Xiaochaihu decoction alleviates viral pneumonia by regulating macrophage polarization

Feng Chen1, Fei Qu1, Yu-Long Shi1, Feng Zhang1*

1Department of Respiratory, Jiaxing Traditional Chinese Medicine Hospital, Jiaxing 314000, China.

*Corresponding to: Feng Zhang, Department of Respiratory, Jiaxing Traditional Chinese Medicine Hospital, No. 1501 Zhongshan East Road, Nanhu District, Jiaxing 314000, China. E-mail: sy2007@yeah.net.

Abstract

Background: In this investigation, we sought to evaluate the benefits of Xiaochaihu decoction (XCHD) on polyinosinic-polycytidylic acid-induced viral pneumonia in mice and elucidate its mechanisms of action. Method: A viral pneumonia model was established in mice using polyinosinic-polycytidylic acid, with mice being intragastrically administered different doses of XCHD. The benefits of XCHD therapy for mice with viral pneumonia were assessed by determining the weight ratio of lung tissue, wet-to-dry, overall protein concentrations, and total cell counts in bronchoalveolar lavage fluid, and hematoxylin and eosin staining of lung tissues. By determining the interleukin-1β levels, interleukin-6, tumor necrosis factor-alpha, nitric oxide, interleukin-10, and interleukin-4, and the mRNA and protein expression of nitric oxide synthase 2, arginase-1, and macrophage mannose receptor 1 in bronchoalveolar lavage fluid, we assessed consequences of XCHD on macrophage polarization with mice suffering from viral pneumonia. Results: XCHD was found to significantly reduce lung tissue wet-to-dry and the total protein content and total number of cells of bronchoalveolar lavage fluid, while ameliorating pathological modifications to the lung tissues of rodents suffering from viral pneumonia, thereby indicating that this medicinal preparation has a healing impact on model mice with viral pneumonia. In addition, XCHD was found to reduce the magnitudes of interleukin-1β, interleukin-6, tumor necrosis factor-alpha, and nitric oxide and the mRNA and protein manifestation of nitric oxide synthase 2, and promote an increase in the levels of interleukin-10 and interleukin-4 and the mRNA and protein expression of arginase-1 and macrophage mannose receptor 1, thereby indicating that XCHD can favorably mediate polarization of macrophages in mice with viral pneumonia. Conclusion: XCHD has notable therapeutic effects on viral pneumonia in mice, the fundamental workings of action of which may be connected to regulation of macrophage polarization.

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**Introduction**

Viral pneumonia arises from a viral infection in the upper respiratory tract that descends and infects the lungs, resulting in lung inflammation and ultimately leading to pulmonary ventilation dysfunction [1]. It is a ubiquitous, rapidly progressive infection associated with a significant rate of mortality. Additionally, the high variability and complex pathogenesis of the virus severely restrict the evolution of effective shots and antiviral medications. Consequently, discovering safe and efficient treatments for viral pneumonia can lessen the risks to the public's health and the strain on associated healthcare systems [2].

For hundreds of years, traditional Chinese medicine (TCM) has amassed vast theoretical and practical knowledge in combating epidemic diseases. TCM is guided by an overarching philosophy and treatment based on syndrome differentiation, providing effective treatment prescriptions in cases in which specific medications have yet to be clinically applied. In accordance with the principle of the same treatment for the same disease, it is essential to strengthen the prevention and treatment capabilities of TCM for viral pneumonia by clarifying its modern scientific connotation in treatment under the guidance of TCM theory. In this context, we sought to clarify the modes of action of the medicinal formulation Xiaochaihu decoction (XCHD) in managing viral pneumonia by evaluating its treatment outcomes for viral pneumonia in mice and examining its underlying molecular effects on macrophage polarization.

**Materials**

**Animals**

Beijing HFK Bioscience Co., Ltd. supplied adult male C57BL/6 mice that were specifically pathogen-free and weighed 20 ± 2 g. The mice were kept in a specifically pathogen-free-grade clean environment, with five mice per cage, at 22 ± 2 °C and 50% ± 15% humidity. They were also kept on a 12-h/12-h light-dark cycle, with free access to food and water. All animal procedures performed in this study were initially conducted by the National Institutes of Health Guide for Care Use of Laboratory Animals and approved by the Animal Care Ethics Committee of Jiaxing Hospital of Traditional Chinese Medicine (approval no. 2023-0023).

