Establishing a dexamethasone treatment regimen to alleviate sulfur mustard-induced corneal injuries in a rabbit model

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ABBREVIATIONS
ANOVA: analysis of variance
COX-2: cyclooxygenase 2
CWA: chemical warfare agents
DEX: Dexamethasone
ECM: extracellular matrix
HCE: human corneal epithelial cells
LSCD: limbal stem cell deficiency
MMP-9: matrix metalloprotease
NM: nitrogen mustard

NV: neovascularization

ROS: reactive oxygen species

SM: sulfur mustard

SPARC: Secreted protein acidic and rich in cysteine

VEGF: vascular endothelial growth factor
ABSTRACT

Sulfur mustard (SM) is an ominous chemical warfare agent. Eyes are extremely susceptible to SM-toxicity; injuries include inflammation, fibrosis, neovascularization (NV), and vision impairment/blindness, depending on the exposure dosage. Effective countermeasures against ocular SM-toxicity remain elusive and are warranted during conflicts/terrorist activities and accidental exposures. We previously determined that dexamethasone (DEX) effectively counters corneal nitrogen mustard toxicity and that the 2 h post-exposure therapeutic window is most beneficial. Here, the efficacy of two DEX dosing frequencies, i.e., every 8 or 12 h (initiated, as previously established, 2 h post-exposure) until 28 days post SM-exposure was assessed. Furthermore, sustained effects of DEX treatments were observed up to day 56 post SM-exposure. Corneal clinical assessments (thickness, opacity, ulceration, and NV) were performed at the day 14, 28, 42, and 56 post SM-exposure timepoints. Histopathological assessments of corneal injuries (corneal thickness, epithelial degradation, epithelial-stromal separation, inflammatory cell, and blood vessel counts) using H&E staining and molecular assessments (COX-2, MMP-9, VEGF, and SPARC expressions) were performed at days 28, 42, and 56 post SM-exposure. Statistical significance was assessed using Two-Way ANOVA, with Holm-Sidak post-hoc pairwise multiple comparisons; significance was established if p<0.05 (data represented as the mean ± SEM). DEX administration every 8 h was more potent than every 12 h in reversing ocular SM-injury, with most pronounced effects observed at days 28 and 42 post SM-exposure. These comprehensive results are novel and provide a comprehensive DEX-treatment regimen (therapeutic-window and dosing-frequency) for counteracting SM-induced corneal injuries.
SIGNIFICANCE STATEMENT:

The study aims to establish a DEX treatment regimen by comparing the efficacy of DEX administration 12 h vs 8 h, initiated at 2 h post-exposure; treatment initiated 2 h post-exposure, and DEX administration every 8 h thereafter was most effective in reversing SM-induced corneal injuries. SM-injury reversal during DEX administration (initial 28 days post-exposure) and sustained (further 28 days after cessation of DEX administration i.e., up to 56 days post-exposure) effects were assessed using clinical, pathophysiological, and molecular biomarkers.
INTRODUCTION

Sulfur mustard (SM), when first reported by Despretz in 1822 (Niemann, 1860), was not envisioned to be deployed as a chemical warfare agent (CWA). Lommel and Steinkopf (1993) suggest its combat potential; thus, SM is also called "Lost" or "S-Lost". Since then, numerous nomenclatures that etch a historical lineage of SM in warfare have arisen. These include unstable Hun Stuff, ‘H’ or ‘HS (Stewart, 2006), distilled and stable ‘HD’ or Pyro (Jahromy et al., 2017) and various “blended” mustard varieties like ‘HQ’ (Gates and Moore, 1946), ‘HT’ or Runcol, and ‘HL’(McCamley, 2007). As research progressed to develop better CWAs, SM was stockpiled and improperly discarded, for example in waterbodies after the World Wars (Geraci, 2008; Wattana and Bey, 2009). To avoid confusion, sulfur mustard will be referred to as SM throughout this study.

Individuals exposed experience a range of symptoms depending upon dosage (concentration/duration) and form (liquid or vapor) of SM, route of exposure, and physiology of individuals exposed. Primary routes of exposure are ocular, dermal, and inhaled (respiratory exposure to the lungs). Exposure can lead to systemic SM toxicity at high doses (Papirmeister et al., 1991; Ghabili et al., 2010; Ghabili et al., 2011). Ocular system is most vulnerable to SM exposure (Rafati-Rahimzadeh et al., 2019), with injuries developing even at 5 mg/min/L doses of SM (Lommel and Steinkopf, 1993; Amata et al., 2002; McNutt et al., 2020). SM exposure may cause discomfort/pain, smarting, inflammation, lacrimation, photophobia, edema, corneal fibrosis, neovascularization (NV), nerve damage, blepharospasm, delayed ulcerative keratitis and limbal stem cell deficiency, partial or complete vision impairment or even death in extreme cases (Balali-Mood and Mehrdad, 2006; Ghasemi et al., 2009; Gordon et al., 2009; Kadar et al., 2009; McNutt et al., 2012; Ghasemi et al., 2013). Cornea, the outer most, transparent layer of the eye,
directly interacts with external environment and hence, is primarily susceptible to injury by external toxins, such as SM.

Threat of SM exposure is ever-present during a conflict or terrorist activity (Saladi et al., 2006; Geraci, 2008; Ganesan et al., 2010; Ghabili et al., 2011) or accidental exposure from stockpiles (Geraci, 2008; Wattana and Bey, 2009) or improperly discarded SM reserves (Missiaen et al., 2010). Effective and targeted countermeasures are warranted against ocular SM-induced injuries. Dexamethasone (DEX), a Food and Drug Administration-approved anti-inflammatory steroidal drug, has been shown to effectively reverse vesicant-induced ocular injuries (Kadar et al., 2009; Kadar et al., 2014; Tewari-Singh et al., 2012; Goswami et al., 2018; Goswami et al., 2022). It is readily available, inexpensive, and does not require medical training/assistance to administer, strengthening it as an ideal therapeutic intervention, especially in a mass casualty situation.

Previously, DEX was shown to treat nitrogen mustard (NM)-induced injuries in ex vivo rabbit corneal culture (Tewari-Singh et al., 2012; Goswami et al., 2018) and a therapeutic window of DEX administration in an in vivo rabbit model of NM-induced ocular injuries (Goswami et al., 2022) was established. Few studies have assessed the efficacy of DEX treatment alone in SM-induced corneal injuries in in vivo rabbit ocular injury models (Amir et al., 2000; Kadar et al., 2009).

Here, efficacy of DEX was assessed at two dosing frequencies, administration every 8 h or 12 h (beginning 2 h post-exposure) until 28 days post SM-exposure was assessed in alleviating late pathology, beginning at 14 days post exposure of SM-induced corneal injuries. Sustained effects of DEX treatments were observed up to day 56 post SM-exposure i.e., for an additional 4 weeks post cessation of DEX administration. Clinical assessment of corneal injury
was performed at days 14, 28, 42, and 56 post SM-exposure, using the following parameters: thickness, opacity, ulceration, and NV. Histopathological (corneal epithelium degradation, epithelial-stromal separation, and inflammatory cell count and blood vessel count in the corneal stroma) and molecular changes (cyclooxygenase-2 [COX-2], vascular endothelial growth factor [VEGF], matrix metalloproteinase-9 [MMP-9], secreted protein acidic and rich in cysteine [SPARC]) were also assessed. Comparisons between the two dosing frequencies were also made to evaluate the differential effects of the DEX dosing frequencies.
MATERIALS AND METHODS

