Influenza A H1N1 induces declines in alveolar gas exchange in mice consistent with rapid post-infection progression from acute lung injury to ARDS

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Background Patients with severe seasonal or pandemic influenza pneumonia frequently develop acute respiratory distress syndrome (ARDS). One clinical diagnostic criterion for ARDS is the P\textsubscript{a}O\textsubscript{2}:F\textsubscript{I}O\textsubscript{2} ratio, which is an index of alveolar gas exchange. However, effects of H1N1 influenza infection on P\textsubscript{a}O\textsubscript{2}:F\textsubscript{I}O\textsubscript{2} ratios and related pathophysiologic readouts of lung function have not been reported in mice.

Methods To develop a method for determining P\textsubscript{a}O\textsubscript{2}:F\textsubscript{I}O\textsubscript{2} ratios, uninfected mice were anesthetized with pentobarbital, diazepam/ketamine, or inhaled isoflurane. Subsequently, they were allowed to breathe spontaneously or were mechanically ventilated. After 15 minutes exposure to room air (F\textsubscript{I}O\textsubscript{2} = 0.21) or 100% O\textsubscript{2} (F\textsubscript{I}O\textsubscript{2} = 1.0), carotid P\textsubscript{a}O\textsubscript{2} was measured. To determine influenza effects on P\textsubscript{a}O\textsubscript{2}:F\textsubscript{I}O\textsubscript{2}, mice were challenged with 10 000 p.f.u./mouse influenza A/WSN/33.

Results P\textsubscript{a}O\textsubscript{2}:F\textsubscript{I}O\textsubscript{2} ratios were abnormally low (<400 mmHg) in spontaneously breathing mice. Mechanical ventilation with positive end-expiratory pressure was required to obtain P\textsubscript{a}O\textsubscript{2}:F\textsubscript{I}O\textsubscript{2} ratios in uninfected mice consistent with normal values in humans (>600 mmHg). At day 2 following infection P\textsubscript{a}O\textsubscript{2}:F\textsubscript{I}O\textsubscript{2} ratios indicated the onset of acute lung injury. By day 6, P\textsubscript{a}O\textsubscript{2}:F\textsubscript{I}O\textsubscript{2} ratios were <200 mmHg, indicating progression to ARDS. Impaired gas exchange in influenza-infected mice was accompanied by progressive hemoglobin desaturation, hypercapnia, uncompensated respiratory acidosis, hyperkalemia, and polycythemia.

Conclusions Influenza infection of mice results in impairment of alveolar gas exchange consistent with rapid development of acute lung injury and progression to ARDS. PaO2:FiO2 ratios may be of utility as clinically relevant and predictive outcome measures in influenza pathogenesis and treatment studies that use mouse models.

Keywords Hypercapnia, mechanical ventilation, PaO2:FiO2 ratio, respiratory acidosis.

Introduction Influenza A viruses cause a highly contagious acute respiratory disease in humans. Despite vaccination and use of antiviral drugs, seasonal influenza-related disease in the United States has been increasing during the last two decades, and now accounts for 200 000 hospitalizations and more than 36 000 excess deaths per year. The 2009–2010 pandemic of influenza A/H1N1/09 virus (swine flu) was estimated to have infected over 60 million people in the United States, resulting in approximately 275 000 hospitalizations and 12 500 excess deaths.

Severe primary influenza pneumonia can progress to acute lung injury and even acute respiratory distress syndrome (ARDS). A significant fraction of patients hospitalized during the ‘swine flu’ pandemic developed ARDS, and histopathology consistent with ARDS has been reported in fatal cases from the 1918 pandemic. ARDS is also reported as an outcome in interpandemic ‘seasonal’ influenza outbreaks, although it may be under-diagnosed. Moreover, development of ARDS has been associated with poor influenza prognosis.

