Title page:

A murine model of vesicant-induced acute lung injury

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Abbreviations
ABG – arterial blood gas
ALI – acute lung injury
ARDS – acute respiratory distress syndrome
BALF – bronchoalveolar lavage fluid
CBC – complete blood cell count
DAMP – damage associated molecular pattern.
DAPI - 4′,6-diamidino-2-phenylindole
FOT – forced oscillation technique.
HMGB1 – high mobility group box 1
IL – interleukin
LIF – Leukemia inhibitory factor
PAO – phenylarsine oxide
PEEP – positive end-expiratory pressure
SIRS – systemic inflammatory response syndrome
VEGFA – vascular endothelial growth factor

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Abstract

Burn injuries including those caused by chemicals can result in systemic effects and acute lung injury (ALI). Cutaneous exposure to Lewisite, a warfare and chemical burn agent, also causes ALI. To overcome the limitations in conducting direct research on Lewisite-induced ALI in a laboratory setting, an animal model was developed using phenylarsine oxide (PAO) as a surrogate for lewisite. Due to lack of a reliable animal model mimicking the effects of such exposures, development of effective therapies to treat such injuries is challenging. We demonstrated that a single cutaneous exposure to PAO resulted in disruption of alveolar-capillary barrier as evidenced by elevated protein levels in the bronchoalveolar lavage fluid (BALF). BALF supernatant of PAO-exposed animals had increased levels of HMGB1, a damage associated molecular pattern (DAMP) molecule. Arterial blood-gas measurements showed decreased pH, increased PaCO₂, and decreased PaO₂, indicative of respiratory acidosis, hypercapnia, and hypoxemia. Increased protein levels of IL-6, CXCL-1, CXCL-2, CXCL-5, GM-CSF, CXCL-10, LIF, Leptin, IL-18, CCL-2, CCL-3, and CCL-7, were observed in the lung of PAO-exposed mice. Further, VEGFA levels were reduced in the lung. Pulmonary function evaluated using a flexiVent showed a downward shift in the PV loop, decreases in static compliance and inspiratory capacity, increases in respiratory elastance and tissue elastance. These changes are consistent with an ALI phenotype. These results demonstrate that cutaneous PAO exposure leads to ALI and that the model can be used as an effective surrogate to investigate vesicant-induced ALI.
Significance Statement:

This study presents a robust model for studying acute lung injury (ALI) resulting from cutaneous exposure to PAO, a surrogate for the toxic vesicating agent Lewisite. The findings in this study mimic the effects of cutaneous lewisite exposure, providing a reliable model for investigating mechanisms underlying toxicity. The model can also be used to develop medical countermeasures to mitigate ALI associated with cutaneous Lewisite exposure.
Introduction

Burn injuries, including those caused by chemicals, can have systemic effects and lead to acute lung injury (ALI) and Acute Respiratory Distress syndrome (ARDS) (Silva et al., 2016). The incidence of ALI/ARDS in burn injury patients is quite high, at 24%, and the mortality rate for these conditions is even higher reaching up to 31% (Wang et al., 2021). Unlike thermal burns, chemical burns are different in terms of mechanisms and pose a much greater risk (Hall, Mathieu and Maibach, 2018). Chemical burns constitute 2-5% of all skin burn injuries (Hall, Mathieu and Maibach, 2018). However, in a study cohort from Iran, they accounted for about 30% of all burn related deaths (Akelma and Karahan, 2019). It not only involves local inflammation but also leads to inflammation in distant organs too known as systemic inflammatory response syndrome (SIRS). Furthermore, the absorption of chemicals through the skin poses an additional risk, as these substances and their byproducts can potentially reach distant organs through the bloodstream. Among the chemicals that cause burns, vesicating warfare agents like lewisite, are of particular concern because of lack of effective antidotes due to limited understanding of the mechanisms involved in the injury.

