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Therapeutic effects of hypothermia on Lewisite toxicity

Peggy Nelson, James R. Hancock, Thomas W. Sawyer *

Medical Therapy Group, Chemical Biological Defence Section, Defence Research and Development Canada-Suffield, Box 4000, Medicine Hat, Alta., Canada T1A 8K6

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Abstract

The cytotoxicity of the arsenical vesicant Lewisite was assessed in first passage cultures of proliferating neonatal human skin keratinocytes. Both munitions grade and distilled Lewisite were extremely toxic with LC_{50} values in the low ng/ml range, with no significant differences between them. This similarity in toxicity was also mirrored with respect to their toxic effects on hairless guinea pig skin. Two-, 4- and 6-min vapour exposures of these agents resulted in similar and severe skin injury that was obvious by 3–5 h post-exposure and almost maximal at 24 h. The toxicity of Lewisite in culture was temperature dependent, with a >10-fold reduction in 24 h LC_{50} values as the incubation temperature was reduced from 37 to 25 °C. However, this cooling induced protection was not persistent. In contrast, cooling of Lewisite exposed hairless guinea pig skin at ~10 °C for as little as 30 min post-exposure resulted in dramatic and permanent protection, with 4 h of cooling almost completely eliminating Lewisite induced skin injury. Further, significant protection was also evident even when cooling was delayed for as long as 2 h post-Lewisite exposure. In an effort to investigate whether cooling might also increase the window in which chelation therapy against this vesicant agent would be useful, we examined the protective effects of the heavy metal chelator dimercaptosuccinic acid (DMSA). Topical application to Lewisite exposed skin was extremely protective, even when delayed for 2 h after Lewisite. Cooling of Lewisite exposed skin for 2 h, followed by DMSA topical application resulted in decreased skin injury compared to either treatment in isolation. It appears that the simple and non-invasive application of cooling measures may provide not only significant therapeutic relief to Lewisite exposed skin, but that it may also increase the therapeutic window in which medical countermeasures against this vesicant agent are useful.

Keywords: Chemical warfare (CW) agent; Dimercaptosuccinic acid (DMSA); Hairless guinea pig; Human skin keratinocytes; Hypothermia; Lewisite (dichloro (2-chlorovinyl) arsine); Therapy

1. Introduction

Lewisite (dichloro (2-chlorovinyl) arsine) is an arsenical vesicant chemical warfare (CW) agent that has been weaponized and stockpiled in several countries (National Academy Press, 1993). Although it is highly toxic both systemically and cutaneously, its primary role appears to be as a mixture component with sulphur mustard (HD), ostensibly to lower the freezing point for use in cold weather military operations. Both agents are highly vesicant in nature. However, the skin lesions produced by Lewisite differ from those of HD in their pathology and development. Topical Lewisite exposure is accompanied by immediate pain, compared to the delayed symptoms caused by HD, and while the blisters produced by Lewisite tend to be much more severe than those produced by HD, they heal faster (Goldman and Dacre, 1989). Thus, use of the mustard/Lewisite mixture would undoubtedly produce immediate casualties

* Corresponding author. Tel.: +1 403 544 4708/5365; fax: +1 403 544 3388.
E-mail address: Thomas.Sawyer@drdc-rddc.gc.ca (T.W. Sawyer).

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with painful and debilitating injuries that would only heal slowly.

In contrast to HD, antidotes to Lewisite do exist. In studies carried out during and prior to World War II, it was identified that thiol groups were attacked by arsenic and that these groups were constituents of the pyruvate–oxidase system (Peters, 1947). Although today it is not clear that perturbation of this particular pathway is responsible for Lewisite's toxicity, the ensuing studies resulted in the successful development of dimercaprol, more commonly known as British Anti-Lewisite, or BAL (Peters, 1947). However, the use of BAL is not without drawbacks. It has limited solubility in water, is relatively toxic (Klaassen, 1980; Oehme, 1972) and the intramuscular injection of BAL dissolved in peanut oil is painful. More recent studies have examined the efficacy of more water soluble and less toxic chelators (Aposhian, 1982; Aposhian et al., 1982, 1984; Inns and Rice, 1993; Inns et al., 1990).

