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A Cutaneous Full-thickness Liquid Sulfur Mustard Burn Model in Weanling Swine: Clinical Pathology and Urinary Excretion of Thiodiglycol†‡

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Key words: sulfur mustard; mass spectrometry; thiodiglycol; clinical pathology; animal model; weanling pigs.

Sulfur mustard (bis(2-chloroethyl)sulfide, HD) is a well-known blistering chemical warfare agent. We have developed a cutaneous full-thickness HD burn model in weanling pigs for efficacy testing of candidate treatment regimens. This report addresses clinical pathology findings and the urinary excretion profile of a major HD metabolite (thiodiglycol, TDG) in this model. Six female Yorkshire pigs were exposed to HD liquid on the ventral surface for 2 h, generating six 3-cm diameter full-thickness dermal lesions per pig. Blood samples were collected throughout a 7-day observation period for hematology and serum chemistry examinations. Urine was collected in metabolism cages. Routine urinalysis was performed and the urine analyzed for TDG using gas chromatography/mass spectrometry. Examination of clinical pathology parameters revealed subtle HD-related changes that are suggestive of a mild hemolytic episode. No other signs of clinically significant systemic toxicities were noted, including bone marrow suppression. Thiodiglycol was detected at the earliest time point tested (6–8 h post-exposure) at levels ranging from 0.66 to 4.98 μg ml^{-1} with a mean of 2.14 μg ml^{-1}. Thiodiglycol concentrations were the highest for half of the animals at this earliest time point and at 24–48 h for the others. By the evening of day 3, the mean level had reached 50 ng ml^{-1}. Mean levels remained 10–40 ng ml^{-1} for the remainder of the 7-day observation period, with the highest individual concentration noted during this period of 132 ng ml^{-1}. Our results are in general agreement with the TDG excretion profiles previously described for rodent models and humans. Urinary excretion of absorbed HD in our weanling pig wound healing model appears to follow the same pattern as is seen in other laboratory animals models. In general, urinary excretion of TDG appears to peak within the first 1–4 days following exposure, with detectable levels after 1 week. Relatively high urinary TDG levels may thus indicate agent exposure within the previous 96 h. Low levels significantly above natural background levels may indicate either exposure to low levels of agent or exposure that occurred more than 4 days prior to collection of the sample. Published in 2000 by John Wiley & Sons, Ltd.

INTRODUCTION

Sulfur mustard (bis(2-chloroethyl)sulfide, HD) is a well-known chemical warfare agent that was first used in World War I by Germany against French troops at Ypres, Belgium in 1917. Its use has been alleged in 11 conflicts since then, including the Iran–Iraq war in the 1980s. It continues to be a significant threat today to both military and civilian populations.[1,2]

Sulfur mustard is an incapacitating agent, causing cutaneous, pulmonary and ocular damage upon contact with liquid or high vapor concentrations. It has been shown to be carcinogenic and mutagenic in animal studies, and epidemiological studies of exposed soldiers and factory workers have implicated it in human cancers.[2] It is known to form a cyclic sulfonium ion, which alkylates nucleophilic cellular sites such as nitrogen bases on DNA strands. The molecular mechanisms of action, chemistry, toxicodynamics and genotoxicity of HD, as well as the pathogenesis and histopathology of HD injuries, have been widely described.[2–6] Following cutaneous exposure, the major target is the rapidly proliferating basal cell. A separation of the epidermis from the dermis occurs at the dermal–epidermal junction, which is dependent upon the loss of integrity of

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basal cells as well as anchoring filaments. Separation appears to occur at the lamina lucida of the basement membrane[5,7–9] and is characterized by fluid-filled blisters in man and microblisters in most animal models, including pigs.[10] High doses of HD can result in cytotoxic effects on the gastrointestinal and hematopoietic systems following systemic absorption.[2,11–13]

Battlefield HD casualties will likely suffer burns of varying degrees of depth. In humans, varying depths ranging from first- to third-degree burns have been noted.[1,12–14] Casualties generally experience multiple sites of injury, with the ultimate severity of a lesion at any particular site dependent upon the dose of the agent, ambient temperature and skin hydration.[1,13] It is anticipated that different treatment regimens will be required for burns of different depths. For example, deep dermal or full-thickness burns may require debridement followed by split-thickness skin grafting,[1,11,13,14] whereas superficial dermal burns may only require debridement, irrigation and topical antibiotics. Recommended therapies of cutaneous HD wounds in humans include debridement, irrigation, topical antibiotics and split-thickness skin grafting, depending on burn depth.[1,11,13,14]

To examine the efficacy of candidate treatment regimens, it is important that second- and third-degree cutaneous HD burns be generated and characterized in an appropriate animal model. The weanling pig was selected as a model because of its many dermatological similarities to humans (epidermal thickness and composition, pelage density, dermal structure, lipid content and general morphology).[15] In addition, pig skin is antigenically closer to human skin than is rodent skin. The percutaneous absorption of many compounds in the pig is similar to that found in humans,[15,16] and this animal has been found to be a good model for human skin metabolic studies.[17] Meyer and co-workers concluded that, among the domestic species, the pig provides the most suitable experimental model for dermatological research on humans.[18]

We have developed a cutaneous full-thickness HD burn model in weanling pigs. We examined the efficacy of split-thickness autologous skin grafting and the use of a temporary wound dressing in improving wound healing in this animal model. This report addresses clinical pathology findings and urinary excretion profiles of a major HD metabolite in this model. The histopathology of the burn lesions and results of the grafting therapy will be addressed in another report.

Sulfur mustard-induced systemic intoxication is normally seen only after very high doses, which result in debilitating ocular, cutaneous or respiratory injuries.[2] Bone marrow, lymphoid tissue and intestinal mucosa are particularly sensitive to very high doses of HD. Leukopenia resulting from bone marrow depression and leukocyte recruitment due to secondary infections has been noted in humans and a variety of animal models.[2,11–13,19–22] Bone marrow depression following moderately severe HD exposure was noted in humans during the recent Iran–Iraq war.[14,23–25] Changes in clinical pathology parameters following subcutaneous HD exposure have been noted in hairless guinea pigs.[19] We had previously examined clinical pathology parameters in male weanling pigs cutaneously exposed to saturated HD vapor and debrided with a pulsed CO_2 laser, and found no indication of systemic toxicities.[26] Slight increases in white blood cell counts were noted, primarily due to increases in the number of neutrophils. Because HD is systemically absorbed following cutaneous exposure, hematology and clinical chemistry examinations were conducted to ascertain any systemic toxicities in our full-thickness HD burn model. Hematological cell counts and selected serum chemistries were performed on blood samples collected before exposure and on days 1, 3 and 7 post-exposure.