Lewisite, a warfare and chemical burn agent, can also cause ALI when exposed to the skin. It poses a significant threat due to its high toxicity and destructive effects in the victims. Lewisite (dichloro(2-chlorovinyl) arsine (C_2H_2AsCl_3)), an arsenic containing organic chemical, is primarily composed of 2-chlorovinylidichloroarsine (L), although there are variations known as Lewisite 1, Lewisite 2, and Lewisite 3. This potent vesicating agent can cause severe injuries and fatalities in humans. Exposure to Lewisite results in intense pain, a burning sensation in the affected areas, systemic capillary injury and leak leading to fluid loss, and the development of pulmonary edema followed by acute bronchopneumonia (Institute of Medicine. Committee to Survey the Health Effects of Mustard et al., 1993; Vilensky and Sinish, 2005).
The precise mechanisms responsible for the immediate effects of cutaneous vesicants like lewisite on the lungs have not been fully understood. Therefore, the objective of this study was to create an in-house model that simulates acute effects of lewisite exposure using phenyl arsine oxide (PAO) as a surrogate. This surrogate model closely mimics typical chemical burn exposures observed in humans. PAO being an organoarsenical compound can penetrate the membrane and form stable ring structures with sulfhydryl groups of proteins. It also binds with thiol groups and inhibits the activity of certain enzymes containing thiol. This makes it very similar to lewisite and other arsenicals in terms of its biochemical reaction profile as described earlier (Li, Srivastava and Athar, 2016; Li et al., 2016). Using this specific injury model, we conducted a comprehensive assessment of the acute pulmonary consequences, while incorporating the recently updated recommendations for characterizing acute lung injury (ALI) in animal models. This model shows promise for the development of medical countermeasures against lewisite and other vesicants, which are recognized as agents of concerns by the NIH Chemical Countermeasures Research program (CCRP).

Methods:

Chemicals
Phenyl arsine oxide (PAO; Cat# P3075; CAS# 637-03-6) obtained as a powder (> 97% purity) was prepared at a stock concentration of 5 mg/ml in ethanol (200 proof) for animal studies and 100 mM for in-vitro studies. All chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless mentioned otherwise.

Animals and exposures.
Animal procedures conducted at the University of Alabama at Birmingham were approved by the Institutional Animal Care and Use Committees (IACUC). Adult male C57BL/6J mice weighing 25 g were obtained from Envigo Co., Indianapolis, IN, and were given unrestricted
access to food (rodent pellet diet) and water (sterile hydropacs). They were housed in a room with a temperature of 25 °C and a 12-hour light/dark cycle with 40-45% humidity in mice cages. Two days before the exposure, the mice were shaved and depilated using Nair (Church and Dwight Co., Ewing, NJ), the hair removing cream (applied for 2-3 min) and thoroughly cleaned using a moist cloth. The mice were weighed and then anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). Prior to PAO exposure buprenorphine (0.05-0.1 mg/kg) was also administered subcutaneously approximately 30 min before anesthesia. A solution of PAO in a volume of 60 μl was prepared according to dose and animal weight and uniformly applied once to rectangular area of 2 x 4 cm² on the dorsal skin of mice, following a method described elsewhere (Li et al., 2016). After exposure, each mouse was isolated in a separate cage and placed in the fume hood for 6 hours. This was done to allow any remaining vapors to dissipate, prevent cross-contamination between mice, ensure the full recovery of each individual mouse, and to facilitate the complete absorption of PAO through the skin. After 6 hours, mice were housed in the designated animal facility area with 2-3 mice per cage (disposable open top cages with woodchip bedding and enrichment with mice enviro-pak). Control animals were similarly exposed to 60 μl of ethanol applied topically and housed in a similar fashion. After 24 hours, the mice were anesthetized under isoflurane (2.5 % in small chamber and 1.5% under nose cone), blood was collected, and the mice were then euthanized.

**Arterial blood gas (ABG) measurements**

Arterial blood gas measurements were performed as described previously (Manzoor et al., 2020). After 24h post exposure animals were anesthetized with isoflurane (2.5% for induction and 1.5% for maintenance) and blood was collected from the descending aorta and analyzed immediately using an EPOC-Blood gas and electrolyte analyzer (Heska, Loveland, CO).

**Complete blood cell counts:**
For complete blood cell counts (CBC), blood was collected from the descending aorta in ethylenediaminetetraacetic acid (EDTA) tubes (BD Biosciences). Complete blood counts were analyzed using a HEMAVET® hematology analyzer (Hemavet 950 FS; Drew Scientific, Dallas, TX). After blood collections, mice were exsanguinated through an abdominal artery incision.