**Medicines and reagents**

XCHD (Bupleuri Radix 30 g, Scutellariae Radix 9 g, Ginseng Radix 9 g, Pinelliae Rhizoma 9 g, Glycyrrhizae Radix 9 g, Zingiberis Rhizoma 9 g, Jujubae Fructus 12 g) was purchased from Jiaxing Hospital of traditional Chinese medicine; polysinosinic-polycytidylic acid (poly(I:C)) (CAS No. 24939-03-5; Lot No. 518188) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China); dexamethasone (DxMS) (CAS No. 50-02-2; Lot No. D8040) was bought out of Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China); tumor necrosis factor-alpha (TNF-α) (Lot No. EK282), interleukin-1β (IL-1β) (Lot No. EK201B), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-4 (IL-4) (Lot No. MAS28466), and nitric oxide (NO) (Lot No. 8098) was bought out of Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China); nitric oxide synthase 2 (NOS2) (Lot No. IQP-1736), arginase-1 (Arg-1) (Lot No. ETI065-8/50 μL), macrophage mannose receptor 1 (CD206) (Lot No. P5857359), RNA extraction kits and antibodies were purchased from Xiamen Senksei Technology Co., Ltd. (Xiamen, China).

**Methods**

**Creation of a viral pneumonia mouse model**

Having initially anesthetized mice with pentobarbital, the mice's incisors and limbs were secured on mouse boards that were 60 degrees inclination, and they were set up on a spotless bench with their heads higher than their tails. The neck was bare and sterilized, with incremental layering of the trachea exposure. The poly(I:C) was aspirated using a 1 mL syringe (dissolved in 50 μL phosphate buffer solution (PBS)) and after inserting the needle entered the trachea diagonally and in the direction of the center by fixing the trachea slightly with the left hand. When there was a perception of falling, the needle was extended slightly forward and parallel. If there was no resistance but air when withdrawing, we established that the needle had been inserted in the trachea. The drug administered at a dosage of 5 mg/kg was inserted into the trachea gradually, and to make sure the entire medication solution had reached the trachea, a tiny amount of air was injected at the conclusion. Immediately after the infusion was completed, the board was placed upright and gently shaken to distribute the drug solution uniformly in the lungs. Upon the cessation of anesthesia and the restoration of conscious respiration, the mice were returned to their cages.

**Preparation of XCHD**

In order to prepare the XCHD, Bupleuri Radix 30 g, Scutellariae Radix 9 g, Ginseng Radix 9 g, Pinelliae Rhizoma 9 g, Glycyrrhizae Radix 9 g, Zingiberis Rhizoma 9 g, and Jujubae Fructus 12 g were mixed with eight times the volume of water and left to soak for 2 h. Thereafter, the suspension was subjected to two 30-min periods of boiling, after which the residue was filtered. Following this double boiling process, the resulting decotion was mixed and concentrated to give 5 g crude drug/mL preparation, which, until it was needed again, was kept at 4 °C.

**Grouping and administration**

After adaptive feeding for 1 week, 60 mice were randomly allotted to one of six groups, namely, the sham operation (Sham), model (poly(I:C)), positive control (DxMS), XCHD low-dose (XCHD-L), XCHD medium-dose (XCHD-M), and XCHD high-dose (XCHD-H) groups. In all but the Sham group, poly(I:C) was injected into the trachea of mice to establish a viral pneumonia model, whereas 50 μL of PBS was instilled into the trachea in the Sham group mice. Having confirmed the efficacy of modeling, mice from the poly(I:C) and Sham groups were administered 0.2 mL of normal saline at 12 h intervals, whereas mice in the positive control group were administered 0.5 mg/kg DxMS time via injection into the abdomen [3]. Mice in the XCHD-L, XCHD-M, and XCHD-H groups were intragastrically administered 5.6 g, 11.2 g, and 22.4 g of the XCHD crude drug/kg, respectively, at 12-h intervals. The administered dosages of XCHD were determined using the formula for daily dosage in humans, which was converted to a dosage for animals. The XCHD-M group mice were treated with the human-equivalent dose, whereas the XCHD-L group mice received half this dose, and the XCHD-H group mice received twice the dose [4]. Following a 24 hour dosing period, an intraperitoneal injection of sodium pentobarbital (50 mg/kg) was used to induce anesthesia in the mice. And using a syringe, blood was drawn from the abdominal aorta. To extract the serum, the collected blood was centrifuged for 15 minutes at 3000 rpm.

