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A Histological Assessment of Lung Injury in Rats Exposed to Inhaled Sulfur Mustard across Dose and Time

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14. ABSTRACT Sulfur mustard (HD) causes severe chemical burns to the skin, eyes, and airways. HD was used as a chemical warfare agent in the Iran/Iraq War. Many surviving HD-exposed casualties continue to suffer from permanent lung injuries. Temporal development of these pathologies is poorly defined, and there is no effective antidote. Rats were exposed to vehicle or nebulized HD for 10 min. at doses up to 3.0 mg/kg. Lung samples were removed at various time points from 3 h to 6 months post-exposure. The most striking histologic lesions were associated with the conducting airways. Epithelial necrosis, clefting, and loss; acute fibrinous inflammatory exudation; and pseudomembrane formation were common early. Lymphocytolysis was common in high-dosed animals. Histologic lesions were also present in the centroacinar region, particularly adjacent to the mainstem bronchioles. From 7-14 days, alveolar histiocytosis with a mixed inflammatory exudate, alveolar (luminal) edema and flooding, and alveolar interstitial edema and inflammation were seen. From third week to 6 months post-challenge, focally extensive subepithelial fibrous proliferations (polyps) occluded airway lumina as much as 50%. Interstitial fibrosis and emphysematous-like change were observed at +2 months. Hemorrhage and fibrin, neutrophilic and histiocytic interstitial inflammation, and prominent (hyperplastic) alveolar epithelial cells were also seen.					
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ABSTRACT

Sulfur mustard (HD) causes severe chemical burns to the skin, eyes, and airways. HD was used as a chemical warfare agent (CWA) in the Iran/Iraq conflict, and more than half of surviving HD-exposed casualties continues to suffer from permanent lung injuries. The mechanisms and timing of the development of these pathologies are poorly defined, and there is no effective antidote. Rats were intubated and ventilated for 10 min with nebulized HD or vehicle to achieve total doses of 0, 0.5, 1.75, 2.25, or 3.0 mg/kg. Lung samples were removed at various time points from 3 h to 6 months post-exposure. Tissue samples were fixed using standard H&E processes. Throughout the disease time-course regardless of HD dose, the most striking histologic lesions were associated with the conducting airways, i.e., the trachea and “mainstem bronchioles” (specifically defined in this study as the intrapulmonary bronchi and the primary bronchioles). Epithelial necrosis, clefting, and loss; acute fibrinous inflammatory exudation; and pseudomembrane formation were common early. By the third week a combination of epithelial attenuation, goblet cell hyperplasia, and squamous metaplasia (often in the same airway) along with a mostly proteinaceous exudate were a noteworthy findings. Also by the third week and extending to 6 months post-challenge, focally extensive subepithelial fibrous proliferations that progressed to well-organized fibroepithelial polyps occluded airway lumina by as much as 50%. Such lesions were only seen in the mainstem bronchioles and never in the terminal bronchioles. Bronchoalveolar lymphoid tissue (BALT) lymphocytolysis was common in high-dosed animals but was not a significant feature 72 hours after challenge. Histologic lesions were also present in the centroacinar region (terminal bronchioles and alveoli), particularly adjacent to the mainstem bronchioles, but lessened in severity towards the periphery of the lobe. Within the first 7-14 days, alveolar histiocytosis with a mixed inflammatory exudate, alveolar (luminal) edema and flooding, and alveolar interstitial edema and inflammation were seen. Acute alveolar changes remained present in some lung lobe sections by 4-6 weeks. Additional findings included hemorrhage and fibrin; neutrophilic and histiocytic interstitial inflammation with rare individual cellular necrosis; and prominent (hyperplastic) alveolar epithelial cells. Interstitial fibrosis and emphysematous-like change were also observed at 2 months and beyond.

INTRODUCTION

Sulfur mustard (NATO designation HD) is a vesicating agent. HD ($C_4H_8Cl_2S$) is also known as Yellow Cross, Lost, S-Lost, H or Yperite. It was first used as a chemical warfare agent (CWA) in 1917 and more recently during the Iraq-Iran conflict in 1983-1988. HD vapor was responsible for about 80% of the chemical exposure deaths during World War I (Hurst and Smith, 2008). HD is relatively easily synthesized from common industrial chemicals. Although an oily liquid with a moderately low vapor pressure, HD can be aerosolized or heated to produce vapor and as such can travel long distances during windy conditions. As a vapor, it is 5.4 times as dense as air, hugs the ground, and can be persistent in the environment from days to several weeks. It can remain a threat for some time in various aqueous environments and has caused skin blisters when handled many years after submergence (Aasted et al., 1985; Wulf et al., 1985). Sulfur mustard is an alkylating agent and reactive molecular cross-linker (Wheeler, 1962). Chemical reactions involving HD can produce free radicals (Brimfield et al., 2012) and oxidative stress, which can escalate via an inflammatory chain-reaction (Laskin et al., 2010; Balali-Mood and Hefazi, 2005). The reactive chemical nature of HD is due to the cyclization formation of an episulfonium ion which can combine with numerous nucleophilic sites. HD has been shown to be toxic in a range of *in vitro* (Meier, et al., 1996; Sawyer, 1999; Atkins et al., 2000; Ray et al., 2008; Pohl et al., 2009) and *in vivo* experimental models (Vijayaraghavan et al., 1991; Gold and Scharf, 1995; Anderson et al., 1996; Casillas et al., 1997; Rao et al., 1999; van Helden et al., 2004; Dillman et al., 2005; Fairhall et al., 2010).

HD can be readily absorbed into tissues with much of it finding its way into the circulation, thereby causing systemic effects. The target organs of HD toxicity are ocular, dermal, and lung tissue surfaces. Bone marrow, a site of cell proliferation, is also a target of HD toxicity (Dacre and Goldman, 1996). While exposure through ocular and dermal routes is incapacitating, most fatalities occurring from exposure to HD are a result of inhalation. Symptoms of exposure are delayed, with a latency of ~24 hours for inhalation exposures. HD forms fluid-filled blisters, and associated pain may take several hours. Following dermal exposure, pain is immediate; tissue damage and clinical effects occur hours to days later. Higher exposures have lower latency times to symptoms (acute). Inhaled HD is largely absorbed in the upper airway and rarely penetrates to the lung parenchyma unless the concentration or duration of exposure is sufficiently high. In humans, following wartime inhalation exposure to HD, reports have identified bronchopneumonia, chest tightness, and in long-term survivors, chronic bronchitis, lung fibrosis, productive cough, and chronic obstructive pulmonary disease (Ghanei and Harandi, 2007). Injury can develop slowly, becoming much more intense over time. More detailed clinical manifestations can be found in Willems (1989).

While there is not a direct causative link between HD exposure and cancer from battlefield exposures, workers occupationally exposed to mustard during production had a higher incidence of respiratory, skin and blood cancer (Pechuta and Rall, 1993; Wada et al., 1968). Experimentally, Kan et al. (2008) provided some evidence of a possible causal link between the inhalation of HD

and cancer in rats exposed to a single high dose of HD for 10 min. This current study was designed to satisfy an information gap by testing an animal exposure model to investigate the effects of a single inhalation challenge to HD over a 6-month time period. These data provided evidence that longer-termed responses after a single challenge to HD are manifested in pathological responses that support the human experience years after battlefield exposure to HD.

MATERIALS AND METHODS

Animals. Adult Male Sprague-Dawley (SD) rats (Charles River Laboratories, Wilmington, MA) weighing 250-300 g on arrival were used. Animals were housed individually under standard conditions with 12 h light/dark cycle and food and water were available *ad libitum*. The study protocol was approved by the Institutional Animal Care and Use Committee, USAMRICD, Aberdeen Proving Ground, MD. Research at the USAMRICD is conducted in compliance with the *Animal Welfare Act* as amended (2007) and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, published by the National Academy Press, 1996.

Challenge Agent and Dose. Male rats (240-270 g) were anesthetized (i.m.) with 10 mg/kg xylazine and 90 mg/kg ketamine. All exposures occurred over a 10 min exposure period. The entire exposure system was confined within an inhalation exposure chamber (glovebox). Exposure doses were based on earlier published findings that 350 µg of vaporized HD over 50 min will produce lung injury in spontaneously breathing rats (Anderson et al., 1996). Aged-matched naïve rats and those administered aerosolized saline via the exposure system for 10 min were control animals.

