Rat model of smoke inhalation-induced acute lung injury

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ABSTRACT
Background Acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) is a lethal disease with limited therapeutic options and an unacceptably high mortality rate. Understanding the complex pathophysiological processes involved in the development of ALI/ARDS is critical for developing novel therapeutic strategies. Smoke inhalation (SI) injury is the leading cause of morbidity and mortality in patients with burn-associated ALI/ARDS; however, to our knowledge few reliable, reproducible models are available for pure SI animal model to investigate therapeutic options for ALI/ARDS without the confounding variables introduced by cutaneous burn or other pathology.

Objective To develop a small animal model of pure SI-induced ALI and to use this model for eventual testing of novel therapeutics for ALI.

Methods Rats were exposed to smoke using a customized smoke generator. Peripheral oxygen saturation (SpO2), heart rate, arterial blood gas, and chest X-ray (CXR) were measured before and after SI. Wet/dry weight (W/D) ratio, lung injury score and immunohistochemical staining of cleaved caspase 3 were performed on harvested lung tissues of healthy and SI animals.

Results The current study demonstrates the induction of ALI in rats after SI as reflected by a significant, sustained decrease in SpO2, and the development of diffuse bilateral pulmonary infiltrates on CXR. Lung tissue of animals exposed to SI showed increased inflammation, oedema and apoptosis as reflected by the increase in W/D ratio, injury score and cleaved caspase 3 level of the harvested tissues compared with healthy animals.

Conclusion We have successfully developed a small animal model of pure SI-induced ALI. This model is offered to the scientific community as a reliable model of isolated SI-induced injury without the confounding variables of cutaneous injury or other systemic pathology to be used for study of novel therapeutics or other investigation.

INTRODUCTION
Since Ashbaugh et al first described acute respiratory distress syndrome (ARDS) in 1967, clinicians and researchers have investigated several therapeutic options for the failing pulmonary system without significant effect on overall mortality. ARDS is the most severe clinical manifestation of acute lung injury (ALI). A recent review of clinical trial studies regarding ARDS in adults included 177 articles with 25,966 subjects and found a decreasing trend in mortality in prospectively designed studies over time. However, a significant decline in overall mortality has yet to be seen despite the wide adoption of low-volume lung protective ventilation made popular by the ARDSNet trial. The current mortality rate of ARDS remains high, ranging from 30% to 45%.

Smoke inhalation (SI) injury is the major cause of respiratory failure in critical burn injury patients leading to increased morbidity and mortality. Although major advances have been made in the treatment of cutaneous burn, relatively few advances have been made for pulmonary complications of inhalation injury. SI injury involves damage to the respiratory tract and lung parenchyma from smoke and toxic substances as well as heat. With such a multimodal mechanism of injury, it is imperative for the scientific community to have reliable, reproducible small and large animal models of isolated SI injury. The development of such models is essential to investigation of therapeutic strategies by separating lung pathology from cutaneous and systemic injury.

Large animals have commonly been used for the development of lung injury models as these animals can be ventilated and monitored for a prolonged duration and can mimic the human clinical situations and body habitus. However, large animals are not always cost effective, especially in the early
stages of therapeutic investigation. Enormous personnel expenditure and costs are required to perform survival studies in these animals.\textsuperscript{9} Second, at present not enough study has been performed for the genetic manipulation in large animal models when compared with small animal models.\textsuperscript{10} Rats are widely used to study various diseases in the preclinical set up as they are small and easy to control. In many cases, the rats physiology mimics human condition and is easy to monitor.\textsuperscript{10} When compared with mouse models, serial blood samples are more easily obtained from rats for determining the induction of ALI/ARDS.\textsuperscript{10} Rats have also been proven to be an excellent model for the study of different types of lung injuries and also for the development of treatment strategies against these injuries.\textsuperscript{11}

In the present study, our aim is to develop a rat model of isolated, pure wood SI-induced ALI that is reproducible and reliable. Using this isolated SI small animal injury model will enable our group to study potential therapies without confounding variables.

