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Therapeutic Effects of Cooling Swine Skin Exposed to Sulfur Mustard

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Recent world events have highlighted the need for effective medical therapies for chemical weapon injuries. Of the chemical weapon agents, perhaps one of the most widely used, both historically and most recently in the Iran-Iraq War, is sulfur mustard (HD). No effective antidotes exist for this vesicant agent and, to this day, HD casualties are treated entirely symptomatically. Previous work carried out in this laboratory has indicated that cooling HD-exposed tissue may ameliorate the resultant injury. To further examine this, an anesthetized domestic swine model was used to investigate whether alteration of skin temperature had any effect either visually or histopathologically on the development and progression of HD-induced skin lesions over 7 days. Exposure of swine skin to HD vapor resulted in lesions whose severity was exposure time related (4, 8, 12, and 16 minutes). Postdecontamination heating of skin above ambient temperature (~39°C) resulted in worsening of the lesion, whereas postdecontamination cooling (~15°C) for between 2 to 4 hours postexposure lessened the severity of HD-induced injury. The authors conclude that the early, noninvasive and simplistic act of cooling HD-exposed skin may have a salutary effect on the severity of HD-induced cutaneous lesions.

Introduction

The Iran-Iraq and Persian Gulf Wars, the recent terrorist attacks in Japan, and most recently the events of September 11, 2001 have focused attention on the very real possibility of chemical warfare (CW) agent use during either military[1–4] or terrorist[5–8] activity These events, in conjunction with the ongoing efforts by several nations to decommission existing CW stocks[9,10] and incidences of accidental exposure[11,12] highlight the need for effective medical treatment of weapons of mass destruction. The classical CW agents most likely to be used include the organophosphate nerve agents and sulfur mustard (bis-2-chloroethyl sulfide; HD). Whereas effective medical countermeasures and antidotes exist for the treatment of organophosphate nerve agent exposure, this is not the case with the vesicating agent, HD. Although a great deal has been discovered about this strong alkylating agent, how it exerts its vesicant activity has eluded researchers and definitive therapeutic measures against its effects are not available. Thus, medical treatment of HD-induced "burns" is largely symptomatic and consists primarily of measures designed to control the pruritus, discomfort, and pain caused by these lesions as well as in preventing subsequent infection.[13–16]

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Previous work in this laboratory[17–19] has shown that a class of drugs related to L-arginine confers significant concentration-related protection against the toxicity of HD in cell culture. The protective effects were therapeutic in nature, because administration of these drugs up to several hours after HD exposure resulted in protection that was quantitatively similar to that obtained with pretreatment regimens. Furthermore, the therapeutic window for these drugs was dramatically increased by lowering the temperature of the cultures after HD exposure.[20] Preliminary work during these same studies also indicated that the cooling of HD vapor-exposed skin in hairless guinea pigs was effective in reducing the severity of the resultant lesions. In this report, we detail initial results of investigations into the effect of temperature on HD-induced skin lesions in domestic swine considered by some to be a superior animal model for studying the pathophysiology of HD-induced cutaneous injury.[21]

Methods

Animals

Castrated male York-Landrace cross pigs (~20 kg) were purchased from a single local supplier and housed indoors in the Defense Research and Development-Suffield vivarium. The pigs were allowed to acclimatize prior to experimental use. In conducting this research the authors adhered to the *Guide to the Care and Use of Experimental Animals* and *The Ethics of Animal Experimentation published by the Canadian Council on Animal Care.*

Anesthesia

Animals were fed until the evening before surgery and allowed tap water ad libitum until the time of the experiment. The animals were not premedicated. General anesthesia was induced by mask with isoflurane (5%) in oxygen. Postinduction, the animals were transferred to a heated operating table, placed in the supine position, and intubated orally. Anesthesia was maintained with isoflurane (2.0%) in oxygen. The time from exposure to HD to eduction from general anesthesia was constant in all animals (360 minutes). Concomitantly with the anesthetic, all animals received IV normal saline (sodium chloride 0.9%, Abbott Laboratories Ltd., Montreal, Quebec, Canada) at a rate of 9.02 mL kg^{-1} h^{-1} ± 0.75 (mean ± SD, $N = 21$) via a volumetric infusion pump (Travenol FloGard 8000, Travenol Laboratories, Deerfield, Illinois).

