Astragaloside IV inhibits inflammation caused by influenza virus via ROS/NLRP3/Caspase-1 signaling pathway

Xiaoli Huang[1], Yifan Zhou[1], Yi Li[1], Ting Wang[1], Yandong Chen[1], Yuanhong Zhou[1], Xiaolin Zhou[1], and Qiang LIU[1]

[1]Sanxia University First Clinical Hospital

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Abstract

Astragaloside IV (AS-IV) is the most active monomer in the traditional Chinese herbal medicine Radix Astragali, which has a wide range of antiviral, anti-inflammatory, and anti-fibrosis pharmacological effects, and shows protective effect in acute lung injury. This study utilized the immunofluorescence, flow cytometry, ELISA, RT-qPCR, Western blot and Hematoxylin and eosin(H&E) staining methods to investigate the mechanism of AS-IV in reducing viral pneumonia caused by influenza A virus (IAV) in A549 cells and BALB/c mice. The results showed that AS-IV suppressed ROS production in influenza virus-infected A549 cells in a dose-dependent manner, and subsequently inhibited the activation of NLRP3 inflammasome and Caspase-1, decreased IL-1β and IL-18 secretion. In BALB/c mice infected with Poly (I: C), oral administration of AS-IV can significantly reduce Poly (I: C)-induced acute pneumonia and lung pathological injury. Which provides a new therapeutic strategy for influenza virus infection.

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Author Listing

Xiaoli Huang [1, 3, #], Yifan Zhou [1, 3, #], Yi Li [2, 3], Ting Wang [1], Yandong Chen[1], Yuanhong Zhou [1], Xiaolin Zhou [1], Qiang Liu [1, 3, *]

[1] Department of Infectious Diseases, The First College of Clinical Medical Science, China Three Gorges University & Yichang Central People's Hospital, Yichang 443003, China

[2] Department of Cardio-Thoracic Surgery, The First College of Clinical Medical Science, China Three Gorges University & Yichang Central People's Hospital, Yichang 443003, China

[3]Central Laboratory, The First College of Clinical Medical Science, China Three Gorges University & Yichang Central People's Hospital, Yichang 443003, China

[#]First authors: Xiaoli Huang and Yifan Zhou contributed equally to this work and share the first authorship.

*Corresponding author.

Co-author email address:

Xiaoli Huang, *1007707237@qq.com* ; Yifan Zhou, 1465503273@qq.com; Yi Li, *okle1993@qq.com*; Ting Wang,*huanghhh7237@foxmail.com*; Yandong Chen,*3419558250@qq.com;*Yuanhong Zhou,*1051285305@qq.com;*Xiaolin Zhou,

liyi1051285305@foxmail.com.

Correspondence information

Qiang Liu, Ph.D., Professor E-mail address: *liuqiang@ctgu.edu.cn*

Mailing Address: Yichang Central People's Hospital, NO.183 Yiling Road, Yichang, 443003, P. R. China.

Tel: +86 13720181669 Fax: 0717-6482302

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Abstract: Astragaloside IV (AS-IV) is the most active monomer in the traditional Chinese herbal medicine *Radix Astragali* , which has a wide range of antiviral, anti-inflammatory, and anti-fibrosis pharmacological effects, and shows protective effect in acute lung injury. This study utilized the immunofluorescence, flow cytometry, ELISA, RT-qPCR, Western blot and Hematoxylin and eosin(H&E) staining methods to investigate the mechanism of AS-IV in reducing viral pneumonia caused by influenza A virus (IAV) in A549 cells and BALB/c mice. The results showed that AS-IV suppressed ROS production in influenza virus-infected A549 cells in a dose-dependent manner, and subsequently inhibited the activation of NLRP3 inflammasome and Caspase-1, decreased IL-1β and IL-18 secretion. In BALB/c mice infected with Poly (I: C), oral administration of AS-IV can significantly reduce Poly (I: C)-induced acute pneumonia and lung pathological injury. Which provides a new therapeutic strategy for influenza virus infection.

Keywords: Influenza virus, Inflammation, Astragaloside IV, ROS, NLRP3, Caspase-1

1. Introduction

Influenza virus is a major respiratory pathogen which remained the world's most deadly communicable disease[1]. Studies have shown that the prognosis of viral pneumonia caused by influenza virus is related to the interaction between the virus and the host [2-4]. In mild cases, the hosts can effectively remove pathogens and result to a good prognosis [2]. However, in severe cases, the violent inflammatory response causes extensive lung tissue damage and hypoxemia which may progress to acute respiratory distress syndrome (ARDS). As a result, the mortality rate for severe influenza infection ranges from 10% (seasonal influenza) [3] to 65% (pandemic influenza) [4]. Early antiviral treatment can improve the prognosis of influenza patients. But Dobso et al. found that antiviral treatment alone could not effectively reduce the mortality of severe influenza patients [5]. HeroldS et al. further confirmed that the high mortality rate of severe influenza is related to the excessive inflammatory response of the host lung [2]. Thus, excessive host inflammatory response to influenza virus is the main cause of death in patients with severe influenza.