Important routes of detoxification of HD include hydrolysis to form thiodiglycol (TDG) and conjugation with glutathione.[2] Levels of free TDG and acid-labile conjugates have been measured in urine samples collected from both laboratory animals exposed to HD[27–29] and human casualties.[30–34] Using electron ionization gas chromatography/mass spectrometry in our study, TDG concentration (free plus bound) was determined in urine samples collected twice a day through day 7.

MATERIALS AND METHODS

Animal model

Six female weanling Yorkshire pigs (*Sus scrofa*, 8–11 kg) were used (Isler Genetics, Inc., Prospect, OH). They were quarantined upon arrival for 7 days and screened for evidence of disease before use. They were maintained under an AAALAC-accredited animal care and use program. Animals were supplied tap water *ad libitum* and fed a total of ca. 20% of their body weight of a laboratory swine grower (PMI Feeds, Inc., St. Louis, MO) twice a day. Animals were housed individually in 4 × 4 ft. raised nursery pens with plastic-coated wire-mesh flooring (Palco, Inc., Belleplaine, IA). The animal holding room was maintained at 50–80°F with 50 ± 20% relative humidity using at least 10 complete air changes per hour of 100% conditioned fresh air. Animal rooms were maintained on a 12-h light/dark, full-spectrum lighting cycle with no twilight.

Sulfur mustard exposure

The animals were exposed to undiluted HD on the ventral surface, generating six 3-cm diameter full-thickness dermal lesions per pig. The HD was obtained from the US Army Edgewood Research, Development and Engineering Center (Aberdeen Proving Ground, MD). The purity was 97.5 mol.% as determined by NMR spectroscopy. About 18–24 h before agent exposure, each animal was sedated with an intramuscular combination of xylazine · HCl (100 mg ml^{-1}, Ben Venue Laboratories, Inc., Bedford, OH) and Telazol® (250 mg of tiletamine · HCl and 250 mg of zolazepam · HCl, Fort Dodge Laboratories, Inc., Fort Dodge, IA). Five milliliters of xylazine was used to reconstitute Telazol® and the resulting solution was dosed at 0.044 ml kg^{-1} body weight. Under sedation, the ventral surface was depilated using Nair® AG (Carter Products, Division of Carter-Wallace, Inc., New York, NY). After 7 min the Nair® was removed with wetted gauze applied with gentle pressure, followed by gentle washing with a soap solution (1:20 Ivory® liquid) and rinsing with warm water.

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On the morning of agent exposure (day 0), the animals were sedated with 0.044 ml kg^{-1} of an intramuscular combination of Telazol® (250 mg of tiletamine · HCl and 250 mg of zolazepam · HCl) reconstituted with xylazine · HCl (100 mg ml^{-1}), intubated with a size 4–5 endotracheal tube and anesthetized to effect with isoflurane using an anesthesia machine (Anesco, Inc., Georgetown, KY). Prior to intubation, atropine sulfate (AMVET Scientific Products, Yaphank, NY) was administered intramuscularly at 0.04 mg kg^{-1} body weight to control excessive salivation. Six sites were set up on the ventral surface: three sites per side parallel to and ca. 1 in. lateral to the teat line and located between the axillary and inguinal areas. A plastic template was used for even spacing and consistent anatomical positioning of the sites among animals. Each site was delineated by placing small dots at each corner using a permanent marker. Tape assemblies (2″ × 2″) were prepared out of double-sided carpet tape and duct tape, with $1\frac{1}{8}$″ holes punched through the center of each tape assembly. A circle of Whatman No. 2 glass-microfiber filter paper ($1\frac{1}{2}$″ in diameter, Whatman, Hillsboro, OR) was sandwiched between the carpet tape and the duct tape, centered over the $1\frac{1}{8}$″ hole. A small bead of cyanoacrylate adhesive was placed along the periphery of the hole on the duct tape and a rubber O-ring (31 mm inner diameter, Hewlett Packard, Wilmington, DE) was glued onto the template. Templates were then placed onto the ventral surface of the depilated pig, one centered in each of six exposure sites. The pig was then placed in an agent hood on its back, supported by a stainless-steel pig sling. A therapeutic heating pad (Gaymar Industries, Inc., Orchard Park, NY), set at 41°C, was placed under the animal during the exposure period to minimize hypothermia. Using a 500-μl glass Hamilton syringe (Hamilton Company, Reno, NV), 400 μl of undiluted HD was placed on each filter paper. A solid PTFE cap liner (0.015″ thick, sized for a 28-mm cap, Thomas Scientific Company, Swedesboro, NJ) was placed over the filter paper, followed by an appropriately sized rubber stopper to occlude the site and ensure complete contact of the wetted filter paper with the skin. The purpose of the O-ring was to keep the PTFE disk and rubber stopper in place. After all six sites were dosed and cap liners and rubber stoppers put in place, rubber tile floats (one per side) were placed on top of the rubber stoppers to ensure an even downward pressure on all sites. Vetrap™ bandaging tape (3M Animal Care Products, St Paul, MN) was then wrapped around the pigs to secure the floats in place. All exposures lasted for 2 h. At the end of the exposure period, the Vetrap, tile floats, rubber stoppers, cap liners and tape assemblies were removed and the sites were cleansed with absorbent material wetted with lukewarm water. The animals were kept under the agent hood for ca. 24 h, until off-gassing levels of HD were deemed safe by analysis using a Minicams™ (O.I. Analytical, CMS Field Products Group, Birmingham, AL).

On day 2 the animals were sedated with xylazine · HCl, tiletamine · HCl and zolazepam · HCl and anesthetized to effect with isoflurane as described above. Three of the six experimental sites on each animal were debrided to a depth of 0.75 mm using a compressed nitrogen-gas-driven dermatome (Zimmer® Air Dermatome, Cat. No. 8801, Zimmer Inc., Columbia, MD). Sites for debridement were rotated to prevent site-specific biases. Following the debridement procedures and while the animals were still under the effects of anesthesia, two 8 mm diameter biopsies were collected from each experimental site for histopathological analysis of lesion depth, wound severity and debridement depth (results to be presented elsewhere). All experimental sites remained uncovered.

Clinical pathology

Blood samples were collected from the anterior vena cava just prior to agent exposure and on days 1, 3 and 7 post-exposure. Only the 24-h samples were collected in sedated animals. Ten milliliters of blood was collected at each time point and distributed into serum separation tubes and tubes coated with an anticoagulant (ethylenediamine tetraacetate, EDTA). The following hematology parameters were immediately measured on whole blood: red blood cell (RBC) counts, platelets, differential white cell counts, hematocrit, hemoglobin, mean cell volume (RBC), mean cell hemoglobin and mean cell hemoglobin concentration. An Abbott Cell-Dyn Model 3500 hematology analyzer (Abbott Division, Abbott Park, IL) was utilized to measure these hematology parameters. Serum was frozen and stored at −70°C until the following clinical chemistry parameters were measured: electrolytes (chloride, sodium, potassium, calcium, phosphorus), blood urea nitrogen (BUN), creatinine, BUN/creatinine ratio, glucose (hexokinase), alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine phosphokinase (CPK), amylase, total protein (TP), albumin, globulin and albumin/globulin ratio. A Hitachi Model 704 using Boehringer Mannheim reagents (Boehringer Mannheim, Indianapolis, IN) was utilized to measure these serum chemistry parameters. Each pre-exposure sample served as the animal's reference control.

