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Original Article

Oxygen free radical involvement in acute lung injury induced by H5N1 virus in mice

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Background Acute lung injury is an important cause of death in humans infected with H5N1. It has been found that oxygen free radicals (OFRs) are elevated in lung tissue during influenza virus infections. In this study, we used a mouse model to explore the role of OFRs in acute lung injury caused by H5N1 viral infection.

Methods Four- to six-week-old male specific pathogen-free BALB/c mice were inoculated intranasally with 10^5 50% tissue culture infective doses ($TCID_{50}$) of highly pathogenic A/Chicken/Hebei/108/2002 (H5N1) viruses and were then given 1000 IU of lauric acid modified superoxide dismutase (LA-SOD) by intraperitoneal injection, starting 2 days post-infection and continuing for 6 days.

Results The extent of lung injury and the concentration of OFRs were higher, and the SOD activity was lower in H5N1 virus-infected mice than that in uninfected control mice on days 3, 6, and 7 post-inoculation. Weak amelioration of clinical signs, a minor decrease in the total mortality and the extent of lung injury, and the lower OFRs concentration were seen in the LA-SOD treatment group, but a reduction in lung virus titers was not observed in the LA-SOD treatment at all time points.

Conclusions The LA-SOD treatment has a mild inhibitory effect on H5N1 influenza virus infection in mice. OFRs, therefore, might play an important role in the pathogenesis of acute lung injury induced by H5N1 virus.

Keywords Acute lung injury, H5N1 avian influenza A virus, oxygen free radical, superoxide.

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Introduction

H5N1 avian influenza virus causes severe and often fatal disease in humans. As of April 12, 2012, a total of 602 cases, with 335 deaths, have been reported by the World Health Organization, giving a fatality rate of 58·97%.[1] Patients infected with H5N1 influenza virus present with clinical signs of influenza-like illness and then develop rapidly progressive pneumonia leading to acute lung injury (ALI).[2,3] ALI is an important cause of 'death in patients with H5N1 infection.[4,5] To treat this disease effectively, we must, therefore, determine the mechanism leading to the development of ALI.

It has been suggested that the pathogenicity of influenza virus infection in mice is determined, at least in part, by the overreaction of the host immune response rather than a direct effect of viral multiplication.[5] The strong inflammatory response in the lung is marked by extensive infiltration by inflammatory cells such as polymorphonuclear neutrophils and macrophages,[6–9] and these cells are also important cellular sources of oxygen free radicals (OFRs) in the mouse lung during influenza virus infection.[10,11] OFRs attack polyunsaturated fatty acids in biomembranes and induce lipid peroxidation, which is a principal cause of lung injury. Malondialdehyde (MDA) is the direct product of lipid peroxidation and is an indicator of oxidative damage; thus, levels of MDA in the lung can reflect the degree of lipid peroxidation in the body and indirectly act as a marker of the degree of lung injury.[12,13] In addition, it has been proposed that reactive oxygen species (ROS) are produced in the lung as a result of the infection, and these OFRs could increase local inflammation and thereby contribute to pulmonary tissue damage.[14] This has been demonstrated in mouse

models, which can be protected against potentially lethal influenza virus infection by the injection of superoxide dismutase (SOD), a specific superoxide radical scavenger.[10,11,15] It seems that OFRs play an important role in the pathogenesis of influenza virus infection. However, there is no direct evidence of OFR involvement in severe H5N1 infection, and their role needs further evaluation.

An H5N1 virus-induced mouse model of acute respiratory distress syndrome (ARDS) has been described previously by our research group.[16] The model replicates most of the clinical and pathological changes observed in human ARDS induced by H5N1 infection. In this study, we used this model to determine whether OFRs are involved in ALI induced by H5N1 influenza virus and to assess the role of OFRs in ALI.

Materials and methods

Ethics statement

The study was approved by the Animal Care Committee of China Agricultural University (Beijing, People's Republic of China). All animal procedures followed the ethics guidelines of the National Research Council Guide for the Care and Use of Laboratory Animals (1985).

Virus and cell culture

The avian H5N1 influenza virus (A/Chicken/Hebei/108/2002, HB/108) used in this study was isolated from infected chickens in Hebei province of Northern China in 2002. The virus was propagated in the allantoic cavities of 10-day-old embryonated SPF chicken eggs, and virus stocks were stored at −80°C. The complete viral genomic sequences (DQ343152, DQ349116, DQ351860-DQ351861, DQ351866-DQ351867, DQ351872-DQ351873) can be obtained from GenBank. The virus caused 100% (8/8) mortality in 4-week-old SPF chickens within 2 days of intravenous injection with 0·2 ml of infectious allantoic fluid at 1:10 dilution. Our previous studies have shown that this virus is highly lethal to mice and causes typical ARDS in BALB/c mice.[16] All manipulations of live viruses were conducted in biosafety level 3 facilities.

