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Toxicology Studies on Lewisite and Sulfur Mustard Agents: Modified Dominant Lethal Study of Sulfur Mustard in Rats

Final Report

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Occupational health standards have not been established for sulfur mustard (HD) [bis(2-chloroethyl)-sulfide], a strong alkylating agent with known mutagenic properties. Little, however, is known about the mutagenic activity of HD in mammalian species and data regarding the dominant lethal effects of HD are ambiguous. The purpose of this study was to determine the dominant lethal effect in male and female rats orally exposed to HD. The study was conducted in two phases; a female dominant lethal phase and a male dominant lethal phase. Sprague-Dawley rats of each sex were administered 0.08, 0.20, or 0.50 mg/kg HD in sesame oil 5 days/week for 10 weeks. For the female phase, treated or untreated males were mated with treated females and their fetuses were evaluated at approximately 14 days after copulation. For the male domi-

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nant lethal phase, treated males cohabited with untreated femal (during 5 days of each week for 10 weeks) and females were sacrificed for fetal evaluation 14 days after the midweek of cohabitation during each of the 10 weeks. The appearance and behavior of the rats were unremarkable throughout the experiment and there were no treatment-related deaths. Growth rates were reduced in both female and male rats treated with 0.50 mg/kg HD. Indicators of reproductive performance did not demonstrate significant female dominant lethal effects, although significant male dominant lethal effects were observed at 2 and 3 week post-exposure. These effects included increases of early fetal resorptions and preimplantation losses and decreases of total live embryo implants. These effects were most consistently observed at a dose of 0.50 mg/kg, but frequently occurred at the lower doses. Although no treatment-related effects on male reproductive organ weights or sperm motility were found, a significant increase in the percentage of abnormal sperm was detected in males exposed to 0.50 mg/kg HD. The timing of these effects is consistent with an effect during the postmeiotic stages of spermatogenesis, possibly involving the generally sensitive spermatids.

FOREWORD

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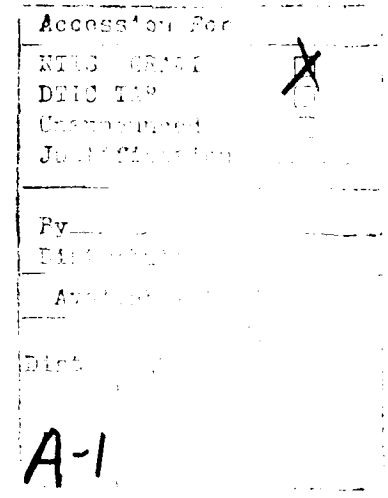

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EXECUTIVE SUMMARY

Chemical warfare agents present an obvious risk to individuals suffering acute exposure, but they may also present long-term environmental or occupational health hazards for workers in operations involving these chemical agents. Occupational health standards have not been established for sulfur mustard (HD) [bis-(2-chloroethyl)-sulfide] a strong alkylating agent with known mutagenic and suspected carcinogenic properties. Sulfur mustard is used in a number of research laboratories, stored in depot sites throughout the country and occasionally transported to distant sites. The destruction of current stockpiles of sulfur mustard by the U.S. Army in the near future could create additional environmental and occupational risk. To establish a data base for setting environmental and occupational standards, we have conducted studies to evaluate the toxicity, mutagenicity, and reproductive effects of sulfur mustard using in vitro and in vivo study systems.

The purpose of this study was to determine the dominant lethal effect in rats orally exposed to HD. Because chemically induced dominant lethal mutations can be obscured by perturbations in reproductive competence that are unrelated to genetic change, a modified dominant lethal test was performed in both male and female rats. In addition, motility, population size and morphology were measured in sperm obtained from the cauda epididymis.

The study was conducted in two phases: 1) the female dominant lethal phase and 2) the male dominant lethal phase. Phase I evaluated female dominant lethal effects of 10-week exposed females mated to exposed and unexposed males during a 3-week post-exposure mating period. Phase II evaluated the dominant lethal effects and sperm morphology and function of the male rat. The dominant lethal studies were conducted over a 10-week post-exposure period following 10 weeks of exposure.

Solutions of HD were prepared for administration by diluting the neat agent with sesame oil. Sprague-Dawley rats of each sex, 6-7 weeks old, were gavaged with either 0, 0.08, 0.20, or 0.50 mg/kg of sulfur mustard 5 days/week for 10 weeks. A constant dosage volume of 1.67 ml/kg of body weight was given. Dominant lethal effects were evaluated at the end of the gavaging period.

The appearance and behavior of the rats were unremarkable throughout the experiment and there were no treatment-related deaths. Growth rates were reduced in both female and male rats treated with the high level of HD. Indicators of reproductive performance did not demonstrate significant female dominant lethal effects in the rat at any of the HD doses studied. On the other hand, significant male dominant lethal effects were observed in HD-

exposed male rats mated to untreated females at 2 and 3 weeks following a 10-week exposure. These effects, which included increases of early fetal resorptions and preimplantation losses and decreases in total live embryo implants, were most consistently observed at a dose of 0 50 mg/kg, but frequently occurred at the lower doses. Although no treatment-related effects on male reproductive organ weights or sperm motility were found, a significant ($P<0.05$) increase in the percentage of abnormal sperm was detected in males exposed to 0.50 mg/kg HD. The timing of dominant lethal effects is consistent with an effect during the postmeiotic stages of spermatogenesis, possibly involving the generally sensitive spermatids.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Chemical warfare agents present an obvious risk to individuals suffering acute exposures and may also present certain long-term environmental or occupational health hazards for workers in operations involving these chemical agents. These materials are used in a number of research laboratories, stored in depot sites throughout the country and occasionally transported to distant sites. In addition, stockpiles of agents are scheduled for destruction by the U.S. Army in the near future, creating an additional potential for environmental and occupational exposure. Although considerable information is known concerning the acute effects of these materials, little information is available on the long-term hazards of these materials, including reproductive effects. Segments of the population that may be particularly sensitive include the chronically ill, the young and old and the unborn. It is this concern that has prompted these studies to identify the potentially toxic, mutagenic and reproductive effects of chemical agents and to establish a data base for the development of hazard evaluations and occupational health standards for these chemicals.

The two general categories of vesicants are typified by lewisite [dichloro(2-chlorovinyl)arsine] and sulfur mustard (HD) [bis(2-chloroethyl) sulfide] (Cassarett and Doull, 1986). Contact with these chemicals produces severe skin burns. Recently, a renewed interest in these chemicals was generated by the release of a United Nations report that contained substantial evidence that Iraq was manufacturing and using these agents as chemical warfare agents (Marshall, 1984).

The mustard compounds (both sulfur and nitrogen) are biochemically related to a group of cytotoxic alkylating agents, including the ethylenimines, sulfonic esters, epoxides and n-alkyl-n-nitroso compounds (Wheeler, 1962). These chemicals react rapidly with certain functional groups of proteins (OH, NH2, and SH) to alter their metabolic activity. In aqueous solutions, both sulfur and nitrogen mustard hydrolyze to form cyclic sulfonium or immunium forms, respectively, which, in turn, will react with nucleophilic sites. The sulfur mustard reaction proceeds more rapidly to the reaction with nucleophiles than does nitrogen mustard and is independent of the concentration of nucleophiles present (Fox and Scott, 1980). The cytotoxic, mutagenic, and carcinogenic properties of mustard compounds have been studied extensively (Fox and Scott, 1980), but most of these data relate to nitrogen mustard because sulfur mustard is a more toxic and chemically reactive vesicant.

Relevant chemical and physical properties of sulfur mustard are summarized in Table 1. In aqueous solutions, sulfur mustard

rapidly hydrolyzes to form a cyclic sulfonium salt, ß-chloroethyl-ethylenesulfonium chloride. This salt reacts with water to form ß-chloroethyl ß-hydroxyethyl sulfide and hydrochloric acid. Subsequent hydrolysis of the sulfide, presumably through the intermediation of a second sulfonium salt, forms thiodiglycol (Anslow et al., 1948). These workers have investigated the toxicity of these derivatives of sulfur mustard and a number of other intermediates isolated from hydrolysates of sulfur mustard. They found that two of the derivatives, ß-chloroethyl ß-hydroxyethyl sulfide and thiodiglycol, were relatively nontoxic.

TABLE 1. Relevant Chemical and Physical Properties of Sulfur Mustard, Bis(2-Chloroethyl)Sulfide[a]

CAS #:	505-60-2
RETCS #:	WQ0900000
Structural formula:	$Cl-CH_2-CH_2-S-Cl-CH_2-CH_2$
Molecular weight:	159.1 g
Density at 25°C:	1.3 g/ml
State:	Colorless,oily liquid
Vapor pressure at 20°C:	0.072 mm
Decomposition temperature:	149-177°C
Solubility in water at 25°C:	0.68 g/L
Hydrolysis	
Rate (T1/2 at 25°C, pH 7):	8.5 min
Products:	Thiodiglycol,chloride
Army Abbreviation	HD

[a]Rosenblatt et al., 1975; Windholz, 1983.

Few values are available in the literature for the LD_{50} of sulfur mustard. Table 2 includes LD_{50} data for sulfur mustard administered to mice, rats and rabbits. Haskin (1948) reported that extensive edema occurred at the site of administration of nitrogen mustard (ip and subcutaneous) and that diarrhea, dysprosium, and anorexia were common observations. Death occurred in rats within 3 to 4 days after administration at dose levels of 1.8 to 3.1 mg/kg and within 5 to 19 days of administered doses of 1 to 1.2 mg/kg.

TABLE 2. LD_{50} Values of Various Routes of Administration for Sulfur and Nitrogen Mustard

Chemical	Route of Administration[b]	LD_{50} (mg/kg) Rat	Rabbit	Mouse
Sulfur mustard	IV	0.7	1.1	8.6
	SC	1.5	2.0	2.0
Nitrogen mustard	IV	1.1	---	---
	SC	---	---	1-4
	IP	1.8-2.5	---	4.4
	Oral	---	---	10-20

[a]D. V. Sweet, 1987.
[b]IV = intravenous; SC = subcutaneous; IP = intraperitoneal.