HD Treatment

After the animals achieved steady-state anesthesia, HD vapor exposures were carried out. All test groups consisted of at least three animals each. Distilled HD was prepared to more than

98% purity by the Chemical Biological Defense Section, Defense Research and Development-Suffield. Mirror-image target sites were delineated on either side of the epigastric midline using felt markers, and the exposures were carried out for 4, 8, 12, and 16 minutes using vapor cups (1 5-cm diameter, Fig. 1). At the end of the exposure, the vapor cups were removed, and the sites were decontaminated using the Canadian reactive skin decontamination lotion. Complete decontamination was confirmed by sampling the air over the exposure sites using an AP2C chemical agent monitor. After decontamination was confirmed, one (control) side was left exposed to ambient room temperature (~22°C), whereas the other (test) side was cooled to ~15°C to 20°C for various time periods (2–6 hours) using ice packs filled with crushed ice. The application of ice was delayed from the usual 4 minutes to 30 minutes in one group of animals subsequently cooled for 330 minutes In one group of animals, the test sites were slightly warmed to ~39°C for 4 hours. Thermocouples placed under the packs were used to assess skin temperature. Animals were allowed to emerge from anesthesia in their pens. The lesions resulting from exposure to HD were assessed visually and photographically at 7 hours postexposure and then daily for 7 days. At the end of the experimental period, the animals were humanely sacrificed with 10 mL of IV sodium pentobarbital (540 mg/mL, Bimeda-MTC, Cambridge, Ontario, Canada), and full-thickness skin samples were excised for histology.

Histology

Excised skin samples were immediately placed in 10% neutral-buffered Formalin and fixed for at least 24 hours before processing. The samples were then trimmed into tissue cassettes, processed into paraffin blocks, sectioned, mounted on glass slides, and stained with hematoxylin and eosin using standard techniques. Slides were examined by a board-certified pathologist who was blinded as to the treatment that had been administered to each group of samples. Observations were made of each anatomic area of the skin sections and recorded on a histology laboratory worksheet. A scoring system was devised based on the lesions observed, and the lesions were graded according to their nature and severity. Upon completion of the histopathological assessment, the treatment code was revealed, and the results were organized according to treatment group.

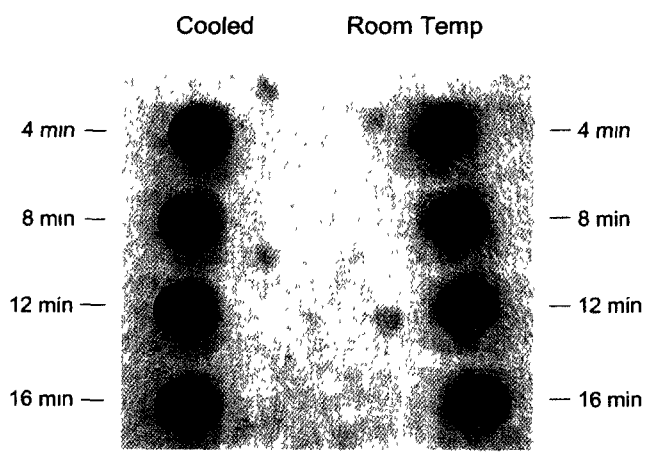

Fig 1 Topical HD vapor exposure of anesthetized swine Anesthetized swine were placed in the dorsal recumbent position on a heated operating table, and mirror-image target sites were delineated on either side of the epigastric midline using a felt marker Exposures were carried out for 4, 8, 12, and 16 minutes using vapor cups (1.5-cm diameter). At the end of the exposure, the vapor cups were removed and the sites were decontaminated One side was left at room temperature and the other side was used to investigate the effects of skin surface temperature on the HD-induced lesions

Results

Visual Assessment

Twelve- and 16-min HD vapor exposures of swine epigastrum produced erythema by 3 hours postexposure. By 6 hours, HD at all exposure times (4, 8, 12, and 16 minutes) produced erythema whose intensity was exposure time related. At 24 hours, erythema to the 4-minute exposure sites was somewhat resolved, whereas the 8-, 12-, and 16-minutes exposure sites became more precisely demarcated and had darkened in color intensity. The apparent severity of the lesion was clearly exposure time related, although this relationship varied considerably from animal to animal, with some animals displaying obvious scab-like lesions at the 12- and 16-minute exposure sites. The severity of the skin lesions appeared to become maximal at 48 to 72 hours post-HD exposure and thereafter appeared to begin to heal