Chemicals and reagents. DEX (cat #10000106054) 0.1% for topical ocular application was obtained from Bausch and Lomb, Rochester, NY. The vapor cap (cat #300-1006-020) for SM exposure was obtained from Caplugs Evergreen, Buffalo, NY and the rubber O-ring was procured from MSC Industrial Supply Company, Melville, NY was glued onto the vapor cup using gel-based glue from Loctite, Düsseldorf, Germany. The hematoxylin (cat #HHS32) and eosin (cat #HT 110116) stains were obtained from Sigma Aldrich, St. Louis, MO. The primary antibody used in immunohistochemistry (IHC) for COX-2 (cat #160112) was procured from Cayman Chemical, Ann Arbor, MI. The primary antibodies for MMP-9 (cat #ab58803), VEGF (cat #ab28775), and SPARC (cat #225716) were obtained from Abcam, Cambridge, MA. The primary antibody used as negative control, mouse IgG antibody, was procured from N-Universal, DAKO, Santa Clara, CA. For IHC, 3, 3′-diaminobenzidine (DAB) peroxide substrate kit (cat #SK-4100) was procured from Vector Labs, Inc. Burlingame, CA. The secondary fluorochrome attached antibody (cat #A11008) used in SPARC immunofluorescence (IF) was obtained from Invitrogen, Waltham, MA. The IF mounting media (with DAPI; VECTASHIELD Vibrance, cat # H-1800) was obtained from Vector Labs, Inc. Burlingame, CA.

Animals and study design. New Zealand white rabbits (n=33; males), weighing between 2.5-4.0 kg and a minimum of 3 months old, were obtained from Charles River Laboratories (Wilmington, MA). Upon arrival, animals were inspected to ensure good health and quarantined for a minimum of 8 days before proceeding to SM exposures. Animals were provided food and water ad libitum. Rabbits were housed individually, at 16-22°C, ~50% relative humidity, and a 12 h light/dark/day cycle.
Rabbits were randomly divided into two groups: Group 1 (n=6/timepoint; treatment group) and Group 2 (n=5/timepoint; control group). Animals in group 1 received SM exposure in both eyes, as detailed below. Thereafter, 2 h post SM-exposure, DEX was administered every 8 h (left eye; DEX 8h treatment group) or every 12 h (right eye; DEX 12h treatment group) for 28 days post SM-exposure and its effects were studied for an additional 28 days after cessation of DEX administration. In group 2, left eyes served as control (control group) whereas, right eyes were exposed to SM vapor. Group 2 did not receive any DEX treatment. Schematic representation of the study is provided in Fig. 1. The timepoints of sacrifice were day 28, day 42, and day 56 post SM-exposure.

**SM exposure.** SM exposures and DEX treatments were performed at MRIGlobal (Kansas, MO). All experimental protocols and animal procedures used in this study were approved by the Institutional Animal Care and Use Committee at MRIGlobal before commencement of the study. The pre-exposure pain management procedures were performed as described in Goswami et al 2021. Briefly, buprenorphine-SR (SQ; 0.05-0.1 mg/kg) was used for pain management. SM-exposures were performed inside a chemical hood, under the influence of anesthesia (ketamine \([≤60 \text{ mg/kg}]\) and xylazine \([≤5 \text{ mg/kg}]\) cocktail). SM exposures were performed per the protocol described in the study by McNutt et al., (2021). Briefly, neat SM (10 µL) was applied to Whatman #2 filter paper seated in a 14-mm vapor cap that was inverted on a glass slide for 1 min, and then transferred onto the SM exposure eye for 90 s. Control eyes received a sham (no SM) exposure; vapor cap was placed on the control eye for 90 s to mimic conditions of SM exposure. Each cap was single use. Following the exposure, each eye was rinsed with 10 mL saline solution (2 min post-exposure). Once off-gassing was completed, the animals were
returned to their cages, observed until sternal recumbency, and provided food and water ad
libitum.

**DEX treatment.** DEX (0.1%) administration was performed per the protocol described in
Goswami et al., (2022). Briefly, eye lids were softly restrained to keep the eyes open. Two drops
of DEX were delivered to the central cornea and the animal was held in this position for ∼5 s for
complete absorption of DEX, before releasing it and allowing it to blink normally. DEX
treatment was initiated 2 h post SM-exposure. Thereafter, DEX was administered either every 8
h (left eye; DEX 8h treatment) or every 12 h (right eye; DEX 12h treatment) for 28 days post
SM-exposure. Sustained effects of DEX treatment were determined for up to 56 days post SM-
exposure. DEX administration every 8 h and 12 h daily would be referred to as the DEX 8h
treatment and DEX 12h treatment, respectively, in this study for clarity.

Clinical parameters were evaluated on the day 14, 28, 42, and 56 post SM-exposure
timepoints. Timepoints of animal euthanization and harvest of ocular tissues were at day 28, 42,
and 56 post SM-exposure, for evaluation of biological and molecular parameters. Animals were
euthanized following humane and approved protocols. Corneal tissues were harvested,
sequentially fixed in 10% buffered formalin, and dehydrated in 70-100% ethanol, followed by
paraffin embedding and sectioning (5 μm thick sections). Hematoxylin and eosin (H&E) staining
was performed for the assessment of biological parameters (three slides/tissue). Additionally,
IHC was performed (three slides/tissue) to assess the levels of molecular markers COX-2, MMP-
9, and VEGF. IF was performed (three slides/tissue) to assess the levels of SPARC. Clinical,
biological, and molecular parameters were assessed as described previously (Tewari-Singh et al.,
2016; Goswami et al., 2019; Goswami et al., 2021; Goswami et al., 2022) in brief below.
Assessment of clinical parameters. Detailed ophthalmic examinations were performed using slit lamp microscopy on sedated rabbits on the day 14, 28, 42, and 56 post SM-exposure timepoints. Corresponding digital pictures were taken to document efficacy of the two different DEX treatment frequencies, as well as to determine the SM-induced injury progression in the SM-exposed eyes without DEX treatment.

Corneal thickness was assessed, using ultrasonic pachymetry measurements (TOMEY SP-3000 Pachymeter, Phoenix, AZ), from five different corneal regions and the average corneal thickness was calculated (reported in μm). Corneal opacity was used to assess corneal-stromal injury upon SM exposure and was determined by scoring loss of corneal transparency. Corneal ulceration was determined by the uptake of fluorescein staining and was scored as either 0 signifying no fluorescein uptake or 1 signifying fluorescein uptake was present. Corneal NV was determined by quantitating the extent of new vessel growth in the cornea. Each cornea was divided into four equal quadrants and NV in each of the quadrants was estimated individually; the average was used as the NV score. The scoring was based on the invasive progression of the vessel (s) within the quadrant of origin. Scoring was done from 0 to 4 with a score of 0 signifying no NV, 1 signifying longest NV up to ~25% of the corneal radius, 2 signifying longest NV between 26% to 50% of the corneal radius, 3 signifying longest NV between 51%-75% of the corneal radius, and 4 signifying longest NV >75% of the corneal radius.

Histopathological assessments. Corneal tissues were harvested, fixed, paraffin embedded, and sectioned (5 μm thick sections) as described previously (Tewari-Singh et al., 2016; Goswami et al., 2019; Goswami et al., 2021; Goswami et al., 2022). Three slides per tissue were used for H&E staining, following the protocols described in our previous studies (Tewari-Singh et al., 2016; Goswami et al., 2019; Goswami et al., 2021; Goswami et al., 2022). Briefly, sections were
treated with xylene, re-hydrated and stained with H&E, then dehydrated, cleared with xylene, and mounted using mounting media with a glass cover slip. The H&E-stained slides were used for assessment of the biological parameters, specifically, total corneal thickness, epithelial degradation, and epithelial-stromal separation as well as estimations of inflammatory cell count and blood vessel count. Corneal thickness, assessed from 10-12 randomly selected regions, was averaged to get the sample value, which was averaged to generate the group score. Percent epithelial-stromal separation and epithelial degradation were measured throughout the length of the cornea for each slide; averaged measurements for each group were used.