Work in these laboratories has identified that cooling HD exposed cells decreases cytotoxicity and also effectively reduces the severity of HD induced skin lesions (Mi et al., 2003; Nelson et al., 2002; Sawyer and Risk, 1999). In this report, we investigate the protective efficacy of cooling Lewisite exposed cells and tissue, as well as its possible role in increasing the therapeutic window of the heavy metal chelator dimercaptosuccinic acid (DMSA).

2. Methods

2.1. Lewisite purity and analysis

Distilled Lewisite (DL) was distilled on-site from munitions grade Lewisite (ML) of undetermined origins. Samples of both DL and ML were prepared as 0.1 mg/ml solutions in dichloromethane. The samples were analysed by GC–MS (Agilent 5973N under EI conditions: 70 eV, 0.035 mA, 230 °C) using a 15 m × 0.25 mm i.d. J&W DB-35MS capillary column and the following temperature programme: 40 °C (2 min), 10 °C/min 280 °C (5 min). All injections (1 μl) were cool on-column at 43 °C. The mass spectrometer was scanned from 40 to 400 Da at 2.08 scans/s.

2.2. Human skin keratinocyte culture

Primary cultures of human skin keratinocytes were prepared from neonatal foreskins. Tissue was obtained on the day of circumcision and incubated at 4 °C for 24 h in 25 U/ml dispase (Collaborative Research, Bedford, MA). The epidermis was removed and the cells segregated by a further 5 min incubation in 0.25% trypsin at 37 °C. The trypsin was deactivated by the addition of 20% serum in medium and the cell suspension was centrifuged. The cell pellet was resuspended in Keratinocyte Serum Free Medium (KSFM, Gibco BRL, Grand Island, NY) and filtered through 70 μM nylon mesh. Seventy-five square centimetre flasks were seeded at 5×10^5 cells/8 ml KSFM supplemented with gentamicin (5 μg/ml) and Fungizone (0.25 μg/ml) and incubated in a 37 °C humidified incubator in a 5% CO_2/95% air atmosphere. Cultures were re-fed every 2–4 days, as required. Only first passage cultures were used for experimental purposes; these were seeded from log growth primary cultures at a density of 1000 cells/well in 96-well Costar multiwell plates.

2.3. Chemical treatment and cytotoxicity studies

On the day of chemical treatment, actively proliferating cultures (3–4 days in vitro, ~30–40% confluent) were treated with freshly prepared treatment medium so that the desired final Lewisite concentration was reached at 0.25% ethanol (v/v). The viability of Lewisite-exposed cultures was determined at various time points after exposure using alamarBlue (AccuMed International Inc., Westlake, OH). At the time of assay, alamarBlue was added (10%, v/v) and the cultures were allowed to incubate with the dye for the last 2–3 h of the treatment time period. The absorbances (570–600 nm) were then read on a Thermomax titerplate reader (Molecular Devices, Sunnyvale, CA). Median lethal concentration (LC_{50}) values were determined graphically from experiments utilizing six wells per data point. All experiments were performed at least three times using cells from different donors each time. Studies investigating the effects of temperature on Lewisite toxicity were carried out in humidified CO_2 incubators set at 25, 31 or 37 °C.

2.4. Animal studies

Male hairless guinea pigs (strain Crl: IAF(HA)-hrBR) were acquired from Charles River Laboratories (St. Constant, Que., Canada). The animals were acclimated for at least 1 week 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. On the day of use, the animals were restrained and placed under ketamine anaesthesia. Three target sites approximately 2 cm apart were selected along the dorsal midline and exposed to Lewisite vapour for 2, 4 or 6 min (head to tail, ~13 mm diameter vapour cup). This model system was adapted from that of Mershon et al. (1990), who showed that 2–8 min HD vapour exposures (using slightly larger vapour cups) in this animal model consistently produced microblistering of the epidermis. After exposure, animals designated as room temperature were left to off-gas in the fume hood. Animals designated as low temperature were allowed to off-gas for 2 min and then the exposure sites were covered with a refrigerated cool pack for 5 min to cool the entire dorsal region. The cool packs were replaced with 3 in. lengths of dialysis tubing (1.6 cm diameter) which covered the exposure sites for varying time periods. The dialysis tubing was inflated with

cooled water recirculated from a refrigerated water bath using 1/8 in. tubing run through a peristaltic pump. Surface skin temperature was monitored using thermocouples attached to an Omega 412B-T sensor (Omega Engineering, Inc., Stamford, CT). Experiments assessing the efficacy of chelation therapy against Lewisite induced lesions used DMSA dissolved in dimethylsulphoxide (DMSO, 50 mg/ml). Each Lewisite exposure site was treated topically with 50 μl of DMSA solution or DMSO vehicle, either 2 min or 2 h after treatment. After 1 h, residual DMSA/DMSO was gently rubbed off with tissue paper and the treatment sites washed under warm running water. The animals were photographed at 5 h post-Lewisite exposure and every day thereafter for the next 7 days.

