TOPICAL THERAPEUTIC STRATEGIES FOR THE TREATMENT OF CORNEAL FIBROSIS IN VETERINARY SPECIES

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By
ALLISON FUCHS
Dr. Elizabeth A. Giuliano, Thesis Supervisor
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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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Presented by Allison Fuchs,

A candidate for the degree of Master of Science,

And hereby certify that, in their opinion, it is worthy of acceptance.

Dr. Elizabeth Giuliano

Dr. Rajiv Mohan

Dr. Mahesh Thakkar

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LIST OF ABBREVIATIONS

1. ECM: Extracellular matrix
2. MMPs: Matrix metalloproteinases
3. TGF-β1: Transforming growth factor-beta-1
4. VEGF: Vascular endothelial growth factor
5. TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling
6. MMC: Mitomycin C
7. SAHA: Suberanilohydroxamic acid
8. HDACi: Histone deacetylase inhibitor
9. EGF: Epidermal growth factor
10. HCF: Human conjunctival fibroblast
11. αSMA: Alpha-smooth muscle actin
12. A2-P: L-ascorbic acid-2-phosphate
13. BSS: Balanced salt solution
14. mMs: Modified McDonald-Shadduck
15. IOP: Intraocular pressure
16. H&E: Hematoxylin-eosin
17. DAPI: 4',6-Diamidino-2-Phenylindole
18. qRT-PCR: Quantitative real-time polymerase chain reaction
19. ANOVA: Analysis of variance
20. CCT: Central corneal thickness

ABSTRACT

Purpose: To determine the safety and efficacy of a novel combination of TRAM-34 and ascorbic acid applied topically *in vivo* in naïve and alkali injured rabbit corneas.

Methods: Twelve New Zealand rabbits were randomly assigned into 2 groups (6 rabbits/group). Naïve healthy eyes were treated OD BIDx5 days with either treatment (combination TRAM-34 25μM (Tocris Biosciences, Bristol, UK) and ascorbic acid 10% (TVC)) or control (BSS). Rabbits underwent an axial corneal wound OS using an established model. Groups were treated OS BIDx5 days. Degree of corneal opacity, ocular health, safety, and efficacy were determined utilizing the Fantes grading scale and modified McDonald-Shadduck (mMS) scoring system. Immunohistochemical and microscopy techniques evaluated corneal fibrotic markers at study conclusion (day 28).

Results: Combination therapy was well tolerated in all eyes, with no significant differences in mMS scores, IOP, or central corneal thickness (CCT) between treatment and control groups. Significant differences in mMS scores between groups were found at day 1 ($p=0.0001$), 2 ($p=0.0001$), 3 ($p=0.0001$), 4 ($p=0.0285$), 14 ($p=0.0041$) and 28 ($p=0.0002$). Significant differences in Fantes scores were detected between groups at day 7 ($p=0.001$), 14 ($p=0.0027$), and 28 ($p=0.0001$). Significant differences in CCT between groups were found at days 7 ($p=0.036$), 14 ($p=0.0495$), and 28 ($p=0.0487$). Laboratory testing of corneal tissues demonstrated decreased fibrosis in treatment versus control groups at day 28.

Conclusions: Novel bi-modal TVC topical therapy was well tolerated and demonstrated improved corneal wound healing and reduction in fibrotic changes in TVC treated rabbits compared to controls.

CHAPTER 1

INTRODUCTION

Corneal fibrosis: comparative ophthalmology at its finest

Corneal haze or fibrosis is a common sequela to corneal injury, infection, and ocular surgery in all mammalian species. Corneal disease is often perpetuated through secondary infection of either bacterial or fungal origin. The long-term sequelae of corneal damage is frequently significant permanent visual loss [1-5]. This can have a major impact on quality of life for both human and veterinary patients. People with corneal scarring often suffer major limitations to their daily life. In our veterinary species, corneal fibrosis is a significant and commonly encountered problem, whereby an animal's vision is severely damaged by corneal fibrosis secondary to corneal trauma or infection. This visual impairment results in decreased performance, especially critical in our working, service, and performance animals (police and military dogs, agility performance dogs). In our large animal patients, vision loss can necessitate the animal being retired from their function. Finally, visual impairment presents a danger to both the animal and the owners/handlers particularly in large animal patients.

Corneal wound healing and major players

Corneal wound healing is a complex process involving numerous cytokines, activation of keratocytes, transdifferentiation of fibroblasts to myofibroblasts, angiogenesis, and increased extracellular matrix (ECM) deposition, increased secretion of matrix metallic proteinases (MMPs), and altered gene expression of genes such as the Smad family [3, 5-7]. The cytokine transforming growth factor beta (TGFβ) has been shown to play a major role in the formation of corneal fibrosis via activation of fibroblasts and differentiation of activated fibroblasts to

myofibroblasts [6, 8, 9]. It is well established that the persistence of opaque myofibroblasts in the corneal stroma leads to corneal haze and stromal opacity, and that a reduction in myofibroblasts correlates with improved corneal transparency following corneal injury [5, 6, 10, 11]. Other causes of corneal opacity depending on the underlying etiology may include deposition of lipid and mineral in the cornea due to chronic inflammation and abnormal corneal vascularization, which may leak lipid and amyloid [12-15].

Animal models of corneal wound healing

A variety of models have been utilized to study corneal wound healing in a laboratory setting, including *in vivo* cell cultures of corneal cells, *ex vivo* corneal wound models, and *in vitro* models in several species. Mice have been commonly used due to availability of reagents for many common corneal markers [16-18]. Rabbits have become a commonly used laboratory animal in recent years. It is important to note that they are not a perfect animal model correlate, as rabbits are able to regenerate corneal endothelial cells to an extent, while humans have no regenerative endothelial cell capabilities [19]. For certain types of ocular injury, rabbits are more resistant than humans. However previous studies have established normalized doses and a similar progression of injury as observed in exposed humans for some types of corneal injury, such as sulfur mustard toxicity [12, 13, 20, 21]. Rabbits have anatomical and physiological features that make them ideal for ocular toxicology research however, including large cornea to sclera ratio, and a greater similarity to human eyes than other laboratory animals such as mice or rats [13].

TRAM-34 and ascorbic acid and the eye

Tram-34 (1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole) is a selective inhibitor of intermediate-conductance calmodulin/calcium-activated K+ channels ($K_{Ca}3.1$) [22].

These channels are expressed in mitochondrial and cytoplasmic membranes and are known to assist with regulation of cell cycle progression and proliferation [23]. $K_{Ca}3.1$ channels are upregulated in response to injury [24-26]. Previous studies have shown activation of $K_{Ca}3.1$ to be important in the development of fibrosis in various organ systems such as the lung, liver, and kidney [26-28]. Recently, the peer reviewed literature has demonstrated the role of $K_{Ca}3.1$ in corneal cell proliferation and its importance in corneal fibrosis [24]. *In vitro* corneal cell culture experimentation has demonstrated that $K_{Ca}3.1$ mediates the TGFβ-1 induced proliferation and differentiation of fibroblasts to myofibroblasts [24, 25]. Inhibition of $K_{Ca}3.1$ by TRAM-34 downregulates these processes, and thus may present a therapeutic target for treatment and prevention of corneal fibrosis [29].

Ascorbic acid is abundant in the corneal epithelium of various species and has antioxidant properties as well as protective effects in corneal disease [30]. Application of topical ascorbic acid has been associated with improved corneal epithelial wound healing *in vivo* and is thought to aid in reconstruction of epithelial basement membranes as well as upregulating corneal epithelial stem cell formation [31]. Studies in rabbits *in vivo* have shown reduction of corneal neovascularization with the use of topical ascorbic acid via reduction of vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP9) [32].

CHAPTER 2

LITERATURE REVIEW

The majority of work on corneal fibrosis (haze) has been focused on transformation of normal clear corneal collagen from fibroblasts to myofibroblasts via the activation of TGFβ. An early change that occurs when there is injury to the corneal stroma is apoptosis of damaged keratocytes [3, 5, 33, 34]. This keratocyte apoptosis indicative of stromal damage can be detected with the TUNEL (terminal deoxynucleotidyl transferase-mediated dTUP nick end labeling) assay, as well as transmission electron microscopy [35, 36]. Release of cytokines leads to activation of surrounding keratocytes and induces migration of bone-marrow derived cells from the limbal blood vessels, which both generate myofibroblasts [5, 11, 35-37].