**Estimation of inflammatory cell count and blood vessel count.** Stromal region of the H&E-stained corneas was used for the estimation of inflammatory cell count and total number of blood vessels, as previously described (Goswami et al., 2019; Goswami et al., 2021; Goswami et al., 2022). Scoring of inflammatory cell count ranged from 0 to 4 with a score of 0 signifying no inflammatory cell, 1 signifying between 1-50 inflammatory cells, 2 signifying between 50-100 cells, 3 signifying between 100-500 cells, and 4 signifying >500 inflammatory cells in the corneal stroma.

**Immunohistochemistry for determining levels of COX-2, VEGF, and MMP-9 proteins.** IHC was performed to assess the levels of COX-2, VEGF, and MMP-9 proteins in the corneal epithelium of the rabbit eye sections per the methods previously reported (Tewari-Singh et al., 2016; Goswami et al., 2019; Goswami et al., 2021; Goswami et al., 2022). Briefly, sections were sequentially treated with xylene, re-hydrated, underwent epitope retrieval (heat mediated), and endogenous peroxidases blocking. Thereafter, he sections were incubated overnight at 4°C with the respective primary antibodies for COX-2, VEGF, MMP-9, or the negative control (rabbit IgG), followed by incubation with secondary and tertiary antibodies. The staining was visualized
using DAB and counter-staining the nucleus with hematoxylin. For all the IHC estimations a score of 0 to 4 was used to denote the intensity of brown color. A score of 0 signified no staining, 1 signified light staining, 2 signified moderate staining, 3 signified high staining, 4 very high (maximum intensity) staining.

**Immunofluorescence for determining SPARC levels.** IF staining was performed to estimate the levels of SPARC protein in the corneal epithelium of the rabbit cornea sections. Sections were sequentially treated with xylene, re-hydrated, permeabilized (0.3% Triton X-100) after epitope retrieval (using a decloaking chamber), blocked for non-specific binding, and incubated overnight at 4°C with the primary antibodies for SPARC. Next, slides were incubated in the dark with fluorochrome-conjugated secondary antibodies and mounted. The images were captured using Nikon Eclipse Ti2 inverted confocal microscope with NIS elements AR version 5.20 (green colored staining; cytoplasmic SPARC protein) and counter-staining the nucleus with DAPI (blue). For all the quantification of SPARC staining, the QUPath software (Ver 0.3.2) was used. Visualization of SPARC staining was done using 5-6 sections per animal at 600X magnification (n=3 per group).

**Statistics and data analysis.** All parameters were scored as described in each of the respective materials and methods sections. Percentage reversals were calculated using the increase/decrease induced by SM exposure (from the control) and then determining the reversal by DEX ([SM-DEX]/[SM-C] X 100). Two-Way analysis of variance (ANOVA) was used to determine statistical significance between groups (p<0.05), accounting for treatment (SM exposure or DEX administration) and day (study timepoints for the respective parameters analyzed). All post-hoc pairwise multiple comparisons were performed using Holm-Sidak method. All statistical tests
were performed using SigmaPlot version 15. Data are represented as the mean ± standard error of the mean (SEM).
RESULTS

In our previous study, we have shown the acute effects of SM-induced corneal injuries in the *in vivo* rabbit model. The primary signs of SM injuries become apparent around 6 h post-exposure and optimum injuries are observed around the day 7 and day 14 post-exposure timepoint and the effects were sustained up to day 28 post-exposure, the study endpoint (Goswami et al., 2021).

We reported histopathological alterations in corneal structure, decrease in keratocytes, increased inflammatory cells, NV, and expressions of COX-2, MMP-9, VEGF, and cytokines such as interleukin-8, which marked the acute injury effects. In the present study, we assess the effect of DEX treatment at two different dosing frequencies on the delayed SM injuries, beginning from day 14 up to day 56 post-exposure. The results are detailed in the following sections.

**Effect of DEX treatments on clinical parameters**

SM exposure led to the development of corneal injuries as determined by increased opacity, ulceration, and NV of the SM exposed corneas as compared to the unexposed controls. DEX treatment at both the twice daily (DEX 12h) and thrice daily (DEX 8h) regimens was effective in preventing the SM-induced damage to the corneas as a function of time until the DEX administration was discontinued, i.e., at day 28 post SM-exposure. The sustained effects of DEX treatment were also observed until the study endpoint of day 56.

**Corneal thickness.** SM exposure led to thickening of the rabbit corneas, as determined by the pachymetry measurements (in µm) that was statistically significant (omnibus SM exposure effect) in our studies (p<0.001). Pairwise analysis revealed a significant thickening upon SM exposure as compared to the controls at the day 14, 28, and 42 post SM-exposure timepoints. The average corneal thickness of the SM exposed corneas was greater than that of the control corneas at the day 56 post SM-exposure timepoint as well; however, the difference between the
two groups was not statistically significant at this timepoint in the pairwise analysis. Additionally, the effect of SM exposure was not consistent for all the study timepoints, with statistically significant differences observable 4 weeks after the SM exposure i.e., between day 14 and day 42, day 14 and day 56, day 28 and day 42, and 28 and day 56 post-exposure timepoints (p<0.001 for all pairwise analysis). Corneal thickening is indicative of an SM-induced inflammatory response, as also indicated from our previous studies on characterizing injuries in NM and SM exposed rabbit corneas (Goswami et al., 2021; Goswami et al., 2022). The differences indicate a decline in SM induced corneal thickening 28 days after SM exposure. Statistically significant omnibus treatment effects were observed, indicating reversal of SM-induced corneal thickening upon DEX treatment at both dosing frequencies in our study (p<0.001; Fig. 2A). DEX 8h treatment significantly and effectively decreased the SM-induced corneal thickening at the day 14 (~90% reversal), day 28 (~100% reversal), and day 42 (~97% reversal) post SM-exposure timepoints, determined using pairwise analysis. The effect of DEX 8h treatment was continued at the and day 56 timepoint as well (~68% reversal); however, the effect was not statistically significant at the study endpoint between the two dosing frequencies as determined using pairwise analysis.

The DEX 12h treatment also reduced corneal thickening significantly until the DEX administration was discontinued i.e., at day 14 (~77% reversal) and day 28 (~71% reversal) timepoints; however, the effects of DEX 12h treatment were not sustained post termination of the treatment i.e., at the day 42 and 56 post SM-exposure timepoints (Fig. 2A). Omnibus treatment effects, determined using the Two-Way ANOVA, also indicated a significant difference between the DEX 8 and DEX 12h treatments as well as between the control and DEX 12h groups (p<0.001). This could imply that DEX 8h treatment more effectively reversed SM-
induced corneal thickening as compared to the DEX 12h treatment.