Madin–Darby canine kidney (MDCK) cells were obtained from the American Type Culture Collection (CCL-34) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin). The cells were maintained at 37°C in 5% CO2.

Lauric acid modified SOD (LA-SOD)

Lauric acid modified SOD (Tianjin Institute of Life Sciences Applications, China), a specific superoxide radical scavenger, was used in this study. It has a long half-life (about 8 hour *in vivo*), high specific activity ($\geq$20 000 Unit/mg), stability, percutaneous absorption, and low molecular weight characteristics.[17] It was dissolved in 0·9% sterile physiological saline at the appropriate concentrations and stored at 4°C.

Mice and viral infection

Four- to six-week-old male specific pathogen-free BALB/c mice were purchased from the Beijing Laboratory Animal Research Center (China) and housed in microisolator cages ventilated under negative pressure with HEPA-filtered air. During the experiment, the mice were given standard mouse chow and water *ad libitum*.

For the experiments, the mice were lightly anesthetized with diethyl ether and inoculated intranasally with 10^5TCID$_{50}$ (50% tissue culture infective dose) of HB/108 virus stocks diluted in phosphate-buffered saline (PBS) to a volume of 50 μl. Control mice were inoculated intranasally with an equivalent dilution of allantoic fluid from uninfected eggs under the same anesthetic regimen.

Experimental design and LA-SOD administration

To determine whether OFRs are involved in the pathogenesis of ALI induced by H5N1 virus infection *in vivo*, we conducted two experiments. In the first, we assessed the effect of LA-SOD on mortality and clinical features in H5N1-infected mice. For this purpose, BALB/c mice were divided randomly into four groups of 20 each: a LA-SOD-treated infected group (virus inoculation and daily LA-SOD injection), a LA-SOD control group (control inoculation and daily LA-SOD injection), an infected group (virus inoculation and daily PBS injection), and a control group (control inoculation and daily PBS injection). According to previous studies[10,18] and our unpublished data, 1000 IU LA-SOD was used as the daily dose, dissolved in 100 μl sterile physiological saline. This was administered by intraperitoneal injection on days 2–8 after H5N1 infection. The control mice received an intraperitoneal injection of 100 μl sterile physiological saline on the same days. Symptoms, body weight, food intake, and mortality were monitored daily for each group for 14 days.

In the second experiment, the effect of LA-SOD on the development of the disease induced by H5N1 virus was studied. BALB/c mice were divided randomly into three groups of 50 each: a LA-SOD-treated group, an infected control group, and an uninfected control group. Because the two control groups (untreated uninfected and LA-SOD-treated uninfected) provided identical data and exhibited similar clinical signs in the first experiment, we used only one control group in the second experiment. At the indicated time, the mice were sacrificed, and lung weight, lung wet-to-dry weight ratio, and lung histopathology were assessed. Concentrations of free radicals (reactive oxygen species (ROS) and malondialdehyde (MDA)), viral replication and antioxidase (SOD) activity in the lungs were also measured.

Assessment of lung water content

Three mice from each group were euthanized on days 3, 5, 6, 7, 9, and 14 post-inoculation, the whole lung weights were recorded, and the lung wet-to-dry weight ratios were calculated. In brief, whole lungs were excised, and the right lungs were separated from the left. The right lungs were weighed to obtain their wet weight and then dried in an oven at 80°C for 2 days to obtain their dry weight and to calculate wet-to-dry weight ratios. The left lungs were divided into the two lobes (the upper lobe and the lower lobe) and were used to observe the histopathological change and titrate the virus titer.

Lung histopathology examination

On days 3, 5, and 7 post-inoculation, parts of the left lungs were fixed in buffered 10% formalin and embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin for light microscopy. The remaining left lungs were used for the determination of virus titers. The histopathology was evaluated on a blind basis by a veterinary pathologist according to our previous described methods.[19]