**Bronchoalveolar lavage fluid (BALF) collection.**

At the end of study (24h) and after blood collection bronchoalveolar lavage (BAL) was performed on whole lungs as mentioned previously (Manzoor et al., 2020). Briefly, the trachea was cannulated and a total of 2×1ml of Dulbecco’s phosphate-buffered saline (DPBS, without calcium and magnesium) was injected into the lungs of mice via the trachea. The retrieved fluid was then centrifuged at 800× g for 10 minutes at 4°C. Following centrifugation, the resulting supernatant was collected and stored at -80°C until further use.

**Cytokine profiling in lung lysates**

After BAL collection mice lung were perfused through the pulmonary artery using a perfusion pump and lungs were harvested and snap frozen for cytokine analysis. Cytokine profiling in lung lysates was performed as described earlier (Manzoor et al., 2020; Mariappan et al., 2023). Briefly, frozen lung tissue was homogenized in liquid nitrogen and extracted with lysis buffer containing PMSF and protease inhibitor cocktail. Tissue homogenates were centrifuged at 10,000 g for 15 min at 4°C and the supernatant was collected and stored in -80°C until analyzed. Lung cytokines and chemokines were measured by using multiplex bead array system (Procartaplex, Thermofisher, Vienna, Austria), which were incubated with 3 mg/ml protein from lung lysate. The plate was read at Magpix (xMAP) instrument (Luminex Corporation, Austin, TX, USA).
**Western blotting**

Immunoblot of BALF supernatant was performed as mentioned before (Manzoor et al., 2020). 40 μl of BALF supernatant was separated on a precast gradient gel (Bio-Rad laboratories, CA) and transferred to nitrocellulose membrane. Polyclonal antiHMGB1 antibody (NB100–2322; Novus Biologicals, Littleton, CO) was used at a dilution of 1:1000. Membranes were then incubated with HRP-conjugated anti-rabbit antibody (BioRad laboratories, Hercules, CA) for 1hr at room temperature (1:3000 dilution) and detection was done by using Super Signal® West Femto maximum sensitivity substrate (Thermo Fisher, Waltham, MA) on a Gel Doc imaging system (Bio-Rad laboratories, Hercules, CA).

**Histology and immunofluorescence**

For histology, the lungs were filled with 4% buffered formalin through intratracheal instillation. They were then immersed in the same solution and subsequently placed in 70% ethanol before paraffin embedding. The fixed lungs, embedded in paraffin, were cut into 5 μm sections using a microtome (Lieca biosystems, IL). Hematoxylin and Eosin (H&E) staining was performed on these sections for histological examination and lung injury scoring, following a previously described method (Manzoor et al., 2020; Kulkarni et al., 2022).

For immunofluorescence analysis, 5 μm sections of the lung were deparaffinized and rehydrated. Subsequently, they were incubated in citrate buffer (10 mM; pH 6.0; 0.05% Tween-20) for 20 minutes to retrieve antigens. Non-specific binding sites were blocked using 5% normal goat serum (NGS), followed by overnight incubation (4°C) with anti-MPO antibody (ab208760; Abcam, Cambridge, MA) at a dilution of 1:400 [4]. The next day, the tissue sections were incubated with 594-AlexaFluor conjugated secondary anti-rabbit antibody (Invitrogen) at a dilution of 1:1000 for 1 hour at room temperature. Rabbit IgG control (DAKO/Agilent, Santa Clara, CA) was used as a negative control, following the same specifications. The tissues were
mounted using Vecta shield hard mount containing DAPI, and images were acquired using a Leica microscope.

**Lung mechanics measurements:**
Separate groups of animals were utilized for conducting respiratory mechanics and function assessments using the forced oscillation technique (FOT) maneuver. To induce anesthesia in the animals exposed to ethanol or PAO, a combination of ketamine (100 mg/kg), xylazine (7.5 mg/kg), and acepromazine (1.5 mg/kg) was administered intraperitoneally. Subsequently, an 18G cannula was inserted into the trachea for further procedures. The animals were then subjected to the FlexiVent system (SCIREQ Inc, Montreal, QC, Canada) to evaluate respiratory mechanics. To limit passive breathing, a paralytic agent, pancuronium bromide (0.8 mg/kg), was administered intraperitoneally. Before the commencement of respiratory mechanics assessments, two deep inflation maneuvers at a pressure of 30 cm H\textsubscript{2}O were performed as described earlier (Mariappan et al., 2023). This was done to standardize lung volumes and recruit closed airspaces. Baseline lung function measurements were taken under mechanical ventilation conditions, with a respiratory rate of 150 breaths per minute, tidal volume of 10 ml/kg, and positive end-expiratory pressure (PEEP) set at 3.0 cm of water. The FlexiVent system employed the FX1 module designed for mice and was operated using FlexiWare software version 8.0.