**Collection of bronchoalveolar lavage fluid (BALF)**

Having collected blood from mice, after the animals were put to sleep, the cervical trachea was gradually exposed by cutting open the thoracic chamber. An incision of approximately 1 to 2 mm was made in the trachea, followed by insertion of a gastric gavage needle into the base of the right main bronchus, and the trachea was sewed tightly with the gavage needle in place using surgical sutures. Surgical suturing was also performed for the left lung hilum of mice to ensure an airtight state. Thereafter, using a syringe, 1 mL of saline was slowly injected into the right lungs of mice by connecting the syringe to the intragastric needle ligated to the neck trachea, which then remained in the alveoli for 15 to 30 s. During this period, having ensured no leakage of the saline by careful monitoring, extracting BALF required carefully withholding the syringe. This procedure was repeated three times to acquire 2.5 mL of BALF from each mouse.

**Lung wet weight/dry weight ratio**

Having collected BALF from every mouse, a section of the left lung...
was removed following the determination of the wet mass of the tissue temperature-controlled oven until no more lung weight loss was noted, thereby establishing the dry weight (D) of the tissue. Values of the lung tissue wet-to-dry (W/D) ratio were then calculated to assess the gravity of pulmonary edema.

The lung tissues were stained with hematoxylin and eosin (H&E). The left lung tissues of mice were sectioned into 3 μm sections, embedded in paraffin, fixed in formalin solution, stained with standard H&E stain, and sealed with neutral gum. Under an optical microscope, pathological alterations in the lung tissues of the mice in each group were noted.

**Protein concentration and total cell numbers in BALF**

To obtain the supernatant, the collected BALF was centrifuged for 10 minutes at 4 °C and 3000 rpm, and the overall protein concentration was determined using a commercial kit. The remaining BALF pellet was resuspended in 200 μl of PBS buffer, and 20 μl of the resuspension was brought in to compute the hemocytometer count of all the cells.

**BALF inflammatory factor determinations**

The inflammatory factors TNF-α, IL-10, IL-1β, IL-6, and IL-4 at different levels, in BALF supernatant were determined using the respective enzyme-linked immunosorbent assay kits, and levels of NO were established using a biochemical kit.

**qPCR**

Total RNA was extracted from BALF cell pellets using an extraction kit, and cDNA was subsequently obtained using a reverse transcription kit. To ascertain the mRNA expression of NOS2, Arg-1, and CD206, qPCR was used, with the expression of each target relative to that of the β-actin reference gene determined by the 2^−ΔΔCt technique. Table 1 lists the primer sequences that were employed in the amplification process.

**Western blot analysis**

Collected BALF cell pellets were placed in ice-cold radioimmunoprecipitation assay buffer, and the total protein content was extracted using ultrasonic homogenization, with the concentration of proteins in lysate being quantified using the Bicinchoninic Acid Assay method. Having added protein loading buffer, protein samples were completely denatured by heating at 99 °C for 5 min. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate the denatured protein samples before they were transferred to polyvinylidene fluoride membranes, followed by blocking with 5% skim milk powder solution for 1 h and incubating all through the night at 4 °C with inducible nitric oxide synthase (1:2000), Arg-1 (1:10000), and CD206 (1:2000) antibodies. The following day, after utilizing Tris-buffered saline plus Tween-20 three times to cleanse the membranes, they were treated at room temperature for one hour with a secondary antibody that was horseradish peroxidase-labeled. After additional washing, using an (W); the sample was dried for 48 hours at 80 °C in a electrochemiluminescence reagent, the protein bands were created, and Image J software was used to quantitatively assess the results.