METHODS

Animal preparation

All animal studies were performed in accordance with the University of Nebraska Medical Center/Nebraska Medicine (UNMC/NM; Omaha, Nebraska) Institutional Animal Care and Use Committee standards. We purchased adult male Wistar rats (Charles River Laboratories, Wilmington, Massachusetts) aged 6–8 weeks and weighing 250–290 g. Carotid artery catheters were implanted by the vendor prior to shipment following the vendor’s established protocols. Animals were housed in individual microisolator cages in a temperature-controlled room with a 12 hours light/dark cycle and free access to food and water and were acclimatised for 3 days after arrival. In total, 15 animals were used for the study.

Compressed wood constant feed smoke generator system

Smoke was generated from Douglas fir compressed wood pellets in a Teague Systems compressed wood constant feed smoke (CWCFS) generator system (http://www.teague-ent.com/) (figure 1A, left panel). Wood pellet feeder box with the lid removed is shown in figure 1A, right panel. The CWCFS generated wood smoke by constant feeding of compressed pellets into an incendiary box (figure 1B). Schematic of the smoke generator was provided in figure 1C. The temperature of the incendiary box was monitored by a thermocouple and controlled by adding air from a blower to keep the temperature of the incendiary box constant. Generated smoke was then mixed with ambient air at a mixer. This mixing process functioned to (1) dilute the smoke to a consistent density and (2) cool the smoke prior to venting into the exposure chambers so as not to cause thermal injury to the rats. Excess air/smoke was added or exhausted to a dedicated outlet in order to provide a consistent concentration of smoke in the chamber during the exposure time. Each rat exposure chamber measured 28×24×24 inches (264 L volume). Carbon monoxide (CO\textsubscript{2}), carbon dioxide (CO), temperature and relative humidity was sampled in the exposure chamber during the entire smoke exposure period. The flow through of the exposure chambers was from side to side and designed to mix throughout. The minimum flow rate in the exposure chamber was 66 L/min for 15 changes-per-hour.

Animal model of SI

Animals (n=15) were divided into two groups designated ‘SI’ for those who underwent smoke exposure (n=12), or ‘SHAM’ for rats who did not receive any smoke exposure (n=3). SI subjects were exposed to Douglas fir wood smoke using the smoke generator (figure 1) for 5–6 hours per day for 7 consecutive days, or until the development of ALI as documented by a significant, sustained decrease in peripheral oxygen saturation (SpO\textsubscript{2}) and bilateral infiltrates in chest X-rays (CXR).

To ensure uniform smoke exposure in SI animals, the smoke exposure chambers were monitored throughout the study using a single-channel, light-scattering laser photometer (DustTrak II Aerosol Monitor 8532, TSI Incorporated, Shoreview, Minnesota) and air quality metre (IAQ-CALC Model 7545, TSI Incorporated). The following parameters were recorded throughout the smoke exposure period: smoke concentration (mg/m\textsuperscript{3}), CO\textsubscript{2} concentration (ppm), temperature (°F), relative

Figure 1 Compressed wood constant feed smoke (CWCFS) generator system: (A) CWCFS connected to the rat exposure chambers (A, left panel), and wood pellet feeder box with the lid removed to show the pellets (A, right panel). (B) Empty wood pellet stove (WPS) incendiary chamber with the lid removed (B, left upper panel); WPS as the pellets are being automatically fed into the incendiary chamber (B, right upper panel); and WPS chamber with smoke generated from the pellets (B, lower panel). (C) Schematic of the smoke generator.
humidity (%) and CO concentration (ppm) (online supplemental table S1).

**Haemodynamic measurement**
Heart rate (HR) and peripheral oxygen saturation (SpO₂) were measured using a Kent Physiosuite (Kent Scientific Corporation, Torrington, Connecticut).

**Blood gas analysis**
300 µL of arterial blood was collected from the carotid artery catheter port locked with a heparinised dextrose solution using a syringe as per protocol from the vendor. Blood gas analysis was immediately conducted with an i-STAT VetScan Handheld Analyzer (Abaxis, Union City, California) after blood samples were collected. The following parameters were measured: partial pressure of oxygen (PaO₂), partial pressure of carbon dioxide (PaCO₂), Hydrogen bicarbonate (HCO₃⁻), pH, arterial oxygen saturation (SaO₂), anion gap, base excess/deficit, haematocrit (Hct), haemoglobin (Hb), sodium (Na⁺), potassium (K⁺), chloride (Cl⁻), and glucose (Gluc).