Isoenzymes of CPK were measured using classic electrophoretic techniques. The BB isoform of CPK is found primarily in brain, peripheral nerves, cerebrospinal fluid and various visceral organs. The MM isoform is found in skeletal and cardiac muscle. The MB isoform is found mainly in cardiac muscle, with small amounts in skeletal muscle.

Urine was collected every 12 h throughout the 7-day observation period in metabolism cages, at approximately 6:30 in the morning ('AM' samples) and 6:30 in the evening ('PM' samples). Control samples were collected prior to agent exposure. At each collection time the total volume of urine was measured and the following urinalysis parameters measured using Multistix® 10 SG Reagent Strips (Bayer Corp., Elkhart, IN): glucose, bilirubin, ketone (acetoacetic acid), blood (hemoglobin), protein (albumin), nitrite, leukocytes, pH and specific gravity. Aliquots of urine were immediately frozen and stored at −70°C until analyzed for TDG. No additives or preservatives were utilized.

Determination of thiodiglycol concentration in urine

Sample preparation. Urine samples were prepared and analyzed for TDG concentrations using a modified

version of the procedures published by Jakubowski *et al.*[27] One milliliter of urine was placed into a polypropylene vial. Each sample received 0.1 ml of each of the following solutions prepared in water: 1 mg ml^{-1} thiodipropanol (Aldrich, Milwaukee, WI), 10 μg ml^{-1} deuterated thiodiglycol (d_8-TDG; Ash Stevens, Detroit, MI) and 25 mg ml^{-1} β-glucuronidase (Sigma, St Louis, MO). Each vial was vortexed and allowed to sit inside a hood with the vial lid closed at room temperature for 1 h. The pH was adjusted to 3 using 1 N HCl. Samples were dried under nitrogen at 90°C and then reconstituted with 0.4 ml of ethyl acetate. To derivatize the TDG, 0.4 ml of heptafluorobutyric anhydride (Pierce, Rockford, IL) was added to each vial and allowed to react at room temperature for 1 h with vortexing every 15 min. Samples were centrifuged and the supernatant was transferred into a clean vial. The supernatant was dried under nitrogen at 90°C and reconstituted with 0.2 ml of ethyl acetate. Samples were once again vortexed and centrifuged; the supernatant was then transferred to an autosampler vial containing a glass insert. The resulting solution was analyzed using gas chromatography/mass spectrometry (GC/MS). Pre-exposure control urine samples from each animal were initially analyzed to determine that background levels of TDG were not measurable. The control urine samples were used each day to generate standard curves by adding appropriate amounts of TDG (Sigma, St Louis, MO) in water. Standard curves of TDG were prepared in the range of 0.02–5.0 μg ml^{-1}. The standard curves were produced by plotting the peak area response ratio of TDG/d_8-TDG vs TDG concentration. The linear correlation coefficient (r) for each analysis was 0.998 or better. The lower limit of detection for the assay was ca. 20 ng ml^{-1}.

Gas chromatography/mass spectrometry. Gas chromatographic separations were performed using a Hewlett-Packard (Wilmington, DE) 5890 gas chromatograph. The gas chromatograph was fitted with a 30 m × 0.25 mm i.d. DB-5 bonded phase column of 0.25 μm film thickness (J&W Scientific, Folsom, CA). Helium was used as the carrier gas at a column head pressure of 12 psi. The oven temperature was held initially at 45°C for 1.1 min, programmed from 45 to 110°C at 40°C min^{-1}, programmed from 110 to 125°C at 3°C min^{-1}, programmed from 125 to 265°C at 40°C min^{-1} and held at 265°C for 5 min. Splitless injections of 1 μl volume were made using a Hewlett-Packard 7673A automatic sampler. Other GC conditions were as follows: solvent purge delay, 0.2 min; injection port temperature, 220°C; total flow, 50 ml min^{-1}; transfer line temperature, 265°C; and septum purge, 2 ml min^{-1}. The gas chromatograph was interfaced to a Hewlett-Packard 5970 mass-selective detector. The electron ionization MS operating conditions were as follows: ion source pressure, 1.5×10^{-5} Torr; source temperature, 180°C; electron energy, 70 eV; and electron emission current, 220 μA. Samples were analyzed using selected ion monitoring (SIM). Fragment ions of the derivatized TDG were monitored at m/z 300 and 301; the analagous fragment ions of the d_8-TDG internal standard were monitored at m/z 307 and 309. The dwell time for all ions was 20 ms, resulting in a cycling time of 6.7 cycles s^{-1}.

Statistics

For each serum chemistry, hematology and TDG parameter, the following analysis of variance (ANOVA) model was fitted to the data

$$y_{ij} = \mu + \alpha_i + \tau_j + \epsilon_{ij}$$

where y_{ij} is the reading for animal j on study day i, μ is the overall mean, α_i is a fixed effect for study day i, τ_j is a random effect for animal j and ϵ_{ij} is a random error term. Appropriate contrasts were used to estimate the difference in means between study days. For the serum chemistry and hematology parameters, all pairwise comparisons between study day means were estimated. For the TDG parameters, AM and PM means on each study day were compared with the pretreatment mean (study day 0, AM reading). For each parameter, a Bonferroni adjustment for multiple comparisons was applied to ensure that the probability of making at least one incorrect conclusion of significance was no higher than 0.05. The SAS (V6. 12, SAS Institute, Cary, NC) MIXED procedure was used to fit the statistical models.

RESULTS

Serum chemistry parameters

Mean serum chemistry values are shown in Table 1.

Mean alkaline phosphatase levels showed statistically significant decreases over the course of the experiment. The mean on day 0 was greater than on days 1, 3 and 7. The mean on day 1 was greater than on days 3 and 7. The means on days 3 and 7 were not significantly different.

One animal (97-1-7) showed a large increase in alanine transaminase by day 3, decreasing to baseline levels on day 7. In general, the means on days 1 and 3 were greater than on day 7; however, the changes were not clinically significant. No differences were noted in comparison with the mean on day 0.

Blood urea nitrogen increased by day 1 and remained elevated through day 7. Animal 97-1-7 had the greatest increase in BUN between days 0 and 1.

The mean BUN/creatinine ratio was greater on day 7 than on day 0. The ratios steadily increased for most animals over the course of 7 days; however, the changes were not clinically significant.

