Assessment of Topical Therapies for Improving the Optical Clarity Following Stromal Wounding in a Novel Ex Vivo Canine Cornea Model

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PURPOSE. To evaluate the effect of topical suberanilohydroxamic acid (SAHA) and 5-methyl-1-phenyl-2[1H]-pyridone (pirfenidone) on the degree of corneal haze in the stromal wounded ex vivo canine cornea.

METHODS. Twenty-four corneascleral rims from normal dogs were uniformly wounded with an excimer laser and placed into culture medium with an air–liquid interface. The control group (n = 8) contained placebo-treated corneas. Treatment group 1 (n = 8) received SAHA topically every 6 hours. Treatment group 2 (n = 8) received pirfenidone topically every 6 hours. Each cornea was fluorescein stained and macrophotographed every 6 hours to assess epithelialization rate. All corneas were also macrophotographed weekly to assess optical clarity (haze). Images were analyzed for differences in pixel intensity between wounded (haze) and unwounded (nonhaze) regions, and haze surface area for each cornea was calculated.

RESULTS. The mean epithelialization time was 47.25 hours in the control group, 45.00 hours in the SAHA group, and 43.50 hours in the pirfenidone group, revealing no significant difference (P = 0.368). The median difference in pixel intensity between haze and nonhaze areas was 21.5 in the control group, 8.0 in the SAHA group, and 8.0 in the pirfenidone group, which is significant (P < 0.01). The median haze surface area was 12.96 mm² in the control group, 5.70 mm² in the SAHA group, and 5.92 mm² in the pirfenidone group, which is significant (P < 0.01).

CONCLUSIONS. Stromal-wounded ex vivo canine corneas exhibited greater optical clarity when treated with SAHA and pirfenidone than when placebo treated at 21 days. There was no significant difference in epithelialization rate between groups. Corneal contour was correlated with geographic haze distribution.

Keywords: ex vivo, SAHA, corneal haze, pirfenidone, fibrosis

Disruption of the corneal epithelium and stroma is a common occurrence in human beings and veterinary patients, whether due to environmental trauma or surgical iatrogenia following photorefractive or phototherapeutic keratotomy. The biological response to this tissue disruption can result in significant subepithelial stromal haze, often due to fibrosis.1–4 Because the cornea is responsible for 65% to 75% of the light refraction necessary for clear vision in mammals, subepithelial haze can have a significant impact on visual acuity. For this reason, the development of safe and effective topical therapies that mitigate the formation of stromal fibrosis continues to be an extremely important area of research.5–10

Upon initiation of the corneal wound healing process, quiescent keratocytes inhabiting the corneal stroma transform into fibroblasts and myofibroblasts.1–4,11 In addition, bone marrow–derived precursor cells are attracted to the wound site and transdifferentiate into myofibroblasts as well.12 Myofibroblasts are crucial to survival and repair of the wounded cornea due to their unique ability to secrete extracellular matrix material, produce adhesions in the surrounding stroma, and contract wound edges through the expression of alpha smooth muscle actin (αSMA).13–16 At the same time, myofibroblasts contribute substantially to fibrosis and corneal haze through decreased production of cytoplasmic crystallins and increased deposition of type I collagen and fibronectin, which lack the fibrillar organization and volume fraction necessary for appropriate light transmission through the corneal stroma.15,17,18 One of the most important potentiators of myofibroblast formation from both keratocyte and bone marrow–derived precursors is transforming growth factor β (TGF-β).19–22

Suberanilohydroxamic acid (SAHA) is a histone deacetylase inhibitor (HDAC), which has gained recent interest for its ability to block TGF-β signal transduction, thereby decreasing myofibroblast transformation in vitro.8,9,23,24 Given the evidence that TGF-β is important for both keratocyte-derived and bone marrow–derived myofibroblast formation, SAHA is hypothesized to act upon both pathways.19,20,22 Through its effect on the balance of histone acetylation and deacetylation, SAHA is able to exert epigenetic control over the proliferation and differentiation of cells in a multitude of tissues.25,26 This
Effect of SAHA and Pirfenidone on Corneal Fibrosis

has led to its extensive clinical use against T-cell lymphoma in humans (for which it is Food and Drug Administration approved), as well as Sezary syndrome, gastrointestinal multiforme, and myelodysplastic syndromes.\textsuperscript{27–31} If effectively adapted for topical use, SAHA may be a potent and safe therapeutic option for preventing subepithelial haze.