Corneal myofibroblast development has been recognized as being modulated by TGFβ both *in vitro* and *in vivo* for many years [38, 39]. It is important for corneal wound healing due to induction of cellular proliferation and differentiation, extracellular matrix production, and immune modulation, which are critical to stromal replacement when there is a corneal wound [5, 11, 37, 40-42]. Modulation of these effects, however, helps prevent fibrosis and the subsequent effects on vision [1]. The effects of TGF-β1 are facilitated via various intracellular signaling pathways including the Smad-dependent intracellular signaling pathways [9]. The Smad protein family is classified based on function and is composed of three categories including receptor-regulated Smads (R-Smads), common mediator Smads (co-Smads), and inhibitor Smads (I-Smads) [7, 9, 43].

Classically, mitomycin C (MMC) has been the drug of choice for the prevention of fibrosis formation after surgical insult to the cornea as well as in conjunctival bleb formation after glaucoma surgery. While MMC is effective in prevention of postoperative corneal haze in

many patients, its safety profile is controversial [44, 45]. MMC treatment causes apoptosis, reduced proliferation, and sub-epithelial stromal loss [44, 45].

Suberanilohydroxamic acid (SAHA) is a histone deacetylase inhibitor (HDACi), which has been investigated in multiple studies for its ability to block TGF-b signal transduction and thereby reduce corneal fibrosis. SAHA is an FDA approved drug under the name Vorinostat for the treatment of cutaneous T-cell lymphoma in humans [43].

TRAM-34 was designed as an immunosuppressant medication with selective blockade of the intermediate-conductance calmodulin/calcium-activated K+ channels ($K_{Ca}3.1$) [22]. It is in the same family as the antimycotic Clotrimazole, which also has efficacy as an immunosuppressant due to non-selective channel-blocking activity, but comes with significant and limiting potential for side effects on the GI tract, urinary tract, hepatic enzyme elevations, and elevations in plasma cortisol due to induction of cytochrome P450 in human patients [22, 46]. TRAM-34 was synthesized via the same route as clotrimazole with modifications that allow it to specifically target the $K_{Ca}3.1$ channel [22, 46]. Although initially developed to target the channels in T-lymphocytes, TRAM-34 has subsequently been discovered to have efficacy in numerous tissues that express $K_{Ca}3.1$, including brain microglia, vascular endothelium, liver, lung, and now ocular tissues [24-29, 46-50].

One study investigating TRAM-34 for use in pulmonary fibrosis has demonstrated that the $K_{Ca}3.1$ channel modulates TGFβ-1-dependent myofibroblast differentiation in this disease [28]. Roach *et al* demonstrated that TRAM-34 successfully blocked TGFβ-1-dependent α-SMA protein expression, and thus prevented myofibroblast formation. Similar results were found in a study by Freise *et al* examining the use of TRAM-34 for liver fibrosis [26]. In the aforementioned study, TRAM-34 application *in vitro* effectively reduced TGFβ-1-induced

activation of collagen-1, α-SMA, and TGFβ, and *in vivo* reduced fibrosis-induced portal perfusion pressure [26].

Due to the wide distribution of $K_{Ca}3.1$ in tissues and the apparent efficacy of TRAM-34 in selective blocking of these channels leading to reduction of fibrosis in multiple tissues, investigation of safety and efficacy in ocular tissues was a logical progression. One early study on TRAM-34 in the cornea actually examined its effect on angiogenesis due the upregulation of $K_{Ca}3.1$ channels induced by epidermal growth factor (EGF), which is another mediator of corneal wound healing [29, 51]. In this publication by Yang *et al*, TRAM-34 was used both *in vitro* and *in vivo* to alkali wounded mouse corneas. Results indicated that TRAM-34 as successful in suppressing EGF-induced corneal angiogenesis without delaying corneal wound healing [29]. The aforementioned study did not examine markers of fibrosis.

Our laboratory has performed two studies evaluating the role of the calcium-activated potassium channel (KCa3.1) in the development of ocular fibrosis and its potential as a target for therapy [24, 25]. In the *in vitro* portions of these studies, KCa3.1 was shown to be expressed in human corneal epithelium, stroma, and endothelium, as well as in human conjunctival cell cultures. The first study examined the use of TRAM-34 for conjunctival fibrosis in primary human conjunctival fibroblast (HCF) cultures and treated the HCFs with TRAM-34 at doses of 0, 1, 5, 10, 25, or 50 μM for up to 7 days to evaluate for cellular toxicity [25]. There was a moderate decrease in cellular viability at the 50μM dose based on trypan blue exclusion assay, but the 25μM and lower doses were well tolerated [25].

In a second study performed by our group, *in vivo* studies on wild type and KCa3.1-/- mice demonstrated a reduction in markers of corneal fibrosis including alpha-smooth muscle actin (α-SMA), collagen I, collagen IV and TGFβ1 in KCa3.1-/- mice. These deficient mice also

had a decreased number of α-SMA positive cells after alkali injury and a reduction in corneal fibrosis clinically, suggesting that the loss of KCa3.1 channels inhibits corneal fibrosis and may represent a therapeutic target [24]. In the same publication, TRAM-34 was administered to human corneal fibroblasts at a dose of 25 μM for 24 and 72 hours with only a moderate decrease in cell viability, and a demonstrably lower expression of pro-fibrotic genes mainly attributed to inhibition of TGFβ1 [24].

Several previous studies have been published on the use of 10% ascorbic acid *in vivo* and its safety and efficacy in treatment of corneal disease [30-32, 52]. One such study evaluated the efficacy of ascorbic acid at concentrations of 10mg/mL, 1mg/mL, and 0.5mg/mL applied topically twice daily for 1 week after induction of corneal neovascularization via surgical placement of a stromal suture in rabbits [32]. Evaluation of surface area affected by neovascularization showed that there was a significantly lower ratio of cornea with neovascularization at the 10mg/mL concentration of ascorbic acid compared to the control and other treatment groups. Two markers of angiogenesis, VEGF and matrix metalloproteinase-9 (MMP-9) were also quantified in the rabbit corneas. The treatment groups showed significantly lower concentrations of both VEGF and MMP-9. In all groups, treatment was well tolerated. This data suggests that a 10mg/mL solution of ascorbic acid is the appropriate concentration to achieve the desired clinical result [32]. A recent study used an *in vivo* mouse model of corneal epithelial scraping, with subsequent application of a single dose of topical 10% ascorbic acid versus saline in the control group [31]. Their data showed a significantly smaller epithelial defect area in the treatment group compared to the control at 48- and 72-hours post-debridement [31]. The aforementioned study also examined cultured mouse corneal epithelial cells for markers of stemness after exposure to L-ascorbic acid-2-phosphate (A2-P), a stable ascorbic acid derivative

[31]. Cultured corneal epithelial cells treated with A2-P showed increases in markers of stemness including p63, ABCG2, and pluripotent stem cell markers SOX2 and OCT4 [31].

CHAPTER 3

EXPERIMENTAL PURPOSE AND HYPOTHESIS

A wide variety of topical therapeutics have been studied for the treatment of corneal fibrosis in veterinary and physician ophthalmology. Many of these therapeutic strategies including gene therapy, mitomycin C, pirfenidone, vorinostat, and others have demonstrated that reduction of TGFβ will reduce myofibroblast formation, thus inhibiting corneal fibrosis [1, 43, 53-61]. However, some of these therapeutic modalities can have negative side effects and very few are approved for clinical use at present [44]. Both TRAM-34 and ascorbic acid have previously been shown to be safe and well tolerated when administered to the cornea topically with minimal effects or cellular toxicity [24, 25, 31, 32, 52, 62]. Previous studies have elucidated some of the mechanisms of action of both TRAM-34 and ascorbic acid on their corneal wound healing and anti-fibrotic pathways [24, 25, 30, 62]. The aims of this study were first to establish the ocular tolerability of a novel combination formulation of TRAM-34/ascorbic acid when applied topically to normal rabbit corneas *in vivo*; and second, to determine the efficacy of TRAM-34/ascorbic acid in the prevention of corneal fibrosis and neovascularization in an *in vivo* rabbit corneal injury model.