Study Design. Male SD rats were anesthetized with 90 mg/kg ketamine and 10 mg/kg xylazine intramuscularly. Rats were placed in supine position, and a modified and polished smooth-bore glass pasture pipette was inserted into the trachea using a modified laryngoscope. The glass insert was held in place with mildly adhesive tape, and the rat was then transferred to the exposure chamber. A one-time exposure to HD was delivered using a closed-to-atmosphere inhalation system via mechanical ventilation. The respiration rate was determined, and the HD was delivered by a small animal ventilator (Harvard Model 683, Harvard Apparatus, Holliston, MA) set for a tidal volume of 2.5-3 mL and an average respiratory rate of 75 breaths/minute. Because conscious rats can control their respiration when subjected to an irritant environment, anesthetized and ventilated procedures were used to standardize the minute volume, respiration rate x tidal volume, for each exposed rat. HD was delivered from a closed circuit standard nebulizer system (Aeroneb, Aerogen Inc., Marietta, GA). The nebulizer was in series between the ventilator and the glass endotracheal insert; thus, HD aerosol was administered by the action of mechanical ventilation. To produce an aerosolized exposure stream, HD was diluted in a 75% ETOH + 25% sterile water solution for ease and consistency of delivery. During the course of exposure, a sample from the aerosol

stream was taken for MINCAMS quantitative analysis. The Aeroneb has a volume of 10 mL and can deliver about 0.22 mL/min at a mean particle size of 1.6 μ m, well within the range for peripheral lung deposition. This allowed for an even and consistent delivery of HD to the peripheral airway by bypassing the nasal passages, a site of detoxification, and assisted the rat in ventilation in the event that agent irritation affected the breathing pattern. Exhaled gases from the rat were routed via a unidirectional Rudolph valve (Hans-Rudolf Inc., Shawnee, KS) into a flask neutralized with 50 ml of 5% bleach, then through an activated charcoal canister, and finally exited the system through the exhaust HEPA filter. Following HD exposure and after a 10 min room air off gas time period, rats were extubated, placed in recovery posture, and observed during recovery.

Perfusion, Necropsy and Tissue Sample Collection. At designated time points after HD-exposure, the rats were deeply anesthetized as described, and a ventral midline incision was made to expose the thoracic cavity. Rats were perfused with 500 mL of 0.89% normal saline (4.5 grams sodium chloride in 500 mL distilled water) and 250 mL of 10% neutral buffered formalin (Sigma) at a perfusion rate of 40-60 mL/min using a Cole Parmer Masterflex perfusion pump (Model 77200-60). Perfusate was injected into the left ventricle to perfuse the brain and injected into the right ventricle to perfuse the lungs. Immediately following perfusion, the lungs and brain were harvested and placed into 10% neutral buffered formalin (NBF) at biosafety level 2 (BSL-2) for 24-72 h at 4°C. To properly fix the lungs, fixative was slowly instilled into the trachea using a blunted 18 gauge needle until the lungs were inflated.

Histopathology Materials and Methods. After 24-72 h, two cross-sections from each lung lobe (right cranial, right middle, right caudal, left, and accessory lobes) and distal trachea (slightly rostral to the tracheal bifurcation) were trimmed, processed, and paraffin embedded according to standard protocol. Sections submitted for routine histopathology were cut on a rotary microtome at 5 μ m, mounted on glass slides, and stained with hematoxylin and eosin (H&E). Histologic lesions were graded on the extent of severity and the total distribution of lesions using the following scale: 0=no change; 1=trace (1-10%); 2=moderate (10-50%); and 3=severe (>50%).

RESULTS

At all time points, the most noteworthy pulmonary and tracheal histologic lesions were seen in those animals challenged with higher doses, particularly at 2.25 mg and 3.0 mg/kg, although changes were present in lower-dosed animals. However, while not all high-dosed animals had significant histologic lesions, nor were all lung lobes equally affected. Lesions were observed in all lung lobes. Generally, the most striking changes were seen in the right middle, right caudal, and left lobes in association with the respiratory epithelium and subepithelium of the conducting airways that included the trachea, intrapulmonary bronchi (absence of cartilage but close association to bronchus-associated lymphoid tissue-BALT), and primary bronchioles. For the purpose of consistency and organization, “mainstem bronchioles”

will be used to denote the changes observed in the intrapulmonary bronchi and primary bronchioles unless otherwise specified. Neither the tracheal bifurcation nor the extrapulmonary bronchi was submitted for examination in this study and, therefore were not examined histologically. Histologic lesions were present in the centroacinar region (terminal bronchioles and alveoli) but lessened in severity towards the periphery of the lobe.

At 3-6 h, multifocal individual tracheal epithelial necrosis was the most noteworthy histologic change (**Figure 1B**). Subepithelial hemorrhage and cellular debris with neutrophilic inflammation were seen occasionally, along with individual chondrocyte necrosis. A cellular exudate composed of inflammatory cells and sloughed epithelial cells could be found in the tracheal lumen.

In the lung, perivascular (and peribronchiolar) edema with minimal neutrophilic inflammation was observed. Varying in severity and cell type (both neutrophilic and mononuclear), peribronchiolar edema and inflammation, respectively, were a consistent finding as far out as 10 weeks post-challenge. Severe bronchiolar-associated lymphoid tissue (BALT) lymphocytolysis, as early as 3 h and extending to 72 h, was seen in some but not all animals. When BALT lymphocytolysis was present, the adjacent airway epithelium was generally affected, consisting of focally extensive individual (single-cell) bronchiolar epithelial necrosis; the remaining bronchiolar epithelium was histologically normal (**Figure 2**).

By 24 h and extending through 72 h, greater than 50% of the tracheal epithelium was affected with one or more of the following changes: single cell and nests of necrotic epithelial cells, clefting/separation from the basement membrane (**Figure 3B**), and ulceration. A brightly eosinophilic fibrillar and cellular pseudomembrane occasionally formed along the denuded surface and partially occluded the tracheal lumen (**Figure 3A**). Subepithelial hemorrhage and inflammation and individual chondrocyte necrosis (**Figure 3C**) were also more extensive. In the lungs, individual bronchiolar epithelial necrosis became more apparent with occasional fibrinous and cellular exudation and pseudomembrane formation in the secondary bronchi and mainstem bronchioles (**Figures 4A and 4B**). At 72 h (not shown), there was a mild increase in bronchiolar goblet cells which progressively became more noticeable between 7 and 14 days post-challenge. Additionally, in other areas, there was attenuation of the bronchiolar epithelium.

Transmigration of a few neutrophils through the bronchiolar epithelium and periobronchiolar connective tissue was also noted as early as 24 hours. Within the terminal bronchioles and alveoli, alveolar histiocytosis, characterized by 2-4 mononuclear cells (macrophages) with abundant eosinophilic vacuolated cytoplasm, was common, although a mixed neutrophilic exudate was occasionally observed. Other early findings observed in the most affected lung lobes included alveolar (luminal) edema, alveolar interstitial edema and inflammation, alveolar epithelial cell hypertrophy, and, rarely, alveolar hemorrhage and fibrin (**Figures 5A, 5B, and 5C**).

Between 2-3 weeks after HD challenge, focally extensive sub-epithelial proliferations of loosely packed fibroblast-like cells elevating the ulcerated bronchiolar epithelium were observed in the mainstem bronchioles of a few animals (**Figures 6A and 6B**). Bronchiolar epithelial necrosis, ulceration, and loss with attenuation and/or goblet cell hyperplasia (often in the same airway) were more common findings. A fibrinous and cellular bronchiolar exudate (with occasional hemorrhage) was also observed. Although minor edema was present, alveolar neutrophilic exudate was more common and occasionally admixed with hemorrhage and fibrin. Neutrophilic and histiocytic alveolar interstitial inflammation and alveoli lined by plump epithelial cells (suggestive of type II hyperplasia) were accompanying histologic lesions. In a few areas, rare foci of spindled cells (focal alveolar fibrosis) also mildly expanded the alveolar interstitium. The tracheal epithelial changes resembled those seen in the mainstem bronchioles, although individual chondrocyte necrosis was not as readily observed.