Pirfenidone (5-methyl-1-phenyl-[2][1]H]-pyridone) is a non-
peptide pharmacologic agent, which has demonstrated significant efficacy as an inhibitor of TGF-\(\beta\).\textsuperscript{32–34} Though its exact mechanism of action has not yet been elucidated, pirfenidone may also have an inhibitory effect on platelet-derived growth factor (PDGF) and connective tissue growth factor (CTGF), and promote fibroblast apoptosis via suppression of nuclear factor (NF)-kappa B, all of which may inhibit myofibroblast proliferation.\textsuperscript{35–39} It has gained widespread use for treatment of idiopathic pulmonary fibrosis.\textsuperscript{40–42} It also has come into use for other conditions where an antibacterial and antioxidant are of utility, such as renal fibrosis and hepatic cirrhosis.\textsuperscript{43–44} In the eye, pirfenidone has been shown to inhibit the activity of human Tenon's fibroblasts in vitro,\textsuperscript{45} and has also been tested as an adjunctive postoperative antibacterial for strabismus surgery in rabbits.\textsuperscript{46} In addition, it has been evaluated as a postoperative anticarring agent for use in glaucoma surgery in a lagomorph model, and it was recently reported to successfully inhibit TGF-\(\beta\)-induced equine corneal fibrosis in vitro.\textsuperscript{47} Its inhibitory effects on TGF-\(\beta\) and PDGF may be efficacious in decreasing myofibroblast formation from both keratocyte and bone marrow–derived precursors.\textsuperscript{48}

While pirfenidone and SAHA have shown promise in lagomorph models and in vitro studies, their respective effects on corneal clarity following regimented topical administration have not been established. To address the challenges of evaluating the effect of SAHA and pirfenidone on subepithelial haze, a novel canine ex vivo cornea model was developed. The model described herein is unique in that it is the first to reliably and reproducibly form a quantifiable scar in the canine. In addition, the use of the canine cadaver cornea is beneficial as the curvature, thickness, and diameter make it an excellent translational model.\textsuperscript{48} Importantly, corneas in this model also exhibit greater clarity and less edema than those of some ex vivo models previously published.\textsuperscript{49,50} This preserves the tissue architecture and maintains similar healing behavior to a cornea in a live animal, without the need for expensive and time-consuming live animal colonies. This model also permits reproducibly quantitative and time- and dose-dependent assessments of wound healing, which would not be possible with live animals, and eliminates the need to create painful corneal wounds in animal subjects.

**Materials and Methods**

All animals used in experiments reported herein were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and in a protocol that was reviewed and approved by the University of Florida Institutional Animal Care and Use Committee.

**Harvesting and Wounding of Corneal Tissue**

Twenty-four globes were aseptically enucleated from 12 adult dogs, which were euthanized for reasons unrelated to this study. All globes were determined to be free of gross anterior segment abnormalities based on biomicroscopic examination (Kowa SL-15; Kowa American Corp., Torrance, CA, USA). Each globe then received an axial 6.0-mm diameter, 250-\(\mu\)m deep stromal excimer laser phototherapeutic keratectomy (PTK) wound with no transition zone (Nidek EC5000; Nidek, Inc., Freemont, CA, USA).

Following wounding, corneoscleral rims were aseptically harvested from each globe by making a circumferential incision 4 mm posterior to the limbus, and removing the uveal tract with gentle manual traction.

**Ex Vivo Air-Liquid Interface Model**

To provide a scaffold for each corneoscleral rim, a black polyurethane elastomer (Smooth-Cast ONYX; Smooth-On, Inc., Macungie, PA, USA) was molded into a conical frustum with a base radius of 20 mm, an apical radius of 15 mm, and a height of 10 mm. Each identical black scaffold was then centered in the floor of one well of a 6-well polystyrene nonculture-treated microplate ( Falcon; Corning, Inc., Corning, NY, USA) and permanently affixed using methyl cyanoacrylate adhesive (Krazy Glue; Westerville, OH, USA). The finished scaffold-microplate assembly was then allowed to cure for 24 hours before being sterilized with ethylene oxide.

Each wounded corneoscleral rim was placed epithelium side-up onto a black scaffold in a separate culture well, with an air bubble between the endothelium and scaffold to maintain a convex corneal curvature. Culture wells were then filled with liquid medium until the fluid line reached the level of the limbus. Each microplate containing 6 cornea samples was covered, placed on a rotating plate at 24 rpm (UltraCruz; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and incubated at 37°C in a humid atmosphere containing 5% CO₂. For the first 24 hours, all corneoscleral rims were cultured in a starter medium containing Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture with L-glutamine at a ratio of 1:1 ( Gibco, Thermo Fisher Scientific Co., Waltham, MA, USA), supplemented with 10% fetal bovine serum ( Gibco, Thermo Fisher Scientific Co.), HEPES buffer ( Gibco, Thermo Fisher Scientific Co.), dextran 40 ( Sigma-Aldrich Corp., St. Louis, MO, USA), chondroitin sulfate ( Sigma-Aldrich Corp.), and fortified with 10% antimicrobial solution ( streptomycin, penicillin, and amphotericin; Thermo Fisher Scientific Co.). Thereafter, all corneoscleral rims were cultured in maintenance medium, which was identical to the starter medium, except fortified with only 1% antimicrobial solution ( streptomycin, penicillin, and amphotericin).

