Published in final edited form as:

Pediatr Pulmonol. 2015 February ; 50(2): 115–126. doi:10.1002/ppul.23043.

Intratracheal Heparin Improves Plastic Bronchitis Due to Sulfur Mustard Analog

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Summary

Background—Inhalation of sulfur mustard (SM) and SM analog, 2-chloroethyl ethyl sulfide (CEES), cause fibrinous cast formation that occludes the conducting airways, similar to children with Fontan physiology-induced plastic bronchitis. These airway casts cause significant mortality and morbidity, including hypoxemia and respiratory distress. Our hypothesis was that intratracheal heparin, a highly cost effective and easily preserved rescue therapy, could reverse morbidity and mortality induced by bronchial cast formation.

Methods—Sprague Dawley rats were exposed to 7.5% CEES via nose-only aerosol inhalation to produce extensive cast formation and mortality. The rats were distributed into three groups: non-treated, phosphate-buffered saline (PBS)-treated, and heparin-treated groups. Morbidity was assessed with oxygen saturations and clinical distress. Blood and bronchoalveolar lavage fluid (BALF) were obtained for analysis and lungs were fixed for airway microdissection to quantify the extent of airway cast formation.

Results—Heparin, given intratracheally improved survival (100%) when compared to non-treated (75%) and PBS-treated (50%) controls. Heparin-treated rats also had improved oxygen saturations, clinical distress and airway cast scores. Heparin-treated rats had increased thrombin clotting times, factor Xa inhibition, and activated partial thromboplastin times, indicating systemic absorption of heparin. There were also increased red blood cells (RBCs) in the BALF in 2/6 heparin-treated rats compared to PBS-treated control rats.

Conclusions—Intratracheal heparin 1 hr after CEES inhalation improved survival, oxygenation, airway obstruction, and clinical distress. There was systemic absorption of heparin in rats treated intratracheally. Some rats had increased RBCs in BALF, suggesting a potential for intrapulmonary bleeding if used chronically after SM inhalation.

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Conflict of interest: None.

Conference Presentations: Data presented in poster abstract form at Counter ACT Conference 2013 and American Thoracic Society Conference 2013.

Supporting Information: Additional supporting information may be found in the online version of this article at the publisher's web-site.

Keywords

pulmonology; plastic bronchitis; heparin; sulfur mustard; inhalation injury; CEES

Introduction

Sulfur mustard (bis (2-chloroethyl) sulfide) is an alkylating vesicant used as a chemical warfare agent, reportedly causing more than 90% of all documented chemical warfare gas casualties in the last century.¹ Sulfur mustard (SM) remains a threat, despite last being used in the Iran-Iraq War (1979–1988), because it is stockpiled in multiple countries around the world, including the United States, the former Soviet republic, North Korea, Syria, and potentially others. SM has acute and chronic harmful effects on the eyes, skin, bone marrow, and respiratory tract.¹ The most acutely threatening injury is damage to the respiratory tract. Veress et al. replicated the acute respiratory injury of SM in a rat SM analog (2-chloroethyl ethyl sulfide [CEES]) inhalation model.² The CEES rat model is consistent with previous reports of human SM inhalation, described as “airway obstructive, necrotic debris/mucosa with ‘pseudomembranes’ in the large airways of victims”^{1,3,4} and this closely resembles airway injury caused by authentic SM in rats. The acute airway obstruction secondary to SM and CEES are predominantly due to fibrin casts formed by clotting elements leaking into airways from the bronchial circulation.^{2,5} The severe conducting airway obstruction frequently leads to acute respiratory failure, requiring intubation, mechanical ventilation and potentially causing death.^{1,6} The CEES model correlates clinically to SM inhalation because the rats experience decreased oxygen saturations, increased respiratory distress and death.⁴

The fibrin casts formed after SM and CEES inhalation mimic a type of the disorder called “plastic bronchitis,” which occurs in children, a rare disease process, now seen most frequently after palliative Fontan surgery for cyanotic congenital heart diseases.^{7–14} However, plastic bronchitis also occurs with cystic fibrosis,¹⁵ asthma,¹⁶ acute chest syndrome from sickle cell disease,¹⁷ and inhalational burns,¹⁸ among other diagnoses. The branching bronchial casts seen in plastic bronchitis in children can partially or completely occlude the tracheobronchial tree, potentially leading to acute respiratory failure, similar to that seen in the airways after SM inhalation.^{19–21} Treatment of plastic bronchitis in children has been based mainly on anecdotal case reports, including those of use of inhaled²² and systemic corticosteroids,²² mucolytics,²³ Fontan fenestration,^{19,24} thoracic duct ligation,²⁵ lobectomy,²⁶ percussive vest therapy, extracorporeal tracheal synchronization,⁸ bronchoscopic therapy,^{27–29} and heart transplant.³⁰ These therapies can be very invasive, expensive and/or variably effective, depending on the etiology of the airway casts.

