Special Section on Medical Countermeasures

Establishing a Dexamethasone Treatment Regimen To Alleviate Sulfur Mustard–Induced Corneal Injuries in a Rabbit Model

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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-hour postexposure therapeutic window is most beneficial. Here, the efficacy of two DEX dosing frequencies [i.e., every 8 or 12 hours (initiated, as previously established, 2 hours after exposure)] until 28 days after SM exposure was assessed. Furthermore, sustained effects of DEX treatments were observed up to day 56 after SM exposure. Corneal clinical assessments (thickness, opacity, ulceration, and NV) were performed at the day 14, 28, 42, and 56 post–SM exposure time points. 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 after 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 ± S.E.M.). DEX administration every 8 hours was more potent than every 12 hours in reversing ocular SM injury, with the most pronounced effects observed at days 28 and 42 after 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 dexamethasone (DEX) treatment regimen by comparing the efficacy of DEX administration at 12 versus 8 hours initiated 2 hours after exposure. DEX administration every 8 hours was more effective in reversing sulfur mustard (SM)-induced corneal injuries. SM injury reversal during DEX administration (initial 28 days after exposure) and sustained [further 28 days after cessation of DEX administration (i.e., up to 56 days after 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. Lommel and Steinkopf (Pechura and Rall, 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”

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ABBREVIATIONS: COX-2, cyclooxygenase 2; DEX, dexamethasone; IF, immunofluorescence; IHC, immunohistochemistry; MMP-9, matrix metalloproteinase; NM, nitrogen mustard; NV, neovascularization; SM, sulfur mustard; SPARC, secreted protein acidic and rich in cysteine; VEGF, vascular endothelial growth factor.
mustard varieties like HQ (Gates and Moore, 1946), HT or Run- col, and HL (McCamley, 2007). As research progressed to de- velop better chemical warfare agents, SM was stockpiled and improperly discarded (e.g., in waterbodies) after the World Wars (Geraci, 2008; Wattana and Bey, 2009). To avoid con- fusion, sulfur mustard will be referred to as SM throughout this study.

Individuals exposed experience a range of symptoms, de- pending upon dosage (concentration/duration) and form (liquid or vapor) of SM, route of exposure, and physiology of individu- als 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, 2011). The ocular system is most vulnerable to SM exposure (Rafati-Rahimzadeh et al., 2019), with injuries developing even at 5 mg/min per L doses of SM (Pehchura and Rall, 1993; Amata et al., 2003; McNutt et al., 2020). SM exposure may cause discomfort/pain, smarting, in- flammation, 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 Hefazi, 2006; Ghasemi et al., 2009; Gordon et al., 2009; Kadar et al., 2009; McNutt et al., 2012; Ghasemi et al., 2019). The cornea, which is the outermost, transparent layer of the eye, directly interacts with external environment and is hence primarily susceptible to injury by external toxins such as SM.

Threat of SM exposure is ever present during a con- flict 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 improper- ly discarded SM reserves (Missiaen et al., 2010). Effective and targeted countermeasures are warranted against ocular SM-induced injuries. Dexamethasone (DEX), a US Food and Drug Administration–approved anti-inflammatory steroidal drug, has been shown to effectively reverse vesicant-induced ocular injuries (Kadar et al., 2009, 2014; Tewari-Singh et al., 2012; Goswami et al., 2018, 2022). It is readily available, inex- pensive, and does not require medical training/assistance to administer, strengthening it as an ideal therapeutic interven- tion, 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 or 12 hours (beginning 2 hours after ex- posure) until 28 days after SM exposure, beginning at 14 days after exposure of SM-induced corneal injuries. Sustained effects of DEX treatment were assessed for an additional 28 days follow- ing termination of DEX administration in alleviating late pathol- ogy. Sustained effects of DEX treatments were observed up to day 56 after SM exposure (i.e., for an additional 4 weeks after cessation of DEX administration). Clinical assessment of cor- neal injury was performed at days 14, 28, 42, and 56 after SM exposure using the following parameters: thickness, opacity, ulceration, and NV. Histopathological (corneal epithelium degrada- tion, 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), and 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 topi- cal 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 proc- eeded from MSC Industrial Supply Company (Melville, NY) and 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 #ab25775), and SPARC (cat #225716) were obtained from Abcam (Cambridge, MA). The primary antibody used as negative control, mouse IgG antibody, was procured from N-Uiversal (DAKO, Santa Clara, CA).