NOD-like receptor thermal protein domain associated protein 3 (NLRP3) inflammasome is a high molecular weight polyprotein complex whose formation is strictly regulated by initiation and activation signals [6]. The NLRP3 inflammasome plays a key regulatory role in the innate immune system, recognizing signals from a variety of microorganisms (such as bacteria and viruses), both endogenous and exogenous (crystalline particles, etc.) [6]. After the NLRP3 inflammasome is activated, it can activate the downstream effector Caspase-1 to mediate pyroptosis. Tate et al. found that the delaying administration of NLPR3 inhibitors in the early stage of influenza virus infection could significantly improve the survival rate of mice and reduce lung injury[7]. Ong et al. suggest that controlling the activation of the NLRP3 inflammasome at a reasonable level is necessary to maintain homeostasis[8]. Reactive oxygen species (ROS) is a key factor regulating NLRP3 activation [9]. Heid ME et al. have shown that increased ROS can activate NLRP3 inflammasome-mediated cell damage [10]. Further, increased ROS can also promote the spread of viruses between cells and increase the body's susceptibility to respiratory viruses [11-13].

Radix Astragali , the root of *Astragalus membranaceus* var. *mongholicus* (Bunge) P.K. Hsiao. is one of the most popular herbs with multiple functions such as anti-inflammatory[14] and antiviral effects [15]. The study of Liang et al. showed that *Radix Astragali* injection significantly inhibited the proliferation of H1N1 and improved the survival rate of cells infected with the H1N1 virus [16]. Khan et al. demonstrated that aqueous extract of *Radix Astragali* has antiviral activity and can be used to treat avian influenza virus infection [17]. In addition, *Radix Astragali* has been shown to possess anti-hepatitis B virus activities both *in vitro* and *in viro* [18] and exhibits therapeutic effect on myocarditis caused by Coxsackie virus [19]. Astragaloside IV (AS-IV) is the main active component of *Radix Astragali* , which has strong antioxidant, anti-fibrosis, anti-inflammatory, and antiviral effects [20]. AS-IV can reduce lung injury caused by various pathological factors by reducing ROS [21,22]. AS-IV can improve pulmonary fibrosis induced by Bleomycin-Induced Pulmonary Fibrosis by inhibiting ROS [22,23]. AS-IV alleviates the progression of Chronic Obstructive Pulmonary Disease (COPD) by inhibiting the production of ROS and inflammatory factors [24].

Recently, Zhang et al. reported that in H1N1 infection, AS-IV attenuates the secretion of the inflammatory factor IL-1β by enhancing autophagy [25]. However, the specific mechanism of action of AS-IV in influenza virus infection remains unclear. In many diseases, AS-IV reduces inflammation by inhibiting the production of ROS. Therefore, in this study, we combined the antioxidant properties of AS-IV to further explore the possible mechanism of AS-IV in influenza viral pneumonia and develop effective anti-influenza viral pneumonia drugs from the perspective of reducing viral inflammation and body damage.

2. Material and methods

2.1. Cells and viruses

Human lung carcinoma cell lines (A549 cells) (ATCC, Manassas, VA, USA) were grown at the condition of 37 °C with 5% CO2 after adding High glucose Dulbecco's modified Eagle's medium (DMEM) Nutrient Mix (10% FBS, 1% penicillin and streptomycin). Influenza A virus (IAV) (A/PR/8/34) was obtained from the Institute of Virology (Wuhan, China) and propagated in the allantoic cavity of 9-day-old embryonated eggs for 48 h at 37 °C and then for 12 h at 4 °C, after which allantoic fluid was collected. The hemagglutination titer of the virus in the allantoic fluid was detected by the hemagglutination method, and the allantoic fluid with a high hemagglutination titer was filtered and stored. Dilute the virus stock solution with DMEM (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}), add it to the A549 cells that grow into a monolayer, and incubate at 37 for 2 h. At the end of the infection, the viral fluid was replaced by the maintenance medium containing DMEM, 100 U/mL penicillin and streptomycin, 1.2% BSA, and 1 μg/mL trypsin (used to activate the virus). After continued incubation for 72 h, the TCID50 (median tissue culture infective dose) of A/PR/8/34 was calculated by the Reed-Muench method. A viral titer of 100 plaque-forming units (PFU) (PFU= 0.7×TCID50) was used in subsequent*in vitro* experiments. All virus experiments were conducted in the Biosafety Level 2 laboratory of the Central Laboratory of Yichang Central People's Hospital.

