Reduning plus ribavirin display synergistic activity against severe pneumonia induced by H1N1 influenza A virus in mice

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**Abstract**

**OBJECTIVE:** To investigate synergistic effect of Reduning (RDN) injection plus ribavirin against severe pneumonia induced by H1N1 influenza A virus in mice.

**METHODS:** We established a mouse model of severe pneumonia induced by influenza A virus by infecting Balb/c mice with CA07 virus. We randomly assigned the infected mice into four groups, and treated them with normal saline (NS group), RDN (injection, 86.6 mg/kg), ribavirin (injection, 66.6 mg/kg) or double Ribavirin plus RDN group, the same dosage as used in the single treatments) for 5 d.

Lung index and lung pathology were recorded or calculated in terms of the curative effective. Cytokines, NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome related protein including caspase-associated recruitment domain (CARD) domain Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), caspase-1 and NOD-like receptor family, pyrin domain containing 3 (NLRP3), and reactive oxygen species were simultaneously investigated.

**RESULTS:** RDN plus ribavirin treatment, not RDN or ribavirin alone, provided a significant survival benefit to the influenza A virus-infected mice. The combination treatment protected the mice against severe influenza infection by attenuating the severe lung injury. The combined treatment also reduced the viral titers in mouse lungs and lung index, downregulated their immunocytokine levels, including IL-1β and IL-18, and downregulated the NLRP3, especially the transcription and translation of caspase-1. Meanwhile NS group had significantly higher reactive oxygen species (ROS) expression which could was dramatically reduced by the treatment of RDN plus ribavirin.

**CONCLUSION:** Our study showed that RDN combined with ribavirin could protect the mice, and reduce the lung immunopathologic damage caused by severe influenza pneumonia. The mechanism could be that it reduced ROS produce and inhibited NLRP3 inflammasome activation so that mainly lower the downstream inflammatory cytokines IL-1β and IL-18.
INTRODUCTION

H1N1 influenza A virus first emerged in Mexico in April 2009 and quickly spread worldwide by human-to-human transmission. By June 2009, the World Health Organization (WHO) declared the outbreak to be a H1N1 pandemic. As of August 2010, more than 18,449 deaths from the 2009 H1N1 flu pandemic were reported in over 214 countries or communities according to the WHO. The main concern about an influenza A pandemic is its ability to cause severe pneumonia and mortality in the affected population. A number of antiviral medications used for the clinical management of influenza A, such as neuraminidase inhibitors (e.g. oseltamivir and zanamivir) and broad-spectrum antiviral agents (e.g. ribavirin), have WHO approval for treating or preventing influenza virus infections. These antiviral medications are effective in most patients with such infections. However, antiviral drug resistance has long been a problem for antiviral medications, and that may limit the clinical efficacy of such drugs in future. In view of this, many studies have been performed to test the efficacy of various combinations of antiviral agents. However, the potential clinical benefits of various treatment combinations remain elusive.

Traditional Chinese medicine (TCM) plays an important role in the Chinese National Health System. Chinese herbs are recommended for preventing and treating influenza in China by the Chinese Guidelines for Diagnosis and Treatment of Influenza (2011) (www.ncbi.nlm.nih.gov/pmc/articles/PMC3256530), a document released by the Ministry of Health of China. A series of Chinese herbs are recommended for the treatment of Influenza A virus infections, including naturally extracted herbal products and patented Chinese medicines. Some studies have confirmed the effectiveness of Chinese herbs for treating naturally-acquired influenza infections. However, there is little information regarding the effects of Chinese herbs on severe influenza infections.

Reduning (RDN), a patented TCM injection used for ‘clearing bodily heat and bodily detoxification’, has been reported to have a promising effect in influenza and pneumonia treatment. It is prepared from extracts of Qinghao (Herba Artemisiae Annuae), Jinyinhu (Flos Lonicerae) and Zhizhi (Fructus Gardeniae), a 75-component mixture that includes iridoids, lignans, phenolic acids, flavonoids, caffeoylquinic acid derivatives, 4 sesquiterpenoids and 3 coumarin compounds, and other components. It has been extensively used to treat infectious diseases, especially pulmonary infectious diseases characterized by pyrexia. Some studies have shown that RDN can ameliorate influenza virus-induced lung inflammation by disturbing the nuclear factor kappa-B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, and also relieve oxidative stress injury.