Although there is no definitive therapy for plastic bronchitis or antidote for SM, Veress and Rancourt, respectively, have previously shown that fibrinolytics such as tissue plasminogen activator (tPA), and anticoagulants like tissue factor pathway inhibitor (TFPI), administered into the airway, can effectively reverse and/or decrease formation of the fibrous airway-obstructive casts, improving morbidity and mortality after SM inhalation.^{4,31} Currently, these two proteins are relatively expensive, difficult to manufacture and require refrigeration to maintain stability. Because there are no medications that people can currently be given in

the field to prevent or reverse sulfur mustard inhalational injury after being exposed, we sought to determine if heparin could be used to prevent airway obstruction, hypoxemia, and mortality due to SM inhalation.

Heparin is an attractive anticoagulant therapy. Heparin was one of the first manufactured anticoagulants and is now relatively inexpensive compared to other newer anticoagulants. It is readily available and can be stored at room temperature for easy transport. Nebulized heparin has been shown to decrease signs of acute lung injury in sepsis after inhalational burns in a sheep model by decreasing lung edema, cell infiltrates, and airway cast formation.²² Aerosolized heparin (5,000 IU UHF), given every 4 hr, has also been used off-label in combination with *N*-acetylcysteine in children that suffered inhalational burns, with improvement in mortality, as well as decreased frequency of atelectasis and reintubation when compared to untreated controls.¹⁸ Nebulized heparin, administered with this same dosing regimen, also was reported to be beneficial in a child with plastic bronchitis secondary to Fontan physiology, with improvement in hypoxemia, secretion expectoration and dyspnea.²³

The promising success of aerosolized heparin in other fibrin cast models led us to hypothesize that heparin could improve morbidity and mortality associated with acute fibrous airway obstruction (plastic bronchitis) seen after SM inhalation. Our first objective was to test this hypothesis by exposing Sprague-Dawley rats in our SM analog (2-chloroethyl ethyl sulfide [CEES]) model and giving either heparin or control phosphate-buffered saline (PBS) intratracheally 1 hr after exposure. Endpoints used to assess benefit of the heparin intervention were percent survival, pulse oximetry, clinical distress, airway cast scores, as well as blood gas measurements at study termination. Our second objective was to assess for any systemic effects of intratracheal heparin in our model by evaluating bronchoalveolar lavage fluid (BALF) for red blood cells and assaying plasma for inhibition of coagulation factors by heparin.

Materials and Methods

Drugs

Unfractionated heparin sodium for injection (10,000 USP units/mL) was purchased from Sigma. The heparin was diluted to 400 U/kg concentration in 1 × PBS and kept at room temperature. For the dose range study, heparin was diluted to 200, 300, and 400 U/kg. The pH of the diluted heparin was 7, tested with litmus paper. The pH of the PBS control was 7, also.

Chemicals

2-Chloroethyl ethyl sulfide (CEES, 8.41M) was obtained from TCI America (Portland, OR).

Animal Care

The Institutional Animal Care and Use Committee (IACUC) of University of Colorado, Denver approved this study. Adult male (275–325 g) Sprague-Dawley rats (Harlan Co., Indianapolis, IN) were used.

