Nitrogen mustard-induced *ex vivo* human cornea injury model and therapeutic intervention by dexamethasone

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ABBREVIATIONS

ANOVA: analysis of variance

COX-2: cyclooxygenase 2

CWA: chemical warfare agents

DLL: delta like canonical Notch ligand 1

DEX: Dexamethasone

ECM: extracellular matrix

ERBB4: receptor tyrosine-protein kinase erbB-4
FGF: fibroblast growth factor
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

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ABSTRACT

Sulfur mustard (SM), a vesicating agent first used during World War I, remains a potent threat as a chemical weapon to cause intentional/accidental chemical emergencies. Eyes are extremely susceptible to SM toxicity. Nitrogen mustard (NM), a bifunctional alkylating agent and potent analog of SM, is used in laboratories to study mustard vesicant-induced ocular toxicity. Previously, we showed that SM-/NM-induced injuries (in vivo and ex vivo rabbit corneas) are reversed upon dexamethasone (DEX) treatment, an FDA approved, steroidal anti-inflammatory drug. Here, we optimized NM injuries in ex vivo human corneas and assessed DEX efficacy. For injury optimization, one cornea (randomly selected from paired eyes) was exposed to NM: 100 nmoles for 2 h or 4 h, and 200 nmoles for 2 h, and the other cornea served as a control. Injuries were assessed 24 h post NM-exposure. NM 100 nmoles exposure for 2 h was found to cause optimal corneal injury (epithelial thinning [~69%]; epithelial-stromal separation [6-fold increase]). In protein arrays studies, 24 proteins displayed ≥40% change in their expression in NM exposed corneas compared to controls. DEX administration initiated 2 h post NM exposure and every 8 h thereafter until 24 h post-exposure reversed NM-induced corneal epithelial-stromal separation [2-fold decrease]). Of the 24 proteins dysregulated upon NM exposure, 6 proteins (DLL1, FGFbasic, CD54, CCL7, endostatin, ERBB4) associated with angiogenesis, immune/inflammatory responses, and cell differentiation/proliferation, showed significant reversal upon DEX treatment (Student’s t-test; p≤0.05). Complementing our animal model studies, DEX was shown to mitigate vesicant-induced toxicities in ex vivo human corneas.
SIGNIFICANCE STATEMENT

NM exposure-induced injuries were optimized in an ex vivo human cornea culture model and studies were carried out at 24 h post 100 nmoles NM exposure. DEX administration (started 2 h post NM exposure and every 8 h thereafter) reversed NM-induced corneal injuries. Molecular mediators of DEX action were associated with angiogenesis, immune/inflammatory responses, and cell differentiation/proliferation, indicating DEX aids wound healing via reversing vesicant-induced neovascularization (DLL1 and FGF basic) and leukocyte infiltration (CD54 and CCL7).
INTRODUCTION

Mustard vesicant sulfur mustard (SM) is a very potent bifunctional alkylating agent that was used in chemical warfare during World War I (Dacre and Goldman, 1996). Since then, SM has been reportedly used in several conflicts and skirmishes worldwide (Saladi et al., 2006; Geraci, 2008; Ganesan et al., 2010; Ghabili et al., 2011). It is a highly sought-after chemical threat agent for warfare and terrorist activities due to its ease of manufacturing and deployment; thus, known as the king of battle gases. Stockpiles of SM are known to exist and accidental exposure from old stocks or improperly discarded SM reservoirs has been reported (Geraci, 2008; Wattana and Bey, 2009; Missiaen et al., 2010). Since SM is tightly regulated as a category 1 chemical, its prototype nitrogen mustard (NM) is used in laboratory settings. NM is also a potent vesicant and a bifunctional alkylating agent, with similar toxicity and vesicating properties as SM (Calvet et al., 1999; Mishra et al., 2022).

Exposure to mustard alkylating agents most commonly occurs via ocular and dermal routes or through inhalation. Eyes are very susceptible to vesicant toxicity, due to rapid cellular turnover and their high-water content (Rafati-Rahimzadeh et al., 2019). Ocular mustard vesicant exposure can cause mild discomfort, redness, inflammation, itching, pain, neovascularization (NV), and vision impairment, depending upon the vesicant dose, exposure duration, and physiological characteristics of the individuals exposed (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 transparent outer tissue layer that is the ocular barrier to the external environment, while allowing light to enter the eye, is primarily susceptible to injury by external threats. Research has shown that ocular mustard exposure causes damage to the cornea in the rabbit model (Tewari-Singh et al., 2012, Goswami et al., 2019, 2021).
Currently, the threat of exposure to mustard vesicants is realistic and very high, with the history of its recent use in Syria, and looming threat of conflicts worldwide (Koblentz 2019). There are no proven treatment modalities or countermeasures for ocular mustard insults in humans. Dexamethasone (DEX), a potent steroidal, anti-inflammatory drug that is FDA approved for ocular use as an anti-inflammatory drug, has been shown to effectively reverse vesicant-induced corneal injuries in animal and cell culture models (Amir et al., 2000; Kadar et al., 2009; Tewari-Singh et al., 2012; Goswami et al., 2018, 2022). Moreover, DEX is easy to use (topical eye drops), inexpensive, and readily available. These properties make it an ideal therapeutic modality in case of mass causality/emergency situations.

