A mouse model of swine influenza virus H9N2 infection with acute lung injury

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Summary. – BALB/c mice inoculated intranasally with A/swine/HeBei/012/2008/ (H9N2) virus (SIV), showing acute lung injury (ALI)/acute respiratory distress syndrome (ARDS), were observed for morbidity (lung histopathology, lung coefficient, lung wet/dry weight (W/D) ratio, arterial blood gas characteristics and inflammatory cells in bronchial alveolar lavage fluid (BALF)) and mortality. The results showed that, (1) on days 1–4 post infection (p.i.), mice appeared depressed and showed ruffled fur, reduced food intake, weight loss and hypoxemia with a decreased arterial partial oxygen pressure and an increased partial carbon dioxide pressure. (2) From day 4 p.i., mice began to die and showed pulmonary edema and hemorrhage and inflammatory cells in the alveolar exudate. The lung coefficient and lung W/D ratio significantly increased. (3) On days 3–8 p.i., inflammatory cells, especially alveolar macrophages and polymorphonuclears (PMNs) in BALF significantly increased. (4) The mortality rate reached 62.5%. This study established a successful animal model of ALI induced by infection with H9N2 SIV which may help in further investigations of the pathogenesis of human ALI/ARDS induced by H9N2 SIV infection.

Keywords: swine influenza virus; H9N2 subtype; acute lung injury; mouse

Introduction

Acute lung injury (ALI) is a type of a clinical syndrome comprising of inflammation and increased permeability in the lungs that result in characteristic pathology of diffuse lung cells damage, edema and micro-atelectasis, and disorders typical for gas exchange. ALI is observed during various conditions including severe infection, trauma, shock, and toxic poisoning. It is identified by the presence of injured alveolar epithelial and endothelial cells that lead to diffuse interstitial lung disease and alveolar edema, as well as acute low oxygen respiratory insufficiency (Bernard et al., 1994; Bernard, 2005). Studies indicate that the mortality rate of ALI/ARDS is 40%~60% so far (Fagan et al., 1999; Hudson and Steinberg, 1999). In recent years, both severe acute respiratory syndrome and human-avian influenza viruses have caused serious lung infections and ALI, and also increase in the incidence of ARDS and high mortality. Hence, infections, especially the viral infections, have become one of the important causes of ALI/ARDS, and have drawn the attention of the scientific community worldwide.

H9N2 subtype influenza virus is one of the main virus subtypes responsible for increased morbidity and mortality in the poultry. Human infections with H9N2 virus which displayed mild respiratory disease signs were recorded in 1999 and 2003 in Hong Kong and mainland of China (Butt et al., 2005; Lin et al., 2000). Genetic analysis showed that H9N2 virus had partial genetic information similarity with
that of H5N1 virus which gave rise to human deaths in Hong Kong in 1997 (Lin et al., 2000). Further studies showed that the H9N2 virus had specific receptor which bound to human respiratory epithelial cells (i.e. Leu 226) (Wan and Perez, 2007). In addition, studies have confirmed the ability of the H9N2 virus to cross the species barrier from the poultry to mammals and its higher positive infection rate among pigs in China (Cong et al., 2007, 2008; Yu et al., 2008). Meanwhile, H9N2 subtype virus and humanized H3N2 subtype virus were found to be transmitted to pigs simultaneously (Peiris et al., 2001) which provided the environment for the recombination of H9N2 subtype virus. Hence, it seems that H9N2 subtype virus circulating in pigs has potential threat to cause novel influenza pandemics in human in future. H9N2 SIv has important significance in public health and in studies on pathogenic mechanisms of H9N2 SIv in mammals. Especially, the studies on lung injury mechanisms will be of important significance for the prevention and treatment of potential human infections in the future.

Since there was so far no study on H9N2 SIv infection of mammals with pulmonary impairment, we attempted to analyze such an infection of mice and thus lay foundation for further studies on the mechanism of pulmonary impairment in mammals infected with H9N2 SIv.

Materials and Methods

Virus. A/swine/HeBei/012/2008 (H9N2) virus was inoculated into 10-day-old SPF chicken embryos (Beijing Laboratory Animal Research Center, China) and was consecutively blind passaged for 3 generations. The allantoic fluid was collected and stored at -70°C.

