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Isoflurane and Pentobarbital Anesthesia for Pulmonary Studies Requiring Prolonged Mechanical Ventilation in Mice

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Mechanical ventilation can be used in mice to support high-risk anesthesia or to create clinically relevant, intensive care models. However, the choice of anesthetic and inspired oxygen concentration for prolonged procedures may affect basic physiology and lung inflammation. To characterize the effects of anesthetics and oxygen concentration in mice experiencing mechanical ventilation, mice were anesthetized with either isoflurane or pentobarbital for tracheostomy followed by mechanical ventilation with either 100% or 21% oxygen. Body temperature, oxygen saturation, and pulse rate were monitored continuously. After 6 h, mice were euthanized for collection of blood and bronchoalveolar lavage fluid for evaluation of biomarkers of inflammation and lung injury, including cell counts and cytokine levels. Overall, both isoflurane and pentobarbital provided suitable anesthesia for 6 h of mechanical ventilation with either 21% or 100% oxygen. We found no differences in lung inflammation biomarkers attributable to either oxygen concentration or the anesthetic. However, the combination of pentobarbital and 100% oxygen resulted in a significantly higher concentration of a biomarker for lung epithelial cell injury. This study demonstrates that the combination of anesthetic agent, mechanical ventilation, and inspired oxygen concentrations can alter vital signs and lung injury biomarkers during prolonged procedures. Their combined impact may influence model development and the interpretation of research results, warranting the need for preliminary evaluation to establish the baseline effects.

Abbreviations: BALF, bronchoalveolar lavage fluid; ICAM1, intracellular adhesion molecule 1; LLD, lower limit of detection; RAGE, receptor for advanced glycation end-products; SpO_2, oxygen saturation

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Introduction

In research that uses mice, mechanical ventilation can be used to control respiration and support oxygen exchange, thereby helping to maintain normal physiology during surgical procedures and prolonged anesthesia.[35] In addition, the use of mechanical ventilation provides clinical relevance to critical care models in mice by including an intervention that is commonly used in human patients. However, despite its substantial benefits, mechanical ventilation can have deleterious effects. The injurious effects of lung overdistention due to either high-pressure or high-volume ventilation are well documented.[35,43] However, the routine use of even lung-protective, low-volume ventilation (6 to 12 mL/kg)[18] can produce time-dependent, progressive lung inflammation in mice.[42] In addition, when high inspired oxygen concentrations are used with mechanical ventilation, excess production of reactive oxygen species leads to cell injury, dose- and time-dependent lung inflammation, and loss of alveolar–capillary barrier function.[1,14] Therefore, mechanical ventilation and hyperoxia could alter research outcomes in mice and confound some studies by producing underlying lung inflammation.

Another concern with mechanical ventilation in mice is that, unlike in humans, it always requires full anesthesia. The choice of anesthetic influences the analgesia, hemodynamic stability, thermoregulation, and even patterns of inflammation in mice.[8,35,47,48] Volatile anesthetics are generally considered safer for rodent anesthesia than injectable anesthetics.[8,29] However, inhalants have direct contact with lung epithelium and influence pathophysiologic responses of the lung. The overall effects of volatile anesthetics are anti-inflammatory and cytoprotective and thus potentially preserve alveolar–capillary barrier function and protect against lung injury.[6,9,30,34] Although injected anesthetics do not contact lung epithelium initially, they are immunomodulatory[4] and can trigger distinct patterns of inflammation in the lung.[24,33,42] Therefore, the choice of anesthetic may influence the development of lung injury and, in some cases, confound studies on inflammation.[28,31]

The combination of anesthesia, mechanical ventilation, and hyperoxia triggers a complex array of inflammatory responses.[14] Because ventilator settings, inspired oxygen concentrations and anesthetics are not standardized across studies,[18] their combined effects should be evaluated to optimize the interpretation of results derived from mouse models of lung injury. This need became apparent when our laboratory began studies to determine the role of atelectrauma (i.e., the cyclic opening and closing of airways) on the pathophysiology of acute respiratory distress. During sudden airway opening, liquid plugs or menisci rupture, producing the lung sounds known as 'crackles.'[12] This forceful rupture also produces mechanical stress on airway epithelium that may, in theory, cause damage that contributes to the disease process. To study repeated mechanical

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stress, we proposed a model of atelectasis induced by delivering low-volume mechanical ventilation without positive end-expiratory pressure, allowing cyclic airway collapse and reopening. We planned to compare this experimental condition to a control condition in which open airways were maintained by using mechanical ventilation with positive end-expiratory pressure. Prior to beginning those studies, we performed preliminary studies to evaluate the effects of anesthetic agents and oxygen concentrations during 6 h of mechanical ventilation in the control, open-airway model without atelectrauma. Our goal was to develop a safe anesthetic protocol that had few, if any, effects on the lung.

