

A Novel Topical Ophthalmic Formulation to Mitigate Acute Mustard Gas Keratopathy In Vivo: A Pilot Study

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Purpose: This pilot study investigated the in vivo therapeutic potential and tolerability of a multimodal ophthalmic formulation, topical eye drops (TED), for acute mustard gas keratopathy (MGK) using a rabbit model.

Methods: Twenty New Zealand White rabbits were used. Only right eyes of 18 rabbits (oculus dexter [OD]) received single sulfur mustard gas (SM) vapor injury, whereas contralateral eyes were left untreated or received TED for tolerability evaluation. Two rabbit eyes received no treatment and served as age-matched naive control. The four groups were: Naive (oculus sinister [OS] untreated eyes; n = 9); TED (OS treated only with TED BID for 3 days; n = 9); SM (OD exposed to SM vapor; n = 9); and SM+TED (OD exposed to SM+TED BID for 3 days; n = 9). Ocular examination in live rabbits were performed utilizing slit-lamp biomicroscopy, Fantes grading system, fluorescein staining, Schirmer's tests, pachymetry, and applanation tonometry. Cellular and molecular changes in rabbit corneas were assessed after humane euthanasia on day-3 and day-7 with histopathological and real-time polymerase chain reaction PCR techniques.

Results: TED to rabbit eyes was found tolerable in vivo. SM-exposed eyes showed significant increase in Fantes scores, central corneal thickness (CCT), Schirmer's test, epithelium-stroma separation, and corneal edema. TED mitigated clinical symptoms by reducing corneal edema, Fantes scores, CCT, and Schirmer's test. Further, TED decreased SM-induced corneal haze, inflammatory and profibrotic markers, transforming growth factor-TGF- β 1 and cyclooxygenase-2COX-2, and damage to corneal structure, including epithelial-stromal integrity.

Conclusions: The developed multimodal eyedrop formulation, TED, has potential to mitigate acute MGK effectively in vivo.

Translational Relevance: TED is effective against MGK

Introduction

Sulfur mustard gas (SM) was first used as a chemical weapon during World War I.¹ Since then, SM has been used in many wars, including the Iran-Iraq war and, most recently, in the Syrian War.² The use of SM as a weapon of mass destruction is an imminent threat because of availability in stockpile and

ease of production in large quantities. Hundreds of thousands of tons of SM and other chemical weapons are disposed along the coastlines of Russian, United States, Japanese, and European borders, and remain a threat to environmental and human health in these areas.³ SM is an oily noxious gas, and can easily penetrate mucous membranes, causing debilitating wounds in the skin, lungs, and eyes, compromising the quality of life for decades.¹⁻⁵ The eye is the

most vulnerable to SM exposure and exhibits dose-dependent damage.^{6,7} SM is a potent alkylating agent and rapidly penetrates into the eye up on contact, and causes severe corneal injury, ocular pain, and acute vision loss within hours.^{8–11} Clinically, it causes a grade -III or -IV clinical ocular pathologies characterized by severe ocular pain, inflammation, recurrent corneal epithelial erosions, ulcerations, epithelial-stromal separation, haze/fibrosis, and neovascularization called mustard gas keratopathy (MGK).^{6–11} MGK results in progressive corneal degeneration, with biphasic acute and delayed-onset manifestations via multiple mechanisms.^{11,12}

Safe and effective therapies for the mitigation of MGK are currently lacking. Mild symptoms of MGK are manageable, however, outcomes in patients vary considerably.¹³ Amphoteric rinsing solutions and doxycycline hydrogels have been shown to exhibit moderate clinical benefits.^{14,15} Corneal rinsing immediately after exposure helps to reduce the concentration of SM on the eye. Doxycycline has shown some encouraging results in treating respiratory, integumentary, and ocular SM damage. A combination of dexamethasone, doxycycline, and silibinin has been shown to reverse the nitrogen mustard (SM analog)-induced corneal injuries in vivo.¹⁶ Several studies have demonstrated that the clinical utility of a steroid-based therapy is limited by therapy's duration with a recurrence of clinical signs following the withdrawal of the steroid.^{17–19} In addition, steroid use in patients can cause severe side effects, including glaucoma.²⁰ Recently, the potential success of concurrent topical steroids with other agents, such as nonsteroidal anti-inflammatory drugs (NSAIDs), zinc-desferrioxamine, and others, etc. to mitigate SM-induced corneal pathology has been reported.^{21,22} However, these formulations rely heavily on a steroidal component. In fact, most of the current topical therapies available for the treatment of MGK are steroid-based drugs. Thus, the development of nonsteroidal topical therapy is utmost required for the treatment of MGK.

In an attempt to improve therapeutic options for mustard-induced ocular injury, we formulated a combination of U.S. Food and Drug Administration (FDA)-approved nonsteroidal drugs designed to mitigate ocular damage after exposure to SM and named “topical eye drops” (TED). The formulation of TED contains 4 FDA-approved drugs (0.5% ketorolac, 25 μ M SAHA, 25 μ M enalapril, and 10% Vitamin C). Ketorolac is a non-steroidal anti-inflammatory drug (NSAID), which works via blocking cyclooxygenase (COX) enzymes, reducing SM-induced ocular pain and inflammation. Ketorolac is commonly used in ophthalmic care as an alternative or adjunctive

therapy in the treatment for post-operative inflammation and pain.^{23–25} Suberoylanilide hydroxamic acid (SAHA or vorinostat) is a histone deacetylase inhibitor (HDACi) and is used clinically to treat cancer in humans.²⁶ Previously, we demonstrated that topical application of SAHA to the injured cornea significantly reduces transforming growth factor beta (TGF- β)-induced corneal fibrosis in vitro and in vivo.^{27–29} Furthermore, we found SAHA as an effective and safe alternative to mitomycin C for preventing scarring after glaucoma filtration surgery³⁰ and corneal fibrosis in vivo.³¹ Enalapril is an angiotensin-converting enzyme (ACE) inhibitor described for its role in the inhibition of vascular endothelial growth factor (VEGF) VEGF-mediated corneal neovascularization in vivo.³² Also, topical enalapril drops in rabbit eyes have been shown to reduce intraocular pressure (IOP).³³ Topical application of ascorbic acid has been shown to attenuate alkali and laser-induced damage to the cornea.^{34–36} Lee et al.³⁷ showed that ascorbic acid decreased corneal neovascularization in rabbits in vivo by decreasing VEGF and MMP-9 levels³⁷. Additionally, ascorbic acid has shown promising results in accelerating corneal epithelial healing and protecting basal epithelial cells in vivo.^{38,39} In the present study, we used a rabbit model of acute SM injury, MGK, and tested the tolerability and effectiveness of the TED formulation for the recovery of ocular damage. Our clinical and pathophysiological investigations indicated that TED is well tolerated at mitigating very acute MGK.

Methods

Animals

The Institutional Animal Care and Use Committees of the University of Missouri, Columbia, Missouri and the MRI Global, Kansas City, Missouri have approved the study. Animals were treated in accordance with the ARVO Statement for Use of Animals in Ophthalmology and Vision Research. For this study, male New Zealand White rabbits from Charles Rivers Laboratories were used. All rabbits were between 2.5 to 4.0 kg and in good health and free from any signs of ocular clinical disease at the time of arrival and during the study. Upon delivery, animals were inspected for signs of ill-health and quarantined in the animal facility for more than two weeks under the Veterinarian supervision. All rabbits were fed certified feed and water ad libitum. The rabbits were housed in environmentally controlled rooms at a temperature between 16°C and 22°C and relative humidity of 50% \pm 20% with a 12-hour light/dark cycle per day.