Although lesions were observed at earlier time points, the most widespread and generally the most severe lesions were present between 4 and 7 weeks. In addition to the previously described bronchiolar epithelial changes, squamous metaplasia was also noted (**Figures 7A, 7B, and 7C**). Also, the bronchiolar exudate appeared less fibrinous and cellular but more proteinaceous compared to that at earlier time points (**Figure 8**). When present, the focal subepithelial proliferation became more organized, partially occluded the airway lumen, and was partially or completely lined by respiratory epithelium resembling a fibroepithelial polyp (**Figure 9A and 9B**).

Unlike the mostly proteinaceous bronchiolar exudate, the alveolar exudate varied from mostly proteinaceous to heavily neutrophilic or a combination of both. Alveolar hemorrhage, fibrin, and hemoglobin crystals admixed with erythrocyte- and hemosiderin-laden macrophages were also routinely seen. In the most affected areas, prominent (hyperplastic) alveolar epithelial cells lined alveolar septae thickened up to 4x normal by neutrophilic and histiocytic inflammation, fibrosis, and occasionally individual cellular necrosis (**Figure 10**). Vascular necrosis with organized fibrin thrombi, affecting small caliber vessels, was also observed in some animals. Tracheal epithelial attenuation, squamous metaplasia, ulceration, and/or loss with fibrinous and cellular exudation continued to be observed.

At 2 months post-exposure, similar histologic lesions were seen in the lung and trachea but were generally not as severe. The most noteworthy changes were the partially or completely epithelial-lined subepithelial fibrous proliferations/polyps, bronchiolar goblet cell hyperplasia, and alveolar interstitial fibrosis (**Figure 11A and 11B**). Between 3 and 6 months, the subepithelial polyps and bronchiolar goblet cell hyperplasia in larger airways remained noteworthy features (**Figure 13A**). When present, the alveolar changes were minimal to mild in severity and consisted of interstitial fibrosis and histiocytosis. Occasionally, in some lung lobe sections, alveolar spaces had united and formed dilated alveolar spaces, a histological finding consistent with emphysematous change. This finding was most noticeable adjacent to mainstem bronchioles partially occluded by the subepithelial fibrous proliferations/polyps.

Likewise, the tracheal epithelial changes were also similar (**Figure 12**), although goblet cell hyperplasia was only occasionally observed. Additionally, subepithelial fibrous proliferation with osseous metaplasia was infrequently found in the trachea of some animals (**Figure 13B**).

Histologic pathological lesions were observed in rats exposed to 2.25 or 3.0 mg/kg HD and sacrificed at the timeframes indicated. Figures 14A-C show scores ranging from 1 day to 6 months post-exposure with emphasis aligned to corresponding temporal changes in mortality. **Figure 14A** shows the temporal effects of HD exposure on tracheal epithelial necrosis. Exposure to HD at 3.0 mg/kg indicates that there was a persistent necrotic condition from 2-8 weeks after exposure, whereas exposure to 2.25 mg/kg had less of an effect, but also indicated that there was a chronic condition that was largely resolved in survivors out to 10 weeks post-exposure. **Figure 14B** shows a dose-dependent delayed response for bronchiolar goblet cell hyperplasia with 2.25 mg/kg peaking in the 6-week timeframe, while for 3.0 mg/kg HD an earlier increase was observed. Perivascular edema was observed in the 2.25 mg/kg group from day 1 to nearly 10 weeks, peaking at 3 and 8 weeks after exposure. At 3 weeks there was a marked decrease in survival in the 2.25 mg/kg group and again at 7-8 weeks post-exposure. A similar but reduced effect was seen in the 3.0 mg/kg group from 3-8 weeks also, matching quite well with increased mortality (**Figure 14C**).

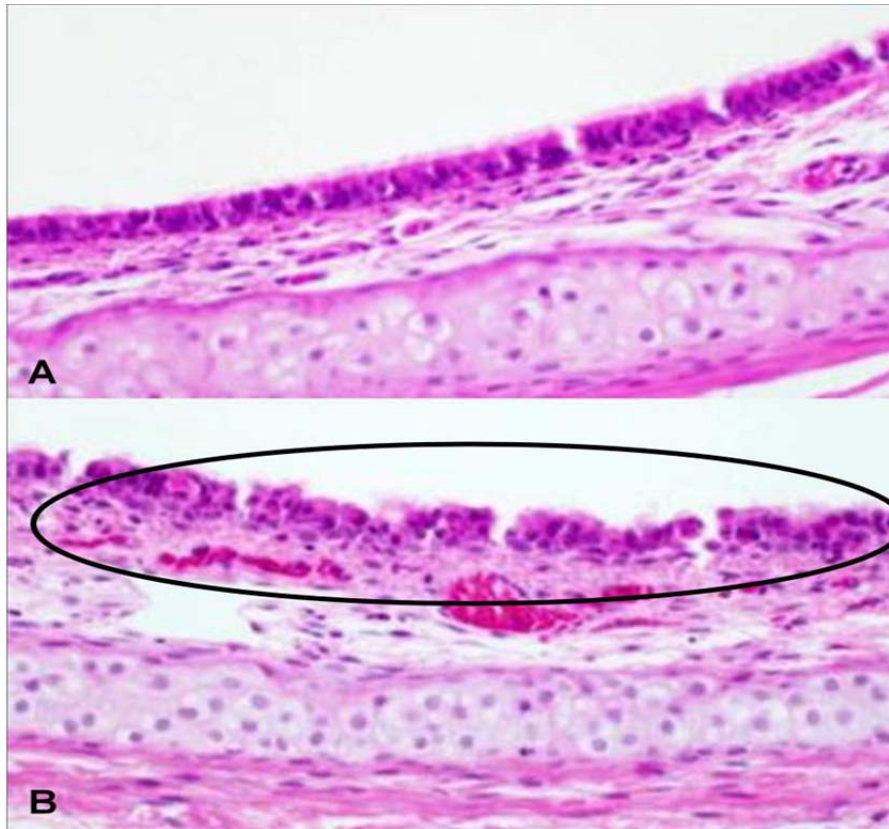


Figure 1. Morphologic changes in the trachea following HD exposure at 7.5 hours (3.0 mg/kg). (A) Normal tracheal epithelium from a non-HD-exposed Sprague Dawley rat. 40x. (B) In the HD-exposed animal are numerous individual and nests of shrunken epithelial cells with hypereosinophilic cytoplasm and pyknotic or karyorrhectic nuclei (consistent with epithelial necrosis). 40x.

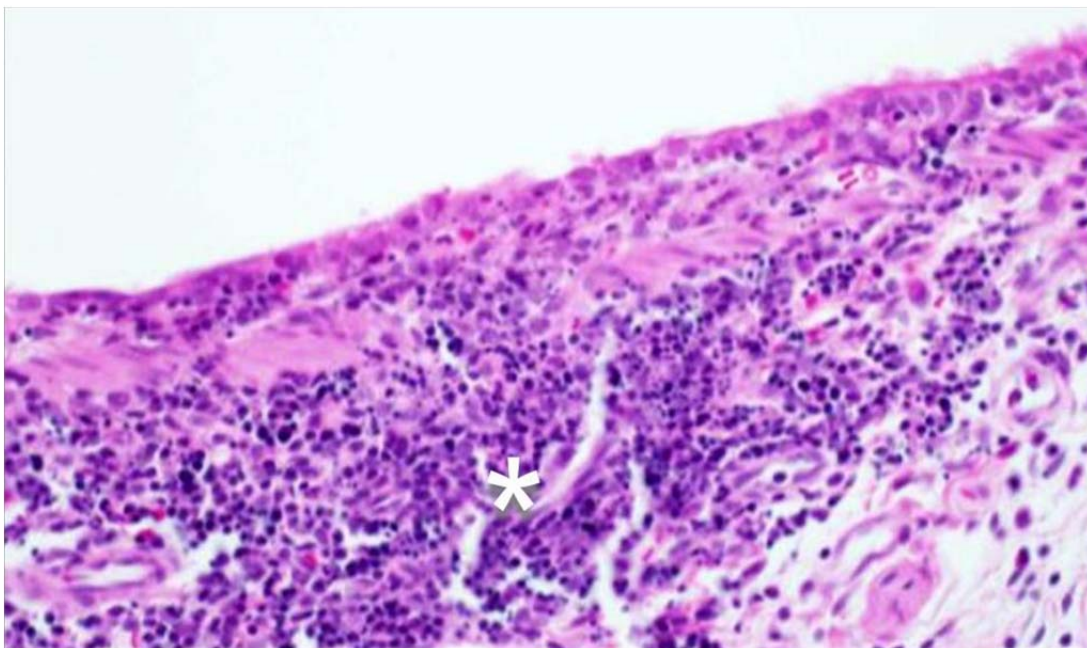


Figure 2. Morphologic changes in the accessory lobe following HD exposure at 7.5 hours (3.0 mg/kg). Histopathology of the BALT and overlying respiratory epithelium in the accessory lung lobe. Note the “rounded-up” lymphoid cells (*), consistent with extensive lymphocytolysis, and the individual necrotic epithelial cells. 40x.

