Combination of ribavirin and Reduning protects mice against severe pneumonia induced by H1N1 influenza a virus

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Abstract

OBJECTIVE: To investigate the effects of ribavirin administration combined with Reduning in a mouse model of influenza A (H1N1)-induced severe pneumonia.

METHODS: Influenza A/Beijing/501/2009 (H1N1)-infected C57BL/6 mice were randomly divided into four experimental groups treated with either a mock injection of phosphate-buffered saline (PBS), ribavirin (66.6 mg/kg daily) or Reduning (86.6 mg/kg daily), or a combination of both, for 7 days. Mice were monitored for clinical signs and survival, and body weight was measured daily for 14 days. Virus titer, lung wet-to-dry ratios, pathology and cytokines including interleukin (IL)-6, IL-10, and interferon (IFN)-γ were assayed on different days.

RESULTS: In the untreated group injected with phosphate buffer saline, all the mice died of the infection. The survival rate of mice treated with Reduning was only 10%, whereas 100% of the ribavirin- and the combination-treated mice survived. Low lung viral loads indicated that ribavirin significantly inhibited virus replication, whereas Reduning did not. Lung wet-to-dry ratios demonstrated that both ribavirin and Reduning, administered together or separately, reduced acute lung edema compared with results in the untreated group. Pathology analyses also showed that treatment with a combination of both drugs relieved pathological lesions, whereas the single drug treatment did not. Levels of IL-6, IL-10 and IFN-γ in mice treated with ribavirin or the combination of both ribavirin and Reduning were all significantly lower than in the untreated group, especially in the combination-treated group. In addition, Reduning administration significantly decreased both IL-6 and IL-10 production but had no effect on IFN-γ.

CONCLUSION: Due to the synergistic effect of antiviral and antiinflammation, the combination of ribavirin and Reduning could be an effective treatment for severe H1N1 which was considered to be significant to delayed antiviral and drug resistant.

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INTRODUCTION

In April 2009, H1N1 swine flu first emerged in Mexico. On August 10th, 2010, the World Health Organization declared influenza A H1N1 (pH1N1) a pandemic. During this period, 214 countries reported confirmed cases of influenza A H1N1; and the disease resulted in 18,449 deaths. Mainland China alone reported a total of 12.8 million cases with 805 deaths and a 0.5% mortality rate. Some severely infected patients developed acute pneumonia, which was the predominant cause of reported deaths. Although the mortality caused by pH1N1 infection was low, 9%-31% of pH1N1 patients required admission to intensive care units, and 14%-46% of these patients ultimately died. To this day, the main treatment strategy for severe pneumonia relies on different types of adjunctive therapies, although those treatments are controversial. The clinical treatment of pH1N1 mainly depends on neuraminidase inhibitors such as oseltamivir, and antiviral compounds such as ribavirin. However, antiviral therapy usually involves a delayed response or has low efficacy. Systemic corticosteroids are often used to treat severe influenza, although several studies have indicated that this may increase the risk of mortality. Chinese medicine has been widely used in the treatment of pH1N1. Some drugs such as Shufengjiedu capsules and Maxingshigan decoctions have shown effectiveness. However, there have been few studies reporting the effects of Chinese medicine on severe influenza. Reduning, one clearing heat and detoxifying Traditional Chinese Medicine (TCM) injection, was mainly used for treatment of acute and severe disease in clinical. Many studies had reported that Reduning could reduce the duration of influenza illness which was attributed to the role in inhibiting NA and anti-inflammation. Compared with antiviral injections, formulations of capsules, decoctions, or granules alone have been unable to provide immediate relief for severe influenza in clinical practice. This study investigated the efficacy of antiviral administration combined with injections of the Chinese medicine Reduning for the treatment of severe influenza.

MATERIALS AND METHODS

Virus, cells and animals
Influenza A/Beijing/501/2009 (H1N1) (BJ501) and Madin Darby Canine Kidney cells were obtained from the Beijing Institute of Microbiology and Epidemiology, State Key Laboratory of Pathogen and Biosecurity. Specific pathogen free (SPF) C57BL/6 female mice, 4-6 weeks old (Experimental Animal Production License No. SCXK 2012-003), weighing 14-16 g, were provided by the Laboratory Animal Center of the Academy of Military Medical Science (Beijing, China).

