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Inhalation Exposure Systems for the Development of Rodent Models of Sulfur Mustard-Induced Pulmonary Injury

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Abstract

Sulfur mustard (SM) is a chemical threat agent for which its effects have no current treatment. Due to the ease of synthesis and dispersal of this material, the need to develop therapeutics is evident. The present manuscript details the techniques used to develop SM laboratory exposure systems for the development of animal models of pulmonary injury. These models are critical for evaluating SM injury and developing therapeutics against that injury. Iterative trials were conducted to optimize a lung injury model. The resulting pathology was used as a guide, with a goal of effecting homogeneous and diffuse lung injury comparable to that of human injury. Inhalation exposures were conducted by either nose-only inhalation or intubated inhalation. The exposures were conducted to either directly vaporized SM or SM that was nebulized from an ethanol solution. Inhalation of SM by nose-only inhalation resulted in severe nasal epithelial degeneration and minimal lung injury. The reactivity of SM did not permit it to transit past the upper airways to promote lower airway injury. Intratracheal inhalation of SM vapors at a concentration of 5400 mg · min/m³ resulted in homogeneous lung injury with no nasal degeneration.

Keywords

Aerosol; Inhalation; Rodents; Sulfur Mustard

INTRODUCTION

Sulfur mustard (Bis(2-chloroethyl) sulfide; SM) is a chemical threat agent that the Department of Homeland Security has classified as a high priority for the development of therapeutic agents to combat SM-induced injuries (DHS, 2007). Sulfur mustard may be encountered in both the battlefield or by civilians through acts of terrorism. It is easily produced and encountered in a combination of chemical forms that include liquids and vapors. Exposure targets of concern include the skin, eyes, and respiratory tract. The work described in this manuscript focuses on the respiratory tract.

Short-term localized effects of inhaled SM in humans include discomfort in the nose and sinuses, sore throat, sneezing, coughing, and respiratory dyspnea. Histological injuries

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include aphonia, tracheobronchitis, alveolar hemorrhaging, mucosal necrosis, and airway obstruction (Balali-Mood and Navaeian, 1986; Sidell and Hurst, 1992; Stepanov and Popov, 1962; Warthin and Weller, 1919; Willems, 1989; Freitage et al., 1991). Bronchopneumonia is a common complication (Willems, 1989). Systemic SM-induced injuries include toxic reduction in lymphoid, bone marrow, and white blood cells; acute gastroduodenitis; and hemorrhagic colitis (Willems, 1989; Balali et al., 1991; Canelli, 1918). Exposures to higher SM concentrations can lead to central nervous system excitation, leading to convulsions (Anslo and Houk, 1946). Delayed effects of SM inhalation in humans include persistent cough, expectoration, and dyspnea, including wheezing, crackles, clubbing, and cyanosis (Hosseini et al., 1989; Balali-Mood, 1986; Afshinniaz and Ghanei, 1995). Chronic bronchitis, asthma, bronchiectasis, and large airway narrowing are the most common complications (Balali-Mood and Hefazi 2005; Bijani and Moghadamnia, 2002; Emad and Rezaian, 1997). These effects have been observed in humans exposed to SM during the Iran-Iraq war of the 1980s (Ghanei et al., 2004; Ghanei and Harandi, 2007).

Despite the substantial risk for exposure, there are currently no approved treatments for SM-induced pulmonary injury. As a result, the development of countermeasure therapeutics is necessary. Currently if an exposure to SM is suspected, the subject is decontaminated with soap and water and given supportive care by treating the resulting symptoms (DHHS, 2007). Sodium thiosulfate can be administered to react with SM to help limit proliferation of the injury, but it has been shown to be far more effective when given prophylactically or very shortly after SM exposure (Foster et al., 1962; Connors, 1966; Connors et al., 1964). However, in most cases it is not practical to treat SM injury until several hours post exposure. Currently, therapies include administration of oxygen and bronchiole dilators such as salbutamol and ipratropium and/or antibiotics to fight the resulting infections (Warthin and Weller, 1989; Aslani, 2000). Each of these therapeutics treats symptoms but do not target initiation or progression of SM-induced lesions and injuries. As such, there is a need to evaluate additional therapeutics through additional targets that may stop the progression (Kehe et al., 2008).

