Dermal Exposure to Vesicating Nettle Agent Phosgene Oxime: Clinically Relevant Biomarkers and Skin Injury Progression in Murine Models

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Abbreviations: CX, Phosgene oxime; SM, sulfur mustard; HD, sulfide; NM, nitrogen mustard; LEW, lewisite; CWAs, Chemical Warfare Agents; CEES, chloroethyl ethyl sulfide.

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Abstract

Phosgene oxime (CX), categorized as a vesicating chemical threat agent, causes effects that resemble an urticant or nettle agent. CX is an emerging potential threat agent that can be deployed alone or with other chemical threat agents to enhance their toxic effects. Studies on CX-induced skin toxicity, injury progression, and related biomarkers are largely unknown. To study the physiological changes, skin clinical lesions and their progression, skin exposure of SKH-1 and C57BL/6 mice was carried out with vapor from 10 µl CX for 0.5 min or 1.0 min durations using a designed exposure system for consistent CX vapor exposure. 1 min exposure caused sharp (SKH-1) or sustained (C57BL/6) decrease in respiratory and heart rate leading to mortality in both mouse strains. Both exposures caused immediate blanching, erythema with erythematus ring (wheel) and edema, and an increase in skin bi-fold thickness. Necrosis was also observed in the 0.5 min CX exposure group. Both mouse strains showed comparative skin clinical lesions upon CX exposure; however, skin bifold thickness and erythema remained elevated up to 14 days post exposure in SKH-1 mice but not in C57BL/6 mice. Our data suggest that CX causes immediate changes in the physiological parameters and gross skin lesions resembling urticaria, which could involve mast cell activation and intense systemic toxicity. This novel study recorded and compared the progression of skin injury to establish clinical biomarkers of CX dermal exposure in both the sexes of two murine strains relevant for skin and systemic injury studies and therapeutic target identification.
Significance Statement

Phosgene oxime (CX), categorized as a vesicating agent, is considered as a potent chemical weapon and is of high military and terrorist threat interest since it produces rapid onset of severe injury as an urticant. However, biomarkers of clinical relevance related to its toxicity and injury progression are not studied. Data from this study provides useful clinical markers of CX skin toxicity in mouse models using a reliable CX exposure system for future mechanistic and efficacy studies.
Introduction

Phosgene oxime (dichloroformoxime, CX; Cl₂CNOH) is a halogenated oxime categorized together with vesicating chemical threat agents. Severe toxicity upon exposure and easy synthesis makes CX a potent threat when employed alone or together with other chemical threat agents to cause incapacitation and death (Augerson, 2000; Patočka, 2011). CX was stockpiled during World War II as a potential chemical weapon to cause instantaneous incapacitation. More recently, on March 20, 2019, an FBI investigation found large amounts of synthesized CX inside a Lawton home in Oklahoma, USA (NEWS9, 2019), highlighting the potential danger of its deployment as an emerging chemical threat.

Vesicating agents sulfur mustard [SM; bis(2-chloroethyl) sulfide, HD]; nitrogen mustard HN2 (NM; 2,2'-dichloro-N-methyldiethylamine), and lewisite [LEW; dichloro(2-chlorovinyl) arsine], cause painful delayed blistering upon dermal exposure and result in severe incapacitating injuries (Augerson, 2000; McManus and Huebner, 2005; Patočka, 2011; Singh et al., 2021). The effect of dermal CX exposure, however, resembles more like an urticant or nettle agent (Rosenbloom et al., 2002; Patočka, 2011). Compared to other vesicating agents, CX penetrates faster and leads to instantaneous onset of toxic effects, including tissue destruction and severe systemic toxicity that could lead to rapid mortality (Patočka, 2011; Tewari-Singh et al., 2017; Goswami et al., 2018). Dermal exposure to CX causes intense itching and rashes resembling breakout of hives with immediate skin irritation, erythema, blanching (whitening), and necrosis (Tewari-Singh et al., 2017).

The toxic effects of dermal exposure to mustard vesicating agents (SM and NM) have been well characterized and clinical biomarkers that could be valuable in diagnosis, and in
evaluating the efficacy of interventions to alleviate skin injury have been reported in various animal models (Smith et al., 1997; Kehe et al., 2009; Tewari-Singh et al., 2009; Shakarjian et al., 2010; Tewari-Singh et al., 2013; Mouret et al., 2015; Tewari-Singh and Agarwal, 2016). However, similar studies to evaluate the clinical lesions and identify relevant injury markers from CX dermal exposures in useful animal models are lacking. Also, the role of sex as a biological variable in vesicant injury is poorly understood.

Our previous pilot study using acute CX dermal exposure in SKH-1 hairless male mice showed that CX exposure causes instant blanching with an erythematous ring, urticaria, and edema (Tewari-Singh et al., 2017). These skin lesions were accompanied by cell death, mast cell degranulation, and inflammatory changes in the skin as well as dilatation of the peripheral vessels, increased red blood cells in vessels of internal organs leading to mortality (Tewari-Singh et al., 2017). However, the assessment of these immediate skin lesions and injury progression/healing and associated pathophysiology as compared to other vesicating agents, are further needed. Additionally, variability in the CX vapor exposures was observed since its melting point is around 40°C, and an exposure system that maintained CX in a liquid state was needed. Hence, comprehensive studies were designed to evaluate the injury progression and pathophysiology of both exposure-duration and time dependent CX exposures to identify biomarkers of injury and establish reliable animal models.