In our previous studies, we showed that DEX could reverse NM-induced damage in ex vivo (Tewari-Singh et al., 2012; Goswami et al., 2018) and in vivo rabbit corneas at different therapeutic windows of treatment initiation (Goswami et al., 2022). These results were translated to an in vivo rabbit ocular SM exposure model. DEX treatment, beginning at 2 h post exposure, reversed SM-induced injuries in corneas at both twice daily and thrice daily dosing frequencies, though DEX administration thrice daily was more effective than twice daily (Mishra et al, 2023). Thus, the first part of the current study focused on the development of an ex vivo human cornea model of NM exposure. Ex vivo human corneas were subjected to three NM exposure conditions, and corneas were cultured for 24 h post NM exposure. The concentration of 100 nmoles for 2 h was optimized as the NM exposure condition and further, NM-induced injury was assessed as a function of time for up to day 5 post-exposure. Parameters assessed included histopathological (H&E staining) and molecular (protein arrays) biomarkers. The second part of the study focused on assessing the efficacy of DEX treatment, established in our previous studies i.e., beginning at 2 h post NM-exposure and every 8 h thereafter until the study endpoint. It is imperative to
translate the results obtained in our *in vivo* and *ex vivo* animal studies, to human samples, as although animal models are useful for basic research, it is necessary to validate the results in human samples. It would be unethical to perform ocular NM/SM exposures on human subjects and human samples exposed to these chemical warfare agents (CWAs) are not readily available. Hence, the best method to validate our findings was to optimize NM exposure induced injury biomarkers and time points in *ex vivo* human corneas and assess the efficacy of the previously established DEX treatment regimen in rabbit models (Mishra et al., 2023). The study outline is provided in Figure 1. Thus, the aim of the study is to optimize histopathological and molecular biomarkers of injury in an *ex vivo* human corneal culture model of NM-exposure as well as assess the efficacy of DEX treatment in this model.
MATERIALS AND METHODS

Chemicals and reagents. Human corneas were obtained from donors consented for research from Lions World Vision Institute, Tampa, FL and Saving Site, Kansas City, MO. NM (mechlorethamine hydrochloride; cat #122564) was procured from Sigma-Aldrich, St. Louis, MO. DEX (0.1%) was procured from Bausch and Lomb (cat #10000106054). For culturing human corneas, antibiotic-antimycotic (ABAM) solution (cat #15240062) and minimum essential medium (MEM; cat #11095-080) were obtained from Thermo Fisher Scientific, Waltham, MA. Calf skin collagen (cat #C9791), acetic acid (cat #A-6283), agar (cat #A1296), and insulin-transferrin-selenite supplement (ITS; cat #I3146) were bought from Sigma-Aldrich, St. Louis, MO. Hematoxylin (cat #HHS32) and eosin (cat #HT 110116) stains were procured from Sigma Aldrich, St. Louis, MO. The protein array kits were obtained from R&D systems, Minneapolis, MN (cat #ARY026).

Ex vivo human cornea culture. Corneas were obtained, and all experiments were performed after approval from the Colorado Multiple Institutional Review Board of the University of Colorado, Anschutz Medical Campus (UCD-AMC). Corneas were obtained from individuals of both sexes, aged 20-70 years. The death to preservation time for the corneas was <24 h. The corneal epithelium showed 100% mild or mild to moderate diffuse exposure and the corneal stroma was either compact only or clear and compact, before shipping to UCD-AMC. Complete media for culturing the corneas was prepared by adding ABAM and ITS to MEM. Dissected human corneas (along with a conjunctival rim) were obtained in OptiSol-GS from the respective institutions. Upon arrival, corneas were washed three times with ABAM solution. Calf skin collagen (1 mg/ml) in 0.1 N acetic acid, 1% agar, and ITS were added to MEM to make MEM agar, which was microwaved and filtered through a 0.22 μm syringe filter for dissolving agar and
sterilization, respectively. The MEM agar was allowed to cool down to 37-39°C and poured onto the inner surface of the corneas, stabilized in the mold, and was allowed to solidify to keep the corneas in shape. Thereafter, corneas were placed in 6-well culture plates, with complete culture media (~1 ml), and incubated at 37°C in a humidified 5% CO₂ incubator. Corneas were cultured for 24 h before NM-exposure. Culture media (20 µl) was added dropwise to the center of each cornea every 8 h to keep the tissue moist.