Drugs and reagents
Ribavirin Injection (Batch No. 1003167, 0.1 g/2 mL) was obtained from the Shandong Lu Kang Chen Xin Pharmaceutical Co, Ltd. (Shandong, China). Reduning (Batch No. 1009022, 6 g/10 mL) was purchased from the Kang Yuan Pharmaceutical Co, Ltd. (Hunan, China). The mouse inflammatory cytokines cytometric bead array was obtained from BD Biosciences (Franklin Lakes, NJ, USA).

Therapeutic efficacy of different drugs or combination against pH1N1 in mice
To evaluate the therapeutic role of ribavirin, Reduning or both against BJ501 infection, following intraperitoneal (i.p.) anesthetization with pentobarbital sodium, C57BL/6 mice received 2 x 10^4 TCID50 of BJ501 intranasally (i.n.) on day 0 as shown previously, followed by i.p. injections of Ribavirin (86.6 mg·kg^-1·d^-1), ribavirin (66.6 mg·kg^-1·d^-1), a combination of both, or a mock injection phosphate buffer saline (PBS) once a day for 7 days post infection (in order to achieving therapeutic drug concentration once infection, each group were administered 12 h before infection. Mice in each group (n = 10) were monitored daily for survival, clinical signs and body weight. Mice from each group were euthanized on different days, and the lungs were collected as described below.

The viral load in the lung tissue of mice
Lung tissues from five mice selected from each group were collected at 4 days post-infection (DPI). The titers of viruses in the lung tissue were determined by the cell culture infective dose 50% (CCID50) assay in Madin Darby canine kidney cells.

Acute pulmonary edema (wet-to-dry ratio)
Assessment of acute pulmonary edema was performed at 4 DPI by calculating the lungs’ wet-to-dry ratios from the weights of wet lungs and the dry weight obtained after heating the tissues at 68 °C for 24 h.

Histological examination
Following pentobarbital sodium anesthesia, two to four mice from each group were sacrificed at 5 DPI. Lungs were fixed in formalin and embedded in paraffin. Ultrathin sections were obtained and stained with hematoxylin-eosin. Lung histopathology was determined by light microscopy (Olympus, Japan).

Cytokine and chemokine measurement
For cytokine measurements, five mice from each group were euthanized and bronchoalveolar lavage fluids (BALFs) were collected at 2 and 4 DPI. BALFs were assessed for interleukin (IL)-6, IL-10 and interferon (IFN)-γ using the cytometric bead array. Array analysis was performed using FCAP Array software.
Statistical analyses
All data are shown as means ± standard deviation ( x ± s ). Survival data were analyzed by Kaplan-Meier survival analysis measurements at single time points and statistics performed using analysis of variance. If they reached significance, data were further analyzed by a two-tailed Student’s t-test. All statistical tests were conducted using GraphPad Prism 5.0 software (Software MacKiev, San Diego, CA, USA). P < 0.05 indicates statistical significance.

RESULTS
Administration of ribavirin and Reduning ameliorated pH1N1-induced acute influenza symptoms
At 14 DPI, none of the mice that received the mock injection survived BJ501 infection (0%). The survival rate was significantly improved when mice were given ribavirin (100%), or a combination of ribavirin and Reduning (100%). However, administration of only Reduning resulted in a low survival rate (10%) (Figure 1A). Loss in body weight in the PBS group was as high as 40% with continuous decline until death. However, mice treated with ribavirin or both ribavirin and Reduning showed a weight loss of only 15%, which stopped declining at 5 DPI and improved at 8 DPI. The therapeutic efficacy of Reduning alone was very low; weight loss in this group was 25%, with a late recovery in weight that began at 10 DPI (Figure 1B). When compared to the mice treated with Reduning alone, both Ribavirin and the combination Ribavirin and Reduning significantly decreased the body weight loss induced by BJ501 infection, especially the combination group (Figure 1C). Acute lung pneumonia, assessed by lung pathology and wet-to-dry weight ratio, was also improved when mice were treated with either of the single drugs or both drugs. The pathological observations showed that both drug treatments improved lung pathological lesions. At 5 DPI, lungs of PBS-injected mice showed congestion, edema and hemorrhage with diffuse inflammatory cell infiltration, shedding and necrosis of alveolar epithelial cells as well as bronchial epithelial cell degeneration. The lungs of the mice treated with both ribavirin plus Reduning displayed significant improvement with reduced edema, local alveolar septal thickening and inflammatory cell infiltration. The lung lesions of the mice treated with either ribavirin or Reduning were alleviated when compared with the PBS-treated group but not to the extent of both drugs administered together (Table 6 and Figure 2). Similarly, wet-to-dry ratios of mice treated with ribavirin alone or ribavirin and Reduning together were significantly reduced compared with the PBS-treated group (Table 1). Notably, the wet-to-dry ratio of mice treated with Reduning alone was also significantly reduced (Table 1).