In the current study, we report the assessment of skin injury progression and clinical consequences of 0.5 min and 1.0 min CX dermal exposures in both the sexes of hairless (SKH-1) and haired (C57BL/6) mice (Fig. 1A). SKH-1 hairless mice have been most widely used in dermatologic research, including wound healing and skin carcinogenesis, and in skin toxicity studies from vesicating agents (Hwang et al., 2006; Benavides et al., 2009; Tewari-Singh et al.,
2009; Jain et al., 2011; Cléry-Barraud et al., 2013; Tewari-Singh et al., 2013; Jain et al., 2014; Mouret et al., 2015). C57BL/6 mice were used in the current study along with SKH-1 mice to compare the effect of dermal CX exposure in both the murine models. Since most genetically modified mice are available on a C57BL/6 background, this study is highly valuable to establish clinical markers in this mouse strain for further mechanistic studies using knockout mouse models.

CX is a colorless crystalline solid at room temperature with melting point around of 40°C and a boiling point of 128°C. A modified exposure system was designed to keep the test environment around 40°C, resulting in more consistent vapor exposures, which is critical to develop novel in vivo models for the evaluation of CX skin lesions (Fig. 1B). The gross skin lesions and quantitative clinical markers of CX skin injury progression in useful experimental mouse models will be helpful to gain insight into the accompanying pathophysiology involved in CX skin injury to identify therapeutic targets.

Materials and Methods

**CX synthesis and exposure system:** CX was synthesized with 95% purity at MRIGlobal, Kansas City, MO, as described previously (Tewari-Singh et al., 2017). CX is corrosive and volatile, and requires a temperature of around 40°C to be in the liquid state. For dermal toxicity assessment of CX vapor in the mice model using filter caps, the chemical must be applied to the filter paper in liquid form for accurate mass loading. The goal of the vapor exposure model is to expose dorsal skin of mice to CX using gaseous CX generated from a filter paper moistened with liquid CX. Gaseous CX (vapor) was chosen for our model because reported human exposures to CX could occur mainly via exposure to CX in vapor form. To maintain the reproducibility of CX
vapor concentrations over the exposure time, the test environment must be maintained at a constant temperature. Therefore, an exposure system was designed at MRIGlobal using a containment box to regulate and maintain the test environment at a constant temperature so that CX remains in liquid state (around 40°C) for more accurate and consistent mice dermal exposures to CX vapor (Fig. 1B). The system is equipped with a temperature regulated hot plate for maintaining the CX test article as a liquid for filter cap application. A positive displacement glass tip micropipette was utilized for accurate filter mass loading of CX onto filter caps preceding dermal application. To maintain the test environment at a constant temperature, the glove box was modified for the introduction of heated air that is introduced into the system through a delivery pipe at controlled rates with a temperature regulated heat gun. The internal temperature of the glove box was maintained at approximately 40°C and monitored with a Type K thermocouple with digital temperature display to ensure a constant temperature regulated test environment. The glove box was placed in a secondary chemical fume hood for safety. It was verified that the designed exposure system generates the desired/consistent CX vapor concentration for exposures, which was checked prior to the exposures. MRIGlobal standard operating procedures and approval from Institutional Animal Care and Use Committee as well as safety procedures were followed for the CX exposures in accordance with the Guide for the care and use of laboratory animals.

CX exposure and clinical assessments: Male and female SKH-1 hairless and C57BL/6 mice (age: 4-6 weeks) were obtained from Charles River Laboratories (Wilmington, MA). The C57BL/6 mice were shaved 2 days before exposure using a clipper. The mice were singly-housed in polycarbonate Tecniplast caging with wire top (Tecniplast, Phoenixville, PA) throughout the acclimation/quarantine and throughout the study to protect skin integrity. Mice
were pre-medicated with buprenorphine SR and anesthetized 30 minutes later by an IP administration of a Ketamine and Xylazine cocktail. Once anesthetized, the mice had double-sided carpet tape placed on their back for placement of the vapor caps and secured to a rectangle of cardboard. The animals were then moved into a temperature-controlled glove box for dose administration. Randomly selected mice were exposed topically (n=5/group) to vapor from filter saturated with 10 µL of liquid CX with one cap placed on each side of the dorsal midline of the back, for a duration either 0.5 min or 1.0 min. The exposure and study paradigm is detailed in figure 1A. There is no human data available on exposure to CX and published data on CX and dermal toxicity is scarce. The exposure amount and exposure durations doses were chosen based on mustard vesicant SM animal exposures and our pilot studies. The CX vapor exposure for 1 min using 10 µl liquid was estimated to be 2.04 mg/kg which would be stable for the duration of exposure (NCBI, 2023). The mice remained under the chemical fume hood a minimum of 24 hours post-exposure with CX; for earlier study time points, mice were observed and clinical measurements taken under the hood. Hydrogels were used to provide water while animals were in the chemical fume hood.