**Treatment Groups**

The 24 excimer-wounded corneoscleral rims were randomly assigned to one of the following three groups: the control group contained eight cornea samples, which were treated topically with 0.05 mL of sterile maintenance media; treatment group 1 contained eight cornea samples, which were treated topically with 0.05 mL of maintenance media supplemented with 25-\(\mu\)M SAHA ( SML0061; Sigma-Aldrich Corp.); and treatment group 2 contained eight cornea samples which were treated topically with 0.05 mL of maintenance media supplemented with 1.08 mM of pirfenidone (P2116; Sigma-Aldrich Corp.). The concentrations of SAHA and pirfenidone chosen in this study were extrapolated from dose response and viability studies previously reported.\textsuperscript{8,23,33,45} All corneas were treated once every 6 hours until the end of the 21-day study period. Prior to treatment, all medium was evacuated from each culture well. Treatment solutions were allowed to remain on each cornea for 5 minutes, and all samples were then thoroughly rinsed with PBS (Sigma-Aldrich Corp.) before filling each well with fresh culture medium. At least once daily, all corneas were examined by slit-lamp biomicroscopy assess the character of the epithelium and anterior stroma.
Experiment 1: Epithelialization Rate

Fluorescein Dye Test. At time 0 and every 6 hours thereafter, all 24 corneas were stained with sodium fluorescein (Bio-Glo; HUB Pharmaceuticals, LLC, Rancho Cucamonga, CA, USA) after evacuating the media from each culture well as described above. Staining and photography were repeated in this way until all corneas were negative for stain retention (Fig. 1).

Macrophotography. Each cornea was then macrophotographed in a darkened room with cobalt blue illumination (Kowa SL-15; Kowa American Corp., Torrance, CA, USA). Images were obtained using a digital single lens reflex (dSLR) camera with a macro lens prefocused to a 1:1 reproduction ratio (EOS Rebel XSi; Canon USA, Inc., Melville, NY, USA), mounted on a tripod with a fixed lens working distance of 15.5 cm for each cornea. For image capture of all corneal wounds, the camera was set to manual exposure with an International Standards Organization (ISO) sensitivity of 400, an aperture setting of f/32, and an exposure time of 1/250th of a second. For each cornea, samples were stained with a 1:1000 monoclonal anti-SMA mouse antibody (clone IA4, F3777; Sigma-Aldrich Corp.). Samples were counterstained with blue-fluorescent 4′,6-diamidino-2-phenylindole (DAPI) nucleic acid stain (62248; Thermo Fisher Scientific, Inc.), and examined under a diaminobenzidine (DAB) histochemistry. On day 21 of the study period, all corneas were removed from culture and placed into Davidson’s fixative for a minimum of 72 hours. Then, three sections were collected, measuring 6 mm in diameter and approximately 300-μm thick. To confirm the morphology of α-SMA in the haze area of each cornea, all 24 corneas were stained with 1:1000 monoclonal anti-α-SMA mouse antibody (clone IA4, F3777; Sigma-Aldrich Corp.). Samples were counterstained with blue-fluorescent 4′,6-diamidino-2-phenylindole (DAPI) nucleic acid stain (62248; Thermo Fisher Scientific, Inc.), and examined under a fluorescence microscope (EVOS FL Cell Imaging System; Thermo Fisher Scientific Co.).

The same immunostained corneal samples were trimmed a second time, stripping the epithelium, and preserving approximately 100 μm of the anterior stroma. The morphology of α-SMA-positive cells was then characterized using a combination of fluorescence and light reflecting confocal laser scanning microscopy (Leica Microsystems, Inc., Buffalo Grove, IL, USA).

Experiment 2: Optical Haze Measurement

Macrophotography and Hypersomotic Treatment. At time 0 and once every 7 days until day 21 of the study period, all 24 corneas were macrophotographed in room light, using the dSLR apparatus described above with the addition of a lens-mounted macroflash (Fig. 2). For this phase of the study, the camera was set to manual exposure with an ISO sensitivity of 400, an aperture setting of f/32, and an exposure time of 1/250th of a second. Prior to macrophotography on day 21, each corneal section was immersed in 50% dextrose (VetOne; Boise, ID, USA) for 10 minutes to eliminate any accumulation of stromal edema, which could contribute to optical haze.

Image Processing. To optimize the contrast between the haze and background (haze free) regions of each day, 21 corneal images were converted to grayscale with computer software (ImageJ) using the red channel data only. For each grayscale image, three random modal measurements of pixel intensity were acquired in the periligmal or paraxial cornea outside of the wound area (hereafter referred to as background pixel intensity). Then, three modal measurements of pixel intensity were acquired within the 6-mm axial wound area (hereafter referred to as haze pixel intensity). The mean of each set of three measurements was then calculated, and the numerical difference between the mean background pixel intensity and the mean haze pixel intensity was established as an indicator of the “optical brightness” of the haze for each cornea. To determine the haze surface area of each axial cornea, a threshold (i.e., bandpass) filter was then applied to each image using the mean background pixel intensity as the minimum value and a set pixel intensity of 150 as the maximum value (Fig. 3). All pixels in the wound area, which contained intensity values within this range were included in the haze surface area measurement. A lasso tool was then used to mark the wound-associated haze area and exclude any flash artifact on each cornea (as these artifacts carry pixel intensity values high enough to be erroneously included in the haze area measurement by the computer software, confounding data analysis). In this way, the surface area of the wound-associated haze for each cornea was generated in pixels, and then converted to millimeters squared (Fig. 4). Differences in haze surface area, as well as differential haze pixel intensity (i.e., haze brightness) for the control group and each experimental group were assessed using the Kruskal-Wallis 1-way ANOVA.