2.2. Cell Viability Assay

The cytotoxic effects of AS-IV (Solarbio, CAS:84687-43-4, HPLC[?]98%, Beijing, China) on A549 cells were detected by Cell Counting Kit-8 (CCK-8, CK04, Dojindo, Japan). In brief, the A549 cells were seeded in 96-well plates at a density of 1 x 10^4 cells per well, and AS-IV was diluted to the following concentrations: 1000, 500, 250, 125, 62.5, 31.2 and 0μ M added to the cells. After 72h, 10 μ L CCK-8 was added to each well immediately and continued to incubate for 2h. The cell viability was calculated by reading the optical density (OD) value at 450nm with a microplate reader (Thermo Fisher Scientific). The effect of AS-IV on the viability of A549 cells infected with the influenza virus was also detected by the cck8 method. A549 cells were seeded in 96-well plates with partitions, and the experiment was set as the negative control group (NC), IAV group, and IAV+ AS-IV group. 2 h after virus infection, the cells were cleaned with PBS solution twice, and then AS-IV solution was added to IAV+ AS-IV group, and maintenance medium was added to the other groups. OD values were determined after continuous culture for 24 h.

2.3. Immunofluorescence Staining

1×10^5 A549 cells were seeded in 48-well plates with partitions, and the experiment was set as NC group, IAV group, and IAV+ AS-IV group. 2 h after virus infection, the cells were cleaned with PBS solution twice, and then AS-IV solution was added to IAV+ AS-IV group, and maintenance medium was added to the other groups. After a certain time, the cells were fixed with 4% paraformaldehyde for 30 min and then blocked with 1% bovine serum albumin + 0.2% TritonX-100 for 1 h. Influenza A Nucleoprotein/ NP antibody (Sino Biological, Cat: 11675-MM03T, Beijing, China; 1:200) was then added to the cells and incubated overnight at 4°C. The primary antibody was then absorbed and abandoned, fluorescein Dye488-conjugated goat anti-mouse antibody was supplied by (Biqdoo-Bio, B100812, Wuhan, China; 1:200) was added and incubated in the dark for 1h, and finally Hoechst (Beyotime, C1022, Shanghai, China) was added to stain the nucleus for 15min. The expression of NP was observed under the fluorescence microscope. Use Photoshop (2021) to merge images.

2.4. Measurement of TAC, SOD, GPX, CAT and MDA

5×10^5 A549 cells were seeded in 6-well plates with partitions, the experiment was set as NC group, IAV group, and IAV + AS-IV group, which were given corresponding treatment, and cells were collected 24 h later. According to the manufacturer's instructions, the levels of TAC (Beyotime, S0121, Shanghai, China), GPX (Beyotime, S0058, Shanghai, China), CAT (Beyotime, S0051, Shanghai, China), SOD (Beyotime, S0101S, Shanghai, China) and MDA (Beyotime, S0131S, Shanghai, China) were detected using the kits.

2.5. Flow Cytometry

A549 cells were seeded in 6-well plates with partitions, the experiment was set as NC group, IAV group, and IAV+ AS-IV group, which were given corresponding treatment for 24 h. The cells are then digested and collected with trypsin (GENOMBIO, Fujian, China). The ROS assay kit (Beyotime, S0033S, Shanghai, China) was used to detect the intracellular total ROS levels. Fluorescence probe DCFH-DA was diluted in serum-free medium at 1:1000 to a final concentration of 10 μ mol/L. The cells were suspended in diluted DCFH-DA at a cell concentration of 5 million/ml and incubated in a cell incubator at 37°C for 20 min. Invert and mix every 3-5 minutes so that the probe is in full contact with the cells. The cells were then washed three times with PBS to fully remove DCFH-DA that had not entered the cells. DCFH-DA was hydrolyzed by esterase in cells to produce DCFH, and DCFH was oxidized by ROS species to produce DCF with fluorescence. Finally, DCF was detected by a flow cytometer (CytoFLEX; Beckman). Because the fluorescence spectrum of DCF is very similar to that of FITC, the parameter setting of FITC was used to detect DCF in this experiment.

