The Ocular Surface 16 (2018) 154–162

Contents lists available at ScienceDirect

The Ocular Surface

journal homepage: www.theocularsurface.com

Original Research

# Nitrogen mustard-induced corneal injury involves the sphingomyelin-ceramide pathway

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ARTICLE INFO

*Article history:*
Received 26 September 2017
Received in revised form
24 October 2017
Accepted 7 November 2017

*Keywords:*
Cornea
Depth of injury
Metabolomics
Nitrogen mustard
Sulfur mustard

ABSTRACT

*Purpose:* Nitrogen mustard (NM), which simulates the effects of sulfur mustard (SM), is a potent vesicant known to cause irreversible corneal damage. This study investigates the mechanisms by which NM induces corneal damage by examining the impact of NM exposure on the morphology and lipidome of the cornea.

*Methods:* Intact *ex vivo* rabbit eyes were placed in serum-free DMEM organ culture. NM (0, 1, 2.5, 5 or 10 mg/ml) was applied to the central cornea for 5, 10 or 15 min using a 5 mm filter disk and subsequently rinsed with DMEM. Corneas were then cultured for 3, 24, or 48 h before being fixed for morphological analysis or for 24 h before being snap frozen for lipidomic analysis.

*Results:* No morphological changes were detected 3 h after NM exposure. Twenty-four h after exposure, 1 mg/ml NM caused erosion of the corneal epithelium, but no damage to the underlying stroma. Damage caused by 2.5 mg/ml NM extended almost two thirds through the corneal stroma, while 5 mg/ml completely penetrated the corneal stroma. An altered lipid profile occurred 24 h after corneas were exposed to NM. Specific sphingomyelins, ceramides, and diacylglycerols were increased up to 9-, 60- and 10-fold, respectively.

*Conclusions:* NM induces concentration- and exposure time-dependent damage to the cornea that increases in severity over time. Alterations in the sphingomyelin-ceramide pathway may contribute to the damaging effects of NM exposure.



## 1. Introduction

Sulfur mustard (SM, bis[2-chloroethyl]sulfide) is a potent, highly reactive, lipophilic vesicant [1–3]. It is highly volatile at room temperature and, due to its lipophilicity, is a very persistent contaminant of the environment, especially of the soil [4].

Sulfur mustard is a chemical warfare agent, which was first used during World War I (WWI). It has been the most widely used chemical weapon and was used heavily during the Iran-Iraq war [5]. Existing large stockpiles of SM pose a continuous risk to military and civilian populations residing near their sites of storage, in addition to their potential use in warfare and terrorism [4].

Sulfur mustard can cause injury to organs (including the skin, eyes, and respiratory tract) that can take several months to heal [6,7]. When used as a volatile chemical weapon, the eye is the most susceptible to SM-related injuries compared to the target organs, the skin, and respiratory tract [8]. Due to its highly alkylating properties, SM exerts a local toxic effect on the eyes by rapidly reacting with major cellular molecules [9]. The severity of the ocular injury depends on the concentration and the time of exposure [10]. Symptoms may occur up to 12 h post-exposure. Usually, an acute phase occurs initially, manifesting clinically as photophobia, inflammation of the anterior segment, and corneal erosions. This may be followed by irreversible corneal injuries that progress to vision impairment and, potentially, blindness [11].

Studies investigating ocular SM exposure have reported histopathological changes of the cornea, including ulceration, keratocyte death, inflammation, stromal neovascularization, and blistering [12–14]. The mechanisms by which these deleterious effects occur

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https://doi.org/10.1016/j.jtos.2017.11.004
1542-0124/

have been proposed to include induction of oxidative stress and lipid peroxidation, and cell damage associated with SM alkylation of DNA. This has also been linked to glutathione (GSH) depletion possibly leading to oxidative stress and lipid peroxidation [15–18].

However, the concentration or the time of the exposure needed to trigger the pathophysiological effects after SM exposure have not been fully elucidated yet, leading to unsuccessful treatments. The present study uses nitrogen mustard (NM), which has structural and toxicological properties that are similar to SM and can been used as a surrogate [19–21]. The aim of the present study was to (i) characterize corneal damage induced by (NM), in the *ex vivo* isolated rabbit eye, and (ii) assess the impact of NM exposure on the metabolic profile of the cornea using untargeted lipidomics.

## 2. Methods and materials

### 2.1. Methods

#### 2.1.1. Isolated rabbit eye testing

The methods used for these studies were based on a previous published model for the detection of ocular irritation in isolated rabbit eyes [22]. Eyes with intact eyelids from rabbits aged 8–12 weeks were received from a "slaughter house" from Pel Freez Biologicals (Rogers, AR), and shipped overnight on ice in serum-free Dulbecco's Modified Eagle's Medium (DMEM). Upon receipt, eyelids were surgically removed, and the eyes were subsequently washed in sterile, serum-free and phenol red-free DMEM with low glucose (pH 7.2) (Sigma-Aldrich, St. Louis, MO) supplemented with antibiotic/antimycotic agents (Life Technologies, Grand Island, NY). Eyes were then placed in a 12-well tissue culture plate (Costar, Corning Inc, Corning, NY), cornea side up. Approximately 1 ml phenol red-free DMEM was added to each well without submerging the cornea. To restore corneal transparency and normal thickness, eyes were then incubated for 2 h in a humidified, 5% $CO_2$ tissue culture incubator maintained at 37 °C. Eyes were then examined using a dissecting microscope (Zeiss Discovery V12 Stereo Microscope (Gottingen, Germany)) for signs of damage; any corneas showing ocular lesions were removed from the study.

