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ORIGINAL ARTICLE

Polygonum chinense Water Decoction Lessens Acute Lung Injury in Mice Induced by Influenza Virus

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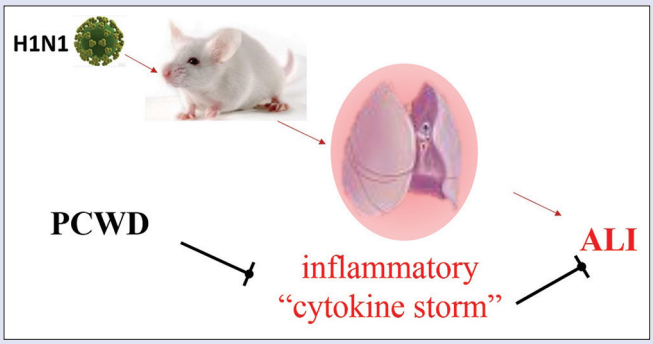
ABSTRACT

Aim/Background: In traditional Chinese medicine, *Polygonum chinense* has been used to treat influenza. However, little information is available from current studies regarding the anti-influenza pharmacological activities of *P. chinense* water decoction (PCWD) and its underlying mechanisms. The present study aimed at investigating the treatment of PCWD on acute lung injury (ALI) induced by the H1N1 influenza A virus (IAV) and its underlying mechanisms. **Materials and Methods:** Mice were infected with IAV. PCWD (300 and 600 mg/kg/day) and ribavirin (100 mg/kg/day) were orally administrated to mice. Mice survival rate was observed for 15 days after inoculation. On day 5 after virus infection, serum and lung tissues of mice were collected for the analysis of lung index, virus titers, cytokines, histopathology, and immunohistochemistry. **Results:** PCWD significantly lessened ALI and improved survival rate induced by H1N1. PCWD also decreased the 5th day lung index and ameliorated the injuries, inflammatory cells infiltration, and lung edema. PCWD reduced the level of tumor necrosis factor-α and interleukin-6. PCWD obviously decreased hemagglutinin titer in the lungs. Immunohistochemistry showed that PCWD obviously inhibited TLR-4 and p-NFκB p65 expression. **Conclusion:** PCWD can alleviate ALI induced by IAV through inhibiting inflammation.

Key words: Acute lung injury, anti-inflammation, H1N1, *Polygonum chinense* water decoction, survival rate

SUMMARY

- This is the first study showing the effects of *Polygonum chinense* water decoction and its signaling pathways.



Abbreviation used: PCWD: *Polygonum chinense* water decoction; ALI: Acute lung injury; IAV: H1N1 influenza A virus; SARS: Severe acute respiratory syndrome; LD_{50}: Median lethal dose.

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INTRODUCTION

The World Health Organization estimated that approximately one billion people were infected and up to 500,000 people died from influenza each year.[1] The most severe cases were presented with pneumonia with acute lung injury (ALI) that then progressed to severe respiratory failure and acute respiratory distress syndrome (ARDS). The disease resembled the disease in patients infected with highly pathogenic avian influenza A virus or severe acute respiratory syndrome virus.[1,2] However, influenza continues to evolve and antiviral drugs are usually not given in the early stage of virus infection, thus rendering the antiviral therapy ineffective. It is suggested that the host immune response has the potential advantage of exerting less-selective pressure on viral populations.[3-6]

Polygonum chinense Linn. is a perennial herb and belongs to the family of *Polygonaceae*. In Southwest China, *P. chinense* has been widely used for the treatment of inflammatory diseases and infections.[7-9] In this study, we used the *Polygonum chinense* water decoction (PCWD) in the BALB/c mice model of H1N1 virus infection to explore whether virus-induced severe pneumonia could be treated and to investigate the possible mechanism.

MATERIALS AND METHODS

Plant materials and preparation of *Polygonum chinense* water decoction

The dried whole plants of *P. chinense* Linn. were purchased from Guangzhou medical company (Guangzhou, China) in December 2012 and authenticated by Prof. Rongkui Liu. A voucher specimen (YPA2L0003) has been deposited in the Department of Infectious Disease, Ji'nan Traditional Chinese Medicine Hospital, Ji'nan, China. The dried herbs 1 kg were ground into fine particles and extracted using 8 L water for 2 h with a heating reflux method. The extract solution was filtered

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and concentrated using a rotary evaporator. The total phenolic acids in the extract were 2.24% determined at 312 nm by an ultraviolet spectrophotometer, using ellagic acid as the reference solution. Then, the extract was dried to obtain a powder on an atomizing drier named PCWD.