Immunohistochemistry. On day 21 of the study period, all corneas were removed from culture and placed into Davidson’s fixative for a minimum of 72 hours. Then, three corneas were randomly selected from the control group and each treatment group (n = 9 corneas total). Centering on the wound area of each selected cornea, a circular disk-shaped section of axial, anterior tissue was collected, measuring 6 mm in diameter and approximately 300-μm thick. To confirm the presence and distribution of α-SMA in the haze area of each cornea, samples were stained with a 1:1000 monoclonal anti-α-SMA mouse antibody (clone IA4, F3777; Sigma-Aldrich Corp.). Samples were counterstained with blue-fluorescent 4′,6-diamidino-2-phenylindole (DAPI) nucleic acid stain (62248; Thermo Fisher Scientific, Inc.), and examined under a fluorescence microscope (EVOS FL Cell Imaging System; Thermo Fisher Scientific Co.).

The same immunostained corneal samples were trimmed a second time, stripping the epithelium, and preserving approximately 100 μm of the anterior stroma. The morphology of α-SMA-positive cells was then characterized using a combination of fluorescence and light reflecting confocal laser scanning microscopy (Leica Microsystems, Inc., Buffalo Grove, IL, USA).

Figure 1. Successive macrophotographs of an excimer-wounded, SAHA-treated ex vivo canine cornea stained with fluorescein dye under cobalt blue light. Photographs depict the progressive stages of epithelialization at 6 hours postwounding (A), 18 hours postwounding (B), 24 hours postwounding (C), and 30 hours postwounding when the wound was declared healed (D).
During the study period, an abnormal pattern of subepithelial haze was noticed on all corneas, which was crater-like, with a central ring of opacity surrounded by radial spicules. This was contrary to the generalized ‘cloud’ of haze within the photorefractive keratectomy (PRK) area, which has been observed in prior studies.\textsuperscript{51} It was also noticed that the air pocket between each corneal endothelium and its urethane conical frustum diminished over the first 7 days of the study period, resulting in a sinking-down of each cornea onto its scaffold and grossly altering its curvature. It was therefore hypothesized that the abnormal pattern of subepithelial haze was a sequela of the altered corneal curvature during the timeline of haze formation. To test this hypothesis, a pilot group of canine corneas was cultured, using a second novel scaffold design.

Sixteen globes were enucleated from eight additional dogs, which were euthanized for reasons unrelated to this study. The corneoscleral rim from each globe was harvested using the method previously described. One cornea from each dog (left or right) was selected at random to receive an axial 6.0-mm diameter, 250-\textmu m deep, stromal excimer laser phototherapeutic keratectomy wound with no transition zone (\(n = 8\) wounded corneas). The other cornea from each dog remained unwounded (\(n = 8\) unwounded corneas). Each corneoscleral FIGURE 2. Three representative ex vivo canine cornea images from each treatment group are shown for comparison, 21 days postwounding. Photographs from the placebo-treated group (left column) depict a bright, crater-like region of axial subepithelial haze. Note that photographs from the SAHA-treated group (center column) and pirfenidone-treated group (right column) display subjectively less axial subepithelial haze, though all three treatment groups display a low level of diffuse background haze associated with corneal edema.

**Experiment 3: Dome-Shaped Conformer Pilot Study**

During the study period, an abnormal pattern of subepithelial haze was noticed on all corneas, which was crater-like, with a central ring of opacity surrounded by radial spicules. This was contrary to the generalized ‘cloud’ of haze within the photorefractive keratectomy (PRK) area, which has been observed in prior studies.\textsuperscript{51} It was also noticed that the air pocket between each corneal endothelium and its urethane conical frustum diminished over the first 7 days of the study period, resulting in a sinking-down of each cornea onto its scaffold and grossly altering its curvature. It was therefore hypothesized that the abnormal pattern of subepithelial haze was a sequela of the altered corneal curvature during the timeline of haze formation. To test this hypothesis, a pilot group of canine corneas was cultured, using a second novel scaffold design.

Sixteen globes were enucleated from eight additional dogs, which were euthanized for reasons unrelated to this study. The corneoscleral rim from each globe was harvested using the method previously described. One cornea from each dog (left or right) was selected at random to receive an axial 6.0-mm diameter, 250-\textmu m deep, stromal excimer laser phototherapeutic keratectomy wound with no transition zone (\(n = 8\) wounded corneas). The other cornea from each dog remained unwounded (\(n = 8\) unwounded corneas). Each corneoscleral

**FIGURE 3.** Day-21 images of two placebo-treated ex vivo canine corneas (top and bottom row) are shown to demonstrate image analysis. The color channels from each raw image (A) are first separated and only the red color channel is retained (B). Three separate measurements of modal background pixel intensity (B) and three separate measurements of modal wound haze pixel intensity (C) are acquired. These measurements are used to establish the lower boundary of a threshold filter, which effectively labels each pixel involved in the wound-associated haze area (D).
corneas were wounded with the excimer laser and placed onto agarose domes for 24 hours, in culture conditions previously described. Wounds were 6 mm in diameter and 155 μm in depth. Ten of 30 corneas were cultured in maintenance medium (control group), 10 corneas were cultured in maintenance media containing 25-μM SAHA, and 10 corneas were cultured in maintenance media containing 1.08-mM pirfenidone. At 24 hours post wounding, all 30 corneas were removed from culture for immediate RNA extraction.