General Health observations were carried out least once daily and clinical observations were recorded prior to challenge, at 30 min, 4-6 hours post CX exposure and twice daily thereafter. Skin pictures were taken using a digital camera, skin bi-fold thickness was measured using digital calipers as described previously (Tewari-Singh et al., 2009). Clinical assessments (erythema, edema, necrosis) were carried out at the mentioned time points using modified Draize Scoring as per our previous report (Tewari-Singh et al., 2017) (Fig. 1A). Mouse pulse oximeter [MouseOx Plus, Starr Life Sciences (Oakmont, PA)] was used to measure the physiologic parameters (Heart rate and Respiratory (Breath) rate) using a small collar sensor
attached to the back of the neck in conscious/anesthetized mouse and sedated with Isoflurane (1-2%). A rectal probe was used to measure the body temperature. These parameters were recorded using the MouseOx Plus software according to the manufacturer’s instructions. Pulse Ox was performed in only a subset of animals due to the procedural and personnel limitation. Mice were sacrificed at the desired time points and samples were collected for further analyses.

**Statistical Analyses:** Kaplan-Meier Log-rank test was used to compare the survival curves. Statistically significant differences between groups were determined by student t-test or one-way ANOVA followed by the Tukey test for multiple comparisons (GraphPad Prism 8 software). Data are presented as mean ± SE, and a p-value of < 0.05 was considered significant.

**Results**

**Effect of dermal CX exposure on mice survival and physiological parameters**

The modified exposure system maintained the temperature at ~ 40°C, keeping CX in a liquid state resulting in consistent vapor exposure (Fig. 1). Our previous pilot study with acute dermal CX exposure in mice showed that CX causes instant toxic effects and can lead to mortality (Tewari-Singh et al., 2017). In the present study, survival and related physiological parameters were evaluated after 10µl dermal CX exposure for 0.5 min and 1.0 min in hairless (SKH-1) and haired (C57BL/6) mice. CX exposure led to instant blanching at the vapor cap exposure site in both the mouse strains. Dermal CX exposure for 1 min resulted in 90% mortality in SKH-1 mice (80% mortality in male mice and 100% mortality in female mice) within 24 hours of its exposure (Fig. 2 A). Mortality was not observed in the 0.5 min CX exposure group and the mice survived the complete experimental duration of 2 weeks (Fig. 2 A). In C57BL/6 mice, 1 min CX exposure resulted in 50% mortality (60% mortality in male mice and 40%
mortality in female mice; Fig. 2 B). Like SKH-1 mice, 0.5 min CX exposure did not result in mortality in C57BL/6 mice by 24 hours after exposure (Fig. 2 B). 0.5 min CX exposure did not result in mortality in either mouse strain, while 1.0 min CX exposure resulted in significant mortality in both the strains.

**Respiratory Rate**

In the 0.5 min CX exposure group, a decrease in respiratory rate (pre=195.74 brpm; 30 min: 131.88 brpm) was observed at 30 min post CX-exposure in the SKH-1 mice with an increase at later time points (162 brpm) but remained lower than measurements taken before CX exposure (Fig. 3A). In the C57BL/6 mice, a similar trend was observed with the decrease being maximum at 30 min post CX-exposure (pre=143.55; 30 min= 55.1 brpm) and increasing slightly thereafter (2 hours= 95.94; and 8 hours= 100.27) (Fig. 3A); however, the values remained much lower than pre-exposure levels. CX-exposed mice were not significantly different from controls at any time point and fold change from control in heart and breath rate were not significantly different in C57BL/6 mice when compared to SKH-1 mice.

The 1.0 min CX exposure led to a sharp decrease in the respiratory rate of the mice within 30 min of exposure (Fig. 3B). This decrease in respiratory rate was observed in both SKH-1 and C57BL/6 mice, with the decrease being significant in SKH-1 mice at all the time points following 1 min CX exposure (Fig. 3B). In the SKH-1 mice, respiratory rate decreased at 30 min (pre=195.74 brpm; 30 min: 127.09 brpm) and was lowest at 2 hours (81.00 brpm) before increasing at 8 hours (122.5 brpm) (Fig. 3B). In the C57BL/6 mice also, respiratory rate decreased sharply at 30 min (pre=143.55 brpm; 30 min: 71.77 brpm) and increased at 2 hours (93.00 brpm) before decreasing again at 8 hours (52 brpm) (Fig. 3B) but these changes were not statistically significant. The 0.5 min CX exposure did not induce significant changes in
respiratory rate in either mouse strain, while 1.0 min CX exposure caused significant decreases in the respiratory rate the the SKH-1 mice.

**Heart rate**

In the 0.5 min CX exposure group, the decrease in heart rate was observed in both the mouse strains; however, it was more significant in C57BL/6 mice. In SKH-1 mice, a significant decrease in heart rate was observed only at 2 hours post CX-exposure and increased thereafter at 8 hours post-exposure (pre=472.92; 2 hours=318.27; Fig. 3C). In C57BL/6 mice, the decrease in heart rate was significant at both 30 min and 2 hours post CX-exposure before increasing at 8 hours (pre=573.33; 30min=196; 2 hours=365.44; 8 hours=487.05; Fig. 3C). Table 1 and 2 represent a comparison of the physiological parameters post 0.5 min and 1.0 min CX exposures, respectively, in male SKH-1 and C57BL/6 mice. Due to limited female numbers and no significant sex difference observed, data on heart and respiratory rate in only male mice presented here.