CHAPTER 4

EVALUATION OF A NOVEL COMBINATION OF TRAM-34 AND ASCORBIC ACID FOR THE TREATMENT OF CORNEAL FIBROSIS *IN VIVO*

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[1] Fuchs, AL., Balne, PK., Giuliano, EA., Sinha, NR., Mohan, RR. Evaluation of a novel combination of TRAM-34 and ascorbic acid for the treatment of corneal fibrosis *in vivo.* PLoS One. 2022;17(1):e0262046.

Materials and methods

Animals

Twelve healthy 2 to 3-month-old female New Zealand white rabbits (Charles River Laboratory Inc., Wilmington, MA) weighing 2.5 to 3 kg were utilized for this study. All studies were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research and were approved by the University of Missouri Institutional Animal Care and Use Committee. Following the 3R rule (reduce, replace, and refine) animal rule to keep the number of animals in experiments as low as possible, both eyes of 12 rabbits were utilized. Twenty-four eyes were divided into 4 groups. Group-1 (left eyes: injury cohort) received alkali (n = 6). Group-2 (right eyes: naïve cohort) received BSS onto the normal eye (n = 6). Group-3 (left eyes: therapy cohort) received alkali injury and eyedrop twice daily for 5 days (n = 6). Group-4 (right eyes; safety cohort) received eyedrop twice daily onto the na.ve eye for 5 days (n = 6). All rabbits underwent a complete ophthalmic examination by an American Board of Veterinary Ophthalmology approved senior ophthalmology resident prior to onset of the study, including slit lamp biomicroscopy (SL-15 Kowa Company, Ltd, Tokyo, Japan), indirect ophthalmoscopy (Wireless indirect ophthalmoscope, Keeler Instruments Inc., Broomall, PA, USA and pan retinal 2.2 indirect lens, Volk Optical Inc., Mentor, OH, USA). All rabbits were determined to be free of ocular disease.

In vivo corneal wound model

Using an established corneal wound model, corneal alkali wounding was induced in the left eye of each rabbit with the contralateral eye serving as a naïve control [63]. After initial clinical examinations and extraocular imaging, rabbits were anesthetized by intramuscular

injection of ketamine hydrochloride 50mg/kg (MWI, Boise, ID) and xylazine hydrochloride 10mg/kg (Akorn, Lake Forest IL). Briefly, proparacaine hydrochloride 0.5% (Alcon, Fort Worth, TX) was topically applied to the cornea and a wire eyelid speculum was placed. A 7mm-diameter filter paper was soaked in 0.5N sodium hydroxide (NaOH) solution and then applied onto the axial cornea for 30 seconds while visualized under a surgical microscope (Leica Wild Microscope MEL53; Leica, Wetzlar, Germany). Following removal of the filter paper, the wounded cornea was immediately and copiously rinsed with sterile balanced salt solution (BSS) to remove residual alkali solution. Fluorescein stain (Flu-Glo, Akorn, Inc., Buffalo Grove, IL, USA) was applied to verify corneal burns.

Tram-34/Ascorbic Acid Preparation and Treatment

The combination drop was prepared by solubilizing TRAM-34 (25μM) and ascorbic acid (10%) in BSS and adjusting the pH using either hydrochloric acid or sodium hydrochloride (NaOH) to achieve a pH approaching 6.4 as assessed by an electronic pH meter at room temperature under sterile conditions

Corneal Health and Corneal Haze Analysis

Corneal health was evaluated prior to study initiation and at regular intervals throughout the study period. Using slit-lamp biomicroscopy, ocular health was graded according to the modified McDonald-Shadduck (mMs) scoring system [64] and corneas were imaged using a slit-lamp biomicroscope fitted with a digital imaging system (Kowa, portable Vk-2 Version 5.5) as previously described, as well as a stereomicroscope (Leica MZ16F, Leica Microsystems Inc., Buffalo Grove, IL) equipped with a digital camera (SpotCam RT KE, Diagnostic Instruments Inc., Sterling Heights, MI) [61, 65]. Scoring was performed daily for the first 5 days, then at days 7, 14, and 28. Ophthalmic testing including Schirmer tear testing (Fischer Scientific, Pittsburgh,

PA, USA), fluorescein staining, applanation intraocular pressure (IOP) measurements (Tono-Pen AVIA, Reichert Technologies, Depew, NY, USA), pachymetry (Accutome AccuPach VI, Keeler Instruments Inc., Broomall, PA, USA), and extraocular imaging was performed at days 0, 3, 7, 14, and 28.

Corneal haze scoring was performed according to the established Fantes grading scale [40]. Haze was scored by three independent examiners (AAF, PKB, SK) masked to the treatment group. In summary, grade 0 is a clear cornea; grade 0.5 is considered clear with trace haze on tangential illumination; grade 1 is minimal haze on direct or diffuse illumination; grade 2 is mild haze easily visible on direct focal slit lamp examination; grade 3 is moderate opacity which partially obscures iris detail; grade 4 is severe opacity that completely obscures iris details at the site of corneal injury.

Intraocular Pressure Measurement

Intraocular pressure recordings were measured at day 0, 3, 7, 14, and 28 using an applanation tonometer. All measurements were performed under general anesthesia after application of topical anesthetic with the rabbits in lateral recumbency and the eye being measured facing up. All IOP measurements were performed between 9am and 11am to minimize diurnal variations.

Euthanasia and Tissue Collection

Rabbits were humanely euthanized with intravenous pentobarbital 150mg/kg (SomnaSol, Henry Schein Animal Health, Dublin, OH, USA) while under general anesthesia on day 28 post-injury. Corneas were harvested and halved using sharp dissection. One half of the corneal sections were immediately placed in 24x24x5 mm molds (Fischer Scientific, Pittsburgh, PA, USA) containing optical cutting temperature compound (Tissue Plus O.C.T., Fisher HealthCare,

Houston, TX, USA) and snap frozen. Frozen tissue blocks were maintained at -80°C until further processing. The remaining half of the corneal section was placed in a pre-labeled cryo-vial and stored immediately at -80°C until further processing for gene expression studies.

Histopathology and Immunofluorescence Studies

Serial corneal sections (8 μm) were prepared from cryo-preserved corneal tissues using a cryostat (HM525 NX UV; Microm GmbH, Walldorf, Germany), placed on labeled glass microscope slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA, USA), and stored at −80°C until staining. Hematoxylin-eosin (H&E) staining was performed as previously described for histopathologic examination [2, 66]. Tissue sections for immunofluorescence studies were immunostained for α-SMA using established methods to evaluate for the presence of myofibroblasts in tested corneal tissue [67, 68]. In brief, tissue sections were incubated at room temperature for 30 minutes with 2% bovine serum albumin and then probed with mouse monoclonal anti-α-SMA antibody (1:200 dilution, M0851; Dako, Carpentaria, CA, USA), and incubated at room temperature for 60 min, followed by overnight incubation at 4 ^{o}C.
The sections were then incubated with Alexa-Fluor 488 goat anti-mouse IgG secondary antibody (1:1000 dilution, A11001; Invitrogen, Carlsbad, CA, USA) for 1 hour in darkness at room temperature. Antifade Mounting Medium containing 4',6-Diamidino-2-Phenylindole (DAPI) (H1200, Vector Laboratories, Inc. Burlingame, CA, USA) was used to stain the nucleus and mount corneal sections. The stained corneal sections were imaged using a fluorescence microscope (Leica) and the α-SMA positive cells were quantified in six randomly selected, non-overlapping full thickness central corneal columns, extending from anterior to posterior stromal surfaces.

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

Total RNA was extracted from tissues using the RNeasy kit (Qiagen, Valencia, CA), according to the manufacturer's protocol and stored at -80ºC until analysis. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using One Step Plus Real-Time PCR system (Applied Biosystems, Carlsbad, CA) according to manufacturer's instructions as previously described [24, 25, 68]. This reaction mixture was run at universal cycle (95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 60 s) following manufacturer's instructions. GAPDH was used as a housekeeping gene for α-SMA, fibronectin, and collagen-3, with the primer sequences described in Table 1.

The fluorescence threshold value (Ct) was calculated to detect signal differences in association with an exponential increase of PCR products in the log linear phase. Relative expression/fold change over the corresponding values for the control was calculated by the 2-ΔΔCt method. Two to three independent experiments were executed, each sample was run in triplicate, and the average fold changes in mRNA levels were calculated.

Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using a commercially available software (GraphPad Prism 6.0, GraphPad Software, La Jolla, CA, USA). Kolmogorov-Smirnov's test was used to determine whether data are normally distributed. Data that were not normally distributed were transformed using the natural log function. Unpaired t-test was performed for α-SMA qPCR safety study. A one-way or two-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test was performed for clinical scoring and tissue processing data, respectively. Results were considered significant at $p \leq 0.05$.

Results

In vivo safety and toxicity

Unwounded rabbit eyes of the Group-2 (na.ve cohort) and Group-4 (safety cohort) had mMs scores of zero throughout the study, indicating no ocular irritation (Fig 1). Additionally, Group-2 (naïve cohort) and Group-4 (safety cohort) eyes showed no significant differences in the central corneal thickness (CCT) (Fig 2A) or IOP (Fig 2B) as well as any fluorescein uptake (data not shown) at all tested times in the study. Also, molecular analysis of fibrotic marker, α-SMA, with qRT-PCR did not find significant differences in the corneas of the Group-2 (naïve cohort) and Group-4 (safety cohort) (Fig 3). The comparisons of mMs scores of the wounded eyes with eyedrop (Group-3: therapy cohort) versus without eyedrop (Group-1: injury cohort) exhibited significantly lower mMs scores on day-14 ($p = 0.0041$) and day-28 ($p = 0.0002$) (Fig 1). The IOP and CCT analysis of these two groups are shown in Fig 5A and 5B, respectively.

Corneal morphology

H&E staining of unwounded eyes of Group-2 (naïve cohort) and Group-4 (safety cohort) showed no morphological differences in corneal tissues (Fig 4A and 4B). Conversely, H&E stained corneal tissues of wounded eyes of Group-1 (injury cohort) that had no eyedrop demonstrated re-epithelialization with keratinization of the epithelial layer, as well as stromal edema and disorganization of the collagen layers within the stroma (Fig 4C). By contrast, H&E stained wounded corneal tissues of Group-3 (therapy cohort) that received eyedrop showed return of organized stromal collagen fibrils with minimal to no corneal edema, as well as return to normal full thickness epithelium (Fig 4D).

In vivo efficacy

Corneal wounding and ocular health evaluations were performed to evaluate the efficacy of the eyedrop. All employed corneas were healthy prior to the initiation of the study. Following

wounding, all corneas developed significant opacity consistent with severe edema and inflammation and were fluorescein stain positive (data not shown). Corneal wounds had epithelialized in all rabbits by day 5 of the study without complication from infection or self-trauma based on negative fluorescein staining and slit-lamp clinical examination. Likewise, IOP between Group-1 (injury cohort) and Group-3 (therapy cohort) eyes was not significantly different during the study (Fig 5A). Comparison of CCT in Groups 1 and 3 was also performed. The eyedrop treated Group-3 eyes showed significantly decreased CCT at days 7, 14, and 28 ($p<0.05$) than the non-eyedrop treated Group-1 eyes (Fig 5B).

In vivo reduction of fibrosis

Rabbit eyes that received eyedrop after injury (Group 3: therapy cohort) demonstrated a markedly increased transparency based on significant differences in Fantes scores compared to untreated injured corneas (Group 1: injury cohort) at days—7 ($p = 0.001$), 14 ($p = 0.0027$), and 28 ($p = 0.0001$) (Fig 6). Slit lamp (Fig 7A) and stereomicroscopic images (Fig 7B) revealed marked differences in corneal haze between injured eyes receiving eyedrop (Group-3: therapy cohort) and the untreated injured eyes (Group-1: injury cohort).

Pro-fibrotic gene expression and immunohistochemistry

Alkali wounded eyes (Group-1: injury cohort) demonstrated significant upregulation of profibrotic markers compared to na.ve corneas (Group-2: naïve cohort) via qRT-PCR, including α- SMA ($p<0.0001$), collagen-3 ($p = 0.011$), and fibronectin ($p<0.0001$) as shown in Fig 8. The corneas of wounded rabbit eyes that received eyedrop (Group-3) showed significantly decreased ($p<0.0001$) expression of α-SMA at day 28 compared to the non-treated corneas (Group-1) (Fig 8A). A similar trend was observed for other tested profibrotic markers collagen-3 (Group-3 vs Group-1, $p = 0.0482$; Fig 8B) and fibronectin (Group-3 vs Group-1, $p<0.0001$; Fig 8C). To test if

the eyedrop affects expression of profibrotic α-SMA protein, immunofluorescence staining was performed. A significant increase in α-SMA-positive cells ($p = <0.0001$) in Group-1 wounded corneas (Fig 9B) compared to the Group-2 naïve corneas (Fig 9A) was observed. The eyedrop-treated corneas of Group-3 (Fig 9C) showed significantly reduced α-SMA+ cells compared to untreated injured corneas of Group-1 ($p<0.0001$). The quantification of α-SMA+ cells in groups 1–3 is shown in Fig 9D.

Discussion

Corneal wound healing entails a complex cascade of interrelated signaling pathways and cytokines. Formation of corneal haze has been shown to be a result of normal corneal healing pathways which activate and transform fibroblasts to myofibroblasts and induce ingrowth of blood vessels [5, 11, 37, 69]. Our laboratory and others have demonstrated that corneal clarity can be preserved with inhibition of profibrotic and angiogenic factors during the wound healing process, in particular TGFβ and VEGF [1, 4, 24, 32, 43, 44, 53-56, 67, 70, 71]. Our study has demonstrated that the combination of TRAM-34 and ascorbic acid, when applied topically to wounded rabbit corneas, significantly decreases corneal haze *in vivo*. This was proven by reduction in clinical parameters including Fantes scores, mMs scores, and CCT. Testing for reduction of fibrotic markers collagen-III, fibronectin, and α-SMA also all demonstrated statistically significant reductions in the presence of these markers. The treatment was well tolerated in unwounded control eyes with no evidence of deleterious clinical side effects based on mMs scoring, CCT, and IOP. Twice daily dosing was used in this study to good effect, but it is possible that treatment intervals in higher mammals (dogs, cats, horses, people) may require more frequent application due to differences in tear film and blink rates.

TRAM-34 was developed as a possible therapeutic alternative to agents such as clotrimazole for treatment of ion channel activity disorders including sickle cell disease, and to avoid the systemic toxicity associated with clotrimazole's inhibition of cytochrome P450 enzymes [22, 46, 72, 73]. Since its development, it has been utilized primarily as a research tool in the study of the intermediate conductance Ca^{2+}-activated K^+ channels in various organs including lung, liver, and kidney as well as in certain tumors [23, 26-28, 46-48, 72]. More recent studies have demonstrated the role of KCa3.1 in the development of fibrosis via activation of TGFβ [27, 48, 74]. Several *in vitro* and *in vivo* studies have shown that TRAM-34 inhibition of the KCa3.1 channel effectively inhibits TGFβ activation and ultimately reduces fibrosis in the lung, liver, and kidneys [26-28].

In ocular tissues, selective blockade of KCa3.1via application of TRAM-34 has been shown to downregulate TGFβ-activated pro-fibrotic gene expression in both conjunctiva and cornea, and thus reduce activation and differentiation of fibroblasts to myofibroblasts [24, 25]. Another study demonstrated that application of TRAM-34 to alkali-wounded mouse corneas may be beneficial in prevention of corneal angiogenesis via inhibition of epidermal growth factor (EGF) [29]. One study from our group specifically examined the ocular toxicity of TRAM-34 in primary human conjunctival fibroblast cultures by treating the fibroblasts with TRAM-34 at doses of 0, 1, 5, 10, 25, or 50 μM for up to 7 days to evaluate for cellular toxicity [24]. In the aforementioned study by Anumanthan *et al*, there was a moderate decrease in cellular viability at the 50μM dose based on trypan blue exclusion assay, but 25μM and lower doses were well tolerated. Our study supports ocular tolerability of this therapy *in vivo* in the rabbit model with twice daily dosing.

Ascorbic acid has been evaluated for use topically in rabbits at various concentrations ranging from 10mg/mL to 0.5mg/mL after induction of corneal neovascularization via surgical placement of a stromal suture [32]. Lee et al showed significantly reduced presence of markers of angiogenesis VEGF and MMP9 in treated groups compared to controls, as well as a lower ratio of corneal surface area of neovascularization in the treatment groups at 10mg/mL ascorbic acid [32]. Additional data from a recent study using an *in vivo* mouse model of corneal epithelial scraping, with subsequent application of a single dose of topical 10% ascorbic acid, showed a significant improvement in corneal re-epithelialization in the treatment group [31].