Several anesthetic combinations are available for use in mice,[15,18,48] but we opted to examine monoanesthesia to reduce the number of potential drug effects. Isoflurane was chosen for its broad safety margin during long periods on anesthesia[8,11,38] and prior recommendations of its suitability for effective evaluation of airway inflammation in rats.[40] Pentobarbital was chosen because of its prior use in mechanical ventilation studies[18] and its reportedly lower impact on the immune system.[20,24,28,31] We performed mechanical ventilation with 100% oxygen, which is considered advantageous for rodent anesthesia.[5] 100% oxygen was compared with 21% oxygen, which we have used previously for mouse anesthesia[46] and which would allow comparison of 2 anesthetics and 2 oxygen concentrations over a 6 h exposure time. We hypothesized that the combinations of anesthetic agents and oxygen concentrations would cause variation in select biomarkers of lung inflammation and cell injury associated with mechanical ventilation. The aims of our study were to determine the safety and efficacy of the anesthetic regimens for prolonged mechanical ventilation in mice and to establish their effects on the biomarkers of inflammation and lung injury we used in our studies.

Materials and Methods

Study design. Mice (n = 6 per group) were anesthetized with either pentobarbital or isoflurane for tracheotomy and mechanical ventilation with either 21% or 100% O_2. Vital signs were recorded every 15 min. After 6 h of mechanical ventilation, the mice were euthanized and blood and bronchoalveolar lavage fluid (BALF) were collected. Cell counts and cytokine concentrations were analyzed for markers of inflammation and lung injury. All studies were approved by the University of Michigan IACUC and were conducted in accordance with the *Guide*[16] and Public Health Service assurance in an AAALAC-accredited facility.

Animals. Conforming with the sex and strain proposed for model development in a funded grant, male C57BL/6J mice (catalog no. 000664; age, 7 to 8 wk) were obtained from Jackson Laboratories (Bar Harbor, ME). The vendor reported that the mice were SPF for the following agents: ectromelia virus, Hantaan virus, lactate dehydrogenase elevating virus, lymphocytic choriomeningitis virus, minute virus of mice, mouse adenovirus, mouse cytomegalovirus, mouse hepatitis virus, mouse norovirus, mouse parvovirus, mouse rotavirus, mouse thymic virus, pneumonia virus of mice, polyoma virus, reovirus, Sendai virus, Theiler murine encephalomyelitis virus, *Bordetella* spp., *Citrobacter rodentium*, *Corynebacterium bovis, Corynebacterium kutscheri, Helicobacter* spp., *Mycoplasma pulmonis*, *Salmonella* spp., *Streptobacillus monoliformis*, *Encephalitozoon cuniculi*, pinworms, and fur mites.

The mice were group housed (n = 5 per cage) on corncob bedding (Bed-o'Cobs, The Andersons, Maumee, OH) in autoclaved, polysulfone microisolation cages that were individually ventilated (Allentown Caging, Allentown, NJ). Each cage contained 6 g of crinkled paper (Enviropak, WF Fisher and Son, Branchburg, NJ) for enrichment. The mice were acclimated for 1 wk prior to experimentation under standardized conditions. Mice had unrestricted access to food (Laboratory Rodent Diet 5001, PMI LabDiet, St. Louis, MO) and water and were maintained on a 0600:1800 light:dark cycle and constant temperature (72 ± 2 °F [22.2 ± 1.1 °C]) and humidity (30 to 70%).

Anesthesia. Mice were randomly assigned to groups receiving either pentobarbital or isoflurane. Pentobarbital (60 mg/kg; Nembutal sodium, Akorn, Lake Forest, IL) was given intraperitoneally, with redosing of 1/4 to 1/2 of the original dose approximately every 40 min. Isoflurane (Fluriso, VetOne, Boise, ID) was used for box induction at 5% and then reduced to 1.2 to 1.4% by mask and continued via tracheotomy tube.

Tracheotomy. A ventral midline skin incision was used to expose the salivary glands and underlying muscles which were retracted to expose the trachea. Two, 4-0 silk ligatures (Look Surgical Suture, Corza Medical, Westwood, MA) were placed around the trachea, and a transverse incision was made between the tracheal rings. A 20-gauge intravenous catheter (Safelet Cath, NIPRO Medical, Miami, FL) was inserted and secured by the ligatures.

Ventilation. Immediately after tracheostomy, a mechanical ventilator (RoVent, Kent Scientific, Torrington, CT) was attached to the catheter for volume-controlled delivery of either 21% (room air) or 100% oxygen (rate, 100 breaths/min; tidal volume, 7 mL/kg; positive end-expiratory pressure, 4 cm H_2O) for 6 h. Ventilator settings, including rate, were held constant throughout the study, consistent with the parameters that would later be required in atelectrauma studies. Each mouse received 0.1 mL of saline subcutaneously (Hospira, Wake Forest, IL) every hour during anesthesia. Anesthetic depth was titrated to prevent spontaneous respiration.

Vital monitoring. A homeothermic control system (RightTemp, Kent Scientific) set to 37 °C maintained and recorded body temperature, while a pulse oximeter (MouseStat, Kent Scientific) recorded pulse rate and oxygen saturation from a hindpaw. Values were recorded every 15 min and averaged for each hour of anesthesia.