The American/European Consensus Conference (AECC) and subsequent groups defined ARDS as a clinical syndrome characterized by acute onset of severely impaired alveolar gas exchange. Clinically, the capacity for gas exchange is defined by the ratio between arterial O\textsubscript{2} tension (P\textsubscript{a}O\textsubscript{2}) and the fraction of inspired O\textsubscript{2} (F\textsubscript{I}O\textsubscript{2}). P\textsubscript{a}O\textsubscript{2}:F\textsubscript{I}O\textsubscript{2} (P:F) ratios ≤200 mmHg in the presence of positive end-expiratory pressure (PEEP) are considered diagnostic of ARDS. Less severe hypoxemia (P:F ≤ 300 mmHg) is considered evidence of acute lung injury.
Recently, limited evidence of ARDS has been reported in mice infected with H5N1 and H9N2 influenza A strains. However, this diagnosis was primarily based upon mortality rates and postmortem analyses, rather than such AECC criteria as P:F ratios. We have shown that mice infected with a lethal dose of a mouse-adapted influenza A H1N1 viral strain develop decreased peripheral oxygen saturation, increased lung water content, and reduced alveolar fluid clearance rate, all of which are indicative, but not diagnostic of, ARDS. We have also found that influenza infection results in a progressive increase in pulmonary edema (as determined by magnetic resonance imaging) and reduced pulmonary compliance, both of which are AECC diagnostic criteria for ARDS. However, effects of influenza on P:F ratios, which are essential to a definitive clinical diagnosis of ARDS, have not been accurately determined in the mouse model. We postulated that we could develop a novel method, analogous to that used in human ICUs, that would allow us to accurately determine P:F ratios in mice. Furthermore, we hypothesized that this method would allow us to demonstrate that influenza A H1N1-infected mice develop pathophysiologic alterations in alveolar gas exchange consistent with progression from acute lung injury to ARDS.

Methods

Animals
All studies used pathogen-free, 8- to 12-week-old BALB/cAnNCr mice of either sex. Mice were maintained in sterile caging on standard ventilated racks and provided with ad libitum sterile food and water, as well as appropriate environmental enrichment with Nestlets™ (Ancare, Bellmore, NY, USA). For all studies, data for each group were derived from a minimum of two independent experiments. All mouse procedures were approved by the Institutional Animal Care and Use Committee at The Ohio State University.

Study design for development of P:F ratio measurement method
Uninfected mice were anesthetized using either injectable or inhaled agents. Groups of mice were then allowed to breathe spontaneously or tracheotomized and subjected to mechanical ventilation for 15 minutes. During this period, mice inhaled either room air or 100% O₂. After 15 minutes, carotid arterial blood samples were collected, PₐO₂ values measured, and P:F ratios calculated. See Figure 1 for a summary of experimental groups.

Anesthesia protocols
Mice were anesthetized using one of the following methods:
1. Pentobarbital sodium (50 mg/kg, i.p.);
2. Diazepam (5 mg/kg, i.p.) followed by ketamine (200 mg/kg, i.p.) 6 minutes later;
3. 4% isoflurane delivered by nose cone (reduced to a maintenance level of 1-5% following induction).

Mechanically ventilated mice also received post-induction pancuronium bromide (0.4 mg/mouse; Gensia Pharmaceuticals, Irvine, CA, USA) to limit spontaneous respiratory movements. Each anesthetized mouse was placed on a Deltaphase® isothermal heating pad (Braintree Scientific, Braintree, MA, USA) to maintain body temperature during the P:F measurement procedure.

Ventilation regimen
Unventilated mice were allowed to breathe spontaneously through a nose cone. For mechanical ventilation studies, mice were tracheotomized and the trachea cannulated with a cut-down 18-gauge I.V. catheter. Using a Model 687 volume-controlled mouse ventilator (Harvard Apparatus, Holliston, MA, USA), mice were ventilated at 160 breaths/minute and 4 cm H₂O ventilator pressure, with a tidal volume of 200 µl (~8 ml/kg body weight) in the presence of 0 or 8 cm H₂O PEEP.

FₐO₂
Animals were exposed to either room air (FₐO₂ = 0.21) or 100% O₂ (FₐO₂ = 1.0), administered via the nose cone (spontaneously breathing mice) or the tracheal cannula (mechanically ventilated animals). Both gases were delivered from compressed gas cylinders via a step-down regulator with a final flow rate of 2 L/min.