A novelty of this study is preparation and evaluation of eyedrop consisting of water-soluble vitamin, ascorbic acid, and a highly selective and potent inhibitor of the intermediate-conductance Ca2+-activated K+ channel (KCa3.1), TRAM-34, that does not block cytochrome P450. Previous studies of topical TRAM-34 found it highly effective in preventing fibrosis in ocular and non-ocular systems in vitro and in vivo [24, 25, 29]. Likewise, ascorbic acid has been previously evaluated in rabbits in vivo and shown great success in treating corneal ulcers and improving corneal healing [30, 31, 52, 75]. To the best of authors knowledge, a combination of these agents has never been tested previously. This is the first study formulating, preparing, and evaluating the safety and efficacy of a bimodal eyedrop consisting of TRAM-34 and ascorbic acid in rabbits in vivo. Another strength of the study is use of alkali dosing in rabbit that produces fibrosis in the cornea without significant neovascularization.

There are certain limitations to this study. For example, no direct comparisons of the antifibrotic effect of combination eyedrop with TRAM-34 or ascorbic acid alone were performed, the changes in cellular and molecular parameters in corneal tissues were evaluated only at one time point (28 days), and minimal efforts were made to characterize underlying

mechanisms. Additionally, we did not evaluate effects of TRAM-34 on corneal epithelial and stromal fibroblast cells despite the fact that potassium channels could modulate cellular proliferation, an important factor in corneal wound healing. Our future studies will address these limitations.

We observed downregulation of multiple fibrotic markers associated with of TGFβ-mediated fibrosis, including collagen III, fibronectin, and α-SMA, in this study's therapy cohort (group 3). The reduction of these markers supports our conclusion that our therapy targets TGFβ and has antifibrotic properties in rabbit corneas. In this study, anti-angiogenic markers such as VEGF and MMP9 were not specifically tested, as that was not the primary aim of the current research. However, future studies may examine other markers to help further delineate the mechanisms of both TRAM-34 and ascorbic acid and their combined effect on corneal stromal wound healing.

Conclusion

The combination TRAM-34 and ascorbic acid applied topically was well tolerated and effective in prevention of corneal fibrosis through inhibition of TGFβ-mediated fibroblast migration and myofibroblast differentiation. Further studies are needed to determine the safety and efficacy in other species as well as the optimal dosing regimen. Additionally, further study is required to examine the efficacy of this therapy in treating an established fibrotic corneal lesion.

APPENDIX 1: TABLES

Table 1: Quantitative real-time PCR primer sequences.

Gene	Primer sequence (5'-3')		Tm (ºC)
α-SMA	**Forward**	TGG GTG ACG AAG CAC AGA GC	60
	Reverse	CTT CAG GGG CAA CAC GAA GC	60
Fibronectin	**Forward**	CGC AGC TTC GAG ATC GTG C	60
	Reverse	TCG ACG GGA TCA CAC TTC CA	60
Collagen-III	**Forward**	AGA ACA CGC AAG GCT GTG AGA CTA	60
	Reverse	CCA ACG TCC GCA CCA AAT TCT TGA	60
GAPDH	**Forward**	GCC TCA AGA TCA TCA GCA ATG CCT	60
	Reverse	TGT GGT CAT GAG TCC TTC CAC GAT	60

APPENDIX 2: FIGURES

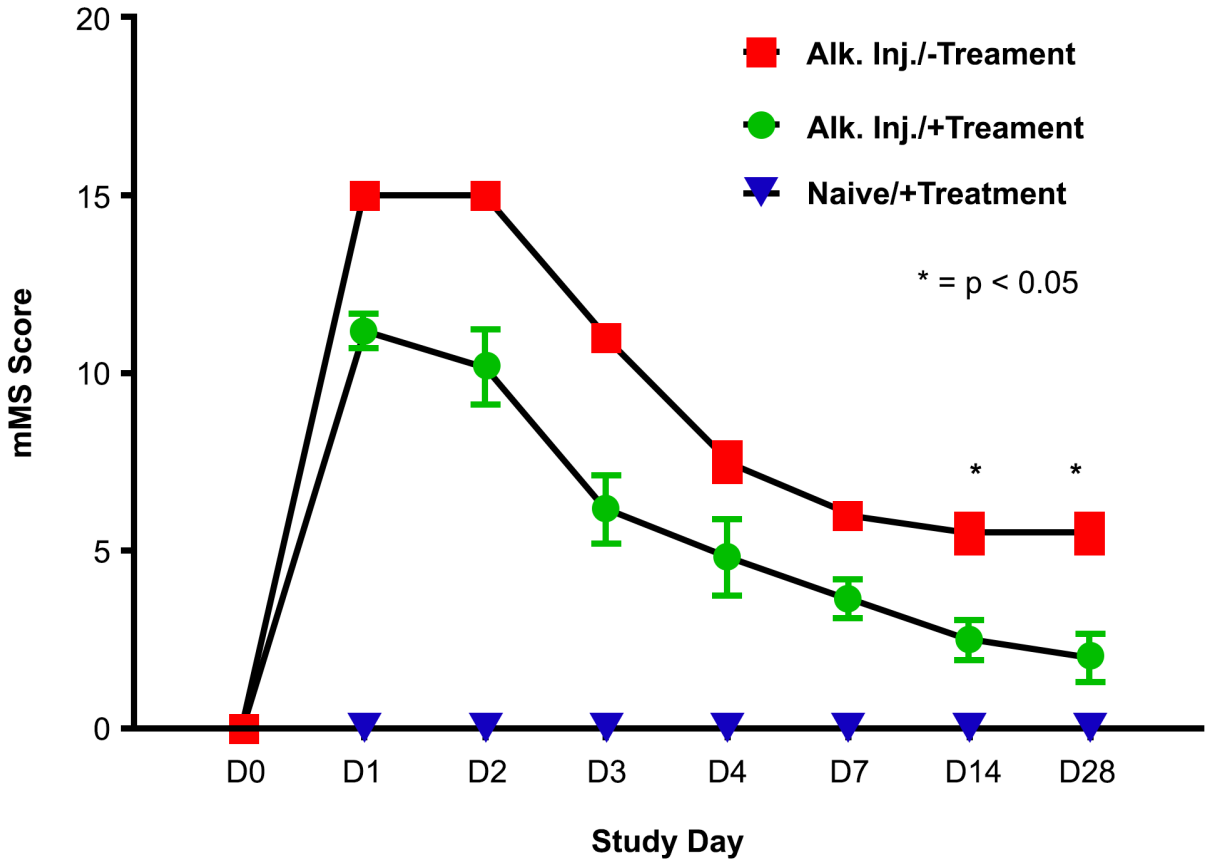

Fig 1. **Modified McDonald Shadduck Scoring.** There was no significant difference in mMS scores between treatment and control groups at days 1 through 7. There was a significant difference ($p<0.05$) between treatment and control groups at day 14 ($p=0.0041$) and day 28 ($p=0.0002$) as denoted by the asterix.