Euthanasia and sample collection. After 6 h of mechanical ventilation and while under deep anesthesia, the mice were euthanized by exsanguination from the caudal vena cava through a laparotomy incision. Blood was collected in tubes containing EDTA. Bronchoalveolar lavage was performed through the tracheostomy catheter by serial infusion and retrieval of 2, separate, 1-mL volumes of HBSS without Ca_2Cl, Mg_2SO_4, or phenol red (Thermo Fisher, Waltham, MA) with heparin (Sargent Pharmaceuticals, Schaumburg, IL) diluted 1:100 into HBSS. Lungs were insufflated and fixed with 1 mL of 10% buffered formalin. The trachea was tied off with 4-0 silk suture, and lungs were then placed in 10% formalin for histologic analysis.

Sample processing and analysis. A CBC was performed (Hemavet 950, Drew Scientific, Miami Lakes, FL). The remaining blood was centrifuged (2000 × g, 5 min), and plasma was removed and stored at −80 °C for later cytokine analysis. BALF samples were centrifuged (600 × g, 5 min), and the supernatant from the first 1-mL sample retrieved from each mouse was stored at −80 °C. The cell pellets from the 2 BALF samples were pooled for total counts on a hemacytometer (Hausser Scientific, Horsham, PA). Viability was determined by diluting cells 1:2 with trypan blue (Thermo Fisher). Cytospin slides were loaded with 1×10^5 cells, centrifuged (700 × g, 5 min), and stained (Diff-Quick, Baxter, Detroit, MI). Differential cell counts (300 cells) were obtained by using light microscopy.

Histology. The lungs were fixed in formalin, embedded, sectioned, and stained (hematoxylin and eosin). The sections were evaluated under light microscopy and scored by an observer (MJH) who was blind to the experimental treatment. A semiquantitative scoring system was used to assess the numbers of immune cell infiltrates in the airways (alveoli and bronchioles) above normal background resident populations (0, none; 1, a few scattered cells [minimal]; 2, increased clusters of cells [mild]; 3, frequent adjacent alveoli that contained cells [moderate]; 4, large sections of airways containing numerous cells [severe]). The overall composition of the cellular infiltrates, when present, was recorded as a descriptor of the primary immune process.

Cytokine and albumin ELISA. Cytokines in thawed plasma (dilution, 1:10) and lung lavage fluid (dilution, 1:2) were measured by using sandwich ELISA kits according to the manufacturer's instructions. Albumin was measured in thawed plasma (dilution, 1:250,000) and lung lavage fluid (dilution, 1:1000). The assays used and their lower limit of detection (LLD) were: Mouse IL-6 DuoSet ELISA kit (LLD, 15.6 pg/mL; R and D Systems, Minneapolis, MN); Mouse Intracellular Adhesion Molecule-1 (ICAM-1) DuoSet ELISA kit (LLD 125 pg/mL; R and D Systems); Mouse Receptor for Advanced Glycation End-products (RAGE) DuoSet ELISA kit (LLD, 125 pg/mL; R and D Systems); Mouse IL-1β Platinum ELISA (LLD, 7.8 pg/mL; Thermo Fisher); Mouse TNF-α Platinum ELISA (LLD, 31.3 pg/mL; Thermo Fisher); and Mouse Albumin ELISA kit (LLD, 1.23 ng/mL; Bethyl Laboratories, Montgomery, TX).Absorbance was read on a spectrophotometer (Biotek, Winooski, VT) at a measuring wavelength of 450 nm for all ELISAs except IL-1β and TNF-α (reference wavelength 630 nm).The data were analyzed by using KC4 software (Biotek).

Statistical analysis. All analyses were performed with Prism (GraphPad Software, La Jolla, CA). The Shapiro–Wilk test was used to assess normality (α = 0.05). Data were expressed as mean ± SE or median with range, depending on the results of normality testing. Vital signs were compared by using 2-way repeated-measures ANOVA. Cytokine data acquired from more than one run of an ELISA were normalized by scaling between 0 and 1.0. Inflammation and lung injury biomarkers were compared by using either one-way ANOVA or the Kruskal–Wallis test, followed by post hoc testing with either the Tukey or Dunn multiple-comparisons test, respectively. A P value of less than 0.05 was considered significant.

Results

Evaluation of anesthesia. All mice (n = 6 per group) survived approximately 6.5 h of anesthesia, which included an initial period of instrumentation aided by mask delivery of the assigned oxygen concentration (0 h of ventilation) followed by 6 h of intubated, mechanical ventilation (Figure 1). The depth of anesthesia was titrated to achieve absence of the pedal response throughout the anesthetic period and lack of spontaneous respiration during mechanical ventilation. To achieve that depth, redosing of pentobarbital was required an average of 5 times per mouse in the 21% O_2 group and 6 times per mouse in the 100% O_2 group. The concentration of isoflurane was maintained at 2% for initial instrumentation, followed by an average of 1.35% isoflurane for the 21% O_2 group and 1.42% isoflurane for the 100% O_2 group.

In all mice, body temperature was controlled by using an autoregulating system (Figure 1A). Prior to initiation of mechanical ventilation, the mean body temperature was 37.2 ± 0.3 °C for all mice anesthetized with isoflurane and 36.6 ± 0.2°C for mice anesthetized with pentobarbital. However, the values were not significantly different among the study groups, and mean values differed by less than 1.5 °C among the groups. By the second hour of mechanical ventilation, body temperatures in all groups had converged around 37 °C and remained stable throughout the remainder of the period of anesthesia.