Initial experiments examining the effects of cooling HD-exposed tissue used commercially available freezer packs that were frozen before use, wrapped in damp gauze, and then placed over the exposure sites. During these conditions, skin temperatures were frequently recorded between 5°C and 10°C, and no salutary effects of cooling were detected. Indeed, more often then not, an apparent increase in the severity of the lesions was obvious. This approach was therefore abandoned, and an alternative cooling regimen using ziplock bags filled with crushed ice was adopted. Damp gauze was placed over the exposure sites and the ice-filled bags were secured on top of the gauze. Using this methodology, skin temperatures were maintained between 13°C and 18°C, and the cooling of HD-exposed tissue was clearly effective in reducing the severity of the resultant lesions. Cooling for 2 and 4 hours appeared to be equally and maximally protective (Fig. 2), whereas 6 hours of cooling appeared to be less effective than shorter cooling times. A 30-minute delay prior to 5.5 hours of cooling reduced the severity of HD-induced lesions but was less effective than 2 to 6 hours of immediate cooling. Slight warming of HD exposure sites to ~39°C appeared to increase the severity of skin injury.

In an effort to more objectively assess the effects of skin temperature on HD-induced lesions, different end points such as erythema, pallor, induration, circumscription, exudation, bleeding, and scabbing were considered as equally weighted factors and given a score of between one and three. Depending on the time after exposure, these factors were summed. For example, during the first 24 hours, where the only signs were erythema and circumscription, these scores were summed. At 7 days, when erythema was not apparent but when the lesions became more serious, a different set of end points describing whether the lesions were circumscribed, indurated, scabbed, or undergoing epidermiolysis were summed. Table I shows that when using this type of scoring scheme, significant protective effects were obtained at 24 hours after a 12-minute HD exposure with 2 hours of cooling and after 12- and 16-minute expo-

TABLE I

VISUAL ASSESSMENT OF THE EFFECTS OF TEMPERATURE ON HD VAPOR-INDUCED SKIN LESIONS AT 24 HOURS POSTEXPOSURE

	HD Vapor Exposure Time			
	4 Minutes	8 Minutes	12 Minutes	16 Minutes
Control	0 25 ± 0 50	1 75 ± 1 04	3 25 ± 0 50	4 00 ± 0 82
Cool 2 hours	0 00 ± 0 00	0 13 ± 0 25	1 88 ± 1 03*	3 50 ± 0 58
Control	1 0 ± 1 00	2 17 ± 0 76	3 00 ± 1 00	4.67 ± 0 58
Cool 4 hours	0 00 ± 0 00	0 83 ± 0 76	1 50 ± 1 50*	3.67 ± 0 58*
Control	0 60 ± 0 82	2 0 ± 0 61	3 80 ± 0 84	4 80 ± 0 84
Cool 6 hours	0 20 ± 0 45	1 10 ± 0 74	2.70 ± 1.48	3 80 ± 0.84
Control	0 0 ± 0 00	1 17 ± 0 76	2 67 ± 1 15	4 67 ± 0 58
30-minute cooling delay/4 hours	0 00 ± 0 00	1 33 ± 1 53	2 17 ± 1 61	3 67 ± 1 15
Control	0 83 ± 1 04	1 67 ± 2 08	4 33 ± 1 53	5 00 ± 1 73
Warm 6 hours	1 33 ± 2 31	2 50 ± 3 04	3 17 ± 2 75	4 67 ± 1 15

At 24 hours, erythema and lesion circumscription were the only evident signs of HD-induced injury The lesions were scored on a scale of 1 to 3, and the score for each exposure site was summed Each pig served as its own control, and therefore a paired t test was used for statistical analysis Asterisks denote significant differences in lesion severity from room temperature control lesions ($p < 0.5$) Data represent the mean ± SD of at least three experiments

sures with 4 hours of cooling. Although a variety of different scoring regimens were used in an attempt to obtain scores objectively reflective of tissue injury (and reflective of the obvious protective effects of cooling), when the scores were subjected to statistical analysis, no consistent statistically significant difference could be obtained showing that skin temperature had an effect on HD-induced skin injury. However, scoring regimens such as the one used for the data in Table I did show similar trends to that detected without scoring, namely, that (a) the longer the HD exposure, the worse the resultant injury; (b) cooling HD-exposed skin improves the prognosis of the resultant injury, with 2 and 4 hours of cooling being optimal; and (c) warming HD-exposed skin increases the severity of the lesions, especially at longer time points.