**Statistical analysis**

The SPSS Statistics 17.0 program was used to do statistical analysis. Utilizing a t-test, measurement data that fit into a normal distribution were examined, with values expressed as x ± s. t-tests were also used for comparative analyses of the data collected from two groups. P values less than 0.05 are considered to be suggestive of statistically significant differences.

**Results**

**Effects of XCHD therapy on mice suffering from viral pneumonia**

Mice in the Sham group were observed to have shiny smooth hair and bright lively eyes, were responsive and active, and made steady weight gain. On the other hand, the mice belonging to the viral group were characterized by sparse matted hair, a hunched posture, dull or fatigued eyes, reduced reactivity, impaired mobility, and continuous weight loss. In contrast to the model group, mice in the DXMS and medium- and high-dose XCHD groups had smooth hair, bright eyes, a rapid response, and made a relatively steady gain in weight. To advance further investigate the potential anti-viral therapeutic consequences of XCHD, we obtained measurements for the lung tissue’s W/D ratio and the total protein concentration and total cell numbers in the BALF of mice from each group. In contrast to the group under control, there was a significant increase in the W/D ratio in model group mice (P < 0.01) (Figure 1a). However, in contrast to the model group’s mice, mice receiving the DXMS or high-dose XCHD treatments were found to have a significantly reduced W/D ratio (P < 0.01) (Figure 1b). The model group had a considerably higher concentration of BALF total protein than the control group (P < 0.05) (Figure 1b), although significantly reduced in the DXMS (P < 0.01) and low-dose XCHD groups (P < 0.05) (Figure 1b). Furthermore, the BALF total cell count of the mice in the model group was significantly higher than that of the mice in the control group (P < 0.01) (Figure 1c), whereas significant reductions were observed in both high-dose XCHD and DXMS groups (P < 0.01) (Figure 1c) compared with the model group mice.

**Pathological modifications to the lungs of mice in each treatments group**

H&E tissue staining of the lung tissues of mice in the control group revealed an intact bronchial epithelial structure, with normal alveolar separation, an absence of interstitial edema, and no apparent inflammatory cell exudate (Figure 2). In contrast, the model group’s mice’s lung tissues were observed to have a disrupted bronchial epithelial structure, characterized by the presence of numerous inflammatory cell infiltrates. However, DXMS and low- and high-dose XCHD therapies were discovered to considerably lessen the histological alterations in the lung tissues of mice infected with a virus that causes pneumonia.

<table>
<thead>
<tr>
<th>Primer</th>
<th>5’ &gt; 3’</th>
<th>Amplification length/bp</th>
</tr>
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<tbody>
<tr>
<td>β-actin</td>
<td>Forward GATATCGCTGCGCTGGTGTCG</td>
<td>132</td>
</tr>
<tr>
<td>NOS2</td>
<td>Forward GAGCAACTACTGCTGGTGGT</td>
<td>178</td>
</tr>
<tr>
<td>Arg-1</td>
<td>Forward ACATTGCGCTTGCGAGACTA</td>
<td>109</td>
</tr>
<tr>
<td>CD206</td>
<td>Forward ATGGATTCGGCCCTGAAACAGCA</td>
<td>71</td>
</tr>
</tbody>
</table>

Table 1 The primer sequences utilized in this investigation

NOS2, nitric oxide synthase 2; Arg-1, arginase-1; CD206, macrophage mannose receptor 1.
Figure 1 Changes in lung W/D weight ratio and total protein content and total cell numbers in the BALF of mice in each treatment group. (a) W/D; (b) BALF total protein content; (c) BALF total cell count. Compared with the normal group, ***P < 0.01, *P < 0.05; compared with the model group, P < 0.01, P < 0.05. W/D, wet-to-dry; BALF, bronchoalveolar lavage fluid; poly(I:C), polyinosinic-polycytidylic acid; DXMS, dexamethasone; XCHD-L, Xiaochaihu decoction low-dose; XCHD-M, Xiaochaihu decoction medium-dose; XCHD-H, Xiaochaihu decoction high-dose.