The mean oxygen saturation during anesthesia exceeded 96% in both of the isoflurane groups and in the group that had pentobarbital and 21% O_2 (Figure 1 B). The oxygen saturation was variable in the pentobarbital group with 100% O_2, with 2 mice having very low initial readings that were corrected during mechanical ventilation. After 2 h of mechanical ventilation, the oxygen saturation was similar among the pentobarbital–100% O_2, isoflurane–100%, and isoflurane–21% O_2 groups. At the end of mechanical ventilation, the pentobarbital–21% O_2 group had a mean oxygen saturation that was significantly (P< 0.05) lower than in the other groups, although values remained above 96%.

Overall, mean pulse rates appeared to be influenced by the type of anesthesia and the inspired oxygen concentration (Figure 1 C). Prior to mechanical ventilation, pulse rates were significantly (P < 0.05) higher in both the isoflurane–21% O_2 and isoflurane–100% O_2 groups compared with either the pentobarbital–21% O_2 or pentobarbital–100% O_2 group. During mechanical ventilation, differences were detected between the isoflurane groups based on inspired oxygen concentration, with isoflurane–100% O_2 significantly (P < 0.05) higher than

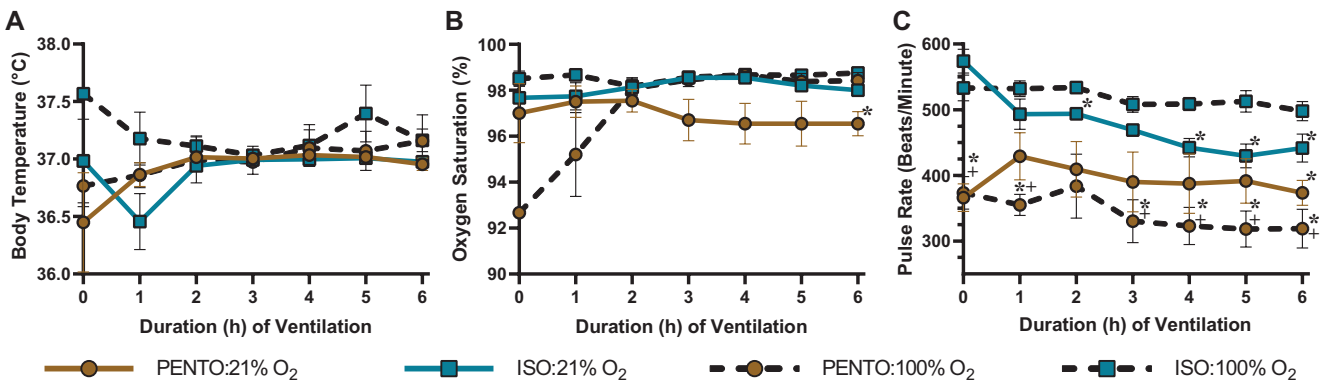

Figure 1. Vital signs during 6 h of ventilation. Mice were anesthetized with either intraperitoneal pentobarbital (PENTO) or inhaled isoflurane (ISO) followed by mechanical ventilation with either 21 or 100% O_2. (A) Body temperature, (B) oxygen saturation, and (C) pulse rate were recorded every 15 min and averaged for each hour. Data are presented as mean ± SEM (n = 6 mice per group). *Value differed (P < 0.05) from that for ISO:100% O_2 group; +value differed (P < 0.05) from that for the ISO:21% O_2 group.

isoflurane–21% O_2 at the later time points. The pentobarbital groups did not differ based on oxygen concentration. When the 2 anesthetics were compared over 6 h, isoflurane–100% O_2 produced significantly ($P < 0.05$) higher pulse rates than pentobarbital–100% O_2, but the differences between isoflurane and pentobarbital at 21% oxygen were not significant. The pentobarbital–100% O_2 group consistently yielded the lowest pulse rates.

Evaluation of inflammation. Inflammation was assessed in systemic circulation and airways by using cell counts and cytokine concentrations.

Systemic inflammation. Automated CBC counts indicated that the inspired oxygen concentration affected monocyte counts in isoflurane-anesthetized mice, with significantly higher counts in the isoflurane–100% O_2 group compared with isoflurane–21% O_2 (Figure 2A). The counts of neutrophils and lymphocytes (Figure 2B and 2C) were not significantly different among groups. Normalized plasma IL-6 concentrations (Figure 2D) were more variable in the isoflurane groups than in the pentobarbital groups and these groups were not significantly different. The values for TNFα and IL1β were below the limits of detection of the assays.

Airway inflammation. BALF counts of macrophages (Figure 3A) and neutrophils (Figure 3B) did not differ among the groups. Median BALF IL6 concentrations did not differ among groups. The values for TNFα and IL1β were below the limits of detection for the assays.