Measurement of arterial PO₂
After 15 minutes anesthesia, the left carotid artery was ligated cranially and a 200 µl blood sample collected using
a 0.5 ml Monovette® heparinized blood gas syringe (Sarstedt Inc., Newton, NC, USA). PaO2 and PaCO2 were measured using an EG6+ cartridge in an iSTAT® blood gas analyzer (both Abbott Laboratories, Abbott Park, IL, USA). This cartridge also provided measurements of arterial O2 saturation (SaO2), pH, plasma [Na+], plasma [K+], plasma [HCO3-], and hematocrit. Samples with SaO2 < 85% on 100% O2 were rejected as being improperly cannulated and/or ineffectively ventilated.

Preparation of influenza A/WSN/33 (H1N1) viral inocula
All infection studies used H1N1 influenza A/WSN/33, grown in embryonated chicken eggs. Infectivity was determined by plaque-forming assay 48 hours after inoculation of the NY3 fibroblast cell line.15 Absence of mycoplasmal and endotoxin contamination of virus preparations were confirmed using the Mycoplasma PlusSM PCR kit (Stratagene, La Jolla, CA, USA) and a Limulus amebocyte lysate gel clot assay (Lonza, Walkersville, MD, USA), respectively.

Infection of mice
BALB/c mice were infected intranasally with 10 000 plaque-forming units of influenza A/WSN/33 in 50 μl PBS with 0.1% BSA under light isoflurane anesthesia.

Measurement of viral titers
Mice were euthanized by i.p. injection of ketamine (8.7 mg/100 g BWT) and xylazine (1.3 mg/100 g body weight) and exsanguinated. Viral titers were determined from serial dilutions of lung homogenates by plaque-forming assay in NY3 cells.15

Preparation of tissues for histopathologic evaluation
Following euthanasia, lungs were inflated with 10% formalin to a standard fixation pressure (25 cm H2O) and fixed. Hematoxylin- and eosin-stained 3-μm sections prepared from paraffin-embedded lung tissues were reviewed for pathologic changes by a veterinary pathologist.

Immunohistochemistry
Influenza viral antigens in deparaffinized lung tissue sections were detected using goat anti-influenza A strain USSR (H1N1) antiseraum (1301, 1:500; Virostat, Portland, ME, USA). Bound antibody was detected as previously described.15

Preparation of histopathologic images
Representative lung tissue sections were scanned with a Scanscope® CS slide scanner (Aperio Technologies, Vista, CA, USA), visualized with ImageScope software (Aperio Technologies), and composed in Adobe Photoshop (San Jose, CA, USA). Images were adjusted in brightness for a more uniform appearance. These adjustments do not obscure, eliminate, or misrepresent any information presented in the original slides.

Statistical analyses
Descriptive statistics were calculated using Instat 3.05 (GraphPad Software, San Diego, CA, USA).13 Gaussian data distribution was verified by the method of Kolmogorov and Smirnov. Differences between group means were analyzed by anova, with a post hoc Tukey–Kramer multiple comparison post-test. Correlations were calculated by Pearson’s linear correlation analysis. P < 0.05 was considered statistically significant. All data are presented as mean ± SEM.

Results
P:F ratios are abnormally low in anesthetized, spontaneously breathing healthy mice, but are normal in mechanically ventilated animals
To replicate the conditions used in previous studies,10,11 we anesthetized healthy, normal mice with pentobarbital, diazepam/ketamine, or isoflurane, allowed them to breathe spontaneously, and exposed them to room air (FiO2 = 0.21). As expected, we found all three anesthetic regimens resulted in abnormally low P:F ratios, although isoflurane had the least impact on this parameter (Figure 2A). All animals were also moderately hypercapnic (mean, PaCO2 30–50 mmHg) and mildly desaturated, but were not acidic (data not shown). In contrast, when anesthetized mice were tracheotomized and subjected to a standardized regimen of mechanical ventilation (8 ml/kg tidal volume, 160 breaths/minute, with 8 cm H2O PEEP) on room air, calculated P:F ratios were consistent with normal values in humans (≥600 mmHg) irrespective of method of anesthesia (Figure 2B). Moreover, hypercapnia was attenuated (mean PaCO2, 20–30 mmHg; data not shown). SaO2 values of 100% and normal plasma pH values (~7.4) were also found in mechanically ventilated mice exposed to room air (data not shown). However, pentobarbital did not consistently induce surgical anesthetic depth, and some mice remained responsive to toe-pinch throughout the study period. This rendered carotid arterial blood sampling very challenging, particularly in mechanically ventilated animals. Moreover, it was incompatible with current recommendations for surgical anesthesia in rodents. We therefore elected not to use this particular anesthetic regimen in subsequent studies.