**Statistical analysis**
All statistical analyses were performed by using an unpaired, nonparametric Mann-Whitney t-test in Prism 9.5 (GraphPad, La Jolla, CA), unless otherwise indicated. Data reported are either as mean values with standard error of the mean (SEM), or as box and whiskers where the line represents the mean, and the whiskers extend to the largest and smallest values. p<0.05 was considered significant. Using pilot data, an a priori F-test was conducted to determine the
number of animals for the experimental groups.
Results:

Dose-response studies were conducted to determine the amount of phenylarsine oxide (PAO) needed to induce injury. Administration of doses at 6, 7.5, and 12 mg/kg in mice resulted in a dose-dependent elevation of BALF protein levels (Supplemental Figure 1). The 12 mg/kg dose showed significant mortality (data not shown), leading to the selection of the 7.5 mg/kg dose.

Cutaneous PAO exposure causes systemic effects.

Cutaneous injuries, such as thermal and chemical burns, can have systemic effects on the body. Changes in hematological parameters have been reported in burn victims (Dinsdale et al., 2017; Sen et al., 2019). In addition, we have previously demonstrated changes in lewisite-exposed mice (Manzoor et al., 2020). To investigate whether cutaneous exposure to PAO leads to systemic effects, complete blood cell counts were conducted. Figure 1 demonstrates significant decreases in white blood cell (WBC) and lymphocyte counts in the PAO-exposed group compared to the control group 24h after exposure. Although there were no changes in neutrophil numbers, the neutrophil-to-lymphocyte ratio, an independent prognostic indicator of systemic inflammation and organ failure (Younan et al., 2019), was elevated. Additionally, PAO-exposed mice exhibited significant increases in red blood cell count (RBC), hemoglobin (Hb) levels, and hematocrit (HCT) levels compared to the control group (Figure 1E, 1F, and 1G, respectively). Platelet counts decreased in the PAO-exposed group Figure 1H), while mean platelet volume (MPV) increased (Figure 1I).

Cutaneous PAO exposure impairs gas exchange, alters electrolyte and blood glucose levels.

ARDS and multiorgan failures (MOF) are frequently reported in burn patients, which also show hypoxemia due to impaired gas exchange (Sio et al., 2010). In our previous study, impaired gas exchange was also observed in mice exposed to cutaneous lewisite (Manzoor et al., 2020). To assess whether cutaneous exposure to PAO (Phenylarsine Oxide) leads to Acute Lung Injury
(ALI), gas exchange was examined. Cutaneous PAO exposure resulted in significant reductions in arterial blood pH compared to mice exposed to cutaneous Ethanol (EtOH), as depicted in Figure 2A. Moreover, the partial pressure of carbon dioxide (PaCO₂) in arterial blood showed a substantial increase in the PAO-exposed group compared to the control, as shown in Figure 2B. Additionally, exposure to PAO caused a significant decrease in the partial pressure of arterial oxygen (PaO₂) when compared to the control group, as illustrated in Figure 2C. However, there were no notable changes in blood bicarbonate levels. Furthermore, the PAO-exposed group exhibited increased levels of potassium and calcium, while there were no significant alterations in the levels of sodium or chloride. Blood glucose levels were significantly reduced in the PAO-exposed group when compared to the control group.

**Acute lung injury induced by cutaneous exposure to PAO.**

To evaluate ALI in mice exposed to cutaneous PAO, lung injury parameters were assessed. That cutaneous PAO causes injury to the skin is demonstrated in Figure 3A. Disruption of the alveolar-capillary barrier is frequently observed in ALI, detected by increased protein leak in the BAL. As shown in Figure 3B, there was significant increase in protein in the BALF supernatant of the PAO-exposed mice when compared to the EtOH controls.