**Optimizing NM exposure condition.** After 24 h and before NM-exposure, corneas were assessed for any observable damage. NM was freshly prepared just before the exposure in culture media at different concentrations 100 nmoles and 200 nmoles (in 20 µl culture media). NM exposure was performed at three different conditions: 100 nmoles for 2 h, 100 nmoles for 4 h, and 200 nmoles for 2 h. These NM exposure conditions were selected from our previous studies on NM toxicity to *ex vivo* rabbit corneal culture (Tewari-Sigh et al., 2012). One cornea (from each pair) was exposed to NM (selected at random) and the other cornea served as control (media only). After the exposure period of 2 h or 4 h, each cornea was washed three times with the culture media and the culture media was changed. Thereafter, the corneas were cultured up to day 5 post NM-exposure. The time points of corneal harvest and histopathological assessments were day 1 (24 h), day 3, and day 5 post NM-exposure. At each sample harvest time point, agar was removed, corneas were collected; one part was fixed in 10% phosphate-buffered formalin and embedded in paraffin for histological assessments and another part was flash frozen (in liquid nitrogen) and stored at -80°C until further protein experiments.

**NM exposure and DEX treatment.** Once the NM exposure conditions were optimized as 100 nmoles of NM for 2 h, DEX efficacy studies were performed. Both eyes (in pairs) were exposed to 100 nmoles NM for 2 h; thereafter, the corneas were washed three times with fresh culture
media and fresh culture media was added. DEX (0.1%) treatment was initiated 2 h post NM exposure and was administered every 8 h thereafter, as this was found to be the best therapeutic window of treatment in our previous studies (Goswami et al., 2022). DEX (20 µl) was administered dropwise at the center of the corneas, in one eye per pair, selected randomly and the other eye served as the injured positive control (NM only) where 20 µl complete culture media was administered dropwise. Time points of cornea harvest and histopathological assessments were day 1 (24 h), day 3, and day 5 post NM-exposure. At each sample harvest time point, agar was removed, and corneas were collected and either fixed in 10% phosphate-buffered formalin and embedded in paraffin for histological assessments or flash frozen (in liquid nitrogen) and stored at -80˚C until further protein experiments.

Assessments of histopathological parameters. Corneal tissue harvested at day 1, 3, and 5 post NM-exposure time points were formalin-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). A minimum of three slides per tissue were used for histopathological assessments using hematoxylin and eosin (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, xylene was used for paraffin removal and clearing slides after which the sections were re-hydrated and stained with H&E. Further, the sections were dehydrated again, cleared with xylene, and mounted. The H&E-stained slides were used for assessment of the biological parameters, specifically, epithelial thickness and epithelial-stromal separation. Percent epithelial-stromal separation was measured throughout the length of the cornea (approx. 7-8 mm length) for each slide; averaged measurements for each group were
used. Epithelial thickness was also measured throughout the length of the corneal epithelium and the averaged value was used (µm).

**Lysate preparation.** Molecular parameters were assessed from whole cornea samples i.e., corneal epithelium and stroma. Briefly, the corneal tissue was washed in cold phosphate-buffered saline (PBS) and minced into smaller pieces using a surgical blade. Tissues were further minced in M tubes, using gentleMACSTM Octo Dissociator, in lysis buffer. The lysate was collected and incubated on ice till the froth subsided (15 min), subjected to three cycles of freeze (-80°C, 20 min)-thaw (37°C for 1 min)-vortex, and centrifuged (4°C; 14,000 rpm for 15 min). The supernatant was collected.

**Protein array.** Protein arrays were performed per the manufacturer’s protocol. Briefly, for the protein array, array membranes were blocked for non-specific activity using array buffer 6 and incubating for 1 h at room temperature on a rocking platform shaker. Next, the volume of each lysate (~120 mg protein) was adjusted to 1.5 ml using array buffer 4 (0.5ml) and array buffer 6. The lysate samples were incubated with the array membranes on a rocking platform overnight at 4°C. The next morning, array membranes were washed three times with wash buffer. Further, the array membrane was incubated with the detection antibody cocktail for 1 h, at room temperature on a rocking platform shaker. Next, the membrane was washed three times with wash buffer. Thereafter, the membrane was incubated with streptavidin-HRP for 30 min, at room temperature on a rocking platform shaker. After washing three times with wash buffer, the protein concentration was visualized using the chemi reagent mix, enclosing the membrane in an autoradiography film cassette and exposing to X-ray films.