Animals and main reagents

Male BALB/c mice of about 15–17 g were purchased from Shanghai SLACCAS Laboratory Animal Co., Ltd. (Shanghai, China). All experimental protocols were approved by the Animal Experiment Committee of Ji'nan Traditional Chinese Medicine Hospital (Approval No. AECTCM20160208).

Mice (rabbit) anti-TLR-4a and mice (rabbit) anti- p-NF-κB p65 were obtained from Abcam (Shanghai, China). Mice tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 ELISA kits were purchased from Shanghai Boatman Biotech Co., Ltd. (Shanghai, China). Ribavirin (No. 140629) was obtained from WeeBeyonnd Scientific and Trade Co. Ltd. (Shanghai, China).

Viruses

A mouse-adapted strain of influenza virus A/FM/1/47 (H1N1) supplied by the Shanghai Center for Disease Control and Prevention (China) and stored in aliquots at −70°C. For each experiment, a new aliquot was thawed to ensure the sample's integrity.

Survival experiments

Mice were randomly divided into five groups of 10 mice each. All groups except the control group were infected intranasally with influenza virus A/FM/1/47 H1N1 under isoflurane anesthesia at a $6 \times LD_{50}$ (median lethal dose) dose in a volume of 30 μL per mice. The treatment was initiated 2 h after virus challenge. Mice were gavaged once daily for 7 days with distilled water (control group, model group), 300 mg/kg/day of PCWD (PCWD 300 group), 600 mg/kg/day of PCWD (PCWD 600 group), and 100 mg/kg/day of ribavirin (Ribavirin group). The survival of mice was monitored for 14 days after the virus challenge.

Lung injury experiments

Mice were randomly divided into five groups of six mice each. All mice except the control group were infected intranasally with influenza virus A/FM/1/47 H1N1 under isoflurane anesthesia at a $3 \times LD_{50}$ dose in a volume of 30 μL per mice. The treatment was initiated 2 h after virus challenge. Mice were gavaged once daily for 4 days with saline solution (control group, model group), 300 mg/kg/day of PCWD (PCWD 300 group), 600 mg/kg/day of PCWD (PCWD 600 group), and 100 mg/kg/day of ribavirin (Ribavirin group) and were then sacrificed by euthanasia at the 5th day to the harvest lung. The lung tissues were weighted and the lung left lobes were suspended in phosphate-buffered saline (PBS)-buffered formalin for pathological observation and the rest of the lung was stored at −70°C for the analysis of cytokine/chemokine.

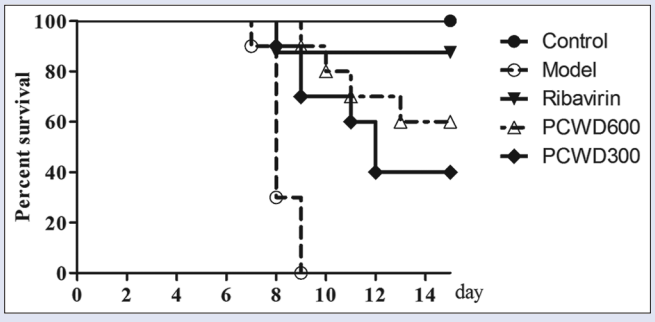

Figure 1: Effect of *Polygonum chinense* water decoction on survival rate of H1N1 influenza A virus (IVA)-infected mice

Histological examination

Histological examination of the lung tissues from each group was performed to evaluate the severity of H1N1-induced lung injury. Tissue specimens were fixed in PBS-buffered formalin for 24 h, embedded in paraffin, sliced into 4 μm-thick sections, and stained with hematoxylin and eosin for pathological observation.

Assay for pulmonary viral titer

Viral load was presented as hemagglutinin (HA) titer according to a previous study.[10] In brief, each lung was homogenized in PBS (10%, w/v) and then serially diluted twofold. Fifty microliter of homogenate dilution and 1% suspension of chicken red blood cells in PBS were added to each well of V-bottom microplate. The suspensions were mixed and incubated at room temperature for 30 min. HA was then added and the endpoint of titration was the final dilution of homogenate that agglutinated red blood cells. The HA titer was calculated.

Measurement of inflammatory cytokines

Anti-mice TNF-α and interleukin-6 antibodies were used to coat the 96-well filtration plates. Lung homogenates were prepared at a concentration of 100 mg tissue/ml PBS and then centrifuged for assay using specific sandwich ELISA kits according to the manufacturer's instructions.