For IHC, 3’,3’-diaminobenzidine (DAB) peroxide substrate kit (cat #SK-4100) was procured from Vector Laboratories, Inc. (Burlingame, CA). The sec- ondary fluorochrome attached antibody (cat #AI1008) used in SPARC immunofluorescence (IF) was obtained from Invitrogen (Waltham, MA). The IF mounting media (with 4’,6-diamidino-2-phenylindole; VECTASHIELD Vibrance, cat #H-1800) was obtained from Vector Laboratories, Inc.

Animals and Study Design. New Zealand white rabbits (n = 33; males), weighing between 2.5 and 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 quar- antined 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-hour light/ dark cycle.

Rabbits were randomly divided into two groups: group 1 (n = 6 per time point; treatment group) and group 2 (n = 5 per time point; control group). Animals in group 1 received SM exposure in both eyes, as detailed below. Thereafter, 2 hours after SM exposure, DEX was ad- ministered every 8 hours (left eye; DEX 8-hour treatment group) or ev- ery 12 hours (right eye; DEX 12-hour treatment group) for 28 days after 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 time points of sacrifice were day 28, day 42, and day 56 after SM exposure.

SM Exposure. SM exposures and DEX treatments were per- formed 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 preexposure pain management procedures were per- formed 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 mg/kg) and xylazine (≤5 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 minute and was then transferred onto the SM exposure eye for 90 seconds. Control eyes received a sham (no SM) exposure; vapor cap was placed on the control eye for 90 seconds to mimic conditions of SM.
Tissue harvest ad libitum. Served until sternal recumbency, and provided food and water ad libitum. Each cap was single use. After the exposure, each eye was rinsed with 10 ml saline solution (2 minutes after exposure). Once off-exposure, each eye was assessed as described previously (Tewari-Singh et al., 2016; Goswami et al., 2019, 2021, 2022). Brieﬂy, sections were treated with xylene, rehydrated, and stained with H&E and 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 biologic 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 was assessed using ultrasonic pachymetry measurements (TOMMY 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 that 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% and 50% of the corneal radius, 3 signifying longest NV between 51% and 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, 2021, 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, 2021, 2022). Brieﬂy, sections were treated with xylene, rehydrated, and stained with H&E and 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 biologic 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 to 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, 2021, 2022). Scoring of inﬂammatory cell count ranged from 0 to 4, with a score of 0...
signifying no inflammatory cell, 1 signifying 1–50 inflammatory cells, 2 signifying 50–100 cells, 3 signifying 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, 2021, 2022). Briefly, sections were sequentially treated with xylene, were rehydrated, and underwent epitope retrieval (heat mediated) and endogenous peroxidase blocking. Thereafter, the 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 counterstaining the nucleus with hematoxylin. For all of 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, and 4 signified 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, were rehydrated, permeabilized (0.3% Triton X-100) after epitope retrieval (using a declaoying chamber), blocked for nonspecific 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 counterstaining the nucleus with 4',6-diamidino-2-phenylindole (blue). For all of the quantification of SPARC staining, the QuPath software (v0.3.2) was used. Visualization of SPARC staining was done using five to six 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] × 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 time points 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 (S.E.M.).