SM Exposure

All the SM exposure procedure was performed at MRI Global facility in Kansas City, MO. Twenty rabbits were used for the study. A randomly selected one eyes of eighteen rabbits, were dosed with SM after anesthesia via a subcutaneous administration of ketamine (up to 60 mg/kg) and xylazine (up to 5 mg/kg). Two rabbits (four eyes) were left untreated to serve as an age-matched naive control for the study. A clinical eye examination looking for potential defects or abnormalities was performed and buprenorphine HCl (0.05-0.1 mg/kg) was administered, 30-60 minutes before anesthesia for pain management. Once the rabbit was anesthetized, ocular vapor goggles were secured around the animal's head for SM vapor exposure inside the hood line. The animals were placed on top of an absorptive pad and positioned on a shelf unit within the chemical hood for SM exposure. All animals received a vapor exposure of SM at a target concentration of 200 mg-min/m³ for 8 minutes. Animals remained on the shelf unit throughout the exposure period until a sufficient wash-out of the SM in the goggles was achieved. After the target SM vapor exposure was completed, the goggles were removed after 2 minutes, and both eyes were rinsed with balanced salt solution (BSS) to decontaminate the exposed area in the eyes. Animals were recovered from anesthesia post-SM exposure in the chemical fume hood for up to 2 hours. Once the animal's eyes have been washed and the rabbits were approved for removal by the attending veterinarian, the rabbits were placed in a clean container for transport to another laboratory for the duration of the study. In addition to buprenorphine administration, the lights in the animal rooms were dimmed or turned off to aid in pain management for a time to be determined by the attending veterinarian observations.

TED Preparation and Administration to the Eye

The aqueous formulation of TED was prepared under sterile conditions by adding the final concentrations of compounds, which include: 25 μM SAHA (Cat. No 10009929, Cayman, Ann Arbor, MI), 25 μM of enalapril (Cat. No 555250 Toronto Research Chemicals, North York, ON, Canada), 0.5% ketorolac (Cat. No K1136, Millipore Sigma, St. Louis, MO), and 10% Vitamin-C (Cat. No A7506, Millipore Sigma, St Louis, MO) to water.

For TED administration, the rabbits were gently restrained with their heads turned slightly to allow the eye to face the ceiling in order to be dosed. The upper and lower eyelids were held open with the fingers of one

hand, while the other hand delivered two drops of TED onto the eye. The animals were held in this position for approximately 5 seconds before being released and allowing them to blink normally. After SM exposure, eighteen animals were split into four groups to study the safety and effectiveness of TED: group-I (Naive) consisted of the untreated eyes (oculus sinister [OS]; n = 9); Group-II (TED) comprised eyes with TED treatment twice a day for 3 days without SM (OS; n = 9) for safety study; Group-III (SM) comprised eyes with SM vapor exposure only (oculus dexter [OD]; n = 9); and Group-IV (SM+TED) included eyes with SM vapor exposure followed by TED treatment twice a day for 3 days beginning 2 hours post-SM vapor exposure, (OD; n = 9). Two rabbits of similar age whose eyes did not receive any treatment served as age-matched naive controls (n = 4; 2 OS and 2 OD). The details of the research design, such as treatment to eyes, sample size, timing of treatment, and clinical examinations timing, are provided in [Table 1](#).

Clinical and Biomicroscopy Evaluations

The clinical evaluation and biomicroscopy imaging procedures were performed on live animals after SM exposure at regular intervals (pre-SM exposure and post-SM exposure on day-3 and day-7) under general anesthesia. Slit-lamp microscope (Kowa, SL-15 portable slit-lamp, Torrance, CA) coupled with a high definition digital imaging system (Kowa, portable VK-2 Ver. 5.50) was used to record clinical ocular health assessment.⁴⁰ Additionally, eyes were examined with a stereo-microscope (Leica MZ16F, Leica Microsystems Inc., Buffalo Grove, IL) equipped with a digital camera (SpotCam RT KE, Diagnostic Instruments Inc., Sterling Heights, MI) to access and record the levels of ocular damage from SM exposure.⁴⁰ All in vivo clinical examinations were performed by at least two independent investigators (RT, PB, SG, LM, JR, or MK) in a masked manner following Roper-Hall classification providing prognostic guidelines based on the level of corneal involvement and limbal ischemia.⁴¹ Eyes were kept moist during the entire procedure with BSS to prevent corneal desiccation.

Corneal epithelial defects were observed with a commercial ophthalmic fluorescein-stain (Altafluor Benox, Sigma Pharmaceuticals, North Liberty, IA).^{40,42} The epithelial defects were assessed under a cobalt light blue filter and recorded under a green fluorescence filter using a stereo-microscope equipped with an image-capturing system (Leica MZ16F, Leica Microsystems Inc.) and with a digital camera (SpotCam RT KE, Diagnostic Instruments Inc.).^{40,42} The clinical findings were documented by photography and scored by a minimum of two independent

Table 1. Detailed Research Design Showing Various Groups, Animal Identification, Sample Size, Treatments, Imaging-Timing, and Study-Time

Groups	Treatment to the Eye	Study Time	Sample Size	Used Eye	Animal ID	Treatment Start-Time	Treatment Stop-Time	Clinical Eye Examinations Times
Gr-(Nave)	No treatment	Day-3	n = 4	OS	rt01	na	na	Pre-SM, day-3
				OS	rt02	na	na	Pre-SM, day-3
				OS	rt03	na	na	Pre-SM, day-3
				OS	rt04	na	na	Pre-SM, day-3
Gr-(TED)	TED (without SM exposure)	Day-3	n = 4	OS	rt10	2h post-SM	day-4	Pre-SM, day-3
				OS	rt11	2h post-SM	day-4	Pre-SM, day-3
				OS	rt12	2h post-SM	day-4	Pre-SM, day-3
				OS	rt13	2h post-SM	day-4	Pre-SM, day-3
Gr-(SM)	SM vapor	Day-3	n = 4	OD	rt01	na	na	Pre-SM, day-3
				OD	rt02	na	na	Pre-SM, day-3
				OD	rt03	na	na	Pre-SM, day-3
				OD	rt04	na	na	Pre-SM, day-3
Gr-(SM+TED)	SM vapor + TED	Day-3	n = 4	OD	rt10	2h post-SM	day-4	Pre-SM, day-3
				OD	rt11	2h post-SM	day-4	Pre-SM, day-3
				OD	rt12	2h post-SM	day-4	Pre-SM, day-3
				OD	rt13	2h post-SM	day-4	Pre-SM, day-3
Gr-(Nave)	No treatment	Day-7	n = 5	OS	rt05	na	na	Pre-SM, day-3
				OS	rt06	na	na	Pre-SM, day-3, day-7
				OS	rt07	na	na	Pre-SM, day-3, day-7
				OS	rt08	na	na	Pre-SM, day-3, day-7
				OS	rt09	na	na	Pre-SM, day-3, day-7
Gr-(TED)	TED (without SM exposure)	Day-7	n = 5	OS	rt14	2h post-SM	day-4	Pre-SM, day-3, day-7
				OS	rt15	2h post-SM	day-4	Pre-SM, day-3, day-7
				OS	rt16	2h post-SM	day-4	Pre-SM, day-3, day-7
				OS	rt17	2h post-SM	day-4	Pre-SM, day-3, day-7
				OS	rt18	2h post-SM	day-4	Pre-SM, day-3, day-7
Gr-(SM)	SM vapor	Day-7	n = 5	OD	rt05	na	na	Pre-SM, day-3, day-7
				OD	rt06	na	na	Pre-SM, day-3, day-7
				OD	rt07	na	na	Pre-SM, day-3, day-7
				OD	rt08	na	na	Pre-SM, day-3, day-7
				OD	rt09	na	na	Pre-SM, day-3, day-7
Gr-(SM+TED)	SM vapor + TED	Day-7	n = 5	OD	rt14	2h post-SM	day-4	Pre-SM, day-3, day-7
				OD	rt15	2h post-SM	day-4	Pre-SM, day-3, day-7
				OD	rt16	2h post-SM	day-4	Pre-SM, day-3, day-7
				OD	rt17	2h post-SM	day-4	Pre-SM, day-3, day-7
				OD	rt18	2h post-SM	day-4	Pre-SM, day-3, day-7
Age-matched Nave control	Both eyes left untreated	Day-3	n = 2	OS	rt19	na	na	Pre-SM, day-3
				OD	rt19	na	na	Pre-SM, day-3
				OS	rt20	na	na	Pre-SM, day-3, day-7
				OD	rt20	na	na	Pre-SM, day-3, day-7

