2012

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Recommended Citation

Tandon A, Tovey JCK, Waggoner MR, et al. Vorinostat: A Potent Agent to Prevent and Treat Laser-induced Corneal Haze. *J Refract Surg*. 2012;28(4):285-290. doi:10.3928/1081597X-20120210-01.

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HHS Public Access

Author manuscript

J Refract Surg. Author manuscript; available in PMC 2015 July 20.

Published in final edited form as:

J Refract Surg. 2012 April ; 28(4): 285–290. doi:10.3928/1081597X-20120210-01.

Vorinostat: A Potent Agent to Prevent and Treat Laser-induced Corneal Haze

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Abstract

PURPOSE—This study investigated the efficacy and safety of vorinostat, a deacetylase (HDAC) inhibitor, in the treatment of laser-induced corneal haze following photorefractive keratectomy (PRK) in rabbits in vivo and transforming growth factor beta 1 (TGFβ1) -induced corneal fibrosis in vitro.

METHODS—Corneal haze in rabbits was produced with −9.00 diopters (D) PRK. Fibrosis in cultured human and rabbit corneal fibroblasts was activated with TGFβ1. Vorinostat (25 μm) was topically applied once for 5 minutes on rabbit cornea immediately after PRK for in vivo studies. Vorinostat (0 to 25 μm) was given to human/rabbit corneal fibroblasts for 5 minutes or 48 hours for in vitro studies. Slit-lamp microscopy, TUNEL assay, and trypan blue were used to determined vorinostat toxicity, whereas real-time polymerase chain reaction, immunocytochemistry, and immunoblotting were used to measure its efficacy.

RESULTS—Single 5-minute vorinostat (25 μm) topical application on the cornea following PRK significantly reduced corneal haze ($P<.008$) and fibrotic marker proteins (α-smooth muscle actin and f-actin; $P<.001$) without showing redness, swelling, or inflammation in rabbit eyes in vivo screened 4 weeks after PRK. Vorinostat reduced TGFβ1-induced fibrosis in human and rabbit corneas in vitro in a dose-dependent manner without altering cellular viability, phenotype, or proliferation.

CONCLUSIONS—Vorinostat is non-cytotoxic and safe for the eye and has potential to prevent laser-induced corneal haze in patients undergoing PRK for high myopia.

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The authors have no financial or proprietary interests in the materials presented herein.

AUTHOR CONTRIBUTIONS

Study concept and design (D.J.G., R.R.M.); data collection (A.T., J.C.K.T., M.R.W., A.S., J.W.C., D.J.G., Y.L., G.S.S., R.R.M.); analysis and interpretation of data (R.R.M.); drafting of the manuscript (A.T., J.C.K.T., M.R.W., A.S., R.R.M.); critical revision of the manuscript (J.W.C., D.J.G., Y.L., G.S.S.); obtained funding (G.S.S., R.R.M.); supervision (G.S.S., R.R.M.)

Approximately 80% of Americans older than 12 years have refractive errors.[1] Laser eye surgeries such as photorefractive keratectomy (PRK), LASIK, and laser epithelial keratomileusis are frequently used to correct refractive errors and reduce dependency on spectacles or contact lenses.[1–3] Photorefractive keratectomy is considered safest among refractive surgeries but is often associated with postoperative corneal haze in some cases.[2,3] Extensive research revealed that excessive cytokine and growth factor activity in the stroma following PRK induces abnormal corneal wound healing, extracellular matrix deposition, keratocyte transformation to myofibroblasts, and haze formation in the cornea.[4–10] Among many cytokines, transforming growth factor beta 1 (TGFβ1) has been identified to play a major role in haze development, triggering transformation of quiescent keratocytes into corneal fibroblasts and myofibroblasts.[6–10] Selective modulation of TGFβ1 has emerged as an effective strategy to control laser-induced corneal haze.[7–10]

Histone acetyltransferase and histone deacetylase (HDAC) are enzymes involved in epigenetic regulation of DNA transcriptional activity via acetylation-deacetylation of histone proteins including TGFβ1.[11–14] Histone deacetylase inhibitors are shown to reduce TGFβ1-induced collagen synthesis, myofibroblast formation, and fibrosis in many tissues including the cornea.[12–14] In line with our hypothesis that epigenetic modulation is a novel and effective approach to treat corneal haze, we found significant inhibition of TGFβ1-mediated human corneal fibroblast transformation to myofibroblasts in vitro and PRK-induced corneal haze in rabbits in vivo by a potent HDAC inhibitor, trichostatin-A.[14] Unfortunately, it is not approved for human use; however, in 2006 an analog of trichostatin-A, vorinostat (suberoylanilide hydroxamic acid) was approved by the United States Food and Drug Administration for medical use. Currently, vorinostat is used clinically to treat cancer in human patients. The purpose of this study was to evaluate the usefulness of vorinostat in preventing postoperative PRK corneal haze by testing its efficacy and toxicity using in vivo PRK corneal haze rabbit and in vitro TGFβ1-induced corneal fibrosis models.