Electron spin resonance (ESR) spectroscopy

Reactive oxygen species production in the lung was measured by electron spin resonance (ESR) spectroscopy, which was performed as described previously.[20,21] On days 3, 6, 7, and 12 post-inoculation, approximately 100 mg of lung tissue was collected quickly and ground in 0·1 M phosphate buffer (pH 7·4) containing 0·32 M sucrose, 10 mM HEPES, 10 mM phenyl-t-butyl nitrone (PBN), 2 mM diethylenetriaminepentaacetic acid (DPTA), 0·05% Tween, and 80·5 mM thioaethylenglycol in ice water in the presence of quartz sand. After centrifuging at 13 000 ***g*** for 20 minutes, 10 μl each of 0·5 M $Na_2S_2O_4$, 0·3 M $FeSO_4$, and 0·6 M diethyldithiocarbamate (DETC) was added to 450 μl of the supernatant, and the mixture was maintained at 37°C for 1 hour. ESR spectra were obtained at room temperature using a Bruker ER200D-SRC spectrometer following the method of Cao *et al.*[20] The conditions for ESR were as follows: X-band, 100 kHz modulation with 3·2 G amplitude, microwave power 20 mW, central magnetic field 3385 G with scan 400 G, and scan time 200 second. The intensity of the ESR signals in tissue was calculated by dividing the height of the signal by the number of milligrams of tissue extracted. The signal intensity was expressed as the signal height in millimeters per milligram of lung tissue.

Determination of MDA concentration and SOD activity in the lung

On days 3, 6, 7, and 12 post-inoculation, three mice from each group were selected randomly. After euthanasia, the lungs were collected immediately and homogenized with 0·9% sterile physiological saline. The homogenate was centrifuged at 4500 ***g*** for 10 minutes, and then, the supernatant was collected and divided into two, one aliquot was used to measure SOD activity and the other to measure MDA concentration. SOD activity was measured according to the method of Sun *et al.*,[22] by determining the inhibition of nitroblue tetrazolium (NBT) reduction with xanthine–xanthine oxidase as an $O2^-$ generator. One unit of SOD is defined as the amount of protein that reduces the rate of NBT reduction by 50%. The results were expressed as units per milligram of protein (U/mg protein).

Lung MDA levels were determined by the method of Hermann and Cheeseman[23] based on the reaction with thiobarbituric acid at 90–100°C. After cooling, the absorbance was read at 532 nm. The concentrations were expressed as nanomoles per milligram of protein (nmol/mg protein).

Virus titration

Virus titration was performed as described by Price *et al.*[24] Parts of the left lungs were homogenized in 1 ml of cold DMEM. Solid debris was removed by brief centrifugation before the homogenates were titrated for viral infectivity using a 50% cytopathic effect assay on MDCK cells with initial dilutions of 1:10. Median tissue culture infective dose ($TCID_{50}$) was calculated by the method of Reed and Muench[25] and expressed as mean $\log_{10}TCID_{50}$ per gram of tissue ± standard deviation. The threshold of virus detection in the MDCK assay is about 10^2 $TCID_{50}$/g tissues.

Statistical analysis

All data were expressed as the mean ± SD. Statistical analysis was performed using SPSS version 17·0 (SPSS Inc, Chicago, IL, USA). The one-way analysis of variance (ANOVA) followed by a post hoc Tukey test or unpaired two-tailed t-test was used to evaluate the statistical significance of differences between two groups. The survival data were analyzed by Kaplan–Meier method, followed by a long-rank test for pairwise analyses. A *P*-value less than 0·05 was considered statistically significant.

Results

Clinical features and mortality after infection with H5N1

To assess the effect of LA-SOD on the mortality and clinical features of H5N1-infected mice, an experiment was performed on four groups of 20 mice each. These were identified as follows: a LA-SOD-treated infected group (virus inoculation and daily LA-SOD injection), a LA-SOD control group (mock inoculation and daily LA-SOD injection), an infected control group [virus inoculation and daily phosphate-buffered saline (PBS) injection], and a non-infected control

group (mock inoculation and daily PBS injection). The two infected groups exhibited similar clinical signs (inactivity and ruffled fur) on day 3 post-inoculation. By day 6, more severe clinical signs of the infected mice were present, including inactivity, ruffled fur, poor appetite, hunched posture, and signs of labored respiration and respiratory distress. The body weights of the two infected groups decreased by 3·7–15·2% from its initial level, and the food intake was significantly decreased on days 6–8 post-inoculation ($P < 0·05$) compared with the two non-infected control groups (Table 1). The mice in the two infected groups began to die from day 5 post-inoculation, reaching a plateau on day 8 post-inoculation. As shown in Table 1, the food intake, survival rates, and the body weight were higher in the LA-SOD-treated infected mice than those in the infected control mice on days 6–9 post-inoculation, but there was no statistically significant difference between the two infected groups ($P > 0·05$). Therefore, weak inhibition of the mortality rate, body weight loss, and the food intake loss were observed in LA-SOD-treated mice. None of the non-infected control mice showed any clinical signs or died, and their body mass and food intake increased over the course of the experiment, which suggested that treatment with LA-SOD did not have side effects.