Histopathological Assessment

There was considerable variation noted in the severity of the lesions within each treatment group. In general, the earliest observable effect of HD was reactive change in the cells of the basal layer of the epidermis. However, in most specimens examined, these early effects were overwhelmed by necrosis, erosion, ulceration, and suppurative inflammation of the epidermis. In some cases, ulceration extended into the dermis. HD exposure followed by room temperature resulted in considerable intersample variation in the presence or absence of ulceration at 4 and 8 minutes of exposure. With 12 minutes of exposure, there was ulceration in all samples, and with 16 minutes of exposure, there was ulceration in the majority of samples. In contrast, when HD exposure was followed by cooling for either 2 or 4 hours, there was amelioration of the ulcerative and inflammatory effects in the 4-, 8-, and mostly 12-minute exposure samples. Postexposure cooling had minimal effect in reducing either ulceration or inflammation in the 16-minute exposure samples.

When HD exposure was followed either by room temperature or by cooling for 6 hours, the results were qualitatively similar but not as quantitatively differentiated. The majority of the 4-minute HD-exposed samples were normal, whereas after the 8-minute exposure there was wide variability ranging from normal to ulceration. Inflammatory reaction was evident in the superficial dermis only in most cases. Following exposures of 12 and 16 minutes, all samples showed some reaction, most with ulceration. The majority of the samples demonstrated an inflammatory reaction in both the superficial and deep dermis. In contrast, when HD exposure was followed by a 6-hour period of warming, there was an increase in the number of samples that demonstrated reactive or ulcerative changes into the 4- and 8-minute exposure times. When there was a 5.5-hour postexposure cooling period that was delayed for 30 minutes, the results within each exposure group exhibited more variation

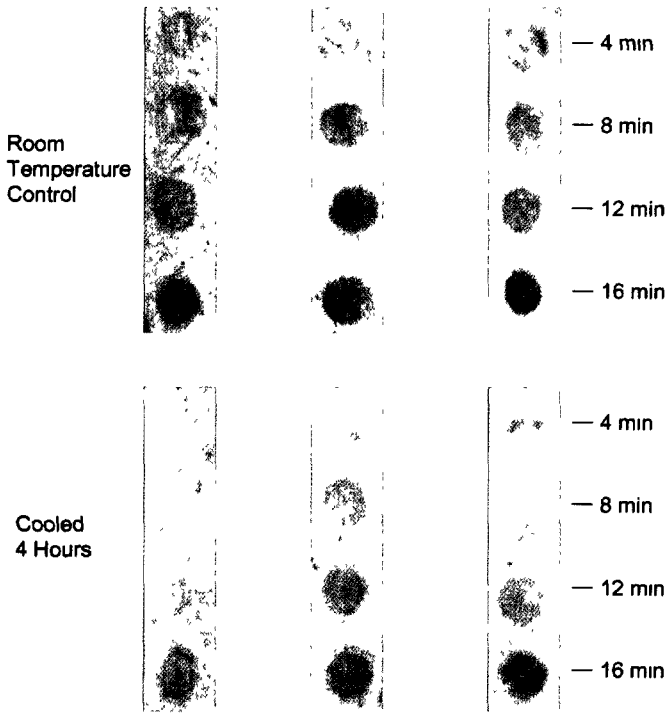

Fig 2 Effect of cooling HD vapor-exposed swine skin HD vapor exposures were carried out on either side of the epigastric midline for 4, 8, 12, or 16 minutes before vapor cup removal and decontamination Subsequently, one side was cooled for 4 hours (−15°C–20°C), whereas the other was left at room temperature The animals were allowed to come out of the anesthetic, and the resultant lesions were assessed daily The figure depicts the appearance of HD-induced lesions at 48 hours on three different animals Note the consistent but variable protective effects of cooling

than when cooling was started 4 minutes postdecontamination. In summary, histopathological assessment of the samples indicated that (a) the longer the HD exposure, the worse the resultant injury; (b) immediate cooling of HD-exposed skin improves the prognosis of the resultant injury, with 2 and 4 hours of cooling being optimal; and (c) warming HD-exposed skin increases the magnitude of the lesions, especially at longer time points.