Immunohistochemical analysis

Four-μm-thick, formalin-fixed, paraffin-embedded tissues were immunostained using the streptavidin-biotin-horseradish peroxidase method. The deparaffinized sections were rehydrated through a graded series of alcohol and then microwaved in EDTA antigen retrieval buffer (pH 8.0) at 97°C for 12 min to unmask antigen epitopes. The sections were treated with 3% hydrogen peroxide methanol solution for 10 min to block endogenous peroxidase and then incubated with 100 μL serum for 30 min at 37°C. The serum was removed and the sections were incubated with primary antibody (TLR-4, p-NF-κB p65) dilution overnight at 4°C and then with ready-to-use HRP-labeled secondary antibody at 37°C for 30 min each. The tissues were stained in 3'3-diaminobenzidine substrate and then counterstained with hematoxylin, dehydrated, and mounted.

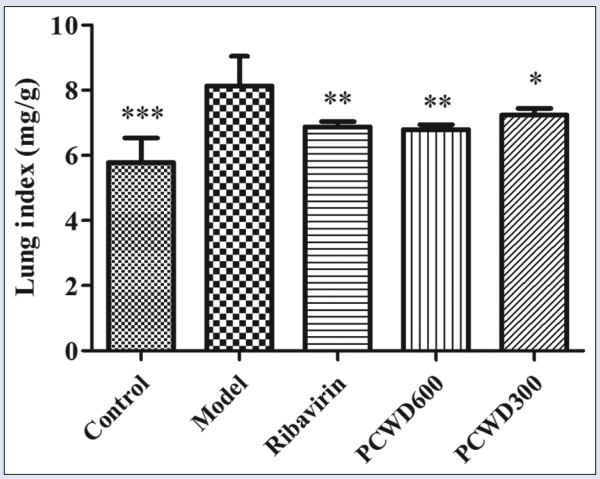

Figure 2: Effect of *Polygonum chinense* water decoction on lung index of H1N1 influenza A virus-infected mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus model. Data were expressed as mean ± standard deviation, $n = 6$

Statistical analysis

The significance between different groups was analyzed by one-way analysis of variance (ANOVA) with Dunnett's posttest. Differences in HA titer between the groups were analyzed using a two-way ANOVA with Bonferroni post-test. The significance between survival curves was analyzed by Kaplan–Meier survival analysis with a log-rank test.

RESULTS

Polygonum chinense water decoction improved survival rate

BALB/c mice showed signs of piloerection, lethargy, and weight loss on the 3rd day after infection with the H1N1 virus and some of them died from 7 to 9 days. Figure 1 shows that no mice survived in the model group (0/10), while the survival rate at the 14th day was 40% for the PCWD 300, 60% for the PCWD 600, and 90% for the Ribavirin group, respectively, which were significantly higher than the model group.

Mice were infected intranasally with the influenza virus at a 6 × LD_{50} dose and then treated with distilled water (control group, model group), PCWD at a dose of 300 and 600 mg/kg/day (PCWD 300 group, PCWD 600 group), or ribavirin at a dose of 100 mg/kg/day (Ribavirin group). Survival of mice was monitored daily for 15 days after a viral infection, and the survival rate was calculated (n = 10).