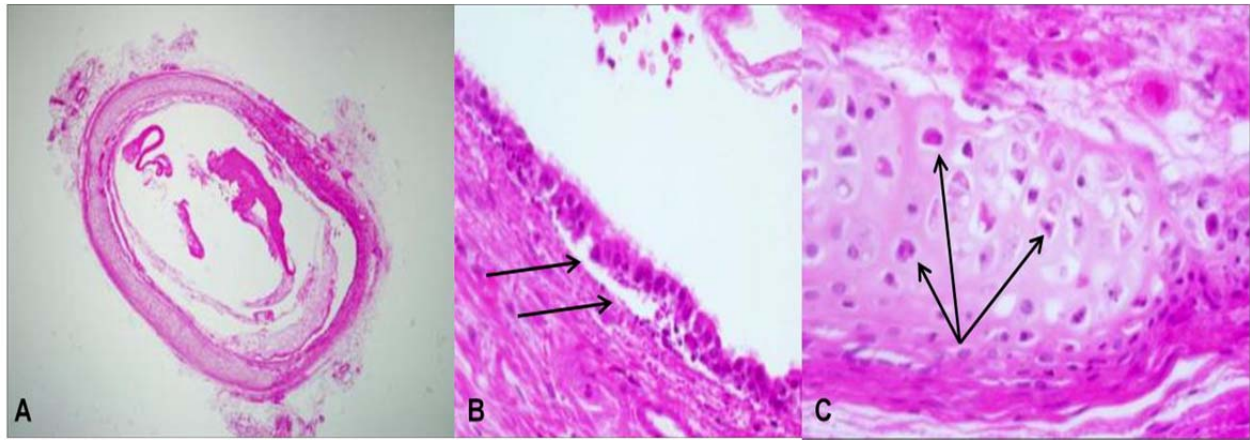


Figure 3. Histomorphologic changes in the trachea following HD exposure at 24 hours. (A) A fibrinous and cellular pseudomembrane and plug partially occlude the tracheal lumen. 4x. **(B)** Separation and clefting of necrotic epithelium from the underlying basement membrane (arrows). 40x. **(C)** Numerous brightly eosinophilic and necrotic chondrocytes are present in the tracheal cartilage (arrows). 60x.



Figure 4. Histomorphologic changes in the left lobe following HD exposure at 48 hours (2.25 mg/kg). (A) A fibrinous and cellular exudate and pseudomembrane partially (arrows) occlude the bronchiolar lumen. 10x. **(B)** Note the ulceration and loss of respiratory epithelium (*) adjacent to the BAL. 20x

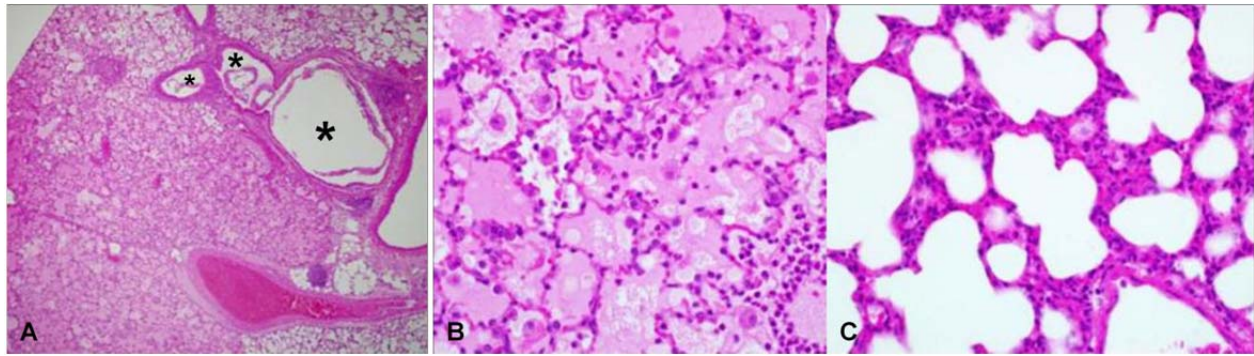


Figure 5. Histomorphologic changes in the left lobe and right cranial lobe following HD exposure at 48 hours (2.25 and 3.0 mg/kg). (A) Severe alveolar proteinaceous edema and flooding adjacent to the mainstem bronchioles. Note the fibrinous and cellular exudate that partially occludes the bronchiole lumen (*). 4x. (B) Proteinaceous edema admixed with variable numbers of foamy alveolar macrophages and non-degenerate neutrophils fill the alveolar lumina. 40x. (C) Although there is no evidence of alveolar edema or exudation, the alveolar interstitium is expanded 3-4x normal by neutrophilic and histiocytic inflammation and edema. 40x.

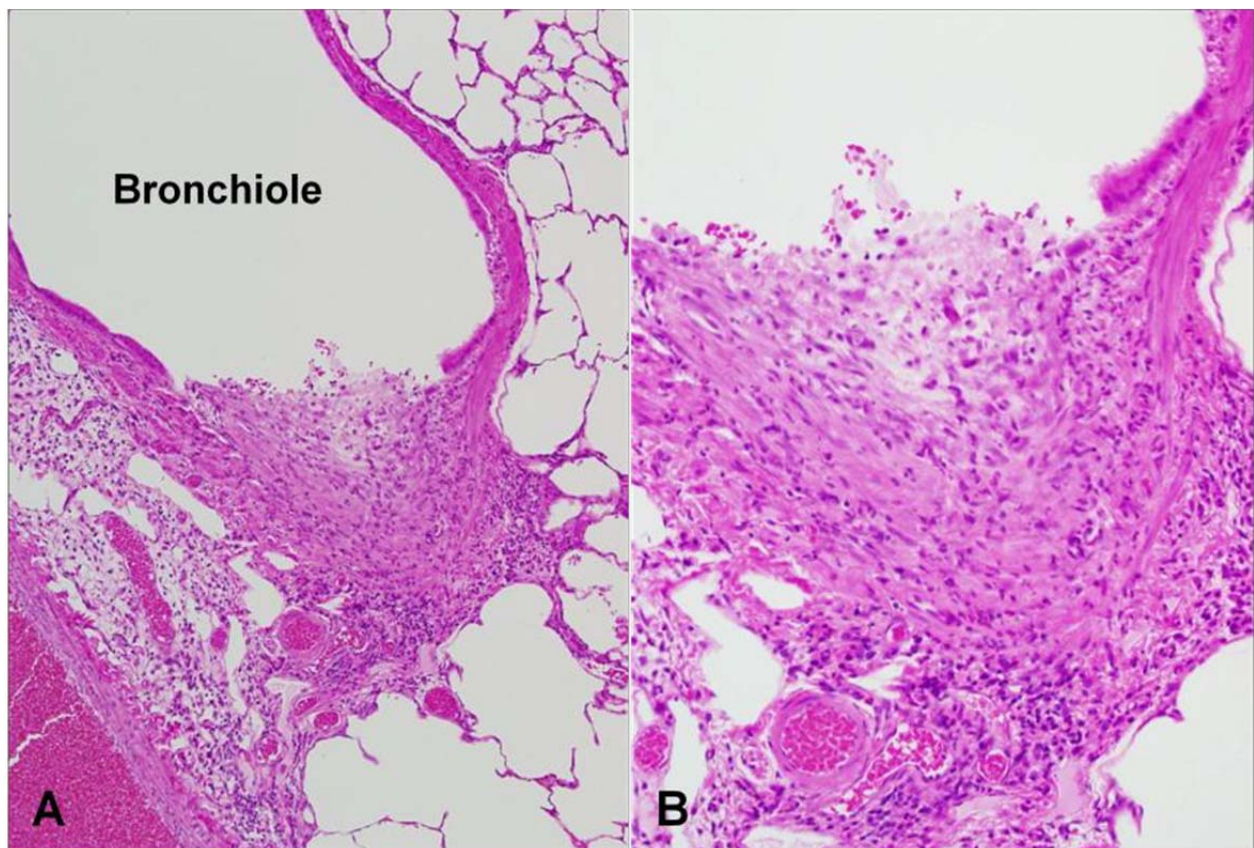


Figure 6. Histomorphologic changes in the left lobe following HD exposure at 21 days (3.0 mg/kg). (A, B) The bronchiolar subepithelial proliferation composed of fibroblast-like cells admixed with few inflammatory cells elevates the ulcerated epithelium. 10x, 20x.