Inhalational Exposure to CEES

Rats were anesthetized with combination of ketamine (75 mg/kg), xylazine (7.5 mg/kg), and acepromazine (1.5 mg/kg) and placed in polycarbonate tubes with sealing plungers. Tubes were attached to nose-only inhalation system (CH Technologies, Westwood, NJ), and compressed air with the aerosolized 7.5% CEES was administered for 15 min. CEES was administered using a Razel syringe pump (Razel Scientific, St. Albans, VT) connected to a BioAerosol nebulizing generator (DAN 3; CH Technologies). After 15 min CEES exposure, rats were removed from the polycarbonate tubes and observed during recovery from anesthesia.²

Intratracheal Medication Delivery

At 1 hr after CEES inhalational exposure, rats were placed in isoflurane induction chamber and given 3 min of 5% isoflurane in 100% oxygen. Rats were briefly assessed for moderately deep sedation and adequate respiration quality. Each rat was placed supine on an intubation table and a laryngoscope with neonatal blade was used to directly visualize vocal cords for intubation. A microsprayer (PainCentury, Wyndmoor, PA) was inserted immediately inferior to the vocal cord, for the duration of Heparin or PBS control administration (10 sec). After the microsprayer was removed, the rats were recovered in an upright and vertical position. Animals were replaced into their cages only after awakened from anesthesia (~1–3 min). Heparin or PBS administration was repeated via the above procedure at 5 and 9 hr after exposure to CEES.

Noninvasive Oxygen Saturation Monitoring

MouseOx Plus (Starr Life Sciences, Oakmont, PA) with the XL CollarClip Sensor was used to obtain oxygen saturations in unanesthetized rats before exposure, then every 2 hr starting at 4 hr after the CEES exposure.

Clinical Distress Scoring

Respiratory quality, stridor/wheezing and activity level were assessed and each scored at levels of 0–3, with higher number representing the greatest distress (see Supplement Fig. S1). The three category scores were added to obtain a cumulative score (maximum 9). A score of 10 was given to deceased or euthanized rats.

Euthanasia

Animals were euthanized if oxygen saturations were less than 70% and clinical distress score greater than 7 because rats have previously been unable to recover from this level of morbidity after CEES exposure. Rats were euthanized with a combination of ketamine (75 mg/kg), xylazine (7.5 mg/kg), and acepromazine (1.5 mg/kg) and aortic blood plasma samples were obtained prior to exsanguination.

Bronchoalveolar Lavage Fluid (BALF)

Tracheas were cannulated at euthanasia and two 5 ml 0.9% saline lavages were performed. BALF was centrifuged and cell pellet reconstituted in $1 \times$ PBS. Total red blood cell (RBC) counts were performed using a hemacytometer.

Lung Fixation

Tracheas were cannulated at euthanasia and lungs were fixed at 20 cm H₂O with 4% paraformaldehyde in PBS.

Airway Cast Scoring

Cast scoring was performed with previously described microdissection techniques by our lab group.² Fixed lungs were separated into five lobes by cross-sectioning each lobar bronchus at the take-off site from the central airway bronchus (see Supplement Fig. S2A). Each cut bronchial lumen was visualized by angling it perpendicular to the dissecting microscope and a digital picture (Olympus C-750 camera, Olympus Imaging America, Inc., Center Valley, PA) was taken of each lobar opening. Percent of airway occlusion by the cast, if present, was determined from the digital image using Image-J program (1.44p, NIH, USA) (see Supplement Fig. S2B). The first gravity-dependent lobe (when animal in prone position) of each lobar bronchus was also assessed and scored with the same protocol. The percent occlusion was converted to a raw nominal cast score with scale from 0 (0% occlusion) to 7 (100% occlusion). Each lobar score was weighted based on percent of lung volume supplied by that bronchus (see Supplement Fig. S2C). The five weighted lobe scores were summed to obtain the main or dependent cast scores respectively, for each animal (scores 0–7).

Arterial Blood Gas Measurements (ABG)

Blood collected from the descending aorta was placed into a calibrated test card (EPOC-BGEM Test Card) and analyzed for pH, partial pressure of carbon dioxide (pCO₂), partial pressure of oxygen (pO₂), and lactate using the EPOC-vet Blood Analysis system (Epocal Inc., Ottawa, Canada).

Blood Plasma Collection

Blood was collected from anesthetized rats (ketamine [75 mg/kg], xylazine [7.5 mg/kg], and acepromazine [1.5 mg/kg]) prior to euthanasia at study termination and anticoagulated with 1:9 part calcium citrate 3.2%, centrifuged at 3,000 rpm for 15 min. Plasma was collected and frozen to –80°C for storage.

Thrombin Time

Thrombin time coagulation assay was performed in a 96-well plate using 50 µl of plasma, 90 µl of 0.9% sodium chloride, 10 µl of a solution of 0.0125 units of activated rat thrombin (Sigma), and then added to 50 µl of 30 nM calcium chloride. Clotting was defined as a sustained elevation of absorbance without fluctuation of greater than 10% in absolute absorbance. Clot time was analyzed by SpectroMax 340 using absorbance over 20 min at 37°C, assessed at 20 sec intervals.