HMGB1, a prominent damage-associated molecular pattern (DAMP), is released by cells either actively or passively in response to stress or cell death (Chen, Kang and Tang, 2022). Previously, we and others have demonstrated the involvement of HMGB1 in Acute Lung Injury (ALI)(Andersson, Ottestad and Tracey, 2020; Manzoor et al., 2020; Mariappan et al., 2023). Consistent with an ALI phenotype, there was increase in levels of HMGB1 in the BAL supernatant (Figure 3C). To further investigate lung injury histological sections of the lung stained with hematoxylin and eosin were examined. Histological evaluation revealed moderate lung injury, which was characterized by edema in alveolar interstitium, increased numbers of macrophages and interstitial neutrophils (Figure 3D). Infiltration of neutrophils were further
confirmed with myeloperoxidase (MPO) staining, a marker of neutrophil (Figure 3E). Quantitation of lung injury using a previously described method (Matute-Bello et al., 2011) showed significant increase in the PAO-exposed group when compared to the ethanol controls (Figure 3F).

**Cutaneous PAO exposure alters lung function.**

After observing decreased blood oxygenation, disruption of the alveolar-epithelial barrier, and increased lung inflammation in animals exposed to cutaneous PAO, we sought to investigate potential impairments in lung function. Burn victims often show reduced lung and chest compliance (Bakowitz, Bruns and McCunn, 2012). To achieve this, we utilized the flexiVent system and performed measurements using the forced oscillation technique (FOT).

To examine the mechanical changes occurring in the lung, we generated pressure-volume (PV) curves using pressure-driven maneuvers. The PV loop of mice in the PAO group exhibited a downward shift, indicating a stiff lung (Figure 4A). Furthermore, the static compliance and inspiratory capacity were significantly reduced in the PAO group compared to the control group exposed to ethanol (Figure 4B, and 4C). In FOT measurements, we observed no changes in the baseline values of resistance of the respiratory system (Rrs). However, the baseline elastance of the respiratory system (Ers; Figure 4D) and tissue elastance (H; Figure 4E) showed significant increases in the PAO-exposed group.

**Cutaneous exposure to PAO alters the cytokine levels in the lung.**

To gain further insight into the lung injury resulting from cutaneous exposure to PAO in mice, a multiplex assay was conducted to determine the concentrations of lung cytokines. The analysis revealed significant increases in the levels of various pro-inflammatory cytokines in the lung tissue of the PAO-exposed group. These cytokines included IL-6, CXCL-1, CXCL-2, CXCL-5, GM-CSF, CXCL10, LIF, leptin, and IL-18 (Figure 5A-I). Additionally, a single topical application
of PAO led to a notable elevation of several chemokines in the lung tissue. In the PAO-exposed group, there were significant increases observed in the chemokines MCP-1 (or CCL2), MCP-3 (or CCL7), and MIP-1α (or CCL3) (Figure 5J-L). These chemokines serve as signals that recruit leukocytes to the site of injury. Additionally, VEGFA, an angiogenic growth and repair factor was significantly decreased in the PAO group (Figure 5M).
Discussion

Effective therapies to mitigate injury caused by exposure to Lewisite is lacking due to limitations in conducting direct research on Lewisite-induced ALI in a laboratory setting. To overcome this limitation, we used phenylarsine oxide (PAO), a less toxic arsenical compound, as a safer substitute for Lewisite in laboratory-based ALI studies. PAO shares several similar chemical properties with Lewisite, allowing it to mimic the mechanisms of action and pathological consequences observed in Lewisite-induced ALI. By utilizing PAO as a surrogate, one can investigate the underlying mechanisms and develop potential therapeutic interventions for ALI associated with toxic substances like Lewisite. In this study, we have conducted a thorough characterization of an ALI model induced by cutaneous PAO exposure.

Cutaneous exposure to PAO resulted in evident systemic injury, as indicated by an increased neutrophil-to-lymphocyte ratio, which is a recognized risk factor and prognostic indicator of overall survival in patients with ARDS and other diseases. Furthermore, it is associated with the severity of inflammation (Gunay et al., 2014; Kain et al., 2019). Additionally, we observed an elevation in total red blood cell (RBC) count, hemoglobin (Hb) and hematocrit (HCT) in mice exposed to PAO, which could result from hypoxemia or possible dehydration. Thrombocytopenia along with an increase in mean platelet volume is often observed in disseminated intravascular coagulopathy (DIC) and inflammation, which are common in humans with ARDS (Wang et al., 2014; Schmoeller et al., 2017; Korniluk et al., 2019).

Disruption of the alveolar-capillary membrane as observed by the increased BALF protein in the PAO group is typical of ALI. Impairment in gas exchange in the PAO-exposed group, leading to hypoxemia is possibly due to disruption of the alveolar-capillary membrane.