Experiments on mice. Eighty 6–8-week-old SPF BALB/c female mice (Beijing Laboratory Animal Research Center, China) were divided into 2 groups of 40 mice each. The mice were intranasally inoculated with H9N2 SIv (0.1 ml, containing 1×10^3ID50) and control mice by the same dose of noninfectious allantoic fluid, which had been diluted 1:5 by sterile saline buffer. The inoculated mice were separated and kept in the bio-safety level 3+ laboratories which had been diluted 1:5 by sterile saline buffer. The inoculated mice were monitored daily for morbidity by measuring the weight, food intake, clinical signs as well as mortality. The study was approved by the Animal Care Committee of Hebei North University (Zhangjiakou, China).

Histopathology. Four mice of each group were weighed and sacrificed on days 2, 4, 6, 8, and 14 p.i. Left lobes of lungs were fixed either in 10% formalin or 4% paraformaldehyde and embedded in paraffin or Epon, respectively. The samples for microscopic examination were stained with hematoxylin and eosin. The samples for ultrastructural examination were stained with uranyl acetate and lead citrate, and were examined in a transmission electron microscope operated at 80 kV (Hitachi model H-7650, Hitachi corp., Japan).

Lung coefficient and lung W/D ratio. The mice were sacrificed as described above, and the whole wet lungs were weighed to assess lung coefficient. The W/D ratios were determined by weighing the right lung before and after oven desiccation at 80°C. W/D ratio was used as an indicator of lung edema (Majeski et al., 2003).

Blood gas analysis. Blood gas analysis was conducted as described by Fagan (Fagan et al., 2004). Briefly, four mice of each group were anaesthetized slightly with pentobarbital sodium and arterial blood samples (0.4 ml) were collected in the heparinized syringe by percutaneous left ventricular puncture. Blood gas analysis was conducted with an IL/1620 full-automatic blood gas analyzer at once (Fagan et al., 1999).

BALF inflammatory cells. Another three mice of each group were sacrificed on days 2, 4, 6, 8, and 14 p.i. BALF was collected and cell count was done as described by Xu and co-workers (Xu et al., 2006).

Statistical analysis. All data were expressed as means ± SD. Statistical analysis was performed with the SPSS statistical software package for Windows, version 13.0 (SPSS, Inc., USA). Differences between groups were examined for statistical significance by two-tailed Student t test. The p-values less than 0.05 were considered statistically significant.

Results

Clinical and gross pathological observations

On day 2 p.i., infected mice showed slight depression, inappetence, inactivity and ruffled fur. By the day 3 p.i., most mice presented abrupt decrepitation, clinical signs of respiratory disease, including visual signs of labored respiration and respiratory distress. They also exhibited more severe inappetence, emaciation, with weight decreased to about 80% compared to that of normal mice (Fig. 1). First mice died on day 3 p.i. and overall 62.5% of mice (5 of 8) died during the experiment. Lungs of the infected mice were highly edematous, with diffuse areas of hemorrhage and congestion. No obvious gross lesions were observed in other organs, including brain.

Histopathology of lungs

On day 2 p.i., lung histopathology was characterized by interstitial edema around the small blood vessels and bronchiole with inflammatory cell exudate. Neutrophil cells, erythrocytes, plenty of mucinous exudates, and edema fluid were observed in the alveolar lumen (Fig. 2b). On day 4 p.i., alveolar walls were thickened, the alveolar lumen diminished, and pulmonary interstitium broadened. In addition, obvious edema was present in interstitium around vessels and bronchiole, and parts of bronchial epithelium necrosed and sloughed. Moreover, plenty of erythrocytes appeared...
in alveolar lumen and interstitium (Fig. 2c). On day 6 p.i., plenty of inflammatory cells were exuded and bronchopneumonia developed (Fig. 2d). In comparison, lungs from mock-infected mice had no apparent histopathological lesions (Fig. 2a).

**Electron microscopy of lung tissues**

Ultrastructural observation by transmission electron microscopy showed cell edema, cytomembrane rupture, and cytoplasm showed vacuolar degeneration. The cell nucleus edema with nucleolar margination was obvious. Edema also appeared on mitochondria where the cristae were shortened, matrix was reduced, and cytoplasm showed vacuolar degeneration (Fig. 3a). The alveolar lumen also showed evidence of hemorrhage with abundant erythrocyte. Evidence of

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**Fig. 1**

Effect of SIV infection on body weight of mice

Results of body weight for the eight H9N2 SIV-infected mice (●) compared to the group of mock-infected mice with same dose of noninfectious allantoic fluid (■). The mice were monitored daily for 14 days p.i.