PulseOx measurements showed a sharp, significant decrease in heart rate was observed in both SKH-1 and C57BL/6 mice at 30 min post 1 min CX-exposure (SKH-1; pre=472.92 bpm; 30 min: 239.15 bpm, C57BL/6; pre=573.33 bpm; 30 min: 265.92 bpm; Fig. 3D). In SKH-1 mice, the heart rate decreased further at 2 hours post 1 min CX exposure (bpm; 225.27 bpm) before increasing at 8 hours (366.35 bpm), although still significantly lower than the pre-exposure levels (Fig. 3D). In the C57BL/6 mice, the heart rate increased slightly at 2 hours post CX-exposure (353.78 bpm), but remaining significantly lowered, before decreasing further at 8 hours (282.33 bpm; Fig. 3D). Both the CX exposure durations caused significant decreases in heart rate in both the strains of mice.
Further, a sudden and significant drop in body temperature [pre-exposure body temperature ~32°C; at 30 min (temperature drop of 5.6 °C) and 2 hours (temperature drop of 4.7 °C) following CX exposure] was observed in SKH-1 mice before increasing at later time points (data not shown).

**Dermal CX exposure caused erythema, edema, necrosis, and increased skin bi-fold thickness in mice**

Exposure to toxic agents like vesicants and urticants can lead to the activation of inflammatory response and infiltration of immune cells that involves reddening of the skin (erythema), accumulation of fluid (edema) and necrosis (Tewari-Singh et al., 2013; Mouret et al., 2015; Tewari-Singh et al., 2017). Clinical lesions parameters such as skin bi-fold thickness, erythema, edema, and necrosis were evaluated after 0.5 min and 1.0 min CX exposure in mice.

**0.5 min CX exposure**

CX exposure for 0.5 min caused blanching, edema, erythema, pigmentation, urticaria (green arrow), erythematous ring (red arrow), and necrosis (yellow arrow) in hairless and haired mice (Fig. 4). Necrosis started appearing at 24 hours, increased at day 3 and 7 before decreasing at day 14 post CX exposure. These lesions were observed in both male and female mice; however, erythema was more prominent in the female mice of both mouse strains (Fig. 4A and B). Immediately after exposure, blanching was observed after the exposure cap removal at the exposure site surrounded by an erythematous ring with edema in both the mouse strains. At 24 hours post CX exposure, tissue necrosis started to appear at the exposure site and by day 3 post exposure the necrotic area increased covering completely the exposure site. By day 7 post exposure, a crust formed separating the necrotic tissue from the underlying skin. By day 14 the
tissue/wounds started to heal, leaving a small scab in the SKH-1 female mice. However, the healing was slower in male SKH-1 mice as compared to female mice, and a crust and scab were present at day 14 post CX-exposure. A significant difference in the wound healing was not observed in the C57BL/6 male and female mice. Evaluation of these lesions showed a significant increase in skin-bi fold thickness, edema, erythema, and necrosis post dermal CX exposure in both the mouse strains (Fig. 5). A significant increase in skin bi-fold thickness was observed at all the study time points in both male and female SKH-1 mice (Fig. 5A). In the C57BL/6 mice, both male and female mice had significantly increased skin bi-fold thickness up to 7-day post-exposure, which decreased and was similar to the control mice group at 14-day post exposure (Fig. 5B).

Significant edema was observed in both SKH-1 and C57BL/6 mice that resolved by Day 7 post CX exposure (Fig. 5C and D). Maximal edema was observed at 2 hours in the female SKH-1 and at 24 hours post-exposure in the male mice (Average edema score of 1.125 in female mice at 2 hours and 1.5 for male mice at 24 hours post CX exposure; Fig. 5C). In the C57BL/6 mice, maximal increase in edema was observed at 2 hours post CX exposure in both male and female mice (average edema score of 1.27 and 1.67, respectively, in female and male C57BL/6 mouse; Fig. 5D). Thereafter, a gradual decrease in edema was observed at subsequent time points, although it was still significant until day 3 post CX-exposure. On day 7 post CX exposure, the edema resolved in both the mouse strains (Fig. 5C and D).

A significant increase in erythema was observed only in the female mice of both the mouse strains (Fig. 5E and F). The increase in erythema was maximal at 2 hours post CX exposure (average erythema score was 1 in both SKH-1 and C57BL/6 female mice at 2 hours post CX exposure). In the female SKH-1 mice, a reduction in erythema was observed at 24 hours
post exposure. At later time points the erythema in SKH-1 mice increased again; however, it was not statistically significant (Fig. 5E). Similar to SKH-1 mice, erythema was slightly reduced by 24 hours in female C57BL/6 mice, although it was still significantly higher than the control, by day 3 post exposure the erythema resolved completely (Fig. 5F).

Both male and female SKH-1 and C57BL/6 mice showed similar necrosis pattern upon 0.5 min CX exposure with significant necrosis beginning at 24 hours after its exposure with a maximal increase in score at day 7 post exposure (Fig. 5G and H, Table 1). At day 7 post CX exposure, average necrosis score of 3.8 and 3.4, respectively, was recorded in male and female SKH-1 mice, and the average necrosis score of 4 in both male and female C57BL/6 mice (Fig. 5G and H). A decrease in necrosis was observed at day 14 post CX exposure in male and female mice of both the strains; however, it was still significantly higher compared to the control mice (Fig. 5G and H and Table 1).