MATERIALS AND METHODS

In Vitro Studies

Culture Conditions and Viability Assay—Donor human and rabbit corneas were used to generate primary corneal fibroblasts using minimal essential medium (MEM) supplemented with 10% serum. Corneal fibroblasts grown in the presence of TGFβ1 (1 ng/mL) under serum-free conditions produced myofibroblasts. Short- and long-term vorinostat toxicity was examined by incubating cultures for 5 minutes and 48 hours, respectively. Cultures were seeded at 3×10^4 cells/well in 48 well culture plate in MEM 10% serum medium. When cells reached approximately 75% to 80% confluence, medium was switched to serum-free medium, and cells were incubated with/without vorinostat (0 to 25 μm) for 5 minutes or 48 hours, allowed to reach ~90% confluence, trypsinized, and stained with 0.4% trypan blue solution. Toxicity was determined by counting blue and white cells following manufacturer instructions.

Quantitative Real-time Polymerase Chain Reaction—Total ribonucleic acid (RNA) and complementary deoxyribonucleic acid (cDNA) were prepared as described

previously.[10,14] Real-time polymerase chain reaction (PCR) containing SYBR green, cDNA, forward/reverse primers for smooth muscle action (SMA)/fibronectin, and β-actin as housekeeping gene was performed as reported previously.[10,14]

Immunoblotting—Protein lysates were prepared and quantified by Bradford assay as reported previously.[10,14] Samples were resolved on 4% to 12% sodium dodecyl sulfate polyacrylamide gel, transferred onto polyvinylidene fluoride membrane, incubated with SMA and β-actin or β-tubulin antibodies followed by alkaline phosphatase-conjugated anti-mouse secondary antibodies and Nitro-blue tetrazolium chloride and 5-Bromo-4-chloro-3′-indolyphosphate p-toluidine (NBT-BCIP) developing reagents.

Quantification and Statistical Analyses—Smooth muscle actin-positive cells in six randomly selected areas in corneal sections were counted and standard error means were calculated as reported previously.[10,14] Statistical analysis was performed with two-way analysis of variance (ANOVA) and Bonferroni multiple comparisons for real-time PCR, one-way ANOVA followed by Tukey multiple comparisons for cellular viability, and one-way ANOVA with Wilcoxon rank sum test for corneal haze. The value $P<.05$ was considered significant.

In Vivo Studies

Haze Generation, Vorinostat Treatment, Microscopy, and Tissue Collection—The Institutional Animal Care and Use Committee approved the study and animals were treated in accordance with the Association for Research and Vision in Ophthalmology Statement for the use of animals in ophthalmic and vision research.

Twelve female New Zealand white rabbits were anesthetized with intramuscular ketamine (50 mg/kg) and xylazine (10 mg/kg) and one drop of 0.5% proparacaine was topically instilled in the cornea. To induce haze, −9.00-diopter (D) PRK with a 6-mm ablation zone was performed on the central corneal stroma with an excimer laser.[10,14] Vorinostat (25 μm) or balanced salt solution was topically applied on the stroma for 5 minutes immediately after PRK. Contralateral eyes served as controls. Corneal health and haze levels in rabbits were gauged with slit-lamp and stereo-microscopy in a masked manner as reported previously.[10,14] Rabbits were euthanized 4 weeks after PRK (pentobarbitone, 150 mg/kg) and corneas were subsequently excised and snap frozen in optical coherence tomography compound. Tissue sections (7 μm) were cut and maintained at −80°C.

Immunofluorescence Studies to Quantify Fibrosis and Toxicity—Immunostaining and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL assay [ApopTag kit, Chemicon; Millipore Corp, Billerica, Massachusetts]) were performed by incubating rabbit corneal sections with α-SMA antibody or Alexa594-conjugated phalloidin or TdT Enzyme followed by secondary antibody or anti-digoxigenin conjugate, and mounting in DAPI-containing medium following previously reported protocols.[10,14]

RESULTS

Effect of Vorinostat on Corneal Fibroblast Viability and TGFβ1-induced Myofibroblast formation in vitro

Figure 1 shows vorinostat did not alter cellular viability of corneal fibroblasts significantly in a dose-dependent manner after 5 minutes of treatment. Rabbit corneal fibroblasts on 48-hour vorinostat incubation under similar conditions showed similar results (data not shown).

Figure 2 shows the effect of single 5-minute vorinostat treatment on mRNA levels of SMA and fibronectin. Transforming growth factor beta 1-treated human corneal fibroblast produced a 9-fold increase in SMA ($P<.001$) and 3-fold increase in fibronectin mRNA levels ($P<.01$). Vorinostat treatment of 10 and 25 μm reduced TGFβ1-induced mRNA levels of SMA 80% ($P<.001$) and 86% ($P<.001$) and fibronectin mRNA 54% ($P<.001$) and 64% ($P<.001$), respectively.