Evaluation of cell injury and barrier function. To examine the effects of anesthesia and oxygen concentration on cellular components of the lung, we evaluated BALF biomarkers that are indicative of injury on the apical aspect of epithelial cells near intracellular junctions (ICAM-1) and at the basal membrane (RAGE). The normalized BALF ICAM-1 concentrations (Figure 4A) were significantly ($P < 0.05$) higher in the pentobarbital–100% O_2 group as compared with the pentobarbital–21% O_2 and isoflurane–100% O_2 groups. RAGE concentrations were not different among groups (Figure 4 B). Albumin in BALF was measured as an indicator of endothelial integrity and vascular leakage (Figure 4 C). The median BALF concentrations of albumin were numerically highest in the pentobarbital–100% O_2 group, but differences did not reach significance ($P = 0.061$).

Histology. Light microscopic examination of lung sections revealed no evidence of lung injury or overt damage to epithelial cells in any of the groups. We found no evidence of bronchiolar infiltrates in any of the mice and saw only minimal and sporadic infiltrates within alveoli. The alveolar infiltrates were composed predominantly of a few neutrophils and histiocytes (macrophages). Neither the numbers of infiltrates nor their character substantially differed between groups. All mice examined had scattered neutrophils in pulmonary blood vessels. Lung scores did not differ significantly among groups, and none of the sections had a cumulative score above 1.0.

Discussion

The results of this study suggest that either isoflurane or pentobarbital can be used to provide effective and safe anesthesia in mice for 6 h of mechanical ventilation with either 100% or 21% oxygen. Throughout the procedure, body temperature was tightly controlled (Figure 1A) by using a far-infrared warming device and warmed inspired gases. Because the warming device autoregulates based on rectal temperature, we cannot determine whether the anesthetic or inspired oxygen concentration influenced the amount of thermal compensation needed to maintain

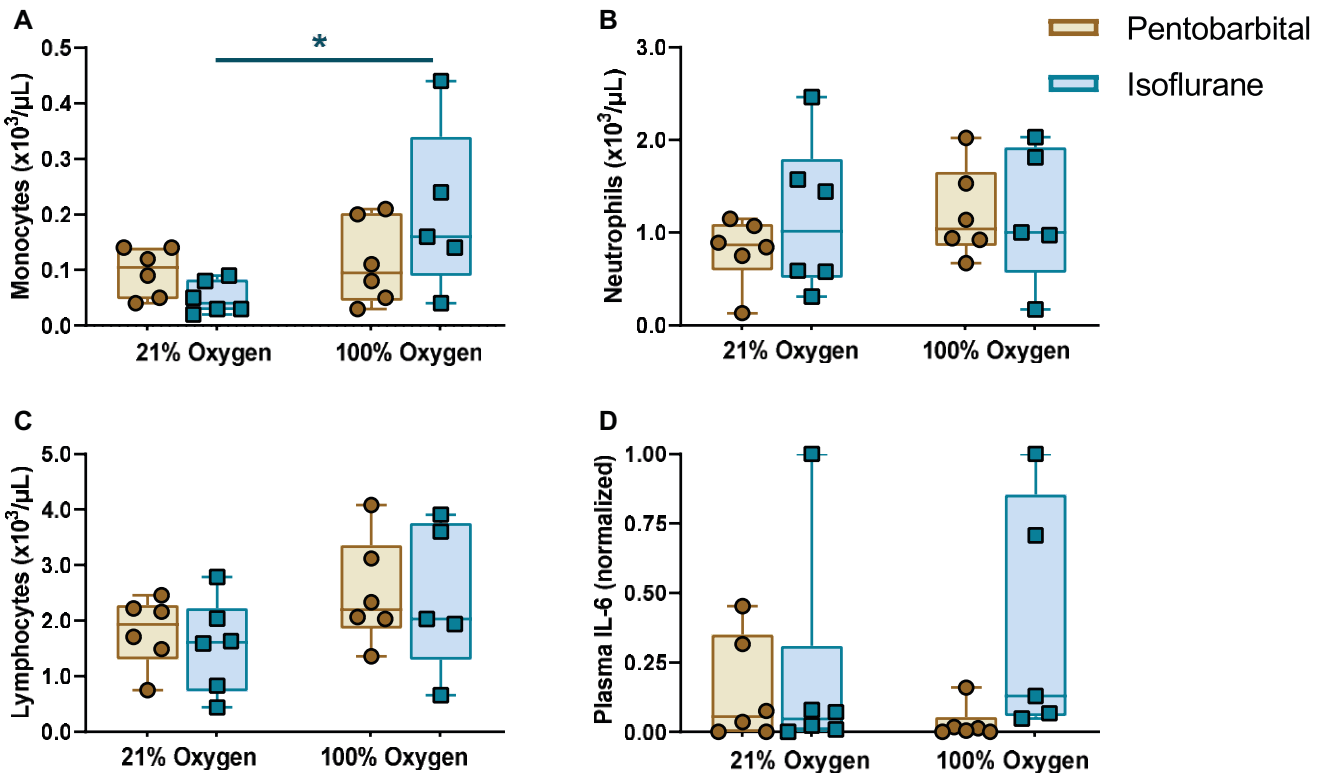

Figure 2. Systemic inflammation after 6 h of mechanical ventilation. After anesthesia with either pentobarbital or isoflurane and ventilation with either 21 or 100% oxygen, blood was collected for automated cell counts of (A) monocytes, (B) neutrophils), and (C) lymphocytes. (D) IL6 in plasma was quantified by ELISA. Data are presented as individual counts. Boxes indicate the interquartile range and median. Whiskers indicate the range of all values. *$P < 0.05$ between indicated groups ($n = 5$ or 6 mice per group).