A summary of the comparison of clinical parameters between SKH-1 and C57BL/6 post 0.5 min CX exposure mice is presented in Table 1. Overall, CX exposure for 0.5 min caused lesions in mice of both sexes and strains. Although erythema was more prominent in females, the SKH-1 females displayed faster visual wound healing pattern than their male counterparts.

**1 min CX exposure**

CX skin exposure for 1.0 min resulted in blanching and urticaria (green arrow) and erythematous ring (red arrow) in both the mouse strains, appearing instantly after CX exposure (Fig. 6A and B). The blanched skin was clearly visible, and edema and erythema were observed up to 24 hours post exposure (Fig. 6A and B).

These lesions were associated with a significant increase in the skin-bi fold thickness, edema, and erythema post dermal CX exposure (Fig. 7). A significant increase in skin bi-fold
thickness was observed at 0.5 hours, 2 hours and 8 hours post CX-exposure in SKH-1 male mice (Fig. 7A). Similarly, in the C57BL/6 mice, a significant increase in skin bi-fold thickness was observed in both male and female mice at 2 hours and 8 hours post dermal CX exposure (Fig. 7B). The maximum increase in skin bi-fold thickness was observed at 2 hours post exposure in both the strains (SKH-1 mice: control=0.77mm; male=1.38mm; female=1.25mm, C57BL/6 mice: control=0.85mm; male=1.50mm; female=1.70mm) (Fig. 7A and B). An increase in edema was observed as early as 30 min post dermal CX exposure (earliest observation time point). In the SKH-1 mice, maximal increase in edema was observed at 8-24 hours post CX exposure, while in the C57BL/6 mice maximal increase was observed much earlier at 2 hours post exposure (Fig. 7C and D). The average edema score in the CX-exposed group was 1.4 in the SKH-1 male mice at 8 hours post exposure and 1.1 in the C57BL/6 male mice at 2 hours post exposure. C57BL/6 female mice showed higher CX-induced edema compared to male mice (Average edema score in female mice was 1.85 compared to 1.1 in male mice at 2 hours post CX exposure) (Fig. 7D). At 8 hours post-exposure, edema reduced in both male and female C57BL/6 mice but was still significantly higher in female mice. Edema reduced further at 24 hours and was parallel in both the male and female mice (Fig. 7D).

Further, our data showed that CX exposure induced erythema in both SKH-1 and C57BL/6 mice. The increase in erythema was significant and maximal at 2 hours post exposure in both male (average erythema score = 0.67) and female (average erythema score = 1) SKH-1 mice (Fig. 7E). At subsequent time points, a decrease in erythema was observed (Fig. 7E). In the C57BL/6 mice, the increase in erythema was significant at 2 and 8 hours post CX exposure before a decrease at 24 hours in both male and female mice (Fig. 7F). C57BL/6 male mice showed increased erythema compared to SKH-1 male mice (Fig. 7E and F, Table 2). Necrosis
was not observed upon 1 min dermal CX exposure at these time points in both male and female
hairless and haired mice. A summary of the comparison of clinical parameters between SKH-1
and C57BL/6 post 1 min CX exposure mice is presented in Table 2. CX exposure for 1 min
resulted in erythema, edema, and increased skin bi-fold thickness within 2 hours of exposure in
male and female mice of both the mouse strains.

**Discussion**

Vesicating chemical agents with a potential to be used in warfare and terrorist activities,
are known to cause edema, erythema, and delayed vesication upon dermal exposure (Kehe et al.,
2009; Tewari-Singh et al., 2009; Shakarjian et al., 2010; Jain et al., 2011; Cléry-Barraud et al.,
2013; Tewari-Singh et al., 2013; Jain et al., 2014; Mouret et al., 2015; Tewari-Singh and
Agarwal, 2016). Compared to other vesicating agents, CX penetrates faster and can cause more
severe damage to the skin tissue leading to instant pain, tissue destruction, systemic toxicity, and
rapid mortality. However, its toxicity has not been well studied. This pioneering study was
carried out for developing reliable and reproducible CX skin injury novel murine models which
could be employed for defining the pathophysiology of CX and for identifying molecular targets
to counteract CX toxicity. Since no human exposure data or skin injury animal model
development studies are available for CX, the dose of 10 µl CX for skin exposure was chosen
based on the 10 µl (1.4 mg/kg for 6 min) vesicant SM vapor murine model development
(Ricketts et al., 2000; Joseph et al., 2011; Cléry-Barraud et al., 2013; Joseph et al., 2014). CX
penetrates faster and causes instant injury and systemic toxicity as compared to other vesicating
agents, hence lower exposure durations were selected based on our pilot studies. The 0.5 min and
1 min CX exposures caused acute toxic effects and acute lethal effects in both the mouse models,
respectively. To accomplish this goal, a valuable CX exposure system was designed in collaboration with MRIGlobal to obtain consistent CX vapor cutaneous exposure in mice.