**Corneal opacity.** Statistically significant treatment effect was observed for the effect of SM exposure (p<0.001). A significant and marked SM-induced corneal opacity (clouding of the cornea) was observed starting at day 14 and sustained until day 42 post SM-exposure as compared to the control group (Fig. 2B), with pairwise analysis. At the day 56 post SM-exposure timepoint, the corneal opacity score of the SM exposure group was higher than that of the control group; however, the increases were not significant (Fig. 2B). Corneal opacity compromises vision and can lead to impaired vision or even blindness if not treated in a timely manner. All treatments were found to have similar effects for all study timepoints, with no statistically significant difference observed for corneal opacity on different timepoints in any study groups. Effect of both DEX 8 and DEX 12 treatments was also found to be significant in reversing SM-induced corneal opacity (Fig. 2B). DEX 8h treatment was found to significantly reverse SM-induced corneal opacity when DEX administration was ongoing at day 14 (~84% reversal) and day 28 (~86% reversal). These effects were maintained post cessation of DEX administration, with near complete reversal in corneal opacity observed at day 42 and day 56 post SM-exposure timepoints though the effects were statistically significant only for the day 42 timepoint (Fig. 2B). DEX 12h treatment also reduced corneal opacity, but the reversal was statistically significant only for the day 14 timepoint (~61% reversal). Difference between the corneal opacity scores for DEX 8h and DEX 12h treatments as well as control and DEX 12h treatments were found to be statistically significant (p<0.001), indicating that DEX 8h treatment was more effective in reversing corneal opacity than DEX 12h treatment.

**Corneal ulceration.** A significant effect of SM exposure independent of the timepoint of analysis was observed for corneal ulceration in our study (Fig. 2C). In pairwise analysis, similar
to corneal thickness and opacity, SM exposure also induced significant corneal ulceration at day 14, 28, and 42 post SM-exposure timepoints. Though ulceration in the SM-exposed group was higher than in the control group at the day 56 post SM-exposure timepoint, although, not statistically significant, in pairwise analysis. Corneal ulceration is caused due to the damage to the corneal integrity upon SM exposure. The ulceration may lead to permanent scaring and impaired vision. Additionally, damage the damage also make the cornea and the eye overall, more prone to infections and secondary insults.

Only DEX 8h treatment was found to be statistically significant in reversing SM-induced corneal ulceration. Pairwise comparisons showed that DEX 8h effectively and significantly reversed SM-induced ulceration in the rabbit corneas at the day 14 (~71% reversal), day 28 (~86% reversal), and day 42 (~100% reversal) post SM-exposure timepoints. The sustained effects of DEX administration were observed at the day 56 post SM-exposure timepoint as well, though the reversal was not statistically significant. The DEX 12h treatment also reduced corneal ulceration, as compared to the SM-exposure group; however, the therapeutic effect for this parameter was not statistically significant for any of the timepoints in the study. Moreover, the difference between corneal ulceration scores for the DEX 8h and DEX 12h treatments as well as DEX 12h treatment and control group was also statistically significant, indicating greater efficacy of DEX 8h treatment in reversing SM-induced corneal ulceration.

Corneal NV. SM exposure was observed to significantly induce the growth of new blood vessels in the cornea for all the timepoints i.e., at day 14, 28, 42, and 56 post SM-exposure timepoints (Fig. 2D). The effect of SM exposure had a significant interaction effect with the timepoint post post-exposure (p<0.001), with a steady increase in NV observed from day 14 to day 56 post-exposure timepoints. Pairwise analysis revealed a significant difference in NV at day 14 post
SM-exposure (p<0.001 for day 14 vs day 28, day 14 vs day 42, and day 14 vs day 56 NV scores). Both the DEX 8h and DEX 12h treatments significantly and markedly prevented SM-induced NV in the rabbit corneas for all the study timepoints. For DEX 8h, the preventive effect at day 14 (~100%), day 28 (~96%), day 42 (~95%), and day 56 (~67%) were comparative to those for the DEX 12 treatment at day 14 (~94%), day 28 (~93%), day 42 (~82%), and day 56 (~67%) post SM-exposure timepoints. Thus, for the NV parameter, DEX administration at both dosing frequencies had a similar statistically significant effect that was sustained even 4 weeks post cessation of DEX administration.

In this comprehensive assessment of the clinical parameters, the DEX 8h treatment was more effective in reversing SM-induced corneal injuries. The effects of DEX were not only apparent at the day 14 and day 28 post SM-exposure timepoints i.e., during the continued DEX administration, but they were also significantly sustained 14 days post cessation of DEX administration i.e., at the day 42 post SM-exposure timepoint, for all parameter in the DEX 8h treatment group. At the 4 weeks post DEX treatment suspension timepoint i.e., day 56 post SM-exposure, the sustained effects of DEX treatment could still be observed; however, the decline in DEX efficacy was apparent and the differences between the DEX treatment groups and SM exposed groups were not significant (Fig. 2). It is important to note that a differential effect of SM toxicity was also observed for corneal thickness and NV, with decrease in corneal thickening at the day 42 and 56 post-exposure timepoints and an increase in corneal NV from the day 14 to day 56 post-exposure timepoints. Though the effect of timepoint post-exposure was not observed for any of the DEX treatment groups, we should be mindful that the DEX efficacy is dependent on SM-induced toxicity and thus, especially for reversal of corneal thickness om day 42 and 56
post SM-exposure timepoints, the results may be indicative of not only DEX treatment effect but also an inherent decline in SM-induced thickening.

**Effect of DEX treatments on histological parameters**

After the analyses of the clinical parameters, the histopathological assessments of corneal thickness, epithelial degradation, and epithelial-stromal separation upon SM-exposure as well as with DEX 8h and 12h treatments were performed in H&E-stained samples at days 28, 42, and 56 post SM-exposure timepoints. These parameters provided insights into SM-induced damage caused to the corneal structure and integrity, that could be associated with clinical signs of SM toxicity observed in the previous section. SM exposure led to increases in all the histopathological parameters assessed, as compared to the respective controls. Both DEX 8h and 12h treatments were effective in attenuating the SM-induced histopathological injuries, with DEX 8h treatment being more potent than the 12h treatment (Fig. 3).

**Corneal thickness.** Significant interaction between the treatment and timepoint post-exposure was observed (p<0.01) for SM and DEX 8h groups, indicating that the effect of SM exposure as well as effect of DEX treatment (DEX 8h) was significant different at different study timepoints. SM exposure led to an increase in the corneal thickness as compared to the controls at all the study timepoints; however, the effect of SM-exposure was found to be significant at day 28 and day 56 post SM-exposure only (Fig. 3A). Both DEX 8h and 12h treatments were found to effectively counter SM-induced corneal thickening, as observed in the H&E-stained corneal sections. The effect of DEX 8h treatment was found to be significantly different on all three study timepoints (day 28 vs day 42 and day 42 vs day 56, p<0.05; day 42 vs day 56, p<0.001).

DEX 8h treatment significantly and effectively mitigated SM-induced increase in corneal thickness with near complete reversals observed at day 28 and day 42 post SM-exposure
timepoints. A ~54% reversal in corneal thickening was observed with DEX 8h treatment at the day 56 post SM-exposure timepoint. DEX 12h treatment was also effective in reversing SM-induced increase in corneal thickness i.e., at day 28 (~96% reversal), day 42 (~73% reversal), and day 56 (~61% reversal) post SM-exposure timepoints. Both DEX 8h and DEX 12h treatments were statistically significant only on the day 28 post exposure timepoint, as compared to the SM treated group in pairwise analysis.

**Epithelial degradation.** Epithelial degradation indicated the damage to the corneal epithelial layers, including cell shrinking, nuclear pyknosis, sloughing-off the epithelium. This parameter indicates the physical damage caused to the outermost layer of the cornea, that is in constant contact with the environment and serves as the primary line of ocular defense. Significant interaction was observed between the treatment and the study timepoints (p<0.001) within the SM exposure and DEX 12h treatment. Pairwise analysis revealed that the day 28 vs 42 (p<0.05), day 28 vs day 56 (p<0.001), as well as day 42 vs day 56 (p<0.001) scores were found to be significantly different. Pairwise analysis for treatment effects revealed that SM caused significant degradation of the corneal epithelium at the day 28 and day 42 post SM-exposure timepoints (Fig. 3B). Epithelial degradation was significantly and markedly decreased upon DEX 8h treatment at the day 28 (~73% reversal) and 42 (~82% reversal) post SM-exposure timepoints. The DEX 12h treatment decreased SM-induced epithelial degradation only at the day 42 post exposure timepoint (~40% reversal) and this effect was not statistically significant compared to the SM treated group. Additionally, the scores of DEX 8h and 12h treatments as well as DEX 12h and control groups was found to be significantly different (p<0.001), indicating that DEX 8h treatment more effective than the DEX 12h treatment.