The development of therapeutics to treat the chemically induced injury includes the development of relevant animal models that enable assessment of therapeutic intervention (efficacy), mode of action, and safety. The Food and Drug Administration has provided guidance for development of therapeutics according to the Animal Rule (FDA, 2008). This guidance specifies that in order to develop therapeutics when human clinical studies are not practical, drugs may be approved based on the utilization of multiple animal models, understanding of mode of action and relevance across species, and a demonstration of both safety and efficacy. The Animal Rule only has been used for approval of a small number of therapeutics, so there is no clear path toward drug approval. However, the development of small animal models for initial screenings of safety and efficacy of potential therapeutics is an obvious initial step toward identifying efficacious countermeasures against SM.

The objective of this research was to develop and optimize methods to enable development of rodent models of SM-induced pulmonary injury. This manuscript describes the development of a facility along with aerosol generation and exposure system trials conducted to optimize the type and location of injury. Subsequent manuscripts are in process that will fully describe the developed animal model. The overarching goal was to achieve a platform to screen therapeutic efficacy and safety in rodents. A rodent lung injury with similar characteristics to those reported in humans was desired. Several approaches were attempted to meet this objective. The results of these trials are described, including an initial assessment of the pathology that was established while optimizing the laboratory system. The step-wise efforts led to the development of vapor and aerosol atmospheres that were delivered either by nose-only inhalation or directly to the lung through an intubation tube.

METHODS

Preparation and Characterization of SM

SM (Bis(2-chloroethyl) sulfide) was synthesized by proprietary methods. The identification and purity of SM was characterized by gas chromatography/mass spectroscopy (GC/MS, Agilent, Santa Clara, CA), proton nuclear magnetic resonance imaging (^1H NMR, Bruker, Billerica, MA), and MiniCAMS GC/flame ionization detector (FID) (OI Analytical, Pelham, AL). The GC/MS ionization pattern in the spectrum was consistent with the structure of SM. ^1H NMR analysis indicated a single, pure compound with chemical shifts consistent with the structure of SM. GC/FID also showed a single peak in an extended chromatogram intended to identify the presence of reaction byproducts or SM breakdown products; none were detected. The purity of SM was determined to be greater than 99%.

Configuration of the Vesicant Exposure Suite

The exposure suite floor plan (Figure 1) consists of two ante rooms located on either side of a central exposure room. The air pressure in the central exposure room was maintained at a negative pressure to the ante rooms that are maintained at a negative pressure with respect to the main hallway to ensure test agent was confined to the exposure room in the unlikely event of a spill or leak. All SM work was conducted inside the exposure room, with the ante rooms serving as procedure and storage areas. Entrance into the exposure suite is granted via key-card access limited to a maximum of 10 individuals.

All work is conducted in stainless steel/Lexan glove boxes maintained at negative pressure with respect to the exposure room. The glove boxes each contain a pass box to ensure the safe transfer of materials. Each glove box exhaust and all of the transit lines were directed through a charcoal filter to remove any SM present. The glove box system was configured with an audio-visual alarm system to sound if the glove box pressure increased to ambient or greater.

A central SM atmosphere generation glove box was connected by a series of valves to three other glove boxes used to expose animals by various routes of administration. All delivery systems were composed of stainless steel or anodized aluminum unless otherwise noted. Air flows were controlled and monitored using calibrated rotameters. A separate glove box was dedicated to the delivery of potential therapeutics. A limited access storage cabinet was located in the exposure room for storage of synthesized SM and precursors. The analytical equipment for monitoring exposure atmospheres and industrial hygiene was located in the exposure room.

SM Vapor Generation

SM vapors were generated with a custom built stainless steel J-tube (McClellan and Henderson, 1995). The J-tube was filled with glass beads and blanketed with a heat jacket and a temperature controller to maintain an operating temperature of 160°C (Figure 2). Nitrogen, supplied by gas cylinder, was used as the carrier gas through the J-tube at a flow rate of 2.1 L/min. Neat SM was injected into the J-tube via syringe through a rubber septum. The syringe speed was controlled by a syringe pump (Kent Scientific, Torrington, CT), with the speed determined by the desired vapor concentration.