observers (RT, PB, SG, LM, JR, or MK) in a masked manner. The size of the corneal epithelial defect in rabbit eyes was digitally computed by counting pixels of the defect areas in images taken with a stereomicroscope at 7.1x magnification using Photoshop software (Adobe, San Jose, CA) following the procedure reported earlier.⁴³

Ultrasonic pachymeter (Accutome, AccuPach VI Pachymeter, Malvern, PA) was used to assess the central corneal thickness (CCT) and edema after SM exposure at day-3 and day-7 with the rabbits under general anesthesia. A handheld tonometer (Tono-Pen AVIA Tonometer, Scottsdale, AZ) was used to record changes in intraocular pressure (IOP) at all tested times.⁴⁰ Schirmer Tear Test (STT) Strips (Fisher Scientific, Pittsburgh, PA) were used to quantify tear volume at each time-point in live animals.

Corneal Tissue Collection, Histopathological examinations, and Immunofluorescence

At the study termination, animals were humanely euthanized on day-3 and day-7 after all final clinical examinations were completed as described in Table 1. Animals were anesthetized prior to intravenous pentobarbital (SomaSol, Euthanasia-III Solution, Henry Schein, Dublin, OH) (150 mg/kg) administration for euthanasia. The corneas were collected using sharp dissection and placed in 15 × 15 × 5 mm molds (Fisher Scientific, Pittsburgh, PA) with optical cutting temperature (OCT) compound (Sakura Finite, Torrance, CA, USA).^{42,43} The molds were immediately snap-frozen in a liquid nitrogen container immersed in a cryo-cup containing 2-methylbutane, and blocks stored at -80°C until further use. The corneas were cut in two halves: one half was used for histology studies, while the other half was used for molecular studies. For histology studies, serial corneal sections (8-μm thick) were prepared using a cryostat (HM525 NX UV; Microm GmbH, Walldorf, Germany), and placed on labeled glass microscope slides (Superfrost Plus; Fisher Scientific), and stored at -80°C until analysis.

For histopathological examinations, hematoxylin & eosin (H&E) staining was performed following standard technique.⁴⁰ Briefly, cryo-frozen corneal sections were incubated for 15-20 minutes at room temperature and then washed in PBS for 10 minutes and dipped in hematoxylin for 5 minutes followed by a rinse in running tap water, 1 dip in 1% acid-alcohol, and 10-15 dips in 0.3% ammonia water. The slides were incubated in 95% alcohol followed by eosin. The tissues were then dehydrated in absolute alcohol, cleared in CitriSolv solution (Decon Laboratories, King of

Prussia, PA), and finally mounted in cytooseal (Richard-Allan Scientific, Kalamazoo, MI). The images of H&E stained tissues were captured with a bright-field microscope (Leica) equipped with a digital camera and imaging software (SpotCamRT KE; Diagnostic Instruments, Sterling Heights, MI, USA). The level of corneal thickness was digitally measured by counting pixels of corneal length showing thickness in three randomly selected non-overlapping regions at 100X magnification using Photoshop software following the procedure reported earlier.⁴⁵ Immunofluorescence staining was performed to measure the expression of integrin-β4 and counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), a blue stain of nuclei. For immunofluorescence, corneal sections were first blocked with 2% bovine serum albumin at room temperature for 30 minutes. Protein-specific mouse monoclonal primary antibody, anti-integrin-β4 (1:200 dilution, sc-13543 Santa Cruz Biotechnology, Dallas, TX), was probed and incubated for 4 hours, and then incubated with Alexa-Fluor 594 goat anti-mouse IgG secondary antibody (1:1000 dilution, A21135; Invitrogen, Carlsbad, CA, USA) for 1 hour at room temperature. A digital imaging software (SpotCamRT KE; Diagnostic Instruments, Sterling Heights, MI, USA) associated with the fluorescence microscope (Leica) was used to image-capture of stained corneal sections.

RNA Extraction, cDNA Synthesis, and Quantitative Polymerase Chain Reaction (PCR)

For molecular studies, corneal tissues were minced in a tissue lyser (TissueLyser LT, Qiagen, Valencia, CA) in RLT buffer (Qiagen, Valencia, CA, USA) and total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The reverse transcriptase enzyme kit was used to synthesize first-strand cDNA (Promega, Madison, WI).^{40,42,44} The One Step Plus Real-Time PCR system (Applied Biosystems, Carlsbad, CA) was used for quantitative PCR (qPCR). A 20 μl reaction mixture containing 2 l cDNA, 2 l forward and reverse primers (200 nM each), and 10 l of 2X All-in-One PowerUp SYBR green master mix (Applied Biosystems) was run at a universal cycle (95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 60 seconds) as previously reported.^{40,42,44} The forward and reverse gene-specific primer sequences are for cyclooxygenase-2 (Cox-2), Forward-5'-CACGCAGGTGGAGATGATCTAC-3', Reverse-5'-TTCCTGGCCCCACAGCAA-3', TGF-β1, Forward-5'-TGGACACCAACTACTGCTTCAGCTC-3' and Reverse-5'-CAGGTCCTTGCGGAAGTCAATGTA-

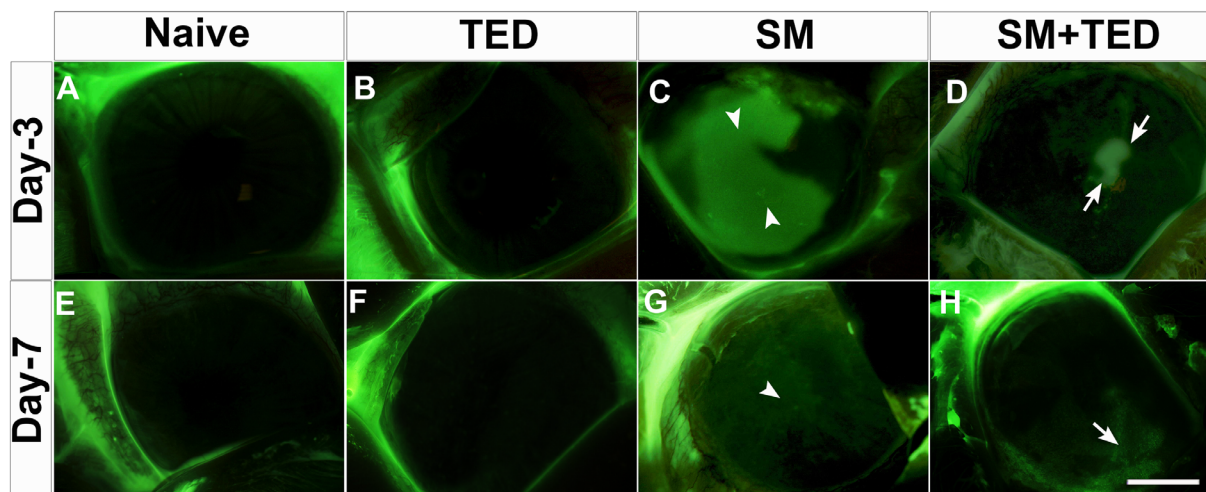


Figure 1. Representative fluorescein dye test images showing the extent/area of corneal epithelial erosion/defect in rabbit eyes. SM-vapor severely damaged corneal epithelium at day-3 (C, arrowheads) and day-7 (G, arrowhead), and topical TED effectively reduced SM toxicity on day-3 (D, arrows) and day-7 (H, arrow). TED dosing to SM-unexposed eye did not show any damage to the corneal epithelium at day-3 (B) or day-7 (F), and corneal health was akin to the naive eyes at day-3 (A) and day-7 (E). Scale bar: 2.0 mm.