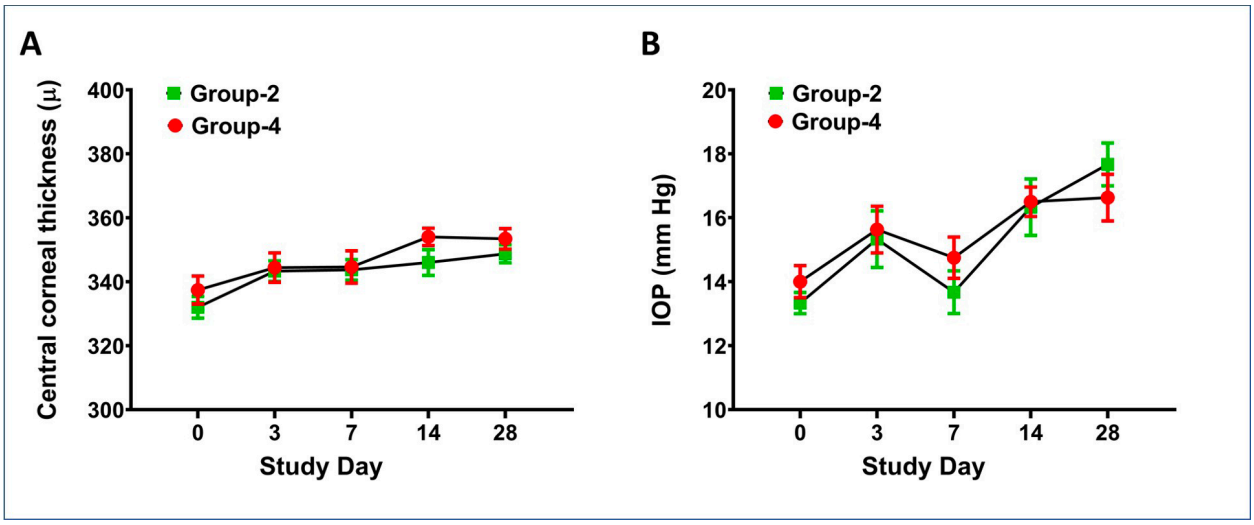

Fig 2. Central corneal thickness and intraocular pressure - safety. No significant difference in CCT (A) or IOP (B) in unwounded right eyes treated with the TVC drop was found at any time point.

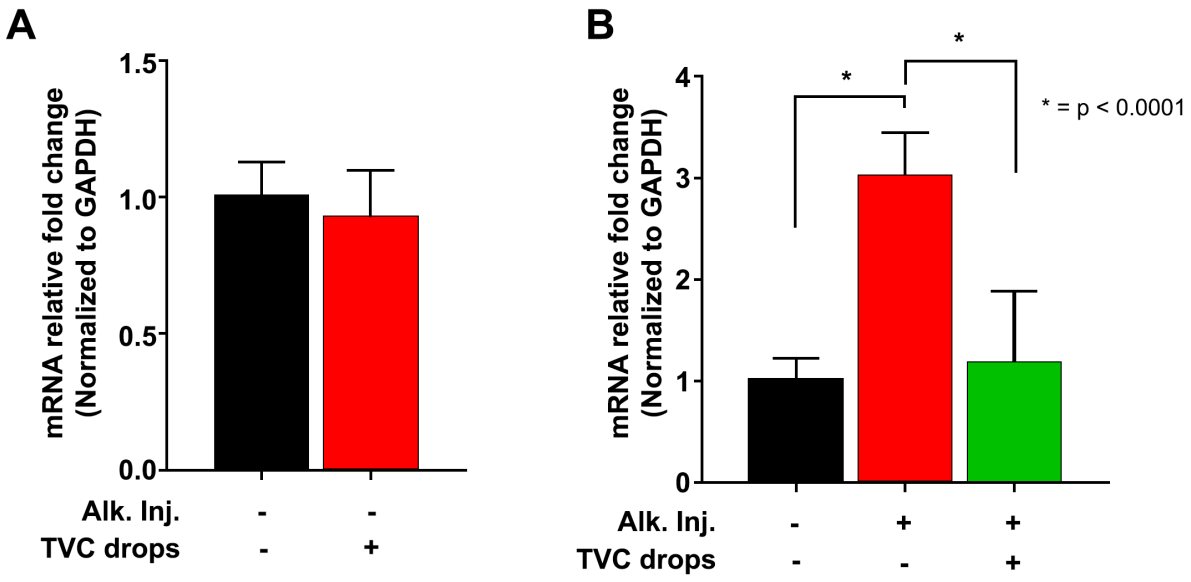

Fig 3. α-SMA – safety and efficacy. α-SMA gene expression was not significantly different between unwounded treatment groups and controls (A). There was a significant difference between wounded eyes treated with BSS versus naïve eyes and eyes treated with TVC. Wounded eyes treated with the TVC drop had α-SMA expression approaching that of naïve corneas (B).

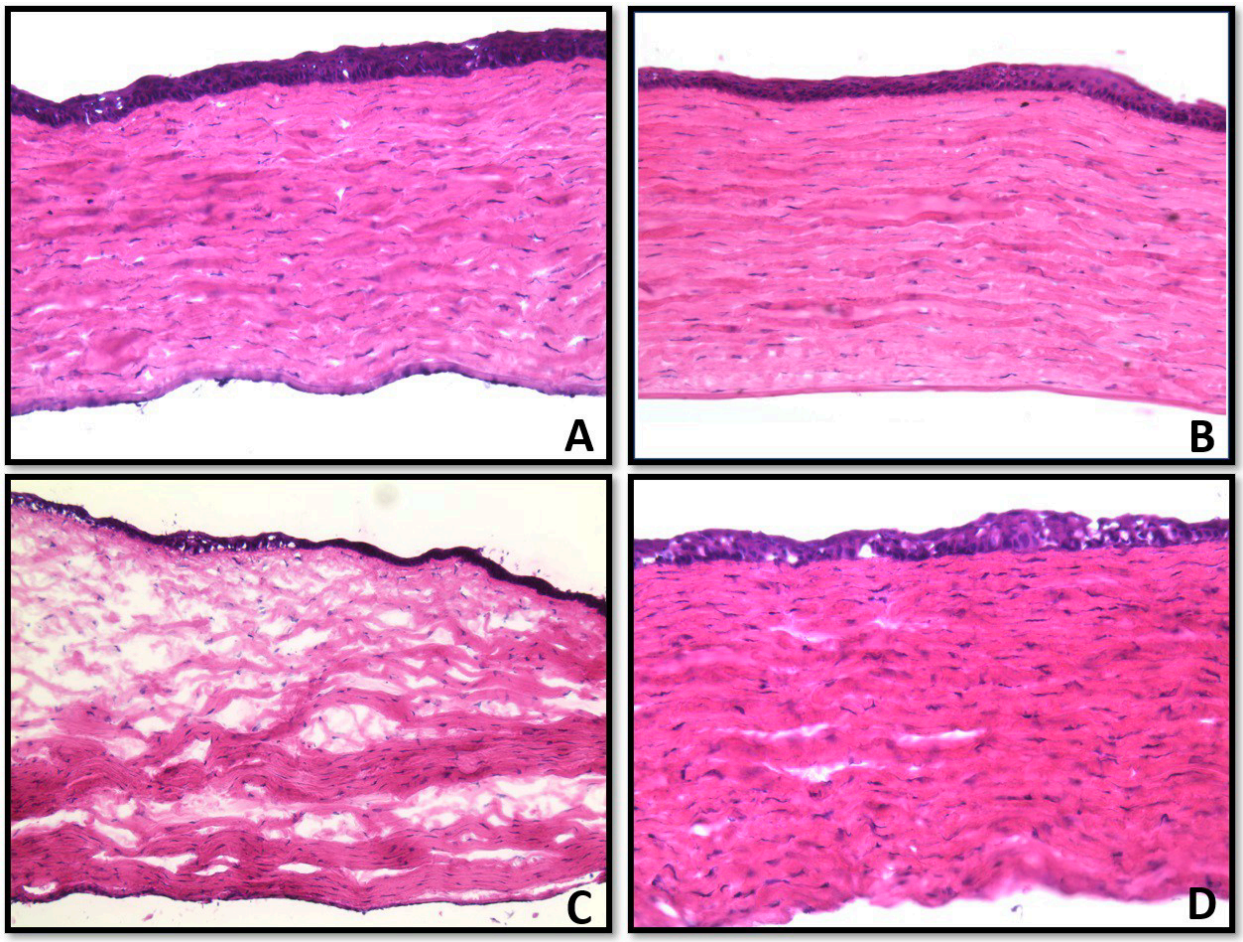

Fig 4. Histopathology. Representative H&E stained corneal tissue sections showing toxicity and efficacy in vivo. Corneal sections of naïve Group-2 (A), unwounded eyedrop-treated Group-4 (B), alkali wounded Group-1 (C), and alkali wounded and eyedrop-treated Group-4 (D) eyes. Group-2 (A) and Group-4 corneas showed normal corneal morphology. Alkali injury led to significant stromal edema and disorganized collagen, as well as a thin keratinized epithelial layer (C) and eyedrop treatment markedly improved corneal pathology through better collagen fibrils organization, less stromal edema, and re-epithelialization with a full-thickness epithelium (D). Magnification 20x.