**Chest X-rays**
CXR was obtained for animals in both ventral-dorsal (VD) and lateral (Lat) views before and after SI. CXR was taken by trained radiology technicians working for UNMC/NM.

**Wet-to-dry weight (W/D) ratio**
Animals were sacrificed on completion of the smoke exposure with lethal intraperitoneal injection of ketamine/xylazine followed by bilateral thoracotomy, and lung tissue was harvested. A portion of lung tissue from each animal was placed in a dry plastic boat and weighed (wet weight) with a precision scale. The tissue was then dried in an incubator at 60°C for 5 days and weighed again (dry weight). The W/D ratio was calculated as the ratio of the wet weight to the final dry weight as described elsewhere. The remaining lung tissue was placed immediately after harvest in 10% neutral formalin buffer for histological analysis.

**Lung injury score**
The lung tissue in 10% neutral formalin were dehydrated in graded concentrations of ethanol solution and cleared in xylene. The tissue samples were then paraffin embedded, sectioned with 4 µm thickness, and stained with H&E. An independent pathologist performed a double-blinded examination of the tissue slides under a light microscope. Ten fields of each lung tissue section were examined at magnification of ×400. The severity of the lung injury was scored by the criteria of alveolar oedema, intra-alveolar haemorrhage, and leucocyte infiltration. Alveolar oedema and intra-alveolar haemorrhage were scored on a scale from 0 to 3, where 0 = absence of extravascular leukocytes; 1 ≤ 10 leukocytes; 2 = 10–45 leukocytes; 3 ≥ 45 leukocytes.

**Cleaved caspase 3 immunohistochemical analysis**
Immunostaining for cleaved caspase 3 (Abcam, catalogue #ab4051; Cambridge, Massachusetts) was performed on rat SI and SHAM formalin-fixed, paraffin-embedded lung tissues sections at UNMC Tissue Sciences Facility using automated Ventana Discovery Ultra (Roche Diagnostics, Indianapolis, Indiana) as per manufacturer’s protocol. Briefly, tissue slides were deparaffinised in histoclear and rehydrated in descending grades of ethanol. After deparaffinisation, tissue antigen retrieval was performed with Ventana Discovery CCI solution followed by treatment with 3% hydrogen peroxide (H₂O₂) to block endogenous peroxidase activity. Slides were incubated in cleaved caspase 3 antibody (1:200 dilution) for 1:200 dilution at 37°C for 32 min. After washing in 1× wash solution, sections were incubated with anti-rabbit HQ at 37°C for 16 min followed by anti-HQ HRP and H₂O₂ at room temperature for 16 min and 4 min, respectively. Sections were developed with chromogen (Discovery ChromoMap DAB kit) for 8 min, counterstained with haematoxylin and dehydrated with ethanol and histoclear. Specimens were processed on the same day to eliminate any variability in conditions. An independent pathologist performed a double-blinded examination of the tissue slides under a light microscope. A total of 2000 cells were counted at magnification of ×400 and the percentage of cleaved caspase 3 positive were calculated.

**Statistical analysis**
All results were expressed as the mean±SEM. Differences between groups were compared using one-way analysis of variance followed by a post-hoc comparison using Tukey’s multiple comparison test to generate adjusted ‘p’ values using GraphPad Prism V.8 (GraphPad Software, San Diego, California). A p<0.05 was considered statistically significant.

**Patient and public involvement**
Patients and members of the public were not involved in the design, conduct and analyses of the study.