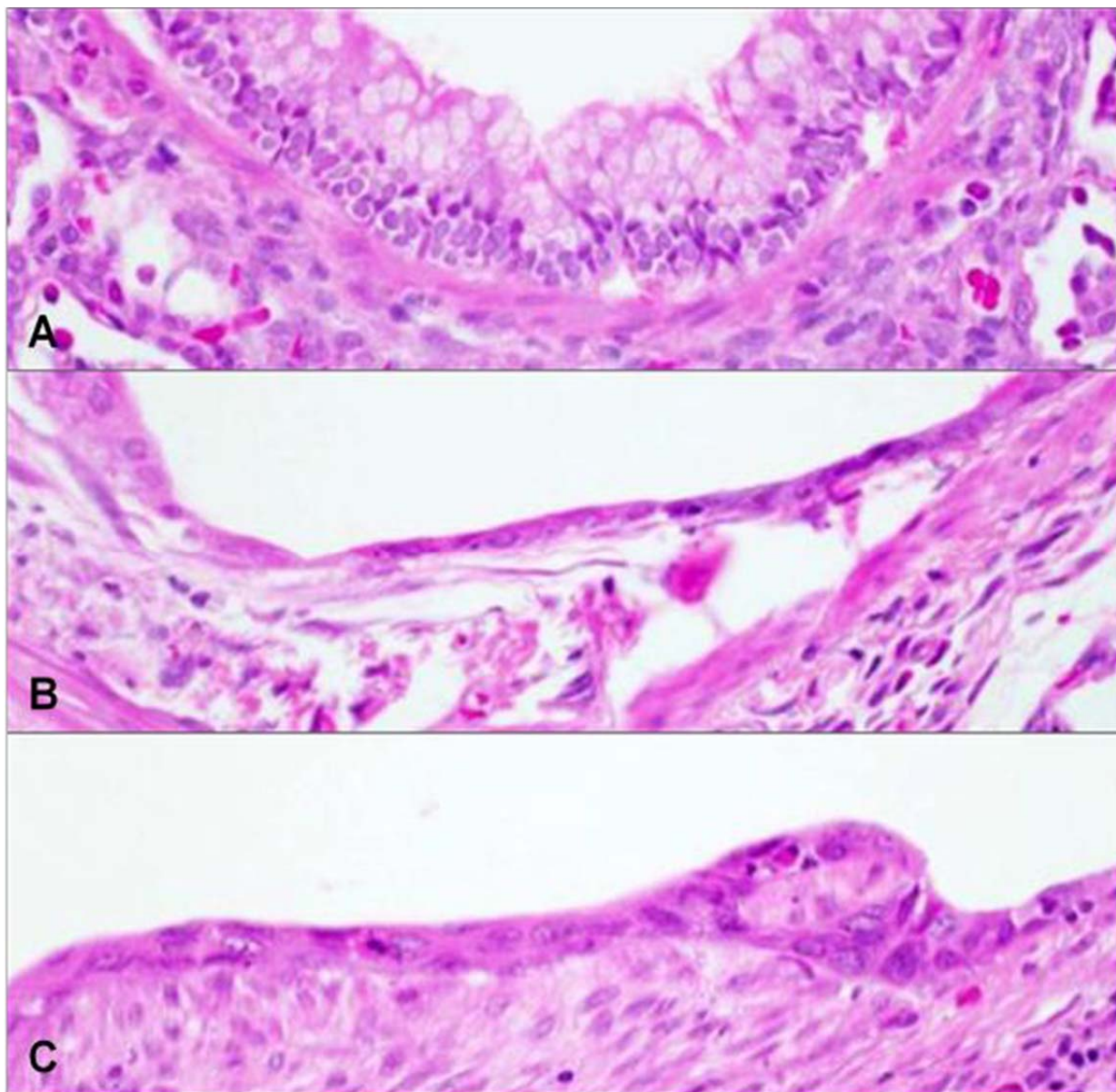


Figure 7. Variation in the histomorphologic changes in the bronchioles of the right caudal lobe following HD exposure at 28 days (3.0 g/kg and 1.75 mg/kg). (A) A marked increase in the number of bronchiole goblet cells (*) (3.0 mg/kg). 40x. **(B)** The respiratory epithelial cells are attenuated with loss of cilia (arrows); few individual necrotic epithelial cells are also present (3.0 mg/kg). 40x. **(C)** The respiratory epithelial cells are attenuated with loss of cilia and pile up to three layers thick (arrows) (1.75 mg/kg). 40X.

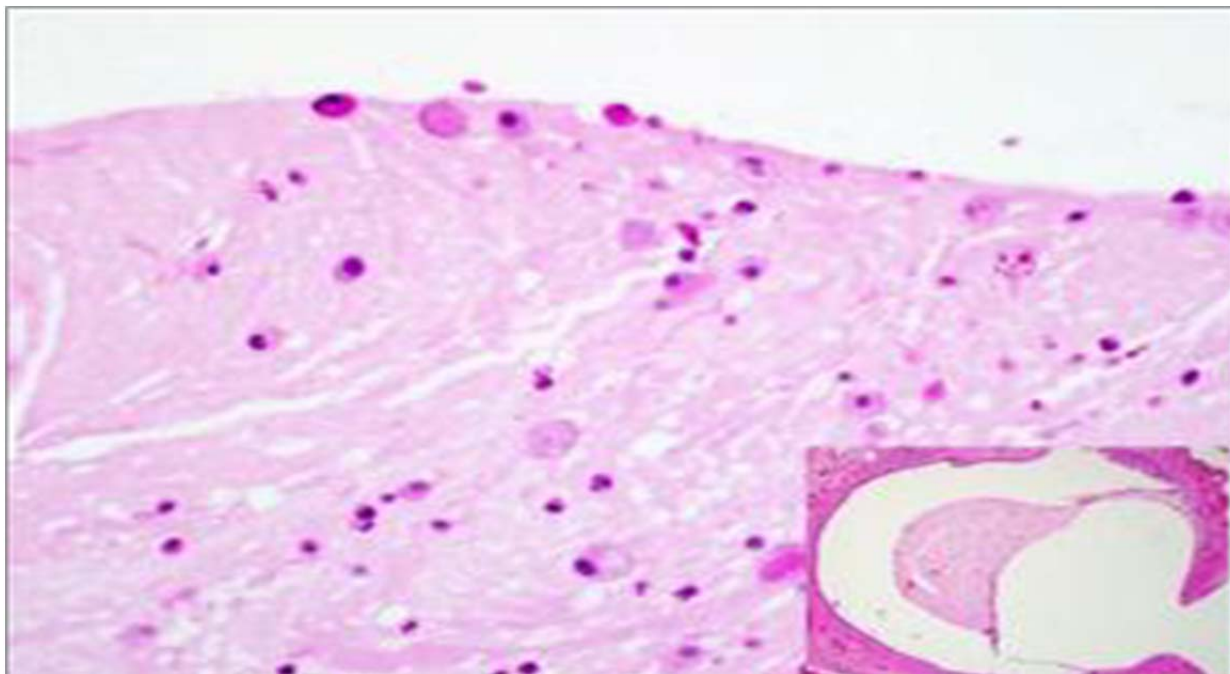


Figure 8. Histomorphologic changes in the right caudal lobe following HD exposure at 28 days (3.0 mg/kg). When present by 4 weeks, the bronchiolar exudate was primarily proteinaceous with few macrophages and neutrophils and minimal hemorrhage. 40x. **Inset.** The proteinaceous exudate partially occludes the bronchiole lumen.