**Estimation of differential protein expression between groups.** The positive signals (black dots) that developed on the X-ray film were identified using the transparency template provided.
with the array. The estimation of density (densitometric analysis) for the protein spots was done using Image Studio™ Lite software (LI-COR Biosciences). The reference spots’ (three pairs) density was averaged and used for data normalization.

**Statistics and data analysis.** All the parameters were scored as described in each of the respective materials and methods sections. Student’s t-test (two-tailed) was used to determine statistically significant. The effects were deemed significant if the p-value obtained was <0.05 (n=3-4 per cohort). Data are presented as mean ± standard error of mean (SEM). Two control samples were used for protein array experiments, due to the limited availability of human corneas and lack of control over the conditions of the samples obtained.
RESULTS

Dose response studies: optimizing NM-induced injury in ex vivo human corneal culture.

Human corneas were subjected to three varying NM exposure conditions, specifically 100 nmoles of NM for 2 h or 4 h, and 200 nmoles of NM for 2 h (n=2-3 per condition). NM-induced injuries for all the three exposure groups were assessed at 24 h post NM exposure (Figure 2). From the analysis of the H&E-stained sections, optimum injuries were obtained with the 2 h exposure of 100 nmoles of NM, where decreased epithelial thickening as compared to the controls was observed. The other two exposure conditions caused detrimental damage to the human corneal epithelium leading to near complete denudation (Figure 2A). There is inherent wear and tear of the corneal epithelium in cadaveric human corneas, observable in all the samples, as eyes undergo a thorough cleaning before corneal harvesting and in part due to the degradation caused by the NM exposure. This denudation when coupled with higher NM concentration or prolonged exposure conditions leads to detrimental damage to the corneas that is not suitable for DEX efficacy studies.

Further, protein arrays were employed for determining the protein profiles to assess NM-induced injury. Protein arrays were performed for one sample each for the 100 nmoles 2 h and 100 nmoles 4 h NM exposures (Figures 2C and D), owing to the limited availability of the human cornea samples. At the molecular levels, both exposures seemed comparable, as assessed using protein array analysis. Thus, as the histopathological damage in the 100 nmoles NM exposure for 2 h was more conducive to mild-moderate damage and comparable with the results from studies in ex vivo and in vivo animal models of ocular vesicant exposure (Tewari-Singh et al., 2012; Goswami et al., 2016; Goswami et al., 2019; Goswami et al., 2021; Goswami et al.,
2022; Mishra et al., 2023), it was determined to be the ideal injury condition. Thus, further time response and DEX treatment studies were performed with the 100 nmoles NM exposure for 2 h. **Time response studies: optimizing NM-induced injury time points in ex vivo human corneal culture.**

**Histopathological parameters.** Next, with the standardized concentration of 100 nmoles and exposure duration of 2 h with NM, we assessed the NM-induced injury as a function of time at day 1, day 3, and day 5 post NM exposure (n=3-4 per cohort). Histopathological analyses and H&E-stained sections showed increased epithelial degradation with NM exposure, starting at day 1 post NM exposure (Figure 3A). At day 1 post-exposure, the average thickness of the epithelium in the control group was 44 µm, with minor degradation of the topmost layer. NM exposure led to statistically significant corneal thinning (69% decrease) with extensive degradation leading to the average thickness being 16 µm at day 1 post-NM exposure (Figure 3A). Near complete denudation of the corneal epithelium was observed in NM exposed samples for the day 3 (Figure 3B) and day 5 (data not shown) time points, which could be attributed to NM exposure as well as prolonged culturing of the corneas; notably, even control samples showed similar patterns further suggesting the limitations of prolonged culturing of corneas. There was a time-dependent thinning observed in the control corneas at day 3 (average thickness 12 µm) and day 5 (average thickness 5 µm). Owing to extensive epithelial degradation in day 3 and day 5 samples post NM exposure, epithelial-stromal separation could also be assessed only for the day 1 post NM exposure time point that showed a 6-fold increase, compared to controls (Figure 3C).

**Molecular parameters.** Molecular biomarkers assessed using protein arrays (n=2-3 per cohort) showed a change of ≥40% upon NM exposure in the expression of 24 molecules (of the total 87
assessed in the array; Table 1) on day 1 (Figure 4A) and 37 molecules on day 3 (Figure 4B) post-exposure time points. The molecular markers could not be assessed in day 5 samples as the protein quality obtained was not good enough to pass the array quality control. This degradation was likely due to the difficult sustainability of the human corneas in cultures as the human corneas degrade over time when maintained ex vivo.