In the present study, we evaluated changes in different physiological and clinical parameters after 0.5 and 1.0 duration CX skin exposures in hairless (SKH-1) and haired (C57BL/6) mice. Although, the number of mice in the study was limited (due to high exposure cost at contract laboratory), results indicate that 1 min CX exposure was lethal and caused mortality in both the mouse strains and that SKH-1 mice could be more susceptible to mortality compared to C57BL/6 mice. Also, because we did not observe significant differences between the clinical results of male and female SKH-1 mice in the pilot study, therefore, we considered females just for one time study point to compare with males. In this study, sex related differences were not significantly apparent in the clinical and physiological parameters we measured. However, observations suggest that there could be faster wound healing in female mice, which could be apparent in molecular or biological events related to their immune or other responses, that are currently being assessed.

CX exposure for 1 min caused a sudden drop in physiological parameters such as respiratory rate, heart rate, and body temperature within 30 mins of exposure. The decrease in respiratory rate and heart rate was sharper in C57BL/6 mice and could be a contributing factor for the early mortality observed in these mice. 0.5 min CX dermal exposure didn’t lead to a significant decrease in respiratory rate, but a significant decrease in heart rate was observed in both the mouse strains. However, all the SKH-1 mice exposed to CX for 0.5 min survived till the study endpoint (14 days). In our previous study, mast cell activation and histamine release were observed after acute CX skin exposure in SKH-1 mice that caused urticaria and allergy-like skin lesions (Tewari-Singh et al., 2017). Mast cell activation is one of the key steps in the allergic
reaction initiation cascade (Metcalf et al., 2009; Leyva-Castillo et al., 2019) and it is a
trademark of anaphylaxis that includes sudden allergic reactions and changes in the physiological
parameters (Rutkowski et al., 2012; Reber et al., 2017). The sudden and significant changes in
physiological parameters in the 1.0 min CX exposed mice could be the reason for the mortality
observed in both strains of mice. A more significant decrease in heart rate and respiratory rate in
SKH-1 mice compared to C57BL/6 could explain the enhanced mortality observed in these mice
upon CX exposure. Additionally, our previous studies with NM have shown that dermal
exposure in SKH-1 mice leads to more increased epidermal thickness, and macrophages and
mast cell infiltration compared to C57BL/6 mice (Jain et al., 2014; Tewari-Singh et al., 2014). In
the 0.5 min CX exposure, these could be contributing to the differences observed in the clinical
effects, including increased erythema (day 7 and 14 post exposure) and skin bi-fold thickness
(day 14 post exposure) in SKH-1 mice compared to C57BL/6 mice; however, this remains to be
investigated.

Together, our present study corroborates the earlier reports of CX skin exposure leading
to instantaneous symptoms and death due to its higher penetration compared to other mustard
vesicants (Augerson, 2000; Patočka, 2011; Tewari-Singh et al., 2017; Goswami et al., 2018;
Singh et al., 2021). Skin exposure with vesicating agents causes inflammation and an increase in
skin thickness, edema, erythema, and necrosis is observed (Goldman and Dacre, 1989; Dacre and
Goldman, 1996; Smith et al., 1997; Kehe and Szinicz, 2005; Shakarjian et al., 2010; Jain et al.,
2011; Tewari-Singh et al., 2013; Kumar et al., 2015; Tewari-Singh and Agarwal, 2016; Goswami
et al., 2018; Nair et al., 2021). Results from our prior report showed that maximum edema,
erythema, skin bi-fold thickness were at 2 hours post-acute CX exposure (Tewari-Singh et al.,
2017). Similarly, skin edema was also observed in both 0.5 min and 1.0 min CX exposure
durations and peaked at 2/24 hours. Moreover, necrosis was only observed in 0.5 min CX skin exposure-duration which gradually increased and peaked at day 7 and reduced by 14 days post-exposure. Overall, the clinical lesions were found to be comparable in both SKH-1 and C57BL/6 mice (Table 1 & 2) which is in line with previous studies from us and others with dermal mustard and arsenical vesicant exposures showing parallel clinical and pathophysiological skin injury features in SKH-1 and C57BL/6 mice (Tewari-Singh et al., 2013; Jain et al., 2014; Tewari-Singh et al., 2014; Srivastava et al., 2022). However, the timeline for healing in female and male mice following CX exposure could be further assessed. Use of both hairless and haired mice in chemical/vesicant agent studies is reported (Kim et al., 1996; Smith et al., 1997; Ricketts et al., 2000). The SKH-1 hairless mouse model is the most widely used mouse model in skin research, including wound healing, photobiologic response, and skin carcinogenesis studies. These mice are easy to manipulate and handle, and hair removal and related inflammation and wounding are avoided, and the hair re-growth does not obscure the wound healing response, (Benavides et al., 2009). This mouse model will be extremely useful to study the skin injury progression following CX exposure and for the efficacy studies (Hinz et al., 1989). However, the CX related clinical biomarkers in the C57BL/6 mice would be applicable for target identification and validation studies using the genetically modified mice in this background.