The carcinogenicity of nitrogen mustard is well documented, but relatively few data are available for HD. Studies in mice have shown evidence of skin papillomas following subcutaneous HD treatment and lung tumors after intravenous injection or inhalation of HD (Fox and Scott, 1980). Studies conducted by the U.S. Army found little evidence of lesions in rabbits, guinea pigs and dogs after being exposed to HD vapor for up to 52 weeks. Treatment-related skin tumors were observed in rats exposed to 0.1 mg/m^3 HD vapor for as few as 12 weeks (McNamara et al. 1975). In an initiation-promotion study using a mouse-skin model, HD was not found to be an active initiator of cancer (Berenblum and Shubik, 1949). However, Japanese factory workers, who were involved in the production of chemical agents and who were potentially exposed to unknown quantities of various chemical agents including HD during World War II, show evidence of an increased incidence of respiratory and gastrointestinal tract cancers (Wada et al., 1968; Norman, 1975; Manning et al., 1981; Yamakido et al., 1985).

The teratogenic potential of HD was studied in rats exposed to two concentrations of inhaled HD (0.001 and 0.1 mg/m^3) during each of the 3 weeks of gestation or throughout the entire gestation period (McNarmara et al., 1975). No evidence of dose-related fetal mortality or gross abnormalities was noted. Teratology studies, following the segment II teratology protocol, were recently

conducted in rats and rabbits by Hackett et al. (1987). Rats were exposed to 0.5-2.0 mg/kg HD by gastric intubation from 6 to 15 days of gestation (dg) and were killed on dg 20. No evidence of a teratogenic response to HD was observed since fetal effects occurred only at doses exhibiting signs of maternal toxicity. Likewise, fetal development of rabbits exposed to 0.4-0.8 mg/kg HD between 6 and 19 dg was not affected even though maternal mortality was induced at the highest dose. These results suggest that HD is not teratogenic in rats and rabbits since fetal effects were observed only at dose levels that induced frank maternal toxicity.

As a bifunctional alkylating agent, HD is capable of reacting chemically with DNA yielding 7-alkylguanine as its principal alkylation product. Approximately 25% of these alkylations result in the formation of the DNA cross link, diguanine-7-ethylmethylamine. DNA cross links are implicated in the production of chromosomal aberrations and chromosomal rearrangements (Bodell et al., 1985; Tokuda and Bodell, 1987). Sulfur mustard is a known clastogen which produces all of the types of chromatid aberrations commonly seen with ionizing radiation. Conversely, very few, if any chromosome type aberrations have been observed after HD treatment. Some investigators feel that this observation suggests that only one strand of the DNA helix is affected by the cross-link (Fox and Scott, 1980).

Mustard agents have been found to produce mutagenic effects in a wide variety of animal species and test systems. Reviews on the genetic toxicology of nitrogen mustard and HD have summarized the known effects of these agents in biological systems (Auerbach, 1949; Auerbach, 1976; Fox and Scott, 1980). Dominant lethal, sex-linked recessive and autosomal lethal, and visible mutations as well as major rearrangements and chromosomal aberrations have been reported in the fruit fly. The mutagenic potential of HD was recently evaluated in the standard plate incorporation version and the preincubation modification of the Salmonella/microsomal (Ames) assay (Stewart, 1987; Stewart et al., 1989). Sulfur mustard induced point mutations in tester strain TA102 and frameshift mutations in TA97 but showed little or no mutagenicity against strains TA98 or TA100. Sulfur mustard has been reported to induce a linear increase in the mutation of L5178Y cells as determined by reversion from asparagine dependence (Capizzi et al., 1973). Jostes et al. (1989) investigated the *in vitro* genotoxicity of sulfur mustard using the Chinese hamster ovary cell (CHO) line. Exposures to micromolar amounts of HD were highly toxic and resulted in marked chromosomal damage and rearrangement as determined by sister chromatid exchange. It also appeared that HD was mutagenic at the HGPRT locus in CHO cells.

Relatively little is known concerning the mutagenicity of HD in mammalian species. Chronic inhalation exposure of male rats to

sulfur mustard (0.1 mg/m3) was reported to produce significant dominant lethal effects, but exposure of pregnant females to the same concentrations for a shorter time interval failed to induce fetal malformations (Rozmiarek et al., 1973). McNamara et al. (1975) subsequently concluded from these same data that there were no differences between the control and experimental groups and no evidence of mutagenesis. It is difficult to resolve the apparent conflict between the conclusions of these two reports, but the fetal mortality values presented in the McNamara report suggest at least a trend for a significant dominant lethal effect. Complete control data are missing from the report and statistical evaluation of the results is not presented, but percentages of fetal deaths at week 12 were 4.12, 4.24, and 21.05 for controls, 0.001 and 0.1 mg/m^3 exposure groups, respectively.

Data regarding the dominant lethal effects of HD in mammalian species are ambiguous and comprehensive information is not available to evaluate the potential risk from long-term occupational or environmental exposures. The purpose of this study was to determine the dominant lethal effect in male and female rats orally exposed to HD. A modified dominant lethal test was performed to extend the exposure period to the approximate length of the sperm cycle. In addition, motility, population size and morphology was measured in sperm obtained from the cauda epididymis.

MATERIALS AND METHODS

SULFUR MUSTARD

Procurement and Characterization

The sulfur mustard used in these studies was 2,2', dichlorodiethyl sulfide, also known as bis(2-choroethyl)sulfide or distilled mustard (HD).

The sulfur mustard was supplied by the U.S. Army Medical Research Institute for Chemical Defense (USAMRICD), Chemical Surety/Safety Office, Aberdeen Proving Ground, Edgewood Arsenal MD from lot No. HD-U-4244-CTF-N-1, previously designated Lot No. ICD-HD-1. The material was prepared August 31, 1981 and analyzed for purity September 4, 1984 by Captain William Beaudry and Linda Szafdraniec (Research Directorate Chemical Research) by nuclear magnetic resonance. Purity, calculated on a weight basis, was 97.3%. There were two impurities with concentrations of 1.2% (assumed to be dithiane) and 1.5% (identity unknown). Material from this lot has been proposed as the standard analytical reference for USAMRDC and USAMRDC has agreed to retain aliquots of this material to comply with the requirements of Good Laboratory Practices (GLP).

A shipment of 25 ml of HD (in two ampules) was delivered on March 7, 1985 by a team from the U.S. Army Technical Escort Unit. The ampules were inspected and found to be intact. Subsequently the HD was transferred from the ampules into 30-ml Wheaton bottles, sealed and stored in secondary unbreakable containers in a refrigerated storage container at approximately 6°C.

Selection and Characterization of Diluent

Sulfur mustard is relatively insoluble (680 mg/L) and also is rapidly hydrolyzed in water; therefore, sesame oil was employed as the diluent for dosing solutions in this study. This selection was not only based on the chemical and physical properties of the compound, but also on the lack of a toxic response of the vehicle when introduced into the stomach of the animal. Corn oil is commonly the vehicle used for the administration of water-insoluble compounds; however, Hackett et al. (1987) concluded from data in the literature that corn oil may not be appropriate for reproductive studies because of its high steroid content and recommended using sesame oil in their studies of the teratogenicity of sulfur mustard. Sesame oil contains no preservatives, appears to be stable when stored under proper conditions, is relatively low in steroids and is readily available.

The sesame oil (Hain Pure Food Company, Los Angeles, CA) used in this study was purchased locally in one quart bottles and numbered according to lot and bottle. Peroxide analyses of each lot of sesame oil was performed at the beginning of the study or when purchased and periodically throughout the study to provide a measure of oxidation as an indication oil rancidity. The method measures the ability of the oil to oxidize aqueous iodide. Only oil in which the peroxide content was less than 10 meq/kg was used in the study. The results of the peroxide analyses of the sesame oil used are given in Table 3. The amount of peroxide in the sesame oil was well within the acceptable limits of 10 meq/kg set forth in the protocol.

TABLE 3. Analyses of Sesame Oil for Peroxide

Lot No.	Date Purchased	Assay Date	Container Number	Peroxide meq/kg
51564-6	10-06-86	03-02-87	11	8.1
51565-09	03-04-87	03-13-87	3	6.8
		05-01-87	12	7.9
51566-30	04-10-87	05-01-87	5	2.3
		06-29-87	12	2.6
51566-38	06-16-87	06-29-87	2	0.8
		11-24-87	12	0.7
51566-55	08-31-87	11-24-87	2	3.6
		11-24-87	12	3.5

Preparation of Solutions for Administration

The HD dosing solutions administered to the animals were prepared in advance and stored in a refrigerator at approximately 6°C for not more than 3 weeks. We have previously shown that HD is stable in the sesame oil for at least 3 weeks (Sasser et al., 1989). The general procedure was to determine in advance the amount of neat HD needed, based on the volumes to be prepared and the final concentrations desired. This volume was then removed

from the bottle of neat HD and thoroughly mixed into a known volume of sesame oil. Aliquots of this intermediate concentration were then diluted further to give the final concentration needed for the dosing solutions. Aliquots of the final solutions were placed in Wheaton bottles with teflon-lined sepa and aluminum caps. Each Wheaton bottle contained sufficient volume of HD-sesame oil for 1 day's use. The bottles were labeled with the name and the concentration of the agent (HD) and placed into a secondary unbreakable container which was identified by chemical name, concentration, lot number and date prepared.

Analytical Procedures

Methods were developed for the assay of HD in sesame oil by gas chromatography, using a capillary column and flame-ionization detection. The assay was complicated by the high boiling points of some components in sesame oil. As a result, the temperature of the capillary-column inlet had to be maintained at 200°C. The procedure consisted of diluting 0.50 ml of the HD-sesame oil sample with 0.50 ml of 18.7 ng/ul 2,4-dichlorotoluene (DCT) in isooctane, contained in a 1.5-ml automatic sampler vial with a Teflon-lined crimped-top cap. The DCT was used as an internal standard for the assay. A Hewlett-Packard 5840A gas chromatograph and 7672 automatic sample changer were used with a capillary DB-5 column (J & W Scientific, Folsom, CA). The method can detect as low as 0.01 mg/ml of HD.

