above-mentioned studies and our findings indicate that ALA may be able to prevent and/or reduce fibrosis in the human body, by inhibiting the pathways of inflammation, revascularization, and the accumulation of ECM.

In the literature it has been reported that topically instilled trehalose onto eyes after simple conjunctival incision or trabeculectomy,\(^{49}\) intraoperative subconjunctival implantation of a sustained-release dexamethasone implant,\(^{50}\) at a concentration of 0.2 mg/mL, or postoperative subconjunctival injections of Ilomastat\(^{17}\) and postoperative use of 0.5% pirfenidone eye drops\(^{16}\) prolongs bleb survival and may also act as a potential adjuvant treatment when MMC alone is inadequate.

The weaknesses of our study are as follows: we determined the doses of ALA as 5 x 1 d independently, due to the lack of reference to the use of ALA as drops in the literature. We used hematoxylin and eosin staining for the identification of inflammatory and endothelial cells in our study but instead we could have used specific immune staining. Only the bleb function was evaluated in our study; a more detailed evaluation could be done by adding the bleb area/calculated volume and bleb vascularity; to the study.

In conclusion, this study shows that peroperatively and postoperatively topically applied ALA may regulate scar tissue formation at the site of trabeculectomy by blocking collagen synthesis, delays tissue regeneration and provides a more functional and permanent fistula in rabbit eyes. ALA can be used as a potential adjuvant agent when MMC alone is inadequate. Clinical studies are needed to investigate the safety of the topical application of ALA in human eyes, and its long-term efficacy in reducing scar tissue formation in the postoperative period of trabeculectomy.

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