**Epithelial-stromal separation.** The separation of the corneal epithelium from the underlying
stoma is indicative of the damage to or dissolving off the basement membrane. This injury is distinct from epithelial degradation, as the epithelium is physically separated, distinct from the damage to epithelium cells. A significant treatment and timepoint interaction effect was observed for this parameter as well, with difference within the SM exposure and DEX 12h treatment groups at the level of timepoints (p<0.001). Significant separation of the corneal epithelium from the stroma was observed upon SM exposure at day 28 and 42 post SM-exposure timepoints, as compared to the controls (Fig. 3C), in pairwise analysis for treatment effects. The epithelial-stromal separation was significantly decreased at the day 28 (~75% reversal) and 42 (~91% reversal) post SM-exposure timepoints upon DEX 8h treatment whereas the DEX 12h treatment significantly reduced epithelial-stromal separation only at the day 42 (~60% reversal) post SM-exposure timepoint. Significant difference in the DEX 8h and DEX 12h groups was also observed at the day 28 post exposure timepoint (p<0.001). DEX 12h group was significantly different from the control group on day 28 and 42 post SM-exposure. These results suggest that DEX 8h treatment was more effective than DEX 12h treatment in reversing SM-induced epithelial-stromal separation.

Thus, the overall assessment of the histopathological parameters indicates that SM exposure caused significant corneal injury and both DEX treatments were effective in reversing the SM-induced histopathological damage. As for the clinical parameters, DEX 8h treatment was more effective than the DEX 12h treatment in reversing SM-induced injuries. The difference between DEX 8h and DEX 12h in reversing the SM-induced epithelial degradation was significantly different at the day 28 and 42 post-exposure timepoints. Additionally, the difference in the epithelial-stromal separation was also significant between DEX 8h and DEX 12h at the day 28 post-exposure timepoint. Moreover, the difference between the DEX 12h and control
groups was significant at day 28 for epithelial degradation and epithelial-stromal separation and at day 42 for all histopathological parameters. This implies that though the DEX 12h treatment was effective in reversing SM-induced injuries, it could not revert corneas to near control the conditions.

Effect of DEX treatments on biological parameters

SM-exposure was found to increase the inflammatory cell count in the corneal stroma, indicating increased inflammatory cell infiltration upon SM-exposure, as well as the number of blood vessels as compared to the controls (Fig. 4). Both DEX 8h and 12h treatments were shown to effectively reverse the SM-induced increase in the number of inflammatory cells and blood vessels in the corneal stroma. DEX 8h treatment was found to be more effective in decreasing the inflammatory cells, whereas both the 8h and 12h treatments were found to be equally effective in decreasing the blood vessel growth.

Inflammatory cell count. Inflammatory cell numbers indicate the inflammatory milieu of the cornea, as any injury response is accompanied by inflammation and wound healing responses. We have previously shown that corneal mustards insults are associated with increased inflammatory mediators (Goswami et al., 2018; Goswami et al., 2019; Goswami et al., 2021; Goswami et al., 2022). A significant interaction of treatment and timepoint post exposure was observed for the inflammatory cell count (p<0.001). Pairwise analysis for the treatment effects showed that SM exposure led to significant and massive influx of inflammatory cells, as indicated by their increased numbers, in the corneal stroma at the day 28 and 42 post SM-exposure timepoints (p<0.001; Fig. 4A). Pairwise analysis for the effect of timepoint showed that SM exposure had significantly differential effects on the level of study timepoints (day 28 vs day 56 and day 42 vs day 56; p<0.001), which could be due to substantial spontaneous decrease in
inflammatory cell counts at day 56 post-exposure timepoint.

Both DEX 8h and 12h treatments significantly reduced the inflammatory cell influx at the day 28 (DEX 8h: ~84% and DEX 12h: ~40% reversal); however, at the day 42 post-exposure timepoint, the reversal was significant with DEX 8h treatment only (DEX 8h: ~94% and DEX 12h: ~30% reversal), with sustained effects of DEX treatment apparent at day 56 that were not statistically significant. DEX 8h treatment was found to be significantly more effective than the 12h treatment in decreasing the influx of inflammatory cells into the corneal stroma at day 28 and 42 post SM-exposure timepoints. Additionally, as with the clinical and histopathological parameters, the difference between the inflammatory cell counts of control and DEX 12h was statistically significant at both the day 28 and day 42 post exposure timepoints, further supporting the greater efficacy of the DEX 8h treatment over the 8h treatment.

**Blood vessel count.** Blood vessels count also showed a significant interaction effect between the levels of treatment and timepoint (p<0.001). Pairwise analysis for SM exposure showed significant increase in the growth of new blood vessels in the corneal stroma at the day 28 and 56 post SM-exposure (Fig. 4B) as compared to the control group. Additionally, there was statistically differential effect of SM exposure on the day 28 vs day 42 and day 28 vs day 56 post-exposure timepoints (p<0.001). This could be due to the decrease in the number of blood vessels observed from the day 28 to day 56 post-exposure study endpoint, though the blood vessel count was significantly increases in the SM exposed groups as compared to the controls. Both DEX 8h and 12h treatments significantly reduced the number of blood vessels at the day 28 (DEX 8h: ~99% and DEX 12h: ~95% reversal) and day 56 (DEX 8h: ~77% and DEX 12h: ~73% reversal) post SM-exposure timepoints, indicating significant sustained effects of DEX treatment even 4 weeks post cessation of DEX administration. The DEX treatments caused
apparent reversals in the growth of new blood vessels at the day 42 post SM-exposure timepoints as well (DEX 8h: ~99% and DEX 12h: ~94% reversal); however, the effects were not statistically significant. The effect of both dosing frequencies was found to be comparative in hampering the growth of new blood vessels in the corneal stroma.

Effect of DEX treatments on molecular parameters

SM exposure significantly increased the expressions of COX-2, MMP-9, and VEGF proteins in the rabbit cornea, as determined using IHC at all the study timepoints i.e., day 28, 42, and 56 post SM-exposure (Fig 5). Both DEX 8h and 12h treatments markedly rescued the SM-induced increases in COX-2, MMP-9, and VEGF expressions; however, DEX 8h treatment showed a marginally greater decrease in COX-2, MMP-9, and VEGF expressions, though the difference between the DEX 8h and 12h treatments was not statistically significant.