Results of samples analyzed using this method are presented in Table 4. In most cases the theoretical and analyzed values were essentially the same especially at the higher concentrations. Some deviation between theoretical and analyzed values was seen at the low concentration. This may have resulted from a lack of precision of the method or could be the result of degradation by the sesame oil as the percentage of oil increased. When samples were repeatedly analyzed over a period of time, no evidence of degradation was seen up to 52 days (Sasser, 1989b).

ANIMAL MAINTENANCE

Male and female rats of Sprague-Dawley derivation were obtained from Charles River Laboratories, Inc., Raleigh, NC facility at 4 weeks of age and quarantined in isolation for about 3 weeks until a health evaluation was completed. The Sprague-Dawley rat was selected because it has been used in a number of previous reproductive studies at PNL including gavage studies of HD, thereby providing a data base for dose determinations. During quarantine the rats were group housed, separated by sex, in stainless-steel wire bottom cages placed on automatic flush racks with an automatic watering system. Untreated female rats used for breeding in the

Table 4. Sulfur Mustard Dose Levels and Solution Concentration for Samples Analyzed for Dominant Lethal Study.

Date Prepared	Date Analyzed	Dose Level (mg/kg)	HD Concentration (mg/ml) Theoretical	Analyzed
04-14-87	04-24-87	0.50	300	0.30
		0.20	120	0.12
		0.08	50	0.05
08-18-87	08-28-87	0.50	300	0.30
		0.20	120	0.14
		0.08	50	0.06
09-02-87	09-12-87	0.50	300	0.30
		0.20	120	0.12
		0.08	50	0.05

phase II male dominant lethal study were procured from the same source at 7 to 10 weeks of age.

The environmental conditions specified for the animal rooms were temperatures of 72±3°F, relative humidity of 50±15%, and a lighting cycle of 12 hours on and 12 hours off. Certified Rodent Chow (#5002, Ralson Purina®, St. Louis, MO.) and drinking water was provided *ad libitum*. Drinking water supplied to the animal rooms was passed through a reverse-osmotic purification unit containing two particle filters and a carbon filter.

Ten rats from each shipment were subjected to a health screen 2 to 3 weeks after receipt. The serum of each animal was tested for antibody titers to selected pathogens. Selected organs including lungs, liver, kidney, heart and intestine were histologically examined. There were no significant findings.

Following isolation the rats were weighed and randomly assigned to the appropriate treatment group by sex and weight by means of a formal randomization statistical package (see Statistical Methods). Each animal was assigned an individual identification number using a metal ear tag. The animals were individually housed in wire bottom cages on flush racks during

dosing and breeding phases of the experiments and cage cards were used to indicate the animal number, treatment group and dose. Indivdual animals were weighed immediately prior to initiation of chemical treatment and at weekly intervals throughout the dosing period. The rats were observed daily for signs of behavioral change, mortality and moribundity.

ADMINISTRATION OF SULFUR MUSTARD

Solutions of the appropriate concentration of HD in sesame oil were administered to the animals by intragastric intubation, 5 days per week for 10 weeks. The dominant lethal assay was modified to a 10-week exposure scheme to allow for chronic exposure conditions because of the expected high toxicity of HD. Animals were not dosed on holidays unless a minimum of 4 doses per week could not otherwise be achieved. Individual dose levels, based on the animal weight, were calculated weekly and the HD was administered in a constant volume of 1.67 ml/kg of body weight. Vehicle control animals were given an appropriate volume of sesame oil.

Dose levels selected for this study were based on data from several previous studies at PNL including two long-term studies. In a 13-week subchronic study, a dose of 0.3 mg/kg significantly reduced weight gain in both sexes compared to controls and produced lesions in the forestomach without any mortality. No HD related mortality occurred during a two-generation reproductive study at doses as great as 0.4 mg/kg, although weight gain was reduced and a dose-related forestomach lesions were observed (Sasser et al., 1989a). Since it was desired to select doses such that the highest dose induced toxicity but not mortality and the lowest dose was without effect, dose levels were set at 0.08, 0.2 and 0.5 mg/kg of body weight.

Oral exposure was specified by the sponsor for this study. The expected routes of environmental exposure are inhalation, dermal exposure, or ingestion, either direct or from swallowing inhaled material. Oral exposure was selected over inhalation, dermal application and subcutaneous or intraparietal injection for a number of reasons. It was considered impractical to expose by inhalation because of the potential hazards to personnel, technical aspects of generating the agent and the cost of a long-term inhalation exposure. Direct application to the animal was not desirous because of hazards incurred while handling the animals and the possible development of lesions after long-term exposure which could affect the translocation of material to the body. Injection of the material was ruled out because of the potential of local lesions from multiple injections of the agent.

EXPERIMENTAL DESIGN

The design of the studies in terms of treatment pairs and number of animals used is shown in Table 5. This study was conducted in two phases; 1) the female dominant lethal phase and 2) the male dominant lethal phase. Phase I evaluated female dominant lethal effects of exposed females mated to exposed and unexposed males as well as sperm morphology of selected males. Phase II evaluated the dominant lethal effects, and sperm morphology and function of the male rat.

Table 5. Breeding Regimen for Male and Female Rats.

	BREEDING GROUPS	
	FEMALES	MALES
Phase I	Vehicle Control	Vehicle Control
	0.08 mg/kg HD	Vehicle Control
	0.20 mg/kg HD	Vehicle Control
	0.50 mg/kg HD	Vehicle Control
	0.08 mg/kg HD	Untreated
	0.20 mg/kg HD	Untreated
	0.50 mg/kg HD	Untreated
	0.08 mg/kg HD	0.08 mg/kg HD
	0.20 mg/kg HD	0.20 mg/kg HD
	0.50 mg/kg HD	0.50 mg/kg HD
	75 mg/kg IMS[a]	Untreated
	100 mg/kg IMS	Untreated
Phase II	Untreated	Vehicle Control
	Untreated	0.08 mg/kg HD
	Untreated	0.20 mg/kg HD
	Untreated	0.50 mg/kg HD
	Untreated	100 mg/kg EMS[b]

[a]Isopropyl methanesulfonate
[b]Ethyl methanesulfonate

Female Dominant Lethality (Phase I)

Three groups of 8-week old Sprague-Dawley rats each consisting of 40 randomly selected females and 10 randomly selected males were administered 0.08, 0.20, or 0.5 mg/kg HD by gastric intubation for 5 days/week for ten weeks. Another 20 females and 40 males were used as vehicle controls and a fifth group of 20 females was treated with isopropyl methanesulfonate (IMS) as positive controls. After 10 weeks of gavaging with HD, 10 male and 10 female breeding pairs were randomly assigned to each of the 12 breeding groups described in Table 5, with the addition of 50 untreated males. To assess possible dominant lethal effects in female rats, a 19-day breeding interval was utilized. This interval is equivalent to approximately four to five estrus cycles.

Each female was caged overnight with the randomly-assigned male from the appropriate treatment group. The following morning, the females were examined for evidence of copulation by the presence of a vaginal plug or presence of sperm in vaginal lavage samples. This procedure was repeated daily for 19 days or until copulation was detected. If copulation was not evident after each 7-day breeding period, the male was replaced with another male from the same group.

The positive control animals were untreated until the end of the 10-week gavaging period when they were subdivided into two groups of 10 rats each and injected intraperitoneally with a single dose of either 75 or 100 mg of IMS per kg. Two days post injection, each female was mated during a 16-day breeding period to one untreated male as described above.

All pregnant animals were killed by CO_2 asphyxiation approximately 14 (13-16) days after copulation was detected. At necropsy, the uteri was examined for number of viable embryos and deciduoma and the number of ovarian corpora lutea was counted. Cytological evaluation of the estrus cycle of females showing no evidence of copulation after 14 days of cohabitation was performed for 7 days, but no dose-related effect on copulation was noted.

Ovaries were collected from the remaining 10 virgin control and 10 HD-treated females of each dose group at sacrifice immediately after termination of the 10-week dosing period. These tissues were stored in NBF for evaluation of ovarian function if evidence of dominant lethality were observed in female animals

Male Dominant Lethality (Phase II)

Following a 3-week quarantine period, 8-week old male Sprague-Dawley rats were divided into four groups of 20 randomly selected animals and treated with 0 (sesame oil), 0.08, 0.20, or

0.50 mg/kg of HD by gastric intubation 5 days/week for 10 weeks. The 10-week exposure period was chosen to permit a complete spermatogenic cycle. In addition, 20 randomly selected untreated males were intraperitoneally injected with 100 mg/kg of ethyl methanesulfonate (EMS) at the end of the 10-week treatment period. EMS is commonly used as a positive-control agent to induce dominant lethal mutations in males, but IMS is more effective and the recommended agent for females (Generoso and Russell, 1969: Generoso et al., 1971; Generoso, personal communications).

At the end of the treatment period, 10 male rats from each treatment group each cohabitated with two 9- to 10-week old untreated virgin female Sprague-Dawley rats for five days each week during a 10-week breeding period. The males were allowed a 2-day rest between breeding weeks to enhance subsequent matings; two new females were provided each week. The females were sacrificed 14 days after the midweek of cohabitation to determine the number and status of implants. The ovaries were removed and the corpora lutea counted Males were sacrificed 2 weeks after the completion of the 10-week breeding period for testicular and sperm morphology evaluations. This recovery period allowed the recovery of sperm production to that of non-breeding status.

The remaining 10 male rats from each treatment group were used for sperm morphology or histological studies. At the end of the treatment period, 10 rats each from the vehicle control and the 0.50 mg/kg groups were sacrificed for sperm morphology evaluations. Testes and epididymides of the 10 rats from the 0.08 and 0.20 mg/kg groups were fixed in Bouins solution for future histological examination. Ten positive controls were sacrificed 35 days after EMS treatment, the time of maximum response for EMS.