2.5. Statistical analysis

Data was analysed using one way analysis of variance (ANOVA) followed by post hoc Tukey's HSD multiple comparison test using SAS JMP software (SAS Institute, Carey, NC).

3. Results

In these studies the definition of purity for the chromatographic analysis of the test agents is peak area of the analyte to the total area, and the total area is based on all detectable (volatile) compounds soluble in dichloromethane. Using these criteria, the make-up of distilled and munitions grade Lewisite was very similar; ML consisted of 84.3% dichloro (2-chlorovinyl) arsine, 4.6% bis (2-chlorovinyl) chloroarsine and 10.7% unknown, while the distilled product was comprised of 79.9% dichloro (2-chlorovinyl) arsine, 0.9% bis (2-chlorovinyl) chloroarsine and 17.9% unknown.

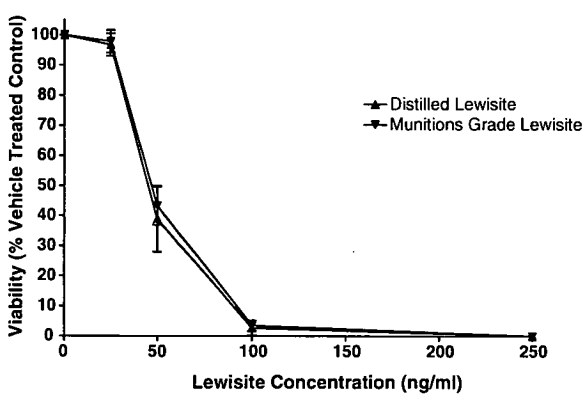

Fig. 1. Concentration response of Lewisite in human skin keratinocytes. Proliferating cultures of first passage neonatal human skin keratinocytes were treated with either distilled or munitions grade Lewisite and incubated at 37 °C. At 48 h, cell viability was assessed using alamarBlue. Data represents the mean ± standard deviation of three different experiments using tissue from three different donors.