While there were no statistically significant differences among the means for creatine phosphokinase, there was one animal (97-50-14) that showed a clinically significant increase on day 7. Examination of the different isoforms for this animal revealed a high percentage of the MM isoform, indicative of muscle damage. Examination of the MB isoform results for all animals did not reveal any clinically significant damage to heart muscle. Examination of the BB isoform results showed increases by day 1 or 3 in four of the six pigs, with the highest percentage noted for pig 97-1-7 on day 3 (Fig. 1). There were no statistically significant differences among any of the means for this parameter, however.

Table 1. Descriptive statistics of serum chemistery parameters by study day (mean ± SD)

	Study day			
Parameter	0 (n = 5)	1 (n = 6)	3 (n = 6)	7 (n = 6)
Alanine transaminase (U l^{-1})	73.6 ± 18.5 AB	76.5 ± 19.7 A	74.3 ± 19.4 A	59.8 ± 12.6 B
Albumin; (ALB g dl^{-1})	3.3 ± 0.2 AB	3.3 ± 0.2 AB	3.4 ± 0.3 A	3.0 ± 0.1 B
Alkaline phosphatase (U l^{-1})	343.4 ± 23.9 A	293.8 ± 32.0 B	245.0 ± 19.6 C	252.5 ± 17.5 C
Amylase (U l^{-1})	7753.6 ± 1960.5 A	6299.7 ± 1812.5 B	5880.5 ± 1669.5 B	6467.3 ± 1776.6 B
Aspartate transaminase (U l^{-1})	50.2 ± 5.7 A	60.2 ± 17.0 A	44.5 ± 29.1 A	49.3 ± 11.8 A
Blood urea nitrogen (BUN; mg dl^{-1})	10.0 ± 3.0 A	16.2 ± 3.9 B	16.5 ± 2.3 B	16.5 ± 1.8 B
Calcium (mg dl^{-1})	9.5 ± 0.2 AB	9.1 ± 0.5 A	9.7 ± 0.6 AB	9.9 ± 0.5 B
Chloride (mEq l^{-1})	78.2 ± 5.9 A	82.3 ± 4.5 AB	87.8 ± 7.4 B	77.5 ± 3.5 A
Creatine phosphokinase (CPK; U l^{-1})	636.0 ± 153.0 A	915.3 ± 501.7 A	473.3 ± 136.0 A	1451.0 ± 1883.3 A
MM isoform of CPK (%)	70.8 ± 14.2 A	72.5 ± 9.5 A	65.7 ± 6.8 A	71.9 ± 13.7 A
MB isoform of CPK (%)	9.3 ± 7.4 A	6.8 ± 5.4 A	8.4 ± 4.7 A	10.1 ± 7.1 A
BB isoform of CPK (%)	19.8 ± 7.2 A	20.7 ± 7.2 A	25.9 ± 10.3 A	18.0 ± 6.7 A
Creatinine (CRE; mg dl^{-1})	0.9 ± 0.1 A	1.1 ± 0.1 A	1.1 ± 0.2 A	0.9 ± 0.1 A
Globulin (GLOB = TP − ALB; g dl^{-1})	1.0 ± 0.2 A	1.0 ± 0.1 A	1.0 ± 0.2 A	1.2 ± 0.2 B
Glucose (hexokinase; mg dl^{-1})	129.8 ± 24.4 A	138.0 ± 25.5 A	158.5 ± 17.7 A	150.8 ± 35.3 A
Lactate dehydrogenase (U l^{-1})	599.2 ± 42.8 A	597.2 ± 83.7 A	652.7 ± 199.6 A	642.5 ± 144.4 A
Phosphorus (mg dl^{-1})	10.2 ± 1.2 A	9.9 ± 1.1 AB	8.4 ± 0.8 B	10.4 ± 0.9 A
Potassium (mEq l^{-1})	4.3 ± 0.4 A	3.7 ± 0.6 A	5.6 ± 1.8 A	5.1 ± 1.1 A
Sodium (mEq l^{-1})	142.0 ± 2.0 A	141.2 ± 2.0 A	144.8 ± 4.2 A	143.3 ± 2.4 A
Total protein (TP; g dl^{-1})	4.2 ± 0.4 A	4.3 ± 0.2 A	4.4 ± 0.4 A	4.2 ± 0.2 A
BUN/CRE ratio	10.7 ± 2.5 A	15.7 ± 5.0 AB	15.6 ± 3.6 AB	18.1 ± 1.8 B
ALB/GLOB ratio	3.4 ± 0.4 A	3.5 ± 0.6 A	3.6 ± 0.6 A	2.5 ± 0.4 B

Letters indicate significant differences (means for study days sharing at least one common letter were not significantly different at an overall 0.05 level, using a Bonferroni adjustment for multiple comparisons within a given parameter).

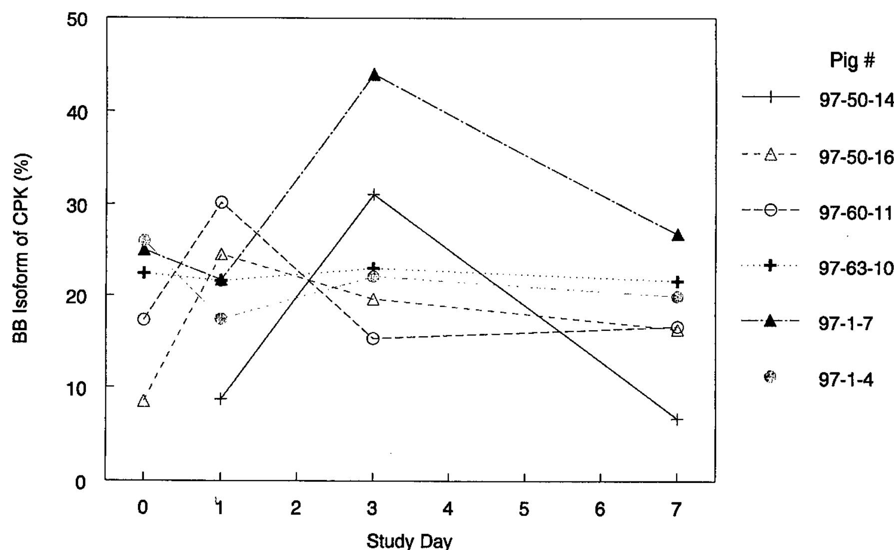

Figure 1. Profiles of the BB isoform of creatinine phosphokinase (present in brain, peripheral nerves, cerebrospinal fluid and various visceral organs) of six weanling swine exposed to sulfur mustard. Increases are seen by day 1 or 3 in four of the six animals.

There were no statistically significant changes in mean creatinine, lactate dehydrogenase or aspartate transaminase levels. Pig 97-1-7, however, showed large increases on day 3 for these parameters.

The mean chloride level was greater on day 3 than on days 0 and 7, but not significantly different from that on day 1.

