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Original Article

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_aO_2:F_iO_2 ratio, which is an index of alveolar gas exchange. However, effects of H1N1 influenza infection on P_aO_2:F_iO_2 ratios and related pathophysiologic readouts of lung function have not been reported in mice.

Methods To develop a method for determining P_aO_2:F_iO_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_iO_2 = 0·21) or 100% O_2 (F_iO_2 = 1·0), carotid P_aO_2 was measured. To determine influenza effects on P_aO_2:F_iO_2, mice were challenged with 10 000 p.f..u./mouse influenza A/WSN/33.

Results P_aO_2:F_iO_2 ratios were abnormally low (≤400 mmHg) in spontaneously breathing mice. Mechanical ventilation with positive end-expiratory pressure was required to obtain P_aO_2:F_iO_2 ratios in uninfected mice consistent with normal values in humans (≥600 mmHg). At day 2 following infection P_aO_2:F_iO_2 ratios indicated the onset of acute lung injury. By day 6, P_aO_2:F_iO_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. P_aO_2:F_iO_2 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, P_aO_2:F_iO_2 ratio, respiratory acidosis.

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Introduction

Influenza A viruses cause a highly contagious acute respiratory disease in humans.[1] 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.[2] 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.[3]

Severe primary influenza pneumonia can progress to acute lung injury and even acute respiratory distress syndrome (ARDS).[4] A significant fraction of patients hospitalized during the 'swine flu' pandemic developed ARDS,[5] and histopathology consistent with ARDS has been reported in fatal cases from the 1918 pandemic.[6] 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.[2,7]

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.[8,9] Clinically, the capacity for gas exchange is defined by the ratio between arterial O_2 tension (P_aO_2) and the fraction of inspired O_2 (F_iO_2). P_aO_2:F_iO_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.[8]

Recently, limited evidence of ARDS has been reported in mice infected with H5N1 and H9N2 influenza A strains.[10,11] 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.[12,13] 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.[14] 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% O_2. After 15 minutes, carotid arterial blood samples were collected, P_aO_2 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.

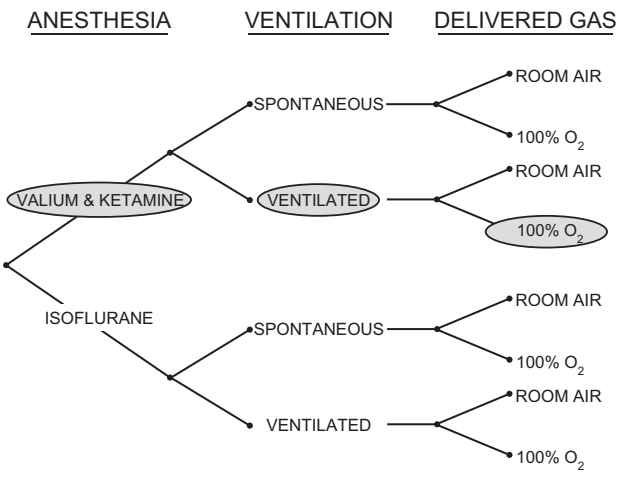

Figure 1. Anesthesia and ventilation strategies used to identify optimal conditions for P:F ratio measurement in mice. Optimal measurement conditions identified are denoted by ovals.

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.[13] Using a Model 687 volume-controlled mouse ventilator (Harvard Apparatus, Holliston, MA, USA), mice were ventilated at 160 breaths/minute and 4 cm H_2O ventilator pressure, with a tidal volume of 200 μl (~8 ml/kg body weight) in the presence of 0 or 8 cm H_2O PEEP.

F_iO_2

Animals were exposed to either room air (F_iO_2 = 0·21) or 100% O_2 (F_iO_2 = 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 PO_2

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). P_aO_2 and P_aCO_2 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 O_2 saturation (S_aO_2), pH, plasma [Na^+], plasma [K^+], plasma [HCO_3^-], and hematocrit. Samples with $S_aO_2 < 85\%$ on 100% O_2 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 *Plus*™ 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 H_2O) 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) antiserum (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. $P < 0{\cdot}05$ 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 ($F_iO_2 = 0{\cdot}21$). 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, P_aCO_2 30–50 mmHg) and mildly desaturated, but were not acidotic (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 H_2O 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 P_aCO_2, 20–30 mmHg; data not shown). S_aO_2 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 F_iO_2 had any impact on measured arterial P_aO_2 values, we exposed both spontaneously breathing and mechanically ventilated valium/ketamine- or isoflurane-anesthetized mice to 100% O_2 ($F_iO_2 = 1{\cdot}0$). Relative to room air values, P:F ratios in