**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 became apparent around 6 hours after exposure, optimum injuries were observed around the day 7 and 14 postexposure time points, and the effects were sustained up to day 28 after 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 after 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, determined by increased opacity, ulceration, and NV of the SM-exposed corneas compared with the unexposed controls. DEX treatment at both the twice-daily (DEX 12 hours) and thrice-daily (DEX 8 hours) 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 after 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 were statistically significant (omnibus SM exposure effect) in our studies (P < 0.001). Pairwise analysis revealed a significant thickening upon SM exposure compared with the controls at the day 14, 28, and 42 post-SM exposure time points. The average corneal thickness of the SM-exposed corneas was greater than that of the control corneas at the day 56 post-SM exposure time point as well; however, the difference between the two groups was not statistically significant at this time point in the pairwise analysis. Additionally, the effect of SM exposure was not consistent for all of the study time points, with statistically significant differences observable 4 weeks after the SM exposure (i.e., between day 14 and 42, day 14 and 56, day 28 and 42, and day 28 and 56 postexposure time points) (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, 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 8-hour 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 time points, determined using pairwise analysis. The effect of DEX 8-hour treatment was continued up to day 56 post SM exposure 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 12-hour treatment also reduced corneal thickening significantly until the DEX administration was discontinued [i.e., at day 14 (~77% reversal) and day 28 (~71% reversal) time points]; however, the effects of DEX 12-hour treatment were not sustained after termination of the treatment (i.e., at the day 42 and 56 post-SM exposure time points) (Fig. 2A). Omnibus treatment effects, determined using the two-way ANOVA, also indicated a significant difference between the DEX 8-hour and DEX 12-hour treatments as well as between the control and DEX 12-hour groups (P < 0.0001). This could imply that DEX 8-hour treatment more effectively reversed SM-induced corneal thickening compared with the DEX 12-hour 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 after SM exposure, compared with the control group (Fig. 2B), with pairwise analysis. At the day 56 post-SM exposure time point, 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...
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.
blindness if not treated in a timely manner. All treatments were found to have similar effects for all study time points, with no statistically significant difference observed for corneal opacity on different time points in any study groups.

Effect of both DEX 8-hour and DEX 12-hour treatments was also found to be significant in reversing SM-induced corneal opacity (Fig. 2B). DEX 8-hour 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 after cessation of DEX administration, with nearly complete reversal in corneal opacity observed at the day 42 and 56 post-SM exposure time points, although the effects were statistically significant only for the day 42 time point (Fig. 2B). DEX 12-hour treatment also reduced corneal opacity, but the reversal was statistically significant only for the day 14 time point (~61% reversal). Difference between the corneal opacity scores for DEX 8-hour and DEX 12-hour treatments as well as control and DEX 12-hour treatments was found to be statistically significant (P < 0.001), indicating that DEX 8-hour treatment was more effective in reversing corneal opacity than DEX 12-hour treatment.

**Corneal Ulceration.** A significant effect of SM exposure independent of the time point 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 the day 14, 28, and 42 post-SM exposure time points. Ulceration in the SM-exposed group was higher than in the control group at the day 56 post-SM exposure time point, 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 scarring and impaired vision. Additionally, the damage makes the cornea and the eye over-all more prone to infections and secondary insults.