Other clinical chemistry results revealed a few statistically significant changes; however, the changes were not clinically significant. The mean albumin level was greater on day 3 than on day 7. The mean amylase level was greater on day 0 than on days 1, 3 and 7. The mean calcium level was less on day 1 than on day 7. The mean globulin level was greater on day 7 than on days 0, 1 and 3. The mean phosphorus level on day 3 was less than those on days 0 and 7, but not significantly different from that on day 1. The mean albumin/globulin ratio was less on day 7 than

on days 0, 1 and 3. No significant differences were seen among the means for glucose (hexokinase), potassium, sodium or total protein.

Animal 97-1-7 showed large increases in several clinical chemistry parameters. This pig had the largest increase in BUN between days 0 and 1, a large increase in alanine transaminase by day 3, the highest percentage of BB isoform of CPK on day 3, large increases in creatine, lactate dehydrogenase and aspartate transaminase levels on day 3 and the highest chloride level on day 3. This animal also had the highest urine TDG concentration found in the sample collected 6–8 h following agent exposure.

Hematology parameters

Mean hematology values are shown in Table 2.

The means of overall white blood cell counts on days 0 and 1 were less than those on days 3 and 7. Mean basophil count on day 7 was greater than on days 0 and 1, but not significantly different from that on day 3. The mean on day 3 was greater than that on day 1. Similar changes were noted for eosinophil counts. Mean monocyte counts on days 0 and 1 were less than on days 3 and 7. Mean neutrophil count on day 0 was less than that on day 3, and the mean on day 1 was less than on days 3 and 7. No significant differences were noted among the means of lymphocyte counts.

The mean platelet count on day 0 was greater than on day 3, and the mean on day 7 was greater than on days 1 and 3.

Although there were no statistically significant differences in mean red blood cell counts, hematocrit or hemoglobin values over the 7 day observation period, decreasing trends were noted in some animals. Mean corpuscular hemoglobin on day 7 was less than on day 1. Mean corpuscular hemoglobin concentration on day 7 was less than on days 0 and 1, but not significantly different from that on day 3. Also, the mean on day 3 was less than that on day 1. There were no significant differences noted among the means for corpuscular volume.

Urinalysis

Urinalysis results are presented in Table 3. Fresh urine samples were frequently noted to have a reddish tint. Dipstick analysis indicated that blood levels in urine rose through the evening of day 3 where 83% of animals had large amounts of blood detected and the remaining 17% had moderate amounts of blood. The blood levels then began to decline toward the end of the study. By the morning of day 3, 67% of the animals had >30 mg dl^{-1} protein in the urine, whereas the remaining 33% all showed trace amounts of protein. Average protein levels remained elevated at the end of the study. No leukocytes were detected in the urine at any time point. Specific gravity appeared to have increased slightly following agent exposure, remaining higher for the remainder of the observation period. Similarly, an increase in the percentage of samples being positive for nitrite was also noted. There did not appear to be any significant changes in glucose, bilirubin, ketone or pH over the course of the experiment.

Urine thiodiglycol levels

The mass spectrum of TDG after derivatization with heptafluorobutyric anhydride is shown in Fig. 2. A typical chromatogram is shown in Fig. 3. Descriptive statistics of TDG parameters by study day are shown in Table 4.

Thiodiglycol (a major *in vivo* degradation product of HD) was detected in urine at the earliest post-exposure time-point tested (6–8 h post-exposure). The highest mean TDG concentration (2.14 μg ml^{-1}, Fig. 4) was noted at that time point, with the highest individual level (4.98 μg ml^{-1}) being detected for animal 97-1-7. Mean TDG concentration decreased to

Table 2. Descriptive statistics of hematology parameters by study day (mean ± SD)

Parameter	Study day 0 (n = 5)	1 (n = 6)	3 (n = 5)	7 (n = 6)
Basophils (10^3 μl^{-1})	0.071 ± 0.047 AB	0.058 ± 0.050 B	0.151 ± 0.065 AC	0.206 ± 0.038 C
Eosinophils (10^3 μl^{-1})	0.072 ± 0.057 AB	0.029 ± 0.032 B	0.179 ± 0.101 AC	0.194 ± 0.079 C
Hematocrit (%)	32.060 ± 2.327 A	30.400 ± 2.498 A	31.180 ± 2.519 A	29.667 ± 2.796 A
Hemoglobin (g dl^{-1})	10.690 ± 0.714 A	10.237 ± 0.830 A	10.146 ± 0.931 A	9.568 ± 0.833 A
Lymphocytes (10^3 μl^{-1})	4.338 ± 0.531 A	3.888 ± 1.579 A	5.862 ± 2.623 A	5.853 ± 2.129 A
Mean corpuscular hemoglobin (pg)	17.260 ± 0.856 AB	17.450 ± 0.766 A	17.320 ± 0.829 AB	16.667 ± 0.873 B
Mean corpuscular Hb concentration (g dl^{-1})	33.340 ± 0.702 AB	33.650 ± 1.117 B	32.520 ± 0.661 AC	32.267 ± 0.314 C
Mean corpuscular volume (fl)	51.800 ± 1.771 A	51.883 ± 1.716 A	53.260 ± 2.387 A	51.783 ± 2.914 A
Monocytes (10^3 μl^{-1})	1.324 ± 0.502 A	1.040 ± 0.309 A	2.076 ± 0.419 B	2.503 ± 0.236 B
Neutrophils (10^3 μl^{-1})	5.284 ± 1.505 AB	4.370 ± 1.319 A	8.934 ± 3.174 C	8.320 ± 0.929 BC
Platelet count (10^3 μl^{-1})	691.400 ± 102.166 AC	634.500 ± 121.070 AB	558.200 ± 112.611 B	785.833 ± 150.121 C
Red blood cell count (10^6 μl^{-1})	6.210 ± 0.628 A	5.857 ± 0.377 A	5.852 ± 0.377 A	5.728 ± 0.378 A
White blood cell count (10^3 μl^{-1})	11.090 ± 1.604 A	9.355 ± 2.353 A	17.200 ± 4.693 B	17.083 ± 1.975 B

Letters indicate significant differences (means for study days sharing at least one common letter were not significantly different at an overall 0.05 level, using a Bonferroni adjustment for multiple comparisons within a given parameter).