COX-2. COX-2 is pro-inflammatory cytokine and serves to increase the production of pain and inflammation causing prostaglandins. A significant interaction effect between the levels of treatment and timepoints was observed for COX-2 expression. Pairwise analysis for the effect of treatment showed that SM exposure led to pronounced and significant increases in COX-2 expressions in the corneal epithelium at all the study timepoints i.e., days 28, 42, and 56 post SM-exposure (Fig. 5A). Both DEX 8h and 12h treatments significantly reduced the SM-induced COX-2 levels at the day 28 (DEX 8h: ~37% and DEX 12h: ~32% reversal) and day 42 (DEX 8h: ~69% and DEX 12h: ~43% reversal) post SM-exposure timepoints. At the day 56 post SM-exposure timepoint, the effect of DEX treatments was still apparent; however, the difference from the SM exposure group was not statistically significant in either group. The effect of both dosing frequencies was found to be comparative in reversing the SM-induced increases in COX-2 expression in the corneal epithelium, at all the study timepoints.
**MMP-9.** MMP-9 is an integral protein for maintaining the structural integrity of the cornea. Overall treatment effects indicated that SM exposure caused significant increases in the expression of MMP-9 as compared to the control corneal epithelium and these increases were significantly reversed by both DEX treatments in a comparative manner. Pairwise analysis for MMP-9 expression showed that SM exposure led to pronounced and significant increases in MMP-9 expressions in the corneal epithelium at all study timepoints i.e., day 28, 42, and 56 post SM-exposure (Fig. 5B). Both DEX 8h and 12h treatments significantly reduced the SM-induced MMP-9 levels at day 42 (DEX 8h: ~53% and DEX 12h: ~35% reversal), and day 56 (DEX 8h: ~89% and DEX 12h: ~73% reversal) post SM-exposure timepoints. At the day 28 post SM-exposure timepoint, the effect of only DEX 12h treatment was significant (~43% reversal; Fig. 5B). The effect of both dosing frequencies was found to be comparable in reversing the SM-induced increases in MMP-9 expression in the corneal epithelium, at all the study timepoints, similar to the COX-2 expression results.

**VEGF.** VEGF plays an important role in angiogenesis and could be the molecular mediator of the clinical (NV) and biological (blood vessel count) effects observed in our study. Following suit with other molecular markers, pairwise analysis showed that SM exposure led to a significant increase in the expression levels of VEGF and both DEX 8h and 12h treatments significantly countered these increases (Fig. 5C), at all study timepoints at day 28 (DEX 8h: ~80% and DEX 12h: ~78% reversal), day 42 (DEX 8h: ~88% and DEX 12h: ~52% reversal), and day 56 (DEX 8h: ~86% and DEX 12h: ~81% reversal) post SM-exposure. These results indicate marked and comparable efficacy of both DEX 8h and 12h treatments until DEX discontinuation and sustained efficacy up to the study endpoint.

**SPARC.** SPARC, along with MMP-9, is associated with maintaining the structural integrity of
the cornea. SM exposure significantly increased the expressions of SPARC proteins in the corneal epithelium at day 28 and 42 post SM-exposure timepoints (Fig. 6), as determined using IF staining. Both DEX 8h and 12h treatments markedly and significantly reversed the SM-induced increases in SPARC expressions at the day 28 and 42 post-exposure timepoints, with DEX 8h treatment showing a near complete reversal at all study timepoints that was greater than the reversals observed with the DEX 12h treatment (day 28: ~100%, day 42: ~94%, and day 56: ~84% reversal). As observed from these results, the effects of DEX 8h treatment were sustained up to 4 weeks post termination of DEX administration and were more pronounced than the DEX 12h effects in reducing SM-induced increases in SPARC expression.
DISCUSSION

Our ongoing studies focus on finding effective therapeutics against vesicant-induced ocular injuries. DEX has emerged as a promising potential sanative from our ex vivo (Tewari-Singh et al., 2012; Goswami et al., 2018) as well as in vivo (Goswami et al., 2022) NM-exposure studies in rabbit models. Although DEX efficacy in mitigating NM-induced injuries could be gauged from these studies, it was important to translate these findings in SM-induced corneal injury. It was also important to establish a treatment regimen of DEX administration. An effective therapeutic window for DEX administration was determined in our previous study (Goswami et al., 2022) for the treatment of NM-induced corneal injuries. Thus, in the present study, we wanted to decipher the best dosing frequency for reversal of SM-induced corneal injuries. Based on clinical, histopathological, and molecular assessments, initiation of DEX administration at 2 h post SM-exposure and treatment with DEX every 8 h thereafter was more potent than treatment every 12 h in reversing SM-induced corneal injuries in our study model (Fig. 7). Effects were most pronounced at the day 28 and 42 post exposure and were sustained until the study endpoint. These results are novel and immensely important. Apart from demonstration of a complete DEX-treatment regimen (therapeutic window and dosing frequency), long-term in-depth evaluation of clinical, histopathological, biological, and molecular parameters was also performed with, correlation of clinical injuries, biological histopathology of the corneal structure, and downstream molecular regulators.

DEX 8h treatment had better reversals in SM-induced corneal injuries from the phenotypic signs to the expression profiles of molecular markers both during the administration of DEX i.e., until the day 28 post SM-exposure timepoint, as well as post cessation of DEX treatment until day 56 post SM-exposure. Sustained effects observed with DEX 8h treatment
could be due to the greater protection conferred by the DEX 8h dosing frequency that mitigated initial damage and thus downstream long-term effects.

Both DEX dosing frequencies had profound effects on reversing SM-induced blood vessel growth, and the effects were also apparent at the molecular, biological, and clinical levels. DEX reversed SM-induced NV, decreased the blood vessel count, and decreased the expression of VEGF significantly and markedly at all study timepoints. VEGF is associated with the initiation of blood vessel generation and plays a vital role in wound healing (Bao et al., 2009). Early prevention of NV and inhibition of growth of existing blood vessels in the cornea is very important. Significant NV and blood vessel invasion of the cornea leads to corneal damage at invasion sites. Even after degeneration of invading blood vessels, opaque areas develop at the vessel entry site in the cornea, leading to vision impairments (Baradaran-Rafii et al., 2011). Therapeutics such as anti-VEGF therapy (Kardar et al., 2014; Gore et al., 2018; Gore et al., 2023) as well as DEX treatment alone (Amir et al., 2000; Kadar et al., 2009; Goswami et al., 2016; Goswami et al., 2018; Goswami et al., 2022) mitigate mustard vesicant-induced NV.

Ocular vesicant exposure is associated with latent pathology that becomes apparent 1-3 weeks post exposure and includes NV, erosions, and edema. Delayed symptoms are attributed to damage at the nucleic acid and protein levels, caused by alkylation. The clinical signs of these insults take time to present clinically. Additionally, the latent phase signifies the time required for the generation of new blood vessels and their ingress into the cornea.

Although both DEX treatments frequencies were effective in reducing SM-induced increases in COX-2, MMP-9, VEGF, and SPARC expressions in the corneal epithelium, DEX 8h was more effective than DEX 12h treatment. COX-2 is primarily associated with generation of an inflammatory response (Chen et al., 2011). Increase in COX-2 expression in the corneal
epithelium paralleled the increase in inflammatory cells numbers in the corneal stroma as well as inflammation associated thickening of the cornea upon SM-exposure. DEX treatment significantly and effectively countered these SM associated inflammatory responses. While the differential effect of the two DEX dosing frequencies was not apparent for the blood vessel growth and inflammatory response associated parameters, it was most prominent in the reversal of SM-induced clinical and histopathological injuries, such as corneal opacity and ulceration, epithelial degradation as well as its separation from the underlying stromal layer. DEX dosing at the 8h frequency seemed more beneficial for the preservation of corneal structures, including the epithelium degradation and detachment.