Only DEX 8-hour treatment was found to be statistically significant in reversing SM-induced corneal ulceration. Pairwise comparisons showed that DEX 8-hour 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 time points. The sustained effects of DEX administration were observed at the day 56 post-SM exposure time point as well, although the reversal was not statistically significant. The DEX 12-hour treatment also reduced corneal ulceration compared with the SM exposure group; however, the therapeutic effect for this parameter was not statistically significant for any of the time points in the study. Moreover, the difference between corneal ulceration scores for the DEX 8-hour and DEX 12-hour treatments as well as the DEX 12-hour treatment and control group was also statistically significant, indicating greater efficacy of the DEX 8-hour 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 of the time points (i.e., at day 14, 28, 42, and 56 post-SM exposure time points) (Fig. 2D). The effect of SM exposure had a significant interaction effect with the time point after exposure (P < 0.001), with a steady increase in NV observed from the day 14 to day 56 postexposure time points. Pairwise analysis revealed a significant difference in NV at day 14 after SM exposure (P < 0.001 for day 14 vs. 28, day 14 vs. 42, and day 14 vs. 56 NV scores). Both the DEX 8-hour and DEX 12-hour treatments significantly and markedly prevented SM-induced NV in the rabbit corneas for all of the study time points. For DEX 8 hours, the preventive effect at day 14 (~100%), day 28 (~96%), day 42 (~95%), and day 56 (~67%) was comparable to those for the DEX 12-hour treatment at the day 14 (~94%), day 28 (~83%), day 42 (~82%), and day 56 (~67%) post-SM exposure time points. Thus, for the NV parameter, DEX administration at both dosing frequencies had a similar statistically significant effect that was sustained even 4 weeks after cessation of DEX administration.

In this comprehensive assessment of the clinical parameters, the DEX 8-hour 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 time points (i.e., during the continued DEX administration), but they were also significantly sustained 14 days after cessation of DEX administration (i.e., at the day 42 post-SM exposure time point) for all parameters in the DEX 8-hour treatment group. At the 4-weeks after DEX treatment suspension time point (i.e., day 56 after 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 postexposure time points and an increase in corneal NV from the day 14 to day 56 postexposure time points. Although the effect of time point after 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 on the day 42 and 56 post-SM exposure time points, 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 Histologic 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 8-hour and 12-hour treatments were performed in H&E-stained samples at the day 28, 42, and 56 post-SM exposure time points. 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 of the histopathological parameters assessed compared with the respective controls. Both DEX 8-hour and 12-hour treatments were effective in attenuating the SM-induced histopathological injuries.
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 time point) and bar graphs (right panel) showing (A) corneal thickness, (B) epithelial degradation, and (C) epithelial–stromal separation.
with DEX 8-hour treatment being more potent than 12-hour treatment (Fig. 3).

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

DEX 8-hour treatment significantly and effectively mitigated SM-induced increase in corneal thickness, with nearly complete reversals observed at the day 28 and 42 post–SM exposure time points. A ~54% reversal in corneal thickening was observed with DEX 8-hour treatment at the day 56 post–SM exposure time point. DEX 12-hour 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 time points]. Both DEX 8-hour and DEX 12-hour treatments were statistically significant only on the day 28 postexposure time point compared with the SM-treated group in pairwise analysis.

**Epithelial Degradation.** Epithelial degradation indicated the damage to the corneal epithelial layers, including cell shrinking, nuclear pyknosis, and sloughing off the epithelium. This parameter indicates the physical damage caused to the outermost layer of the cornea, which 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 time points ($P < 0.001$) within the SM exposure and DEX 12-hour treatment. Pairwise analysis revealed that the day 28 versus 42 ($P < 0.05$), day 28 versus day 56 ($P < 0.001$), and day 42 versus 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 42 post–SM exposure time points (Fig. 3B). Epithelial degradation was significantly and markedly decreased upon DEX 8-hour treatment at the day 28 (~73% reversal) and 42 (~82% reversal) post–SM exposure time points. The DEX 12-hour treatment decreased SM-induced epithelial degradation only at the day 42 postexposure time point (~40% reversal), and this effect was not statistically significant compared with the SM-treated group. Additionally, the scores of DEX 8- and 12-hour treatments as well as DEX 12-hour and control groups were found to be significantly different ($P < 0.001$), indicating that the DEX 8-hour treatment more effective than the DEX 12-hour treatment.