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**Fig. 2**

Effect of SIV infection on lung histopathology of mice

(a) Mock-infection. (b) Mild interstitial edema around small blood vessels and bronchiole and inflammatory cell exudate on day 2 p.i. (c) Moderate bronchioalveolitis with cellular debris in bronchioles and alveoli on day 4 p.i. (d) Severe bronchitis characterized by degeneration and necrosis of bronchial epithelium with intra-luminal cellular debris on day 6 p.i. Magnification 100x.
hemorrhagic necrosis along with PMN cell infiltration was also prominent and seen at different time points (Fig. 3b). Short-stick-shaped and spherical influenza virions could be observed on flat epithelial cell surface of pulmonary alveoli and in alveolar lumen of mice (Fig. 3c).

**Lung coefficient and lung W/D ratio**

The lung wet weight of H9N2-infected mice increased slightly on day 2 p.i., but there was no statistical significance in the W/D ratio. However, the lung W/D ratio on day 4–6 p.i. increased significantly (P <0.01). On day 8 p.i., W/D ratio of the infected mice began to recover progressively, but it still had significant difference (P <0.01). On day 14 p.i., W/D ratio was close to control data (shown in Fig. 4).

As shown in Fig. 5, there was no significant difference of lung coefficient between infected and mock-infected mice on day 2 p.i. However, the lung coefficient of H9N2-infected mice increased dramatically on day 4–8 p.i. (P <0.01). On day 14 p.i., there was no significant difference between H9N2-infected and mock-infected mice (P >0.05) again.

**Arterial blood gas characteristics**

Table 1 shows the results of blood gas analysis. The partial pressure of arterial oxygen (Pao2) and oxygen hemoglobin...
saturation (Sao₂) were slightly decreased, while the partial pressure of arterial carbon dioxide (Paco₂) increased (P < 0.05) and pH value decreased in h9n2-infected mice on day 2 p.i. However, pH value, Pao₂ and Sao₂ of the most infected mice decreased (P < 0.01), and Paco₂ increased significantly on day 4–6 p.i. On day 8 p.i., the results were similar to those of day 6 p.i. Decrease of Pao₂ was only 1/2 of that of control mice, while Paco₂ increased dramatically (Table 2).

**BALF inflammatory cells**

The total number of inflammatory cells in BALF of h9n2-infected mice increased from day 2 p.i., and the increase became obvious on day 4–8 p.i. (P < 0.01). From day 2 p.i., the ratio of lymphocytes and especially PMNs (P < 0.01) in BALF started to increase. By contrast, there was a slight decrease in ratio of alveolar macrophages, which reached the highest level on day 6 p.i. with significant difference in comparison with control mice. From 8 day p.i., the number of PMNs and lymphocytes started to decrease, however, the significant difference compared with controls was still visible.

**Discussion**

Pigs, acting as an intermediate host for avian and human influenza virus, can play a very important role in the epidemic of human influenza. H9N2 SIV is one of the major epidemic subtypes which have caused swine influenza so far, and the recombinant virus containing human-avian-swine influenza viruses has already been described (Sullivan et al., 2010). If the strain acquires the ability to bind human cell surface receptors, there might be a threat of H9N2 swine influenza epidemic.

In this study, BALB/c mice inoculated with A/swine/HeBei/012/2008/ (h9n2) virus displayed inactivity, inapetence and weight loss. From days 3 to 8 p.i., respiratory frequency increased and respiratory distress occurred and resulted in 62.5% mortality. Hemorrhage, severe edema and

<table>
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<th>Days p.i.</th>
<th>Pao₂ (kPa)</th>
<th>Paco₂ (kPa)</th>
<th>pH</th>
<th>Sao₂ (%)</th>
</tr>
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<tr>
<td>2</td>
<td>Infected</td>
<td>11.6±1.08</td>
<td>5.56±0.31</td>
<td>7.39±0.035</td>
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<tr>
<td></td>
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<td>12.39±1.23</td>
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<td>7.36±0.071</td>
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<tr>
<td>4</td>
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<td>9.38±1.45</td>
<td>5.98±0.26</td>
<td>7.26±0.079</td>
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<tr>
<td></td>
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<td>12.17±1.46</td>
<td>5.27±0.31</td>
<td>7.34±0.052</td>
</tr>
<tr>
<td>6</td>
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<td>6.79±1.27</td>
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</tr>
<tr>
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<td>5.16±0.82</td>
<td>7.35±0.027</td>
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<tr>
<td>8</td>
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<td>7.31±0.086</td>
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<tr>
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<td>14</td>
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<td>5.29±0.76</td>
<td>7.37±0.067</td>
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*/**Significant differences with P <0.05/P <0.01, respectively.