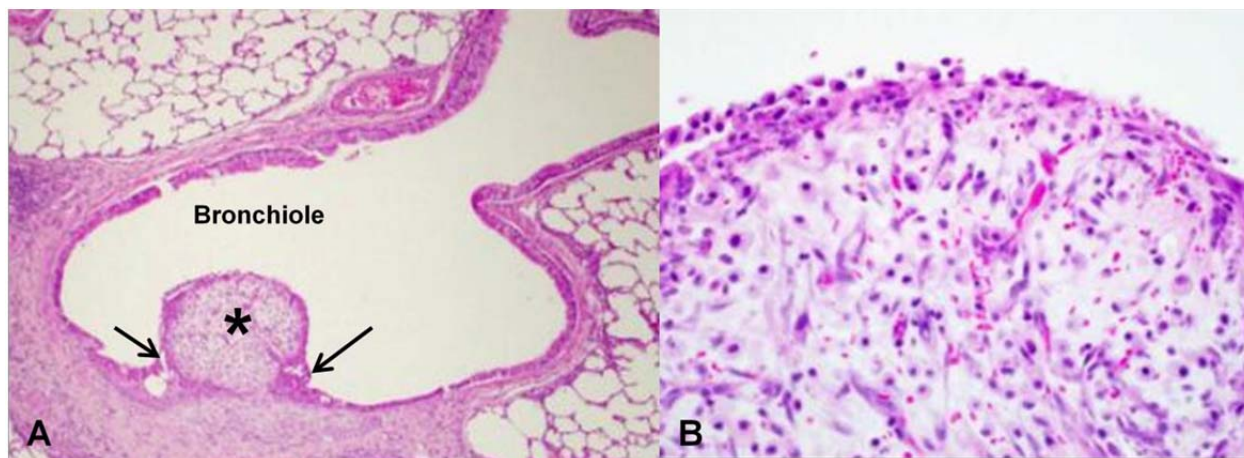


Figure 9. Histomorphologic changes in the right caudal lobe following HD exposure at 31 days (2.25 mg/kg). (A, B) A well-organized, dome-shaped, ulcerated subepithelial proliferation of spindled fibroblast-like cells (*) supported by a loose edematous matrix with small caliber vessels with a mixed inflammatory infiltrate partially occludes the bronchiole lumen. Note how in the ulcerated epithelium lining the growth is contiguous with the bronchiolar epithelium (arrows). The proliferation most resembles a fibroepithelial polyp. 4x, 40x.

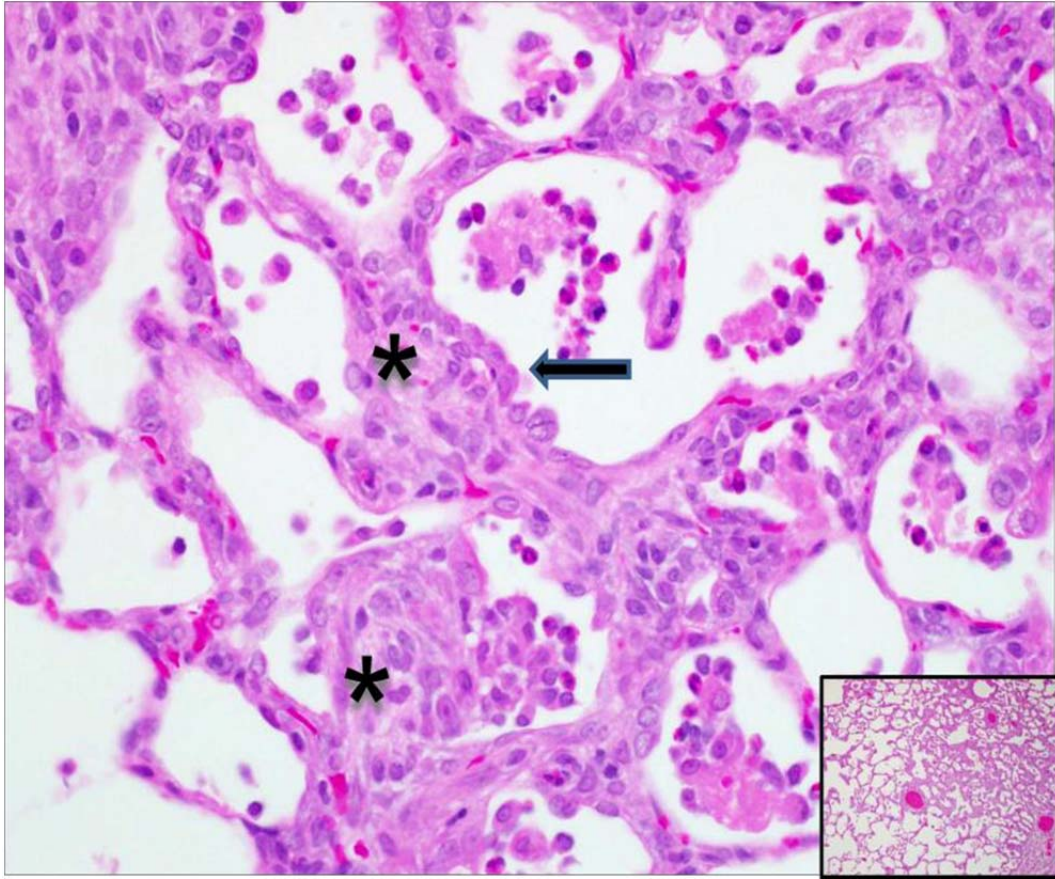


Figure 10. Histomorphologic changes in the left lobe following HD exposure at 28 days (3.0 mg/kg). Alveolar septae are markedly thickened by fibroblast-like cells and mononuclear inflammation (*) and occasionally lined by prominent cuboidal alveolar epithelial cells (arrow). An alveolar exudate composed of alveolar macrophages, neutrophils, and cellular debris partially occludes the airways. 40x. **Inset.** Note the diffuse thickening of the alveolar septae.

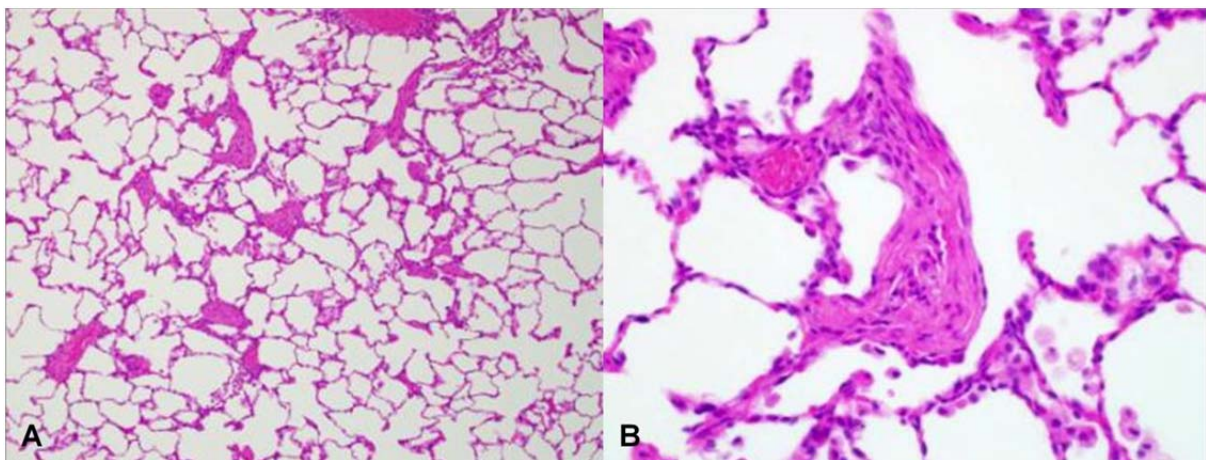


Figure 11. Histomorphologic changes in the right cranial lobe following HD exposure at 2 months (2.25 mg/kg). (A, B) Multifocal alveolar interstitial fibrosis (arrows) with minimal alveolar histiocytosis. 10x, 40x.