3', and GAPDH Forward-5'-GCCTCAAGATCAT CAGCAATGCCT-3', and Reverse-5'-TGTGGTCAT GAGTCCTTCCACGAT-3' was used for the normalization of qPCR data. GAPDH showed no detectable relative fold change at various tested points or between groups. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative gene expression and reported as relative fold-change over the respective control values. The qPCR was performed in triplicate for each sample, and a minimum of three independent experiments were conducted.

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 8.2 software (GraphPad, La Jolla, CA). The Student's *t*-test and one-way analysis of variance (ANOVA) with the Wilcoxon rank-sum test or the Bonferroni multiple comparison post-hoc test were used depending on research design for all clinical data collected. The values of $p < 0.05$ were considered statistically significant. The sample size was determined using the G*Power (3.1.9.4 software, www.psych.uni-duesseldorf.de/abteilungen/aap/gpower3) priori power analysis method to achieve $\alpha = 0.05$; power ≥ 0.9 .

Results

Fluorescein dye staining

The fluorescein dye test demonstrated no corneal epithelial erosion/defect or loss of epithelial barrier

function in naive and TED-only treated rabbit eyes at day-3 (Figs. 1A, 1B) and day-7 (Figs. 1E, 1F). This revealed that the TED dose administered was acceptable and safe to rabbit eye. On the other hand, SM vapor exposure to rabbit eyes caused significant corneal epithelial erosion/defect at day-3 (Fig. 1C; naive vs. SM, $p < 0.0001$), which continued at day-7 (Fig. 1G; naive vs. SM, $p = 0.0002$). TED dosing to the SM-exposed rabbit eyes significantly mitigated corneal epithelial erosion and loss of barrier function at day-3 (Fig. 1D; SM vs. SM+TED, $p = 0.0001$) and day-7 (Fig. 1H; SM vs. SM+TED, $P = 0.0337$). Quantification of corneal epithelial erosion/defect in rabbit corneal sections was performed digitally for each of the four groups. At day-3, measurements were as follows: naive = 0 pixel (Fig. 1A), TED-only = 0 pixel (Fig. 1B), SM = $205,852 \pm 25,310$ pixels (Fig. 1C), and SM+TED = $74,189 \pm 11,086$ pixels (Fig. 1D). At day-7, measurements were as follows: naive = 0 (Fig. 1E), TED-only = 0 (Fig. 1F), SM = $132,239 \pm 30,710$ pixels, and SM+TED = $58,142 \pm 11,484$ pixels.

Slit-Lamp Microscopy

SM vapor exposure to rabbit eyes caused grade - III level corneal injury as observed at day-3 (Fig. 2C) and day-7 (Fig. 2G) in slit-lamp biomicroscopy. On the other hand, TED treatment to SM-exposed rabbit eyes reduced corneal damage and inflammation at day-3 (Fig. 2D) and day-7 (Fig. 2H) to grade - II. This clinical observation suggested that TED is effective in decreasing corneal injury in our pilot investigations. TED dosing to untreated contralateral naive eyes did

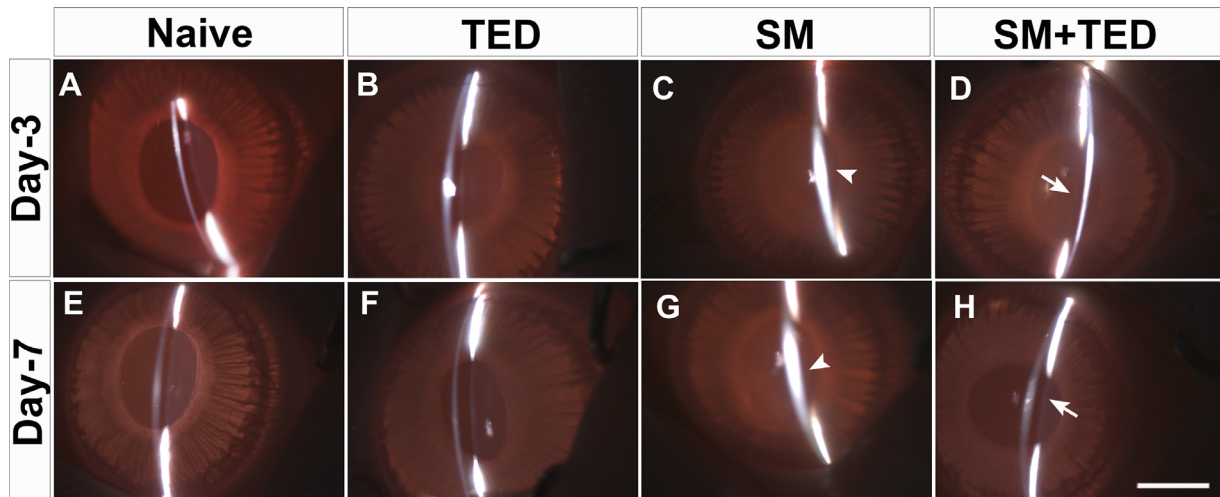


Figure 2. Representative slit-lamp narrow beam images exhibiting that SM-vapor caused inflammation in rabbit cornea at day-3 (C, arrowhead) and day-7 (G, arrowhead). TED decreased SM-induced corneal toxicity in vivo and reduced inflammation at day-3 (D, arrow) and day-7 (H, arrow). TED on SM-unexposed eye did not show any corneal inflammation at day-3 (B) or day-7 (F), and corneal health was similar to the naive corneas at day-3 (A) and day-7 (E). Scale bar: 2.0 mm.