![Table 2 Effect of SIV infection on arterial blood gas characteristics in mice](image)

![Table 2 Effect of SIV infection on total and differential leukocyte counts in mice](image)
plenty of blood-red frothy exudate was found in the lungs of infected mice. Meanwhile, the lung coefficient and lung W/D ratio increased significantly, as a sign of severe lung edema. Histopathological observation confirmed that H9N2 SIV infection caused viral interstitial pneumonia characterized by diffuse and exudative inflammation with obvious alveolar and interstitial edema, significant inflammatory cell exudate in alveolar lumen and interstitium. Plenty of inflammatory cells, erythrocytes, and epithelial debris around bronchus and inside lumen were found as well. The results of this study were consistent with the histopathological features of ALI/ARDS. Meanwhile, these changes are consistent with the histopathological lesions of ALI/ARDS, which were induced by inoculation of CBA/J mice with reovirus 1/L strains by London and his co-workers (London et al., 2002). Others have found that after infection, follicular bronchitis and lymphatic folliculitis around vessels developed from original bronchiolar f lake pneumonia (Fagan et al., 1999). Furthermore, our findings are also consistent with the pathological findings from studies on H9N2 avian influenza virus impact on mice by Deng and colleagues (Deng et al., 2010). In these studies, the infected mice displayed a similar histopathological pattern, including peribronchial patchy pneumonia – bronchiolitis - bronchopneumonia. Hence, the pathological changes in H9N2 SIV infected mice were consistent with that of ALI, that is, the inflammatory cellular infiltration, interstitial and alveolar edema, and hemorrhage were observed in the lungs.

Both, the number of ventilated alveoli and thickness between alveoli-capillary membranes can influence exchange of oxygen in alveoli and erythrocytes in blood. Studies have demonstrated that virus infections lead to impairment of the alveolar epithelium and vascular endothelium, which increases the thickness of alveolar-capillary membranes and influences the function of oxygen exchange, resulting in hypoxia (Headley et al., 1997). Arterial blood gas exchanges were studied to confirm whether the serious histopathological injury of lungs caused by A/swine/HeBei/012/2008 (H9N2) virus infection leads to hypoxemia during the infection process. The results indicated that PaO₂ and SaO₂ of the infected mice decreased significantly, while PaCO₂ increased, indicating the presence of severe hypoxemia in mice (PaO₂ was 6.79 kPa ± 1.27 kPa, lower than 8 kPa, PaCO₂ was 7.45 kPa ± 0.87 kPa, higher than 6.67 kPa). During hypoxemia phase of the illness, the corresponding histopathological changes of the mice lungs showed diffuse alveolar injury and dysfunction of ventilation function between pulmonary alveoli and capillary, which resulted in hypoxemia. Clinical symptoms comprised of increase in respiratory rate, occurrence of respiratory distress, and lung failure. Our results showed that serious progressive hypoxemia occurred in mice during the process of A/swine/HeBei/012/2008/ (H9N2) virus infection.

In this study, after intranasal inoculation of A/swine/HeBei/012/2008/ (H9N2) influenza virus, the mice showed acute onset of the disease, urgent breathing and expiratory dyspnea. Diffuse alveolar injury was the main feature of histopathological changes. Progressive hypoxemia resulted from biased ventilation function. All above mentioned symptoms corresponded with clinical characteristics and pathophysiological changes of ALI/ARDS.

In this report, we described a mouse model of ALI induced by H9N2 SIV, which demonstrated that BALB/c mice become ill when inoculated intranasally with H9N2 virus. Furthermore, studies on cynomolgus macaques infected with new type of H1N1 virus by Herfst et al. also showed that the virus could cause pathological changes in lungs with diffuse alveolar injury as its main feature (Herfst et al., 2010 ), and the changes were similar with that of development process of H9N2 SIV infection in mice. Therefore, the investigations on mice models infected with H9N2 SIV in this study not only makes it possible to provide a basis for the prevention and treatment of H9N2 infections, but also helps to explore the treatment for human ALI induced by new H1N1 virus.

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