SM Aerosol Generation and Particle Size Determination

SM aerosol generation was attempted using two separate approaches. First, an attempt to condense SM vapors by passing them through a 90-cm countercurrent heat exchanger (condenser) that chilled the vapor to approximately 5°C was conducted. This approach was unsuccessful. SM aerosols from ethanol ultimately were generated by compressed jet

nebulization with a Swirler nebulizer (Swirler, Amici Inc., Spring City, PA). Solution concentrations of SM in ethanol ranged from 0.1%–1%. Because the goal of the aerosol was to deliver SM to the deep lung, a small particle size was targeted. Previous studies have indicated that a 0.5-micron aerosol provides enhanced pulmonary deposition in rodents (~10–15%) compared with larger aerosols (Rabbe, 1982). This targeted small particle size was implemented with a nebulizer customized to enhance pulmonary deposition through the creation of small particles. The nebulizer was operated at a pressure of 2100 cm of H₂O.

The concentration of the SM aerosol atmospheres was controlled by the concentration of SM in the nebulizer solution. Aerosol size determination was determined by a time-of-flight analyzer (APS, TSI, Inc., Shoreview, MN). Importantly, this method of aerosol size analysis does not specifically measure the size of SM. Rather, it measures the size of all of the droplets in the aerosol, the majority of which are composed of ethanol in the formulation. An alternative approach to specifically collect and measure SM droplets by a cascade impactor was evaluated. However, spike-recovery of SM onto cascade impactor substrates showed that the agent quickly evaporated, prohibiting the ability to quantitatively (or qualitatively) assess the size and quantity of SM in the aerosol.

Nose-Only Inhalation Exposure System

A schematic depicting the nose-only inhalation exposure glove box is shown in Figure 3. A 48-port nose-only inhalation chamber (In-Tox Products, Moriarty, NM) was operated at an exhaust flow rate of 20.5 L/min. The exhaust was filtered through both a HEPA and charcoal filter before returning to the main exhaust line. The chamber was maintained from 1.25–3.75 cm of water negative by the controlled addition of filtered dilution air. Oxygen and temperature were monitored.

Intratracheal Inhalation Exposure System

A schematic depicting the intubation exposure system is shown in Figure 4, and Figure 5 is a more detailed description of the exposure plenum. SM vapor atmospheres were generated as previously described. This system was fabricated from stainless steel for the distribution and exposure plenums and Teflon-lined Tygon tubing (Saint Gobain, Valley Forge, PA) was used for the vapor delivery lines. Flow from the generator glove box into the intubation exposure glove box was directed through a distribution plenum. The distribution plenum was attached to four exposure lines that transited SM vapor to four individual exposure plenums. The exhaust flow through the distribution plenum was maintained at 20.5 L/min. A small plug of glass wool was placed inside the distribution plenum to help enhance the homogeneity of the vapor prior to its distribution to the exposure plenums. Flows at the exposure plenum were 700 mL/min. These flows were controlled by mass flow controllers operated remotely by a computer interface and custom LabView (version 8.0) application.

Dilution air to the distribution plenum was composed of a mixture of compressed air and medical grade oxygen that transited through an isoflurane (JD Medical, Phoenix AZ) vaporizer to supply a continuous anesthetic into the exposure atmosphere. The flow through the vaporizer was set to approximately 23 L/min and adjusted as needed to maintain negative 0.64–1.27 cm of water at the exposure plenum. The vaporizer output was connected to the SM intubation system and the vaporizer adjusted between 1.5–2.5% isoflurane in the system in order to maintain the breathing rates of rodents (40–50 breaths per minute) placed on the system. The oxygen concentration also was adjusted to maintain 21% oxygen at the exposure plenum. Figure 5 is a schematic depicting the exposure plenum/exposure sled layout.

SM Atmosphere Characterization

Samples were collected from both the nose-only inhalation system and the intubation systems directly from the breathing zone of the animal. Samples were collected by drawing a gas-tight syringe from the systems and injecting the sample onto the MiniCAMS Tenax sorbent. Samples were analyzed by sorbent desorption GC with a flame photometric detector (MiniCAMS, O I Analytical, Pelham, AL). The GC method resulted in a SM chromatographic peak at 125 s. SM vapor concentrations were calculated by comparison of the generated peak area to a standard curve generated on the day of exposure. Two five-point standard curves were created: a low level curve linear from 3.2–12.7 ng and a high level curve linear from 25.4–106 ng. Standards were created in acetone. The R^2 values were greater than 0.98, and each point was calculated to be within 15% of theoretical and 20% at the curve's sensitivity limit of 3.2 or 25.4 ng.