The findings from the present study are in concordance with previous reports showing vesicating agents induced skin lesions are associated with skin inflammation, edema, erythema, and necrosis (Jain et al., 2011; Tewari-Singh et al., 2017). (Tewari-Singh et al., 2013). (Tewari-Singh et al., 2009). Together, previous studies on other vesicating agents showed delayed clinical lesion development after 1.0 min exposure duration; however, data from this study indicates that CX skin exposures caused an instant skin injury, blanching and urticaria compared to other
vesicating agents. However, further long-term toxic effects of acute CX cutaneous exposure remain to be determined. The instantaneous appearance of symptoms upon CX-exposure and the associated mast cell degranulation as shown previously (Tewari-Singh et al., 2017) warrants further investigation into the role of mast cells and associated signaling in CX-induced toxicity, which is being currently evaluated. The CX exposure-induced pooling of blood in various internal organs (Tewari-Singh et al., 2017) suggests its fast penetration causes hematological disturbances, including clotting of blood/ disseminated intravascular coagulation upon CX-exposure and needs to be further explored.

In summary, the data from this study shows that both CX skin exposure durations cause gross skin lesions in both the sexes of the mouse strains that appear within seconds, which is faster than observed with other vesicating agents. Although faster healing is suggestive in female SKH-1 mice compared to male mice, most skin injury lesions are parallel, and their progression is comparable in both the sexes of haired and hairless mice (Tables 1 and 2). Further, 1.0 min CX exposure leads to higher mortality with a sudden decrease in physiological parameters that could serve as pivotal clinical markers for CX-induced skin injury and mortality. Although our exposure model ensures a consistent and reproducible temperature and CX vapor exposure concentration, there are limitations. The instantaneous toxic effect of CX resulted in mortality; however, this needs further analyzed employing a larger sample size. Further studies to evaluate the histopathological effects of CX cutaneous exposure and to decipher the underlying molecular mechanisms are being carried out using both the sexes of these mouse strains to identify therapeutic targets.

Data Availability Statement
The authors declare that all the data supporting the findings of this study are contained within the paper.

**Authorship contribution**

*Participated in research design:* Neera Tewari-Singh, Rajesh Agarwal and Claire R. Croutch.

*Conducted experiments:* William Sosna, Poojya Anantharam, Rick Tuttle, Dinesh G. Goswami, Neera Tewari-Singh, Claire R. Croutch.


*Wrote or contributed to the writing of the manuscript:* Dinesh G. Goswami, Satyendra K. Singh, Ebenezar O.M. Okoyeocha, Andrew K. Roney, Omid Madadgar, Rajesh Agarwal, Neera Tewari-Singh.

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Footnotes

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None

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Figure legends:

**Figure 1. CX exposure paradigm and the modified exposure system designed for exposing mice skin.** CX structure and study paradigm in SKH-1 and C57BL/6 mice (A) and the modified CX exposure system (B). The chamber maintains the CX at constant temperature and in a liquid state during exposures for consistent and reproducible cutaneous exposures. CX, phosgene oxime.

**Figure 2. Dermal CX exposure for 1 min caused mortality in mice.** The dorsal skin of hairless (SKH-1) and haired (C57BL/6) mice was exposed topically to CX vapor for 0.5 or 1.0 min duration using 2 vapor caps (12 mm each); one on each half side of the dorsal skin. Graphs show the effect of dermal CX exposure on the survival in hairless SKH-1 (A) and haired C57BL/6 (B) mice, and that 1 min exposure duration was lethal to both the strains of mice. Graphs show Kaplan-Meier plots for animal survival [pooled male and female data (n=6 for sham and n=10 for the exposure groups)]. P values show the results of a log rank test comparing the control vs. CX (1 min exposure) group. CX, phosgene oxime.

**Figure 3. Dermal CX exposure caused physiological changes in mice.** The Dorsal skin of hairless (SKH-1) and haired (C57BL/6) mice was exposed topically to CX vapor for 0.5 or 1.0 min duration using 2 vapor caps (12 mm each); one on each half side of the dorsal skin. Physiological parameters including respiratory (breath) rate and heart rate using mouse Pulse Oximeter. Graphs show male mice data for respiratory (breath) rate after 0.5 min exposure (A) and 1.0 min exposure duration (B), and heart rate after 0.5 min exposure (C) and 1.0 min exposure duration (D) to CX. Data represent mean ± SE; */#/$, p <0.05 CX vs. control. CX,
phosgene oxime; hashtag, SKH-1 vs. control; asterisk, C57BL/6 vs. control; dollar sign, C57BL/6 vs. SKH-1.CX, phosgene oxime.

Figure 4. CX skin exposure for 0.5 min caused visible skin lesions on the skin of exposed mice. The Dorsal skin of hairless (SKH-1) and haired (C57BL/6) mice was exposed topically to CX vapor for 0.5 min duration using 2 vapor caps (12 mm each); one on each half side of the dorsal skin. CX-induced visible skin lesions were assessed, and digital pictures were taken. Visible skin lesions in SKH-1 (A) and C57BL/6 (B) mice after 0.5 min CX exposure. CX, phosgene oxime; green arrows, blanching and urticaria; red arrows, erythematous ring; yellow arrow, necrosis.