To determine whether increased FiO2 had any impact on measured arterial PaO2 values, we exposed both spontaneously breathing and mechanically ventilated valium/ketamine- or isoflurane-anesthetized mice to 100% O2 (FiO2 = 1.0). Relative to room air values, P:F ratios in
spontaneously breathing mice did not improve significantly in either group following exposure to 100% O\textsubscript{2} (Figure 2C). These animals therefore remained hypoxemic. In contrast, mechanical ventilation on 100% O\textsubscript{2} resulted in normalization of P:F ratios, irrespective of gender (Figure 2D). All mice in this group were normocapnic (mean PaCO\textsubscript{2} < 30 mmHg), with SaO\textsubscript{2} values of 100%, and normal plasma pH values (data not shown). Adequate PEEP is necessary to normalize P:F ratios in mechanically ventilated mice

The above studies showed that P:F ratios are effectively normal when healthy mice are subjected to a standardized regimen of mechanical ventilation, independent of mouse strain (not shown), anesthesia regimen, and FiO\textsubscript{2}. However, P:F ratios in mechanically ventilated mice on 100% O\textsubscript{2} declined significantly in the absence of PEEP (Figure 3A). In these animals, the only ventilation pressure provided was that of the ventilator itself (4 cm H\textsubscript{2}O). Most mice ventilated without PEEP were also severely hypercapnic (Figure 3B). This shows that, as in humans, adequate PEEP is essential to achieving physiologically meaningful P:F ratio measurements.

Influenza A virus infection results in severe hypoxemia, hypercapnia, and acute respiratory acidosis

We hypothesized that severe primary influenza infection might serve as a form of direct lung injury which predisposes...
to development of ARDS. Mice were therefore infected intranasally with 10,000 plaque-forming units of influenza A/WSN/33, which results in severe disease by 6 days post-infection (d.p.i.), with 100% mortality by 8 d.p.i. (a median of 7 days to death). Infection status was confirmed by daily measurement of body weights (not shown) and lung homogenate viral titers. Mean virus titers were $6.2 \pm 0.1$ log plaque-forming units/g at 2 d.p.i. and $5.9 \pm 0.2$ log plaque-forming units/g at 6 d.p.i. Finally, while mock infection for 6 days did not induce histopathology (Figure 4A), influenza infection for 2–6 days resulted in increasingly severe lesions. At 2 d.p.i., mild peribronchial and interstitial neutrophil and mononuclear cell infiltrates were present (Figure 4B), and many bronchial epithelial cells were influenza antigen-positive, but still intact (Figure 4C). By 6 d.p.i., pathology was more severe, with marked areas of bronchial epithelial necrosis and denudation of basement membranes, together with prominent neutrophil infiltration (Figure 4D, E). Bronchial and alveolar Type II epithelial cell immunoreactivity for influenza antigens was stronger than at 2 d.p.i. (Figure 4E, F, respectively). Influenza antigens were also present in alveolar macrophages (Figure 4F).

Based on both our findings in normal animals and our prior experience with this anesthetic regimen, P:F ratios were measured in diazepam/ketamine-anesthetized, mechanically ventilated mice on 100% O2 with 8 cm H2O PEEP. Influenza-infected animals of either gender rapidly developed significant impairment of pulmonary gas exchange (Figure 5A). At 2 d.p.i., hypoxemia was of a severity consistent with diagnosis of acute lung injury in humans (P:F < 300 mmHg). P:F ratios declined slightly further at 4 d.p.i., and by 6 d.p.i. were significantly decreased relative to 2 d.p.i. Indeed, at 6 d.p.i., P:F ratios in most animals were consistent with development of frank ARDS (P:F < 200 mmHg). As a result of these changes in P2O2, the mean alveolar to arterial (A-a) gradient increased from 64 mmHg at day 0 to 348 mmHg at 2 d.p.i. and 470 mmHg at 6 d.p.i. P:F ratios in mice that had been mock-infected with 50 μl virus diluent (PBS with 0.1% BSA) were normal at day 6 (mean, 646 ± 46 mmHg; n = 5).