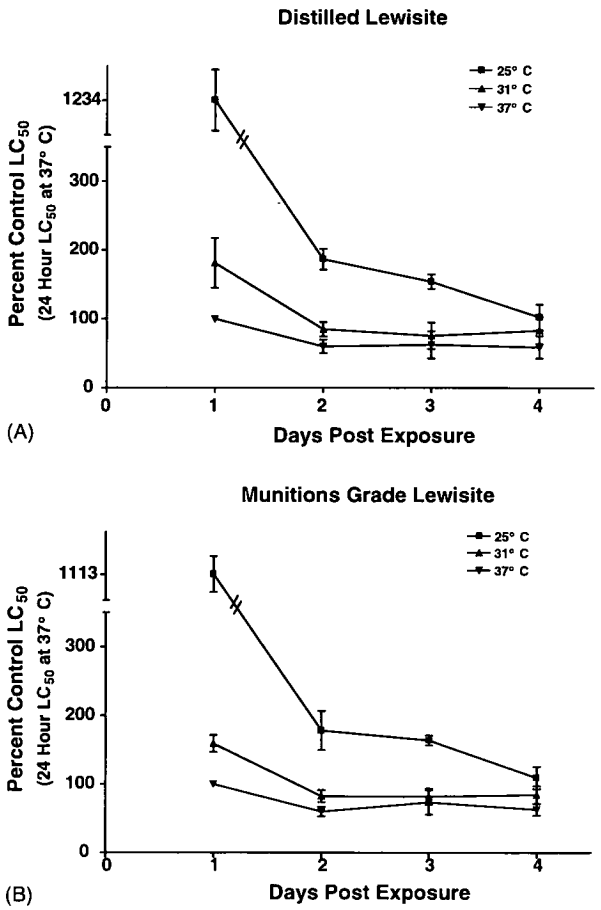

Fig. 2. Effect of incubation temperature on the development of Lewisite toxicity in human skin keratinocytes. Proliferating cultures of first passage neonatal human skin keratinocytes were treated with either distilled (A) or munitions grade (B) Lewisite and incubated at 25, 31 or 37 °C. At 24, 48, 72 and 96 h unique test cultures were assayed for viability using alamarBlue. Data is expressed as a percentage of the LC_{50} obtained at 24 h of exposure at 37 °C and represents the mean ± standard deviation of three different experiments using tissue from three different donors. With both agents, the 24 h LC_{50} values at 25 °C are significantly different from all other data points ($p < 0.05$, Tukey's HSD multiple comparison test).

Both ML and DL were extremely toxic in first passage cultures of proliferating neonatal human keratinocytes, with virtually identical concentration responses and 48 h LC_{50} values (Fig. 1; distilled: $LC_{50} = 45.9 \pm 4.8$ ng/ml; munitions grade: $LC_{50} = 45.9 \pm 3.2$ ng/ml; mean ± S.D., $n = 3$). The toxicity of both agents was found to be profoundly dependent on the incubation temperature during exposure (Fig. 2). At 37 °C, Lewisite toxicity was almost maximal by 24 h, and decreased only slightly during the next 3 days. At 31 °C, 24 h Lewisite toxicity was reduced (but not statistically significant) compared to that obtained at 37 °C. However, by 48 h this protective effect was lost and LC_{50} values were virtually identical

to those obtained at 37 °C. The protective effect of lowered temperature was dramatic when the treated cultures were incubated at 25 °C. The LC_{50} values obtained at 24 h were more than 10 times higher than those at 37 °C. These protective effects were very rapidly lost, however, and by 48 h post-treatment the LC_{50} values obtained for both test articles at 25, 31 or 37 °C were not statistically different.

Vapour exposure of hairless guinea pigs to ML and DL produced lesions that were of equal severity to each other at all test times assessed (Fig. 3). Two-, 4- and 6-min vapour exposures all produced similar gross pathologies, with the 4- and 6-min exposures producing blood scabbing that was approximately equal in severity, and having a larger surface area than the lesions produced by 2-min exposures. The development of the lesions was consistent. By 3–5 h post-exposure, erythema was clearly visible. The lesions progressed rapidly, so that blood scabs were already well formed by 24 h and maximally formed by 48–72 h. Healing and subsequent sloughing of the scabs occurred starting after 5–6 days. The effect of cooling Lewisite exposed skin was investigated using DL. Cooling of Lewisite exposed tissue for 0.5, 2 or 4 h resulted in lesions of significantly reduced severity (as assessed visually) and size in every case at all time frames post-exposure, with the lesions