The proteins elucidated in the study function as essential cogs in the SM-injury machinery (Araj et al., 2020; Araj et al., 2022). Expression of COX-2, MMP-9, and VEGF has been shown to increase upon SM exposure in the corneas of in vivo rabbit model in our previous study (Goswami et al., 2021) and DEX treatment was shown to decrease the expression of COX-2 and VEGF in the corneas of rabbits exposed to NM (Goswami et al., 2022). These results indicate that the effects observed with NM exposure injuries are also observed in DEX treatment of SM-induced corneal injuries. MMRP-9 and SPARC are important for maintenance of structural integrity in the cornea. They regulate structural organization of corneal proteins, especially extracellular matrix (ECM) proteins (Chotikavanich et al., 2009), to maintain cornea transparency and prevent fibrosis. Increase in MMP-9 expression upon SM injury has been reported in previous studies from our lab and other groups (Kadar et al., 2009; Goswami et al., 2021; Horwitz et al., 2014). Anti-MMPs therapies have been shown to ameliorate mustard induced ocular toxicities (Kadar et al., 2009; Horwitz et al., 2014).
The expression profile of SPARC has not been studied in previous studies on SM-exposure or upon DEX administration in the ocular tissue. SPARC is a matricellular protein. Increased expression of SPARC is observed upon corneal injury and corneal epithelial cells secrete SPARC (Mishima et al., 1998). It also has an inverse relationship with structural integrity of tissues; SPARC-null mice have increased wound healing abilities (Bradshaw et al., 2002), indicating its role in injury development and delayed wound healing. Additionally, SPARC increases fibrosis and induces expression of MMP-9 (Venugopal et al., 2019). It is increased in response to NM exposure, along with increased MMP-9 expression (DeSantis-Rodrigues et al., 2021). These findings parallel our results. SM exposure led to increased SPARC expression, leading to corneal damage, and delayed wound healing. DEX treatment decreased SPARC expression in the corneal epithelium, with near complete reversals observed from day 28 until day 56 post exposure in the DEX 8h treatment group. This could be one of the underlying pathways protecting epithelial degradation. Berryhill et al., (2003) reported SPARC accumulation during injury and propose its association with wound healing. Thus, due to the decreased injury upon DEX administration, there is decline in SPARC expression. This is an interesting pathway that is intercepted by DEX, which needs further elucidation.

It is important to note that there is a decline in SM toxicity, especially clinical and biological parameters, at day 56 post exposure. Thus, DEX treatments effects at day 56 post exposure may not be reflective of DEX efficacy and rather of the animal model in the present study. Also, long-term use of DEX may lead to side effects. However, no adverse effects were observed in our study. Additionally, Kadar et al., (2014) also reported no side effects upon long-term DEX treatment (4/day for 4 weeks, beginning 2 weeks after SM exposure in rabbits). Furthermore, in a conflict situation, SM-induced injuries would arguably pose a greater threat to
eyes that prolonged DEX treatment. These limitations are important to acknowledge and be cognizant of while determining the efficacy of DEX in treating SM-induced corneal injuries.

In summary, DEX at both dosing frequencies effectively reversed SM-induced injury in our study. DEX 8h treatment was more potent than DEX 12h treatment. Effects were most pronounced at day 28 and day 42 post SM-exposure. These results are novel, as no other studies have assessed the efficacy of DEX upon SM exposure, evaluating parameters ranging from clinical signs to molecular markers, in depth. Additionally, a comprehensive DEX treatment regimen (therapeutic window and dosing frequency) is also established that is critical in designing further interventions for ocular exposure of SM.
Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Agarwal R., Tewari-Singh N., Agarwal C., Croutch C.R., Petrash M.J., Pantcheva M.B., Araj H

Conducted experiments: Mishra N., Kant R., Kandhari K., Anantharam P

Performed data analysis: Mishra N., Kant R., Kandhari K

Wrote or contributed to the writing of the manuscript: Mishra N., Agarwal R., Tewari-Singh N., Agarwal C., Kant R., Kandhari K., Pantcheva M.B., Petrash J.M., Croutch C.R., Anantharam P, Araj H
REFERENCES


Footnotes

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FIGURE LEGENDS

Fig. 1: Schematic representation of the study design with the parameters analyzed.

Fig. 2: Dosing frequency dependent effect of DEX treatment on SM-induced clinical pathologies. (A) Bar graph showing corneal thickness. Representative slit lamp pictures (left panel) and bar graphs (right panel) showing (B) corneal opacity, (C) corneal ulceration, and (D) corneal neovascularization. DEX administration, initiated at 2 h post SM-exposure and then either every 8 or 12h thereafter for 28 days post SM-exposure; sustained effects observed up to day 56 post SM-exposure. Data presented are mean ± SEM (n=5). #p < 0.05, ##p < 0.01, and ###p < 0.001; for SM, compared to the control group scores and *p < 0.05, **p < 0.01, and ***p < 0.001; for DEX 8h and DEX 12h as compared to SM group scores. Comparisons were also made between the DEX 8h and DEX 12h treatment groups. All statistical analyses were performed using Two-Way ANOVA and post-hoc Holm-Sidak method for pairwise analysis. Absence of control bars indicates a control group value of zero. Yellow arrows, corneal opacity; red arrows, corneal ulceration; and blue arrows, corneal neovascularization. SM, sulfur mustard; DEX, dexamethasone formulation.

Fig. 3: Dosing frequency dependent effect of DEX treatment on SM-induced histopathologies in the corneal epithelium. Representative pictures (left panel; day 28 post SM-exposure timepoint) and bar graphs (right panel) showing (A) corneal thickness, (B) epithelial degradation, and (C) epithelial-stromal separation visualized using H&E staining. DEX administration, initiated at 2 h post SM-exposure and then either every 8 or 12h thereafter for 28 days post SM-exposure; sustained effects observed up to day 56 post SM-exposure. Data presented are mean ± SEM (n=5). #p < 0.05, ##p < 0.01, and ###p < 0.001; for SM, compared to the control group scores and *p < 0.05, **p < 0.01, and ***p < 0.001; for DEX 8h and DEX 12h as compared to SM group scores.
group scores. Comparisons were also made between the DEX 8h and DEX 12h treatment groups. All statistical analyses were performed using Two-Way ANOVA and post-hoc Holm-Sidak method for pairwise analysis. Absence of control bars indicates a control group value of zero. Black arrows, epithelial degradation; red arrows, epithelial-stromal separation. SM, sulfur mustard; DEX, dexamethasone formulation; E, epithelium; S, stroma.

Fig. 4: Dosing frequency dependent effect of DEX treatment on SM-induced increases in inflammatory cell count and blood vessel count in the corneal stroma. Representative pictures (left panel; day 28 post SM-exposure timepoint) and bar graphs (right panel) showing (A) inflammatory cell count and (B) blood vessel count visualized using H&E staining. DEX administration, initiated at 2 h post SM-exposure and then either every 8 or 12h thereafter for 28 days post SM-exposure; sustained effects observed up to day 56 post SM-exposure. Data presented are mean ± SEM (n=5). #p < 0.05, ##p < 0.01, and ###p < 0.001; for SM, compared to the control group scores and *p < 0.05, **p < 0.01, and ***p < 0.001; for DEX 8h and DEX 12h as compared to SM group scores. Comparisons were also made between the DEX 8h and DEX 12h treatment groups. All statistical analyses were performed using Two-Way ANOVA and post-hoc Holm-Sidak method for pairwise analysis. Absence of control bars indicates a control group value of zero. Black arrows, immune cells; red arrows, blood vessels. SM, sulfur mustard; DEX, dexamethasone formulation.