With complementary and alternative medicine being successfully used in clinical practice, and based on the current knowledge about ribavirin and RDN injections, we hypothesized that the combination of these two agents may be an effective strategy for treating severe influenza illness. Our group has previously partly demonstrated the antiviral and anti-inflammation effects of RDN in severe pneumonia induced by influenza A virus. However, the mechanism for RDN against severe pneumonia remains unclear. The question of how RDN functions in severe influenza A-induced pneumonia has recently been a matter of interest for researchers. In this study, we aimed to investigate the synergistic effect of RDN combined with ribavirin, on severe pneumonia induced by H1N1 influenza A virus in mice.

METHODS

Ethics statement

All animal experiments involved in present study were carried out under biosafety level-2 conditions and in strict accordance with the guidelines of the Chinese Regulations of Laboratory Animals (Ministry of Science and Technology of People’s Republic of China) and Laboratory Animal-Requirements of Environment and Housing Facilities (GB 14925-2010, National Laboratory Animal Standardization Technical Committee). All procedures were accredited by the Animal Experiment Committee of Laboratory Animal Center, AMMS, China (IACUC-13-2016-001).

Drugs and reagents

RDN injection (Batch No. 180610, 6 g/10 mL) was purchased from Kang Yuan Pharmaceutical Co., Ltd. (Hunan, China). Ribavirin injection (Batch No. 702026431, 0.1 g/2 mL) was obtained from Shandong Lu Kang Chen Xin Pharmaceutical Co., Ltd. (Shandong, China). The mouse inflammatory cytokine Cytometric Bead Array was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Virus and animals

The influenza A H1N1 virus, A/California/07/2009 (H1N1) (CA07), was kindly provided by the Beijing Institute of Microbiology and Epidemiology, State Key Laboratory of Pathogen and Biosecurity, China. Specific pathogen-free BALB/c female mice, 4-6 weeks old (Experimental Animal Production License No. SCXK 2012-003) and weighing 14-16 g, were obtained from the Laboratory Animal Center of the Academy of Military Medical Science (Beijing, China). All procedures in this study were in accordance with the Principles of the Care and Use of Laboratory Animals.
Study design
To investigate the efficacy of RDN, ribavirin or both against CA07 infection, BALB/c mice were infected intranasally with 2 × 10^7 pfu of CA07 following intraperitoneal (i.p.) anesthesia induced with sodium pentobarbital, and randomly assigned into four groups (n = 6-8 per group). At 0.5 d post infection (dpi), mice were treated (i.p., q.d.) with normal saline (NS group), ribavirin (66.6 mg/kg), RDN (86.6 mg/kg) or both of them (Ribavirin + RDN group) once a day for 5 d post infection, respectively. Another group of mice were infected with PBS, and classified as the normal group (n = 6) (normal group), the infected mice were from each group were euthanized to harvest their lungs for virus load detection, histological examination, lung index calculation or cytokine detection one day after the end of the treatment (at 6.5 dpi).

Viral titer determination
Each lung sample was weighted and homogenized in 1 mL of sterile phosphate-buffered saline (PBS), and subsequently centrifuged at 5000 g for 3 min. The supernatants were harvested for the detection of viral RNA by RT-qPCR. Total RNA of the supernatants was extracted with PureLinkTM RNA Mini Kit (Life Technologies, China), and the primers and probe used in this study were the following: forward, 5'-AACATGGTACCCAGGCGATTGC-3', reverse, 5'-GTGAAAACTGAGGGAGCAATTGAGGTCAGAGTTTCTTT-3', probe, 5'-FA-MAGAGAACTGAGGGAGCAATT-GAGTTTCAG-BHQ1-3'.