Animal Handling and Exposure

Female F344 rats (Charles River Labs, Wilmington MA, 11–13 weeks, 170–190 g) were quarantined for a minimum of 2 weeks prior to use. Animal studies were approved by the LRRRI Institutional Animal Care and Use Committee, conducted in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, and carried out in compliance with the *Guide for the Care and Use of Laboratory Animals* (NRC, 1996). During all periods animals were provided water and food (Harlan-Teklad, Madison, WI) *ad-libitum*. The exception was during the procedures when animals were removed from their standard housing for SM exposures. For nose-only exposure, rodents were conditioned to nose-only tube restraints. Air flow was maintained through the system during addition and removal of rodents from the nose-only exposure chamber. No anesthetic was used for nose-only inhalation. For intubation exposures, rodents were injected subcutaneously with an anesthetic cocktail consisting of acepromazine (0.79 mg/kg), ketamine (39.5 mg/kg), and xylazine (3.95 mg/kg); however, when these animals were intubated and exposed to SM, the anesthesia wore off prior to the conclusion of the exposure. Control atmospheres did not result in a similar observation. As an alternative, animals were anesthetized with isoflurane (5% induction, 2% maintenance). Anesthetized rodents were intubated with a 14-gauge Teflon catheter (approximately 5 cm in length). The catheter was inserted into the trachea to a terminal location approximately half way between the bifurcation of the trachea and the larynx. The placement and seal of the catheter was verified by observing air displacement in a ground glass gas-tight syringe during an inhalation/exhalation cycle. Intubated rodents were transferred to the exposure plenum. An airflow consisting of SM + vehicle or vehicle alone was constantly running through the exposure plenum. Rodents were intubated and exposed to SM on a single occasion and were allowed to recover for observations up to 21 days post exposure. Rodents were not fasted prior to SM exposures. The nose-only exposure system was able to accommodate ~45 rats during a single exposure. The intubation exposure system was able to accommodate 4 rats per exposure.

Off-gassing

After exposure the animals were returned to temporary cages for off gassing. The cages were then coupled to a vacuum line to assist in increasing the air exchange to remove any residual SM that may have sorbed to their fur during exposure. SM concentration in the cages was monitored until the concentration was determined to be less than $1.6 \mu\text{g}/\text{m}^3$, which is lower than the occupational 8-h time-weighted average concentration of $3 \mu\text{g}/\text{m}^3$ as set by the U.S. Army Center for Health Promotion and Preventive Medicine (US Army, 2004). Off-gassing times were between 15 min for the intubation exposures and 90 min for the nose-only inhalation exposures. At the conclusion of the off-gassing period, animals were returned to their normal cages.

Necropsy and Histopathology

Rodents were euthanized with an ip injection of a barbiturate followed by exsanguination. The lungs were inflated with 10% neutral buffered formalin (NBF) via tracheal cannula until the pleura was tense and fixed further by immersion in NBF. The skulls were removed, and nasal passages were flushed with NBF. Skulls were fixed further in NBF and decalcified with formic acid. Standard transverse sections of the rostral and caudal portions of the nasal passages were obtained (4 per rat; Young, 1981). The left, right accessory, and right caudal lung lobes were trimmed sagittally along the axial airways. Tissues were processed routinely, embedded in paraffin, sectioned at 5 μm , and stained with hematoxylin and eosin for evaluation by light microscopy.

RESULTS

Generation of Vapor SM

SM vapor generation conditions were developed by iteration through optimization of operating temperature and SM introduction rate. A summary of several operating conditions and the resulting vapor concentration is provided in Table 1. Initial temperature was set to 220°C, set to approximately the boiling point of SM (217°C). At this temperature SM decomposed in the J-tube and occluded the needle in the syringe. The next trial investigated SM vaporization at 110°C. At this temperature the SM did not degrade, but the vaporization efficiency was poor as an increase in syringe speed did not result in an increase in SM vapor concentration. When the J-tube temperature was raised to 160°C, the vaporization improved without indication of SM degradation. At this temperature higher concentrations of SM vapor were attainable and, in general, increases in the SM introduction rate were proportional to the vapor concentrations. Vapor concentrations from 25–180 mg/m^3 were evaluated in the nose-only and intubated exposure systems.