Optimizing dosing frequency of DEX in NM-induced ex vivo human corneal culture injury model. From the NM dose and time response studies, the 100 nmoles NM 2 h exposure condition and day 1 and day 3 post-exposure time points were selected for DEX efficacy studies. Cultured ex vivo human corneas were exposed to 100 nmoles of NM for 2 h (both eyes in a pair) and only one eye (left or right, selected randomly) received the DEX 8 h treatment, beginning at 2 h post NM exposure, until the study endpoint was employed for assessment of DEX efficacy.

Histopathological parameters. Epithelial thickening did not show sufficient reversal upon DEX treatment at any of the study time points in the study (data not shown), as assessed using H&E-staining. Histopathological analyses for epithelial-stromal separation showed a decrease in separation upon DEX treatment at day 1 (~50% reduction) as shown in Figure 5A. Epithelial separation from stroma could not be assessed on day 3, due to extensive epithelial degradation. Histopathological assessments were performed in 3-4 samples per cohort.

Molecular parameters. Analysis of expression levels of molecules using protein arrays showed that of the 24 molecules that showed ≥40% change in expression upon NM exposure as compared to the controls (Figure 4A; Table 1), 18 molecules showed ≥40% reversal with DEX treatment (Table 2), at day 1 post NM-exposure (n=2-3 per cohort). Thus, 75% of the molecules affected by NM exposure were reversed by DEX treatment at day 1. Notably, 6 molecules were found to have a statistically significant reversal with DEX treatment (Figure 5B) at day 1 post
NM exposure. These included delta like canonical Notch ligand 1 (DLL1), endostatin, receptor tyrosine-protein kinase erbB-4 (ErbB4), fibroblast growth factor (FGF basic), intercellular adhesion molecule 1 (ICAM-1) also known as cluster of differentiation (CD54), and the chemokine CCL7 (Figure 5B).

At day 3 post NM exposure time point, NM exposed samples showed decreased expression in the levels of all the molecules as compared to the DEX treated and control samples. On day 3, 37 proteins had ≥40% change in expression upon NM exposure as compared to the controls (Figure 4B; Table 1), 25 molecules showed ≥40% reversal with DEX treatment (Table 2); thus, reversal in ~68% molecules affected by NM showed reversal. Figure 5C shows the expression levels of the molecules that had ≥40% change in expression upon NM exposure as well as a statistically significant (≥40% reversal) effect with DEX treatment (n=2-3 per cohort).
DISCUSSION

Our present and ongoing studies have been focused on determining targeted and effective treatment modalities for vesicant-induced ocular injuries. DEX has been shown to effectively counter ocular injuries caused by NM and SM exposures in various animal models (Amir et al., 2000; Kadar et al., 2009; Tewari-Singh et al., 2012; Kadar et al., 2014; Goswami et al., 2018; Goswami et al., 2022; Mishra et al., 2023). Thus, it was imperative to validate these findings in a human model. Therefore, we first developed an ex vivo human corneal culture model of NM exposure. Dose response studies showed that exposure to 100 nmoles of NM for 2 h was the optimal exposure condition that caused mild to moderate corneal injury in our model. Thereafter, time response studies were performed and the 24 h (day 1) post exposure time point was determined to be the most suitable for DEX efficacy studies. Epithelial thinning is an indicator of degradation of epithelial layers. Human corneas consist of 5-7 epithelial layers and is 50 µm in thickness. At day 1 post NM exposure, despite some degradation of the top-most layers of the corneal epithelium, significant portions of the lower layers were intact in the control group. In the NM exposed corneas at day 1, there was greater degradation in the corneal epithelium. These results were apparent from the marked corneal epithelium thinning in the NM exposed samples as compared to the control samples. Furthermore, these results were confirmed by the epithelial-stromal separation parameter as well. At the day 3 and day 5 post-exposure time points, irreversible damage was observed in the NM exposed corneas, and even the control group had substantial damage, making these time points unsuitable for performing DEX efficacy studies. This is the first study that reports the development of an NM-exposure ex vivo human corneal culture model with optimized injury time points, to our knowledge. The pathophysiological injury biomarkers validated in the ex vivo human corneas parallel those observed in previous
studies of vesicant-induced corneal injuries (Goswami et al., 2019; Mishra et al., 2022).
Additionally, novel molecular biomarkers of ocular NM exposure were also observed. This model is important not only for our current studies but also in the facilitation of further research on the mechanisms underlying vesicant-induced corneal injuries as well as the development and testing of other therapeutics.