Heparin Assay

Heparin assay was performed on rat plasma samples in a 96-well plate using the Diophen chromogenic substrate Factor Xa inhibition assay purchased from Aniaara (West Chester, OH). Standards were modified to allow detection of as little as 0.00625 units/ml heparin.

Activated Partial Thromboplastin Time (aPTT)

The aPTT assay was performed on rat plasma samples using a STart 4 coagulation analyzer, reagent, and protocol purchased from Stago Diagnostica (Parsippany, NJ).

Statistical Analysis

Prism 5.01 software (GraphPad, La Jolla, CA) was used for statistical analysis. ANOVA with Tukey's post hoc analysis was used for comparison of cast scoring, pulse oximetry, distress composite scores, pH, pCO₂, pO₂, lactate, thrombin times, heparin assays, and aPTT between PBS-treated, non-treated, and heparin-treated groups. Linear regression was used to evaluate correlation between factor Xa inhibition and aPTT. Kruskal–Wallis with Dunn's post hoc analysis was used for comparison of BALF RBC counts. Kaplan–Meier survival curves were analyzed using Mantel–Cox Log-rank test. A *P* value less than 0.05 was considered significant in all statistical analyses.

Results

Effect of Heparin on Airway Obstruction

CEES inhalation exposure was shown by our lab to cause fibrin cast formation in the airway of exposed rats. We developed a cast scoring system that represents the degree of airway obstruction in the main bronchi. The heparin-treated group had a 60% decrease in main bronchial cast scores compared to the non-treated and PBS-treated control groups (1.3 vs. 3.3 and 3.3, respectively, *P* < 0.001) (Fig. 1). There was no significant difference in cast scores between the PBS-treated and non-treated groups. Similar findings were seen in the first proximal “dependent bronchi” cast scores as well with cast scores of 0.9, 3.4, and 3.3, respectively, in the heparin-treated, non-treated, and PBS-treated groups.

Effect of Heparin on Pulse Oximetry

After CEES exposure, our lab's previous studies demonstrated that oxygen saturations decline as fibrin cast obstruction occurs in the airways. Oxygen saturations were consistently higher during the entire study in heparin-treated rats as compared to the non-treated and PBS-treated controls (Fig. 2). At 12 hr after exposure, mean oxygen saturations in heparin-treated rats were improved when compared to the non-treated and PBS-treated controls that survived to the end of the study (90% vs. 51 and 82%, respectively, *P* < 0.05).

Effect of Heparin on Clinical Distress

We previously reported that CEES exposure causes significant respiratory and physical distress after airway fibrin casts form. The clinical score was a composite (respiratory quality, stridor, and activity) used to quantify clinical distress with 0 representing no distress and 10 representing death of the animal. The heparin-treated rats had less clinical distress than the non-treated and PBS-treated rats throughout the study (Fig. 3). The improvement in clinical distress in the heparin-treated group was most pronounced at 12 hr compared to the non-treated and PBS-treated groups (1.1 vs. 4.9 and 4.1, respectively, *P* < 0.01). There was no significant difference of clinical distress scores between PBS-treated and non-treated controls.

Effect of Heparin on Survival

CEES inhalational exposure can cause significant mortality. Survival after CEES exposure in the non-treated group was 75% (11/15). Survival was 100% in heparin-treated rats (17/17) when followed to 12 hr (Fig. 4). When compared to control groups, the heparin-treated rats had greater survival than non-treated rats ($P < 0.05$), with a similar trend of improved survival in the heparin-treated rats when compared to the PBS-treated rats (92% survival).

Effect of Intratracheal Heparin on Arterial Blood Gas Measurements

Heparin-treated rats tended to have improvement in pH (7.41), $p\text{CO}_2$ (36 mmHg), $p\text{O}_2$ (57 mmHg), and lactate (2.0 mmol/l) compared to PBS-treated controls (pH 7.39, $p\text{CO}_2$ 43, $p\text{O}_2$ 52, and lactate 2.3), but none were statistically significant (see Supplement Figs. S3–S6). Heparin-treated rats did have significantly improved pH, $p\text{CO}_2$, $p\text{O}_2$, and arterial lactate, however, when compared to non-treated rats (pH 7.31, $p\text{CO}_2$ 51, $p\text{O}_2$ 44, and lactate 3.1) ($P < 0.01$). PBS-treated were slightly improved in all 4 ABG measurements compared to non-treated rats, but none were statistically different.