Histological examination
Lungs were fixed in 10% formalin. Following the fixation, the specimens were embedded in paraffin, and serially sectioned with a microtome (thickness = 4 μm). The sections were stained with hematoxylin and eosin (HE), and photographed with a digital camera to enable the histopathological changes to be examined. A histological scoring system were adapted to assess the quality of the infiltrates, the degree of luminal exudates, perivascular infiltrates, and parenchymal pneumonia of the HE stained sections.

Real-time PCR
Total RNA, which was extracted from the frozen lung tissue samples using the PureLinkTM RNA Mini Kit (Life Technologies, Shanghai, China) according to the manufacturer’s instructions, was eluted in 50 μL of RNase-free water. RNA was quantified by NanoDrop 1000 Spectrophotometry (Thermo Fisher Scientific, Carlsbad, CA, USA) and diluted to 500 ng/μL. Real-time PCR was conducted in a total volume of 20 μL with the Two-Step PrimeScript™ RT-PCR Kit and the LightCycler®480 system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. The relative quantification of gene expression was assessed by the 2-ΔΔt method.21 and GAPDH served as the internal reference gene. To explore the expression of inflammatory mediators, we measured the mRNA expression of IL-1β, IL-18 and nucleotide-binding oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) inflammasome components including the apoptosis-associated speck-like protein with caspase-associated recruitment domain (CARD) domain (ASC), caspase-1 and NOD-like receptor family NLRP3 in the lung tissues. We set the annealing temperature of RT-PCR at 60 °C. The primers were used are shown in Table 1.

Western blot analysis
Western blot analysis was performed as previously described.31 Total protein was assayed using the previous processed supernatants of homogenizing lungs tissues. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to polyvinylidene fluoride membrane by electrophoretic transfer overnight in TBST (0.1% Tween 20). Blots were blocked in 5% non-fat dried milk for one hour at room temperature and then incubated over night at 4 °C with primary antibodies directed against ASC (1: 1000, Cell Signaling Technology, MA), caspase-1 (1: 500, Adipogen, CA), NLRP3 (1: 500, Adipogen, CA), β-actin (1: 5000, Abcam, Cambridge, UK). The membranes were then treated with enhanced chemiluminescence western blot detection regents (Millipore corporation, Billerica, MA, USA). The binding of specific antibodies was captured by chemiluminescence.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>IL-1β</td>
<td>Forward 5'-CTCCATGAGCTTGTGACAGG-3', Reverse 5'-TGCTGATGACCTACGATGGGG-3',</td>
</tr>
<tr>
<td>IL-18</td>
<td>Forward 5'-GACCTTTGCGTCACTCTCAGG-3', Reverse 5'-CACGCTGTCTTCTTGACACG-3',</td>
</tr>
<tr>
<td>ASC</td>
<td>Forward 5'-CAGTTCTGTCAGAGCAGCACCA-3', Reverse 5'-CTGCTCCAGGTCATCAACCA-3',</td>
</tr>
<tr>
<td>Caspase-1</td>
<td>Forward 5'-ACTGCTACGAGCTTTGCCCTCA-3', Reverse 5'-CTGGGAGCGCAGAATTC-3',</td>
</tr>
<tr>
<td>NLRP3</td>
<td>Forward 5'-ACTGAAAGCCATCTGCTGACAAC-3', Reverse 5'-AACATGGCAGGAGTCTGACAC-3',</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward 5'-GGCAGAAGTCAAGGGCTGAGAATG-3', Reverse 5'-ATGGTGTTGAAGACAGCCATCA-3'</td>
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Cytokine measurement
The whole lung was removed and homogenized in 1 mL of PBS containing protease inhibitors (Thermo Fisher Scientific, Carlsbad, CA, USA). The homogenates were centrifuged at 1400 × g for 15 min, and the supernatants were then passed through a 0.45 μm-pore-sized filter. The filtered supernatants were collected for cytokine analysis with a MILLIPLEX MAP Kit according to manufacturer’s instructions. The data were collected with a Luminex 200 instrument and analyzed by Luminex xPONENT software (Thermo Fisher). Array analysis was carried out using FCAP Array software.