Administration of both ribavirin and Reduning may induce a synergistic antiviral and immunomodulatory effect
Viral load titers in the lungs of mice treated with ribavirin alone or both ribavirin and Reduning were significantly decreased, suggesting that both treatment methods efficiently inhibited virus replication (Table 1). However, there was no difference in lung BJ501 titers between the treatment with Reduning alone and PBS (Table 1). Compared with the PBS group, administration of riba-
Table 1 Wet-to-dry ratios and viral loads of lungs at 4 days post-infection (x ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Wet-to-dry ratios</th>
<th>Virus titer (logCCID 50 /mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 DPI</td>
<td>P value</td>
</tr>
<tr>
<td>PBS</td>
<td>5</td>
<td>7.3±0.7</td>
<td>-</td>
</tr>
<tr>
<td>Reduning</td>
<td>5</td>
<td>6.2±0.3</td>
<td>0.04</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>5</td>
<td>4.7±0.4</td>
<td>0.00</td>
</tr>
<tr>
<td>Ribavirin plus</td>
<td>5</td>
<td>4.6±0.4</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Notes: PBS group: infected mice treated with PBS (1 mL) by i.p. injection for 7 days; Reduning group: infected mice treated with Reduning (86.6 mg·kg⁻¹·d⁻¹) by i.p. injection for 7 days; ribavirin group: infected mice treated with ribavirin (66.6 mg·kg⁻¹·d⁻¹, 1 mL) for 7 days; ribavirin plus Reduning: infected mice treated with both ribavirin (66.6 mg·kg⁻¹·d⁻¹, 1 mL) and Reduning (86.6 mg·kg⁻¹·d⁻¹, 1 mL) for 7 days. Viral loads in lungs were determined in Madin Darby Canine Kidney cells. DPI: days post-infection; PBS: phosphate-buffered saline. All treatment groups compared with PBS group, P < 0.05.

Table 2 Concentrations of IL-6, IL-10 and IFN-γ at 2 and 4 days post infection (pg/mL, ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-6</th>
<th>IL-10</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 DPI</td>
<td>4 DPI</td>
<td>2 DPI</td>
</tr>
<tr>
<td>Naive</td>
<td>18.6±1.2</td>
<td>18.6±1.2</td>
<td>9.12±1.43</td>
</tr>
<tr>
<td>PBS</td>
<td>30.7±3.3</td>
<td>35.4±6.7a</td>
<td>141.9±75.6</td>
</tr>
<tr>
<td>Reduning</td>
<td>24.9±2.2</td>
<td>39.0±6.0</td>
<td>104.1±18.9</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>22.1±3.3</td>
<td>24.0±8.6a</td>
<td>85.6±64.6</td>
</tr>
<tr>
<td>Ribavirin plus</td>
<td>18.4±3.2</td>
<td>17.6±1.3</td>
<td>27.8±7.6</td>
</tr>
</tbody>
</table>

Notes: Naive group: mice treated with PBS (1 mL) i.p. for 7 days; PBS: infected mice treated with PBS (1 mL) i.p. once a day for 7 days; Reduning: infected mice treated with Reduning (86.6 mg·kg⁻¹·d⁻¹) once a day for 7 days; ribavirin: infected mice treated with ribavirin (66.6 mg·kg⁻¹·d⁻¹) and Reduning (86.6 mg·kg⁻¹·d⁻¹) once a day for 7 days. Concentrations of IL-6, IL-10, IFN-γ in bronchoalveolar lavage fluids samples were determined by cytometric bead array. IL: interleukin; IFN-γ: interferon-γ; DPI: days post-infection; PBS: phosphate-buffered saline. PBS group compared with naive group, *P < 0.05 and all treatment groups compared with the PBS group, **P < 0.05.

