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Cutaneous exposure to vesicant phosgene oxime: Acute effects on the skin and systemic toxicity

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ABSTRACT

Phosgene Oxime (CX), an urticant or nettle agent categorized as a vesicant, is a potential chemical warfare and terrorist weapon. Its exposure can result in widespread and devastating effects including high mortality due to its fast penetration and ability to cause immediate severe cutaneous injury. It is one of the least studied chemical warfare agents with no effective therapy available. Thus, our goal was to examine the acute effects of CX following its cutaneous exposure in SKH-1 hairless mice to help establish a relevant injury model. Results from our study show that topical cutaneous exposure to CX vapor causes blanching of exposed skin with an erythematous ring, necrosis, edema, mild urticaria and erythema within minutes after exposure out to 8 h post-exposure. These clinical skin manifestations were accompanied with increases in skin thickness, apoptotic cell death, mast cell degranulation, myeloperoxidase activity indicating neutrophil infiltration, p53 phosphorylation and accumulation, and an increase in COX-2 and TNFα levels. Topical CX-exposure also resulted in the dilatation of the peripheral vessels with a robust increase in RBCs in vessels of the liver, spleen, kidney, lungs and heart tissues. These events could cause a drop in blood pressure leading to shock, hypoxia and death. Together, this is the first report on effects of CX cutaneous exposure, which could help design further comprehensive studies evaluating the acute and chronic skin injuries from CX topical exposure and elucidate the related mechanism of action to aid in the identification of therapeutic targets and mitigation of injury.

1. Introduction

There is a long history of the use of chemical agents as weapons of warfare; however, their development has rapidly escalated since World War I (Vogel, 2007). Among these chemical agents, alkylating vesicants like sulfur mustard [SM; bis(2-chloroethyl) sulfide, HD] have been commonly used as weapons that cause painful blistering lesions and damage mainly to the skin, eyes and respiratory system (Balali-Mood and Hefazi, 2005; Ghabili et al., 2010, 2011; Layegh et al., 2015). Phosgene oxime (dichloroformoxime, CX; Cl_2CNOH) is also categorized as a vesicating agent; however, this halogenated oxime does not form actual blisters but is an urticant, nettle or a corrosive agent (Augerson, 2000; McManus and Huebner, 2005; Bartelt-Hunt et al., 2006; Patocka, 2011). Stockpiled during World War II (WWII), it is a potent chemical weapon and poses a threat of delivery by itself or with other chemical agents to cause surprisingly prompt incapacitation and death (Augerson, 2000; Patocka, 2011). It is said to be one of the few substances that cause violent effects in humans, and yet is one of the least studied chemical warfare agents with no specific antidote available (Augerson, 2000).

In both liquid and vapor forms, CX causes more severe damage than other vesicants due to its fast penetration, immediate pain and tissue destruction (Patocka, 2011). CX is rapidly absorbed through the skin leading to immediate skin irritation, erythema, blanching (whitening), itching hives and necrosis, and it may also cause severe systemic toxicity (Augerson, 2000; Patocka, 2011). Information on its dermal absorption and effects on human skin tissue is limited, and its mechanism of action is unknown. The skin urticaria from CX resembles urticaria caused by allergic and non-allergic reactions to various environmental substances; which are believed to be mainly due to the activation of mast cells and histamine release (Hennino et al., 2006; Jain, 2014). Although the

Abbreviations: COX-2, cyclooxygenase-2; CX, phosgene oxime (dichloroformoxime); MPO, myeloperoxidase; TUNEL, Terminal deoxynucleotidyl transferase (TdT) dUTP NickEnd Labeling; TNFα, Tumor necrosis factor α; MAPKs, Mitogen-activated protein kinases.

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mechanism of action of CX is not known, it has been suggested that CX likely possesses alkylating and nucleophilic properties resembling mustard vesicants, and therefore its effect could be both direct involving corrosive injury, cell death and tissue destruction, and indirect involving inflammatory cells such as mast cells and neutrophils causing delayed tissue injury (Augerson, 2000).

Since CX is known to cause immediate injury, the goal of our study was to examine its acute effects 30 min–8 h post its cutaneous exposure in SKH-1 hairless mice. Results from this study will aid in designing further comprehensive studies to evaluate the acute and chronic injuries from CX topical exposure and elucidate the related mechanism of action. Due to the ability of CX to produce a rapid onset of severe immediate as well as prolonged effects, this study is highly momentous to aid in the identification of therapeutic targets and development of effective therapies against CX cutaneous exposure-induced morbidity and mortality.

2. Materials and methods

2.1. Chemicals and reagents

Hematoxylin, eosin and toluidine blue stains were purchased from Sigma–Aldrich Chemicals Co. (St. Louis, MO). The DeadEnd Colorimetric terminal deoxynucleotidyl transferase (tdt)-mediated dUTP-biotin nick end labeling (TUNEL) staining kit was from Promega (Madison, WI). The detergent compatible (DC) protein assay kit was purchased from Bio-Rad Laboratories (Hercules, CA). Primary antibodies for phosphorylated p53 (Ser15) and total p53 were obtained from Cell Signaling Technology (Beverly, MA). Anti-cyclooxygenase-2 (COX-2) monoclonal antibody was from Cayman Chemical (Ann Arbor, MI). Anti-myeloperoxidase (MPO) and anti-Tumor necrosis factor α (TNFα) antibodies were purchased from Abcam Inc. (Cambridge, MA) and Santa Cruz Biotech (Santa Cruz, CA), respectively. Mouse anti-beta actin monoclonal antibody was purchased from Thermo Fisher Scientific. Goat anti-mouse IgG and anti-rabbit IgG secondary antibody labeled with either IRDye 800CW or IRDye 680LT were obtained from LI-COR Biosciences (Lincoln, NE).