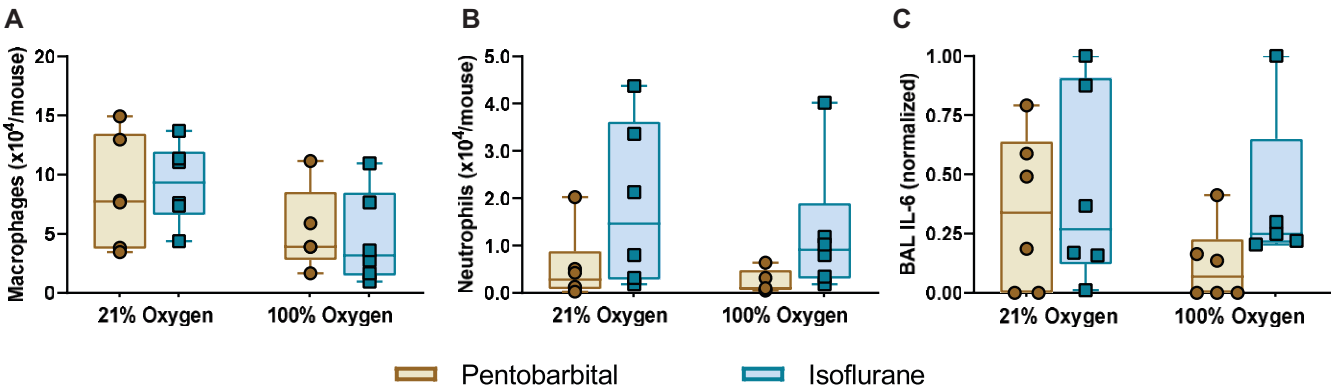

Figure 3. Airway inflammation after 6 h of mechanical ventilation. Bronchoalveolar lavage (BAL) was performed after euthanasia. The cell pellet was counted on a hemocytometer, and differential cell counts were obtained from stained slides read under light microscopy to identify (A) macrophages and (B) neutrophils. (C) The supernatant was evaluated for IL6 by ELISA. Data are presented as individual counts or values (n = 5 or 6 mice per group). Boxes indicate the interquartile range and median. Whiskers indicate the range of values.

stability. Strict control of body temperature is necessary to allow direct comparisons of inflammatory biomarkers, given that secondary hypothermia is known to have confounding effects on cell counts and cytokine levels.[27] In addition to thermal support, fluid therapy was given intraperitoneally to help maintain blood pressure, as has been recommended for mice undergoing mechanical ventilation.[47] We believe that thermoregulation, fluid support, and strict monitoring contributed to the successful outcomes of the 6-h procedures.

Hypoxia has been reported to be a complication in a majority of anesthetized mice and can occur within minutes after induction of anesthesia.[5,41] Therefore, we monitored oxygen saturation continuously via pulse oximetry, with values of less than 95% considered to indicate hypoxia.[8] The first value was recorded when mechanical ventilation was initiated, after approximately 15 min of anesthesia and mask delivery of the assigned oxygen concentration. At that time, SpO_2 exceeded 95% in all mice, except for 2 in the pentobarbital–100% O_2 group. Several factors can contribute to hypoxemia, but in these cases, respiratory depression due to the anesthetics was a likely factor. Both isoflurane and pentobarbital cause respiratory depression,[8,11,41] but hypoxia may be more prevalent with injectable anesthetics.[5] Other possible explanations for hypoxemia in these 2 mice include temporary obstruction of the airway during manipulations and interindividual variation in sensitivity to the anesthetics. We speculate that few mice in our study had low SpO_2 as compared with other studies[2,5,41] because of the anesthetics used, dosing, body temperature, or other factors. In addition, a study in mice anesthetized with high-dose injectable anesthetics showed initial hypoxia that corrected after 10 min of supplemental 100% oxygen.[5] Therefore, more mice might have been hypoxic initially but had resolved prior to the first reading. One drawback of our study was that blood gas and end-tidal CO_2 measurements were not performed due to blood volume requirements and equipment unavailability. This assessment would have allowed more definitive measurement of blood oxygenation in all mice and could have differentiated causation in hypoxic mice. At the conclusion of the study, survival was 100%, and all mice maintained SpO_2 above 95%, although it was statistically lower in the pentobarbital–21% O_2 group than the other groups. In comparison to other studies that reported hypoxia and lower survival rates, the outcome in our study was likely due to the attributes of mechanical ventilation. The fixed respiratory rate likely helped to overcome the depressant effects of the anesthetics, and adequate tidal volume helped to optimize oxygen exchange even at lower inspired oxygen concentrations. In addition, positive end-expiratory pressure would help to prevent dependent alveolar collapse during the prolonged anesthesia and reduce diffusion atelectasis caused by prolonged exposure to 100% oxygen.