Figure 3A shows anti-fibrotic effects of single 5-minute vorinostat treatment on corneal fibroblasts measured with western blot analysis. Transforming growth factor beta 1 treatment significantly increased SMA protein levels and 5-minute 10- or 25-μm vorinostat dose decreased SMA protein by 45% or 50%, respectively (Fig 3B).

Figure 4 shows anti-fibrotic response of long-term (48 hours) vorinostat exposure in human corneas in vitro. The anti-fibrotic effects of vorinostat were dose-dependent as incremental decrease in TGFβ1-induced SMA expression was noted at increasing vorinostat doses with a complete absence of SMA at 10 and 25 μm (data not shown).

Microscopy and Immunohistochemical Measurements of the Effect of Vorinostat on Corneal Haze In Vivo

Figure 5 shows results of single topical application of 25 μm vorinostat on haze reduction in rabbit corneas 4 weeks after PRK evaluated with slit-lamp microscopy. Photorefractive keratectomy-treated rabbit corneas showed a mean haze score of 3±0.7 whereas vorinostat treatment demonstrated significant haze reduction (1.1±0.5; $P<.008$).

Figure 6 shows inhibitory effects of vorinostat on myofibroblast development measured with SMA and f-actin immunohistochemistry. High SMA and f-actin expression in PRK-treated rabbit corneas confirmed myofibroblast and haze formation (Figs 6A and 6C). Smooth muscle actin and f-actin staining was significantly decreased in rabbit corneas (Figs 6B and 6D) that received single 5-minute vorinostat (25 μm) topically immediately after PRK, suggesting that vorinostat is an efficient anti-fibrotic agent for preventing corneal haze. Quantification of SMA in these corneas revealed that vorinostat inhibited SMA by 42% ($P<.001$) in rabbit corneas in vivo (Fig 7).

Vorinostat Toxicity In Vivo

No detection of inflammation, redness, swelling, or discharge in the rabbit eye with slit-lamp microscopy suggests that vorinostat is non-toxic to the rabbit eye. The effects of vorinostat on keratocyte death were analyzed with TUNEL assay. Figure 8 shows TUNEL staining observed in rabbit corneas collected 6 hours or 4 weeks after PRK with/without

vorinostat (25 μm) application. Quantification of TUNEL+ cells in rabbit corneas with/without vorinostat collected 6 hours or 4 weeks after PRK detected no significant differences in TUNEL+ cells, suggesting that topical vorinostat application is safe for the rabbit cornea in vivo. Detection of many TUNEL+ cells in the anterior stroma 6 hours after PRK or in corneal epithelium 4 weeks after PRK is not surprising and is consistent with earlier reports.[5,13]

DISCUSSION

Maintenance of corneal transparency is imperative for normal vision. Infection or injury to the cornea can initiate wound healing, resulting in scarring and vision loss.[4–6] Corneal repair is orchestrated primarily by TGFβ1-mediated excess deposition of extracellular matrix along with the transformation of keratocytes to myofibroblasts, which causes corneal fibrosis and reduces optical clarity.[6–10] The present study provides further support to our central hypothesis that epigenetic modulation in the cornea offers an effective approach to prevent corneal fibrosis in vivo.

The break in the epithelial barrier following refractive laser surgery exposes the stroma to many cytokines and growth factors released from corneal epithelium, tears, and transient inflammatory cell population often leading to excessive corneal healing and haze complication.[6–9] Our recent RNA interference experiments suggest that TGFβ1 induces fibrosis in the cornea via Smad signaling (Mohan et al, unpublished data, September 2011) and histone acetylation is reported to regulate Smad-mediated gene expression.[15] Thus, we tested the anti-fibrotic effect of vorinostat in a rabbit model of PRK-induced corneal haze. Our data demonstrate that a single topical prophylactic vorinostat treatment significantly decreased postoperative PRK corneal haze in vivo in rabbits as detected with slit-lamp microscopy and histological examinations. These results suggest that epigenetic modulation by HDAC inhibition can effectively serve as a means of preventing PRK-induced corneal haze.

At present, steroids and mitomycin C are used for the clinical management of corneal haze.[3,5] The beneficial effects of steroids for inhibiting haze are inconclusive. Mitomycin C topical use after PRK is highly effective and generally provides excellent outcomes; however, it has been associated with multiple severe adverse effects including limbal and scleral necrosis, corneal endothelial damage, and loss of keratocytes.[3,16–19] Our in vitro human cornea and in vivo rabbit cornea toxicity data suggest that vorinostat is safe for corneal application. Our results indicate that a single topical application of this agent has therapeutic potential for treatment of corneal fibrosis in vivo with no major side effects.

This study demonstrates that vorinostat can effectively prevent corneal haze by interrupting the biological effects of TGFβ1 and may have potential clinical applications for preventing corneal haze in patients undergoing PRK for high myopia.