Sperm Morphology and Motility

Sperm motility and sperm head morphology was examined in high dose(0.50 mg/kg) and in vehicle conrol rats immediately after the 10-week exposure and at 5 and 12 weeks post-dosing. Positive control animals were similarly studied at 5 weeks post-dosing. Animals examined immediately after dosing had not been used for breeding; animals examined 12 weeks after termination of dosing were mated to untreated females over a period of 70 days in the male dominant lethal portion of the study, but were rested about 3 weeks before examination.

Immediately after sacrifice, sperm were obtained from the cut distal end of an excised cauda epididymidis and suspended in 3 ml of warm (37°C) egg yolk buffer for motility evaluation. After [illegible] cover application, motile and non-motile sperm were counted in five fields by two technicians. The remaining cauda was minced in

10 ml of warm phosphate-buffered saline (PPS) for 15 minutes. One half ml of the mixed suspension was added to 2 ml of PBS, agitated gently and thoroughly mixed by repeated pipetting. The sperm were heat killed and counted on a hemacytometer. For sperm morphology studies, 1 ml of sperm suspension was transferred to a test tube and stained with 2 drops of eosin Y for 45-60 minutes. Four slides were prepared for each animal and allowed to dry overnight; then coverslips applied. The morphology of at least 500 sperm from each slide was examined under a light microscope and classified as either normal or abnormal. The following types of abnormal sperm were recorded: blunthook, banana, amorphous, pin head, two heads and short sperm-heads (Wyrobek and Bruce, 1975).

STATISTICAL METHODS

The PNL derived computer software program for randomizing animals into experimental groups is based on a single blocking factor for animal weight. Animal weights were ordered from lightest to heaviest: blocks of animal weights were then randomly assigned to the treatment groups and the control group. Block sizes are governed by the number of test groups.

The SAS statistical software and a VAX 11/780 computer were used to calculate all means and standard errors of animal data. Body weights data were analyzed with an analysis of variance model (ANOVA) for unbalanced data. The dose-response relationship of growth data was determined by use of an orthogonal trend test (Zerbe, 1979). The number of implantation sites and intrauterine deaths per litter for each week was analyzed by analysis of variance. When appropriate, proportional data were subjected to arcsin transformations and evaluated by ANOVA (e.g. percentage incidence of resorptions and dead or live fetuses per implant). If the F statistic from the analysis of variance was significant, the Tukey-Kramer multiple comparison test was used to delineate intergroup differences (Tukey, 1953; Kramer, 1957). Dose-response trends were determined by means of orthogonal contrasts (Winer,1971). Values for normal and abnormal sperm heads were expressed as a percentage of the total number of sperm examined for each animal and analyzed by analysis of variance after arcsine transformation as described above. Fertility data were analyzed by the Fisher-Exact test (Snedecor and Cochran, 1980). The litter was used as the basis for analysis of all fetal variables.

RESULTS

No deaths attributable to HD occurred in any of the exposure groups of either phase of the study. The appearance and behavior of female and male animals treated with HD were unremarkable throughout the study except for occasional drooling following dosing by the animals in the 0.50 mg/kg group. No significant gross lesions of the reproductive organs were observed in the males at sacrifice.

Female Dominant Lethality (Phase I)

The mean weekly body weights of the HD-treated female and male rats are presented in Table 6. The rats readily adapted to the exposure environment as control animals of both sexes maintained a typical growth rate during the study (Figure 1). Trend analysis of growth curves showed a significant difference ($P<0.05$) between control females and treated females independent of dose although there were no significant difference among groups based on weekly values. Weekly weights of the 0.50 mg/kg male rats were significantly decreased ($P<0.05$) compared to control values; trend analysis of the growth curves also showed a significant difference between these two groups of animals.

An overall mean pregnancy rate of 86% was achieved; treatment means ranged from 70 to 100 % with no significant differences between treatment groups (Table 7). Indicators of reproductive performance (i.e. number of implants or incidence of intrauterine death) in treated female rats mated to treated or non-treated males did not demonstrate significant differences from control data (Table 7). The number of live implants was consistently greater than 11 fetuses per litter with no indication of a dose-response relationship. Futhermore, there was no increase in the number of dead implants or early resorptions (on a litter basis) associated with treatment. Preimplantation losses were not significantly affected by HD exposure. Isopropyl methanesulfonate was shown to be an effective positive-control agent at the doses used as evidenced by a significant reduction in the number of total and live implants, number of total and number of early resorptions, (75 and 100 mg/kg) and number of preimplantation losses (100 mg/kg) (Table 7).

Male Dominant Lethalilty (Phase II)

The mean weekly body weights of males exposed to 0.50 mg/kg HD were significantly decreased ($P<0.05$) during the 10-week exposure period, beginning after the first week, compared to controls (Table 8). Although the growth rate of the 0.05 mg/kg group did not appear to change until week 13 or 14, the significant effect between treatment groups was lost at week 10 when dosing was

TABLE 6. Weekly Body Weights of Female and Male Rats Exposed to HD (Phase I, Mean ±SE).

Weeks	Sulfur Mustard Dose (mg/kg) 0.0	0.08	0.20	0.50
		FEMALE		
N	20	40	40	40
0	186±3.1	187±1.7	186±1.8	184±1.9
1	210±3.5	206±2.1	203±2.0	198±2.1
2	229±3.2	226±2.2	222±2.4	215±2.8
3	249±4.2	243±2.7	240±2.5	234±2.9
4	263±4.4	254±3.1	251±2.9	250±3.2
5	275±4.6	265±2.9	266±3.2	262±3.7
6	289±6.1	277±3.4	275±3.2	277±3.7
7	296±5.7	285±3.6	281±3.2	284±3.9
8	300±5.7	287±3.6	287±3.3	288±4.0
9	307±5.9	294±3.9	294±3.3	296±4.0
10	300±7.9	301±4.5	303±4.4	302±5.5
		MALE		
N	40	10	10	10
0	274±1.6	274±03.9	274±04.7	275±5.1
1	330±2.5	323±04.8	321±06.2	304±5.0*
2	377±3.3	371±05.5	366±07.7	325±6.0*
3	417±4.4	409±07.3	408±09.1	369±6.7*
4	449±4.6	432±08.0	440±11.0	406±7.1*
5	477±5.4	463±07.8	465±11.9	428±8.4*
6	500±6.0	486±09.0	494±13.4	454±8.4*
7	522±6.6	510±08.6	508±18.5	469±8.3*
8	542±7.3	526±09.1	536±15.3	491±9.1*
9	560±7.6	538±10.6	546±16.3	506±8.7*
10	564±7.6	549±09.4	549±16.1	509±8.2*

*Significantly different from control values ($P<0.05$).

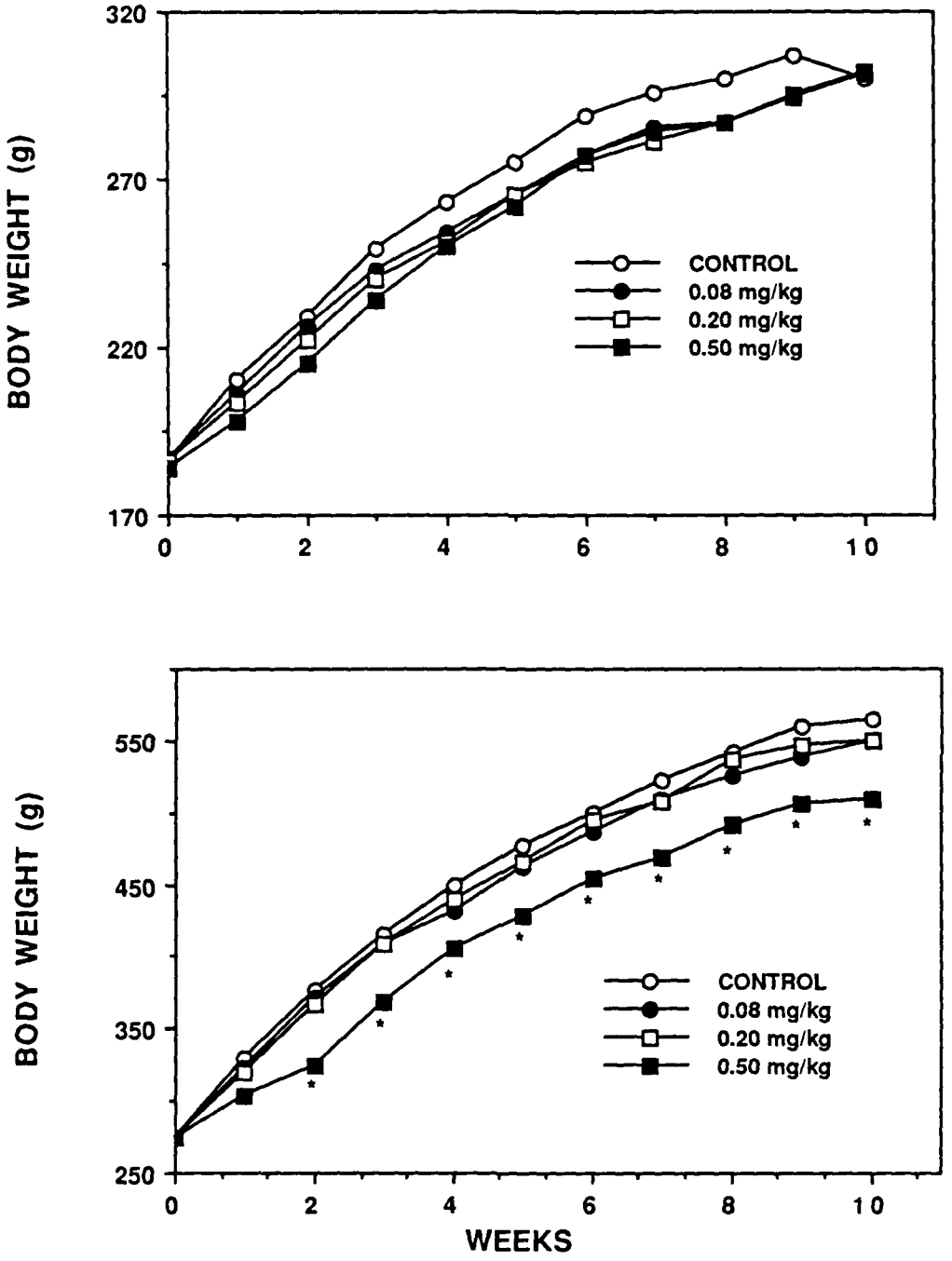

Figure 1. Growth curves of female (upper) and male (lower) rats exposed to 0, 0.08, 0.20 or 0.50 mg/kg HD. *Significantly different from control ($P<0.05$).