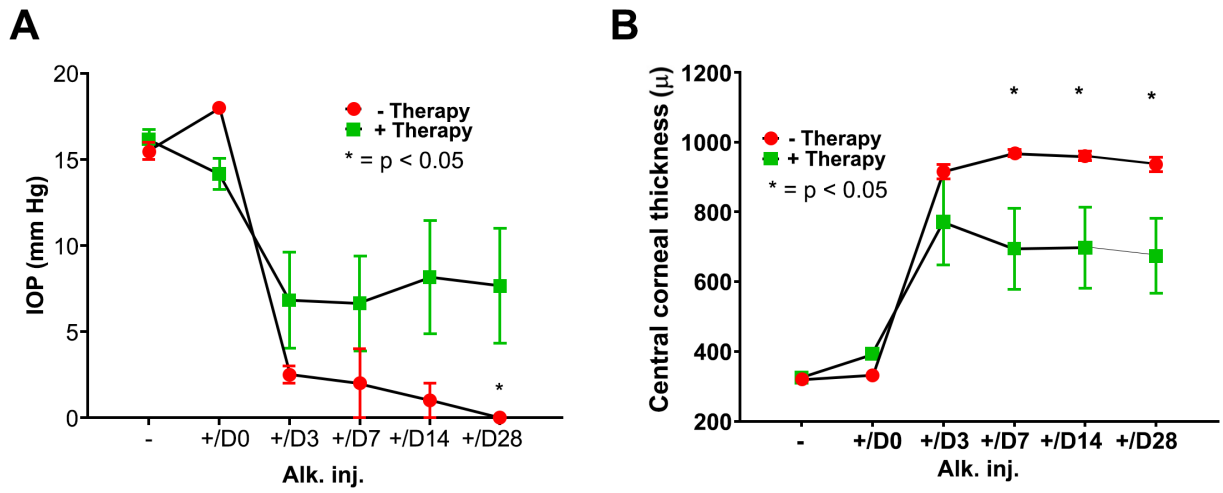

Fig 5. Central corneal thickness and intraocular pressure – efficacy. Intraocular pressure was significantly lower in the BSS treated eyes versus TVC treated eyes at day 28 only as denoted by the asterix (A). CCT was significantly lower in TVC treated eyes versus BSS treated eyes at days 7, 14, and 28 as indicated by the asterix (B).

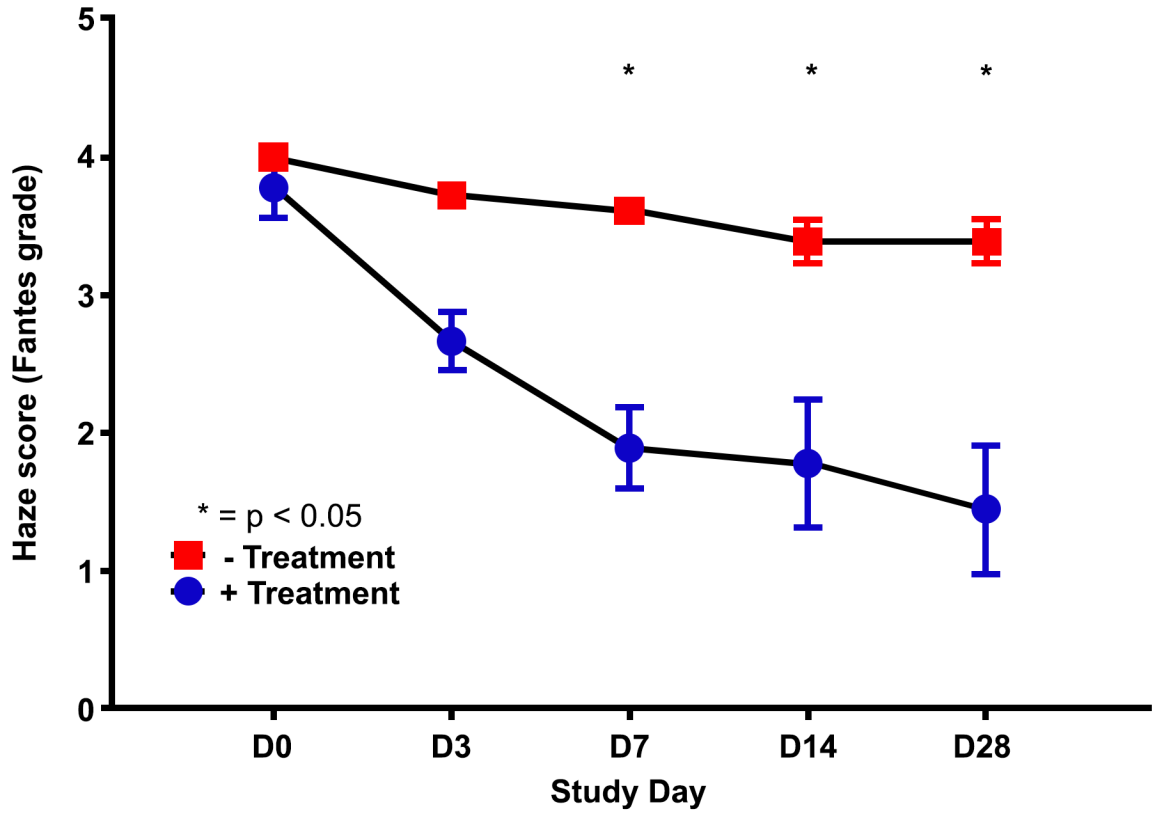

Fig 6. Fantes haze scores. There was a significant difference ($p<0.05$) in Fantes scores between treatment and control groups at day 7, 14, and 28 as indicated by the asterix.

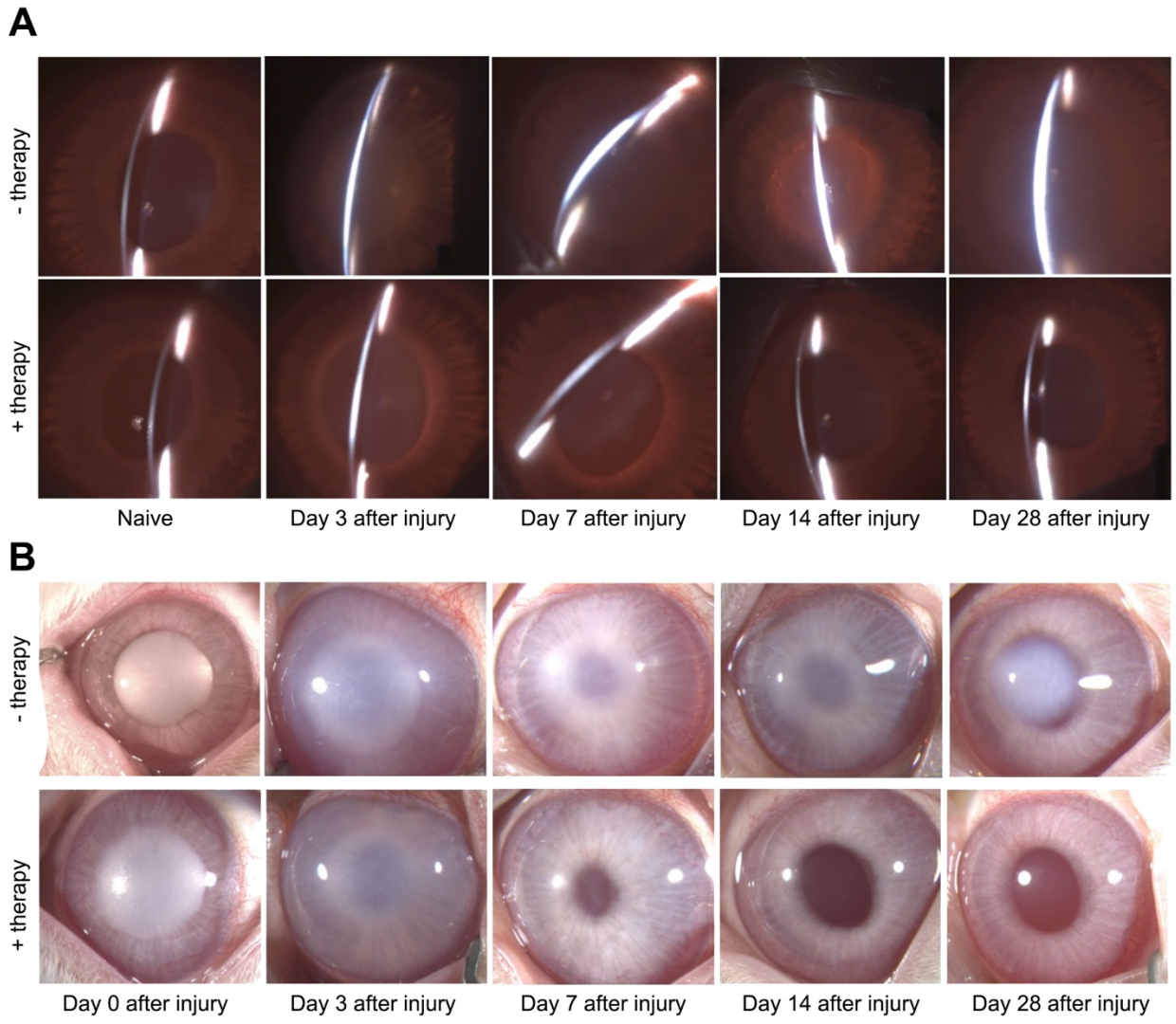

Fig 7. Clinical improvement in TVC treated corneas. Representative slit lamp images and stereo biomicroscopy images demonstrating corneal haze from day 0 through day 28 in control versus treated eyes, indicating an improvement in haze in the treated eyes.