Lung histopathology after infection with H5N1

Similar lung histopathological pattern in the two H5N1 virus-infected groups was observed in Figure 1. On day 3 post-inoculation, the lung lesions of the infected mice were characterized by thickening of alveolar walls (Figure 1A ,B, black arrows), dropout of mucous epithelium, and inflammatory cells adhering to the surface of bronchioles (Figure 1A, B, gray arrows). On day 5 post-inoculation, there was interstitial pneumonia (Figure 1C, D, gray arrows), with severe edema (Figure 1C, D, black arrows), inflammatory cell infiltration of the alveolar walls, and hemorrhage (Figure 1D, white arrows). On day 7 post-inoculation, the virus had caused severe interstitial pneumonia and peribronchiolitis (Figure 1E, F, gray arrows), characterized by edema (Figure 1E, black arrows), extensive lymphocyte infiltration around the area of bronchiolitis, and severe hemorrhage (Figure 1E, F, white arrows). The histopathological change score for LA-SOD-treated infected mice was lower than that of infected control mice,

Table 1. The clinical features and mortality in H5N1-infected mice

		Time (*d*) after Inoculation						
	Groups	3	5	6	7	8	9	14
Food intake (g)	Infected††	3·2 ± 0·3	3·7 ± 0·4	2·3 ± 0·7*	1·8 ± 0·8*	1·8 ± 0·6*	2·4 ± 0·5	3·3 ± 0·3
	LA-SOD treated†††	3·4 ± 0·6	3·5 ± 0·4	2·8 ± 0·5	2·5 ± 0·3*	2·1 ± 0·7*	2·6 ± 0·2	3·1 ± 0·5
	LA-SOD uninfected‡	3·5 ± 0·4	3·8 ± 0·5	3·7 ± 0·6	3·5 ± 0·4	3·4 ± 0·8	3·6 ± 0·7	3·7 ± 0·5
	Control‡‡	3·7 ± 0·3	4·0 ± 0·6	3·2 ± 0·3	3·5 ± 0·4	3·6 ± 0·2	3·4 ± 0·8	3·5 ± 0·6
Body weight *** (%)	Infected††	101·9%±1·2%	102·3%±1·0%	94·2%±2·3%*	89·1%±2·4%**	84·8%±3·8%**	92·2%±1·1%**	100·9%±1·3%
	LA-SOD††	101·8%±0·9%	102·4%±0·7%	96·3%±1·9%*	90·1%±3·0%**	87·3%±2·4%**	96·2%±1·3%*	100·5%±0·5%
	LA-SOD uninfected‡	101·7%±1·4%	102·4%±0·7%	103·1%±0·5%	103·7%±1·7%	104·7%±2·6%	105·4%±2·4%	107·8%±1·2%
	Control‡‡	101·5%±1·0%	102·5%±0·5%	102·8%±0·8%	103·4%±1·8%	104·4%±1·5%	105·6%±1·7%	107·9%±2·0%
Survival rate † (alive/total)	Infected††	100% (20/20)	100% (20/20)	75% (15/20)	65% (13/20)	45% (9/20)	45% (9/20)	45% (9/20)
	LA-SOD treat†††	100% (20/20)	100% (20/20)	90% (18/20)	75% (15/20)	55% (11/20)	55% (11/20)	55% (11/20)
	LA-SOD uninfected‡	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)
	Control‡‡	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)	100% (20/20)

Data are presented as mean ± SD, unless otherwise stated.
*$P < 0·05$.
**$P < 0·01$ compared with the uninfected control groups.
***Body weight was indicated as the percentage of the average initial weight at each time point.
†Survival rate was calculated as the percentage of no. of survivors relative to total number treated at each time point.
††Mice infected with 10^5 $TCID_{50}$ of HB/108 viruses and treated with PBS from days 2 to 8 after H5N1 infection.
†††Mice infected with 10^5 $TCID_{50}$ of HB/108 viruses and treated with LA-SOD from days 2 to 8 after H5N1 infection.
‡Mice infected with a similar volume of allantoic fluid from uninfected eggs and treated with LA-SOD from days 2 to 8 after H5N1 infection.
‡‡Mice infected with a similar volume of allantoic fluid from uninfected eggs and treated with PBS from days 2 to 8 after H5N1 infection.