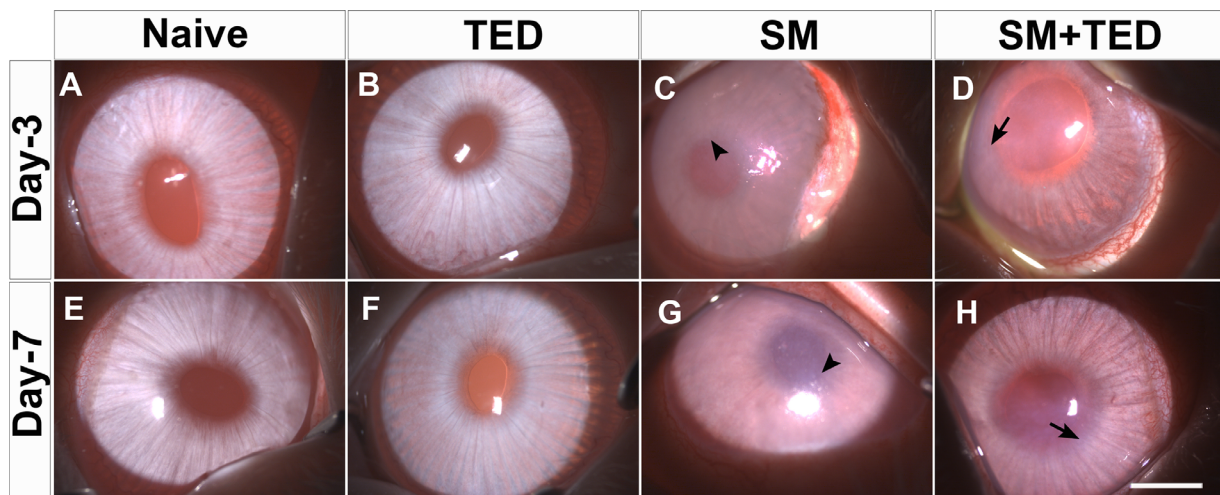


Figure 3. Representative stereo biomicroscopy digital images describing corneal opacity. SM-vapor caused severe corneal haze in rabbits in vivo at day-3 (C, arrowhead) and day-7 (G, arrowhead). TEDs diminished SM-induced corneal haze at day-3 (D, arrow) and day-7 (H, arrow). TED on SM-unexposed eye did not generate any corneal haze or inflammation at day-3 (B) or day-7 (F) and showed ocular health akin to the naive corneas at day-3 (A) and day-7 (E). Scale bar: 2.0 mm.

not cause any ocular inflammation or damage to the cornea at day-3 (Fig. 2B) and day-7 (Fig. 2F) when compared to the naive control at day-3 (Fig. 2A) and day-7 (Fig. 2E). This observation indicated that TED is well tolerated by the rabbit eyes.

Slit-Lamp Broad Beam

SM vapor exposure to rabbit eyes caused severe corneal haze, as observed at day-3 (Fig. 3C) and day-7 (Fig. 3G), and TED application reduced corneal haze formation at day-3 (Fig. 3D) and day-7 (Fig. 3H).

Nonetheless, TED did not abolish corneal haze completely at day-7 (Fig. 3H). The eyes that received TED-only showed no ocular damage at day-3 (Fig. 3B) or day-7 (Fig. 3F) and demonstrated corneal transparency similar to the naive eyes of the rabbits at day-3 (Fig. 3A) and day-7 (Fig. 3E).

The mean corneal haze levels were measured with Fantes score and are represented in the bar graph (Fig. 4). The mean corneal haze was zero for naive and TED-only treated eyes at day-3 and day-7 (Fig. 4). Conversely, significantly high corneal haze levels in the SM-exposed cornea at day-3 and day-7 were observed

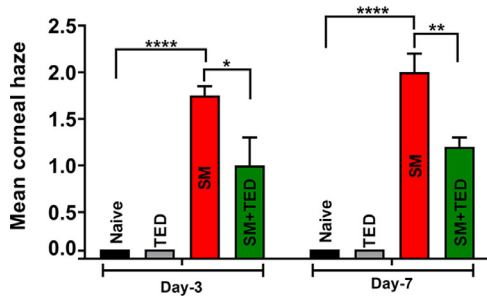


Figure 4. Quantification of mean corneal haze using the Fantes score in naive, TED, SM, and SM+TED. The corneal haze score was significantly increased in rabbit eyes after SM exposure compared to the naive and decreased in the SM+TED as compared to the SM exposure at day-3, and day-7. The TED alone treatment to the cornea did not cause corneal haze and scored 0.00 at the Fantes scale. Results are expressed as Mean \pm SEM (**** $p < 0.0001$ ** $p = 0.0014$, and * $p = 0.0344$, One-way ANOVA).

(naive vs. SM, $p < 0.0001$). Eyes that received TED dosing post-SM exposure exhibited notable reduction in corneal haze (SM vs. SM+TED) at day-3 ($p = 0.0344$) and day-7 ($p = 0.0014$) (Fig. 4) in this pilot study.

Histopathological Evaluation

The histological H&E staining demonstrated normal corneal anatomical features in the untreated naive (Figs. 5A, 5E) and topical TED-only administered rabbit eyes (Figs. 5B, 5F). A lack of detectable corneal epithelial damage or ocular edema in TED-only administered eyes at day-3 (Fig. 5B) or day-7 (Fig. 5F) implied that the tested TED dose was harmless to rabbit eyes. The histological H&E staining of SM vapor-exposed rabbit eyes exhibited dramatic corneal edema at day-3 (Fig. 5C; naive vs. SM, $p = 0.0049$), which continued to the same level until day-7 (Fig. 5G; naive vs. SM, $p = 0.0002$). Contrary to this, eyes administered TED post-SM exposure exhibited remarkably diminished corneal edema at day-3 (Fig. 5D; SM vs. SM+TED, $p = 0.0186$) and day-7 (Fig. 5H; SM vs. SM+TED, $p = 0.0001$). Although, TED initially reduced inflammation significantly, the eyedrops did not completely eliminate corneal edema until day-7 (Fig. 5H).

Digital quantification of corneal tissue thickness for each of the four groups at day-3 was as follows: Naive = 6816 ± 117 , TED-only = 6689 ± 155 , SM = $11,019 \pm 848$, and SM+TED = 7526 ± 1019 ; and at day-7 was: Naive = 6518 ± 179 , TED-only = 6537 ± 199 , SM = $10,129 \pm 520$, and SM+TED = 6323 ± 697 .

Next, we examined the integrity of corneal epithelium and stromal junction in the corneas of all four groups. The epithelium-stroma integrity was fully

intact in the naive control rabbit eyes at day-3 (Fig. 6A) and day-7 (Fig. 6E) and in TED treated contralateral naive eyes at day-3 (Fig. 6B) and day-7 (Fig. 6F). This data suggested that TED is nontoxic for the rabbit eyes and does not endanger epithelium-stroma integrity. The differential comparison of SM vs. SM+TED was recorded for judging the potency of TED treatment. Rabbit eyes exposed to SM demonstrated severe epithelium-stroma separation at day-3 (Fig. 6C) and day-7 (Fig. 6G), and TED treatment after SM exposure led to mitigation and less severe epithelium-stroma separation at day-3 (Fig. 6D) and day-7 (Fig. 6H). TED appeared to show a clear short-term benefit, albeit incomplete, as there remained signs of damage in SM+TED treated corneas (Figs. 6D, 6H).

Afterward, we evaluated the effects of SM, TED, and SM+TED on the corneal epithelial basement membrane that resides between the basal epithelial cells and the stroma employing immunofluorescence staining with Integrin- $\beta 4$ antibody. The SM vapor exposure to rabbit eyes caused severe damage to the basement membrane at day-3 (Fig. 7C) and day-7 (Fig. 7G). A significantly reduced breakage and intact corneal basement membrane were detected in the SM+TED rabbit eyes at day-3 (Fig. 7D) and day-7 (Fig. 7H). The TED treatment to the naive rabbit eyes had no impact on the basement membrane at day-3 (Fig. 7B) and day-7 (Fig. 7F) as integrin- $\beta 4$ staining was similar to naive control eyes at day-3 (Fig. 7A) and day-7 (Fig. 7E). This data divulged that administered TED dosing was benign and tolerable to the rabbit corneal epithelial basement membrane.