DISCUSSION

There is an urgent need for an effective strategy for treatment of severe influenza. Current recommendations for influenza treatment primarily focus on alleviating clinical symptoms and restoring respiratory function.11 Previous autopsy case reports have indicated that lung injury in severe influenza patients included serious interstitial lung injury, hyaline membrane formation, alveolar septal edema, alveolar epithelial cell proliferation, intravascular fibrin thrombosis, and fine bronchial wall necrosis.12 Some pH1N1 cases in teenagers over 18 years of age without any other diseases have displayed histological changes similar to those in

Figure 2 Histological findings from lung tissues. Representative pathological images from the lungs at 5 days post-infection. The lungs of two to four mice from each group were fixed in formalin and embedded in paraffin. Ultrathin sections were obtained and stained with hematoxylin-eosin (× 100).

A: mice treated with PBS showing inflammatory infiltrate and alveolar damage. B: Mice treated with Reduning for 7 days exhibited no difference from the PBS group in terms of histopathology. C: Mice treated with ribavirin for 7 days showed a noticeable reduction in inflammation and tissue damage compared with the PBS group. D: Mice treated with ribavirin and Reduning together for 7 days exhibited the lightest damage compared with anygroup else. PBS: phosphate-buffered saline.
H5N1 infection cases. Thus, the prognosis for severe influenza patients is particularly associated with the severity of lung pathological injuries. Some animal models have validated the fact that virus replication and immune dysregulation with higher levels of proinflammatory cytokines and chemokines play important roles in the pathogenesis of severe influenza. Nowadays, because of the slow progress in developing antiviral agents for influenza, combining available drugs provides an opportunity to generate a more effective treatment than antiviral drugs alone. Adjunctive treatments for influenza, particularly those modulating excessive pro-inflammatory host responses to infection, have been shown to be effective.

As a guanosine analogue, ribavirin is a broad-spectrum antiviral drug that can inhibit both DNA and RNA viruses. There has been no report about the influenza-related viral resistance of ribavirin, which may be related to its unique mechanism of action. A recent study has indicated that the combination of oseltamivir, amantadine and ribavirin has a high degree of synergy in inhibiting viral replication. Also, this regime greatly reduces side effects because of the low dose required for each drug.

Reduning is composed of Artemisia carvifolia, Lonicera japonica Thunb, and Gardenia jasminoides Ellis. Artemisia carvifolia is spicy, bitter and cold, with the function of clearing heat and removing toxins according to the theory of Traditional Chinese Medicine. Reduning exhibits a strong neuraminidase inhibitory activity in vitro neuraminidase inhibitor model. In addition, Reduning can also significantly reduce the levels of IL-8, TNF-α as well as other proinflammatory cytokines in the plasma and BALFs, which may have a role in decreasing the severity of pulmonary edema and improving the pathological changes of acute lung injury.

Our study showed that Reduning alleviated pulmonary edema and suppressed expression of IL-6 and IL-10 in the BALFs of mice infected with BJ501. However, it did not decrease virus titer nor prevent mortality, suggesting limited protection against severe influenza. In contrast, ribavirin as a prophylactic antiviral drug significantly decreased virus titer and reduced lung edema but failed to prevent lung injury. It was found that, even though virus replication had been suppressed by ribavirin, infection remained. This remaining infection could trigger the cytokine storm and drive immunopathological progression. When combined, ribavirin and Reduning showed primarily additive interactions. Mice treated with both drugs had minimal weight loss and lung injury and significant suppression of IL-6 and IL-10 in BALF compared with ribavirin alone. The synergistic effect of the two drugs was clearly more effective than either one administered separately.

This study suggests that Reduning might enhance the activity of ribavirin with an anti-inflammatory mechanism that decreases the production of IL-6 and IL-10 during the early stages of pH1N1(2009) infection. The current data provide an important reference for the combined application of traditional Chinese compounds and antiviral treatments in clinical practice which is helpful for prevention of drug resistant variants in clinical investigations.

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