Immunofluorescence test of frozen section
Autoimmunofluorescence quenching: use a pen to draw a circle around the tissue (to prevent the antibody from flowing away), add autoimmunofluorescence quenching agent into the circle for 5 min, and then rinse with running water for 10 min. Dying: after the frozen sections were slightly dried, reactive oxygen species (ROS) dye solution was added to the ring and incubated at 37 °C in a light-proof incubator for 30 min. 4’,6-diamidino-2-phenylindole (DAPI) retained nuclei: slides were placed in PBS (pH 7.4) and washed by shaking on the decolorization shaker for 3 times, 5 min each. After the sections were slightly dried, DAPI dye was added into the loop and incubated in dark for 10 min at room temperature. Sealed slides: the slides were placed in PBS (pH 7.4) and washed by shaking on the decolorization shaker for 3 times, 5 min each time. The slices were slightly shaken dry and sealed with anti-fluorescence quenching sealant. Microscopy: sections were observed and images were collected under a fluorescence microscope. The nuclei dyed by DAPI are blue under UV excitation, and the positive expression is red labeled with corresponding fluorescein.

Statistics
Data are expressed as box-and-whisker diagrams unless indicated otherwise. All data are expressed as the mean ± standard deviation (x ± s). Survival data were analyzed by Kaplan-Meier survival analysis measurements at single time points. One-way analysis of variance was conducted to test the differences between groups using GraphPad Prism 5.0 software (Software MacKiev, San Diego, CA, USA). A two-sided P < 0.05 was considered statistically significant.

RESULTS
H1N1-induced mortality and infection-related lung pathology in mice
To investigate the efficacy of ribavirin plus RDN, we treated H1N1-challenged mice with ribavirin, RDN or both of them. As shown in Figure 1 A, the survival rate of mice treated with ribavirin (62.5%) or RDN (37.5%) alone was better than that of the NS group (25%), despite no statistical differences. Strikingly, the treatment of RDN plus ribavirin resulted in a significantly greater survival benefit for challenged mice (87.5%), compared with NS (P < 0.05). Further lung index analysis showed that the combined treatment resulted in a significantly lower lung index for challenged mice compared with ribavirin or RDN treatment alone, indicating that reducing lung index may be a direct effect of the combined treatment (Figure 1B).

Figure 1 Combined treatment of ribavirin and RDN reduced the mortality of H1N1 virus infection for mice, and downgraded the lung index and viral titer
A: survival rate; B: lung index; C: virus titer. NS group, injection of normal saline (i.p.,q.d.,153.2 mg/kg) for 5 d post infection; ribavirin group, injection of ribavirin (i.p.,q.d., 66.6 mg/kg) for 5 d post infection; RDN group, injection of RDN (i.p.,q.d., 86.6 mg/kg) for 5 d post infection; The ribavirin plus RDN group, injection of both Ribavirin(i.p.,q.d., 66.6 mg/kg) and RDN (i.p.,q.d., 86.6 mg/kg) for 5 d post infection; NS: normal saline, RDN: Reducing. *P < 0.05 , compared with the NS group; **P < 0.05 , compared with the ribavirin group; ***P < 0.05 , compared with the RDN group.
Meanwhile, as shown in Figure 1C, viral titer in the lungs of mice treated with both ribavirin and RDN is significantly lower than other treatment groups, indicating that the combined treatment has a better efficacy of inhibiting H1N1 virus replication. The histopathological examination showed significant edema, hemorrhage, congestion and diffuse inflammatory cell infiltration, along with shedding, degeneration and necrosis of the alveolar epithelial cells and bronchial epithelial cell degeneration (Figure 2A), indicating that the H1N1 infection model was successfully built. Comparing with the NS-treated mice, mice received combined treatment showed lower level of edema, hemorrhage, congestion and diffuse inflammatory cell infiltration (Figure 2D). The histopathological scores further indicated that the combined therapy lead lowest lung histopathological changes.