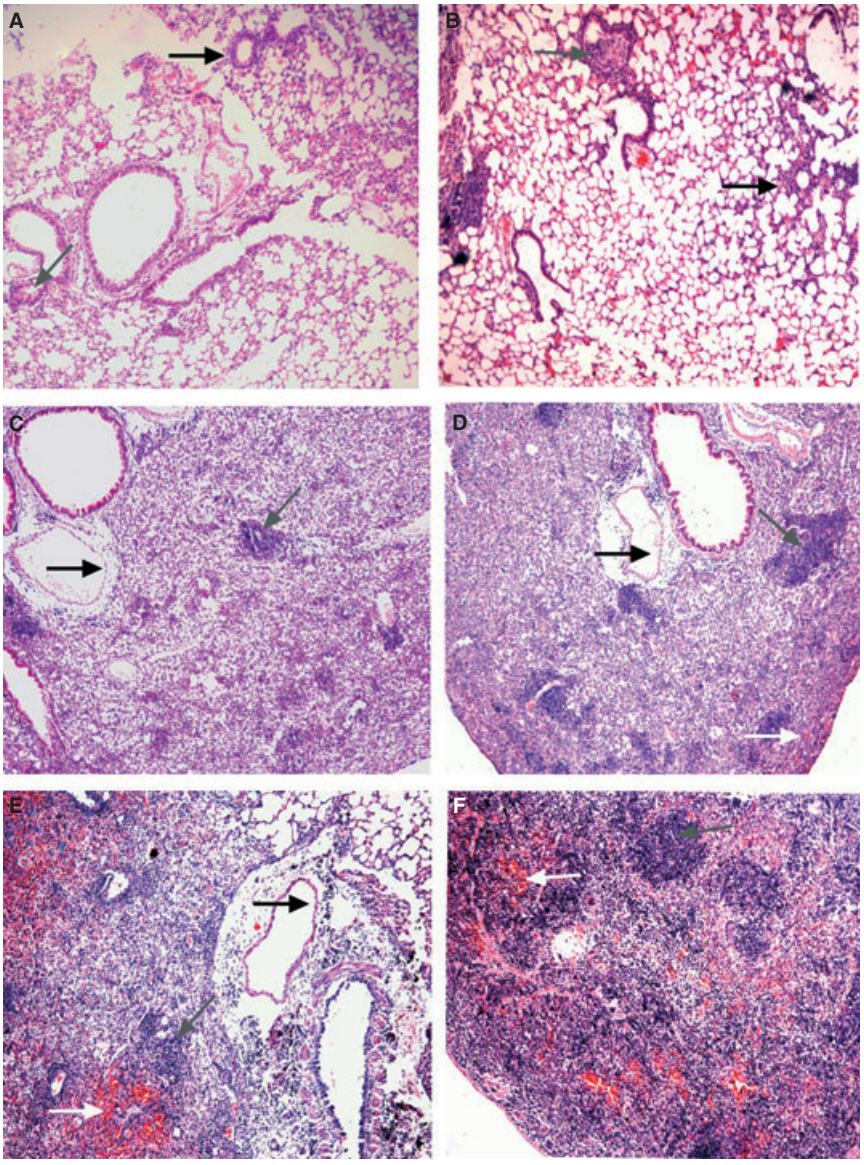

Figure 1. Lung histopathological changes of LA-SOD-treated infected (A, C, and E) and infected control mice (B, D, and F) after infection with HB/108-H5N1 influenza virus. On day 3 post-inoculation, there was thickening of alveolar walls (A and B, black arrows) and dropout of mucous epithelium and inflammatory cells adhering to the surface of bronchioles (A and B, gray arrows). On day 5 post-inoculation, the virus caused interstitial pneumonia (C and D, gray arrows), severe edema (C and D, black arrows), inflammatory cells infiltration of the alveolar walls, and hemorrhage (D, white arrows). On day 7 post-inoculation, the virus caused severe interstitial pneumonia and peribronchiolitis (E and F, gray arrows), characterized by edema (E, black arrows), extensive lymphocyte infiltration around the area of bronchiolitis, and severe hemorrhage (E and F, white arrows). Objective magnification, × 10 (A–E).

but the difference was not statistically significant (data not shown). The results suggest that treatment with LA-SOD might mildly alleviate the histopathological lesions of the lung associated with ALI.

Lung edema after infection with H5N1

To assess the extent of the lung edema, lung weights were recorded, and lung wet-to-dry weight ratios were calculated at different time points during the influenza virus infection. In the infected control group, lung wet-to-dry weight ratios were significantly increased compared with the non-infected control group on days 7–9 post-inoculation ($P < 0{\cdot}01$), reaching a peak on day 7 and declining thereafter. Although lung wet-to-dry weight ratios in the LA-SOD-treated infected group were lower on days 7–9 than in the infected group, there were no statistically significant differences between the two infected groups ($P > 0{\cdot}05$; Figure 2A). The changes in lung weights were similar to the lung wet-to-dry weight ratios (Figure 2B), but the lung weights were significantly reduced in the LA-SOD-treated infected mice compared with