Effect of Heparin on Airway Bleeding

Despite 100% survival among the heparin-treated rats, there was serosanguinous fluid in the trachea noted on necropsy in 2/17 rats. However, BALF comparisons did not show a significant difference in RBC counts between non-treated, PBS-treated, or heparin-treated CEES-exposed groups (Fig. 5). CEES exposure was associated with increased RBC's when compared to naïve rats. The non-treated (555×10^5 RBC), PBS-treated (672×10^5 RBC) and heparin-treated ($8,268 \times 10^5$ RBC) BALF were all higher than naïve rats (0.7×10^5 RBC). The heparin-treated group had 2/6 rats with greater than $6,000 \times 10^5$ RBC's, making this group tend to have higher mean BALF RBC counts. None of the CEES-exposed rats had decreased hematocrits at the end of the study with mean hematocrits of 44.8, 45.9, and 46.7 in the non-treated, PBS-treated, and heparin-treated groups, respectively.

Effect of Intratracheal Heparin on Systemic Heparin Absorption and Coagulation

The mean thrombin clotting time in heparin-treated rats (1,103 sec) was elevated when compared to the non-treated (808 sec) and PBS-treated rats (849 sec), (Fig. 6, $P < 0.001$). All control group samples were clotted by 1,100 sec, but only 22% of the heparin-treated samples were clotted at this time.

A chromogenic factor X inhibition assay was used to quantify the amount of systemically absorbed heparin. The heparin-treated group had an elevated mean serum heparin level (0.125 U/ml) compared to the non-treated (0.043 U/ml) ($P < 0.05$) and PBS-treated groups (0.025 U/ml) (Fig. 7A, $P < 0.01$). The aPTT test, used to evaluate heparin anticoagulation clinically, showed heparin-treated rats' plasma had an elevated aPTT (25.1 sec) when compared to the non-treated (18.7 sec), PBS-treated (19.7 sec) and naïve controls done for comparison (17.3 sec). Figure 7B shows that there was a positive correlation between the elevated plasma heparin and resultant elevation in aPTT.

Effect of Incremental Heparin Doses on Airway Obstruction, Survival, Pulse Oximetry, and Clinical Distress

Incremental doses of heparin (200, 300, and 400 U/kg) were given to assess the most effective dose to improve mortality and morbidity after the SM analog, CEES. The 400 U/kg heparin dose had the best airway cast score, survival, pulse oximetry, and clinical distress score.

Airway obstruction was the most impressive improvement with increasing doses of heparin. The cast scores for the non-treated, 200, 300, and 400 U/kg heparin doses were 3.3, 2.5, 2.0, and 1.5, respectively (see Supplement Fig. S7). All doses of heparin tended to improve survival compared to 75% (6/8) in the non-treated group. There was 83% (5/6) survival in the 200 U/kg group and 100% survival in the 300 (6/6) and 400 U/kg (6/6) heparin-treated groups (see Supplement Fig. S8). There was a dose response improvement in pulse oximetry with 200 U/kg heparin-treated achieving 85% mean oxygen saturations at 12 hr, and 300 and 400 groups both with 90% saturations, compared to 79% saturations in the 6/8 surviving non-treated group (see Supplement Fig. S9). Though there was no statistical difference in clinical distress scores, there was a dose response improvement at 12 hr with the non-treated, 200, 300, and 400 U/kg scoring 5.7, 3.4, 2.1, and 1.2, respectively (see Supplement Fig. S10).