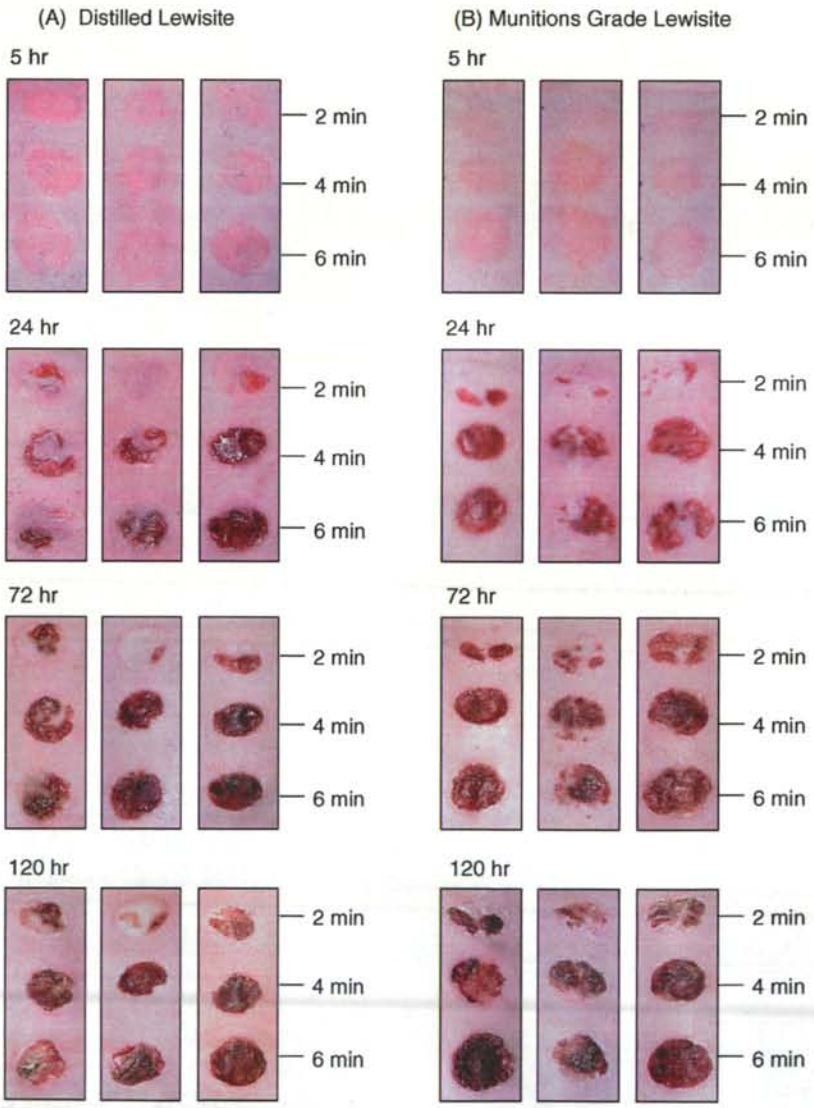

Fig. 3. Effect of topically applied Lewisite vapour on hairless guinea pigs. Animals were exposed to distilled (A) or munitions grade (B) Lewisite vapour for 2, 4 or 6 min. Three animals were used for each treatment and the Lewisite induced lesions were photographed at 5, 24, 72 and 120 h post-treatment.

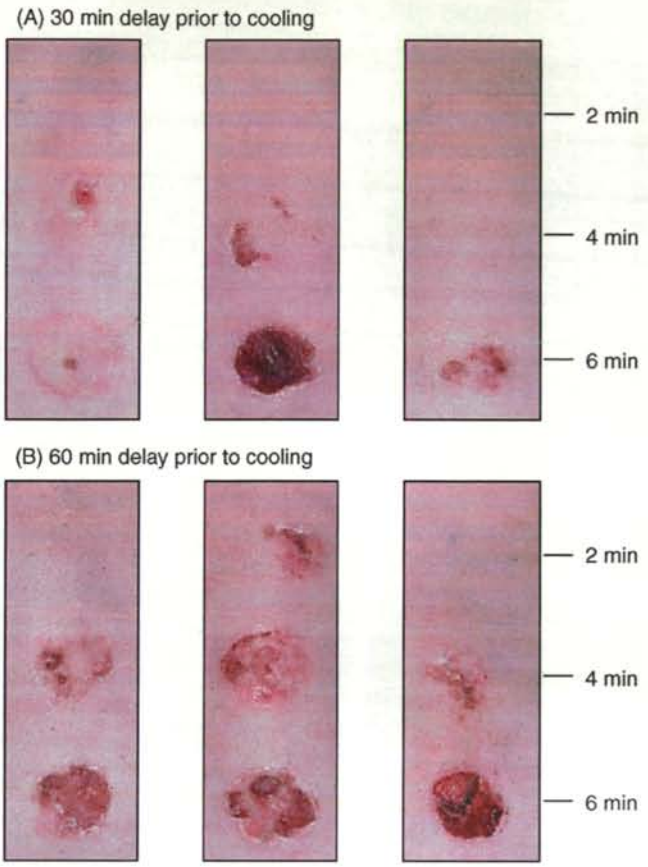

Fig. 5. Effect of delayed cooling on the development of Lewisite vapour induced lesions on hairless guinea pigs. Animals were exposed to DL vapour for 2, 4 or 6 min. At 30 min (A) or at 60 min post-treatment (B), the exposure sites were cooled for 210 and 180 min, respectively at ~10 °C. Three animals were used for each treatment and the photographs depict the appearance of the lesions at 5 days post-treatment.