**mRNA transcription of ASC, caspase-1 and NLRP3**

Previous studies demonstrated that severe acute lung injury after influenza infection is related to excessive NLRP3 inflammasome activation. To confirm whether the lighter lung injury lead by the combined treatment is also related to the NLRP3 inflammasome activation, we examined the gene transcription of its three components, including ASC, caspase-1 and NLRP3. The mRNA transcription of ASC, caspase-1 and NLRP3 was detected by qPCR. As shown in Figure 3, treatment of RDN plus ribavirin significantly reduced the mRNA transcription of ASC, caspase-1 and NLRP3. The level of relative mRNA of ASC and caspase-1 were predominately decreased by single RDN treatment. However, the ribavirin treatment had no significant influence on any of these three gene transcriptions (Figure 3).

**ASC, caspase-1 and NLRP3 in lung homogenates**

NLRP3 inflammasome including ASC, caspase-1 and NLRP3 in lung homogenates was determined by Western blotting. We found that influenza A virus in the lungs of mice could dramatically result in reduction of ASC ($P < 0.05$). The decreased ASC in infected mice could be significantly upgraded by RDN plus ribavirin treatment which was nearly equivalent with that of normal group ($P < 0.05$). Compared with the NS group, treatment with RDN or ribavirin alone showed no significant impact on the activation of ASC. However, NLRP3 secretion in normal group had no significant difference compared with NS group. Moreover, monotherapy or double combination treatment could significantly reduce the transcription and translation of caspase-1 (Figure 4).

**Transcription and secretion of IL-1β and IL-18**

It has been demonstrated that active caspase-1 can further cleave pro-IL-1β and pro-IL-18 into their mature forms, which directly lead lung immunopathological damage. So, following the results we got in last step,
we further investigated the transcription and secretion of these two cytokines. As shown in Figure 5, we found that the concentrations of IL-1β and IL-18 (Figure 5B, 5D), as well as the mRNA transcription of these two cytokines (Figure 5A, 5C), were significantly reduced by the treatment of RDN plus ribavirin.

**Expression of ROS**

As ROS plays an important role in activating the Caspase-1 of the NLRP3 inflammasome, we further investigated the expression of ROS in the lungs of mice treated with RDN, ribavirin or both of them by IFA. The results showed that the infected mice had significantly higher ROS expression levels in the lungs compared with the uninfected group. However, the upgraded ROS expression in the infected mice was significantly downgraded by the treatment of RDN plus ribavirin. Treatments using ribavirin or RDN alone did not have effects of ROS regulation (Figure 6A-D). The integrated optical density (IOD) of each group also confirmed these findings (Figure 6E).
DISCUSSION

Influenza virus is sensed by specific intracellular receptors leading to activation of the innate immune system, which promotes an antiviral response designed to reduce virus replication and eliminate pathogen. Three major classes of pattern recognition receptors (PRRs) involved in influenza virus infection, which initiate various signaling pathways of inducing inflammatory mediators are toll-like receptors (TLRs), retinoic acid inducible gene-1-like receptors (RLRs) and nucleotide and oligomerization domain, leucine-rich repeat-containing proteins (NLRs). Among NLR family members, nucleotide and oligomerization domain, leucine-rich repeat-containing protein family, NLRP3 inflammasome, which primarily assembled by a receptor molecule, the adaptor molecule apoptosis-associated speck-like protein containing ASC, and caspase-1, is now widely studied and thought to be associated with severe influenza illness. The excessive activation of inflammasome recruiting various inflammatory cells into the lung and generating a cytokine storm results in severe pathological injury. Highly inflammatory cytokines of IL-1β and IL-18 attached to the IL-1 family, whose proteolytic activation is under the control of NLRP3 inflammasome are central to accelerate mediating lung inflammation. There is evidence showing that NLRP3 inflammasome activation aims to drive acute lung inflammation conducing to clear virus. While persistent activation of NLRP3 inflammasome may be detrimental and associated with organ and tissue injury. Other cytokines or chemokines, such as IL-8, aiming at recruiting and activating neutrophils and lymphocytes, are as well as critical for adjusting the acute immune response and inflammation.