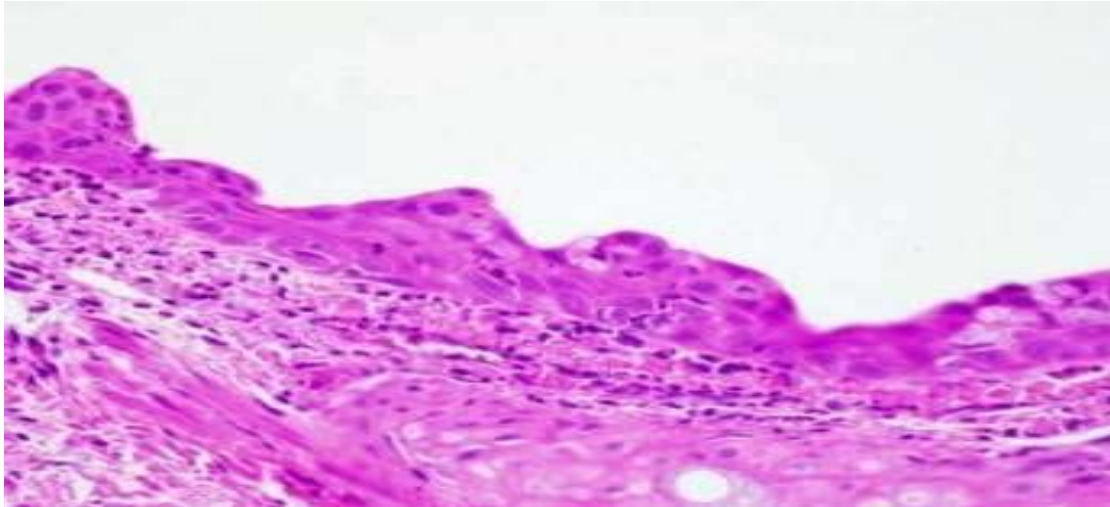


Figure 12. Morphologic changes in the trachea following HD exposure at 3 months (2.25 mg/kg). (A) Diffuse squamous metaplasia with loss of cilia and scattered necrotic epithelial cells. 40x.

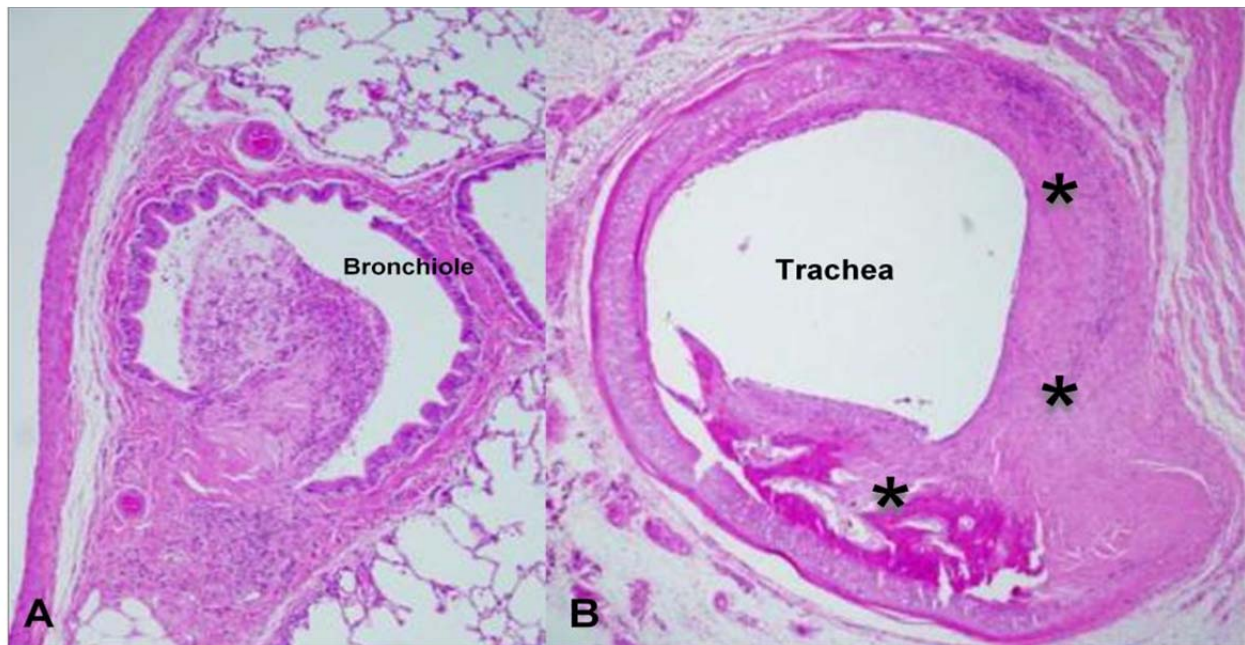


Figure 13. Histomorphologic changes in the left lobe and trachea following HD exposure at 4 months (3.0 mg/kg). (A) Approximately 50% of the bronchiole lumen is occluded by a fibroepithelial polyp. 10x. (B) Marked distortion of the trachea architecture by severe subepithelial fibrous proliferation and osseous metaplasia (*). 40x.

Figure 14 A.

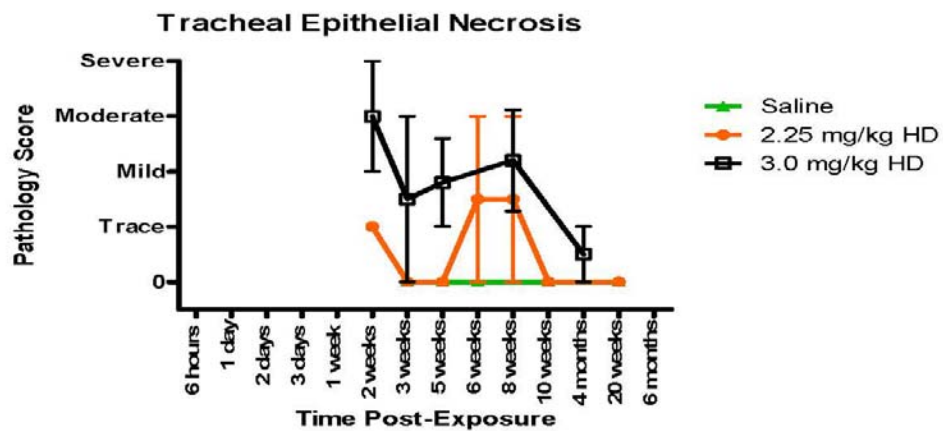


Figure 14 B.

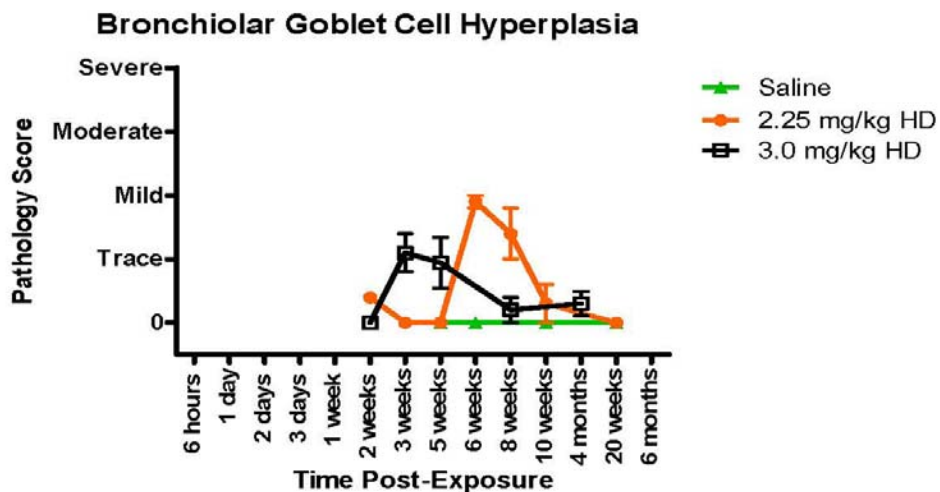


Figure 14 C.