were virtually identical with respect to their toxicity, with LC_{50} values (DL: 220 ± 23 nM; ML: 220 ± 15 nM) that were at least two orders of magnitude more toxic than that of HD in the same test system (39.0 ± 8.8 μM; Sawyer and Risk, 2000). These in vitro results were subsequently paralleled in studies that examined the effects of vapour exposure of both ML and DL to hairless guinea pigs. Although we initially sought to apply the modified Draize scoring system (Draize et al., 1944) that we had used in previous HD studies (Sawyer and Risk, 1999, 2000), this proved not to be feasible. In contrast to HD, where cooling uniformly reduced the severity of the lesion, but not its surface area, cooling of Lewisite induced lesions also reduced the total surface area of the lesion in an irregular fashion. Given the dramatic effects of our treatment regimens, we therefore judged a pictorial documentation of the results to be the most compelling record of our findings. Using this record, there was little doubt that 2-, 4- and 6-min vapour exposures produced lesions of increasing severity, with little difference between the skin injury produced by either agent. It appears that in our hands, simple distillation of the munitions grade Lewisite removes primarily non-volatile constituents having relatively little toxicity.

The dependence of drug action and toxicity on temperature is not a novel concept and a wealth of (mostly older) literature documents these effects (Cremer and Bligh, 1969; Doull, 1972; Fuhrman and Fuhrman, 1961; Weihe, 1973). Although this field does not appear to be as active in the present day, there is still current research that focuses on using hypothermia to increase the therapeutic indices of chemo- and radio-therapies against cancer (Lundgren-Eriksson et al., 1996, 2001), as well as work using hyperthermia as a method to improve the selective toxicity of chemotherapeutics, most especially the bifunctional alkylating agents such as melphalan (Averill and Larrivee, 1998; Takemoto et al., 2003; Urano and Ling, 2002). The effect of temperature on HD toxicity has also been previously investigated and as early as 1918 it was demonstrated that lowered temperature protected goldfish against the toxic effects of HD (Lynch et al., 1918). Additional work in the 1940s was inconclusive,

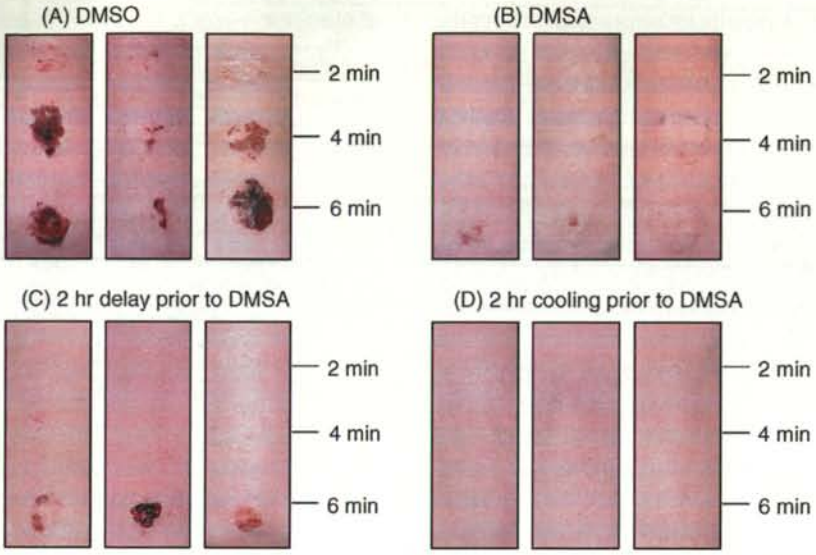

Fig. 6. Effect of DMSA and cooling on the development of Lewisite vapour induced lesions on hairless guinea pigs. Animals were exposed to DL vapour for 2, 4 or 6 min. At 2 min post-treatment, the exposure sites were treated topically with 50 μl of vehicle (DMSO) only (A) or DMSA (50 mg/ml, B) for 1 h. In additional treatment groups, the Lewisite exposure sites were left for 2 h at room temperature and then treated with DMSA (50 μl of 50 mg/ml, C) for 1 h, or cooled for 2 h at ~10 °C prior to DMSA treatment (D). Three animals were used for each treatment and the photographs depict the appearance of the lesions at 5 days post-treatment.