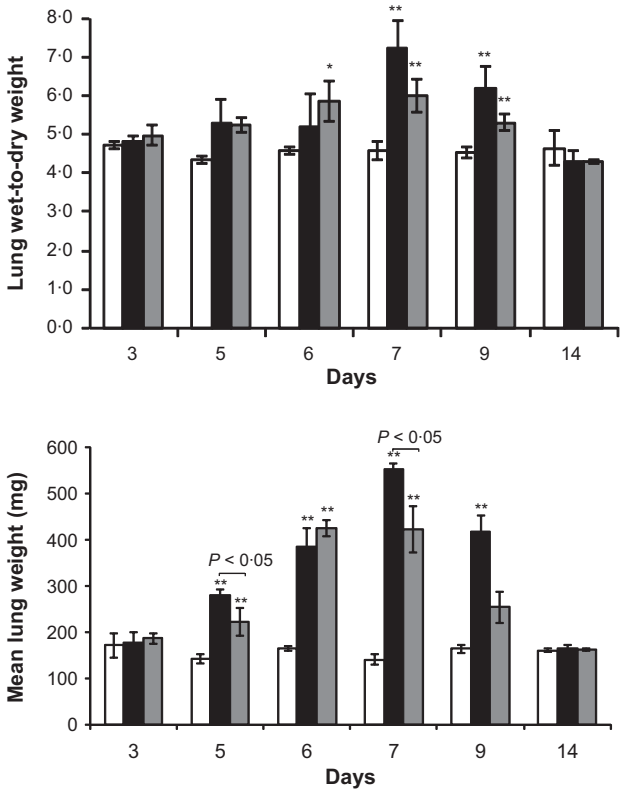

Figure 2. Lung weight and lung wet-to-dry weight ratios after infection with HB/108-H5N1 influenza virus. Lungs were collected at indicated times post-inoculation, lung weights were recorded, and lung wet-to-dry weight ratios were calculated. White bars represent the uninfected control group; black bars, the infected group; and gray bars, the LA-SOD-treated infected group. Bars represent means ± SD of data from three mice. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$ compared with the uninfected control mice.

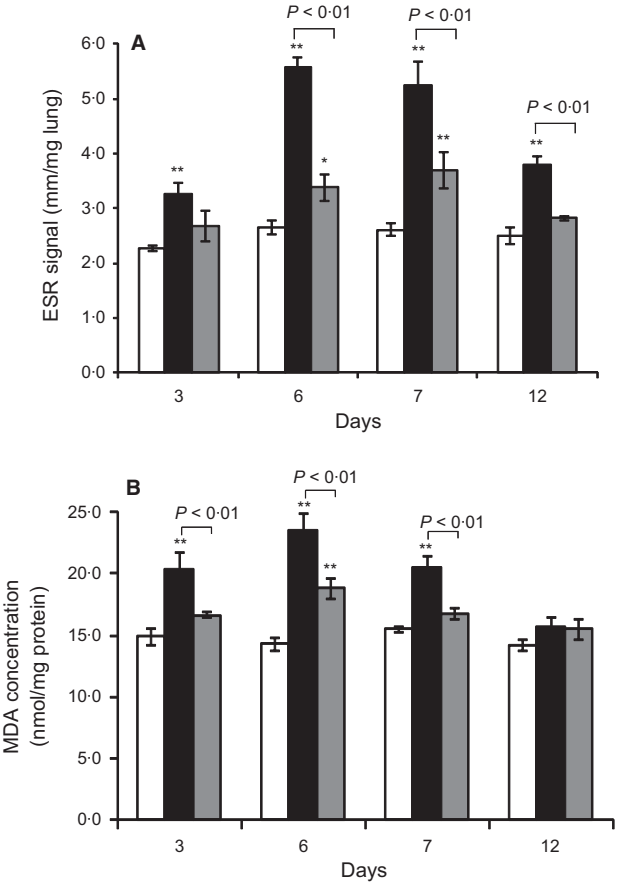

Figure 3. Reactive oxygen species (ROS) and malondialdehyde (MDA) concentrations in mouse lung after influenza virus infection. (A) The amount of ROS generated in the lung was quantified by electron spin resonance (ESR) spectroscopy. White bars represent the uninfected control group; black bars, the infected group; and gray bars, the LA-SOD-treated infected group. The mean ± SD at each time point is shown ($n = 3$). *$P < 0{\cdot}05$, **$P < 0{\cdot}01$ compared with the uninfected control mice. (B) Concentration of MDA is expressed as nmol/mg protein ± SD. White bars represent the uninfected control group; black bars, the infected group; and gray bars, the LA-SOD-treated infected group. The mean ± SD ($n = 3$) at each time point is shown. **$P < 0{\cdot}01$ compared with the uninfected control mice.

the untreated infected mice on days 5 and 7 post-inoculation. The results showed that a trend for alleviating lung edema was present in the LA-SOD-treated mice.