TABLE 7 . Reproductive Measures for the Female Dominant Lethality Study (Phase I, Mean ± SE).

Treatment	HD				HD			HD			IMS	
Male Dose mg/kg)	Vehicle Controls				Untreated			0.08	0.20	0.50	Untreated	
Female Dose (mg/kg)	0.00	0.08	0.20	0.50	0.08	0.20	0.50	0.08	0.20	0.50	75	100
No. Males\Females	10\10	10\10	10\10	10\10	10\10	10\10	10\10	10\10	10\10	10\10	10\10	10\10
Pregnant females												
Number	9	9	9	7	9	9	10	7	10	8	7	9
Percent	90	90	90	70	90	90	100	70	100	80	70	90
No. per litter												
Total Implants	14.33 ±0.73	15.00 ±0.78	13.33 ±1.37	14.43 ±1.65	15.56 ±0.65	16.44 ±0.44	15.00 ±0.92	12.57 ±2.55	15.30 ±0.50	13.75 ±1.98	9.00 * ±2.94	8.33 * ±1.56
Live Implants	13.11 ±0.84	14.22 ±0.83	12.00 ±1.41	12.71 ±2.33	13.00 ±1.3	15.44 ±0.88	13.90 ±0.91	11.86 ±2.37	14.00 ±0.68	11.88 ±1.87	3.14 * ±1.70	4.22 * ±1.10
Total Resorptions	1.22 ±0.55	0.78 ±0.28	1.33 ±0.47	1.71 ±0.92	2.56 ±1.24	1.00 ±0.55	1.10 ±0.35	0.71 ±0.36	1.30 ±0.33	1.88 ±0.58	5.86 * ±1.55	4.11 * ±0.72
Early Resorptions	0.89 ±0.51	0.67 ±0.29	0.33 ±0.17	1.43 ±0.95	2.11 ±1.16	0.78 ±0.55	0.30 ±0.21	0.43 ±0.30	0.80 ±0.29	0.75 ±0.49	4.86 * ±1.52	3.56 * ±0.88
Late Resorptions	0.33 ±0.33	0.11 ±0.11	1.00 ±0.41	0.29 ±0.18	0.44 ±0.18	0.22 ±0.15	0.80 ±0.36	0.29 ±0.29	0.50 ±0.22	1.13 ±0.55	1.00 ±0.72	0.56 ±0.34
No. of Litters with												
Live Implants	9	9	9	6	9	9	10	7	10	8	3 *	8
Early Resorptions	3	4	3	4	6	3	2	2	6	3	6	7
Pre - implant Loss	2	1	5	2	2	1	2	3	2	3	3	9 *

TABLE 7. (Continued)

Percentage of Live Fetuses/litter	91.57 ±3.81	94.78 ±1.91	89.80 ±3.74	81.49 ±13.68	83.79 ±7.74	93.40 ±3.88	92.69 ±2.61	95.71 ±2.20	91.18 ±2.38	87.57 ±3.78	18.04 ±9.50	* 43.89 ±8.51 *
Resorptions/ litter												
Total	8.43 ±3.81	5.22 ±1.91	10.20 ±3.74	18.51 ±13.68	16.21 ±7.74	6.60 ±3.88	7.31 ±2.61	4.29 ±2.20	8.82 ±2.38	12.43 ±3.78	81.96 ±9.50	* 56.11 ±8.51 *
Early	5.65 ±3.27	4.43 ±1.97	2.48 ±1.25	16.87 ±13.90	13.52 ±7.31	5.33 ±3.90	1.56 ±1.09	2.38 ±1.65	5.38 ±1.86	5.25 ±3.50	64.11 ±15.56	* 42.14 ±9.54 *
Late	2.78 ±2.78	0.79 ±0.79	7.72 ±3.46	1.64 ±1.07	2.69 ±1.08	1.27 ±0.84	5.75 ±2.76	1.90 ±1.90	3.44 ±1.58	7.18 ±3.38	17.86 ±14.14	13.97 ±10.93
Litters with												
Live implants	100	100	100	86	100	100	100	100	100	100	43 *	89
Early Resorptions	33	44	22	57	67	44	20	29	60	38	86 *	78
Pre - implant loss	22	11	44	29	22	11	20	43	20	38	43	100*

*Significantly different from control value (<0.05).

TABLE 8. Weekly Body Weights of Male Rats During Exposure and Breeding Periods (Phase II, Mean ±SE).

Week	Sulfur Mustard Dose (mg/kg) 0.0	0.08	0.20	0.50	EMS (mg/kg) 100.0
0	287.9±03.5	291.7±03.4	289.1±05.6	287.0±03.4	292.2±03.8
1	341.9±04.6	341.8±04.8	335.8±05.6	320.8±04.5 *	337.4±07.1
2	386.8±05.1	384.9±05.5	378.1±06.1	343.8±04.9 *	382.5±05.3
3	421.4±06.8	419.5±05.8	413.5±06.4	379.7±06.0 *	421.7±05.7
4	450.4±07.4	451.1±06.4	440.5±07.1	408.3±06.7 *	447.3±06.4
5	473.9±08.2	476.5±07.3	466.2±07.2	435.2±07.3 *	471.4±06.4
6	498.1±09.0	500.4±08.1	487.3±08.4	451.2±08.4 *	495.0±06.7
7	519.3±09.9	524.5±08.4	509.8±08.3	472.9±09.1 *	512.6±07.0
8	532.4±11.1	539.5±08.7	525.1±08.6	486.6±09.6 *	529.5±07.0
9	546.6±11.8	552.2±09.2	537.1±08.1	495.0±09.6 *	544.3±07.5
10#	533.2±16.3	559.3±16.2	542.9±13.1	506.7±12.9	523.8±07.9
11	534.9±16.0	561.4±14.8	545.2±13.5	520.4±13.4	525.6±08.2
12	538.4±15.4	568.4±16.4	557.4±12.9	537.0±13.6	533.8±08.4
13	552.5±15.9	586.4±16.9	577.3±13.3	560.1±13.3	544.4±09.7
14	561.6±15.3	594.9±17.9	587.4±13.8	569.0±14.7	558.0±09.1
15	570.5±16.6	603.8±17.2	598.4±14.9	579.8±13.6	560.5±13.2
16	577.0±18.7	609.0±20.6	604.2±15.4	589.9±15.0	568.7±13.3
17	583.4±17.8	637.0±17.9	613.3±16.4	605.3±15.5	576.9±14.0
18	595.0±18.3	646.6±18.7	622.8±16.8	611.6±15.4	588.9±14.9
19	604.1±19.0	656.6±18.0	633.0±17.9	618.8±17.4	600.4±17.6
20	608.5±19.9	661.7±19.2	640.8±19.4	631.1±17.8	606.6±18.5

*Significantly different from control value ($P<0.05$).

#Final week of HD dosing; beginning of breeding period. EMS was injected at the end of the 10-week period.

discontinued (Figure 2).

An overall mean pregnancy rate of 91% was achieved; weekly means across all treatments ranged from 65 to 100% with no significant differences between treatment groups (Table 9a-9j). The average number of live implants was consistently greater than 13 fetuses per litter with no indication of a dose-response relationship. Indicators of reproductive performance (i.e. number of implants or incidence of intrauterine death) in untreated female rats mated to HD-treated males for each of the 10 post-exposure weeks demonstrated significant differences among exposure groups at weeks 2 and 3 only (Tables 9b and 9c), although a trend for a significant increase in number of late resorptions was observed at week 1 (Table 9a). During the 2nd and 3rd post-exposure weeks the mean number of total and early resorptions per litter was significantly greater ($P<0.05$) than that of controls for the 0.50-mg/kg group. The number of total and late resorptions for the 0.08-mg/kg group was also greater than controls at the 3rd week. Preimplantation losses were also significantly elevated at week 2 ($P<0.05$) for the mid and high dose groups; this difference was only suggestive at week 3 except for the 0.08 mg/kg dose group.

Arcsine transformation of the proportional data demonstrated this response even more dramatically. A significant dose-related increase ($P<0.05$) for percentage of live implants and a dose-dependent decrease ($P<0.05$) for percentage of total and early resorptions were observed at 2 weeks post-exposure. Even though the results were not dose dependent at week 3, significant differences ($P<0.05$) were demonstrated for percentage of live fetuses and percentage of early and total resorptions for the 0.08- and 0.50-mg/kg dosage groups. Except for infrequent occurrences, significant differences were not observed at week 1, and weeks 4 through 10 (Tables 9a and 9d-9j).

EMS (100mg/kg) was shown to be an effective positive-control agent during the first 4 weeks post-exposure as evidenced by a significant ($P<0.05$) reduction in the number of live implants and preimplantation losses and increase in number of resorptions in untreated females mated to treated males (Table 9a-9d).