Generation of Aerosol SM

As indicated previously, initial inhalation trials by nose-only exposure to vapors did not yield the desired pathology for the pulmonary model. It was hypothesized that an aerosol of SM may achieve improved penetration to the lungs. An initial attempt at generating aerosol SM was conducted by attempting to condense vapors at concentrations of $\sim 180 \text{ mg}/\text{m}^3$. This was not successful, as indicated by no measurable particles downstream of the condenser. The inability to condense SM was likely because the vapor concentration was well below the saturation vapor pressure concentration of SM (calculated to be 905 mg/m^3). Trials at substantially higher concentrations of SM were not attempted to conserve agent and minimize risk.

SM aerosols were generated by nebulization of an ethanol SM formulation. The nebulizer produced an aerosol with a mass median aerodynamic diameter at approximately 0.6 microns (Figure 6), and did not change with different SM concentrations (Table 2). This size was targeted to potentially enhance SM penetration to the lung. The SM concentration using nebulization ranged from 10–180 mg/m^3 .

Nose-only Exposure System

The nose-only system was stable once target concentrations were obtained with both an SM aerosol and vapor atmosphere. The time to achieve 90% of target atmospheres was determined to be approximately 5 min. Once steady-state concentration was achieved, atmosphere concentration was within 10% of target. Characterization of the homogeneity among animal ports revealed the relative standard deviation for concentration at different locations on the exposure chamber to be less than 6% for both aerosol and vapor atmospheres.

Intubation Exposure System

The intubation exposure system was designed to deliver exclusively vapor atmospheres as generated by J-tube vaporizer. Similar to the nose-only system, the intubated plenum achieved 90% of the target concentration within 5 min. At the final operating conditions and composition, the intubated system showed good concentration stability (Figure 7) and the homogeneity among parallel exposure plenums showed concentrations within 5%. While the nose-only exposure system showed consistent and stable operation with the default operating conditions, the custom designed intubation system required several iterations to determine the final configuration for system composition and operation. A steady vapor concentration was achieved at 20 L/min; the system was unstable at lower exhaust flow rates. The flows through the exposure plenums also were determined to be important. If flows were less than 500 mL/min the concentrations were not homogenous. When the sample flow was elevated to 700 mL/min the concentrations stabilized.

Several approaches to the construction of the intubated exposure system were attempted. The evaluations investigated the efficiency of SM transit through the custom designed systems. SM, due to its high reactivity, was scrubbed by reaction of material with which the system was constructed. Several types of tubing were attempted, including PolyFlo, Tygon, and carbon impregnated silicon. SM was either not detected or showed significantly reduced concentrations after transit through the 20-cm delivery line. In some cases SM was greatly reduced, and breakdown products (not determined) were observed in the chromatograms. Tygon SE-200 tubing, which contains an internal Teflon lining, was the only tubing evaluated that did not react with SM. The concentration measured at the terminus of the tube showed no decrease in SM or formation of breakdown products.

The composition of the distribution and exposure plenums were important also. Initially, the plenums were composed of plastic materials. SM reacted with the plastic, resulting in reduced concentrations and the formation of breakdown products (not determined). The final plenums were composed of stainless steel, resulting in no interactions with SM.

Pilot Inhalation Studies

Rodent pilot studies were conducted to examine the extent and distribution of pulmonary injury with each exposure configuration. Respiratory tract pathology was used as the important benchmark for development and optimization of the exposure protocol. Preliminary pathology as a function of exposure is reported here. Complete pathology and other characterizations of effects in the model are to be described elsewhere.

Nose-Only Exposure to SM—Animals exposed by nose-only inhalation displayed progressive body weight losses with time post exposure, hunched posture, lethargy, and respiratory distress manifested as labored, open-mouthed breathing and, occasionally, cyanosis. Moreover, animals at necropsy often had marked gas-distention of the gastrointestinal tract, suggesting that significant amounts of air were swallowed. Histopathologically, SM vapor at $3000 \text{ mg} \cdot \text{min}/\text{m}^3$ (60-min exposure at $50 \text{ mg}/\text{m}^3$) resulted in extensive nasal injury, but lung injury was absent to minimal (Figures 8 and 9). After these results, it was postulated that SM may penetrate and deposit more deeply into the lung if it is present as a droplet rather than a vapor. SM in ethanol was nebulized, and aerosol exposures were conducted at $3000 \text{ mg} \cdot \text{min}/\text{m}^3$ (120-min exposure at $25 \text{ mg}/\text{m}^3$). The respiratory tract pathology from the nebulized SM was not different than the vaporized SM (Figures 8 and 9). Thus, respiratory distress following nose-only exposure to SM vapor or aerosol was determined to be an effect of the nasal injury and was not attributable to lung injury.