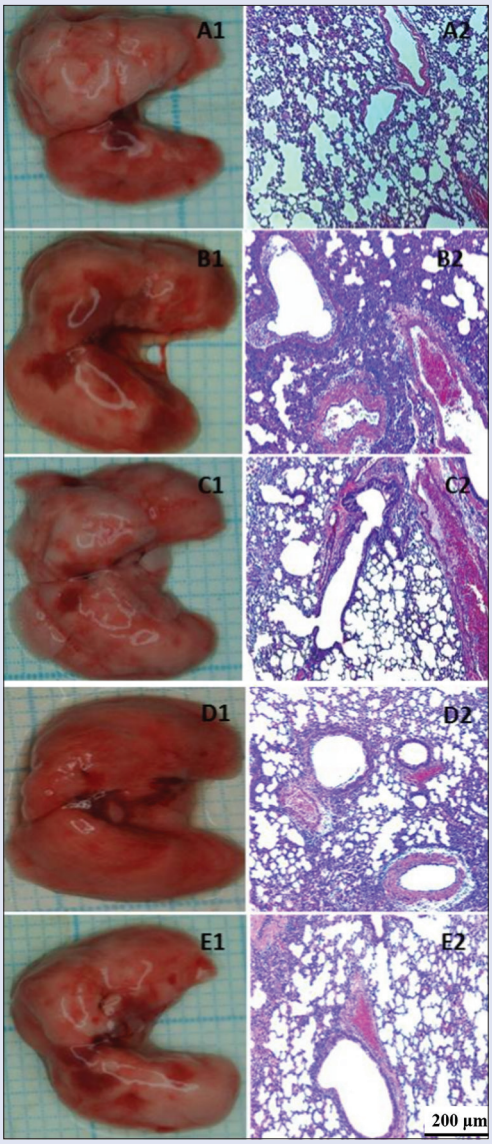

Figure 3: Effect of *Polygonum chinense* water decoction on lung and H and E stained (×200) of H1N1 influenza a virus-infected mice lung tissues collected at day 5 post infection. A: Control group; B: Model group; C: Ribavirin group; D, E: *Polygonum chinense* water decoction 300, 600 group. On day 5 after infection, mice showed pathological damages of acute viral pneumonia. Scale bars, 200 μm

Polygonum chinense water decoction ameliorated pulmonary damage

The main pathological damage observed in mice infected with the influenza virus was viral interstitial pneumonia that occurred on the 5th day after virus inoculation. The lung/body index [Figure 2] in model group mice was increased, indicating that influenza A (H1N1) infection caused swelling of the lung tissues. In the microcosmic view, most infected mice showed severe infiltration of monocytes and lymphocytes, thickened alveolar walls, and exudation of the inflammatory cell into the alveolar space. PCWD markedly ameliorated the pathological injury induced by H1N1 in a dose-dependent manner, as it was found that PCWD at 600 mg/kg/day significantly decreased the number of influenza-related focal lesions and lung consolidation [Figure 3] and lung/body index as well as the infiltration of monocytes and lymphocytes.

Polygonum chinense water decoction decreased pulmonary tissue viral titer

HA is the predominant surface glycoprotein of influenza virus and its expression is positively correlated with copies of the influenza virus. To determine whether PCWD has an influence on virus replication *in vivo*, HA titer and virus titer in lung tissues were measured on day 5. Figure 4 shows that HA in the lungs was significantly higher in model mice than those mice treated with PCWD at a dose of 600 mg/kg/day ($P < 0.05$), as well as the Ribavirin group ($P < 0.001$).

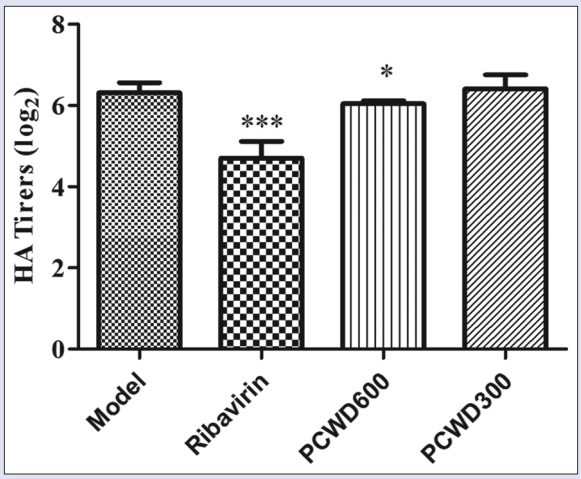

Figure 4: Effect of *Polygonum chinense* water decoction on virus hemagglutinin titer in lungs of H1N1 influenza A virus-infected mice at day 5 postinfection. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus model. Data were expressed as mean ± standard deviation, n = 6

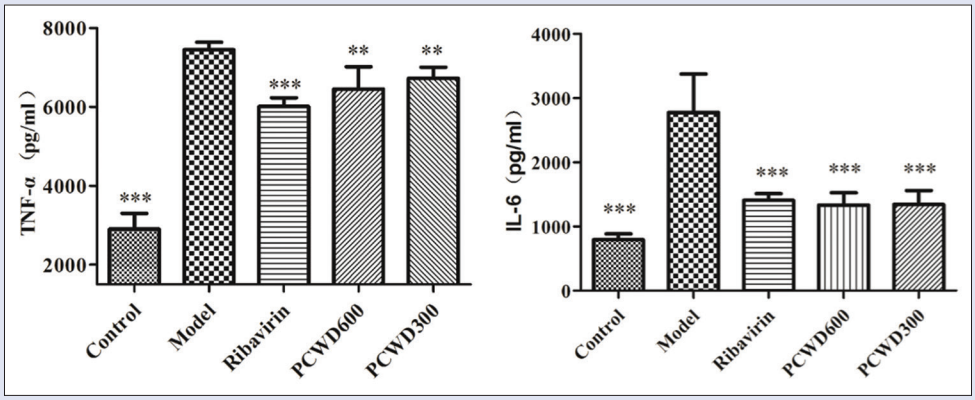

Figure 5: Effect of *Polygonum chinense* water decoction on inflammatory markers in lung homogenates of H1N1 influenza A virus-infected mice at day 5 postinfection. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus model. Data were expressed as mean ± standard deviation, $n = 6$