Acknowledgments

Supported in part by grants RO1EY17294 (R.R.M.), R01EY005587 (G.S.S.) from National Eye Institute, Bethesda, Maryland; 1I01BX00035701 (R.R.M.) from Veteran Health Affairs, Washington, DC; and an unrestricted grant from Research to Prevent Blindness, New York, New York.

The authors thank Heartland Eye Bank, St Louis, Missouri, for providing donor human corneas; Vanessa Lopez, MD and Dr Frank G. Rieger III from the Harry S. Truman VA Hospital, and Chuck W. Hamm, Mason Eye Institute, University of Missouri-Columbia, for their help in slit-lamp microscopy.

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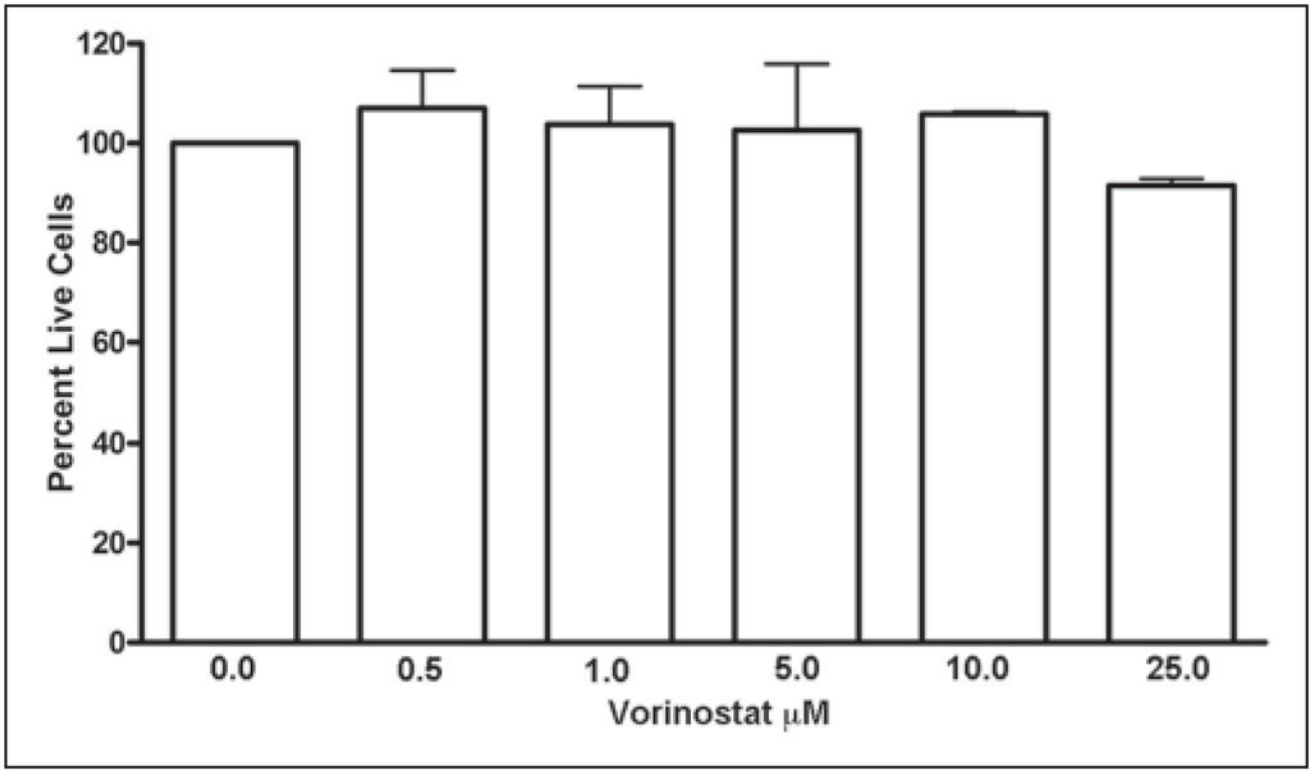

Figure 1.

Dose-dependent effects of vorinostat 5-minute treatment on human corneal fibroblasts viability determined with trypan blue assay. None of the tested doses reduced cellular viability significantly.