Intubated SM Vapor Exposure System—The intubation exposure system was designed to deliver exclusively vapor atmospheres as generated by J-tube vaporizer and was used to deliver SM directly to the lung. Animals exposed via intratracheal tubes, as with nose-only exposure, displayed body weight loss and respiratory distress. When rodents were exposed to $5400 \text{ mg} \cdot \text{min}/\text{m}^3$ (30-min exposure at $180 \text{ mg}/\text{m}^3$) SM vapor by this method, the rats developed substantial multifocal necrosis and degeneration of the mucosal epithelium lining the major pulmonary airways (Figure 10). Nasal injury was not evident. In this exposure scenario, the observed respiratory distress was attributed to the effect of SM on the lung rather than to the upper respiratory tract injury.

Discussion

The goal of this research was to develop methods that would allow for the creation of a laboratory animal model which would mimic pulmonary injury in humans. Human pathology after SM exposure has been described and includes chronic bronchitis, asthma, bronchiectasis, and large airway narrowing (Balali-Mood and Hefazi, 2005; Bijani and Moghadamnia, 2002; Emad and Rezaian, 1997). The final exposure conditions described herein resulted in pulmonary injury that showed acute airway epithelial necrosis and hemorrhaging. These injuries are similar to the pathology observed in human subjects, and this creates a platform for screening the efficacy of potential therapeutics.

As in any simulated exposure, there are many variables to consider for how SM may be encountered in the battlefield or through terrorist activities. SM can be dispersed in a variety of ways, including as part of an explosive device, such as a bomb or a missile, in a military situation. In this case the SM encountered near the explosion will be an aerosol mixture of SM and SM combustion products that are associated with particulate matter (PM) that are part of the debris. This debris can serve as condensation/adsorption sites for the SM, and if inhaled, the SM will likely penetrate to the deep lung as an aerosol. Downstream of the device, or downstream of any major dispersive event, SM is most likely to be encountered as a vapor. Unfortunately, exposure data assessments from previous battlefields or civilian population exposures are lacking. The model described herein represents exposure downwind of the dispersion in areas where concentrations remain substantially high to cause significant injury. It is unclear what role the PM would have in exacerbation of the SM-induced pathology observed here. However, it is likely that a therapeutic evaluated to treat vapor-induced injury also would be effective against SM that is co-associated with PM.

Another caveat of this work is that it is unlikely pure SM would be encountered in most human exposure scenarios. In battlefield or terrorist conditions the purity of the SM would likely not be controlled as it was during this study. As a result, the exposures would likely include SM, thiodiglycol, and oxidation products along with a number of unknown impurities. Furthermore, if the SM were dispersed from a bomb it would include combustion products that may or may not cause injury.

Initially, the pulmonary injury model was attempted using SM vapors and exposure by nose-only inhalation. However, this exposure route yielded severe nasal damage with minimal lung injury. The high reactivity of SM, coupled with the large surface area of the rodent nasal passages, led to an apparent scrubbing of the SM. The highly diffusive properties of a vapor molecule mean that it has ample opportunity to come in contact with the surfaces of the airway. As has been observed with reactive organic compounds such as acetaldehyde, highly reactive gases can leave all of their effects in the nose because they don't reach the lung (Dorman et al., 2008; Teeguarden et al., 2008).

When nose-only vapor inhalation was determined to be unsuitable to yield a lung injury model, the next attempt was to generate a SM aerosol. Because the vapor concentrations

used were below the saturation vapor pressure, SM could not easily be condensed into the aerosol form from a vapor. As an alternative, an aerosol from an ethanol formulation was attempted. The ethanol formulation provided an apparent aerosol with small size (submicron) that was hypothesized to provide a chance for better lung penetration. This hypothesis was proven wrong, as the nebulized SM in ethanol provided near identical pathology to the vaporized SM. A limitation of the aerosol characterization was that it could not be confirmed if SM existed as a vapor or part of a droplet in the aerosol. The particle sizing method was not able to discriminate between aerosols of ethanol or SM. Due to SM's volatility, it could not effectively be captured on an impactor or other device that would allow direct measurement. As a result, it is not possible to confirm if the nebulized aerosols indeed contained aerosols of SM or if the SM evaporated after nebulization. Regardless, the technique did not prove effective in providing an adequate lung injury model.