The 4 combinations of anesthetic agent and oxygen concentration produced distinct differences in pulse rates. Mice anesthetized with isoflurane had higher mean pulse rates than mice anesthetized with pentobarbital. An optimal heart rate of 300 to 450 beats per minute has been suggested for inhalant anesthesia for mice used in other noninvasive procedures,[7] but

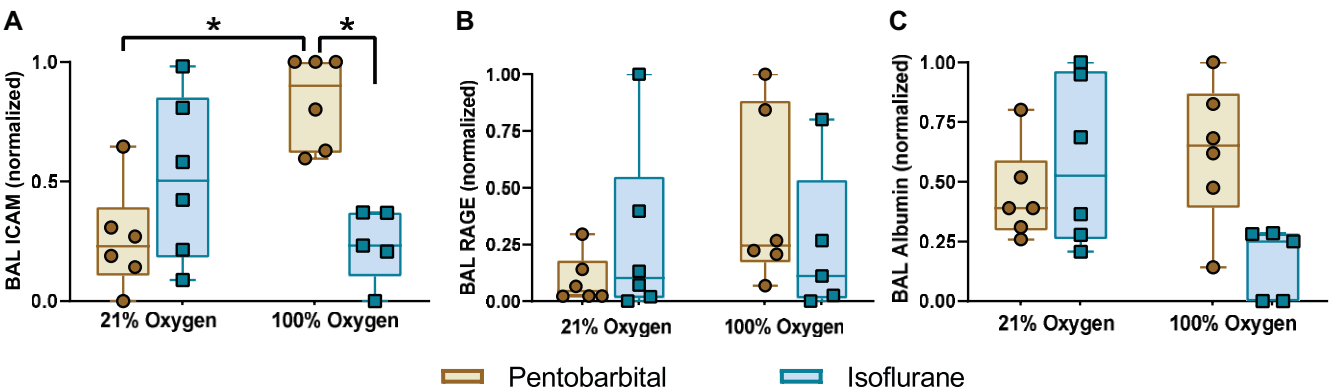

Figure 4. Airway biomarkers for lung injury after 6 h of mechanical ventilation. Lung lavage fluid was diluted and analyzed by ELISA for (A) intracellular adhesion molecule (ICAM), (B) receptor for advanced glycation end-products (RAGE), and (C) albumin. Normalized data are shown as individual counts. Boxes indicate the interquartile range and median. Whiskers indicate the range of values. *P < 0.05 between indicated groups (n = 5 or 6 mice per group).

isoflurane tended to produce higher rates in our study. This finding may indicate a lighter plane of anesthesia[7]; however, the mice did not respond to noxious stimuli or spontaneously override the respiratory rate of the ventilator. Another potential explanation is a reflex increase in heart rate due to the hypotensive effects of isoflurane.[11] The pulse rates from isoflurane-anesthetized mice remained within the wide range of basal heart rates (300 to 840 bpm) reported for unanesthetized mice,[7,45] and other studies have reported similar heart rates (exceeding 500 beats per min) with successful isoflurane anesthesia.[38,41,47] Overall, the pulse rates that we obtained during pentobarbital anesthesia were within or lower than those recommended for inhalant anesthesia in mice. The lower rates could indicate a deep plane of anesthesia[7] or dose-dependent cardiodepressant effects that become relevant when pentobarbital doses exceed 30 mg/kg.[17] Pulse rates were lowest in the pentobarbital–100% O_2 group. This effect might be due to a direct cardiac response to the higher oxygen concentration or an indirect response to the vasoconstrictive effects of 100% oxygen.[36] We did not measure blood pressure during our study, but that information would have helped us to discern the mechanisms behind the different heart rates and could be an important adjunct to monitoring long-term anesthesia.

After establishing the anesthesia protocols, we next determined their effects on biomarkers that we intended to use as outcome measures in our future studies. Overall, few intergroup differences in cell counts or cytokine levels emerged. Blood monocytes were increased in the isoflurane–100% O_2 group as compared with the isoflurane–21% O_2 group, but no differences in the other major biomarkers of inflammation or in lung histology were noted. We had anticipated more and greater differences between the 100% and 21% O_2 groups than were measured in this study. Previous studies have shown that hyperoxia induces inflammation that manifests as increased neutrophils and proinflammatory cytokines in the lung, a condition that can be aggravated further by mechanical ventilation.[14] In addition, significant damage to lung mitochondrial DNA has been seen in mice after only a 2 h exposure to 60% oxygen.[21] The lack of differences in our study may be due to the anti-inflammatory properties of the anesthetics. Blunted systemic concentrations of proinflammatory cytokines have been reported after the administration of isoflurane in mice exposed to lethal doses of endotoxin[10] and in mice with septic peritonitis.[23] Specific to airway responses, exposure to isoflurane reduced BALF neutrophils and pro-inflammatory cytokines in BALF in murine models of primary lung insults,[22,32,37] including hyperoxia,[21] and in models of secondary lung injury.[13,28] Likewise, pentobarbital has been associated with reductions in systemic and pulmonary biomarkers after inflammatory insults such as endotoxin and whole bacteria.[10,33] When directly compared, isoflurane and pentobarbital anesthesia resulted in similar levels of TNF α and neutrophil enzyme activity in lung homogenates from a mouse model of acute respiratory distress induced by zymosan-associated generalized inflammation.[28] Similarly, no differences in BALF neutrophil counts were found when either isoflurane or pentobarbital were used for anesthesia prior to euthanasia of healthy rats.[40] In that study, investigators wondered whether similar agonistic effects on γ-aminobutyric acid receptors might account for the lack of differences between the 2 anesthetics.[40] Similar anti-inflammatory effects would explain the lack of differences attributable to either the inspired oxygen concentration or the anesthetics used in our study. Consequently, we speculate that in our study, 6 h of mechanical ventilation and hyperoxia caused some background airway inflammation that was suppressed to a similar level by both anesthetics.