Gene Expression Analysis

SM vapor exposure to the rabbit cornea demonstrated a significant upregulation of the profibrotic TGF- $\beta 1$ gene (Naive vs. SM, $p = 0.0002$) in rabbit corneas at day-3 (3.42-fold) and day-7 (5.49-fold). TED treatment to SM-exposed eyes led to a significant reduction (2.36-fold) in TGF- $\beta 1$ expression at day-7 (SM vs. SM+TED, $p = 0.0027$) but the decline in TGF- $\beta 1$ level at day-3 was not statistically significant (SM vs. SM+TED, $p = 0.0875$) (Fig. 8A). A similar trend was observed for the inflammatory marker, COX-2 expression. SM vapor exposure significantly upregulated COX-2 expression in rabbit corneas (naive vs. SM, $p < 0.0001$) at day-3 (28.15-fold) and day-7 (3.94-fold), and TED treatment led to a significant reduction in the SM-induced COX-2 levels (Fig. 8B) at day-3 (3.57-fold, $p = 0.0001$) and day-7 (3.20-fold, $p < 0.0001$). TED treatment to the naive rabbit eyes showed no significant changes in TGF- $\beta 1$ (Fig. 8A) or COX-2

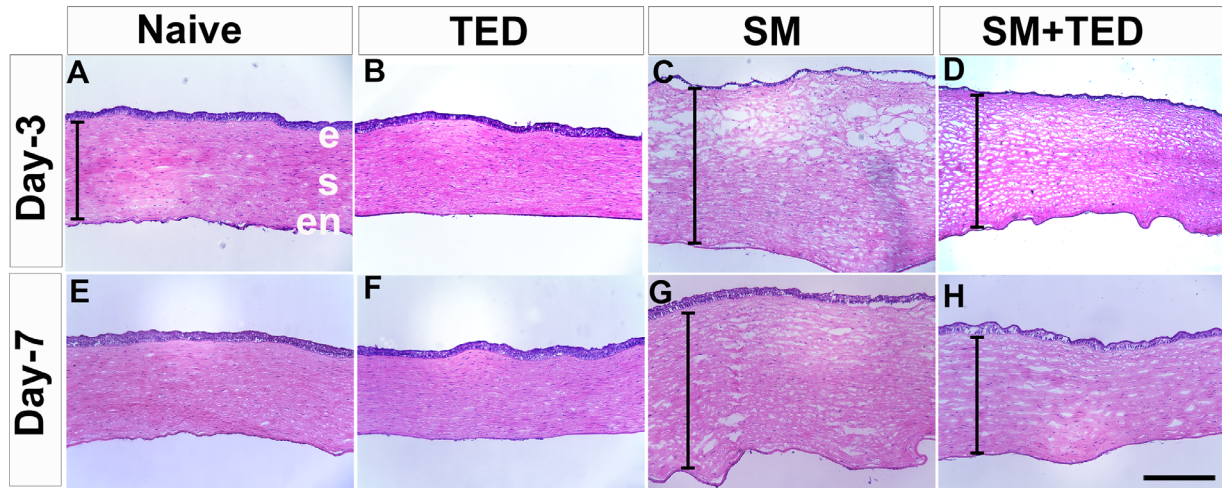


Figure 5. Representative H&E stained images showing that SM-vapor caused severe corneal edema in rabbits in vivo at day-3 (C) and day-7 (G). TEDs attenuated SM-induced edema at day-3 (D) and day-7 (H). TED on SM-unexposed eye did not cause corneal edema or structural abnormalities at day-3 (B) or day-7 (F) and showed corneal morphology akin to the naive corneas at day-3 (A) and day-7 (E). e, epithelium, s, stroma, en, endothelium. Scale bar: 100 μ M.

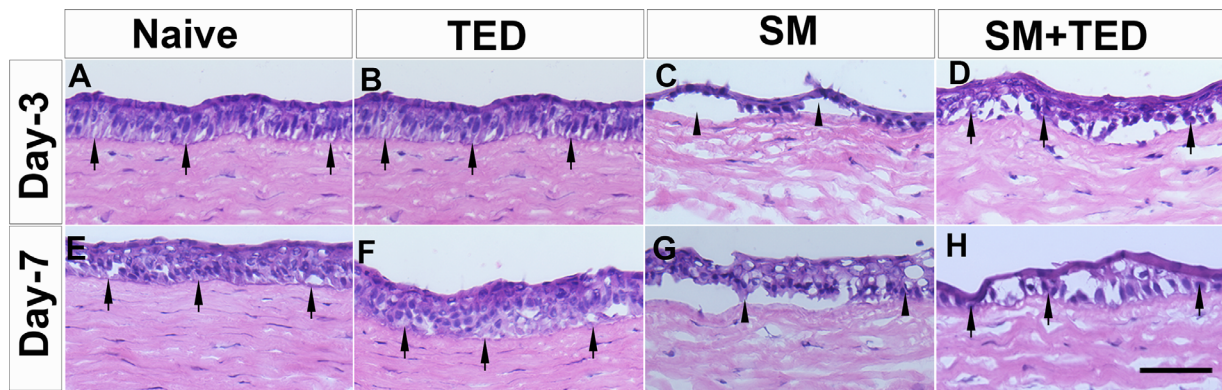


Figure 6. Representative H&E stained images displaying that SM-vapor caused severe epithelium-stroma separation in rabbits in vivo at day-3 (C, arrowheads) and day-7 (G, arrowheads). TEDs diminished SM-induced epithelium-stroma separation at day-3 (D, arrows) and day-7 (H, arrows). TED on SM-unexposed eye did not generate any corneal epithelium-stroma separation at day-3 (B) or day-7 (F) and showed corneal composition analogous to the naive corneas at day-3 (A) and day-7 (E). Scale bar: 100 μ M.

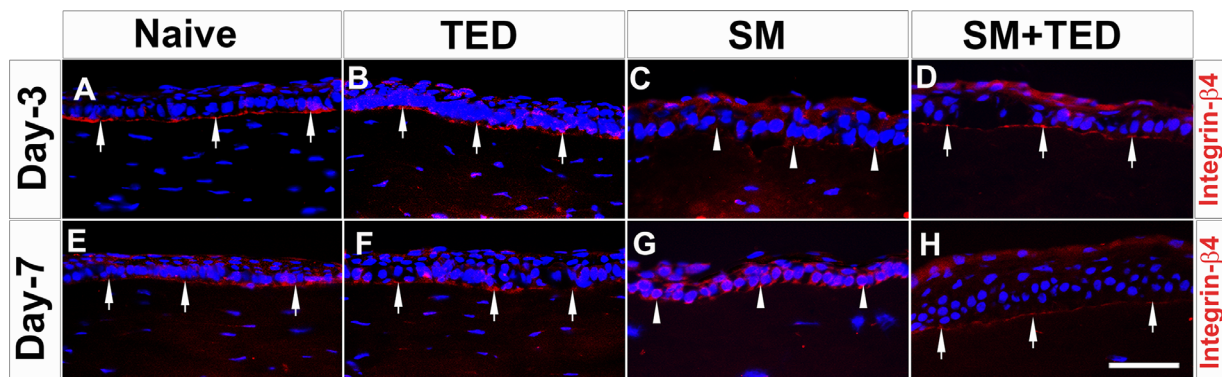


Figure 7. Representative immunofluorescence images stained with anti-Integrin- β 4 antibody indicating that SM-vapor caused severe corneal epithelium-stroma separation in rabbits in vivo at day-3 (C, arrowheads) and day-7 (G, arrowheads). TEDs mitigated epithelium-stroma separation at day-3 (D, arrows) and day-7 (H, arrows). TED on SM-unexposed eye did not alter corneal epithelium-stromal integrity at day-3 (B, arrows) or day-7 (F, arrows) and showed corneal composition analogous to the naive corneas at day-3 (A, arrows) and day-7 (E, arrows). Scale bar: 100 μ M.