The final exposure condition implemented a custom built intubation exposure system that permitted the exposure of SM directly to the lung. In initial trials rodents were anesthetized under an injectable cocktail of acepromazine, ketamine, and xylazine. Animals exposed to the control atmosphere displayed no adverse effects with this cocktail mixture. However, when SM was added to the system the rodents awoke before they were to be removed from the exposure system. An investigation revealed that SM reacts directly with the constituents of this anesthesia cocktail. This observation is in contrast to previous reports by Anderson et al. (1996) and Capacio et al. (2008), who administered doses as low as 30 mg/kg of ketamine and 6 mg/kg of xylazine. The observational difference may be attributed to the difference in exposure concentration but it is a difficult assessment to make as the authors did not quantitate their exposure atmospheres. However, as reported by Capacio et al. (2008), it is currently believed that SM and the cocktail enter into the same compartment such as blood or plasma allowing the components to interact and react, which diminishes the amount of cocktail to be delivered to the central nervous system and thus leads to the premature awakening of the rodent. In the present work, a solution stability study was conducted to confirm this theory, where 12.7 µg of SM was added to 990 µL of acetone with the addition of either 10 µL of water or 10 µL of the anesthetic cocktail administered to the rodents. Aliquots were removed from the reaction every 12 min for 60 min for analysis. Approximately 12 min after the addition 50% of the SM was absent from the reaction system containing the cocktail; by 24 min post addition SM could not be detected by GC/MS. SM in acetone/water did not show significant degradation after 60 min. As an alternative, isoflurane was included in the exposure system to maintain the rodents under anesthesia. Solution chemistry experiments similar to those described above with SM and isoflurane showed that SM did not react with isoflurane.

This manuscript is not the first report of SM inhalation in laboratory animals. The present studies provide additional detail on the approach to the SM administration and, in most cases, provide a superior approach to characterization of the SM. Once more, this is the first report indicating a systematic approach to evaluate the role of aerosol character and exposure technique on resulting pathology. Previous SM injury models have been reported for mice (Pant and Vijayaraghavan, 1999; Vijayaraghavan, 1997), guinea pigs (van Helden et al., 2004), pigs (Fairhall et al., 2008), and rats (Anderson et al., 1996; Capacio et al., 2008).

The mouse studies were conducted with head-only inhalation of SM aerosols generated from acetone. Similar to results presented here, those studies showed little to no pulmonary irritation or pathology (Vijayaraghavan, 1997). In contrast, the same group reported pulmonary toxicity characterized by alveolar hemorrhage, congestion, and mucosecretory cell destruction (Pant and Vijayaraghavan, 1999). Although clinical observations of the exposed animals in the current study and the head-only mouse studies appear to be similar,

the histology of the tissues appears to be significantly different. These differences may be attributed to the exposure concentration, exposure duration, or selected species; but it is not clear why using an identical exposure system in one reported study would result in pulmonary toxicity and one would not. No other recent reports detailing a nose-only or head-only injury were noted.

The guinea pig studies conducted by intratracheal inhalation of SM aerosols generated from a solution of saline resulted in severe acute bronchoconstriction and asthma like symptoms (van Helden et al., 2004). The present study observed the same types of respiratory responses in rats. The intubation inhalation study in pigs only briefly mentioned physiological results but the authors did describe their exposure system in detail.

The rat studies conducted by Anderson et al. (1996) resulted in an injury response at only 6 h post exposure whereas in the present work a similar response was observed ~21 days after exposure. This, again, may be attributed to the exposure concentration, as the authors do not measure and report the vapor concentration explicitly, making it difficult to give an adequate comparison to the present work. The studies conducted by Capacio et al. (2008) also were conducted with an intubated animal using the commercially available FlexiVent™ system. In that case, a nebulizer was filled with a formulation of a known amount of SM and used for a predefined amount of time. The atmospheres in this exposures system were quantitated but had the disadvantage of a co-solvent present, which could compound the interpretation of injury characterization. The current intubated system is configured for a pure vapor that does not have ethanol as a potential confounder. In addition, the current system was fabricated to enable near real-time characterization of the SM vapor concentration. Finally, the plenum permits exposure of multiple animals simultaneously.