Discussion

In this study, we found that intratracheally administered heparin can reduce CEES-induced fibrinous airway obstruction, called plastic bronchitis, and improve morbidity outcome measures, but this resulted in systemic heparin absorption and anticoagulation effects. In our experiments, the heparin-treated rats, after CEES exposure, had decreased airway cast scores, indicating a reduction in airway obstruction. Likely because of the decreased airway obstruction, the heparin-treated rats consistently had less respiratory distress and improved oxygen saturations throughout the study as compared to the PBS-treated control rats. This improved clinical status in rats is important because it translates to less required respiratory support and fewer interventions that may be required to support humans exposed to sulfur mustard (“mustard gas”), such as increased use of supplemental oxygen, positive airway pressure and potentially intubation and mechanical ventilation. The improved hypercarbia, lactic acidosis and oxygenation in the heparin-treated group translated to significant improvement in survival when compared to the non-treated, CEES-exposed group. Even though mortality was not significantly different between the heparin-treated group and the PBS-treated control group at 12 hr, the entire heparin-treated group survived to the end of the study with near-normal pulse oximetry and clinical distress scores, whereas the rats in the PBS-treated group continued to show declining clinical performance throughout the study.

In our experiments, we found that heparin given intratracheally at an optimal dose of 400 U/kg every 4 hr was the most successful regimen for improved morbidity and mortality. Initially, we performed pilot studies with a range of doses of heparin (200–400 U/kg) in order to establish the most efficacious dose. There was decreased survival in the 200 U/kg dose (5/6) compared to the higher 300 and 400 doses with 100% survival, and there was a

trend in improvement in airway cast scores, hypoxemia and clinical distress scores as we increased the dose of heparin to 400 U/kg. These preliminary dose-finding studies suggested that doses of heparin lower than 400 U/kg were inferior in preventing and/or treating the underlying etiology of fibrinous airway obstruction. Other than this initial dose-finding study, lower doses were not rigorously tested for efficacy.

We believe that it was important to give the heparin early after CEES exposure because of heparin's mechanism of action. Heparin binds to antithrombin III and increases its activity 1,000-fold.³⁴ Antithrombin irreversibly binds multiple serine proteases on coagulation factors, most importantly Factor Xa and thrombin. Thrombin is the central component of a developing clot because it not only activates multiple coagulation factors, but it also cleaves fibrinogen to make fibrin. We have shown previously that plastic bronchitis casts in the airway after CEES and sulfur mustard gas are made of fibrin and the airway leak from the systemic circulation's bronchial arteries are the source of fibrin.^{2,5} The improvement in morbidity due to CEES after intratracheally administered heparin is due to increased activity of endogenous antithrombin in inhibiting thrombin. Inhibiting thrombin decreases the hypercoagulable state in the airway occurring after CEES exposure, described previously by our lab, and enhanced antithrombin activity prevents the formation of airway fibrin casts.³⁵

Even though there was an obvious improvement in the heparin-treated group after CEES exposure, intratracheal administration of heparin detectably increased the frequency of airway bleeding after CEES exposure. Heparin is an anticoagulant and, if given at large dose systemically, there can be an increased risk of bleeding in humans and animals. In our experiments, there were pink-tinged secretions noted on necropsy in 2/17 of the heparin-treated rats, indicating the possibility of induced intrapulmonary bleeding. When assessed with bronchoalveolar lavage, we found that 2/6 of the 400 U/kg heparin-treated rats had increased RBC counts in BALF as compared all other heparin-treated, PBS-treated, and non-treated rats, though there was no significant mean RBC difference between treatment groups (Fig. 5).

Presence of RBC's in the airway led us to evaluate for any systemic anticoagulation effects as a potential cause of bleeding after intratracheal heparin administration. Thrombin time is a very sensitive test to evaluate for heparin effect in plasma. The heparin-treated rats had an elevated mean thrombin time when compared to the PBS-treated controls, which indicated that heparin had entered the systemic circulation of the intratracheally heparin-treated rats. The heparin assay, utilizing factor Xa inhibition, confirmed this finding by demonstrating an elevation of factor Xa inhibition in the heparin-treated plasma. We found a mean of 0.125 units/ml of heparin in the serum of the heparin-treated compared to 0.025 units/ml in the PBS-treated control group. Jacques previously showed that there was consistent absorption of intratracheally delivered heparin above a threshold of 1,300 units/kg across species in rats, mice, humans, and dogs.^{36,37} Their group found 1–5 units/ml of heparin in plasma of all four species at all intratracheal doses between 550 and 10,000 units/kg, and these dosages were absorbed systemically within 1.5 hr after administration. We have shown a smaller amount of heparin absorption after CEES exposure, ranging between 0.1 and 0.25 units/ml heparin, at a slightly lower intratracheal heparin dose of 1,200 units/kg over three administrations in a 12 hr period when compared to these prior studies done in naïve humans

and animals. The slight difference in heparin absorption reported previously could potentially be secondary to cellular injury and systemic vascular leak of the bronchial arteries.