Next, we assessed DEX efficacy in the treatment of NM-induced corneal injuries. Histopathological and molecular biomarkers were used to assess DEX efficacy at the day 1 post exposure time point. A significant reversal was observed in the epithelial integrity, as indicated by epithelial degradation and epithelial-stromal separation studies. These results support our previous findings in the rabbit vesicant-injury models (Tewari-Singh et al., 2012; Goswami et al., 2022; Mishra et al., 2023). DEX treatment was not effective in reversing and hindering further thinning of the corneal epithelium after NM exposure in the current study. This result is most likely attributable to the study model and the condition of the human corneas obtained. In our previous studies we have shown that DEX treatment can rescue and prevent vesicant-induced epithelial degradation and thinning in different in vivo and ex vivo rabbit corneal injury models (Tewari-Singh et al., 2012; Tewari-Singh et al., 2016; Goswami et al., 2018; Goswami et al., 2022; Mishra et al., 2023).

As DEX treatment is effective in rescuing and preventing vesicant-induced corneal epithelial degradation in ex vivo culture of rabbit corneas, it can be inferred that ex vivo culturing does not cause significant damage to the corneas, when the corneas being cultured are in pristine condition at the initiation of the culture. However, the condition of the human corneas obtained cannot be controlled by the experimenters. Apart from the wear and tear associated with the harvest of corneas from the donors and testing whether they are suitable for transplant before
being released for research purposes, there is also inherent variability attributable to genetic and epigenetic modification in the human samples as opposed to the animal subjects developed especially for research purposes. There are also differences and damage to the corneas due to lifestyle choices and medical conditions/surgeries/treatment etc., that cannot be controlled in the human samples. Nonetheless, it is still important to validate the findings from animal studies to human samples. Also, since research on CWAs cannot ethically be performed on human subjects, \textit{ex vivo} cultured human corneas are the best available avenue.

Significant molecular markers of NM toxicity and DEX-induced reversal included DLL1, endostatin, ErbB4, FGF, CD54, and CCL7. DLL1 regulates Notch activity and affects angiogenesis (Haung et al., 2018) and neurogenesis in the eye (Nelson et al., 2009). It also regulates inflammatory and immune responses (Radtke et al., 2010; Garis et al., 2021) as well as wound healing (Kimball et al., 2017). Endostatin regulates angiogenesis, and release of cathepsins and metalloproteinases in the eye (Felbor et al., 2000). A significant correlation has also been observed between the levels of endostatin and VEGF in vitreous humor and angiogenesis in diabetic retinopathy (Noma et al., 2002). ErbB4, CD54, and CCL7 are associated with immune cell infiltration leading to subsequent immunological and inflammatory responses. The neuregulin-1 (NRG-1)/ErbB pathway regulates macrophage functioning, fibrosis (Vermeulen et al., 2017), as well as function and repair of the nervous system (Kataria et al., 2019). CD54 triggers leukocyte infiltration (Whitcup et al., 1993).

These molecular markers could be responsible for various clinical and histopathological injury reversals observed upon DEX treatment, in our current and previous studies as well as studies from other groups. DEX treatment has been shown to alleviate inflammation (Amir et al., 2000), NV, and development of delayed signs, such as mustard gas keratopathy (Kadar et al.,
DEX treatment is especially effective in reversing vesicant-induced blood vessel generation. DEX hinders NV, decreases the blood vessel count in the corneal stroma and decreases the expression of VEGF significantly and markedly (Goswami et al., 2018; Goswami et al., 2022; Mishra et al., 2023). VEGF is important for blood vessel initiation and wound healing (Bao et al., 2009). NV causes corneal damage at the site of vessel entry and may cause fibrosis or leave a scar at the site even after vessel degeneration (Baradaran-Rafii et al., 2011). The molecular mediators of NV observed in our study include FGF, DLL1, and endostatin. FGF and DLL1 increase angiogenesis and vessel maturation. Endostatin is a double-edged sword; it may facilitate angiogenesis or inhibit vessel growth leading to apoptosis (Fukai et al., 2002).

Additionally, DEX treatment has also been shown to reduce vesicant-induced corneal inflammation and immune cell infiltration (Goswami et al., 2022; Mishra et al., 2023). Reversal of NM-induced increase in CD54 and CCL7 expression upon DEX treatment observed in our study could be associated with decreased inflammation and immune cell recruitment to the cornea. Figure 6 outlines the association of these molecules in bringing about the therapeutic effects of DEX administration observed in our study.