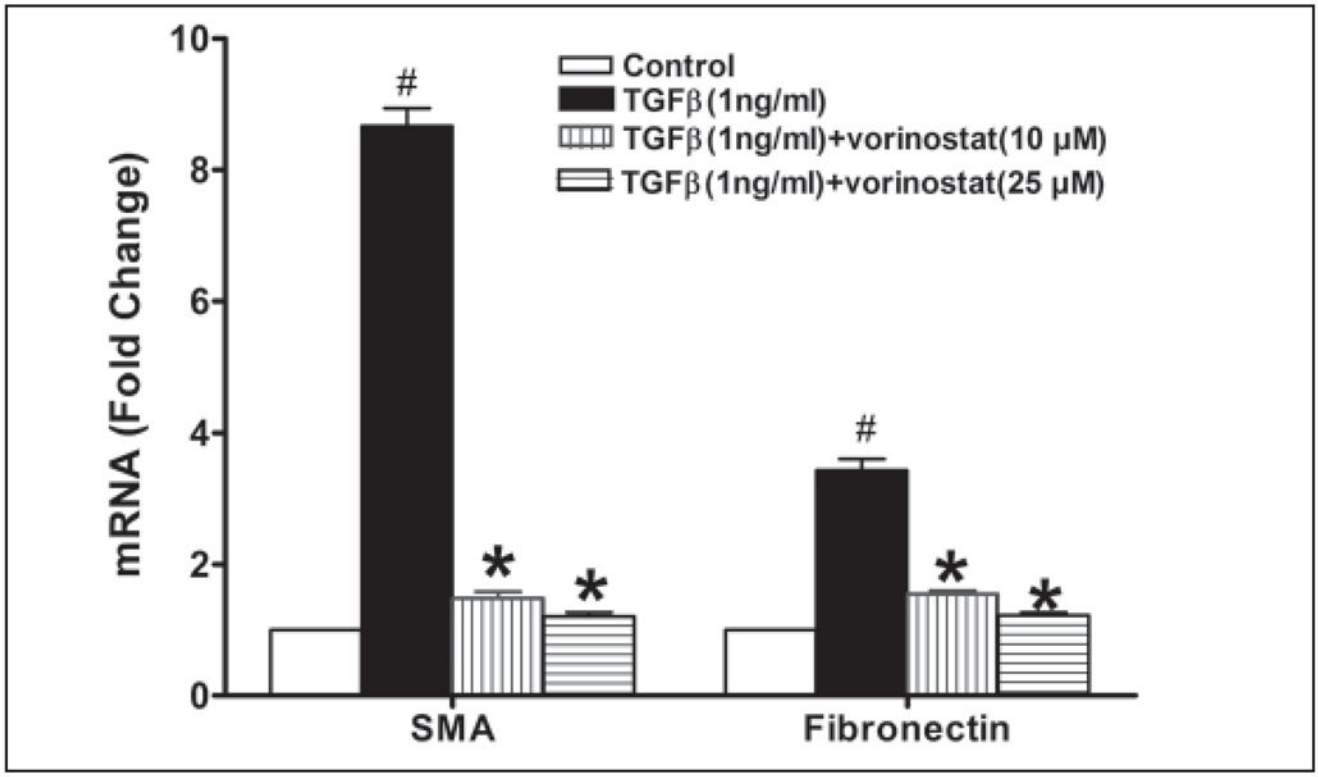

Figure 2.

Effect of vorinostat treatment on smooth muscle actin (SMA) and fibronectin mRNA quantified with real-time polymerase chain reaction. Corneal fibroblast cultures grown in transforming growth factor beta 1 (TGFβ1) under serum-free conditions were exposed to vorinostat (10 or 25 μm) for 5 minutes. Vorinostat significantly reduced mRNA levels of SMA (80% to 86%, respectively) and fibronectin (54% to 64%, respectively). $^{\#}P<.001$ compared to control; $^{*}P<.001$ compared to TGFβ1.