Inflammation is a consistent feature of ALI. Acute lung injury is often accompanied by increase in release of HMGB1, a DAMP molecule. Extracellular HMGB1 has also been linked to the
course of pathogenesis in ALI/ARDS (Venereau et al., 2012). HMGB1 is a non-histone chromatin binding protein that acts as a damage associated molecular pattern (DAMP) molecule in extracellular environment. Release of HMGB1 triggers release of inflammatory cytokines/chemokine and additionally it can bind to toll like receptors and receptors of advanced glycation end products (RAGE) to induce an inflammatory cascade (Zhu et al., 2015). HMGB1 has been shown to cause ALI when injected intratracheally further underscoring its crucial role as a proinflammatory protein (Ueno et al., 2004). Our multiplex data set shows a significant increase in CXCL1 and CXCL5 which are secretory proteins required to direct the recruitment of neutrophils (Koltsova and Ley, 2010; Sawant et al., 2015). In addition, lung resident macrophages upon activation are known to secrete other neutrophil recruitment chemokines like MIP-1α and MIP-2 and we observed a significant increase in their levels in lungs of PAO exposed mice (Zamjahn et al., 2011; Williams and Chambers, 2014). These findings are consistent with our previous findings where we demonstrated that cutaneous PAO exposure induced neutrophil activation and production of neutrophil extracellular traps (NETs) in lung tissue at 6-h time point (Surolia et al., 2021). We also observed significant increase in chemoattractants linked to macrophage recruitment which included, MCP1 and MCP-3 in lung tissues of PAO exposed mice, which are very crucial in the pathogenesis of ALI/ARDS (Seitz et al., 2010; Bein et al., 2021). In addition to IL-6, an increase in IL-18 levels in lung tissue which indicate possible activation of inflammasome complex which have recently been shown to play a significant role in ALI (Dolinay et al., 2012). Taken together, these findings point to a phenotype typical of ALI and ARDS.

Lung injury is commonly accompanied by changes in lung function. A thorough characterization of these changes enables the differentiation and diagnosis of ventilatory disorders. In the PAO group, we observed increased overall elastance, and tissue elastance, while the respiratory and Newtonian resistance (data not shown) remained unchanged. These findings suggest a
dysfunction of the lung itself rather than the airways. The alterations in the pressure-volume (PV) loop, combined with a decrease in inspiratory capacity and static compliance, align with the typical patterns observed in cases of ALI (Kulkarni et al., 2022).

In conclusion, this study presents a model that replicates acute pulmonary toxicity resulting from a single cutaneous exposure to a vesicant, and which closely resembles several pathophysiological characteristics observed in humans diagnosed with ALI/ARDS. Within this study, mice exposed to PAO displayed all four pathophysiological features associated with ALI, including histological evidence of tissue injury, disruption of the alveolar capillary membrane, an inflammatory response, and physiological dysfunction. These findings highlight the relevance of the model in capturing key aspects of ALI and in mimicking the effects of lewisite as described by us earlier (Manzoor et al., 2020; Surolia et al., 2021). The establishment of an animal model utilizing PAO-induced ALI holds significant potential for advancing our understanding of the mechanisms underlying Lewisite toxicity.

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Data availability: All datasets generated or analyzed in the current study will be provided upon requests.

Authorship Contribution:

Participated in research design: Ahmad A, Athar M, Ahmad S, Antony V.

Conducted experiments: Zafar I, Manzoor S, Mariappan N, Ahmad A.

Performed data analysis: Zafar I, Manzoor S, Mariappan N, Ahmad A.

Wrote or contributed to the writing of manuscript: Zafar I, Manzoor S, Ahmad S, Athar M, Antony V, Ahmad A.
Footnotes:

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References:


Legends

Figure 1. Cutaneous PAO exposure alters blood cell counts. Mice were exposed topically to PAO (7.5 mg/kg) or EtOH (control group) and 24 h later blood was collected, and complete blood cell counts were determined using a 5-part differential blood cell count analyzer. (A) White blood cells (WBC), (B) Neutrophils (C) Lymphocytes, (D) Neutrophil-Lymphocyte ratio (NLR), (E) RBC, (F) Hemoglobin, (G) hematocrit percentage (H) Platelet counts, and (I) mean platelet volume (MPV). Values are presented as a scatter plot superimposed on a box and whiskers plot where the line within the box represents the mean, and the whiskers extend to the largest and smallest values (n=26/group).