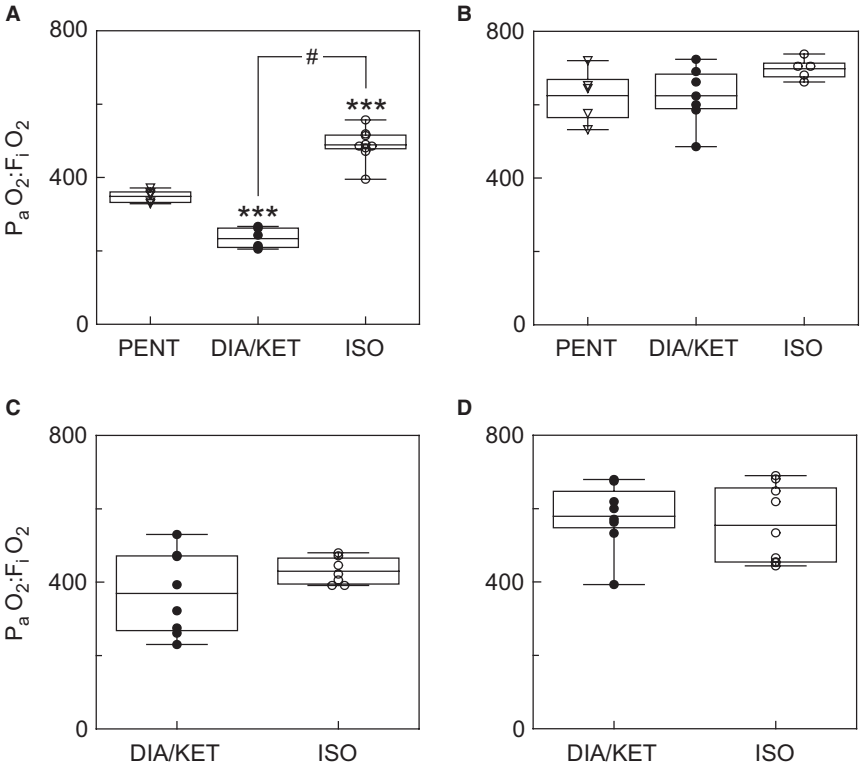

Figure 2. P:F ratios are abnormally low in anesthetized, spontaneously breathing healthy mice, but are normal in mechanically ventilated animals. Calculated P:F ratios in (A) spontaneously breathing mice anesthetized with pentobarbital sodium (PENT; 50 mg/kg, i.p.), diazepam/ketamine (DIA/KET; 5 mg/kg and 200 mg/kg, respectively, both i.p.), or isoflurane (ISO; 1·5%) and exposed to room air (F_iO_2 = 0·21); (B) mechanically ventilated mice anesthetized with PENT, DIA/KET, or ISO and exposed to room air; (C) spontaneously breathing mice anesthetized with DIA/KET or ISO and exposed to 100% O_2 (F_iO_2 = 1·0); and (D) mechanically ventilated mice anesthetized with DIA/KET or ISO and exposed to 100% O_2. n = 5–8 per group. PEEP = 8 cm H_2O for mechanical ventilation studies. ***P < 0·0005, compared with PENT-anesthetized mice. #P < 0·0005, compared with DIA/KET-anesthetized mice. PEEP, positive end-expiratory pressure.

spontaneously breathing mice did not improve significantly in either group following exposure to 100% O_2 (Figure 2C). These animals therefore remained hypoxemic. In contrast, mechanical ventilation on 100% O_2 resulted in normalization of P:F ratios, irrespective of gender (Figure 2D). All mice in this group were normocapnic (mean P_aCO_2 < 30 mmHg), with S_aO_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 F_iO_2. However, P:F ratios in mechanically ventilated mice on 100% O_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_2O). 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