Table 3. Distribution of urinalysis results for each test by study day

		Percentage of samples															
		Study day															
Test	Result	0-AM	0-PM	1-AM	1-PM	2-AM	2-PM	3-AM	3-PM	4-AM	4-PM	5-AM	5-PM	6-AM	6-PM	7-AM	7-PM
Glucose (mg dl^{-1})	Negative	100	100	100	67	83	100	100	100	100	100	100	100	100	100	100	100
	100				17												
	1000				17												
	2000					17											
Bilirubin	Negative	100	100	100	100	100	100	100	100	100	100	83	83	100	100	100	100
	Small											17	17				
Ketone (mg dl^{-1})	Negative	100	100	100	100	100	100	83	100	100	100	100	100	100	100	83	100
	5							17								17	
Blood	Negative	50		60	17	17		33						33		50	17
	Trace	17	33		17	17						67		33	17	33	33
	Small	17	17			33	33			33	67	17	67	17	50	17	33
	Moderate		17	20	17	33		17	17	33	33	17	33	17	33		17
	Large	17	33	20	50		67	50	83	33							
Protein (mg dl^{-1})	Negative	67	33		17	17	17		50		17				17		17
	Trace	33	17	60	83	67	67	33	33	33	33	33	33	33	33	67	67
	30		33	40			17	67	17	67	50	67	67	67	50	33	17
	100					17											
	2000		17														
Nitrite	Positive	17		20	33	33	33	67	67	67	67	83	50	67	67	83	100
Leukocytes	Negative	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
pH	5.0					33				17	33						
	6.0		33	40	17				17	50	17		17				17
	6.5	17		20	33	33	33	17	17	17							17
	7.0	17	17	40	33	17	50	33	50		17	33		17		33	17
	7.5	33	33		17	17		17	17		17	33	50	50	67	50	17
	8.0						17					17	17	17	33		
	8.5	33	17					33		17	17	17	17	17		17	33
Specific gravity (ml)	1.000		17				17					17					
	1.005	50	17	20	17	17	17									17	
	1.010	33	33		33	17	33		17	33	17	17	33				33
	1.015	17					17	17		17	33		17	17	50	33	33
	1.020		17	20	33	33		50	50		17	50	17	33	33	33	17
	1.025			20	17	17	17			17			17	50			17
	1.030		17	40		17		33	33	33	33	17	17		17	17	

1.57 μg ml^{-1} by the morning of day 1 and had fallen to 0.27 μg ml^{-1} by that evening (30–32 h post-exposure). Thiodiglycol levels remained detectable but low for the remainder of the 7 day observation period.

For thiodiglycol concentration, means on study day 0-PM and day 1-AM were significantly greater than the pretreatment (0-AM) mean. Concentrations were the highest on day 0-PM for three animals, on day 1-AM for one animal, on day 1-PM for one animal and on day 2-AM for one animal. Total TDG (TDG concentration multiplied by total sample volume) was the highest on day 0-PM for four animals, on day 1-AM for one animal and on day 2-AM for one animal. For total TDG, the mean on study day 0-PM was significantly greater than the pretreatment mean (Fig. 5). No significant differences were noted for the other study-day times. No statistical comparisons were made for urine volume.

DISCUSSION

We have developed a cutaneous full-thickness HD burn model in weanling pigs to examine the efficacy of candidate treatment regimens in improving wound healing. Female Yorkshire weanling pigs are exposed to undiluted HD on the ventral surface, generating six 3-cm diameter full-thickness dermal lesions per pig. Examination of clinical pathology parameters in this model has revealed subtle HD-related changes suggestive of a mild hemolytic episode. No other signs of clinically significant systemic toxicities were noted during our 7-day observation period, including the bone marrow suppression often seen with high exposures. We noted high levels of TDG in the urine 6–8 h following exposure, with detectable levels after 1 week.

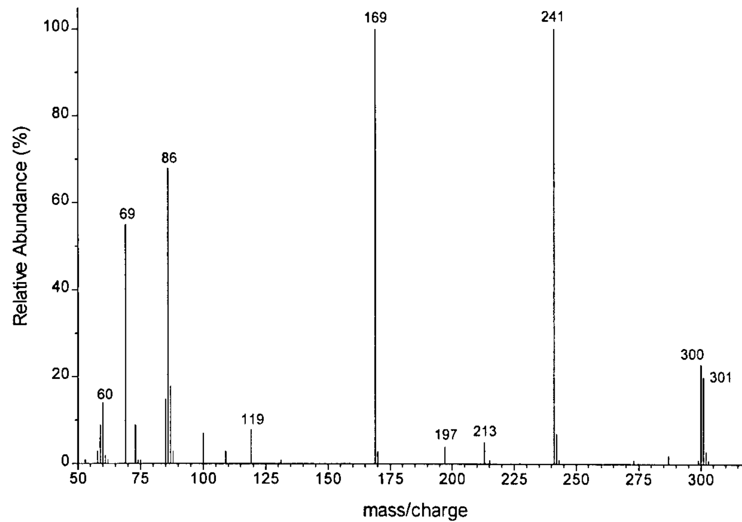

Figure 2. The mass spectra of thiodiglycol (TDG) after derivatization with heptafluorobutyric anhydride. A Hewlett-Packard 5890 gas chromatograph was interfaced to a Hewlett-Packard 5970 mass-selective detector. Samples were analyzed using selected ion monitoring. Fragment ions of the derivatized TDG were monitored at m/z 300 and 301; the analagous fragment ions of the d_8-TDG internal standard were monitored at m/z 307 and 309.