**RESULTS**
**Effect of SI on haemodynamic parameters**
A commonly used classification scheme for human ARDS is the Berlin criteria (ARDS Definition Task Force, 2012), which assumes the subject to be ventilated or on CPAP and receiving a positive end-expiratory pressure of 5 mm Hg or greater. Since our animal model of ALI was not on ventilator support, we chose to use a significant,
sustained decrease in SpO₂ values as a surrogate for the Berlin criteria’s PaO₂/FiO₂ ratio. For purposes of this study, ‘moderate to severe’ ARDS was defined by the presence of bilateral, diffuse pulmonary infiltrates on CXR and a significant (>10%) reduction in SpO₂ values which was sustained for more than 24 hours after the final smoke exposure. At 24 hours following the final smoke exposure, the SI group demonstrated 12%–13% lower SpO₂ values when compared with the baseline and the SHAM group (figure 2A, left panel). The decrease in SpO₂ levels were statistically significant (figure 2A, left panel; p<0.0001). A significant decrease in the delta SpO₂ (ΔSpO₂) level was also observed in SI group compared with SHAM group (figure 2A, right panel; p=0.002). As expected, there was no difference in the SpO₂ levels between baseline and SHAM group (figure 2A, left panel; p=0.9964).

SHAM group animals showed a gradual increase in body mass at the end of the study compared with the baseline value with a total increase of approximately 19 g (figure 2B, left panel; p=0.4546). In contrast, there was no change in body mass in SI group animals when compared with the baseline value (figure 2B, left panel; p=0.826) and in fact showed a reduction in mass of approximately 25 g when compared with the SHAM animals (figure 2B, left panel; p=0.2863) and a marginally significant delta value (figure 2B, right panel; p=0.05).

**Effects of SI on laboratory parameters**

Of the parameters measured by ABG, arterial PaO₂ showed a reduction of up to 13 mm Hg in SI animals compared with either baseline or SHAM animals (figure 3A, left panel); however, statistical significance was not obtained at both total PaO₂ level (figure 3A, left panel; p=0.0826 to 0.9667) and delta PaO₂ (ΔPaO₂) level (figure 3A; right panel; p=0.16). Corresponding to the decrease in pO₂ value, we observed a 10–11 mm Hg increase in the arterial partial pressure of carbon dioxide (PaCO₂) in SI animals compared with baseline and SHAM animals, and these increases were marginally to statistically significant (figure 3B, left panel; p=0.0004 and p=0.0690, respectively). Delta PaCO₂ (ΔPaCO₂) level showed no significant difference between SHAM and SI groups (figure 3B, right panel; p=0.36). In contrast, no change was observed in either PaO₂ or PaCO₂ level between baseline and SHAM animals (figure 3A,B; p=0.9376). Hb and Hct values were found to be significantly increased in SI animals when compared with baseline (figure 3C,D, left panel; p=0.0003). In addition, there were significant difference in ΔHb and ΔHct levels between SI and SHAM groups (figure 3C,D, right panel; p=0.04–0.05). No significant change was observed in either PaO₂ or PaCO₂ level between baseline and SHAM animals (figure 3C,D, left panel; p=0.5115–0.9376). We did not observe significant change in pH, sodium (Na), potassium (K), and ionised calcium (iCa) levels with SI (table 1).

**Effects of SI on lung parenchyma**

SI has been reported to increase capillary leakage. Consistent with previous studies, we observed diffuse, bilateral infiltrates in both the VD and Lat views in CXR after exposure to smoke injury in the SI group of animals (figure 4A, lower panel). In contrast, both lungs were normal in SHAM group of animals (figure 4A, upper panel). Histological examination of lung tissue in SI animals showed an increase in the number of infiltrative leukocytes compared with the SHAM group (figure 4B), with no significant change in either intra-alveolar oedema
or haemorrhage. The lung injury score reflective of infiltrative leukocytes was significantly increased in SI animals compared with SHAM animals (figure 4C; p=0.01). We also observed an increase in the average W/D ratio of lung tissues after SI in SI groups of animals (5.16±0.72) compared with the SHAM group (4.5±0.99); however, the finding was not statistically significant (figure 4D, p=0.39). Studies has reported that acute injury to the lung activates apoptotic pathways.18 19 Interestingly, immunohistochemical staining of cleaved caspase 3 on paraffin embedded lung tissue sections of SI animals showed an increased in the number of apoptotic cells when compared with SHAM animals (figure 4E), and this increase was statistically significant (figure 4F, p=0.0475).