Because heparin absorption was identified, an activated prothromboplastin time (aPTT) was performed to assess for clinical relevance of anticoagulation. There was an elevation in aPTT in the heparin-treated compared to the PBS-treated control group. The aPTT (23.1 sec) in the heparin-treated rat plasma was also elevated as compared to our naïve Sprague-Dawley rats (17.3 sec) as well as the normal range previously reported in Wistar rats (13–19.2 sec).²⁰ By contrast, the PBS-treated aPTT (19.7 sec) was closer to the normal range and not statistically different from naïve plasma. The elevation in aPTT confirms that there was a systemic anticoagulation effect that resulted from administering heparin intratracheally.

After finding systemic heparin absorption, we then attempted to find the source of the blood seen on necropsy. There was a potential risk of blunt trauma to the trachea by the microsprayer during drug delivery, but when assessed by necropsy, no evidence of trauma to the trachea was noted. Intratracheal heparin administration at 200–400 units/kg heparin after higher level CEES inhalation (10%) has been associated with more consistent airway blood seen on necropsy. The central airway bleeding seen with intratracheal heparin after 10% CEES inhalation was associated with increased patterns of microscopic alveolar hemorrhage and ruddy appearance in upper lobes on gross examination (data not shown). This finding suggests that there may be mild patchy pulmonary hemorrhage in the lungs after CEES exposure as a source of bleeding seen in the airway. Microscopic evaluation of the 2/17 rats with apparent central airway bleeding after 7.5% CEES inhalation described here was not possible due to specimen contamination resulting from bronchoalveolar lavage. Despite finding pink-tinted secretions in the airway on necropsy, there were no clinical signs that the airway bleeding caused significant morbidity or mortality. All the heparin-treated rats survived, had decreased airway obstruction, improved pulse oximetry and clinical distress scores, including the rats that had mild signs of central airway bleeding at necropsy and elevated BALF RBC's.

This study is important because it shows that heparin, an inexpensive, easily stored therapy, can be used to improve acute airway obstruction associated with plastic bronchitis and improve survival after sulfur mustard analog inhalation. Because similar findings of plastic bronchitis-induced airway obstruction have been observed in humans after sulfur mustard exposure,⁶ heparin could also improve morbidity in this situation. The concern we have with heparin therapy is the risk for pulmonary bleeding following systemic anticoagulation, as we have shown increased RBC's on BALF as sporadic, potentially untoward effect in this study. During a sulfur mustard gas exposure, if other traumatic injury occurred concurrently during warfare, such as an explosive injury to extremities, cranium, and/or internal organs, this risk of bleeding could also increase after heparin therapy due to the systemic absorption potential of intratracheal heparin. Long-term studies may be required to determine the actual risk of such bleeding potential.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Jorge DiPaola, M.D. and Sally Stables, M.D. for advice on coagulation evaluation portion of the study. Robin Deterling, M.D. for human clinical correlation contribution.

Funding source: National Institutes of Health, Number 2U54-EB015678.

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Fig. 1.

Effect of heparin on airway obstruction by fibrin casts after CEES inhalation. Cast score is a sum of obstruction seen in main bronchi of each lung by microdissection (0 represents no airway cast and 7 represents 100% obstruction). Anesthetized rats were exposed to 7.5% CEES for 15 min. Unfractionated heparin treatment (400 U/kg, intratracheally) with isoflurane anesthesia was given 1 hr after CEES exposure and repeated every 4 hr during a 12 hr study. Control rats received PBS at same intervals under isoflurane anesthesia, or no treatment ("NT"). NT group (n = 9); PBS group (n = 6); Heparin group (n = 11). *** $P < 0.001$ by ANOVA with Tukey's post hoc analysis, all other non-labeled comparisons were not significant.

Fig. 2.

Effect of heparin on pulse oximetry after CEES inhalation. Anesthetized rats were exposed to 7.5% CEES for 15 min. Unfractionated heparin treatment (400 U/kg, intratracheally) with isoflurane anesthesia was given 1 hr after CEES exposure and repeated every 4 hr during a 12 hr study ("IT"). Control rats received PBS at the same intervals with isoflurane anesthesia, or no treatment ("NT"). IT group (n = 15); PBS group (n = 12); Heparin group (n = 18). (* $P < 0.05$ for heparin vs. PBS and heparin vs. NT) by ANOVA with Tukey's post hoc analysis. All other non-labeled comparisons were not significant.