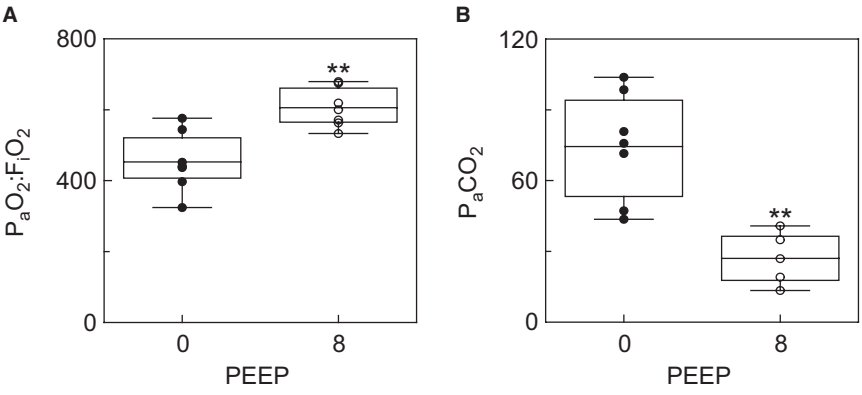

Figure 3. Adequate PEEP is necessary to normalize P:F ratios in mechanically ventilated mice. (A) Calculated P:F ratios and (B) P_aCO_2 values in diazepam/ketamine-anesthetized mice following mechanical ventilation in the presence of 0 or 8 cm H_2O PEEP. n = 7 per group. All studies were performed under 100% O_2 (F_iO_2 = 1·0). *P < 0·05, ***P < 0·0005, compared with 0 cm H_2O PEEP. PEEP, positive end-expiratory pressure.

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).[13] Infection status was confirmed by daily measurement of body weights (not shown) and lung homogenate viral titers. Mean virus titers were 6·2 ± 0·1 log plaque-forming units/g at 2 d.p.i. and 5·9 ± 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% O_2 with 8 cm H_2O 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).[8] 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).[8] As a result of these changes in P_aO_2, 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 S_aO_2 97·2, ±0·9) despite ventilation on 100% O_2 (Figure 5B). Influenza-infected mice were also hypercapnic from 2 to 6 d.p.i. (mean P_aCO_2, >50 mmHg; Figure 5C) and developed acute, uncompensated respiratory acidosis (reduced plasma pH with no accompanying elevation of plasma HCO_3^- 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

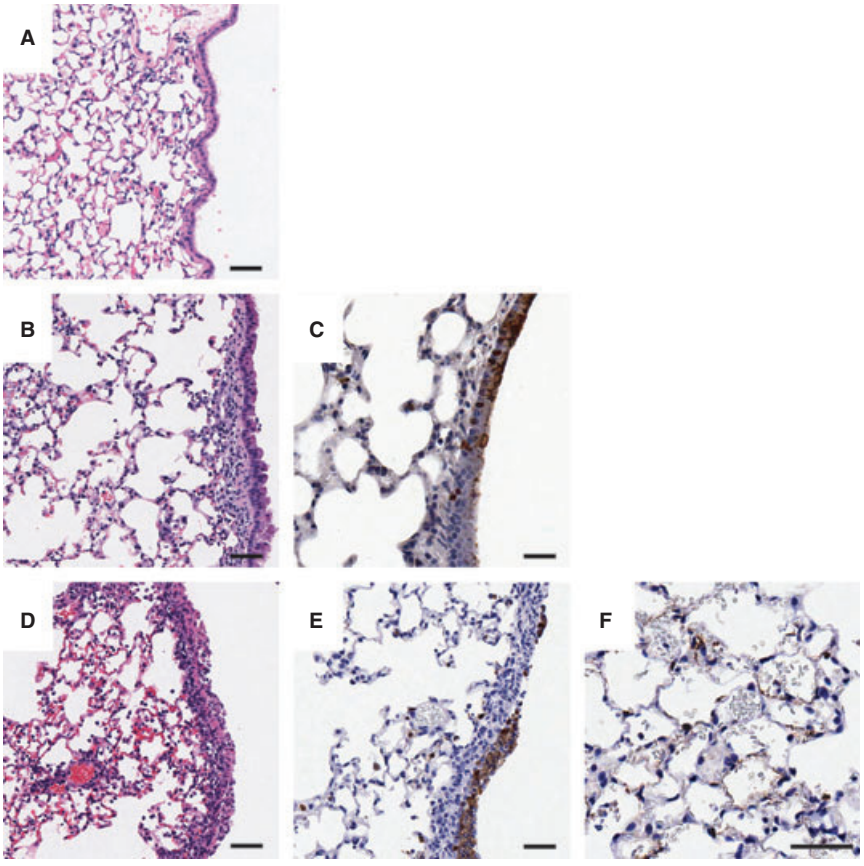

Figure 4. Progression of pulmonary histopathology in influenza A virus-infected mice. (A) Absence of pulmonary histopathology at day 6 after mock infection; (B) histopathology at 2 d.p.i. (20×); (C) bronchial epithelial cell immunoreactivity for influenza antigens at 2 d.p.i.; (D) histopathology at 6 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 20× magnification, except panel F (40×). Scale bar = 100 μm.