**DISCUSSION**

ARDS-related death remains high in critically ill patients, and no significant change in the mortality rate has been reported in last decade despite attempted advances in treatment modalities.2 3 Experimental animal models, critical for the exploration of pathophysiology and of novel therapies against ARDS have contributed significantly in the development of countermeasures against...
SI injury is a major cause of morbidity and mortality in burn patients. Exposure to wood smoke causes denaturation of protein in the airway mucosa, induces inflammation, damages alveolar capillary membranes and increases its permeability, leading to pulmonary oedema. The present study used Douglas fir compressed wood pellets to generate smoke from the smoke generator. Douglas fir wood pellet is mainly composed of cellulose (40%–60%) and lignin (20%–40%), together with gums, resins and variable amounts of water and inorganic matter. Douglas fir wood generates ~1 g fine particulate per kg of wood burned which composed primarily of organic carbon (~80%) and elemental carbon (~8%), and also include ions (chloride, nitrate, sulfate, ammonium etc.) and elements (potassium, silicon, chlorine etc.). Some of the organic compounds found in fine particle mass are levoglucosan and other sugar derivatives, guaiacol and substituted guaiacol, substituted benzene and phenols, PAH and alkyl-PAH and phytosteroids. The current model represents prolonged smoke exposure leading to ALI. Review of current literature for translatability reveals that exposure to indoor air pollution from biomass combustion—as seen during combustion of biomass fuels for cooking and heating used by approximately half of all people in developing countries—is a major source of morbidity and mortality worldwide. As well, the detrimental effect of prolonged exposure to acutely elevated levels of environmental smoke from wildfires has long-standing interest. The levels of particulate matter (PM2.5) observed in the model we present here is similar to, or greater than, levels measured during cooking with open flame indoors or those measured from the environment during mass wildfires. We reported the successful development of a pure SI-induced ALI model in adult rats as reflected by the decrease in peripheral oxygenation, changes in
arterial blood gas changes, development of diffuse bilateral pulmonary infiltrate in CXR, significant increase in inflammatory cells, oedema and haemorrhage in the histology analysis, and the increase of W/D ratio. Previous studies have reported increased apoptosis of lung epithelial cells in ALI/ARDS animal models and patients.\textsuperscript{11,12,27} We also observed significantly increased apoptosis in smoke exposed animal tissues as reflected by the increased in cleaved caspase 3 positive cells compared with SHAM animals.

Large animal model of smoke/burn injury models were developed by delivering smoke directly through the endotracheal tube in ventilated animals.\textsuperscript{26–31} However, in small animals such as mice, rats and rabbits, these animals were forced to spontaneously inhale the smoke in a closed chamber.\textsuperscript{16,17,32–34} These SI models were established on burns/smoke injury together. In recent study by Mercel et al. (2020), rats were intubated under general anaesthesia and smoke was delivered in a closed chamber.\textsuperscript{35}

In the present study, a dynamic method of SI injury was used using a custom-made smoke chamber to deliver smoke to animals without the assistance of sedation and endotracheal intubation for the entire smoke exposure duration. There are two basic types of treatment systems in SI injury: static and dynamic. The majority of research published uses static systems.\textsuperscript{32–34} In a static combustion system, all of the smoke products generated remain in the animal chamber for the duration of the exposure period, leading to an increased probable risk of oxygen depletion. Some static systems require the use of supplemental oxygen in conjunction with the combustion apparatus.\textsuperscript{36,37} In a dynamic system, the smoke combustion products are transferred via pump or blower into the animal chamber and are allowed to flow through and escape.\textsuperscript{38,39} There is some risk of a loss of toxicants (due to the transfer of the smoke) in a dynamic system, but there is a decreased probability of the need for supplemental oxygen due to the higher number of air exchanges. The dynamic CWCFs smoke exposure treatment system used in the current study allowed standardisation of wood smoke treatment and offered increased safety to staff and facilities by not requiring the use of supplemental oxygen. It also decreased the probability of animal death prematurely due to hypoxic conditions. However, we observed a comparable result in lung injury score and wet/dry ratio between published models and the current model and reflected lung injury in patients with ARDS.\textsuperscript{15,35} The difference in timeline of SI might be reflected by the use of isolated smoke injury in the current model. Further study focused on comparison between burn/smoke and pure smoke injury models with and without intubation would help in validating the difference in smoke exposure time between different models.