The total RNA from each cornea was extracted and converted to cDNA using an iScript Select DNA Synthesis Kit (1708897; Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to manufacturer instructions. The forward and reverse primer sequences for αSMA were CGTGGAGGCGTGTCAGAAATAC and GACATCAGGACACACTC, respectively. To quantify TGF-β and CTGF expression in each cornea, real-time PCR was performed using an Applied Biosystems 7300 real-time PCR system (Thermo Fisher Scientific Co.). A 20-μL reaction mixture containing 2-μL cDNA, 2-μL forward primer, 2-μL reverse primer, and 10-μL SYBR select super mix (Applied Biosystems, Thermo Fisher Scientific Co.) was run with 50 cycles at 95°C for 20 seconds. To normalize data, glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used as a housekeeping gene.

The cycle threshold (Ct) for each independent RT-PCR reaction was used to determine the relative gene expression for TGF-β and CTGF between SAHA-treated, pirfenidone-treated, and control corneas by applying the established \(2^{-\Delta\Delta Ct}\) formula. Differences in TGF-β and CTGF expression were evaluated for significance using a Kruskal-Wallis one-way ANOVA, followed by a Nemenyi test when appropriate. Statistical analysis was performed using 

### RESULTS

### Epithelialization Rate: Placebo-, SAHA-, and Pirfenidone-Treated Groups

All 24 excimer-wounded corneas, which were part of the initial control and experimental groups epithelialized fully within 54 hours (with individual epithelialization times ranging from 36–54 hours), as indicated by a negative fluorescein dye test (Fig. 5). The mean ± SD time to epithelialization of the 8 wounded, placebo-treated control corneas was 47.25 ± 5.01 hours (ranging from 42–54 hours). The mean ± SD time to epithelialization of the 8 wounded, 25-μM SAHA-treated corneas was 43.50 ± 5.32 hours (ranging from 36–48 hours). The mean ± SD time to epithelialization of the eight wounded, 1.08-mM pirfenidone-treated corneas was 43.50 ± 5.32 hours (ranging from 36–48 hours). These findings indicate that there is no significant difference in the time to full epithelialization between placebo-, SAHA-, and pirfenidone-treated canine corneas (\(P = 0.368\)).

### Epithelialization Rate: Flat-Topped Scaffold Versus Agarose Scaffold

The mean ± SD time to epithelialization of the eight wounded, placebo-treated corneas cultured on flat-topped scaffolds was 47.25 ± 5.01 hours (ranging from 42–54 hours). By comparison, seven of eight excimer-wounded, agarose-cultured corneas epithelialized fully within 60 hours (with individual epithelialization times ranging from 36–60 hours). The mean ± SD time to epithelialization of these seven corneas was 47.14 ± 8.07 hours. There was no significant
difference in the average time to full epithelialization between wounded corneas in the flat-topped model versus the novel agarose dome model \( (P = 0.171) \). One cornea cultured upon an agarose scaffold failed to epithelialize within 60 hours.

**Haze Formation: Flat-Topped Scaffold Versus Agarose Scaffold**

All wounded canine corneas that survived for 21 days developed observable wound-associated optical haze \( (n = 24 \) control and experimental corneas, and 7 agarose dome-cultured corneas). The corneas that were cultured on flat-topped scaffolds exhibited a focal, crater-like region of optical haze with several linear radiations. In contrast, the canine corneas cultured on the novel agarose dome scaffolds were subjectively characterized by a roughly circular, diffuse, cloud-like pattern of haze, surrounded by a relatively clear periwound (Fig. 6). Slit-lamp biomicroscopy of all tissue samples revealed that the optical haze was localized to the stroma, beginning immediately posterior to the epithelium and extending to the midstroma.

**Differential Haze Pixel Intensity (Flat-Topped Scaffold Model Only)**

For placebo-treated control corneas, the median differential pixel intensity (i.e., the haze pixel intensity—background pixel intensity) ± SD for the wound-associated region of optical haze was 21.5 ± 4.77. For 25-μM SAHA- and 1.08-mM pirfenidone-treated corneas, the median differential pixel intensities ± SD were 8.0 ± 5.69 and 8.0 ± 10.24, respectively. These findings indicate that the wound-associated haze pixels were significantly more intense in the placebo-treated control group than in either the SAHA or the pirfenidone treatment groups \( (P < 0.01) \) (Fig. 7). Furthermore, there was no significant difference in pixel intensity between the SAHA- and the pirfenidone-treatment group \( (P = 0.874) \).