2.2. Dermal exposure to CX

Male hairless SKH-1 mice (4–6 weeks; n = 4) were obtained from Charles River Laboratories (Wilmington, MA) and housed under standard conditions at MRIGlobal. The mice were anesthetized before exposure using ketamine and xylazine (IP) and pain was managed using 0.05–0.1 mg/kg subcutaneous (SQ) injection of buprenorphine 30 min before CX exposure. The mice were exposed to vapor from 10 μl neat CX (synthesized at MRIGlobal) for 4 min using a vapor cap (two 12 mm vapor cap exposures at each side of dorsal skin of mice) at MRIGlobal. The synthesis of CX and all exposures were conducted in compliance with MRIGlobal standard operating procedures (SOPs) and approved Institutional Animal Care and Use Committee (IACUC) as well as safety procedures. Animals were sacrificed 8 h post-CX exposure, and the skin punches and other organs including liver, kidneys, spleen, lungs and heart were harvested and portions of each tissue were either snap frozen or fixed in 10% formalin.

2.3. Clinical observations, measurement of skin bi-fold thickness, edema and necrosis

The clinical observations and pictures including the changes in the CX-exposed skin areas were recorded at pre-CX exposure and at 0.5, 2, 4 and 8 h post-CX exposure. Measurement (in triplicate) of skin bi-fold thickness was carried out using a digital caliper (Tewari-Singh et al., 2009). Dose site observations were recorded for erythema, edema and necrosis using a modified Draize scoring system (Table 1). The death time of any animals, post-CX exposure was also recorded.

Table 1
Draize scoring for erythema, edema and necrosis.

Erythema	Edema	Necrosis
0 = Unchanged from control tissue area 1 = Mild erythema 2 = Moderate erythema 3 = Severe erythema	0 = Unchanged from control tissue area 1 = Mild edema 2 = Moderate edema 3 = Severe edema	0 = Unchanged from control tissue area 1 = Focal Necrosis: focal area(s) of the tissue is (are) necrotic 2 = Mild necrosis: 25–50% of the tissue is necrotic 3 = Moderate necrosis: 50–75% of the tissue is necrotic 4 = Severe necrosis: 75–100% of the tissue is necrotic

2.4. Histopathological evaluation and measurement of epidermal thickness and blood vessels

The formalin fixed skin tissues and organs were processed and embedded in paraffin, and 5 μm sections were cut using a Leica microtome, stained with hematoxylin and eosin (H&E) and evaluated microscopically (Zeiss Axioscop2 microscope, Carl Zeiss, Inc., Germany) as reported earlier (Tewari-Singh et al., 2009; Jain et al., 2014a; Tewari-Singh et al., 2014). The total skin thickness and epidermal thickness was evaluated as reported earlier (Tewari-Singh et al., 2009; Jain et al., 2014a), and the blood vessels were counted in whole length of the skin section (at 400× magnification).

2.5. Measurement of apoptotic cell death

Terminal deoxynucleotidyl transferase (TdT) dUTP NickEnd Labeling (TUNEL) staining was carried out to detect apoptotic cell death in the skin epithelial layer as described previously (Tewari-Singh et al., 2009; Jain et al., 2011, 2014a). Briefly, the brown colored TUNEL positive cells were quantified in 10 randomly selected fields under 400× magnification, and an apoptotic cell index was calculated.

2.6. Staining for mast cells

Toluidine blue staining was carried out to detect mast cells in unexposed (control) and CX exposed skin tissue samples employing a previously reported protocol (Tewari-Singh et al., 2009; Jain et al., 2011, 2014a). Briefly, the skin sections were incubated with toluidine blue working solution (5 ml toluidine blue + 1% sodium chloride) for 2–3 min. The slides were then washed, dehydrated, cleared in xylene and mounted for microscopic evaluation. Mast cells were quantified in 5 randomly selected fields (400× magnification).

2.7. Western blot analyses

Lysates were prepared from control and CX-exposed skin tissue samples and the protein concentration was estimated using Bio-Rad DC protein assay kit. Western blot analyses for phosphor and total p53, COX-2, TNFα and MPO were performed as reported earlier (Tewari-Singh et al., 2010; Kumar et al., 2015). The membrane was re-probed with anti-β-actin antibody for loading control. The bands of the representative blots shown here were scanned using adobe photoshop 6.0 (adobe Systems, Inc., San Jose, CA) and quantified via densitometric analysis of bands using the Image J Program (NIH, Bethesda, MD).

2.8. Statistical analyses

Statistically significant differences between groups were determined by one-way ANOVA using SigmaStat 3.5 software (Jandel