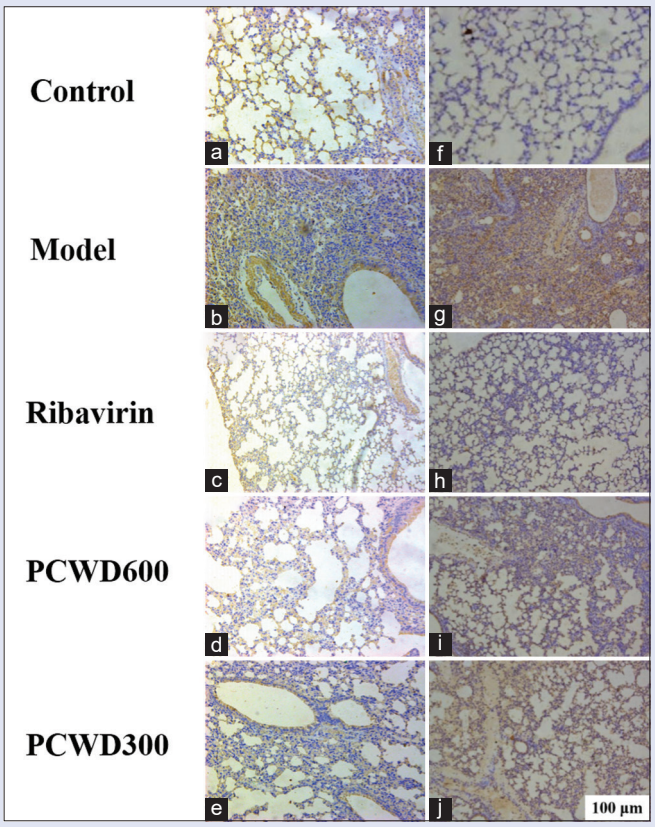

Figure 6: Immunohistochemical expression of TLR-4 (a,b,c,d,e) and p-NFκB p65 (f,g,h,i,j) in the lungs (×400) at day 5 postinfection. Scale bars, 100 μm

Polygonum chinense water decoction decreased inflammatory in the lungs

Influenza virus infection is known to induce inflammatory reaction and it is hallmarked by the production of cytokines. The lungs were collected on the 5th day after infection for the measurement of cytokines. It is shown in Figure 5 that PCWD at a dose of 300 and 600 mg/kg/day significantly inhibited the production of IL-6 and TNF-α.

Immunohistochemistry

Immunohistochemistry with antibodies to TLR-4 and p-NF-κB p65 was assessed. A difference in staining intensity was observed between the noninfected and infected cell areas in lung tissues. Figure 6 shows that H1N1 infection significantly upregulated TLR-4 and p-NF-κB p65 expression in the model group, but their expression could be inhibited by PCWD at a dose of 300 and 600 mg/kg/day.

DISCUSSION

In the present study, we established a mice model of H1N1 virus-induced ALI. The H1N1 virus is characterized by dysregulation of the host immune responses, as indicated by high levels of inflammatory "cytokine storm." Excessive inflammation due to overabundant production of pro-inflammatory cytokines and lung inflammatory infiltrates is considered an important factor in disease pathogenesis.[11] IL-6 expression and TNF-α secretion are directly linked to host morbidity and pulmonary injury.[10] The administration of PCWD decreased the inflammatory cytokine and increased the anti-inflammatory cytokine in the ALI mice. Therapeutic antagonism of TLR-4 signaling would protect against influenza-induced ALI.[6,12] The immunohistochemistry results showed that TLR-4 and p-NF-κB p65 expression could be inhibited by PCWD in the ALI mice.

CONCLUSION

PCWD can be used to treat viral pneumonia, and the underlying mechanism may be related to its modulatory effect on reducing the inflammatory reaction, then reducing the virus reproduction in airway epithelial tissue,[13] and eventually reducing the mortality rate. PCWD treatment remarkably reduced the ALI and systemic inflammation, which may be a promising adjunctive therapy for severe viral pneumonia.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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