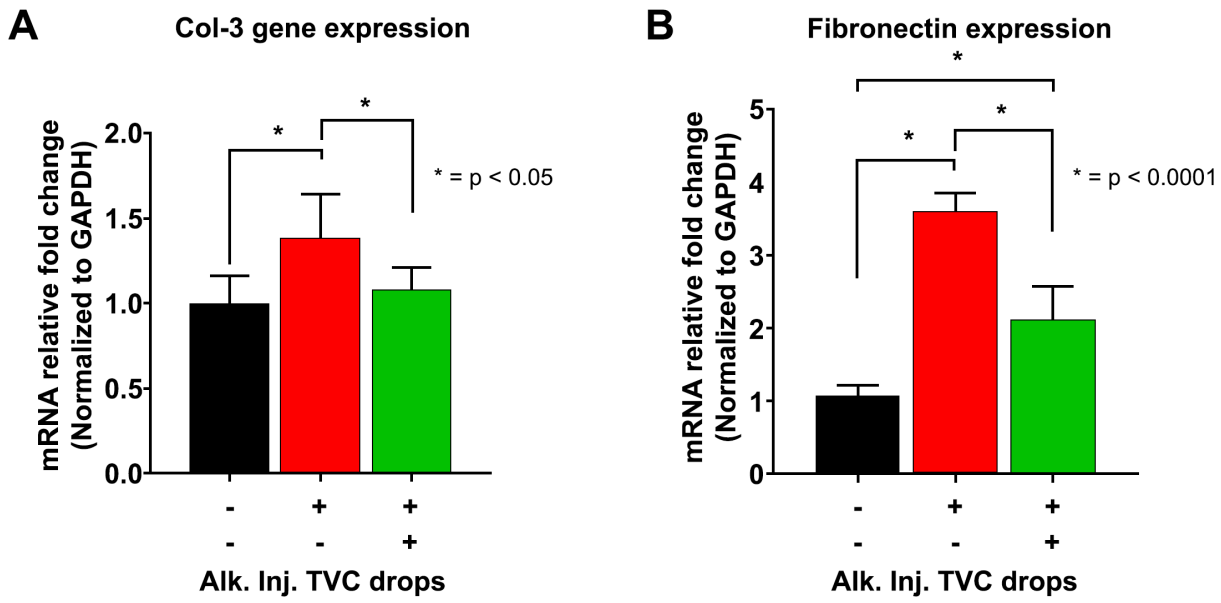

Fig 8. Pro-fibrotic gene expression. Expression of collagen-III was significantly different between untreated wounded corneas and TVC treated corneas. There was no significant difference between naïve and TVC-treated corneas (A). Expression of fibronectin was significantly decreased in wounded corneas treated with TVC versus BSS treated corneas (B).

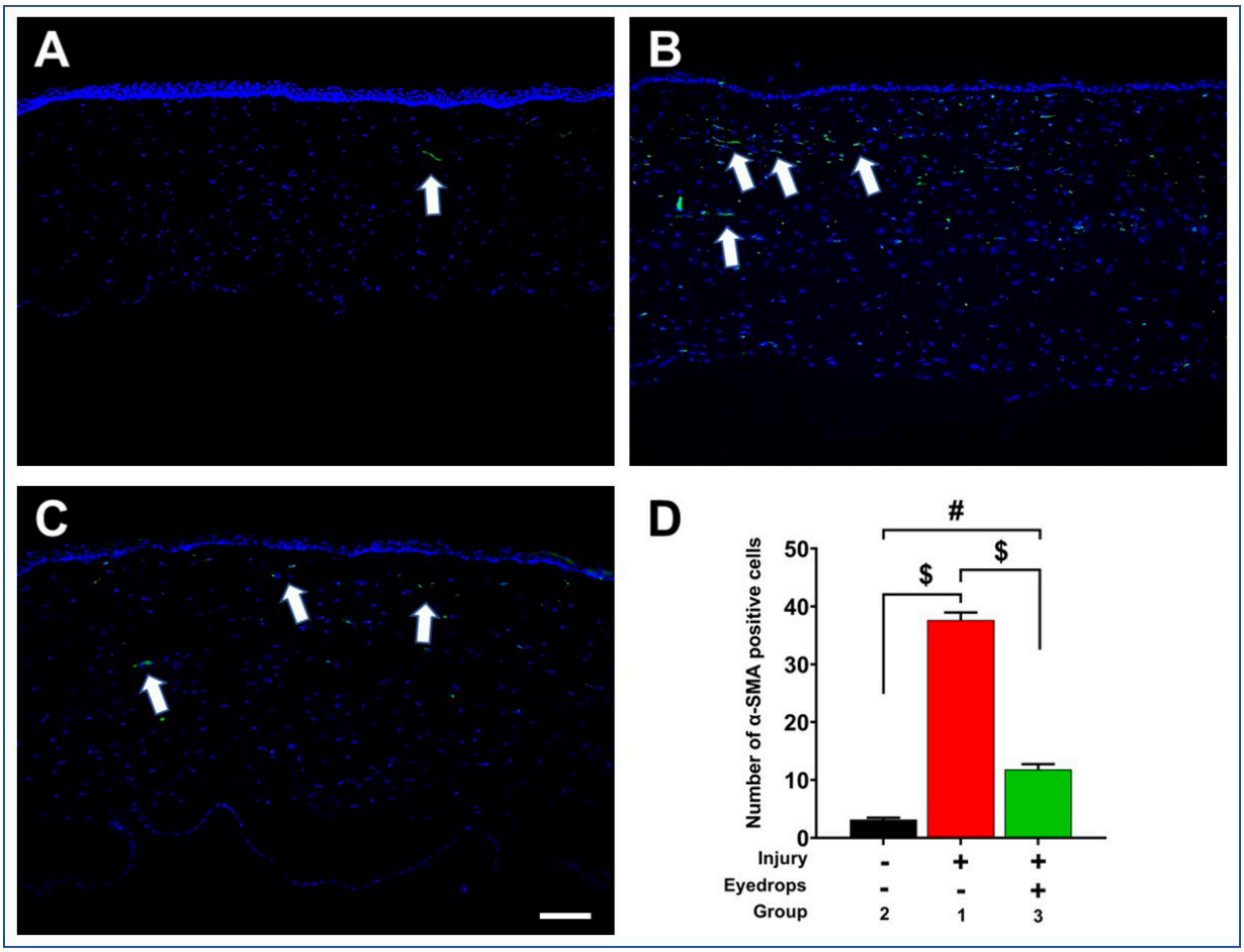

Fig 9. Representative immunofluorescence of α-SMA protein in naïve and wounded eyes -/+ eyedrop. A significantly increased α-SMA+ cells were found in alkali wounded rabbit corneas (B) compared to the naïve corneas (A). Eyedrop treatment after injury significantly decreased α-SMA+ cells in rabbit corneas (C) compared to alkali injured rabbit corneas without eyedrop treatment (B). Panel D shows quantification of SMA+ cells in corneas of 3 groups (n = 6 for each group; # = $p<0.001$; $ = $p<0.0001$;). Scale bar = 50μm.

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