**Epithelial-Stromal Separation.** The separation of the corneal epithelium from the underlying stroma is indicative of the damage to or dissolving off of the basement membrane. This injury is distinct from epithelial degradation, as the epithelium is physically separated and distinct from the damage to epithelium cells. A significant treatment and time point interaction effect was observed for this parameter as well, with difference within the SM exposure and DEX 12-hour treatment groups at the level of time points ($P < 0.001$). Significant separation of the corneal epithelium from the stroma was observed upon SM exposure at the day 28 and 42 post–SM exposure time points, compared with 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 time points upon DEX 8-hour treatment, whereas the DEX 12-hour treatment significantly reduced epithelial-stromal separation only at the day 42 (~60% reversal) post–SM exposure time point. Significant difference in the DEX 8-hour and DEX 12-hour groups was also observed at the day 28 postexposure time point ($P < 0.001$). DEX 12-hour group was significantly different from the control group on day 28 and day 42 after SM exposure. These results suggest that DEX 8-hour treatment was more effective than DEX 12-hour 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 that both DEX treatments were effective in reversing the SM-induced histopathological damage. As for the clinical parameters, DEX 8-hour treatment was more effective than the DEX 12-hour treatment in reversing SM-induced injuries. The difference between DEX 8 hours and DEX 12 hours in reversing the SM-induced epithelial degradation was significantly different at the day 28 and 42 postexposure time points. Additionally, the difference in the epithelial-stromal separation was also significant between DEX 8 hours and DEX 12 hours at the day 28 postexposure time point. Moreover, the difference between the DEX 12-hour 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 although the DEX 12-hour treatment was effective in reversing SM-induced injuries, it could not revert corneas to near control conditions.

**Effect of DEX Treatments on Biologic 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 compared with the controls (Fig. 4). Both DEX 8-hour and 12-hour treatments were shown to effectively reverse the SM-induced increase in the number of inflammatory cells and blood vessels in the corneal stroma. DEX 8-hour treatment was found to be more effective in decreasing the inflammatory cells, whereas both the 8- and 12-hour treatments

(C) epithelial-stromal separation visualized using H&E staining. DEX administration initiated at 2 hours after SM exposure and then either every 8 or 12 hours thereafter for 28 days after SM exposure; sustained effects observed up to day 56 after SM exposure. Data presented are mean ± S.E.M. ($n = 5$). #P < 0.05, ##P < 0.01, and ###P < 0.001; for SM compared with the control group scores and *P < 0.05, **P < 0.01, and ***P < 0.001; for DEX 8 hours and DEX 12 hours compared with SM group scores. Comparisons were also made between the DEX 8-hour and DEX 12-hour treatment groups. All statistical analyses were performed using two-way ANOVA and post hoc Helm-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. E, epithelium; S, stroma.
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 mustard insults are associated with increased inflammatory mediators (Goswami et al., 2018, 2019, 2021, 2022). A significant interaction of treatment and time point after 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 time points (\( P < 0.001 \); Fig. 4A). Pairwise analysis for the effect of time point showed that SM exposure had significantly differential effects on the level of study time points (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 postexposure time point.

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

Blood Vessel Count. Blood vessel count also showed a significant interaction effect between the levels of treatment and time point (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 day 56 post-SM exposure (Fig. 4B) compared with the control group. Additionally, there was a statistically differential effect of SM exposure on the day 28 versus day 42 and day 28 versus day 56 postexposure time points (P < 0.001). This could be due to the decrease in the number of blood vessels observed from the day 28 to day 56 postexposure study endpoint, although the blood vessel count was significantly increased in the SM exposed groups compared with the controls.

Both DEX 8- and 12-hour treatments significantly reduced the number of blood vessels at the day 28 (DEX 8 hours: ~99%, DEX 12 hours: ~95% reversal) and day 56 (DEX 8 hours: ~77%, DEX 12 hours: ~73% reversal) post-SM exposure time points, indicating significant sustained effects of DEX treatment even 4 weeks after cessation of DEX administration. The DEX treatments caused apparent reversals in the growth of new blood vessels at the day 42 post-SM exposure time points as well (DEX 8 hours: ~99%, DEX 12 hours: ~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, determined using IHC at all of the study time points (i.e., days 28, 42, and 56 after SM exposure) (Fig. 5). Both DEX 8- and 12-hour treatments markedly rescued the SM-induced increases in COX-2, MMP-9, and VEGF expressions; however, DEX 8-hour treatment showed a marginally greater decrease in COX-2, MMP-9, and VEGF expressions, although the difference between the DEX 8- and 12-hour treatments was not statistically significant.