On day 3 post NM exposure, the level of molecules was decreased in the NM exposure group as compared to the respective control and DEX treatment groups. The reason for this inversion in expression profile is not apparent and this complete reversal in expression profile is very intriguing. This pattern of expression was observed in both NM exposure and DEX treatment studies. Additionally, the expression levels are comparable for the control and DEX groups on day 1 and day 3 post-exposure time points for most proteins; however, stark anomalies are observed for the NM exposure group. Thus, it can be derived that NM drives this pattern at day 3 post-exposure. One possible reason could be that these are ex vivo cultured samples, and
systemic effects would not be observable once the molecular mediators are released to maintain increased expression or facilitate a prolonged downstream effect. Thus, after the initial surge in protein expression upon NM exposure, there is a decline on day 3 as compared to day 1 in the NM exposure group.

It is important to be cognizant of the fact that although ex vivo human corneas provide several insights into the mechanism of action of NM in causing corneal injury as well as DEX efficacy, several investigations cannot be performed in these samples. These include clinical effects such as NV, infiltration of immune cells, and other associated systemic responses. Moreover, as the corneas are processed (cleaned thoroughly, and checked for transplant quality) before being released for research purposes, there is some epithelial damage introduced even in the control samples. Additionally, ex vivo culturing of human corneas is challenging and leads to deterioration of the sample conditions over time. These samples have limited availability; thus, we do not have control over the gender, age, and condition of the corneas. Thus, further studies in future need to be carried out with larger cohorts to further strengthen our findings.

In conclusion, an ex vivo human corneal culture model of NM exposure was optimized in this study. DEX treatment effectively reversed NM-induced corneal injuries in this model. NM-induced injury and DEX efficacy were observed at the histopathological and molecular levels. The primary molecular mediators of DEX action included DLL1, endostatin, ERBB4, FGF basic, CD54, and CCL7. These proteins are known to play a significant role in angiogenesis, leukocyte infiltration, inflammatory responses as well as cell differentiation and proliferation (Figure 6). Taken together, this study suggests that DEX targets critical pathways of wound healing, particularly reversing vesicant-induced neovascularization (DLL1 and FGF basic) and leukocyte infiltration (CD54 and CCL7), that have not been explored before and are important in
understanding the underlying mechanism of action of DEX to counter vesicant-induced injuries in human cornea.
References:


Data Availability Statement

All the data relevant to the manuscript are presented in Figures 2-6 as well as provided in Tables 1 and 2. No Nucleic Acid and Protein Sequences, Structural Data and Molecular Modeling, and High-throughput Data were part of the present study. Any additional raw data that supports the findings of this study are available on request from the corresponding author.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Agarwal R., Tewari-Singh N., Mishra N., Ammar D.A.,
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Footnotes

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Financial disclosure: No author has an actual or perceived conflict of interest with the contents of this article.
FIGURE LEGENDS

Fig. 1: Schematic representation of the study design.

Fig. 2: Optimization of nitrogen mustard (NM) concentration for inducing injury in *ex vivo* human corneal culture. Assessments of (A) biological (epithelial thinning/degradation and separation from the underlying stromal layer; n=2-3 per cohort) and (B) proteome profiler array layout (C) molecular parameters at 24 h post NM-exposure at different concentrations as well as doses of exposure (D) table of protein fold changes (n=1 per cohort). Red arrows indicate separation of the epithelial membrane from the stroma and black arrows indicate degradation of the epithelial membrane leading to decrease in thickness in the NM exposed corneas. UP indicates expression of protein in the respective NM group only. Down indicates presence of the protein in the control sample only. As the protein is present only in one group (either NM exposure or control), fold change cannot be calculated.

Fig. 3: Determination of nitrogen mustard (NM)-induced injury in *ex vivo* human corneal culture as a function of time. Assessments of corneal epithelial thickening at day 1 (A) and day 3 (B) post-exposure to NM. (C) Assessment of epithelial-stromal separation at day 1 post NM exposure. Condition of NM exposure: 100 nmoles for 2 h. Data are shown as mean±SEM; black arrows indicate degradation of epithelial membrane; red arrows indicate separation of the epithelial membrane from the stroma. Students t-test was performed for determining significance of NM-exposure vs control; p<0.05*; n=3-4 per cohort.