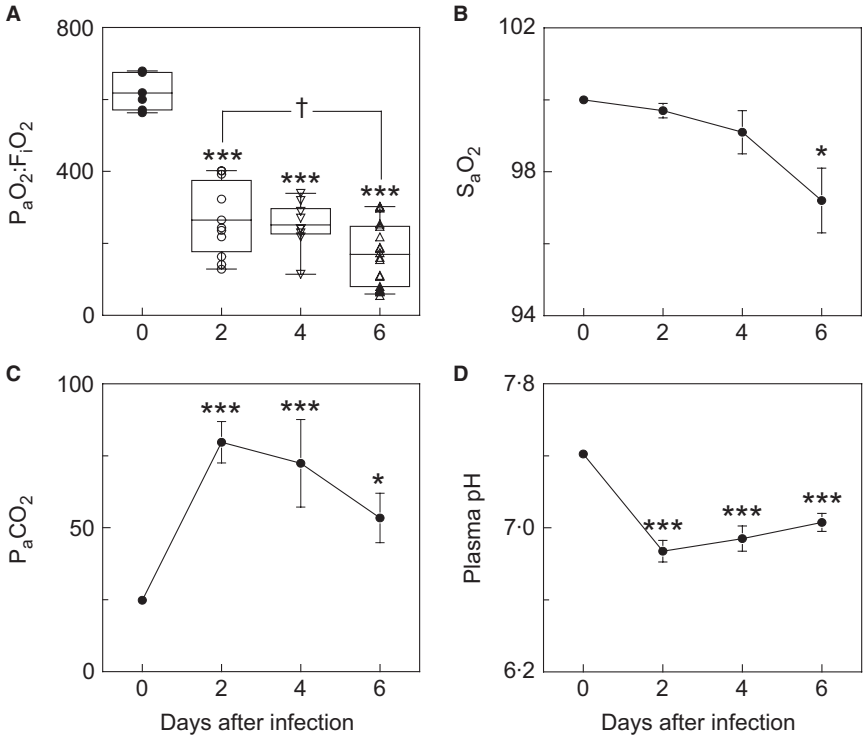

Figure 5. Influenza A virus infection results in severe hypoxemia, hypercapnia, and acute respiratory acidosis. Effect of influenza A virus infection for 2–6 days on: (A) Calculated P:F ratios (mmHg); (B) Arterial O_2 saturation (% S_aO_2); (C) P_aCO_2 (mmHg); and (D) Plasma pH. All mice were anesthetized with diazepam/ketamine and mechanically ventilated on 100% O_2 with 8 cm H_2O 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.

consequence, there was no significant correlation between P_aO_2 and P_aCO_2 over the course of infection. The progressive reductions in P:F ratios and S_aO_2 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).

Influenza A virus infection results in hyperkalemia, metabolic acidosis, and secondary polycythemia

Influenza infection had no effect on plasma [Na^+] (Figure 6A). However, plasma [K^+] increased significantly at 6 d.p.i. (Figure 6B). [HCO_3^-] decreased at 2–4 d.p.i., when acidosis was also most severe (Figure 6D). At 6 d.p.i., when plasma pH had recovered somewhat, some recovery in plasma [HCO_3^-] was detected, suggesting the onset of metabolic compensation of respiratory acidosis. Although infection had no effect on plasma osmolarity (Figure 6C), the hematocrit also progressively increased (Figure 6D). Likewise, erythrocyte hemoglobin content increased from 15·1 ± 0·2 g/dl in uninfected mice to 17·6 ± 0·6 g/dl at 6 d.p.i. (P < 0·0005). The lack of an increase in plasma osmolarity suggests that an increase in hematocrit may reflect increased erythropoiesis in the face of prolonged hypoxemia (secondary polycythemia), rather than hemoconcentration secondary to reduced water intake. Finally, mock infection for 6 days had no effect on plasma [Na^+] (mean, 155 ± 0·4 mm; n = 5), [K^+] (mean, 3·2 ± 0·3 mm), [HCO_3^-] (mean, 18·0 ± 1·9 mm), or hematocrit (mean, 43·8 ± 3·7%).