COX-2. COX-2 is a proinflammatory cytokine and serves to increase the production of pain and inflammation causing prostaglandins. A significant interaction effect between the levels of treatment and time points 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 of the study time points (i.e., days 28, 42, and 56 after SM exposure) (Fig. 5A). Both DEX 8- and 12-hour treatments significantly reduced the SM-induced COX-2 levels at the day 28 (DEX 8 hours: ~37%, DEX 12 hours: ~32% reversal) and day 42 (DEX 8 hours: ~69% and DEX 12 hours: ~43% reversal) post-SM exposure time points. At the day 56 post-SM exposure time point, 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 of the study time points.

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 compared with 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 time points (i.e., day 28, 42, and 56 after SM exposure) (Fig. 5B). Both DEX 8- and 12-hour treatments significantly reduced the SM-induced MMP-9 levels at the day 42 (DEX 8 hours: ~53%, DEX 12 hours: ~35% reversal) and day 56 (DEX 8 hours: ~89%, DEX 12 hours: ~73% reversal) post-SM exposure time points. At the day 28 post-SM exposure time point, the effect of only DEX 12-hour 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 of the study time points, 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 biologic (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 8- and 12-hour treatments significantly countered these increases (Fig. 5C) at all study time points at day 28 (DEX 8 hours: ~80%, DEX 12 hours: ~78% reversal), day 42 (DEX 8 hours: ~88%, DEX 12 hours: ~52% reversal), and day 56 (DEX 8 hours: ~86%, DEX 12 hours: ~81% reversal) after SM exposure. These results indicate marked and comparable efficacy of both DEX 8- and 12-hour 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 the day 28 and 42 post-SM exposure time points (Fig. 6), determined using IF staining. Both DEX 8- and 12-hour treatments markedly and significantly reversed the SM-induced increases in SPARC expressions at the day 28 and 42 postexposure time points, with DEX 8-hour treatment showing a near complete reversal at all study time points that was greater than the reversals observed with the DEX 12-hour treatment (day 28: ~100%, day 42: ~94%, day 56: ~84% reversal). As observed from these results, the effects of DEX 8-hour treatment were sustained up to 4 weeks after termination of DEX administration and were more pronounced than the DEX 12-hour 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 hours after SM exposure and treatment with DEX every 8 hours thereafter was more potent than treatment every 12 hours in reversing SM-induced corneal

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 time point) and bar graphs (right panel) showing (A) COX-2, (B) MMP-9, and (C) VEGF expressions visualized using immunohistochemistry. DEX administration initiated at 2 hours after SM exposure and then either every 8 or 12 hours thereafter for 28 days after SM exposure; sustained effects observed up to day 56 after SM exposure. Data presented are mean ± S.E.M. (n = 5). #P < 0.05, ##P < 0.01, and ###P < 0.001; for SM compared with the control group scores and *P < 0.05, **P < 0.01, and ***P < 0.001; for DEX 8 hours and DEX 12 hours compared with SM group scores. Comparisons were also made between the DEX 8-hour and DEX 12-hour treatment groups. All statistical analyses were performed using two-way ANOVA and post hoc Holm-Sidak method for pairwise analysis. 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 time points; visualized using immunofluorescence. DEX administration initiated at 2 hours after SM exposure and then either every
injuries in our study model (Fig. 7). Effects were most pronounced at days 28 and 42 after 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, biologic, and molecular parameters was also performed with correlation of clinical injuries, biologic histopathology of the corneal structure, and downstream molecular regulators.