and reported that cooling was not a *consistently* effective means to reduce the lesions produced on human skin exposed to HD, although the experimental detail in these reports is very limited (Gates et al., 1946). Recent work has shown that cooling of HD exposed cells and tissue has definite and reproducible effects. While cooling of proliferating neonatal keratinocytes did not prevent HD toxicity, it significantly increased the therapeutic window in which drugs could be effective (Sawyer and Risk, 1999). In addition, permanent and significant protection against the skin lesions produced by HD vapour exposures was achieved by cooling exposed skin in hairless guinea pigs (Mi et al., 2003) and domestic swine (Nelson et al., 2002). Cooling of HD exposed fibroblasts was also shown to alter HD induced cell cycle arrest (Matijasevic et al., 1996, 1998). Consequently, we initiated work to look at the effects of temperature on Lewisite toxicity in proliferating neonatal human keratinocytes. The effects were dramatic and the reduction of incubation temperature from the usual 37 to 25 °C resulted in a log-fold increase in the 24 h LC_{50} values. However, in contrast to HD, where little protection was lost as long as the cultures were held at reduced temperature, by 48 h most of the protective effect of lowered temperature against Lewisite toxicity was eliminated.

The lesions produced by topical vapour exposure of Lewisite to hairless guinea pigs develop much faster and are much more severe than those produced by comparable times of exposure to HD. In light of the in vitro findings where lowered temperature only offered brief respite from Lewisite toxicity, it was therefore most surprising that cooling of Lewisite exposed guinea pig skin yielded dramatic and permanent protection, even with cooling intervals that were totally ineffective against HD. Cooling exposed sites for as little as 30 min (~10 °C) resulted in a significant decrease in both the size and severity of the resultant lesions, while 4 h of cooling all but eliminated injury. Perhaps even more surprising, considering the very rapid development of Lewisite induced skin injury, was the finding that cooling still conferred significant protection when delayed up to 2 h post-Lewisite exposure. Clearly, cooling Lewisite exposed skin has a profound effect on the resultant toxicity.

The final phase of our studies was intended to investigate whether cooling of Lewisite exposed skin would increase the therapeutic window for DMSA chelation therapy. When DMSA was applied to Lewisite exposed sites immediately after exposure, almost total and permanent protection was achieved. This protection did not decrease, even when treatment was delayed for 30 min at room temperature. Once again this was unexpected since Lewisite exposure is known to produce injury very quickly. Delaying DMSA treatment for 2 h still resulted in dramatic protection, but definitive skin injury was easily visible, allowing us to test whether combination therapy (cooling and DMSA) was beneficial. The results of this study were definitive; 2 h of cooling, followed by 1 h of DMSA completely eliminated all visible trace of

Lewisite skin injury—a significant improvement over the results obtained by either therapy alone.

The results of these studies shed new light on possible therapeutic intervention strategies against this extremely toxic CW agent. However, in the absence of a true understanding of Lewisite's mechanism of toxic action, and of radiolabel studies, they are difficult to explain. The in vitro results would appear to indicate that the delaying effects on toxicity conferred by cooling Lewisite exposed cultures are biochemical in nature. It is possible that cooling inhibits the Lewisite induced biochemical pathway(s) responsible for cell death, and/or that temperature insensitive repair pathways are still operable. However, the in vivo results also seem to indicate that these treatments may alter the distribution and movement of a Lewisite depot within the skin. Studies with HD have indicated that this lipophilic CW agent forms depots or reservoirs of live agent that are sequestered in the skin for up to 24 h after topical application (Chilcott et al., 2000). It is conceivable that cooling Lewisite exposed skin serves to immobilize this depot and minimize its biochemical/toxic activity within this organ, while it slowly diffuses into the systemic circulation; this would explain the enhanced protective effects against Lewisite obtained with combination cooling/DMSA treatments.

In conclusion, cooling of exposed cells and tissue is extremely effective in decreasing the toxicity of Lewisite. Although the reasons for this are not clear at this juncture, the results of this work indicate that the safe, simple, non-invasive, easily available and economical treatment of Lewisite casualties should be possible, at least for those with moderate cutaneous vapour exposure. In addition, cooling of Lewisite exposed tissue is also effective in increasing the therapeutic window in which chelation therapy may be utilized, not only from the standpoint of reducing skin injury, but perhaps also in reducing the potentially toxic systemic arsenic burdens posed by cutaneous exposures.

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