At present some studies demonstrated that there are mainly three mechanisms facilitating inflammasome assembly during influenza virus infection, including perturbation of ionic balance, lysosomal rupture and oxidative stress in the form of ROS. ROS plays a positive regulatory role in immune activation which could induce the activation of immune cells and at the same time cause immunopathological injury, in which the proliferation of T lymphocytes is the key to the immune response. Meanwhile, there is an interaction between inflammatory reaction and oxidative stress. On the one hand, inflammatory cells produce ROS and participate in oxidative stress. On the other hand, ROS can also activate NF-κB, leading to increased expression of inflammatory cytokines, chemotaxis of neutrophils and other inflammatory cells, and enhance inflammatory response. Recent studies have found that a large amount of ROS produced in mitochondrial dysfunction is a key regulatory signal for the activation of NLRP3 inflammasomes. After the addition of reactive oxygen ROS inhibitor acetylcystine to cells, intracellular caspase-1 activation level is significantly reduced, and the production of mature IL-1β is also significantly reduced. While overproduction of ROS initiates a signal of NLRP3 inflammasome which widely implicated in severe cases of influenza infection.

In our study, we found that the treatment of ribavirin plus RDN significantly decreased the mortality rate of mice infected by H1N1 influenza A virus, whereas monotherapy with ribavirin or RDN had lower efficacy. Besides, the lung index was much lower, and the pathological damage was lighter in the combination treatment group compared with monotherapy groups or saline group. These results indicate that the treatment of ribavirin plus RDN had a synergistic effect against influenza A-induced death by reducing lung pathological damage.

Increasing evidence supports the concept that tissue damage is highly associated with excessive inflamma-
tion response. To discuss the mechanism of the combination treatment, we detected many kinds of inflammatory cytokines, including IL-1β and IL-18. It showed that combination treatment could only dramatically decrease the transcription and translation of IL-1β and IL-18 which indicated that the effective target of the combination treatment probably lied in down-regulated expression NLRP3 inflammasome. We further detected the mRNA and protein of ASC, caspase-1 and NLRP3. As we predicted, we found that the mice in combination treatment group showed lower transcription level of ASC, NLRP3 and caspase-1 than those mice in monotherapy group. But in terms of protein expression, NLRP3 seemed to be no significance in pathogenicity in this mouse model due to no difference between normal group and NS group. While, ASC protein in all of treatment groups gradually ascended that could not explain the reduction of downstream inflammatory cytokines IL-1β and IL-18. We assumed that the combination treatment mainly reduced caspase-1 activation level to down regulate downstream inflammatory.

ROS production is one of the key mechanisms mediating NLRP3 inflammasome activation. The ROS scavenger N-acetylcysteine can inhibit the activation of intracellular caspase-1, and reduce the production of cytokine IL-1β induced by influenza virus infection. Based on the information, we further tested ROS production in lungs of different group mice to confirm whether the NLRP3 inflammasome activation is caused by the ROS expression change by IFA. Results show that the H1N1-infected mice had significantly higher ROS expression in the lungs compared with the uninfected group, and the upgraded ROS expression in the infected group was significantly downgraded by the treatment of RDN plus ribavirin, indicating that the inhabitation of NLRP3 inflammasome activation may be caused by the down regulation of ROS affected by combination treatment.

One limitation in the present study was that we only focused on signaling pathways related to the nucleotide and oligomerization domain, NLRs. In fact, three major classes of pattern recognition receptors are involved in influenza virus infection, which initiate various signaling pathways or the induction of inflammatory mediators. We should further probe how combination treatment via ROS-NLRP3 inflammasome signaling pathway to reduce inflammatory cytokines and reverse the key enzyme to verify this pathway in vitro or in vivo.

In conclusion, our findings showed that RDN combined with ribavirin protected against lethal pH1N1 infection by inhibiting lung immunopathologic injury. The combined therapy inhibited viral replication and repressed inappropriate aggressive immune responses probably by restraining ROS level and NLRP3 inflammasome activation. This indicated that RDN can be used as a novel treatment alongside existing antiviral therapies in severe influenza. These findings are the first steps towards clinical application of RDN combined with ribavirin against H1N1 influenza A infection. Future studies are needed to provide a detailed immunomodulation timeline and to explore the possibility of resistance to the treatment of RDN combined with ribavirin.

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