Analyses of sperm morphology data obtained from male rats at 0, 5 and 12 weeks post-exposure showed a statistically significant decrease ($P< 0.05$) in the percentages of normal sperm for the 0.50 mg/kg group relative to the control group (Table 10). Complementing these results was a highly significant increase ($P<0.05$) in the percentage of abnormal sperm at all three time points. Blunthook and banana shaped sperm were consistently observed at 0, 5 and 12 weeks, whereas amorphous and short head abnormalities were observed only at 5 or 12 weeks. EMS reduced the percentage of normal sperm and increased the percentage of

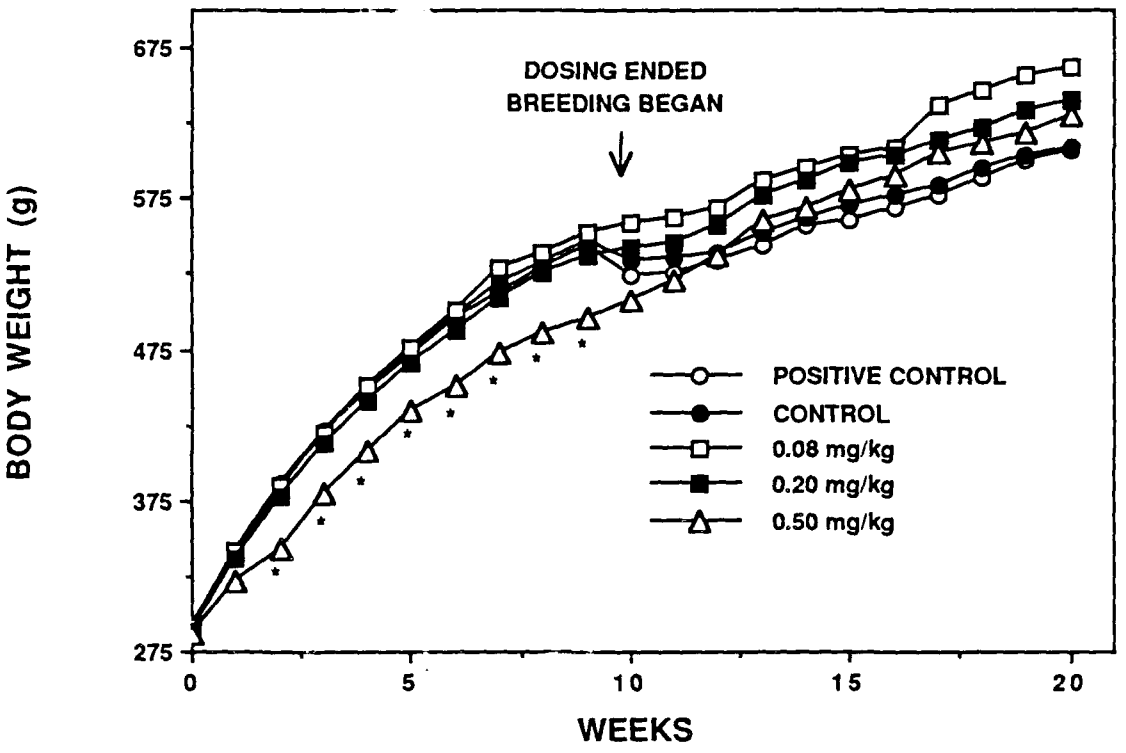

Figure 2. Growth curves of male rats during 10-week exposure and 10-week breeding periods (Phase II). (*Significantly different from control values, $P<0.05$).

blunthook and total abnormal sperm.

Statistical differences were not demonstrated for testis or epididymis weight (absolute or as a percentage of body weight), even though body weights were significantly depressed ($P<0.05$) at post-exposure week 0 by the 0.50 mg/kg dose level. Neither percent motile sperm nor sperm concentration was detrimentally affected by HD exposure.

TABLE 9a . Reproductive Measures for the Male Dominant Lethality Study (Phase II, Mean ± SE).

Post-Exposure Week 1	Sulfur Mustard Dose (mg/kg)				EMS (mg/kg)
	0.00	0.08	0.20	0.50	100.00
No. Males	10	10	10	10	10
Pregnant females					
Number	16	17	19	20	16
Percent	80	85	95	100	80
No. per litter					
Total Implants	14.8±0.8	16.3±0.4	15.4±0.4	16.1±0.5	15.1±0.5
Live Implants	14.0±0.8	15.5±0.4	13.9±0.6	14.3±0.7	11.3±0.6*
Total Resorptions	0.81±0.21	0.82±0.32	1.47±0.47	1.80±0.39	3.75±0.62*
Early Resorptions	0.63±0.15	0.35±0.21	0.79±0.37	0.85±0.30	3.00±0.68*
Late Resorptions	0.19±0.14	0.47±0.24	0.68±0.22	0.95±0.37	0.75±0.41
No. of Litters with					
Live Implants	16	17	19	20	16
Early Resorptions	9	3	5	8	13
Preimplantation losses	10	6	10	14	15*
Percentage of					
Live Fetuses/litter	93.7±2.6	95.2±1.9	90.5±2.9	88.2±2.9	75.4±3.9*
Resorptions/ litter					
Total	6.4±2.6	4.8±1.9	9.5±2.9	11.8±2.9	24.6±3.9*
Early	4.2±1.1	2.2±1.3	5.1±2.3	4.9±1.8	18.8±4.2*
Late	2.2±1.8	2.6±1.3	4.4±1.4	6.9±2.9	5.7±3.2
Litters with					
Live implants	100	100	100	100	100
Early Resorptions	56	18	26	40	81
Preimplantation losses	63	35	53	70	94

*Significantly different from control value (P<0.05).

TABLE 9b. Reproductive Measures for the Male Dominant Lethality Study (Phase II, Mean ± SE).

Post-Exposure Week 2	Sulfur Mustard Dose (mg/kg)				EMS (mg/kg)
	0.00	0.08	0.20	0.50	100.00
No. Males	10	10	10	10	10
Pregnant females					
Number	18	16	19	20	19
Percent	90	80	95	100	95
No. per litter					
Total Implants	15.8±0.7	16.2±0.3	16.0±0.7	15.9±0.4	12.7±0.8*
Live Implants	15.4±0.7	15.1±0.5	14.5±0.7	14.1±0.5	4.2±1.0*
Total Resorptions	0.39±0.16	1.06±0.40	1.42±0.27	1.75±0.43*	8.58±0.98*
Early Resorptions	0.28±0.14	0.75±0.30	1.11±0.21	1.40±0.35*	8.37±0.97*
Late Resorptions	0.11±0.08	0.31±0.25	.32±0.15	0.35±.0.13	0.21±0.10
No. of Litters with					
Live Implants	18	16	19	20	16
Early Resorptions	4	7	13 *	13 *	17*
Preimplantation losses	5	7	14 *	15 *	17*
Percentage of					
Live Fetuses/litter	97.7±1.0	93.4±2.5	91.5±1.6	89.2±2.5*	32.9±7.5*
Resorptions/ litter					
Total	2.3±1.0	6.6±2.5	8.5±1.6	10.8±2.5*	67.1±7.5*
Early	1.7±0.8	4.5±1.7	6.8±1.4	8.7±2.0*	65.0±7.2*
Late	0.7±0.5	2.1±1.7	1.8±0.9	2.1±0.8	2.1±1.1
Litters with					
Live implants	100	100	100	100	84
Early Resorptions	22	44	68	65	89
Preimplantation losses	28	44	74	75	89

*Significantly different from control value ($P<0.05$).

TABLE 9c. Reproductive Measures for the Male Dominant Lethality Study (Phase II, Mean ± SE).

Post-Exposure Week 3	Sulfur Mustard Dose (mg/kg)				EMS (mg/kg)
	0.00	0.08	0.20	0.50	100.00
No. Males	10	10	10	10	10
Pregnant females					
Number	17	17	18	17	18
Percent	85	85	90	85	90
No. per litter					
Total Implants	15.8±0.4	15.8±0.8	15.2±0.4	14.8±0.5	7.4±0.6
Live Implants	15.4±0.3	14.3±0.9	14.3±0.4	13.5±0.7	0.1±0.1*
Total Resorptions	0.41±0.12	1.47±0.31*	0.94±0.21	1.29±0.31*	7.28±0.56*
Early Resorptions	0.18±0.10	0.76±0.20	0.67±0.20	1.24±0.32*	6.39±0.79*
Late Resorptions	0.24±0.11	0.71±0.17*	0.28±0.14	0.06±0.06	0.89±0.51
No. of Litters with					
Live Implants	17	17	18	17	2 *
Early Resorptions	3	10 *	8	11 *	16 *
Preimplantation losses	7	13 *	11	12	18 *
Percentage of					
Live Fetuses/litter	97.5±0.8	90.7±2.0*	93.7±1.4	90.8±2.4*	1.1±0.8*
Resorptions/ litter					
Total	2.5±0.8	9.3±2.0*	6.3±1.4	9.2±2.4*	98.9±0.8*
Early	1.1±0.6	4.9±1.4*	4.5±1.4	8.8±2.4*	85.1±7.8*
Late	1.4±0.6	4.4±1.0*	1.8±0.9	0.4±0.4	13.8±7.5*
Litters with					
Live implants	100	100	100	100	11
Early Resorptions	18	59	44	65	89
Preimplantation losses	41	76	61	71	100

*Significantly different from control value ($P<0.05$).

TABLE 9d. Reproductive Measures for the Male Dominant Lethality Study (Phase II, Mean ± SE).

Post-Exposure Week 4	Sulfur Mustard Dose (mg/kg)				EMS (mg/kg)
	0.00	0.08	0.20	0.50	100.00
No Males	10	10	10	10	10
Pregnant females					
Number	17	20	18	19	18
Percent	85	100	90	95	90
No. per litter					
Total Implants	15.9±0.4	16.2±0.5	16.5±0.4	16.0±0.4	10.9±1.3*
Live Implants	15.4±0.4	15.1±0.5	15.2±0.7	14.9±0.4	3.8±1.3*
Total Resorptions	0.47±0.21	1.10±0.37	1.33±0.47	1.05±0.19	7.17±1.05*
Early Resorptions	0.12±0.08	0.65±0.18	0.94±0.46	0.74±0.17	3.94±0.88*
Late Resorptions	0.35±0.21	0.45±0.29	0.39±0.12	0.32±0.13	3.22±1.13*
No. of Litters with					
Live Implants	17	20	18	19	9
Early Resorptions	2	9*	7	11*	15*
Preimplantation losses	5	11	11	13*	18*
Percentage of					
Live Fetuses/litter	97.1±1.3	93.5±2.0	91.8±3.1	93.4±1.3	25.8±8.1*
Resorptions/ litter					
Total	2.9±1.3	6.6±2.0	8.2±3.1	6.6±1.3	74.2±8.1*
Early	0.8±0.5	4.0±1.1	5.9±3.0	4.5±1.0	47.1±9.3*
Late	2.1±1.3	2.6±1.6	2.3±0.7	2.1±1.0	27.1±8.7*
Litters with					
Live implants	100	100	100	100	50
Early Resorptions	12	45	39	58	83
Preimplantation losses	29	55	61	68	100

*Significantly different from control value ($P<0.05$).