Fig. 4: Optimization of timepoint for nitrogen mustard (NM)-induced injury in *ex vivo* human corneal culture. Assessments of molecular parameters at day 1 (A) and day 3 (B) post-exposure to NM (100 nmoles for 2 h). Data are shown as mean±SEM. Students t-test was performed for determining significance of NM-exposure vs control; p<0.05* and p<0.01**; n=2-3 per cohort.
Fig. 5: Efficacy of dexamethasone (DEX) administration initiated at 2 h post nitrogen mustard (NM)-exposure and every 8 h thereafter in ex vivo human corneal culture. (A) Assessments of corneal epithelial-stromal separation at day 1 post NM exposure. Condition of NM exposure: 100 nmoles for 2 h (n=3-4 per cohort). Data are shown as mean±SEM; red arrows indicate separation of epithelial membrane from the stroma. Assessments of molecular parameters at day 1 (B) and day 3 (C) post-exposure (n=2-3 per cohort). Data are shown as mean±SEM. Students t-test was performed for determining significance of DEX treatment vs NM; p<0.05* and p<0.01**.

Fig. 6: Pathways of DEX efficacy (administration initiated at 2 h post exposure and every 8 h thereafter) in NM exposed ex vivo human corneal culture. DLL: delta like canonical Notch ligand 1; ERBB4: receptor tyrosine-protein kinase erbB-4; FGF: fibroblast growth factor; NM: nitrogen mustard; -R: receptor; VEGF: vascular epithelial growth factor.
Table 1: Significant molecular markers of nitrogen mustard-induced injuries in \textit{ex vivo} human cornea.

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<th>Day1 NM</th>
<th>Day3 Control</th>
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Intensity of expression, as assessed using protein profiler arrays, of proteins that showed ≥40% change in expression upon NM exposure on day 1 and/or day 3 is depicted in the table.

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Table 2: Effect of dexamethasone (DEX) treatment on nitrogen mustard-induced ocular injuries: molecular markers

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<td>GM-CSF</td>
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</table>
The intensity of expression, as assessed using protein profiler arrays, of proteins that showed ≥40% change in expression upon NM exposure and DEX treatment reversal on day 1 and/or day 3 is depicted in the table.

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<th>Day 3</th>
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</tbody>
</table>
STUDY DESIGN

Ex vivo Human Corneal Culture

Cultured for 24 h

NM-injury optimization

Pair-wise analysis

NM group

NM exposure conditions:
- 100 nmoles for 2 h
- 100 nmoles for 4 h
- 200 nmoles for 2 h

Control group

Exposed to normal culture media for 2 h or 4 h

Wash out (with media) 2 h or 4 h post exposure

100 nmoles of NM for 2 h determined as optimum injury condition

DEX efficacy

Pair-wise analysis

NM
- 100 nmoles for 2 h

NM+DEX
- DEX (0.1%), beginning 2 h post NM exposure and every 8 h thereafter

Study time points: Day 1, 3, and 5 post NM exposure

Histopathological assessments
- H&E staining
  - epithelial degradation
  - epithelial-stromal separation

Molecular biomarker
- Protein array

 NM group

Control group

Wash out (with media) 2 h or 4 h post exposure

100 nmoles of NM for 2 h determined as optimum injury condition

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- 100 nmoles for 2 h

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- H&E staining
  - epithelial degradation
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- 100 nmoles for 2 h

NM+DEX
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Study time points: Day 1, 3, and 5 post NM exposure

Histopathological assessments
- H&E staining
  - epithelial degradation
  - epithelial-stromal separation

Molecular biomarker
- Protein array
**A. Biological Parameters**

Control

Control

Control

**B. Proteome Profiler Array Layout**

**C. Proteome profiler array**

**D. Protein Expressions**

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Fig. 3.

A. Day 1: Corneal Epithelial Thickness

B. Day 3: Corneal Epithelial Thickness

C. Day 1: Epithelial-stromal Separation
Fig. 5.

A. Day 1: Epithelial-stromal Separation

- NM
- NM+DEX

Percent Separation

~2-fold

B. Day 1: Protein Array

C. Day 3: Protein Array

CCL7
CD54
FGF basic
ErbB4
Endostatin
DLL1

Average relative expression

NM
DEX

Average relative expression

SPARC
Snail
PDGF-AA
CG α/β
FGF basic
ErbB2
DLL1
VE-Cadherin
E-Cadherin

This article has not been copyedited and formatted. The final version may differ from this version.
Angiogenesis
Vessel Maturation
Endostatin
Apoptosis
FGF
FGFR mediated fibroblast activation
VEGF
VEGFR
DLL1
NOTCH Signaling
Angiogenesis
Wound healing
ERBB4
Endostatin
Apoptosis
Inflammation
Leukocyte Infiltration
CD54
CCL7
Inflammation
Wound healing
CD54
CCL7
Leukocyte Infiltration
Notch Signaling
Angiogenesis
Vessel Maturation
Endostatin
Apoptosis