By 6 d.p.i., mice were desaturated (mean carotid SaO2 97.2, ±0.9) despite ventilation on 100% O2 (Figure 5B). Influenza-infected mice were also hypercapnic from 2 to 6 d.p.i. (mean P2CO2, >50 mmHg; Figure 5C) and developed acute, uncompensated respiratory acidosis (reduced plasma pH with no accompanying elevation of plasma HCO3 levels) as early as 2 d.p.i. (Figure 5D). Hypercapnia and acidosis were less severe at 6 d.p.i., which suggests that metabolic compensation may have begun by this timepoint. As a

Figure 4. Progression of pulmonary histopathology in influenza A virus-infected mice. (A) Absence of pulmonary histopathology at day 6 after mock infection; (B) histopathology at 2 d.p.i. (20x); (C) bronchial epithelial cell immunoreactivity for influenza antigens at 2 d.p.i.; (D) histopathology at 6 d.p.i. (E) bronchial epithelial necrosis and immunoreactivity for influenza antigens at 6 d.p.i.; and (F) bronchoalveolar epithelial and alveolar macrophage immunoreactivity for influenza antigens. All images are shown at 20x magnification, except panel F (40x). Scale bar = 100 μm.
consequence, there was no significant correlation between PaO2 and PaCO2 over the course of infection. The progressive reductions in P:F ratios and SaO2 and increasing hypercapnia in influenza-infected mice are consistent with development of an increasingly larger V-Q mismatch and shunt fraction over the course of infection (approximately 25% by 6 d.p.i., based on standard iso-shunt diagrams).

Figure 5. Influenza A virus infection results in severe hypoxemia, hypercapnia, and acute respiratory acidosis. Effect of influenza A virus infection for 2–6 days on: (A) Calculated P:F ratios (mmHg); (B) Arterial O2 saturation (% SaO2); (C) PaCO2 (mmHg), and (D) Plasma pH. All mice were anesthetized with diazepam/ketamine and mechanically ventilated on 100% O2 with 8 cm H2O PEEP. n = 6, 11, 9, and 18 at 0, 2, 4, and 6 d.p.i., respectively. *P < 0.05, ***P < 0.0005, compared with uninfected mice (day 0). †P < 0.05, compared with mice at 2 d.p.i. PEEP, positive end-expiratory pressure.

Discussion
Alterations in arterial blood gases and, in particular, alterations in gas exchange in ventilated patients receiving 100% O2 are important diagnostic criteria for ARDS.8,9 As PaO2 is merely a function of O2 concentration and solubility (Henry’s Law), there is no physical reason why it should vary by species. However, PaO2 values derived by existing methods in mice have generally been underestimates, which has resulted in overestimation of the degree of alveolar gas exchange impairment following lung insults such as influenza infection. We hypothesized that underestimation of normal mouse PaO2 values in previous studies may have resulted from both the respiratory depressant effect of anesthesia and the tendency of mouse airways to collapse in the absence of PEEP.16 P:F ratios consistent with those in normal humans (P:F ≥600 mmHg) could be measured in normal mice, but only when they were mechanically ventilated at a rate comparable to that of conscious mice and in the presence of PEEP. Under these conditions, P:F ratios were not impacted by either anesthetic choice or FiO2 and mice were normocapnic. Finally, we found that, based on P:F ratios, influenza-infected mice rapidly developed acute lung injury (within 48 hours) and progressed to ARDS within 6 days.
A significant fraction of patients hospitalized during the 2009–2010 H1N1 ‘swine flu’ pandemic developed ARDS, and this syndrome is a common sequel of human infections with influenza A H5N1 (bird flu) strains. Likewise, histopathology consistent with ARDS has been reported in a retrospective study of lung tissues from fatal primary influenza cases from the 1918 pandemic. Although ARDS is less commonly reported as an outcome in interpandemic ‘seasonal’ influenza outbreaks, it has been associated with poor prognosis. Moreover, seasonal influenza may be under-diagnosed as a proximate cause of ARDS. To date, however, development of ARDS (as defined by AECC criteria) has not been demonstrated in influenza-infected mice. This is of particular importance because ‘traditional’ readouts in mouse influenza studies (such as mortality rate and histopathology) have little predictive value clinically.