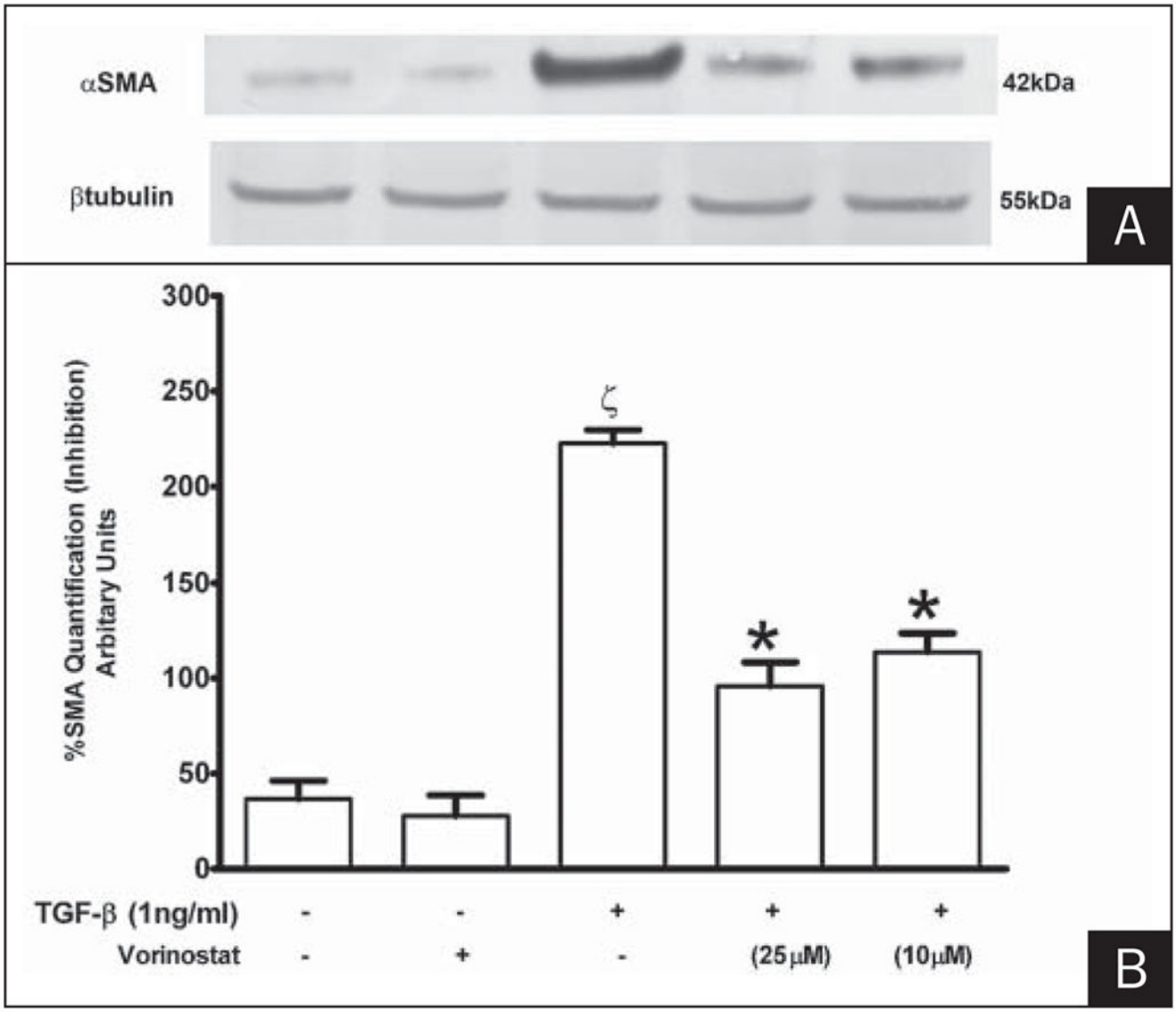

Figure 3.

Effect of vorinostat on smooth muscle actin (SMA) inhibition determined with **A)** western blot and **B)** quantification. Human corneal fibroblasts grown in transforming growth factor beta 1 (TGFβ1) under serum-free conditions and exposed to vorinostat (10 or 25 μm) for 5 minutes showed marked decrease in SMA (45% to 50%, respectively). Beta tubulin was used as a housekeeping gene. $^{\zeta}P<.001$ compared to control; $^{*}P<.001$ compared to TGFβ1.

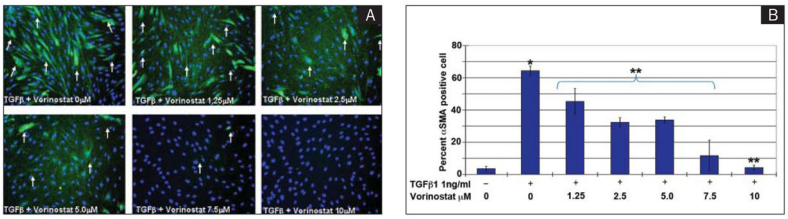

Figure 4.

Dose-response effect of long-term vorinostat exposure on smooth muscle actin (SMA) reduction determined with **A)** immunocytochemistry and **B)** quantification. Rabbit corneal fibroblasts grown to 80% confluence were incubated with transforming growth factor beta 1 (TGFβ1) with/without vorinostat (0 to 10 μm) under serum-free conditions for 48 hours. Vorinostat showed a significant dose-dependent decrease in SMA at all tested doses with highest inhibition at 10 μm. Original magnification ×100. **P*<.05 no treatment vs TGFβ1, and ***P*<.05 TGFβ1 vs vorinostat.

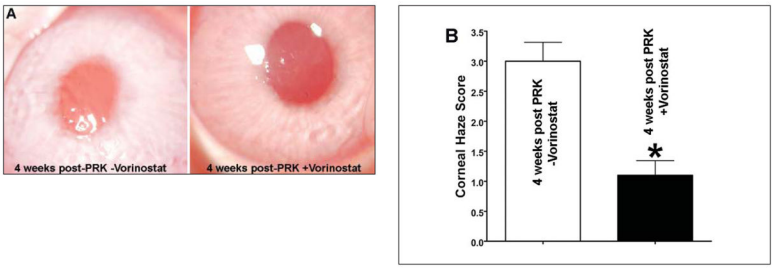

Figure 5.