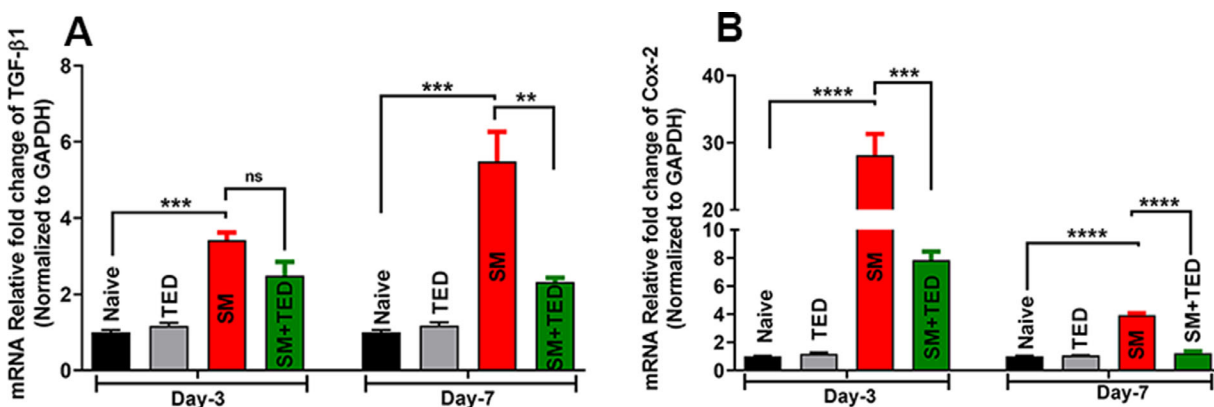


Figure 8. Quantitative gene expression analysis. (A) qRT-PCR analysis results showing upregulation of the TGF- β 1 expression in SM-exposed corneas compared with naive at day-3 and day-7. TED treatment to the SM-exposed corneas downregulates TGF- β 1 expression compared with SM-exposed at day-7 postexposure but not at day-3 ($***P = 0.0002$, $**P = 0.0027$, ns = no significance, one-way ANOVA). No significant changes in the expression of profibrotic marker, TGF- β 1 gene in TED vs. naive rabbits at day-3 and day-7 ($P > 0.05$). (B) qRT-PCR analysis showing significant upregulation of the COX-2 in SM-exposed corneas compared with naive. SM+TED corneas showed downregulation of COX-2 expression compared with SM on day-3 and day-7 postexposure. Results are expressed as mean \pm SEM ($****P < 0.0001$, $***P = 0.0001$, one-way ANOVA). No significant change in the expression of inflammatory marker COX-2 gene in TED versus naive rabbit corneas at day-3 and day-7 ($P > 0.05$).

(Fig. 8B) expression at day-3 and day-7 (naive vs. TED, $P > 0.05$).

CCT, STT, and IOP

Table 2 depicts the observed values of CCT, STT, and IOP at day-3 and day-7 for the four test groups (naive, TED, SM, and SM+TED) and age-matched naive control group. The CCT, STT, and IOP values of the naive and TED groups were found to be in the normal range with no significant changes between these groups. SM vapor exposure caused a significant increase in CCT and STT values ($P < 0.0001$), and TED-treatment demonstrated a reduction in CCT and STT scores at day-3 and day-7 (SM vs. SM+TED). The IOP was not affected significantly in SM-exposed or TED-treated eyes.

Discussion

MGK to the eye due to sulfur mustard (SM) exposure has been well described and documented in human subjects and animal models.^{6–12} In the Iran–Iraq conflict, MGK was a common injury after exposure to SM, leading to corneal dysfunction lasting for several months.⁶ Typical symptoms observed include severe ocular pain, photophobia, excessive tearing, eyelid swelling, blepharospasm, and blurred vision minutes after SM exposure.^{6–12} Slit-lamp examination of MGK commonly shows punctate epithelial

erosions, corneal edema, and anterior chamber inflammation. The acute phase of SM exposure, within 2 to 6 hours postexposure, begins with corneal epithelial defects or sloughing of the surface tissue of the cornea.^{46–50} Corneal edema, iritis, iris vessel dilation, and iris necrosis follow as SM penetrates into deeper corneal tissue.^{46–50} We simulated SM gas injury in our animal model by exposing rabbit eyes to SM vapor of 200 mg-min/m³ for 8 minutes. The median incapacitating (ICT₅₀) dose of SM is 100 to 200 mg-min/m³ based on human clinical studies.^{46–50} The spectrum of SM-induced ocular injury is primarily dependent on the exposure duration and concentration with mild acute toxicity showing mild conjunctivitis to scattered punctate corneal epithelial erosions. Vision loss and permanent corneal scarring can occur with increased SM concentrations and longer durations of SM exposure.^{6–9,12} The current preliminary study demonstrates that within 3 days and 7 days of exposure, rabbit corneal tissue displays corneal erosions, edema, inflammation, and opacity/haze. The pachymetry data showed increased CCT, and STT showed increased tearing. These findings are in alignment with previous studies demonstrating that SM damage to the eye is a dose- and time-dependent phenomenon.^{6–9,12}

The corneal epithelium is the frontline barrier of the eye to prevent any insult from the external environment. The corneal epithelium is highly susceptible to SM injury owing to the high metabolic activity and turnover of the epithelial cells, and comparatively high aqueous–mucous contact between the corneal surface and the tear film.^{10,46–49} In the present study, we

Table 2. Clinical Eye Examination Parameters Determined via Pachymetry, STT, and Applanation Tonometry

Groups	Treatment to the Eye	Pachymetry CCT (μm)			STT Tear Flow (mm)			Applanation Tonometry IOP (mm Hg)		
		Study Time Day-3	Study Time Day-7	Study Time Day-7	Study Time Day-3	Study Time Day-7	Study Time Day-7	Study Time Day-3	Study Time Day-7	
I (Naive)	No treatment	363.0 \pm 5.1	367.4 \pm 4.2	10.0 \pm 0.7	10.2 \pm 0.6	10.7 \pm 0.7	9.0 \pm 0.8			
II (TED)	TED (without SM exposure)	365.0 \pm 5.0 ^{ns}	372.6 \pm 7.7 ^{ns}	9.5 \pm 0.8 ^{ns}	8.4 \pm 0.5 ^{ns}	8.2 \pm 0.9 ^{ns}	8.6 \pm 0.7 ^{ns}			
III (SM)	SM vapor	515.0 \pm 5.6 ^{****}	540.2 \pm 14.0 ^{****}	27.5 \pm 0.5 ^{****}	25.8 \pm 1.3 ^{****}	9.7 \pm 1.0 ^{ns}	8.4 \pm 1.1 ^{ns}			
IV (SM+TED)	SM vapor + TED	420.0 \pm 5.2 ^{****}	412.2 \pm 4.3 ^{****}	16.5 \pm 2.3 ^{****}	16.0 \pm 0.9 ^{****}	10.0 \pm 0.7 ^{ns}	9.0 \pm 1.0 ^{ns}			
Age-matched	Both eyes left	(SM vs. SM+TED)	(SM vs. SM+TED)	(SM vs. SM+TED)	(SM vs. SM+TED)	(SM vs. SM+TED)	(SM vs. SM+TED)			
Naive control	untreated	360.0 \pm 5.2	366.4 \pm 4.1	11.0 \pm 0.4	10.3 \pm 0.4	9.7 \pm 0.6	9.2 \pm 0.5			

**** $P < 0.0001$.

*** $P = 0.0001$.

ns, not significant; data represented in mean \pm SEM.