Effects of XCHD on inflammatory responses in mice with viral pneumonia
In contrast to the group under control, amounts of IL-6 and IL-1β, two inflammatory factors, and TNF-α were all increased in the model group mice’s BALF (P < 0.01) (Figure 3a-3c). In contrast to the mice within the model group, we detected reductions in IL-1β (P < 0.01 and P < 0.05, respectively) (Figure 3a), IL-6 (P < 0.01 and P < 0.05, respectively) (Figure 3b), and TNF-α (P < 0.01) (Figure 3c) after receiving high-dose XCHD and DXMS treatments. There were elevations in the NO levels as compared to the control group (P < 0.01) (Figure 3d) as well as drops in IL-10 and IL-4 levels (P < 0.05) (Figure 3e and 3f) within the model group, whereas in contrast to the model group, there were noteworthy reductions in NO levels and significant increases in the IL-10 and IL-4 levels (P < 0.05) (Figure 3e and 3f) in reaction to DXMS and high-dose XCHD therapy (P < 0.01) (Figure 3d).

Effects of treatment on mRNA and protein expression in the BALF of mice
The results of qPCR analysis revealed increases in the expression of Nos2, Arg-1, and the model group’s CD206 mRNA in comparison to the normal group’s (P < 0.01, P < 0.01, and P < 0.05, respectively) (Figure 4a-4c). Compared with the model collective, we detected a significant reduction inside the phase of Nos2 mRNA in the DXMS and low-, medium- and high-dose XCHD groups (P < 0.01) (Figure 4a), significant increases in the expression of Arg-1 mRNA in the DXMS and medium- and high-dose XCHD groups (P < 0.01, P < 0.05, P < 0.01, and so on) (Figure 4b), and significant increases in the expression of CD206 mRNA in the DXMS and all XCHD groups (P < 0.01) (Figure 4c).

With respect to the corresponding proteins, western blot analysis showed that the model group’s Nos2 protein expression was much higher than that of the normal group (P < 0.01) (Figure 4e), whereas we detected no significant changes in the expression of CD206 or Arg-1 proteins (P > 0.05) (Figure 4f and 4g). In contrast to the model group, we detected significant increases in the expression of Arg-1 (P < 0.05) (Figure 4f) and CD206 in the high-dose groups of XCHD and DXMS (P < 0.01) (Figure 4g), whereas there was a significant reduction in Nos2 expression (P < 0.05) (Figure 4e).

Discussion
In this work, a mouse model of viral pneumonia produced by poly(I:C) was created, using which we examined the effects of administrating the medicinal preparation XCHD. Our findings accordingly revealed notable reductions in the W/D ratio, protein concentrations and the overall number of cells in the lungs of infected mice, indicating that XCHD has promising therapeutic effects for the therapy for mice with viral pneumonia.

Viral pneumonia is characterized by an inflammation of the lung interstitium and parenchyma triggered by viral invasion of the respiratory and alveolar epithelium [5], and this inflammatory response is the main mechanism underlying the occurrence of injury to the alveolar-capillary membrane and barrier destruction, resulting in pulmonary edema [6]. Analyses of lung tissue W/D and the total cell count and total protein content of BALF have been widely used to assess the gravity of viral pneumonia pulmonary edema and the permeability of alveolar-capillary [7], and our findings in the present revealed that all three indicators were all significantly higher in mice suffering from viral pneumonia, thereby suggesting a rise in the permeability of the alveolar-capillary system. In addition, further observations based on the lung tissues stained with H&E presented proof of widespread infiltration of inflammatory cells, bronchial epithelial structural destruction, and lung parenchymal damage. In mice with viral pneumonia, there is extensive alveolar damage,
Figure 3 Variations in the levels of inflammatory factors in mice in each treatment group. (a) IL-1β; (b) IL-6; (c) TNF-α; (d) NO; (e) IL-10; (f) IL-4. In contrast to the typical group, ***P < 0.01, **P < 0.05; in contrast to the model group, *P < 0.01, P < 0.05. XCHD, Xiaochaihu decoction; poly(I:C), polyinosinic:polycytidylic acid; TNF-α, tumor necrosis factor-alpha; DXMS, dexamethasone; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-10, interleukin-10; IL-4, interleukin-4; NO, nitric oxide; XCHD-L, Xiaochaihu decoction low-dose; XCHD-M, Xiaochaihu decoction medium-dose; XCHD-H, Xiaochaihu decoction high-dose.