Concentration of ROS and MDA in the lung after infection with H5N1

As shown in Figure 3A, the concentration of ROS in the infected group increased markedly from day 3 to day 12 post-inoculation compared with the uninfected control group ($P < 0{\cdot}01$). ROS concentrations were significantly higher in the LA-SOD-treated infected group than in the uninfected control mice on days 6–7 post-inoculation ($P < 0{\cdot}05$) and significantly lower than in the infected group on days 6–12 post-inoculation ($P < 0{\cdot}01$).

Similar to the ROS levels, the concentration of MDA in the infected control mice was significantly higher than that in the uninfected control mice on days 3, 6, and 7 post-inoculation ($P < 0{\cdot}01$) and also significantly higher than that in the LA-SOD-treated infected group on days 3, 6, and 7 post-inoculation ($P < 0{\cdot}01$; Figure 3B). The MDA concentration was significantly higher in the LA-SOD-treated infected group than that in the uninfected control mice on day 6 ($P < 0{\cdot}01$; Fig 3B) and declined thereafter. On day 12, there were no significant differences among the three groups. These results show that LA-SOD treatment reduced the concentrations of ROS and MDA in infected mice.

SOD activity in the lung after infection with H5N1

Superoxide dismutase activity in the infected control group was significantly lower than that in the control group on days 6 and 7 post-inoculation ($P < 0{\cdot}01$; Figure 4). SOD activity in the LA-SOD-treated infected group followed a similar pattern to that observed in the infected control mice, with no

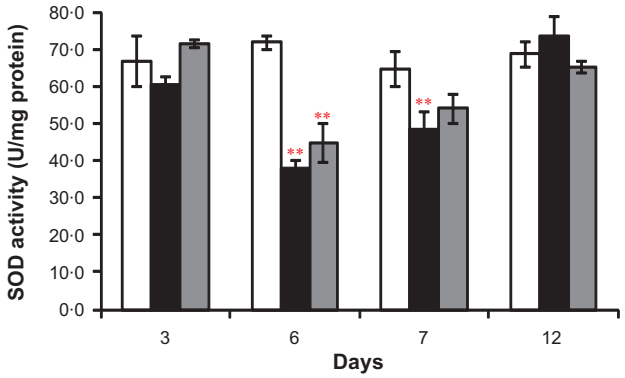

Figure 4. Superoxide dismutase (SOD) activity in lung tissues from influenza virus-infected mice. The SOD activity is expressed as U/mg protein ± SD (the data from three mice). White bars represent the uninfected control group; black bars, the infected group; and gray bars, the LA-SOD-treated infected group. $^{**}P < 0{\cdot}01$ compared with the uninfected control mice.

significant differences between the two groups at the different time points ($P > 0{\cdot}05$).

Virus titer in the lung after infection with H5N1 virus

Patterns of viral replication in the lung were similar in the LA-SOD-treated and infected control groups ($P > 0{\cdot}05$). Viral titers increased on day 3 post-inoculation, reaching a peak on day 7 and then beginning to decline on day 9. Virus was not detected in the lungs of uninfected control mice (data not shown). Thus, treatment with LA-SOD might not affect the amount of virus recovered from infected mouse lungs (Figure 5).

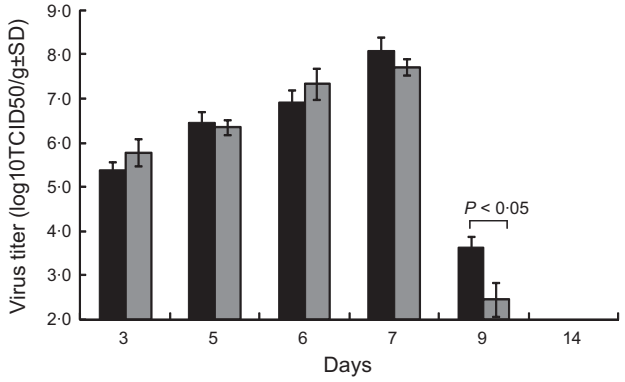

Figure 5. Kinetics of virus replication in the lungs of mice infected with H5N1 virus. After viral infection, three mouse lungs were collected at different time points, and the virus was titrated in Madin–Darby canine kidney cells. Lung titers from three mice per group are expressed as $\log_{10}$ (median tissue culture infective dose)/g ± SD. Black bars represent the infected group, and gray bars, the LA-SOD-treated infected group.

Discussion

Acute lung injury is the principal cause of rapid death in human cases of H5N1 influenza,[26] and OFRs play an important role in the pathogenesis of ALI. Therefore, there is an urgent need to elucidate the role of OFRs in the pathogenesis of H5N1 virus infection. In this study, we used a mouse model to investigate the relationship between OFRs and H5N1 virus-induced ALI.