DEX 8-hour 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 time point) and after cessation of DEX treatment until day 56 after SM exposure. Sustained effects observed with DEX 8-hour treatment could be due to the greater protection conferred by the DEX 8-hour 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, biologic, 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 time points. VEGF is associated with the initiation of

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<th>A. Differential effects of DEX 8h and DEX 12h treatments: overview</th>
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Fig. 7. Comparative overview of DEX 8-hour and DEX 12-hour treatments: The differential effect of DEX 8-hour and DEX 12-hour treatments on all of 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 time points are depicted. NS, gray; *P < 0.05, orange; **P < 0.01, pink; and ***P < 0.001, green for DEX 8-hour and DEX 12-hour treatment group scores compared with SM group scores. Comparisons were also made between the DEX 8-hour and DEX 12-hour treatment groups. All statistical analyses were performed using two-way ANOVA and post hoc Holm-Sidak method for pairwise analysis.

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8 or 12 hours thereafter for 28 days after SM exposure; sustained effects observed up to day 56 after SM exposure. Data presented are mean ± S.E.M. (n = 5). *P < 0.05, **P < 0.01, and ***P < 0.001; for SM compared with the control group scores and *P < 0.05, **P < 0.01, and ***P < 0.001; for DEX 8 hours and DEX 12 hours compared with SM group scores. Comparisons were also made between the DEX 8-hour and DEX 12-hour treatment groups. All statistical analyses were performed using two-way ANOVA and post hoc Holm-Sidak method for pairwise analysis. E, epithelium; S, stroma.
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 are very important. Significant NV and bool 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 (Kadar et al., 2014; Gore et al., 2018, 2023) as well as DEX treatment alone (Amir et al., 2000; Kadar et al., 2009; Goswami et al., 2016, 2018, 2022) mitigate mustard vesicant-induced NV.

Ocular vesicant exposure is associated with latent pathology that becomes apparent 1–3 weeks after exposure and includes NV, erosions, and edema. Delayed symptoms are attributed to damage at the nucleic acid and protein levels caused by alkylating damage. 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 treatment frequencies were effective in reducing SM-induced increases in COX-2, MMP-9, VEGF, and SPARC expressions in the corneal epithelium, DEX 8-hour was more effective than DEX 12-hour treatment. COX-2 is primarily associated with generation of an inflammatory response (Chen and Tansey, 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. Although 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, and separation from the underlying stromal layer. DEX dosing at the 8-hour 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, 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 laboratory and other groups (Kadar et al., 2009; Goswami et al., 2021; Horwitz et al., 2014). Anti-MMP 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 nearly complete reversals observed from day 28 until day 56 after exposure in the DEX 8-hour treatment group. This could be one of the underlying pathways protecting epithelial degradation. Berryhill et al., (2003) reported SPARC accumulation during injury and proposed 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 interpreted by DEX, which needs further elucidation.

It is important to note that there is a decline in SM toxicity, especially clinical and biologic parameters, at day 56 after exposure. Thus, DEX treatment effects at day 56 after 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 per 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 than prolonged DEX treatment. These limitations are important to acknowledge and to 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 8-hour treatment was more potent than DEX 12-hour treatment. Effects were most pronounced at day 28 and day 42 after SM exposure. These results are novel, as no other studies have assessed the efficacy of DEX upon SM exposure in depth, evaluating parameters ranging from clinical signs to molecular markers. 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

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

Authorship Contributions

Participated in research design: Tewari-Singh, Croutch, Pantcheva, Petras, Araj, C. Agarwal, R. Agarwal.

Conducted experiments: Mishra, Kant, Kandhari, Anantharam.

Performed data analysis: Mishra, Kant, Kandhari.

Wrote or contributed to the writing of the manuscript: Mishra, Kant, Kandhari, Tewari-Singh, Anantharam, Croutch, Pantcheva, Petras, Araj, C. Agarwal, R. Agarwal.

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