In summary, the methods to create a pulmonary injury models have been successfully developed to study rodent pulmonary injury to SM. Nasal injury can be studied in isolation of lung injury with a nose-only vapor inhalation system. To investigate lung injury directly, SM is delivered directly to the lung as a vapor through a custom built intubation exposure system. Initial pathology is reported here, and more detailed characterization of the model will be reported elsewhere. The model is currently being utilized to further define the mechanisms and pathogenesis of SM-induced injury, and to screen therapeutics for efficacy.

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FIGURE 1.
Sulfur mustard exposure suite floor plan

FIGURE 2.

J-tube for sulfur mustard vapor generation. Neat sulfur mustard is introduced into the stainless steel J-tube via syringe pump. The J-tube is packed with glass beads and wrapped with heat tape to control the temperature of vaporization. Vaporization is conducted under nitrogen at a flow of 2 L/min.

FIGURE 3.

Inhalation exposure schematic showing a nose-only inhalation system that precedes charcoal/HEPA filters that are used to remove SM from the effluent. Sample ports on either side of the exhaust filter enable evaluation of scrubbing efficiency.

FIGURE 4.

Intubation inhalation exposure system schematic. Vapors are introduced to a central distribution plenum and then diverted to individual exposure plenums that are connected directly to an intubation tube. Rodents breathe on demand from the exposure plenum. Exhaust from the exposure plenum is controlled at 700 ml/min by a computer controlled mass flow controller (MFC). SM is removed from the exhaust by a HEPA and charcoal filtration system.

FIGURE 5.

Exposure sled schematic illustrating the intubation sled that is used to deliver SM directly to the intubated rat. SM vapors traverse through the exposure plenum at 700 ml/min, and the rat breathes from the plenum on demand. Oxygen and SM concentration are measured directly from the exposure plenum.

FIGURE 6. Particle size distribution of nebulized SM ethanol solutions. SM was dissolved in ethanol and aerosolized in a Swirler nebulizer that is designed to deliver submicron aerosols. Particle size, measured by an aerodynamic particle sizer, confirmed that the nebulized material was submicron.

Stability of SM Intubation Vapor Exposure System

FIGURE 7.

Concentration of SM over 80 min, illustrating the rise to target concentration and stability of concentration in the intubation inhalation system.

FIGURE 8.

Transverse sections of the nasal vestibule at the level of the labial surface of the incisors of a control rat (A) and a rat exposed to SM vapor by nose-only exposure (50 mg/m^3 for 60 min) at 8 days post exposure (B). Typically, SM caused severe necrosis and degeneration of the nasal mucosa, particularly in the rostral aspects of the nasal passages. Often, passages were partially occluded with necrotic cell debris and a protein-rich inflammatory exudate containing large numbers of neutrophils. Bars = 250 μm .

FIGURE 9.

Lungs depicting the axial airways from rats exposed to filtered air (control, A) or to SM vapor by nose-only exposure (50 mg/m^3 for 60 min) at 6 days post exposure (B). Airway epithelial injury is not apparent in (B). Bars = $250 \text{ }\mu\text{m}$.

FIGURE 10.

(A) Proximal axial airway of a rat exposed to SM vapor via an intratracheal tube (180 mg/m³ for 21 min) at 4 days post exposure. Necrosis and hemorrhage effaces the mucosal epithelium (arrows) while fibrin, blood, and necrotic cell debris nearly completely occlude the lumen (arrowheads). (B) Proximal axial airway in the lung of a rat exposed to SM vapor via an intratracheal tube (139 mg/m³ for 60 min) at 1 day post exposure. Portions of the mucosal epithelium are sloughed (arrowheads), and hemorrhage is present in the submucosa (arrow). Bars = 100 μm.

TABLE 1

J-tube operating conditions and resulting vapor concentrations

Operating temperature (°C)	Syringe speed (μL/min)	Vapor conc. (mg/m ³) nose-only	Vapor conc. (mg/m ³) intubation
220	1	Occlusion	N/A
110	1	32	N/A
110	5.5	45	N/A
160	0.6	25	N/A
160	5.5	50	N/A
160	0.5	N/A	25
160	3	N/A	100
160	6	N/A	180

TABLE 2

Aerosol solution conditions and resulting aerosol concentrations

Solution concentration (%)	Aerosol conc. (mg/m³)
0.1	10.2
0.5	96.7
1	180