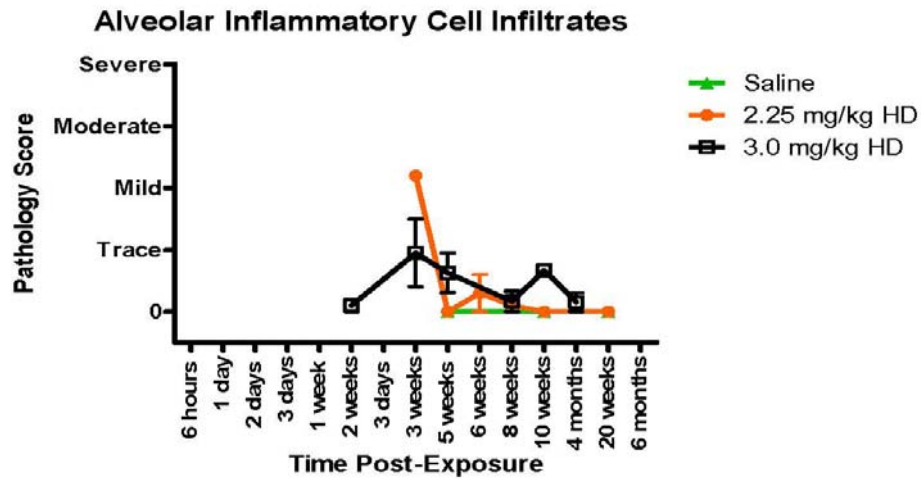


Figure 14 D.

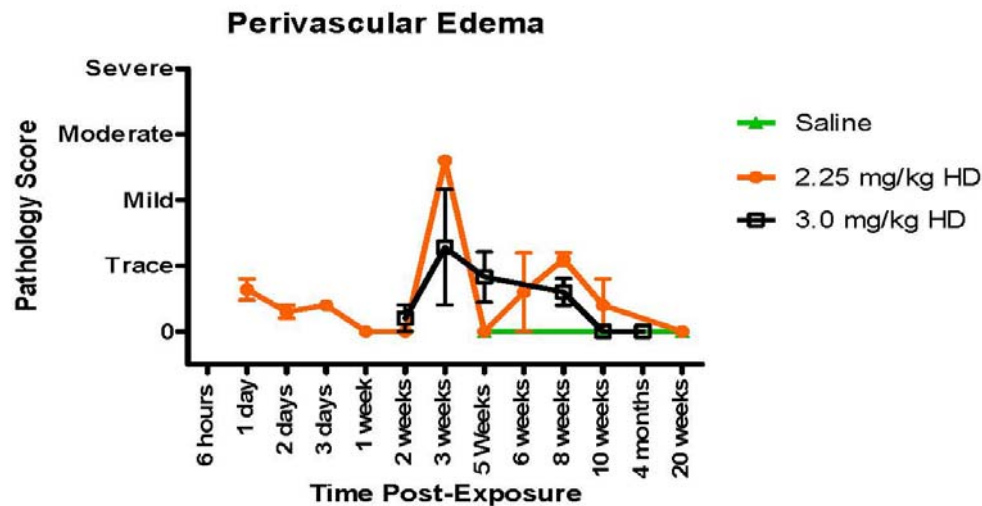


Figure 14. Histopathological lesion scores were observed in rat lung tissue following exposure to HD. **(A)** Tracheal epithelial necrosis indicated that lesion formation was prominent though not severe in 2.25 and 3.0 mg/kg HD-exposed rats from 2-8 weeks after challenge. **(B)** Bronchiolar goblet cell hyperplasia began within 3 weeks after HD exposure in the 3.0 mg/kg group and largely resolved over time, whereas there was a marked change in the presence of lesion formation in the 2.25 mg/kg group. **(C)** Perivascular edema, while a trace to mild, was persistent in both the 2.25 and 3.0 mg/kg groups compared to saline-exposed controls up to 2 months following challenge. Data are plotted as mean $\pm$ SEM. Group sizes ranged from 4-7/time point.

DISCUSSION

Historically, there have been many observations and clinical reports regarding the deleterious effects of exposure to HD. These effects range from ocular abnormalities to dermal impairment as well as compromised respiratory function years after the initial event. In addition to pathological responses leukopenia is also observed. Leukopenia and neutropenia were also observed in primates challenged with HD (2003). Ghanei et al. (2005) have shown that by 48 h after exposure respiratory insufficiency, lung edema and pneumonia are present, and in severe cases of exposure death usually occurs within 4 weeks. Willems (1989) shows that if the white blood cell count is <200 cells/mL death is imminent. Experimentally, Anderson et al. (1996) were one of the first to describe the pulmonary histopathological effects of inhaled-vapor-exposed rats. At 24 h post-exposure they observed severe sloughing of the airways, causing pseudomembrane formation, necrosis, progressive depletion of bronchus-associated lymphoid tissue, multifocal petechial hemorrhages, and fibrosis. These results have been corroborated in later HD inhalation exposure models (Fairhall et al., 2010) and provide a pertinent assessment of the acute response to HD toxicity. The study described herein addressed the chronic conditions over 6 months following a single 10 min exposure to HD. This study describes the histologic lesions in the lung and trachea of Sprague-Dawley rats following inhalation of aerosolized HD. Although varying in severity, pulmonary and tracheal lesions were observed as early as 3-6 hours and continued throughout the 6 months. Even when administered the same dose, not all animals exhibited changes, although histologic lesions were more common in the higher-dosed animals (2.25 and 3.0 mg/kg). There was variation between lobes even in the same animal (i.e., not all lobes affected in the same animal), although the right middle, right caudal and left lobes appeared more affected than the right cranial and accessory lobes. Although uncommon, the accessory lobe was atelectatic in a few HD-exposed animals, limitations were identified in this study. Since the head was removed at necropsy (the brain was not examined histologically as part of this portion of the study), the most rostral aspect of the trachea, to include the larynx, and of mid-trachea was not examined. Although the distal trachea was examined, the tracheal bifurcation was not submitted for histologic evaluation. Since this area is suspected to receive a higher dose of direct aerosol onslaught compared to the more distal lung regions, future HD studies should include a thorough examination of all areas of the trachea (Gopinath and Mowat, 2014).

Special stains to include histochemical and immunohistochemical stains were not performed in this portion of the study. The focally extensive subepithelial “spindled-cell” proliferations first observed as early as 3 weeks post-challenge in mainstem bronchioles appeared to be composed of proliferating fibroblast-like cells. This lesion progressed into well-circumscribed, epithelial-lined, and (partially) lumen-occluding growths that closely resembled fibroepithelial polyps. Similarly, focal areas of spindled-cell proliferations expanded the alveolar interstitium. Fibroblasts are the likely cell of origin for both lesions; hence fibroepithelial proliferation/polyp and alveolar interstitial fibrosis are the preferred terms in this report. However, other spindled cells present in the bronchiole subepithelium and alveoli

(smooth muscle cells, myoepithelial cells) could not be definitively excluded based solely on routine H&E. Trichrome or pentachrome histochemical staining for fibrosis may be used especially to determine the extent of alveolar fibrosis.

Prominent (hyperplastic) alveolar epithelial cells lined thickened alveolar septae in some affected lung lobes. Proliferation of alveolar type II cells (type II hyperplasia) is common following alveolar injury and is likely in this case. Good presumptive evidence for type II hyperplasia includes positive immunoreactivity for surfactant apoprotein A and negative immunoreactivity for CC16 (which identifies Clara cells associated with terminal bronchioles).

In high-dosed animals within the first 48-72 hours following challenge, BALT lymphocytolysis was not uncommon. Also, at these early time points, bronchiole epithelial changes (individual to aggregates of necrotic cells) were first observed in a mainstem bronchiole adjacent to the BALT. Routine histology could not determine if the BALT lymphocytolysis was due to lymphocytes necrosis and/or apoptosis. Special studies to include TUNEL, caspase-3 immunohistochemical staining, or electron microscopy may provide a more definitive answer. Nonetheless, histologic evidence suggestive of a pulmonary bacterial and/or viral infection occasionally seen in immune-compromised animals was not a feature in the lungs of any animals examined.

In summary, the data described herein provide evidence that a viable inhalation HD animal exposure model can duplicate the human exposure condition. Pathological and histological descriptions show the intensity and widespread longer-termed lung damage after a single 10 min exposure to aerosolized HD. While the data failed to provide distinctive indicators of a carcinogenic effect resulting from exposure, it is clear from the pathological profiles that taking this study to a year in duration may be essential to address that concern.

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