Discussion

Alterations in arterial blood gases and, in particular, alterations in gas exchange in ventilated patients receiving 100% O_2 are important diagnostic criteria for ARDS.[8,9] As P_aO_2 is merely a function of O_2 concentration and solubility (Henry's Law), there is no physical reason why it should vary by species. However, P_aO_2 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 P_aO_2 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 F_iO_2 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.

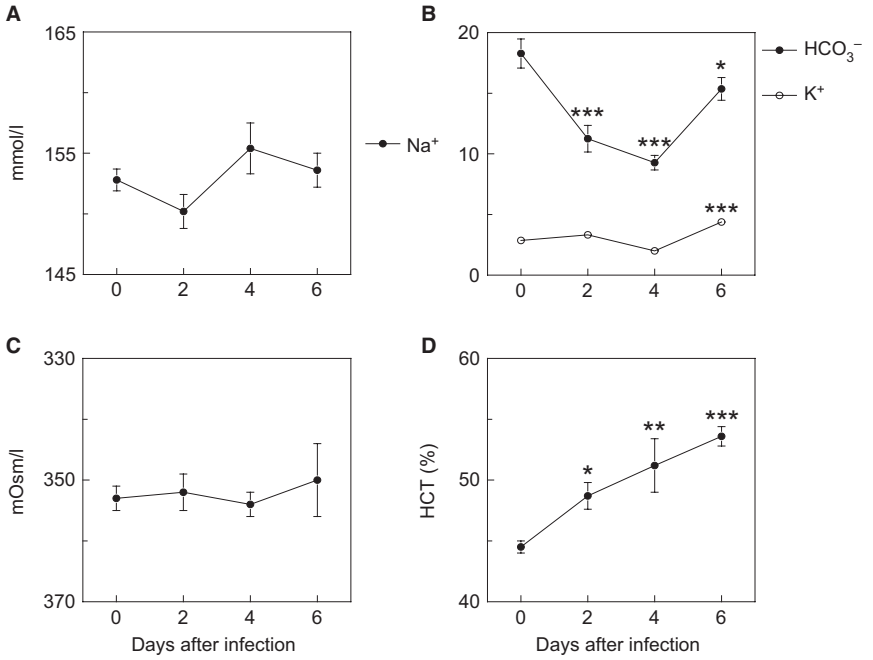

Figure 6. Influenza A virus infection results in hyperkalemia, metabolic acidosis, and secondary polycythemia. Effect of influenza A virus infection for 2–6 days on: (A) Plasma Na^+ concentration (mm); (B) Plasma HCO_3^- and K^+ concentrations (mm); (C) Plasma osmolarity (mOsm/l); and (D) Hematocrit (%). All mice were anesthetized with diazepam/ketamine and mechanically ventilated on 100% O_2 with 8 cm H_2O PEEP. n = 8–12 per group. $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}005$, $^{***}P < 0{\cdot}0005$, compared with uninfected mice (day 0). PEEP, positive end-expiratory pressure.

A significant fraction of patients hospitalized during the 2009–2010 H1N1 'swine flu' pandemic developed ARDS,[5] and this syndrome is a common sequel of human infections with influenza A H5N1 (bird flu) strains.[17] Likewise, histopathology consistent with ARDS has been reported in a retrospective study of lung tissues from fatal primary influenza cases from the 1918 pandemic.[6] Although ARDS is less commonly reported as an outcome in interpandemic 'seasonal' influenza outbreaks, it has been associated with poor prognosis.[2] Moreover, seasonal influenza may be under-diagnosed as a proximate cause of ARDS.[7] 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.[18]

Significant reductions in P_aO_2 have previously been reported in influenza A H5N1- and H9N2-infected mice.[10,11] 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.[13] 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.[13]), rather than the excessive inflammation which has been proposed to play a significant role in severe disease induced by pandemic influenza strains.[19–22]

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_aCO_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_aCO_2, plasma pH, and plasma [HCO_3^-] were reported in a cohort of non-survivors during the 'swine flu' pandemic.[23] 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.[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.[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.[24]

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.