Smoke exposed animals showed decrease in peripheral oxygenation ($SpO_2$) and reduction in the total body mass compared with the baseline and SHAM animals. In addition, there was increase in Hct and Hb levels in these animals. This changes in Hct/Hb value in our model reflected haemococoncentration due to poor water intake in a sick animal who was not receiving any treatment such as IV fluids. We do not believe these changes are reflective of human ARDS, which is most often studied in an inpatient environment and with the benefit of close monitoring and supportive therapies. Rather, these data were included to reflect the severity of illness/injury in our model. Monitoring the respiratory disorder in rats has clear advantages over mouse because of the ability to perform invasive procedure for arterial blood gas sampling and feasibility of documenting the respiratory function for an extended period of time is critical for documenting the level of hypoxemia in animal.\textsuperscript{10}

The pathophysiology of ALI/ARDS in the early exudative phase involves infiltration of inflammatory cells, diffuse alveolar damage and endothelial injury leading to increased lung permeability.\textsuperscript{40–42} Diffuse alveolar damage led to the increase in the cell death of the We also observed marked infiltration of inflammatory cells, intra-alveolar haemorrhage, oedema and increased apoptosis in the histological tissue slides and an increase in the W/D ratio of lung tissues of animals exposed to SI (figure 4B–F). These findings indicate injury to lung tissues and the development of pulmonary oedema. To our knowledge, the present animal model is one of the few reliable models available for pure SI-induced ALI in small animals.

CONCLUSION

We developed a rat model of isolated SI-induced ALI using a custom-made smoke generator. This model will be used for further understanding ALI pathophysiology and for investigating novel therapies, specifically for our future studies involving the utility of oxygen microbubbles as a novel therapeutic strategy in augmenting systemic oxygenation in ALI/ARDS patients.

Contributors Conception and design: TO, KLB. Data collection: HRW, TO, KLB. Analysis and interpretation: PDL, HRW, KLB. Draft the original manuscript: PDL. All authors contributed to the critical revision of the manuscript draft and approval of the final version of the manuscript.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval All animal studies were performed in accordance with the University of Nebraska Medical Centre/Nebraska Medicine (UNMC/NM; Omaha, Nebraska) Institutional Animal Care and Use Committee standards.

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Data availability statement Data are available upon reasonable request from the corresponding author.

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REFERENCES


Table S1: Characteristics of Douglas fir compressed wood pellet smoke in CWCFS<sup>a</sup> smoke chamber and in the SHAM microisolater<sup>b</sup>

<table>
<thead>
<tr>
<th>Characteristics (unit)</th>
<th>Inside smoke chamber&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Outside smoke chamber&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Inside microisolator&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particulate matter concentration (mg/m&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>137.5 ± 7.73</td>
<td>0.075 ± 0.05</td>
<td>NM&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Carbon monoxide (ppm)</td>
<td>0.2 ± 0.13</td>
<td>0.3 ± 0.17</td>
<td>NM&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Carbon dioxide (ppm)</td>
<td>489 ± 24</td>
<td>459 ± 7.01</td>
<td>NM&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Temperature (°F)</td>
<td>74.7 ± 0.28</td>
<td>72.08 ± 0.12</td>
<td>72.08 ± 0.12</td>
</tr>
<tr>
<td>Relative humidity (%)</td>
<td>29 ± 0.4</td>
<td>33.42 ± 0.26</td>
<td>33.42 ± 0.26</td>
</tr>
</tbody>
</table>

<sup>a</sup> CWCFS, Compressed wood constant feed smoke
<sup>b</sup> Smoke chamber (inside and outside) was monitored every 30 minutes during the entire smoke exposure period. Microisolater was monitored daily every 15 minutes.
<sup>c</sup> Values calculated as mean ± SEM.
<sup>d</sup> NM, not measured.