Figure 4 Effects of treatment on mRNA and protein expression in the BALF of mice. (a) Nos2 RNA; (b) Arg-1 RNA; (c) CD206 mRNA; (d) relative protein expression; (e) Nos2 protein; (f) Arg-1 protein; (g) CD206 protein. When comparing the model group, *P < 0.01, P < 0.05, and the normal group, ##P < 0.01, #P < 0.05. BALF, bronchoalveolar lavage fluid; XCHD, Xiaochaihu decoction; poly(I:C), polyinosinic:polycytidylic acid; DXMS, dexamethasone; Nos2, nitric oxide synthase 2; Arg-1, arginase-1; CD206, macrophage mannose receptor 1; XCHD-L, Xiaochaihu decoction low-dose; XCHD-M, Xiaochaihu decoction medium-dose; XCHD-H, Xiaochaihu decoction high-dose.
alveolar cavity fusion, alveolar septal enlargement and collapse, and interstitial edema. These results align with the typical pathology signs and symptoms linked to viral pneumonia, thereby validating the conclusions drawn from our study.

In response to differing microenvironmental inputs, macrophages undergo differentiation to distinct phenotypes associated with diverse biological roles [8]. The two kinds of polarized macrophages that are identified are M2 macrophages (alternatively activated macrophages) and M1 macrophages (classically activated macrophages), which exhibit pro-inflammatory properties, which have anti-inflammatory effects [9]. M1 macrophages express iNOS and CD86, when stimulated by the environment, become active and release large amounts of pro-inflammatory chemicals like TNF-α, IL-1β, IL-6, and NO [10]. M2 macrophages express CD206 and Arg-1 and can release immunological regulating agents, like IL-4 and IL-10, which contribute to inhibiting the inflammatory responses of tissues and cells, thereby facilitating tissue repair and blood vessel formation [11]. Multiple studies have demonstrated [12, 13] that acute viral infection can mediate the polarization of macrophages primarily toward an M1 phenotype, with a concomitant reduction in M2 macrophage-mediated anti-inflammatory effects in lung tissues [14]. Despite the fact that this study, we concentrated on investigating the effects of XCHD on macrophage polarization regulation in viral pneumonia from a protein perspective; numerous studies have adopted immunohistochemistry and immunological approaches to monitor the regulation of macrophage polarization with the aim of ameliorating acute lung injury in mice [15]. We accordingly believe that adopting similar approaches would enable us to gain a more direct assessment of the ability of XCHD to control the polarization of macrophages during the treatment of viral pneumonia. Nevertheless, our findings in this study demonstrated increases in TNF-α, NO, IL-1β, and IL-6 levels and reductions in the levels of IL-10 and IL-4 in the BALF collected from mice with viral pneumonia. However, in response to the administration of high-dose XCHD, we detected notable reductions in levels of IL-1β, IL-6, TNF-α, and NO, and increases in those of IL-10 and IL-4, thereby providing valuable insights into the potential mechanisms underlying the therapeutic effect of XCHD in the treatment of viral pneumonia. Consistently, the findings of our western blot analyses revealed an increase in the expression of NOS2 protein and reductions in the expression of Arg-1 and CD206 proteins in mice with viral pneumonia, whereas high-dose XCHD intervention was observed to reduce expression of the former and increase that of the latter two proteins. On the basis of these findings, we thus propose that the therapeutic efficacy of XCHD can be ascribed, at least in part, to its role in promoting macrophage polarization toward an M2 phenotype, with associated anti-inflammatory effects.

In summary, XCHD shows therapeutic efficacy in the treatment of mice with viral pneumonia, the underlying mechanism of action of which is plausibly connected to a regulation of macrophage polarization.

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