Fig. 5: Dosing frequency dependent effect of DEX treatment on SM-induced increases in protein expression of inflammatory (COX-2), extracellular matrix organizer (MMP-9), and angiogenic markers (VEGF) in the corneal epithelium. Representative pictures (left panel; day 28 post SM-exposure timepoint) and bar graphs (right panel) showing (A) COX-2, (B) MMP-9, and (C) VEGF expressions visualized using immunohistochemistry. DEX administration, initiated at 2 h
post SM-exposure and then either every 8 or 12h thereafter for 28 days post SM-exposure; sustained effects observed up to day 56 post SM-exposure. Data presented are mean ± SEM (n=5). #p < 0.05, ##p < 0.01, and ###p < 0.001; for SM, compared to the control group scores and *p < 0.05, **p < 0.01, and ***p < 0.001; for DEX 8h and DEX 12h as compared to SM group scores. Comparisons were also made between the DEX 8h and DEX 12h treatment groups. All statistical analyses were performed using Two-Way ANOVA and post-hoc Holm-Sidak method for pairwise analysis. SM, sulfur mustard; DEX, dexamethasone formulation; COX-2, cyclooxygenase-2; VEGF, vascular endothelial growth factor; MMP-9, matrix metalloproteinase-9; E, epithelium; S, stroma.

Fig. 6: Dosing frequency dependent effect of DEX treatment on SM-induced increases in expression of SPARC in the corneal epithelium and basement membrane. Representative pictures (left panel) and bar graphs (right panel) showing SPARC expression at (A) day 28, (B) day 42, and (C) day 56 post SM-exposure timepoints; visualized using immunofluorescence. DEX administration, initiated at 2 h post SM-exposure and then either every 8 or 12h thereafter for 28 days post SM-exposure; sustained effects observed up to day 56 post SM-exposure. Data presented are mean ± SEM (n=5). #p < 0.05, ##p < 0.01, and ###p < 0.001; for SM, compared to the control group scores and *p < 0.05, **p < 0.01, and ***p < 0.001; for DEX 8h and DEX 12h as compared to SM group scores. Comparisons were also made between the DEX 8h and DEX 12h treatment groups. All statistical analyses were performed using Two-Way ANOVA and post-hoc Holm-Sidak method for pairwise analysis. SM, sulfur mustard; DEX, dexamethasone formulation; SPARC, secreted protein, acidic and rich in cysteine; E, epithelium; S, stroma.

Fig. 7: Comparative overview of DEX 8h and DEX 12h treatments: The differential effect of DEX 8h and DEX 12h treatments on all the parameters analyzed (A) clinical, histopathological,
and molecular mediators of the neovascularization, inflammatory, and structural pathways (B) at day 28 and day 42 post SM-exposure timepoints are depicted. NS, grey; *p < 0.05, orange; **p < 0.01, pink; and ***p < 0.001, green for DEX 8h and DEX 12h treatment group scores as compared to SM group scores. Comparisons were also made between the DEX 8h and DEX 12h treatment groups. All statistical analysis were performed using Two-Way ANOVA and post-hoc Holm-Sidak method for pairwise analysis. DEX, dexamethasone formulation; COX-2, cyclooxygenase-2; VEGF, vascular endothelial growth factor; MMP-9, matrix metalloproteinase-9; SPARC, secreted protein, acidic and rich in cysteine.
Fig. 1

**Group I**
- Both eyes: SM
- Right eye: DEX 12h
- Left eye: DEX 8h

**Group II**
- Right eye: SM
- Left eye: Untreated [Control]

**Clinical parameters**
- Day 0
- Day 14
- Day 28
- Day 42
- Day 56

**Tissue harvest**
- Corneal epithelium: degradation, separation from stroma
- Corneal stroma: inflammatory cells, blood vessels
- IHC: COX-2, MMP-9, VEGF
- IF: SPARC

**Cornea:**
- Thickness
- Opacity
- Ulceration
- Neovascularization

**SM (10 µl for 90 s)**
A. CORNEAL THICKNESS

- Control
- SM
- SM+DEX 12h
- SM+DEX 8h

B. CORNEAL OPAcity

- Untreated
- SM
- SM+DEX 12h
- SM+DEX 8h

C. CORNEAL ULCERATION

- Untreated
- SM
- SM+DEX 12h
- SM+DEX 8h

D. CORNEAL NEOVASCULARIZATION

- Untreated
- SM
- SM+DEX 12h
- SM+DEX 8h
Fig. 3

A. CORNEAL THICKNESS

B. EPITHELIAL DEGRADATION

C. EPITHELIAL-STROMAL SEPARATION

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**Fig. 4**

**A. INFLAMMATORY CELL COUNT**

Untreated  SM  SM+DEX 12h  SM+DEX 8h

Day 28  Day 42  Day 56

**B. BLOOD VESSEL COUNT**

Untreated  SM  SM+DEX 12h  SM+DEX 8h

Day 28  Day 42  Day 56

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**A. COX-2 EXPRESSION**

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**B. MMP-9 EXPRESSION**

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**C. VEGF EXPRESSION**

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<td><img src="sm.png" alt="Image" /></td>
<td><img src="sm_dex_12.png" alt="Image" /></td>
<td><img src="sm_dex_8.png" alt="Image" /></td>
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<td>56</td>
<td><img src="untreated.png" alt="Image" /></td>
<td><img src="sm.png" alt="Image" /></td>
<td><img src="sm_dex_12.png" alt="Image" /></td>
<td><img src="sm_dex_8.png" alt="Image" /></td>
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Fig. 6

A. Day 28

B. Day 42

C. Day 56
### A. Differential effects of DEX 8h and DEX 12h treatments: overview

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DEX 8h</th>
<th>DEX 12h</th>
<th>DEX 8h vs DEX 12h</th>
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<tbody>
<tr>
<td><strong>Clinical parameters</strong></td>
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<tr>
<td>Corneal thickness</td>
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<td>***</td>
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<tr>
<td>Corneal opacity</td>
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<td>***</td>
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</tr>
<tr>
<td>Corneal ulceration</td>
<td>***</td>
<td>***</td>
<td>*</td>
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<tr>
<td>Corneal neovascularization</td>
<td>***</td>
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<tr>
<td><strong>Histo-pathological parameters</strong></td>
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<tr>
<td>Corneal thickness</td>
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<td>***</td>
<td></td>
</tr>
<tr>
<td>Epithelial degradation</td>
<td>***</td>
<td>***</td>
<td>** ***</td>
</tr>
<tr>
<td>Epithelial-stromal separation</td>
<td>***</td>
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<td>***</td>
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<tr>
<td><strong>Molecular parameters</strong></td>
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<tr>
<td>COX-2</td>
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</tr>
<tr>
<td>MMP-9</td>
<td>***</td>
<td>*</td>
<td>**</td>
</tr>
<tr>
<td>VEGF</td>
<td>*</td>
<td>***</td>
<td>* **</td>
</tr>
<tr>
<td>SPARC</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>Inflammatory cell count</td>
<td>***</td>
<td>**</td>
<td>* ** ***</td>
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<tr>
<td>Blood vessel count</td>
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### B. Differential effects of DEX 8h and DEX 12h treatments on specific pathways

<table>
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<tr>
<th>Pathway</th>
<th>DEX 8h</th>
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<th>DEX 8h vs DEX 12h</th>
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<tbody>
<tr>
<td><strong>Blood Vessel Growth</strong></td>
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<tr>
<td>Corneal neovascularization</td>
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<td>Blood vessel count</td>
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<td>VEGF</td>
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<td>***</td>
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<tr>
<td><strong>Inflammatory response</strong></td>
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<td>***</td>
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<td>Inflammatory cell count</td>
<td>***</td>
<td>***</td>
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</tr>
<tr>
<td>COX-2</td>
<td>***</td>
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<tr>
<td><strong>Structural integrity</strong></td>
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<tr>
<td>Epithelial degradation</td>
<td>***</td>
<td>***</td>
<td>** ***</td>
</tr>
<tr>
<td>Epithelial-stromal separation</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>SPARC</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>MMP-9</td>
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<td>*</td>
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</tbody>
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