Fig. 3.

Effect of heparin on clinical distress after CFES inhalation. Clinical score is a sum of activity, respiratory effort and stridor (0 represents no distress and 10 represents death). Anesthetized rats were exposed to 7.5% CEES for 15 min. Unfractionated heparin treatment (400 U/kg, intratracheally) with isoflurane anesthesia was given 1 hr after CEES exposure and repeated every 4 hr during a 12 hr study ("IT"). Control rats received PBS at same intervals with isoflurane anesthesia, or no treatment ("NT"). NT group (n = 15); PBS group (n = 12); Heparin group (n = 18); (** $P < 0.01$, *** $P < 0.001$ for heparin vs. PBS and heparin vs. NT) by ANOVA with Tukey's post hoc analysis. All other non-labeled comparisons were not significant.

Fig. 4.

Effect of heparin on survival 12 hr after CEES inhalation. Anesthetized rats were exposed to 7.5% CEES for 15 min. Unfractionated heparin treatment (400 U/kg, intratracheally) with isoflurane anesthesia was given 1 hr after CEES exposure and repeated every 4 hr during a 12 hr study. Control rats received PBS at same intervals with isoflurane anesthesia, or no treatment ("NT"). NT group (n = 15); PBS group (n = 12); Heparin group (n = 18). * $P < 0.05$ and ns, not significant by Log-Rank (Mantel-Cox) test.

Fig. 5.

Effect of heparin on red blood cells in bronchial alveolar lavage fluid (BALF) after CEES inhalation. Anesthetized rats were exposed to 7.5% CEES for 15 min. Unfractionated heparin treatment (400 U/kg, intratracheally) with isoflurane anesthesia was given 1 hr after CEES exposure and repeated every 4 hr during a 12 hr study. Control rats received PBS at same intervals with isoflurane anesthesia, or no treatment ("NT"). Naïve group, shown for comparison, were not exposed to CEES or given any treatment. BALF was collected via tracheostomy cannula at necropsy and red blood cells counted directly with microscopy. Naïve group (n = 6); NT group (n = 6); PBS group (n = 6); Heparin group (n = 6); * $P < 0.05$, ** $P < 0.01$ by Kruskal–Wallis with Dunn's post hoc analysis, all other non-labeled comparisons were not significant.

Fig. 6.

Effect of heparin on thrombin clotting time after CEES inhalation. Anesthetized rats were exposed to 7.5% CEES for 15 min. Unfractionated heparin treatment (400 U/kg, intratracheally) with isoflurane anesthesia was given 1 hr after CEES exposure and repeated every 4 hr during a 12 hr study. Control rats received PBS at same intervals with isoflurane anesthesia, or no treatment ("NT"). Blood collected from aorta with ketamine, xylazine, and acepromazine sedation, added to sodium citrate in 1:10 mixture. Plasma separated with centrifugation and rat thrombin added with calcium chloride to start clotting process. Clot time was calculated by spectrophotometer absorbance. NT group (n = 13); PBS group (n = 12); Heparin group (n = 16); * $P < 0.001$ by ANOVA with Tukey's post hoc analysis, all other non-labeled comparisons were not significant.

Fig. 7.

Effect of intratracheal heparin on plasma heparin level and partial thromboplastin time after CEES inhalation. Anesthetized rats were exposed to 7.5% CEES for 15 min. Unfractionated heparin treatment (400 U/kg, intratracheally) with isoflurane anesthesia was given 1 hr after CEES exposure and repeated every 4 hr during a 12 hr study. Control rats received PBS at same intervals with isoflurane anesthesia, or no treatment ("NT"). Blood collected from aorta with ketamine, xylazine, and acepromazine sedation, added to sodium citrate in 1:10 mixture. A: Concentration of heparin in plasma samples evaluated using Biophen hep assay. B: aPTT was calculated by Start 4 coagulometer and correlated to heparin assay. NT group (n = 12); PBS group (n = 12); Heparin group (n = 16); * $P < 0.05$ and ** $P < 0.01$ by ANOVA with Tukey's post hoc analysis, all other non-labeled comparisons were not significant. $r^2 = 0.702$ by linear regression of the correlation.