**Haze Surface Area (Flat-Topped Scaffold Model Only)**

The median total surface area ± SD of wound-associated haze for placebo-treated control corneas was 12.96 ± 2.07 mm². For 25-μM SAHA-treated corneas, the median total surface area ± SD of haze was 5.70 ± 2.23 mm². For 1.08-mM pirfenidone-treated corneas, the median total surface area ± SD of haze was 5.92 ± 2.67 mm². The placebo-treated control corneas exhibited a significantly larger haze surface area than corneas treated with SAHA or pirfenidone \( (P < 0.01) \) (Fig. 8). There was no significant difference in haze surface area between the SAHA- and pirfenidone-treatment groups \( (P = 0.753) \).
Immunohistochemistry: Flat-Topped Scaffold Versus Agarose Scaffold

Tissue samples from the axial wounds of all nine original corneas exhibited strong positive staining for the presence of αSMA. Positive-staining regions universally consisted of a central ring, with linear radial streaks extending toward it from the periphery of the original wound area (Fig. 9). This distribution was consistent with the crater-like areas of greatest optical haze in each tissue sample. All three wounded, agarose-cultured corneas exhibited strong positive staining for αSMA within the circular boundary of the original excimer wound, with few αSMA-staining areas outside of the wound bed (Fig. 10). The three unwounded, agarose-cultured corneas exhibited some background signal, but no definitive pattern of αSMA staining.

Confocal Microscopy

Within the areas of greatest optical haze, many myofibroblasts were present, which exhibited stellate morphology and green-staining intracytoplasmic stress fibers (Fig. 11). Disorganized extracellular matrix material was also present. Reflectance confocal microscopy revealed that the nuclei and cytoplasmic elements of the myofibroblasts, as well as strands within the extracellular matrix, were much more light reflective than the surrounding background corneal tissue.

TGF-β and CTGF RNA Quantification by Real-Time PCR

The expression of TGF-β1 and CTGF mRNA in placebo-, SAHA-(25 μM), and pirfenidone-treated (1.08 mM) lagomorph corneas was quantified via RT-PCR (Fig. 12). In the SAHA-treated group, TGF-β1 mRNA expression was significantly decreased compared with the placebo-treated control group (4.13-fold ± 0.13; P = 0.02). TGF-β1 mRNA expression in the SAHA-treated group was also significantly less than that of the pirfenidone-treated group (2.79-fold ± 0.18; P = 0.03).
was no significant difference in TGF-β1 mRNA expression between the pirfenidone-treated corneas and the control corneas. There was no significant difference in CTGF mRNA expression between any of the SAHA-, pirfenidone-, or placebo-treated control corneas.

**DISCUSSION**

Using the novel canine ex vivo cornea model described herein, we have demonstrated that SAHA and pirfenidone, when administered topically, will significantly decrease the intensity and the surface area of subepithelial haze after sustaining a stromal wound with an excimer laser. In addition, neither of these therapeutic agents were shown to have a deleterious effect on the timeline of corneal wound epithelialization when compared with placebo-treated controls. These findings are important because they demonstrate a dramatic improvement in optical clarity, which is of direct clinical benefit to a potential patient. Furthermore, this effect on optical clarity was attained with topical administration at an interval of one treatment every 6 hours (4 times within a 24-hour period), which represents a reasonable, viable therapeutic regimen in clinical ophthalmology.

While the pathophysiology of fibrosis formation in the wounded cornea is complex and multifactorial, it can be hypothesized that SAHA and pirfenidone mitigate this process by acting against important profibrotic factors during the wound healing response. Upon disruption of the epithelium and epithelial basement membrane (EBM), keratocytes in the underlying stroma undergo apoptosis in response to IL-1, which is released from damaged epithelial cell cytoplasm and stimulated via an autocrine loop. At the same time, quiescent keratocytes nearby are exposed to exogenous growth factors, such as PDGF, which encourage differentiation into a fibroblastic phenotype (characterized by an actin cytoskeleton and motile behavior, to enable migration into the stromal wound). PDGF is released from the EBM after injury and stimulates fibroblast and myofibroblast differentiation. In addition, TGF-β from the damaged epithelium is allowed to come into contact with stromal keratocytes and bone-marrow–derived cells due to disruption of the EBM, resulting in further differentiation of these cells into myofibroblasts. There is recent evidence to suggest that corneal Schwann cells may differentiate into myofibroblasts in a similar manner. While under the influence of TGF-β, myofibroblasts exhibit prolonged protection from apoptosis and sustained profibrotic activity, producing greater quantities of disorganized extracellular matrix material and opaque type-1 collagen. As previously stated, disruption of the EBM is necessary to facilitate contact between IL-1, PDGF, TGF-β, and stromal...
cells. For this reason, the duration of EBM disruption and defective regeneration is thought to be directly proportional to the duration of myofibroblast activity and the ultimate degree of corneal fibrosis that will form. 51,52