In unchallenged physiological states, production and scavenging of OFRs maintain their homeostasis. In disease states, however, OFRs may be generated in quantities sufficient to overwhelm antioxidant enzyme systems such as SOD. Overproduction can lead to various forms of cell damage and death[27] and may aggravate the development of disease.[28,29] In this study, we found that the concentrations of ROS and MDA were significantly higher in the lungs of infected mice than those in the control group on days 3, 6, and 7 after infection with H5N1 virus, but SOD activity in the infected mice was significantly lower than that in the control group on days 6 and 7. These results suggest that increased MDA and ROS levels should be correlated with the reduction in SOD activity in H5N1 virus-infected mice, and we deduced that OFRs may be involved in the pathogenesis of H5N1 influenza-induced pneumonia.

To verify the above hypothesis, we investigated the development of the disease by treating the H5N1-infected mice with LA-SOD. First, LA-SOD treatment weakly ameliorates clinical signs of H5N1 infection, but not to a statistically significant degree. For example, the survival rate, body weight, and the food intake were higher in LA-SOD-treated infected mice than those in the infected control mice (Table 1). Second, LA-SOD has minor effect on ameliorating the histopathological lung lesions associated with ALI, including alveolar and interstitial edema, hemorrhage, and severe bronchiolitis and bronchopneumonia (Figure 1), again, not to a statistically significant degree. Third, the lung weights were significantly reduced in the LA-SOD-treated infected mice compared with the untreated infected mice on days 5 and 7 post-inoculation, and some minor decreases of lung wet-to-dry weight ratios in LA-SOD-treated mice were also observed (Figure 2). Fourth, after administration of LA-SOD, the concentrations of ROS and MDA in the SOD-treated infected group were reduced compared with the infected group on days 3, 6, and 7 post-inoculation, although SOD activity was increased compared with the infected control group on those days. These findings demonstrate LA-SOD treatment is mildly effective in inhibiting the ALI development induced by H5N1 virus and indirectly supports the hypothesis that OFRs might play an important role in its pathogenesis.

It is generally accepted that increasing viral load and the resulting proinflammatory response play key roles in the

pathogenesis of ALI.[30] In this study, we observed that patterns of viral replication in the lung were similar in the LA-SOD-treated and infected control groups. While LA-SOD did not alter lung viral titers in H5N1 virus-infected mice, it did decrease the concentration of free radicals in the lung and mildly ameliorated the degree of lung edema and the mortality of the infected mice. Although survival rate did not differ significantly between the LA-SOD-treated infected and infected control groups, the intensity of the pathologic response was slightly reduced in the former. These results are consistent with those from a previous report demonstrating that injection of recombinant human manganese SOD (Mn-SOD) did not alter viral replication titers in the lung tissue of H1N1-infected mice, but did reduce the damaging effects of the viral load.[18]

Adamantanes and the neuraminidase inhibitors can suppress influenza virus replication.[31] However, the emergence of resistant viruses has decreased the efficacy of antiviral drugs in recent years.[32,33] It has been suggested that elimination of ROS and reduction in viral load could be a new strategy for the treatment of influenza patients.[34,35] Some studies have shown that injecting superoxide radical scavenger (such as SOD, GSH) could alleviate the severity of disease following infection with less virulent influenza viruses, such as H2N2 and H1N1.[10,11,15,18] In addition, the virus virulence and the dose and route of injection may affect the therapeutic efficacy of SOD in influenza in mice. Sidwell *et al.* in their work showed a dose- and route-dependent effect of Mn-SOD on influenza in virus-infected mice.[18] In this study, we used a mouse model to study the role of OFR in severe H5N1 infection and found that LA-SOD treatment had a minor inhibitory effect on ALI disease induced by the H5N1 influenza virus in mice. To achieve the best therapeutic effect, other administration routes, doses, and schedules (early and delayed therapy) should be studied in the future.

In summary, our data indicate that OFRs might play an important role in pathogenesis of ALI induced by H5N1 virus, and the LA-SOD treatment has a mildly inhibitory effect on H5N1 influenza virus infection in mice. The pathophysiology of H5N1 influenza virus infection in mice is significantly different from that in humans. In humans, the disease has a longer duration combined with less severe injuries to the lung, so it is probable that the weak effect of LA-SOD treatment in the present study may be accentuated when it is used for human treatment. We also hope this study will aid research on the pathogenesis of H5N1 viral infection in humans.

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Authors' Contributions

HGM, DCG, and QJ conceived and designed the experiments. HGM, DCG, LZH, ZLH, and XT performed the experiments. HGM, DCG, LZH, BMM, and XT analyzed the data. HGM, DCG, BMM, and QJ wrote the manuscript. HGM, LZH, BMM, DCG, ZLH, XT, and QJ read and approved the final manuscript.

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