Inflammation and mechanical stretch can also lead to direct lung injury that is characterized by damage to epithelial cells and reduced integrity of the alveolar–capillary barrier, with subsequent vascular leakage. Therefore, we examined the effects of the 2 anesthetic regimens on 2 biomarkers for epithelial damage (ICAM-1 and RAGE) and on one for barrier function (BALF albumin). Concentrations of RAGE were similar in BALF from all groups, suggesting that effects on cellular injury at the basal membrane were similar. ICAM-1 is primarily found on the apical surface of type I alveolar cells, particularly near tight junctions, but also in low levels on capillary endothelium.[19,25] ICAM-1 can be shed by proteolytic cleavage from the cell surface into the fluid lining normal alveoli, and injury to the lung increases the levels of soluble ICAM1 in alveolar fluid.[3,26] In our study, ICAM1 levels were highest in the pentobarbital–100% O_2 group, probably due to the effects of both oxygen concentration and the anesthetic. Hyperoxia increases ICAM-1 expression in the lung,[19,44] and increased levels of BALF ICAM-1 occur during hyperoxia.[26] Therefore, the injurious effects of 100% oxygen had the potential to increase ICAM1 in 2 groups in our study. Previous reports have shown that volatile anesthetics such as sevoflurane and desflurane decrease BALF ICAM1 concentrations in lung injury models,[9,34] whereas isoflurane decreases ICAM1 expression on other cell types.[30] Therefore, the pentobarbital–100% O_2 group may have experienced lung injury due to exposure to high oxygen concentration without the cytoprotective benefit of isoflurane. In addition, exposure to isoflurane preserves tight junctions between lung epithelial cells,[6] and several lung injury studies have demonstrated reduced capillary leakage with isoflurane.[6,13,28,32] This effect may explain why BALF albumin levels did not change significantly in our study, as the pentobarbital–100% O_2 group had the highest median level. These results suggest that the type of anesthetic and oxygen concentration used during mechanical ventilation may alter specific markers of lung injury.

In addition to the factors mentioned above, the rationale for using mechanical ventilation and the goals of the study must be considered when choosing the optimal anesthesia and inspired oxygen level. For example, the cytoprotective effects of isoflurane may support the lung during high-risk, prolonged anesthesia. However, those same effects could interfere with creating the epithelial damage required to create a model of acute lung injury. Mouse studies that involve unstable physiology or preexisting disease may require choices other than those we used in the healthy mice in our study. In some cases, the use of balanced or multimodal anesthesia might have advantages over monoanesthesia, and several options are available.[15,18,47] In addition, in critical models, the oxygen concentration should be chosen carefully. Although 21% oxygen may be adequate in some situations,[46] the delivery of 100% oxygen has been advocated for use during rodent anesthesia and may offer a survival benefit when paired with some injectable anesthetics.[5] However, previous studies have shown that hyperoxia may exacerbate preexisting inflammation and lung injury.[1,39] To avoid hyperoxic injury, lower concentrations of oxygen (i.e., 50% or less) can be used for prolonged mechanical ventilation in mice.[14,38,47] Several other strategies have been suggested, including induction with 100% O_2 followed by a gradual decrease in concentration, induction with a lower concentration with a gradual increase over time as oxygen demand increases, and titrating to the lowest inspired oxygen level sufficient to maintain oxygen saturation.[7]

In conclusion, both isoflurane and pentobarbital produced adequate anesthesia and 100% survival for 6 h of mechanical ventilation of mice, regardless of oxygen concentration. Few differences were detected among the anesthetic and oxygen combinations; however, pentobarbital with 100% oxygen produced the highest levels of ICAM-1. These preliminary findings led us to use pentobarbital and 21% oxygen in subsequent studies to avoid the confounding effects of hyperoxia and isoflurane on the development of lung injury in our model. Further studies are needed to characterize acid–base balance and other physiologic parameters during prolonged anesthesia, refine protocols for survival studies, and identify sex-associated differences in lung injury. The results of this study highlight the combined influence of anesthesia, mechanical ventilation, and oxygen concentration on lung tissue and the need to establish the effects of each combination prior to use in research animals.

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