A) Representative slit-lamp microscopy images demonstrating haze density in rabbit corneas treated with photorefractive keratectomy (PRK) with/without vorinostat (25 μm) 4 weeks postoperatively. **B)** Haze quantification showed that a single 5-minute topical application of vorinostat significantly decreased haze in postoperative PRK corneas in vivo. **P*<.008.

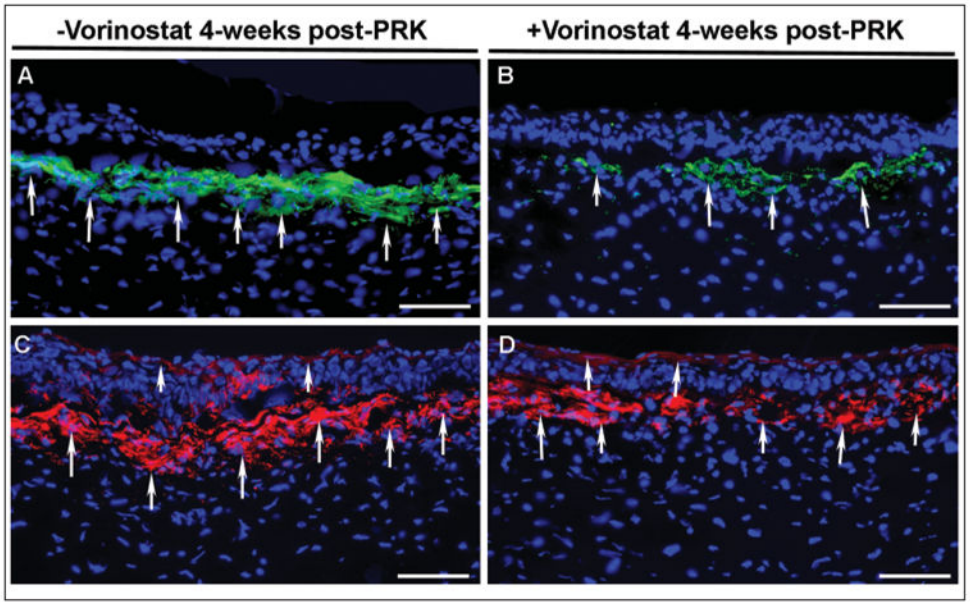

Figure 6.

Representative immunohistochemistry images 4 weeks after photorefractive keratectomy showing **A, B)** smooth muscle actin (SMA) and **C, D)** f-actin levels in corneas treated with/without vorinostat (25 μm). Blue = DAPI-stained nuclei, Green = SMA+, red = f-actin+ cells. Vorinostat-treated corneas showed a significant decrease in SMA (B) and f-actin (D) compared to untreated corneas (A, C). **P*<.001. Scale bar = 100 μm