Significant reductions in P_{a}O_{2} have previously been reported in influenza A H5N1- and H9N2-infected mice. However, these measurements were made in spontaneously breathing animals under ‘moderate’ pentobarbital anesthesia, and in the absence of PEEP. These conditions are clearly neither analogous to those used in a human clinical setting nor consistent with AECC criteria. We found that this regimen resulted in significant hypoxemia, hypercapnia, and desaturation in normal mice. Indeed, normal controls in both reports were relatively hypoxic (mean P:F ratio, 440–450 mmHg). Moreover, as P:F ratios in infected mice were measured under the same conditions as controls, they may have been artificially depressed, resulting in overestimation of lung injury severity. Thus, our studies demonstrate for the first time that mice infected with influenza A H1N1 develop progressive reductions in P:F ratios consistent with current AECC criteria for diagnosis of acute lung injury and ARDS. Importantly, clinically significant alterations in alveolar gas exchange occurred as early as 2 d.p.i. In previous studies, we found only moderate lung damage at this timepoint, as assessed by histopathology, lung water content, and bronchoalveolar lavage fluid protein and LDH levels. This suggests that P:F ratios may be a more sensitive index of lung injury severity than these readouts. Given the lack of lung damage at 2 d.p.i., we hypothesize that this early decline in P:F ratios may result from increased alveolar lining fluid depth (secondary to impaired alveolar fluid clearance, which is present at 2 d.p.i.), rather than the excessive inflammation which has been proposed to play a significant role in severe disease induced by pandemic influenza strains.

In addition to declining P:F ratios, influenza-infected mice rapidly developed severe hypoxemia and hypercapnia, most likely as a result of impaired gas exchange secondary to alveolar edema. P_{a}CO_{2} is largely determined by alveolar ventilation rate, and excess CO_{2} is normally cleared from the lungs by compensatory hyperventilation. However, we did not increase the mechanical ventilation rate following infection and thus prevented this from occurring. Infected animals also exhibited acidosis and hyperkalemia, although increased plasma [HCO_{3}⁻] at 6 d.p.i. resulted in partial compensation of acidosis. Similar alterations in P_{a}CO_{2}, plasma pH, and plasma [HCO_{3}⁻] were reported in a cohort of non-survivors during the ‘swine flu’ pandemic. Hyperkalemia was not reported in that study, but its absence...
may have been a result of aggressive fluid management. These pathophysiologic derangements were also temporally correlated with progressive increases in alveolar fluid clearance impairment, alveolar permeability, and lung water content, as well as decreased lung compliance.\(^\text{13,14}\) Finally, influenza infection also resulted in lung histopathology consistent with postmortem findings in humans with influenza and/or ARDS. These included interstitial edema, neutrophilic inflammatory infiltrates, and diffuse alveolar damage.\(^\text{6,24}\) Although we did not observe hyaline membrane formation, this lesion is generally believed to be the end-result of prolonged mechanical ventilation at high tidal volume.

In conclusion, we have demonstrated that, based on AECC P:F ratio criteria, infection of BALB/c mice with a lethal dose of mouse-adapted 'low-path' influenza A H1N1 virus results in rapid development of acute lung injury (within 48 hours) and progression to ARDS within 6 days. Furthermore, impairment of alveolar gas exchange in infected mice is associated with rapid onset of hypercapnia, acidosis, and secondary polycythemia. We propose that as highly sensitive, clinically relevant indices of influenza-induced lung injury, P:F ratios will be of greater value than histopathology. Finally, our data suggest that agents developed for prevention or treatment for ARDS may also prove to be effective in severe influenza, and vice versa.

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Authors’ contribution
Data collection: Z.P.T. and F.A.; Data analysis and interpretation: I.C.D.; Drafting the manuscript: I.C.D.

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