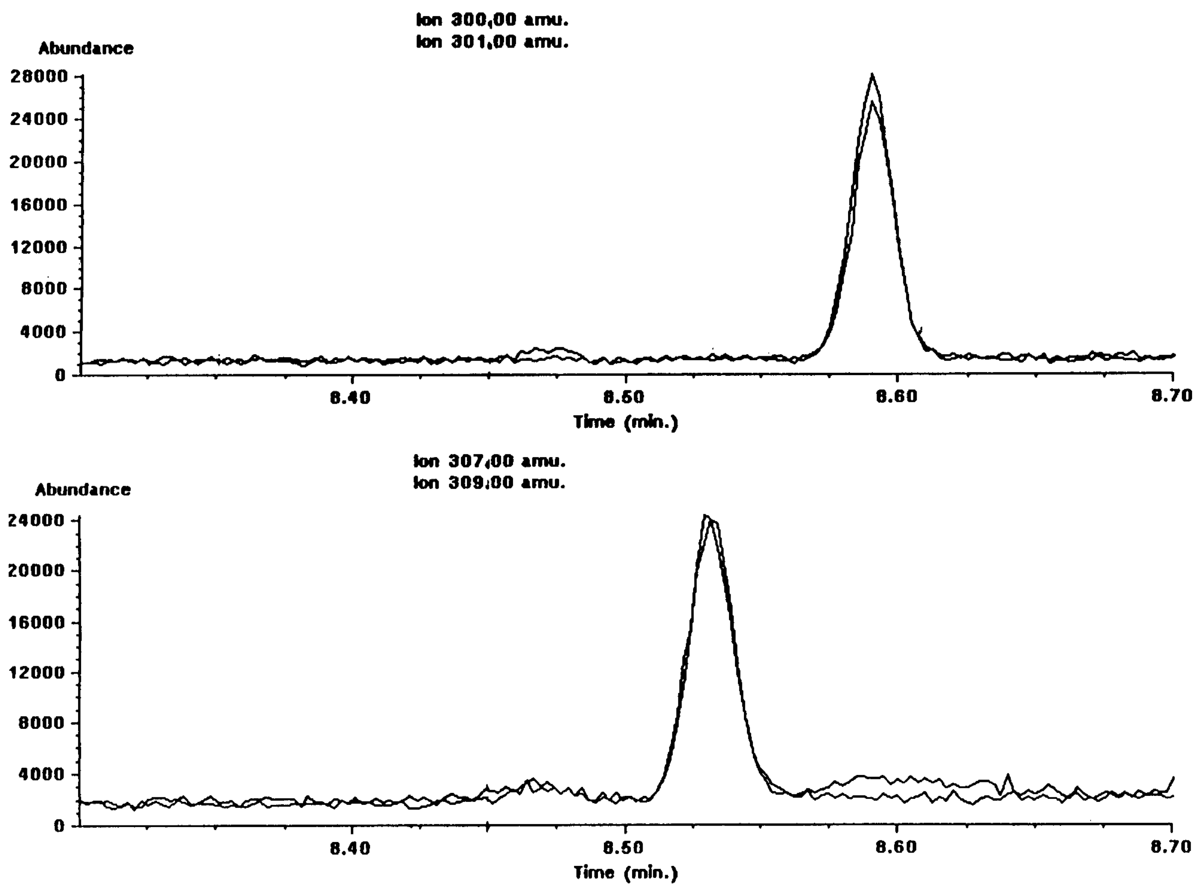

Figure 3. A typical chromatogram shown for animal 97-50-14 (top panel). Thiodiglycol (TDG) concentration was 1.28 μg ml^{-1}. The bottom panel shows the d_8-TDG internal standard.

Table 4. Descriptive statistics of thiodiglycol parameters by study day (mean ± SD)

Study day	*n*	Urine volume (ml)	Thiodiglycol concentration (μg ml^{-1})	Total thiodiglycol (μg)
0-AM	6	94.83 ± 70.56	0.00 ± 0.00	0.00 ± 0.00
0-PM	6	175.17 ± 177.47	2.14 ± 1.66	199.21 ± 124.03
1-AM	5	36.17 ± 24.15	1.57 ± 1.11	61.92 ± 37.01
1-PM	5	210.20 ± 112.85	0.27 ± 0.44	57.41 ± 98.34
2-AM	6	103.75 ± 85.80	0.65 ± 0.71	87.55 ± 166.71
2-PM	5	194.40 ± 144.72	0.21 ± 0.16	26.20 ± 9.54
3-AM	6	95.83 ± 67.69	0.18 ± 0.12	15.29 ± 14.21
3-PM	5	134.40 ± 117.99	0.05 ± 0.05	7.12 ± 7.87
4-AM	6	71.0 ± 37.00	0.04 ± 0.05	3.55 ± 5.81
4-PM	6	189.67 ± 99.80	0.03 ± 0.02	6.24 ± 6.94
5-AM	6	78.83 ± 30.80	0.04 ± 0.05	2.89 ± 3.82
5-PM	6	181.33 ± 104.41	0.02 ± 0.05	2.93 ± 7.16
6-AM	6	114.33 ± 49.56	0.02 ± 0.03	2.48 ± 3.86
6-PM	6	201.33 ± 143.41	0.01 ± 0.02	2.16 ± 5.29
7-AM	6	185.83 ± 91.06	0.03 ± 0.04	4.74 ± 5.42
7-PM	4	247.50 ± 87.86	0.01 ± 0.02	2.80 ± 5.59

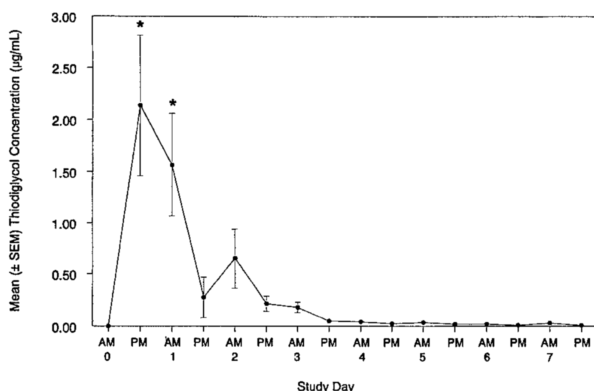

Figure 4. Mean urine thiodiglycol concentrations in weanling pigs exposed to sulfur mustard. Urine was collected every 12 h throughout a 7-day observation period. The means on study day 0-PM (6–8 h following exposure to sulfur mustard) and day 1-AM were significantly greater than that for the pretreatment samples (day 0-AM). Concentrations were the highest on day 0-PM for three animals, on day 1-AM for one animal, on day 1-PM for one animal and on day 2-AM for one animal. Mean levels remained at 10–40 ng ml^{-1} on days 4–7, with the highest individual concentration noted during this period being 132 ng ml^{-1}.

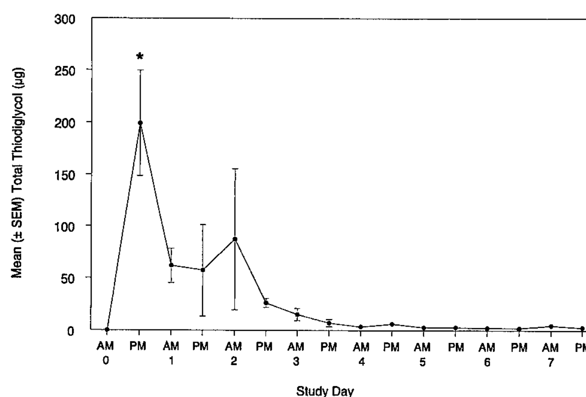

Figure 5. Mean total urine thiodiglycol levels in weanling pigs exposed to sulfur mustard. The mean on day 0-PM was significantly greater than the pretreatment mean (day 0-AM). Total thiodiglycol was the highest on day 0-PM for four animals, on day 1-AM for one animal and on day 2-AM for one animal.

Clinical pathology

In contrast to our current findings, Gold and Scharf[19] found significant leukocyte count suppression in euthymic hairless guinea pigs on days 4, 5 and 6, following an elevation on day 1 after subcutaneous exposure to 20 mg kg^{-1} HD (40% of their calculated median lethal dose). They suggested that the initial leukocytosis was probably due to direct stimulation of the bone marrow and subsequent progressive leukopenia to direct bone marrow injury via cellular DNA alkylation by HD and/or recruitment of leukocytes from the blood to sites of secondary infection. Changes in serum potassium, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration were also noted.