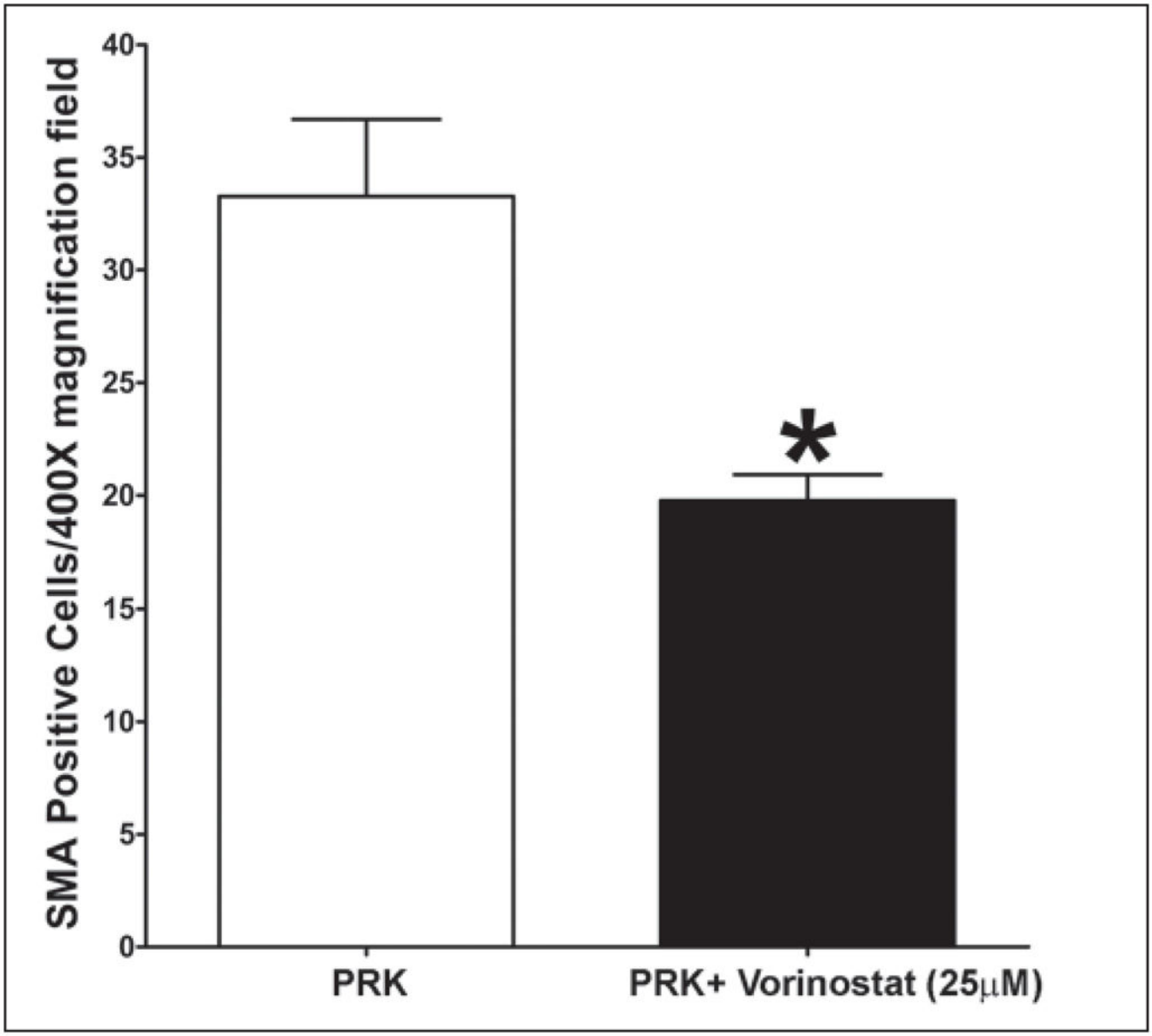

Figure 7.

Quantification of smooth muscle actin (SMA)+ cells 4 weeks after photorefractive keratectomy (PRK) in corneas treated with/without vorinostat (25 μm). Vorinostat treatment significantly decreased SMA+ cells (**P*<.001).

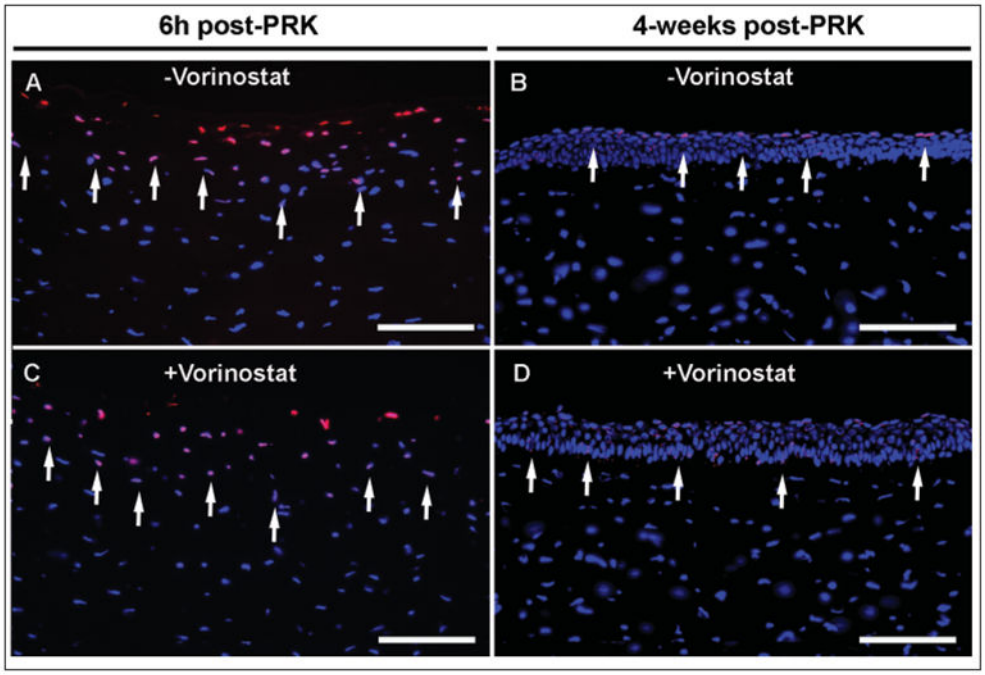

Figure 8.

Representative images showing TUNEL+ cells in rabbit corneas treated with/without vorinostat (25 μm) collected **A, B)** 6 hours or **C, D)** 4 weeks after photorefractive keratectomy (PRK). No significant differences in TUNEL+ cells in rabbit cornea with/without vorinostat collected at early (6 hours) or end-point (4 weeks) suggests that a single 5-minute, 25-μm topical dose of vorinostat is safe for the rabbit eye. The amounts of TUNEL+ cells detected 6 hours after PRK in the anterior stroma or 4 weeks after PRK in the epithelium are consistent with earlier reported findings.[5,13] Red = TUNEL+ cells, Blue = DAPI-stained nuclei. Scale bar = 100 μm