Figure 2. Arterial blood gas, electrolyte and blood glucose measurements in mice exposed topically to PAO. Mice were exposed topically to PAO (7.5 mg/kg) or EtOH (control group) and 24 h later blood was collected from the descending aorta and analyzed for blood gas parameters using the EPOC-Vet Blood analyzer. (A) pH, (B) partial pressure of arterial CO$_2$ (PaCO$_2$), (C) partial pressure of arterial O$_2$ (PaO$_2$), (D) bicarbonate, (E) Potassium, (F) Sodium, (G) Calcium, (H) Chloride, and (I) Glucose. Values are presented as a scatter plot superimposed on a box and whiskers plot where the line within the box represents the mean, and the whiskers extend to the largest and smallest values (n≥12/group).

Figure 3. Single cutaneous application of PAO induced acute lung injury. Mice were exposed topically to PAO (7.5 mg/kg) or EtOH (control group) and 24 h later animals were euthanized. (A) Representative skin images, (B) Protein leak (n≥10/group), (C) immunoblot of high mobility group box (HMGB1) protein along with respective densitometry graph (p= 0.0312 PAO vs EtOH; n=6/group). (D) Representative images of H&E-stained lung tissues showing neutrophils (green arrows), edema in alveolar interstitial (yellow arrows) and monocytes cells (black arrows) in PAO exposed mice (n≥5/group). Images were captured at 400x magnification.
using Leica microscope. (E) Representative images of MPO staining of the lungs demonstrating increased levels in the PAO exposed mice (red color), as compared to EtOH control, and (F) Lung injury score.

**Figure 4. Cutaneous PAO exposure leads to reduced lung function in mice.** Mice were exposed to 7.5mg/kg PAO topically and after 24 hours respiratory mechanics parameters were measured at baseline using forced oscillation technique. (A) Pressure volume (PV) loops were generated using ramp-style pressure volume maneuver and mean PV loops for each group are shown. (B) Static Compliance (Cst) was calculated from the deflating arm of the PV loop. (C) Inspiratory capacity (IC) was measured using deep inflation maneuver. (D) Baseline Elastance of the respiratory system (Ers) and (E) Tissue Elastance (H) were also measured at baseline. Individual values and group mean ± SEM are shown (n=3 per group).

**Figure 5. Cutaneous PAO exposure increased pro-inflammatory cytokines/chemokines in lung lysates.** Mice were exposed topically to PAO (7.5 mg/kg) or EtOH (control group) and 24 h later animals were euthanized. Lung tissues were collected for quantifying the proinflammatory cytokines at 24 h after ATO or saline administration. Lysates were prepared and analyzed for cytokines using a Luminex-based multiplex platform. Concentrations of (A) IL-6 (B) CXCL-1, (C) CXCL-2, (D) CXCL-5, (E) GM-CSF, (F) CXCL10, (G) LIF, (H) Leptin, (I) IL-18, (J) CCL-2/MCP-1, (K) CCL-3/ MIP-1α, (L) CCL-7/MCP-3, and (M) VEGFA. Values are presented as a scatter plot superimposed on a box and whiskers plot where the line within the box represents the mean, and the whiskers extend to the largest and smallest values (n>12/group).
Figure 1

A. WBCs
B. Neutrophils
C. Lymphocytes
D. Neutrophil:lymphocyte ratio

E. RBCs
F. Hemoglobin
G. Hematocrit
H. Platelet
I. Mean Platelet Volume
Figure 2

A. pH

B. PaCO₂

C. PaO₂

D. HCO₃⁻

E. Potassium

F. Sodium

G. Calcium

H. Chloride

I. Glucose
Figure 3

A. Skin

B. Protein Leak in BALF

C. HMGB1

D. H&E staining

E. MPO staining

F. Lung Injury Scoring
Figure 4

A. PV loop

B. Compliance

C. Inspiratory Capacity

D. Respiratory Elastance

E. Tissue elastance
Figure 5

A. IL-6

B. CXCL-1

C. CXCL-2

D. CXCL-5

E. GM-CSF

F. CXCL-10

G. LIF

H. Leptin

I. IL-18

J. CCL-2

K. CCL-3

L. CCL-7

M. VEGFA