In our experiment, a total of 3048 mg of HD was applied onto each animal (based upon a density of 1.27 g ml^{-1} at 25°C). For a 10-kg weanling pig, this equates to ca. 300 mg kg^{-1}, which is a 15-fold increase over the dose administered to hairless guinea pigs by Gold and Scharf.[19] The differences in the results of these experiments are likely due to the difference in route of exposure and species-specific differences in toxicodynamics. Penetration rates, fixative properties, absorption characteristics and distribution of HD have been described previously in humans and pigs.[1–3,35,36]

We noted some changes in hematology and serum chemistry parameters in our weanling pigs during the 7-day observation period. The white blood cell count increase that was noted was mostly the result of an increase in neutrophils. This increase was likely the result of stress (e.g. animal handling), wound healing and possible secondary infections. There were no indications of leukopenia during the 7-day observation period. The decrease in platelet counts on days 1 and

3 was likely to have been the result of the venipuncture conducted for blood collection. Some pigs showed decreasing trends in red blood cell counts, hematocrit and hemoglobin values over the 7-day observation period. A mild hemolytic episode would account for both the hematology results and the blood (hemoglobin) noted in the urine, with the exception that a rise in serum hemoglobin concentration rather than a fall would be expected with intravascular hemolysis. It is likely that the hemolysis occurred within the HD lesions, because we visually noted dermal hemorrhage in most of the sites by day 1. Dermal hemorrhage was also verified by histopathology on day 2 (data not shown). It is possible that the hemolysis could have occurred in the urinary bladder or thereafter. Cytological examination of the urine and histological examination of the urinary bladder were not done in these pigs, so we were unable to determine the source of the blood (hemoglobin). Urine blood levels peaked on day 3 and then began to decline.

The decrease noted in serum alkaline phosphatase levels was not clinically significant. Decreased levels in humans have been associated with hypothyroidism, scurvy and a number of other conditions. The increases in alanine transaminase, indicative of necrosis of hepatocytes, erythrocytes, myocardium or skeletal muscle, were not clinically significant. Examination of creatine phosphokinase and lactate dehydrogenase levels indicated no clinically significant damage to skeletal or cardiac muscle. One pig showed an elevated CPK level on day 7, primarily due to MM isoform increase. Any muscle damage in this animal may have been a result of multiple intramuscular injections rather than HD-induced damage. The BB isoform of CPK, present in brain, peripheral nerves, cerebrospinal fluid and various visceral organs, showed increases by day 1 or 3 in four of the six pigs. The cause of this elevation is unknown. The increases noted in chloride levels on day 3 were likely to be the result of the hydrochloride present in the injectable sedatives administered on multiple occasions during the first 3 days of the experiment. The elevations noted in BUN are minimal and are thought to be pre-renal (e.g. unrelated to kidney function). Only one pig showed an increase in creatinine levels. The slight increases in BUN/creatinine ratio were not clinically significant. The slight increases that were noted in the specific gravity of the urine suggest that the concentrating ability of the kidney was not affected. No clinically significant changes were noted in any of the other serum chemistry parameters examined.

The proteinuria that we noted is thought to be extrarenal in origin and most likely the result of urine hemorrhage (plasma-derived albumin and globulin) or hemolysis (hemoglobin dimers). An increase in nitrites, as was seen in our animals, is normally indicative of Gram-negative bacteria; however, no leukocytes were noted in the urine at any time-point. Fecal contamination of urine samples is the likely explanation for these results, rather than an active urinary tract infection. We did not perform any cultures on the urine samples.

Urine thiodiglycol levels

Biotransformation of HD is via direct alkylation reactions with tissue components, chemical hydrolysis and formation of conjugation products (e.g. with glutathione). Almost all metabolites are excreted in the urine, with TDG being a major metabolite for which tests have been developed on urine samples to verify HD exposure.[3,27] We tested for total TDG (free plus acid-labile conjugates). The conjugates are possibly esters derived from alkylation reactions of HD with free acid groups present in organic acids, proteins or nucleic acids, which are hydrolyzed to TDG under acidic conditions.[28] We detected TDG at the earliest time-point tested (6–8 h post-exposure) at levels ranging from 0.66 to 4.98 $\mu g\ ml^{-1}$ with a mean of 2.14 $\mu g\ ml^{-1}$. The TDG concentrations were the highest for half of the animals at this earliest time point, and at 24–48 h for the others. By the evening of day 3, the mean level had reached 50 $ng\ ml^{-1}$. Mean levels remained at 10–40 $ng\ ml^{-1}$ for the remainder of the 7-day observation period, with the highest individual concentration noted during this period being 132 $ng\ ml^{-1}$. Black *et al.*[28] found a similar excretion profile of TDG in rats topically exposed to 318 μg of HD over a 6-h period. They collected urine before dosing, at 6 and 25 h post-exposure, at 24-h intervals thereafter up to 8 days and on days 15 and 43. Peak levels of free TDG were found in the 6-h samples and peak levels of conjugated TDG at 6–24 h. They noted low levels of both free and conjugated TDG for 8 days but did not detect them at 43 days. They found that more TDG was excreted in conjugated form than in free form, and calculated that the total amount of TDG excreted during the first 8 days accounted for 1.0–1.5% of the applied dose. Jakubowski *et al.*[27] were also able to detect trace levels of total TDG (1–15 $ng\ ml^{-1}$) in rats up to 1 week following subcutaneous exposure to 750 $\mu g\ kg^{-1}$ HD. Thiodiglycol has been found in the urine collected from Iranian casulaties in 1984–1986, with levels averaging 10–100 $ng\ ml^{-1}$,[30,31] although it is unclear how soon after agent exposure the samples were collected or what the severities of the exposures were. Jakubowski *et al.*[32] examined total urinary TDG clearance in humans accidentally exposed to HD cutaneously in the laboratory. In one patient who experienced multiple small skin lesions, they found a maximum urinary clearance of 20 $\mu g\ day^{-1}$ after 3 days, maximum concentrations on day 4 and concentrations $>10\ ng\ ml^{-1}$ for as long as 1 week following exposure. They postulated that HD may be stored in fat and slowly released, or that TDG may be protein-bound and only slowly eliminated. Black *et al.*[33] have also found TDG in urine samples of patients accidentally exposed to HD. Thiodiglycol sulfoxide has been shown to be excreted in greater amounts than the unoxidized form in rats[28] and humans.[33] A natural background of TDG and its sulfoxide has been found in human urine (normally $<1\ ng\ ml^{-1}$ for TDG and 1–12 $ng\ ml^{-1}$ for TDG sulfoxide).[27,33] Levels higher than these have occasionally been found, although the environmental source is unknown. Because of natural background levels, detection of TDG and its sulfoxide does not provide unequivocal proof of exposure to HD.[33,34]

Our results are in general agreement with the TDG excretion profiles previously described for rodent models and humans. Urinary excretion of absorbed HD in our weanling pig wound healing model appears to follow the same pattern seen in other laboratory animal models.

In general, urinary excretion of TDG appears to peak within the first 1–4 days following exposure, with detectable levels after 1 week. Relatively high urinary TDG levels may thus indicate agent exposure within the previous 96 h. Low levels significantly above natural background levels may indicate either exposure to low levels of agent or exposure that occurred more than 4 days prior to collection of the sample.

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