As a histone deacetylase inhibitor (HDAC), SAHA alters the transcription activity of target cells by binding to HDAC and destabilizing the balance between lysine group acetylation and deacetylation. 58,59 By this mechanism, SAHA has recently been shown to mitigate the TGF-β1-induced phosphorylation of Smad2/3 in canine corneal fibroblast cultures. This is an important antifibrotic mechanism, as Smad proteins are involved in the transformation of quiescent keratocytes into fibroblasts and myofibroblasts in the corneal stroma. 60 In human corneal fibroblast cultures, SAHA was shown to significantly increase the expression of 5′TG5-interacting factors (TGIFs), which are important repressors of TGF-β signaling. 61 This culture model also demonstrated a significant decrease in TGF-β1-induced transdifferentiation of fibroblasts into myofibroblasts, and an approximate 97% decrease in zSMA protein. 61 In the lagomorph, SAHA significantly reduced the expression of phosphorylated extracellular signal-regulated kinase (phospho-ERK1/2) independent of the presence of TGF-β1, which may affect the deposition of extracellular matrix in a healing wound. 62,63 While prior data suggest that the significant decrease in optical haze observed in SAHA-treated canine corneas of the present study is most attributable to its TGF-β1-blocking effect on target cells, it is possible that SAHA may also mitigate the expression of TGF-β1 in the wound bed. This is supported by our RT-PCR findings, in which there was significantly less mRNA expression for TGF-β1 in SAHA-treated corneas than in either the pirfenidone- or placebo-treated corneas. Further assessment of the pharmacodynamics of SAHA was beyond the scope of this study. However, the potential of SAHA to decrease TGF-β1 production while also blocking the response of target cells warrants further evaluation to fully elucidate its potency as an antifibrotic medication. Also, while SAHA's effect on EBM restoration has not yet been established, it can be surmised that its TGF-β1 blocking effect on target cells, while also decreasing the amount of TGF-β1 from the corneal epithelium, while also decreasing the amount of TGF-β1 produced.

While less is known of pirfenidone's mechanism of action and molecular target, multiple studies have suggested that it inhibits TGF-β at the level of DNA transcription in tissue cells. 62,63 However, our RT-PCR data do not support this hypothesis, as indicated by no significant difference in TGF-β expression between pirfenidone-treated and control corneas. It is possible that the canine corneas of the present study may have exhibited less optical haze due to pirfenidone's effect on one or more other molecular targets. For instance, pirfenidone may play an important role in the downregulation of PDGF in wounded tissue. 64 Inhibition of PDGF would have a negative effect on the proliferation and migration of fibroblasts, ultimately resulting in less stromal haze after healing. Pirfenidone has also shown an array of other antifibrotic properties, such as CTGF inhibition, 59 NF-kappa B suppression (possibly promoting fibroblast apoptosis), 59 and limitation of reactive oxygen species in target tissues. 66,67 All of these properties have the potential to ameliorate corneal fibrosis, and may have contributed to the results observed in the present study.

In both human and veterinary medicine, there exists a great need for an effective antiscarring therapy, which can be administered topically without detriment to the health and function of ocular tissues. Furthermore, such therapies must also be safe when absorbed systemically. Mitomycin-C (MMC) has been used topically in human patients to decrease corneal haze following surgical wounded for many years. 68 As a potent alkylation agent, MMC prevents the replication of keratocytes and other progenitors of myofibroblasts, thereby mitigating the down-line degree of corneal haze. 69,70 While this drug is an extremely effective inhibitor of fibrosis due to its mechanism of action, its cytotoxic properties raise concerns regarding its widespread use. Several studies have shown that MMC produces a large, long-term acellular zone in treated corneas, devoid of stromal keratocytes. 68,69,71,72 In addition, there are concerns that MMC may cause endothelial cell damage in some patients, though this effect has not been significant in many recent studies. 73-78 The detrimental, long-term effect of MMC on the vital supportive cells of the cornea may predispose patients to future complications with wound healing or corneal function. MMC has not come into widespread use in veterinary patients.

Meanwhile, SAHA has demonstrated a reasonable margin of safety in phase 1 and 2 human clinical trials when given orally or intravenously. 79-83 Adverse effects, such as fatigue, gastrointestinal upset, hyperglycemia, hypocalcemia, anemia, and thrombocytopenia, were found at daily doses between 200 and 800 mg in humans, with dose-limiting toxicity encountered at 800 mg per day. 80 When given topically in the lagomorph, a dose of 25 μM was not associated with any clinical adverse effects, and no significant cytotoxicity within human and lagomorph fibroblast cultures was found based on trypan blue and TUNEL assay. 10 Likewise, pirfenidone has demonstrated a low rate of adverse events when administered systemically, including mild dizziness, nausea, gastrointestinal upset, cold sweat, photosensitivity, and skin rashes. 82-84 When administered topically in a study of the in vivo lagomorph eye, no cytotoxic changes were noted at a concentration of 5 mg/mL. 85 Furthermore, no significant cytotoxicity was noted in a human fibroblast culture based on a trypan blue assay at doses as high as 1 mg/mL. 85 These findings suggest that SAHA and pirfenidone, when administered topically at doses of approximately 25 μM and 1.08 mM, respectively, are expected to be well-tolerated and within an acceptable margin of safety. However, further studies to evaluate the systemic absorption and long-term effects of these topically administered therapies are warranted.