TABLE 9e. Reproductive Measures for the Male Dominant Lethality Study (Phase II, Mean ± SE).

Post-Exposure Week 5	Sulfur Mustard Dose (mg/kg)				EMS (mg/kg)
	0.00	0.08	0.20	0.50	100.00
No. Males	10.00	10.00	10.00	10.00	10.00
Pregnant females					
Number	19	19	18	20	18
Percent	95	95	90	100	90
No. per litter					
Total Implants	15.7±0.9	16.4±0.5	16.3±0.5	15.8±0.7	15.6±0.4
Live Implants	15.0±0.9	15.3±0.6	15.0±0.5	14.8±0.7	13.8±0.6
Total Resorptions	0.74±0.15	1.11±0.25	1.28±0.27	1.00±0.18	1.78±0.47*
Early Resorptions	0.37±0.16	0.63±0.21	0.50±0.17	0.55±0.18	1.22±0.46
Late Resorptions	0.37±0.11	0.47±0.16	0.78±0.26	0.45±0.15	0.56±0.18
No. of Litters with					
Live Implants	19	19	18	20	18
Early Resorptions	5	8	7	7	8
Preimplantation losses	12	12	13	14	12
Percentage of					
Live Fetuses/litter	95.3±1.0	93.1±1.7	92.3±1.6	93.8±1.1	88.7±3.1
Resorptions/ litter					
Total	4.7±1.0	7.0±1.7	7.8±1.6	6.2±1.1	11.3±3.1
Early	2.3±1.0	4.0±1.3	2.8±0.9	3.5±1.2	7.8±3.1
Late	2.4±0.8	3.0±1.0	5.0±1.6	2.7±0.9	3.5±1.2
Litters with					
Live implants	100	100	100	100	100
Early Resorptions	26	42	39	35	44
Preimplantation losses	63	63	72	70	67

*Significantly different from control value ($P<0.05$).

TABLE 9f. Reproductive Measures for the Male Dominant Lethality Study (Phase II, Mean ± SE).

Post-Exposure Week 6	Sulfur Mustard Dose (mg/kg)				EMS (mg/kg)
	0.00	0.08	0.20	0.50	100.00
No. Males	10	10	10	9	10
Pregnant females					
Number	17	18	18	13	15
Percent	85	90	90	65	75
No. per litter					
Total Implants	15.8±0.4	15.6±0.4	15.4±0.7	15.1±0.5	15.9±0.3
Live Implants	14.9±0.6	14.4±0.5	14.4±0.7	14.2±0.7	15.0±0.4
Total Resorptions	0.88±0.28	1.11±0.27	1.06±0.3	0.85±0.27	0.93±0.23
Early Resorptions	0.41±0.19	0.72±0.21	0.39±0.23	0.62±0.21	0.20±0.14
Late Resorptions	0.47±0.17	0.39±0.20	0.67±0.18	0.23±0.12	0.73±0.23
No. of Litters with					
Live Implants	17	18	18	13	15
Early Resorptions	5	9	4	6	2
Preimplantation losses	9	12	11	7	10
Percentage of					
Live Fetuses/litter	93.8±2.3	92.7±1.8	93.4±1.7	93.9±2.0	94.0±1.5
Resorptions/ litter					
Total	6.2±2.3	7.3±1.8	6.6±1.7	6.1±2.0	6.0±1.5
Early	3.0±1.6	4.9±1.5	2.3±1.3	4.5±1.6	1.3±1.0
Late	3.2±1.2	2.4±1.3	4.3±1.2	1.5±0.8	4.6±1.5
Litters with					
Live implants	100	100	100	100	100
Early Resorptions	29	50	22	46	13
Preimplantation losses	53	67	61	54	67

TABLE 9g. Reproductive Measures for the Male Dominant Lethality Study (Phase II, Mean ± SE).

Post-Exposure Week 7	Sulfur Mustard Dose (mg/kg)				EMS (mg/kg)
	0.00	0.08	0.20	0.50	100.00
No. Males	10	10	10	10	10
Pregnant females					
Number	20	19	19	20	19
Percent	100	95	95	100	95
No. per litter					
Total Implants	16.9±0.4	15.6±0.4	17.2±0.4	14.4±0.8*	15.9±0.3
Live Implants	16.0±0.5	14.6±0.5	16.5±0.4	13.6±0.8*	14.8±0.5
Total Resorptions	0.85±0.26	1.00±0.25	0.68±0.22	0.80±0.21	1.05±0.21
Early Resorptions	0.25±0.10	0.53±0.18	0.37±0.16	0.55±0.17	0.37±0.14
Late Resorptions	0.60±0.21	0.47±0.16	0.32±0.15	0.25±0.12	0.68±0.19
No. of Litters with					
Live Implants	20	19	19	20	19
Early Resorptions	5	7	5	8	6
Preimplantation losses	11	12	8	10	14
Percentage of					
Live Fetuses/litter	95.1±1.6	93.6±1.6	96.1±1.3	94.6±1.4	93.2±1.4
Resorptions/ litter					
Total	5.0±1.6	6.4±1.6	3.9±1.3	5.4±1.4	6.9±1.4
Early	1.4±0.6	3.4±1.1	2.1±0.9	3.7±1.1	2.5±0.9
Late	3.5±1.2	3.1±1.0	1.8±0.9	1.6±0.8	4.4±1.2
Litters with					
Live implants	100	100	100	100	100
Early Resorptions	25	37	26	40	32
Preimplantation losses	55	63	42	50	74

*Significantly different from control value (<0.05).

TABLE 9h. Reproductive Measures for the Male Dominant Lethality Study (Phase II, Mean ± SE).

Post-Exposure Week 8	Sulfur Mustard Dose (mg/kg)				EMS (mg/kg)
	0.00	0.08	0.20	0.50	100.00
No. Males	10	9	10	10	10
Pregnant females					
Number	19	16	19	20	17
Percent	95	89	95	100	85
No. per litter					
Total Implants	15.4±0.3	16.6±0.6	16.5±0.4	17.0±0.4*	16.5±0.5
Live Implants	14.8±0.4	15.5±0.5	15.8±0.4	15.7±0.3	15.1±0.6
Total Resorptions	0.58±0.19	1.13±0.30	0.74±0.21	1.35±0.15	1.29±0.28*
Early Resorptions	0.16±0.09	0.44±0.16	0.26±0.15	0.35±0.13	0.35±0.17
Late Resorptions	0.42±0.18	0.69±0.28	0.47±0.18	1.00±0.19	1.00±0.26
No. of Litters with					
Live Implants	19	16	19	20	17
Early Resorptions	3	6	3	6	4
Preimplantation losses	8	10	8	19 *	12
Percentage of					
Live Fetuses/litter	96.1±1.4	93.6±1.7	95.6±1.3	92.1±0.8*	91.6±1.9
Resorptions/ litter					
Total	3.9±1.4	6.4±1.7	4.4±1.3	7.9±0.8*	8.1±1.8
Early	1.0±0.5	2.6±0.9	1.6±0.9	2.0±0.8	2.2±1.1
Late	2.9±1.3	3.8±1.6	2.8±1.0	5.9±1.1	6.2±1.6
Litters with					
Live implants	100	100	100	100	100
Early Resorptions	16	38	16	30	24
Preimplantation losses	42	63	42	95	71

*Significantly different from control value ($P<0.05$).

TABLE 9i. Reproductive Measures for the Male Dominant Lethality Study (Phase II, Mean ± SE).

Post-Exposure Week 9	Sulfur Mustard Dose (mg/kg)				EMS (mg/kg)
	0.00	0.08	0.20	0.50	100.00
No. Males	10	9	10	10	10
Pregnant females					
Number	19	16	17	18	18
Percent	95	89	85	90	90
No. per litter					
Total Implants	16.0±0.8	14.9±1.2	16.2±0.7	16.6±0.3	15.5±0.3
Live Implants	15.1±0.8	13.5±1.2	15.5±0.7	15.7±0.3	14.6±0.3
Total Resorptions	0.84±0.27	1.38±0.27	0.71±0.21	0.94±0.24	0.89±0.27
Early Resorptions	0.63±0.23	0.75±0.23	0.53±0.21	0.44±0.17	0.67±0.28
Late Resorptions	0.21±0.10	0.63±0.27	0.18±0.10	0.50±0.20	0.22±0.10
No. of Litters with					
Live Implants	19	16	17	18	18
Early Resorptions	7	8	6	6	6
Preimplantation losses	9	13	9	11	10
Percentage of					
Live Fetuses/litter	95.2±1.5	88.6±3.1	95.6±1.3	94.4±1.3	94.5±1.6
Resorptions/ litter					
Total	4.9±1.5	11.4±3.1	4.4±1.3	5.6±1.3	5.5±1.6
Early	3.7±1.3	4.8±1.5	3.4±1.4	2.6±1.0	4.1±1.7
Late	1.2±0.6	6.6±3.4	1.1±0.6	3.0±1.2	1.4±0.7
Litters with					
Live implants	100	100	100	100	100
Early Resorptions	37	50	35	33	33
Preimplantation losses	47	81	53	61	56

TABLE 9j. Reproductive Measures for the Male Dominant Lethality Study (Phase II, Mean ± SE).