Under a chemical hood, liquid NM (mechlorethamine hydrochloride, Sigma-Aldrich, St. Louis, MO) was dissolved in sterile water to create various concentrations (0.01–100 mg/ml). Ten μl NM solution or vehicle (sterile water) was applied to a sterile 5 mm diameter #1 filter disc (Whatman International Ltd, Maidstone, England), which was then placed onto the central cornea for various exposure times (10 s - 1 h [Fig. 1A]). Eyes were then rinsed with 20 ml sterile DMEM, placed back into the 12-well tissue culture plate and in the incubator for 3 h. At the end of this period, the cornea (including 2–3 mm of the adjoining sclera) from each eye was surgically removed. Some corneas were then draped over an agar post, (comprised of 2% low melting temperature agarose (SeaPaque Agarose, Lonza, Rockland, ME) in DMEM) (Fig. 1B), fresh sterile media was added to the well without submerging the cornea, and cultured for 24 or 48 h post-exposure period. Corneas were collected at 3 h (i.e., immediately after surgical removal of the cornea), 24 h or 48 h post exposure and fixed overnight in 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in phosphate buffered saline (pH 7.4) (PBS). Each vehicle and NM concentration, exposure duration and post-exposure duration was conducted in at least 3 corneas.

#### 2.1.2. Tissue processing and fluorescent labeling

After overnight fixing, a 3 mm wide, central strip of cornea was

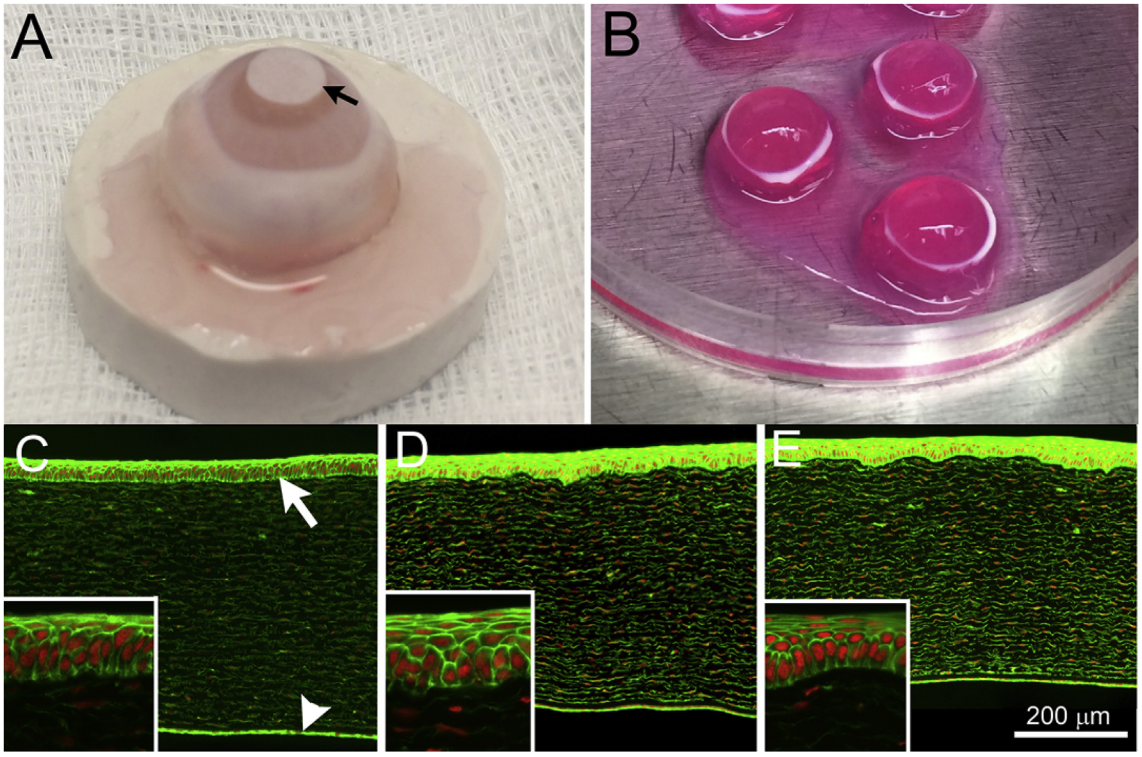


**Fig. 1.** *Isolated rabbit eye model.* (A) Isolated rabbit eyes were exposed to NM by applying a sterile 5 mm diameter filter disk (arrow) impregnated with 10 μl of sterile water (control) or NM solution to the cornea for 5, 10 or 15 min. At the end of the exposure period, the cornea (and 2–3 mm of surrounded sclera) was surgically isolated, placed on top of agar posts and organ cultured at 37 °C (B) for 3, 24 or 48 h. Rabbit corneas exposed to sterile water for 5, 10 or 15 min, showed no differences in epithelial thickness, stromal thickness and stromal cell viability after 3 (C), 24 (D) or 48 h (E) of organ culture. Insets show higher magnification of the corneal epithelium. Live cells and nuclei were identified by phalloidin (green) and DAPI (red) stains, respectively. The corneal epithelium (white arrow) and posterior corneal endothelium (white arrowhead) are shown.