found that the SM exposure to the rabbit eye caused corneal erosions as measured by the clinical fluorescein eye test. Histopathological investigation performed by H&E staining revealed corneal edema and loss of epithelium-stroma integrity. The damage to the corneal epithelial basement membrane was confirmed with Integrin- β 4 immunostaining.⁵¹ The disruption to the corneal epithelium is the principal injury modality to the stroma, which extends two-thirds refractive power of the eye and is composed of tightly packed collagen lamella that makes up 90% of the corneal volume.⁵² The orthogonal arrangement of the collagen fibrils is critical in maintaining corneal transparency, and its functional integrity is highly dependent on the maintenance of a deturgescent state.⁵³ More than a 5% increases in corneal hydration can disrupt vision.⁵² The stromal hydration is primarily controlled by two tissues, at the anterior margin, the corneal epithelium, and at the posterior margin, the corneal endothelium.⁵² Disruption of the proper functioning of either cell barrier allows fluid entry into the stroma, rapidly eliciting stromal edema, disorganizing the collagen lamella, resulting in impaired vision. In this study, the damage to the corneal epithelium upon SM exposure increased stromal hydration making it opaque and edematous due to the disorganized collagen lamellae, as evident by H&E staining and pachymetry.

Currently, there are no definitive treatments or antidotes for the acute phase of MGK. Current therapeutic strategies are based on steroids and immunomodulatory drugs, with more side effects, but otherwise depend on the eye's innate ability to self-repair.¹⁰⁻¹⁴ Since symptoms of the acute corneal damage are distributed throughout the anterior chamber of the eye, we have developed a novel ophthalmic formulation, Topical Eye Drops (TED) using four FDA-approved drugs (0.5% ketorolac, 25 μ M SAHA, 25 μ M enalapril, and 10% vitamin C), as an effective therapeutic strategy for the treatment of SM toxicity. To test the usefulness of TED, we used an in vivo rabbit model of SM vapor exposure because rabbit and human corneas are structurally alike and exhibit nearly similar damage at functionally equivalent doses. The primary objective of this pilot study was to assess the protective effects and tolerability of TED eye drops consisting of four FDA-approved drugs that are known to improve corneal wound repair and reduce ocular pain/inflammation in rabbit eyes in vivo.²³⁻³⁹

SM exposure causes epithelial sloughing and leads to severe ocular clinical symptoms, including pain and persistent vision loss. Pain management is a great challenge in post-SM exposure. Inhibition of COX-1 and COX-2 has been directly implicated in ameliorating inflammation in many tissues including the eye

by the ketorolac, a potent analgesic, antipyretic, and anti-inflammatory agent.²³⁻²⁵ Previous studies have described the effectiveness of ophthalmic ketorolac solution in reducing ocular inflammation, relieving corneal pain, and minimizing discomfort in human patients after cataract and refractive surgeries.^{23,24} Also, it has been shown to reduce corneal inflammation and re-epithelialization without influencing the iNOS and MMP-9 levels in the stroma in vivo in rabbit eyes after an ocular injury from an alkylating agent.²³⁻²⁵ We found that SM vapor exposure to cornea significantly enhanced COX-2 at day-3, which was suppressed by TED application. The inhibition of inflammation was also evident in slit-lamp biomicroscopy examinations of live rabbits and H&E corneal staining. This demonstrates a clear short-term benefit of TED clinically, albeit recovery was incomplete. We predict that this anti-inflammatory mechanism of action of TED plays a role in curbing SM injury to corneal epithelium-stroma and basement membrane based on the H&E and integrin- β 4 staining of the current preliminary study.

TED formulation contains suberoylanilide hydroxamic acid (SAHA also known as vorinostat or Zolinza), which is a histone deacetylase inhibitor and, has been shown to regulate TGF- β 1-induced signaling pathways and impede collagen synthesis, myofibroblast formation, and fibrosis/haze in the cornea, heart, and lung tissues through epigenetic alterations.^{26-31,54} Our previous studies suggested that 25 μ M of topical SAHA treatment inhibits TGF- β 1-induced corneal haze and bleb fibrosis significantly in the rabbit eye in vivo.^{27,30,31} Furthermore, they characterized mechanisms employed by SAHA to exercise anti-fibrotic response.^{28,29} In this pilot study, qRT-PCR data showed an increased level of TGF- β 1 in the SM-exposed corneas, which was reduced after TED application on day-3 and day-7 suggesting the mitigation of TGF- β 1 induced corneal haze up on TED treatment in SM exposed rabbit cornea in vivo. We predict that this could be possibly due to the decline in myofibroblast production by the SAHA present in the TED formulation via activation of epigenetic mechanism based on our previous studies.²⁷⁻³¹

Another active ingredient of TED is enalapril, which is an angiotensin-converting enzyme (ACE) inhibitor currently used for the clinical management of hypertension, congestive heart failure, myocardial infarction, and diabetic nephropathies.³² Enalapril has also been shown to enhance antioxidant activity on hemodialysis patients and glutathione-dependent antioxidant defense in vivo.⁵⁵ Furthermore, enalapril can attenuate the hypertrophic scar in the rabbit ear by reducing the TGF- β expression⁵⁶ and frailty in aging

mice by reducing proinflammatory cytokines interleukin 1α , monocyte chemoattractant protein-1, and macrophage inflammatory protein-1a, and increasing anti-inflammatory cytokine interleukin-10 in mice.⁵⁷ The SM exposure has been shown to trigger oxidative stress through the depletion of glutathione and alter proinflammatory and fibrotic cytokines in nonocular tissues.⁵⁸ We believe that a significant decrease in ocular inflammation observed in the SM-exposed cornea after TED administration is likely from the enalapril and ketorolac present in the TED formulation. More studies are warranted to identify inflammatory pathways and activated antioxidant index in corneal tissues.

Vitamin C (commonly known as ascorbic acid) was also a part of TED formulation. It has been attributed to be protective in the corneal injury sustained from UV irradiation and alkali burns in animal models and human patients.^{34–39} An interesting aspect of vitamin C is its protective role against reactive oxidative stress (ROS) in corneal epithelium.⁵⁹ Vitamin C has also been shown to have a beneficial effect in the reprogramming efficacy for the formation of induced pluripotent stem cells (iPSCs), both in humans and mice.⁶⁰ This unique regenerative property of vitamin C could be attributed to the diminished epithelium-stroma separation in the TED-treated corneas after SM exposure. The exact mechanism of action is not yet known. Our future studies will fill such important knowledge gaps.

There are a number of limitations to this pilot study including a lack of stand-alone test-groups evaluating each drug present in the TED formulation, single TED dosing, two short time-points, and limited safety investigations. Furthermore, SM gas exposure to the eye is known to cause chronic manifestations,^{9–12} and this study did not inspect whether TED-induced protection in the early phase of the SM exposure had any protective effects on chronic manifestations of MGK. Additionally, at this point we do not know whether the observed clinical benefits of TED in mitigating SM ocular toxicity are due to one or more of the components present in TED. Though the drugs used in TED have previously been studied individually for the management of ocular pain and corneal wound healing and fibrosis, they were not specifically studied for SM toxicity in the cornea or MGK. The main focus of this pilot study was to gauge the safety and protective effects of TED against very acute phase MGK caused by SM exposure to the rabbit eyes in vivo.

Conclusions

In conclusion, our pilot study suggests that topical application of TED formulation is well tolerated and

possibly effective in curbing very acute MGK manifestations in rabbit eyes in vivo based on in situ clinical eye examinations in live rabbits and selected biological corneal wound healing investigations.

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