While multiple ex vivo corneal tissue culture models have been described in recent years, to the authors' knowledge this model represents the first to consistently and reliably produce an optical scar in the canine. 85,86 The use of an air/liquid interface has been established as a way to support both stromal keratocyte and epithelial cell health while mimicking an in vivo corneal surface environment. 86 Importantly, the corneas cultured within this model developed a robust optical scar in the absence of corneal innervation, a tear film, or influence from the immune system. For some applications, this could be a significant limitation. However, it is advantageous in the present study because it allows the contributions of keratocytes and myofibroblasts to be evaluated while eliminating outside (and potentially confounding) influences by bone marrow-derived cells. In addition, the effect of topical medications on epithelialization rate could be evaluated without confounding effects from epithelial-neuronal interactions. 87 In this way, therapeutic agents, such as SAHA and pirfenidone, could be evaluated consistently without impact from the systemic factors, which also play important roles in wound healing. Because each sample was cultured upon, and photographed against, a black background, the extent of corneal haze could be objectively quantified in a way that is not typically possible in live subjects. This method effectively eliminates the observer bias commonly associated with grading...
systems, and allows for extremely consistent imaging and measuring conditions.\textsuperscript{88,89} In addition, the photographic procedure used in this study requires less tissue handling than measurement via ultrasound biomicroscopy or confocal microscopy, and uses relatively inexpensive equipment, which requires minimal training for implementation by the vision scientist.

One significant drawback of the flat-topped ex vivo model was the initial air pocket present posterior to the endothelium of each cornea, followed by physical contact with the urethane scaffold after the air pocket dissipated. It is likely that these conditions resulted in damage and/or death of the fragile corneal endothelial cells. This was one likely contributor to the gradual onset of diffuse corneal edema, which was observed in all tissues by the end of the 21-day study period. In addition, recent evidence suggests that a posterior wound healing response is triggered upon disruption of the corneal endothelium.\textsuperscript{90} This could potentially cause fibrosis to form in the posterior stroma, which would confound assessment of anterior stromal haze. In the present study, fibrosis was limited to the anterior- to midstroma in all wounded corneas, based on slit-lamp examination and post hoc immunofluorescent microscopic evaluation. For this reason, it is unlikely that a posterior wound healing response adversely affected data collection in this case.

Another important drawback of the flat-topped ex vivo model was the crater-like pattern of subepithelial haze, which was not analogous to a typical post-PRK scar observed in vivo. After careful assessment of the model, it was hypothesized that this pattern may be due to the use of conical frustum-shaped scaffolds. As previously discussed, corneas settled onto the scaffold in a gravity-dependent way during the first 7 days of the study period, resulting in a slightly convex contour around the limbus, and a concave contour at the axis. The timing of this shape change coincided with the formation of subepithelial haze in wounded tissues. It is known that even small alterations in corneal curvature could result in a distortion of stromal lamellae and unpredictable changes to the formerly parallel arrangement of fibrils.\textsuperscript{91,92} This distortion, while contributing to a decrease of optical clarity on its own, may also significantly impact the migration of fibroblasts into the wound area, possibly forcing them along a path of least resistance. The authors suspected that this biomechanical alteration contributed to the crater-like gross pattern of fibrosis with radial spicules instead of a generalized ‘cloud’ of subepithelial haze in the wound bed. While the anomalous haze pattern did not preclude this ex vivo model from being implemented successfully in the testing of SAHA and pirfenidone, it did have a deleterious effect on the model’s external validity.

To simultaneously test the hypothesis that the crater-like haze pattern was dependent upon corneal curvature, and improve the model for future studies, a second novel ex vivo canine cornea model was developed with an innovative new scaffold design as described previously. With the modified model, low-melting point agarose was used as a conformer, which maintained each cornea’s convex shape throughout the entire 21-day study period. This adaptation resulted in a robust, round, cloud-like area of subepithelial haze, which was consolidated to the 6-mm area of the original excimer laser wound. Due to limitations in study resources, this model was developed as a pilot study only, and testing of SAHA and pirfenidone could not be repeated using the new ex vivo model. However, future use of an agarose dome model may provide a more favorable environment for all layers of the cornea, particularly the corneal endothelium. More in-depth assessment of endothelial cell health was outside the scope of the present study.

Additionally, due to the crater-like haze pattern present during SAHA and pirfenidone trial, traditional histologic sectioning and parafin embedding could not be performed. Depending upon the orientation of sectioning within each cornea, traditional histology would either severely overrepresent or underrepresent the degree of scar present. For this reason, immunohistochemical evaluation of en face samples was chosen. This method offered the benefit of demonstrating that âSMA was localized to the regions of most dense optical haze. However, due to the thickness of the en face corneal tissue and three-dimensional nature of the haze within the wound bed, quantification of the fluorescence signal could not be performed. For this reason, while immunohistochemistry was able to document the presence and distribution of âSMA within the haze area of each cornea, quantification of âSMA in these tissues was not possible.

Overall, the results of this study confirm that SAHA and pirfenidone are both promising, emerging therapies for the mitigation of subepithelial corneal haze. Furthermore, the ex vivo canine cornea model used in this study represents a novel way to consistently and reproducibly create a scar within the corneal stroma and monitor its behavior over time without the cost, potential inconsistencies, and other implications of a live animal colony. In the future, the ex vivo model described herein will be an ideal translational platform for the evaluation of many other topical therapies targeting different molecular pathways within the wounded corneal epithelium and stroma.

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