References

1 Clark N, Lynch J. Influenza: epidemiology, clinical features, therapy, and prevention. Semin Respir Crit Care Med 2011; 32:373, 392.
2 Li G, Yilmaz M, Kojicic M *et al.* Outcome of critically ill patients with influenza virus infection. J Clin Virol 2009; 46:275–278.
3 Girard MP, Tam JS, Assossou OM, Kieny MP. The 2009 A (H1N1) influenza virus pandemic: a review. Vaccine 2010; 31:4895–4902.
4 Yuen KY, Chan PK, Peiris M *et al.* Clinical features and rapid viral diagnosis of human disease associated with avian influenza A H5N1 virus. Lancet 1998; 9101:467–471.
5 Estenssoro E, Rios FG, Apezteguia C *et al.* Pandemic 2009 influenza A in Argentina: A study of 337 patients on mechanical ventilation. Am J Respir Crit Care Med 2010; 1:41–48.
6 Taubenberger JK, Morens DM. The pathology of influenza virus infections. Annu Rev Pathol 2008; 3:499–522.
7 Oliveira EC, Lee B, Colice GL. Influenza in the intensive care unit. J Intensive Care Med 2003; 2:80–91.
8 Bernard GR, Artigas A, Brigham KL *et al.* The American-European consensus conference on ARDS. Definitions, mechanisms, relevant outcomes, and clinical trial coordination. Am J Respir Crit Care Med 1994; 149(3 Pt 1):818–824.
9 Ferguson ND, Davis AM, Slutsky AS, Stewart TE. Development of a clinical definition for acute respiratory distress syndrome using the Delphi technique. J Crit Care 2005; 20:147–154.
10 Xu T, Qiao J, Zhao L *et al.* Acute respiratory distress syndrome induced by avian influenza A (H5N1) virus in mice. Am J Respir Crit Care Med 2006; 9:1011–1017.
11 Deng G, Bi J, Kong F *et al.* Acute respiratory distress syndrome induced by H9N2 virus in mice. Arch Virol 2010; 155:187–195.
12 Ware LB, Matthay MA. Alveolar fluid clearance is impaired in the majority of patients with acute lung injury and the acute respiratory distress syndrome. Am J Respir Crit Care Med 2001; 163:1376–1383.
13 Wolk KE, Lazarowski ER, Traylor ZP *et al.* Influenza A virus inhibits alveolar fluid clearance in BALB/c mice. Am J Respir Crit Care Med 2008; 8:178.
14 Aeffner F, Bratasz A, Flaño E, Powell KA, Davis IC. Post-infection A77-1726 treatment improves cardiopulmonary function in H1N1 influenza-infected mice. Am J Respir Cell Mol Biol 2012; PMID 22679275. [Epub ahead of print].
15 Jewell NA, Vaghefi N, Mertz SE *et al.* Differential type I interferon induction by respiratory syncytial virus and influenza A virus *in vivo*. J Virol 2007; 81:9790–9800.
16 Irvin CG, Bates JH. Measuring the lung function in the mouse: the challenge of size. Respir Res 2003; 4:4–13.
17 Hui DS. Review of clinical symptoms and spectrum in humans with influenza A/H5N1 infection. Respirology 2008; 13(Suppl 1):S10–S13, S10–S13.
18 Bastarache JA, Blackwell TS. Development of animal models for the acute respiratory distress syndrome. Dis Model Mech 2009; 2:218–223.
19 de Jong MD, Simmons CP, Thanh TT *et al.* Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat Med 2006; 12:1203–1207.
20 Cheung CY, Poon LLM, Lau AS *et al.* Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? The Lancet 2002; 9348:1831–1837.
21 Kash JC, Tumpey TM, Proll SC *et al.* Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. Nature 2006; 7111:578–581.
22 Tumpey TM, Garcia-Sastre A, Taubenberger JK *et al.* Pathogenicity of influenza viruses with genes from the 1918 pandemic virus: functional roles of alveolar macrophages and neutrophils in limiting virus replication and mortality in mice. J Virol 2005; 79:14933–14944.
23 Rios F, Estenssoro E, Villarejo F *et al.* Lung function and organ dysfunctions in 178 patients requiring mechanical ventilation during the 2009 influenza A (H1N1) pandemic. Crit Care 2011; 15:R201.
24 Matute-Bello G, Frevert CW, Martin TR. Animal models of acute lung injury. Am J Physiol Lung Cell Mol Physiol 2008; 3:L379–L399.