Post-Exposure Week 10	Sulfur Mustard Dose (mg/kg)				EMS (mg/kg)
	0.00	0.08	0.20	0.50	100.00
No. Males	10	9	10	10	10
Pregnant females					
Number	19	15	18	19	18
Percent	95	83	90	95	90
No. per litter					
Total Implants	16.5±0.4	14.2±1.0	16.1±1.0	15.4±1.2	16.3±0.6
Live Implants	15.5±0.6	12.7±0.9	15.1±1.0	14.5±1.2	15.1±0.8
Total Resorptions	1.05±0.31	1.53±0.38	0.94±0.26	0.89±0.24	1.22±0.26
Early Resorptions	0.53±0.21	0.87±0.29	0.28±0.11	0.47±0.19	0.78±0.27
Late Resorptions	0.53±0.18	0.67±0.25	0.67±0.27	0.42±0.19	0.44±0.15
No. of Litters with					
Live Implants	19	15	18	19	18
Early Resorptions	6	7	5	6	8
Preimplantation losses	9	10	11	10	14
Percentage of					
Live Fetuses/litter	93.1±2.2	90.3±2.4	94.5±1.5	92.3±2.8	90.6±3.1
Resorptions/ litter					
Total	6.9±2.2	9.7±2.4	5.5±1.5	7.7±2.8	9.4±3.1
Early	3.6±1.5	5.5±1.8	1.6±0.6	5.2±2.8	6.7±3.3
Late	3.3±1.1	4.2±1.6	3.9±1.6	2.5±1.2	2.7±0.9
Litters with					
Live implants	100	100	100	100	100
Early Resorptions	32	47	28	32	44
Preimplantation losses	47	67	61	53	78

TABLE 10. Morphologic Classification of Sperm Heads of Rats Exposed to HD (Phase II, % of Total ± SE).

TREATMENT DOSE (mg/kg)	CONTROL 0			HD 0.50			EMS 100
WEEKS	0	5	12	0	5	12	5
NO. OF ANIMALS	10	8	10	9	9	10	10
Normal	99.1±0.17	99.4±0.07	99.2±0.09	97.7±0.32*	97.9±0.14*	98.0±0.17*	97.7±0.30*
Blunthook	0.26±0.05	0.18±0.04	0.30±0.05	1.19±0.17*	0.72±0.09*	0.76±0.05*	1.11±0..17*
Banana	0.35±0.09	0.11±0.02	0.17±0.04	0.57±0.10*	0.46±0.07*	0.38±0.06*	0.69±0.12
Amorphous	0.26±0.07	0.16±0.03	0.16±0.03	0.36±0.06	0.35±0.05	0.50±0.08*	0.32±0.06
Pinhead	0.05±0.01	0.12±0.05	0.15±0.04	0.06±0.02	0.27±0.06	0.20±0.09	0.05±0.02
Double Head/Tail	0.01±0.01	0	0	0	0.01±0.01	0	0
Short Head	0.04±0.02	0.06±0.02	0.05±0.02	0.08±0.03	0.31±0.07*	0.20±0.03*	0.16±0.04
Other	0	0	0	0	0.02±0.01	0.02±0.01	0.01±0.01
Total Abnormal	0.96±0.17	0.63±0.07	0.82±0.09	2.26±0.32*	2.12±0.14*	2.04±0.17*	2.32±0.30*

*Significantly different than control at corresponding week ($P<0.05$)

TABLE 11. Reproductive Organ Weight, Sperm Motility, and Sperm Count of Rats Exposed to HD (Phase II Mean ± SE).

TREATMENT	CONTROL			HD			EMS
DOSE (mg/kg)	0			0.50			100
WEEKS	0	5	12	0	5	12	5
NO. OF ANIMALS	8	10	10	9	10	10	10
Body Weight (g)	564±17	610±22	625±21	484±13*	591±11	648±17*	568±15
Right Epididymal Weight (mg)	642±17	629±21	760±15	577±19	652±10	739±27	722±16
Right Testicular Weight (g)	1.80±0.06	1.78±0.05	1.88±0.03	1.72±0.06	1.77±0.05	1.90±0.06	1.77±0.05
Motile Sperm (%)	85.1±1.1	81.4±2.1	70.9±5.7	82.6±1.1	84.60±1.3	67.5±4.1	84.2±1.9
Sperm Concentration (per µg caudal epididymal tissue)	620±44	419±35	514±18	727±26	408±39	586±36	509±23

*Significantly different than corresponding weekly control group (P< 0.05).

DISCUSSION

The absence of deaths as well as the normal growth curves in control animals of both phases of the study were consistent with their apparent healthy condition throughout the course of the gavaging experiments and during the post-exposure breeding period. The high overall pregnancy rates achieved, 86 and 91% for all animals by treatment in phase I and II, respectively, was indicative of healthy and reproductively sound females and males. The significant decrease of body weight in the HD-treated females (at all doses studied, (phase I) and males (0.50 mg/kg, phase I and II) suggests that an effective dose was administered to induce the desired toxic effect. These results corroborate those of a previously reported 13-week subchronic study in rats gavaged with 0.30 mg/kg HD in which similar reductions in growth were observed.

The lack of a treatment-related effect for the number of implants per litter or for the number of early resorptions per litter in female rats mated to exposed or unexposed male rats indicated that overall the female fertility was not affected by these exposures. In contrast, the treatment-related alterations in the number of implants and resorptions of fetuses in untreated female rats mated to HD-treated males indicates that male fertility was affected by HD, particularly at the highest dose and frequently at lower doses used in the study, and indeed a male dominant lethal effect was demonstrated.

In light of the definite dominant lethal effect in the male rat (phase II), the lack of an effect in the phase I study for exposed males mated to exposed females is of interest. We would not necessarily expect the exposure of females to interfere with the expression of a male dominant lethal effect. However, twice as many females were used for male dominant lethal study as were used for the female dominant lethal study. This may account, in part, for the difference in statistical significance.

Week 2 and 3 of the post-treatment mating was confirmed as the period significantly affected by HD, consistent with results showing that most mutagens elicit their effects during the early weeks (1-5) of the dominant lethal test (Bateman, 1966). This pattern indicates that the effects seen with HD occurred during the postmeiotic stages of spermatogenesis, possibly to the sensitive spermatids. By definition, dominant lethal mutations are self-limiting, but the damage characterized by preimplantation loss of nonviable blastocysts and early embryonic death is strongly associated with structural or chromosomal anomalies in the germ cell and may be indicative of gene or point mutations.

Analyses of the sperm head morphology data obtained for male rats at 0, 5 and 12 weeks post-exposure showed a significant

increase in the total number of abnormal sperm heads and a reduction in percentage of normal sperm. Blunthook and banana shaped sperm heads were the most common abnormality found. Other apparent signs of toxicity affecting male fertility following oral administration of HD were not identified, as epididymal and testicular weight, percent sperm motility and sperm concentration were all unaffected. The male germ cell appears to be sensitive in terms of *in vivo* exposure to subchronic quantities of HD. This assay has been applied to a wide variety of industrial compounds and has shown moderate correlation with *in vitro* assays (Bruce and Heddle, 1979). However, Osterloh et al. (1983) concluded the the assay was not a suitable method to detect male testicular effects of pesticides after studying a wide range of these compounds and finding surprisingly few positive effects. Nevertheless, these results indicate that long-term exposure to HD adversely affects spermatogonia or primary spermatocytes in the rat and further studies may be warranted.

The dominant lethal effects and the abnormal sperm heads observed in this study of the rat are not surprising in light of other mutagenic effects of HD cited in the introduction of this report. Dominant lethal effects, although unclear in a subsequent report, may have occurred in mice exposed to vaporized HD (0.1 mg/M^2), reaching a maximum incidence by 12 weeks of exposure (Rozmiarek et al., 1973). We recently reported that HD induced point mutations in tester strain TA102 and frameshift mutations in TA97 in the Ames test (Stewart, 1987; Stewart et al., 1989) and caused marked chromosome damage and rearrangement and was mutagenic at the HGPRT locus in CHO cells (Jostes et al.,1989). In a study of 174 agents Epstein et al.(1972) found only 16 which unequivocally induced dominant lethal effects; the majority of these are known alkylating agents.

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STUDY DATES

Animal Arrival (Phase I)	02-03-87
Health Screen Complete	02-20-87
Begin Phase I Dosing	02-24-87
End Phase I Dosing	05-01-87
Begin Phase I Breeding	05-05-87
Begin Phase I Sacrifice	05-26-87
Animal Arrival (Phase II)	06-30-87
Health Screen Complete	07-17-87
Begin Phase II Dosing	07-20-87
End Phase II Dosing	09-25-87
Begin Phase II Breeding	09-25-87
Begin Sacrifice of Females (Phase II)	10-12-87
Begin Sacrifice of Males (Phase II)	12-17-87

Data are the property of the U. S. Army and will be archived under the army's direction in approved facilities.

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DOMINANT LETHALITY STUDY OF SULFUR MUSTARD (HD) IN RATS

Quality Assurance Statement

Listed below are the phases and/or procedures included in the study described in this report which were reviewed by the Quality Assurance Unit during the period, 2/1/87 - 12/31/87, specifically for this study and the dates the reviews were performed and findings reported to management. (All findings were reported to the study director or his designee at the time of the review.)

Phase/Procedure Reviewed	Review Date	Date Findings Submitted in Writing to Study Director/Management
Animal Identification	2/19-20/87	3/10/87
Body Weights	2/19-20/87	3/10/87
Clinical Observations	4/06/87	4/07/87
Body Weights	4/06/87	4/07/87
Dosing	4/06/87	4/07/87
Necropsy	5/22/87	5/27/87
Dosing	9/04/87	9/22/87
Necropsy	9/25/87	9/28/87
Sperm Morphology	9/25/87	9/28/87
Health Screen	10/13/87	10/13/87
Necropsy	11/09/87	11/10/87
Data	11/13 & 12/22/87	1/05/88
SMVCE	12/17/87	1/05/88
Data	1/14-18/88	2/01/88
Data	1/14/88	2/01/88
Data	1/14/88	1/20/88
Final Report	7/6,16,17/89	8/23/89

CM Howells
Quality Assurance Auditor

8/23/89
Date

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Date

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