Discussion

Recent world events have highlighted the need for effective medical countermeasures against weapons of mass destruction, including CW agents. Of these agents, two major categories exist that are widely thought to be the most likely used, based on past experience. These include the organophosphate nerve agents, where victims of exposure can be treated effectively by a variety of drug regimens, and the vesicant agents, where treatment modalities are much less effective or nonexistent. Historically, the most commonly used vesicant or blister-causing agent is HD. No antidotes exist for this agent, and treatment is exclusively symptomatic in nature

HD has been the object of exhaustive research since its first battlefield use at Ypres by the Germans in 1917. Nevertheless, its mechanism of toxic action is unknown, a fact that is largely responsible for the absence of effective countermeasures against this agent. Studies in this laboratory have recently demonstrated that moderate cooling of HD-exposed cells in culture significantly reduced the resultant toxicity. Furthermore, although this protection was not persistent in vitro, cooling of HD vapor-exposed hairless guinea pig skin was, unexpectedly, both dramatic and persistent.[20] We elected to pursue these findings in the domestic pig, an animal model that has remarkable anatomic and physiological similarity to human beings with respect to skin.[22,23] Although ethical and humane treatment of these animals dictates administration of a general anesthesia, published research indicates that skin blood flow values at the levels of isoflurane anesthesia that we use (~1.0 MAC), are not significantly different from awake values.[24]

Longer exposure times were required to obtain a "dose-dependent" range of HD-induced skin injury in domestic swine (4, 8, 12, and 16 minutes) as compared with hairless guinea pigs (2, 4, 6, and 8 minutes).[20] Nevertheless, the results of the present study confirmed and extended our preliminary findings with hairless guinea pigs with respect to the protective effects of cooling HD-exposed skin. Although attempts were made to assess skin injury by a variety of means, including visual assessment, statistical treatment of Draize-like[25] scoring schemes, and histopathological assessment, the variable skin response to HD vapor prevented a quantitative assessment of the effect of temperature on HD-exposed skin. However, definite trends were identified using all methodologies. These included (a) the longer the HD vapor exposure, the more severe the skin injury; (b) cooling of exposed tissue ameliorates the resultant injury whether applied immediately after HD exposure (2, 4, and 6 hours of cooling) or delayed (5.5 hours of cooling 30 minutes post-HD exposure); and (c) slight warming of HD-exposed skin exacerbates the resultant lesions.

It is interesting to speculate as to how temperature modulates the severity of HD-induced lesions. Tissue culture studies would appear to indicate that cooling only retards the metabolic processes that ultimately lead to toxicity. Thus, cooling would be expected to slow the development of toxicity and not prevent it, serving only to extend the therapeutic window of opportunity for protective drug regimens. In vitro, these drug regimens exist.[17-19] However, this is not the case in vivo, and the protection conferred by cooling HD vapor-exposed skin in hairless guinea pigs and domestic swine was unexpected, based on the initial in vitro studies. Thus, one is led to the conclusion that cooling HD-exposed skin enables temperature insensitive repair mechanisms to take place, or that cooling results in a greater dilution of HD at the level of the basement membrane, as it more slowly enters the systemic circulation without concomitant skin injury. The former option seems more likely.

In summary, we have shown that the early application of moderate cooling to decontaminated HD-induced cutaneous lesions lessens the severity of the inflammatory and ulcerative responses. Thus, cooling therapy is remarkably similar to that advocated for thermal injuries. This technique should serve as a useful and simple adjunct to the treatments currently proposed for the medical management of HD-induced lesions, including laser debridement and dermabrasion,[26-29] or as a stand-alone treatment. Use of this technique may reduce the requirement for further intervention, which may have associated risks

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