Figure 5. Effect of 0.5 min CX skin exposure on the skin bi-fold thickness, edema, erythema, and necrosis in the skin of mice. The dorsal skin of hairless (SKH-1) and haired (C57BL/6) mice was exposed topically to CX vapor for 0.5 min duration using 2 vapor caps (12 mm each); one on each half side of the dorsal skin. Skin bi-fold thickness was measured using a digital caliper. Edema, erythema, and necrosis were evaluated using modified Draize scoring as previously described. Skin bifold thickness in SKH-1 (A) and C57BL/6 (B) mice, edema in SKH-1 (C) and C57BL/6 (D) mice, and erythema in SKH-1 (E) and C57BL/6 (F) mice observed at various time points after 0.5 mi CX exposure. CX, phosgene oxime; green arrows, blanching and urticaria; red arrows, erythematous ring; yellow arrow, necrosis (n=5 - 15 for SKH-1 and n=7 - 9 for C57BL/6 mice). Data represent mean ± SE; *, p <0.05 CX (Male) vs. control, #, p <0.05 CX (Female) vs. Control.
**Figure 6. CX skin exposure for 1.0 min caused visible skin lesions on the skin of exposed mice.** The Dorsal skin of hairless (SKH-1) and haired (C57BL/6) mice was exposed topically to CX vapor for 1 min duration using 2 vapor caps (12 mm each); one on each half side of the dorsal skin. CX-induced visible skin lesions were assessed, and digital pictures were taken. Clinical lesions in SKH-1 (A) and C57BL/6 (B) mice observed after 1 min of CX exposure. CX, phosgene oxime; green arrows, blanching and urticaria; red arrows, erythematous ring.

**Figure 7. Effect of 1.0 min CX skin exposure on the skin bi-fold thickness, edema, and erythema in the skin of mice.** The dorsal skin of hairless (SKH-1) and haired (C57BL/6) mice was exposed topically to CX vapor for 1 min duration using 2 vapor caps (12 mm each); one on each half side of the dorsal skin. Skin bi-fold thickness was measured by digital calipers. Edema, erythema, and necrosis were evaluated using modified Draize scoring as previously described. Skin bifold thickness in SKH-1 (A) and C57BL/6 (B) mice, edema in SKH-1 (C) and C57BL/6 (D) mice, and erythema in SKH-1(E) and C57BL/6 (F) mice observed at various time points after 1 min CX exposure. CX, phosgene oxime. Data represent mean ± SE; *, p <0.05 CX (Male) vs. control, #, p <0.05 CX (Female) vs. control.
Table 1. Comparison of clinical parameters after 0.5 min CX-exposure in SKH-1 and C57BL/6 mice. Comparison of clinical markers following 0.5 min CX exposure in male and female hairless (SKH-1) and haired (C57BL/6) mice. +, relative increase with respect to control; -, relative decrease with respect to control; M, males; F, females. For respiratory rate: -, decrease of <50 bpm; --, decrease of >50 bpm; N/A, not evaluated. For Heart rate: -, decrease of <100 bpm; --, decrease of 100-200 bpm; ---, decrease of >200 bpm; N/A, not evaluated. For skin bi-fold thickness: +, change of <2-fold; ++, change of >2-fold; N/A, not evaluated. For Edema: +, edema score of <1; ++, edema score of >1; N/A, not evaluated. For Erythema: +, erythema score of <1; ++, erythema score of >1; N/A, not evaluated. For Necrosis: +, necrosis score of <1; ++, necrosis score of 1-2; ++++, necrosis score of >3; ND, not detected; N/A, not evaluated. Underlining indicates statistical significance with p<0.05.
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Table 2. Comparison of clinical parameters after 1.0 min CX-exposure in SKH-1 and C57BL/6 mice. Comparison of clinical markers following 1.0 min CX exposure in male and female hairless (SKH-1) and haired (C57BL/6) mice. +, relative increase with respect to control; -, relative decrease with respect to control; black symbols, males; red symbols, females. For respiratory rate: -, decrease of <100 bpm; --, decrease of >100 bpm. For Heart rate: -, decrease of <100 bpm; --, decrease of 100-200 bpm; ---, decrease of >200 bpm. For skin bi-fold thickness: +, change of <2-fold; ++, change of >2 fold; NA, not evaluated. For Edema: +, edema score of <1; ++, edema score of >1. For Erythema: +, erythema score of <1; ++, erythema score of >1. Underlining indicates statistical significance with p<0.05.

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</table>
Topical exposure to CX vapor using two 12 mm vapor caps, one at each side of dorsal skin of mice.

Figure I
Figure 2
Respiratory (Breath) rate

A
0.5 min CX exposure

B
1 min CX exposure

Heart rate

C
0.5 min CX exposure

D
1 min CX exposure

Time post CX exposure

Pre 30 min 2 hours 24 hours

Pre 30 min 2 hours 8 hours

Heart rate (bpm)

Pre 30 min 2 hours 24 hours

Pre 30 min 2 hours 8 hours

C57Bl/6

SKH-1

Figure 3
Figure 4
Figure 5

SKH-1

Skin bi-fold thickness

C57BL/6

Skin bi-fold thickness

Edema

Erythema

Necrosis

Figure 5
**Figure 6**

A. **SKH-1**

- **Control**
- **30min**
- **2h**
- **8h**
- **24h**

B. **C57BL/6**

- **Control**
- **2h**
- **8h**
- **24h**

**Male**

**Female**
Figure 7