2.6. Enzyme-linked immunosorbent assay (ELISA)

After various treatments, the levels of IL-1β (Beyotime, PI305, Shanghai, China) and IL-18 (Neobioscience, NOV-NR-E10256, Shenzhen, China) in the cell culture supernatant were detected by specific enzyme-linked immunosorbent assay ELISA kits based on the instructions of the manufacturer. The value of absorbance was detected using an enzyme-labeled instrument at 450 nm. The standard curve is made and the measured sample concentration is calculated according to the standard curve.

2.7. Quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

After various treatments, the total RNA of each group of cells was extracted with Trizol reagent and reverse-transcribed according to the instructions of the reverse transcription PCR kit (Servicebio, China). Then, amplification was performed using qPCR SYBR Green Master Mix Reagent (Yeasen Biotechnology, China). The relative expression levels of mRNA in each sample were assessed using the 2^{-Ct}method and normalized to GAPDH expression. Primer design and synthesis are as follows:

NLRP3 forward primer: 5'-GAGGAAAAGGAAGGCCGACA-3'.

NLRP3 reverse primer: 5'-TGGCTGTTCACCAATCCATGA-3'.

Caspase-1 forward primer:5'-AGACATCCCACAATGGGCTC-3'.

Caspase-1 reverse primer: 5'-TGAAAATCGAACCTTGCGGAAA-3'.

IL-18 forward primer: 5'-ACTGTAGAGATAATGCACCCCG-3'.

IL-18 reverse primer: 5'-AGTTACAGCCATACCTCTAGGC-3'.

IL-1β forward primer:5'-GAGCAACAAGTGGTGTTCTCC-3'.

IL-1β reverse primer: 5'- AACACGCAGGACAGGTACAG-3'.

GAPDH forward primer:5'-CGTGGAAGGACTCATGACCA-3'.

GAPDH reverse primer: 5'-GGCAGGGATGATGTTCTGGA-3'.

2.8. Western blot

After modeling, the total protein was extracted by Radio immunoprecipitation assay (RIPA) buffer (Biosharp, BL504A, China), and protein concentrations were measured with a Bicinchoninic Protein Assay Kit (Biosharp, BL521A, China). The protein samples were analyzed in 10-15% SDS-PAGE with a voltage from 80-130 V, and then, transferred to 0.45μm polyvinylidene fluoride (PVDF) membranes (220 mA, 30-60 min). The protein bands were blocked with TBS containing 0.05% Tween-20 and 5% skim milk for 1-3 h at room temperature and incubated overnight at 4 ^{o}C with the following primary antibodies: GAPDH (CST, D16H11, Boston, USA;1:3000), NLRP3 (Abcam, AB 109414, Shanghai, China; 1:500), Pro-Caspase-1 (CST, D7F10, Boston, USA;1:1000), Cleaved Caspase-1(CST, D57A2, Boston, USA;1:500). Next, wash off excess primary antibodies with TBST. HRP-conjugated secondary antibody (Servicebio, GB23303, Wuhan, China;1:3000) was incubated with horseradish peroxidase conjugate at room temperature for 1h. After washing the membranes again with TBST, the bands were analyzed with an ECL system (P10501; Applygen) and Image J software (Bio-Rad).

2.9. Mice protection and lung lesion assay

Balb/c mice (, , 18-19g, 8 weeks old) were purchased from Animal Center of China Three Gorges University (Hubei, China). All mice were exposed to alternating light and dark for 12 hours, and food and water were provided free of charge at the animal center.

The protective effect of AS-IV on mice was observed by simulating IAV infection with poly (I: C) (Yuanye Bio-Technology Co., Ltd, 24939-03-5, Shanghai, China) attack. The mice were randomly divided into normal control group (NC), poly (I: C) group, poly (I: C) + AS-IV (25mg/kg) group, poly (I: C) + AS-IV (50mg/kg) group, and poly (I: C) + DEX (5mg/kg) group (n=6 for each group). Poly (I: C) group and drug therapy groups received poly (I: C) (100 μg/mice, dissolved in 50 μl PBS) nasal drops for 3 days to establish pneumonia mice models, while the NC group received 50 μl of PBS nasal drops for 3 days. Then the mice were given different concentrations of AS-IV, Dexamethasone (DEX, Solarbio, CAS: 50-02-2, purity >98%, Beijing, China), or PBS, administered by intragastric administration every day for 5 days. All the mice on day 6 and sacrificed on day 6, and the lung tissues were harvested and weighed. The lung index was expressed as the ratio of mean lung weights to mean body weights. Finally, the lung tissues of mice in each group were stained with hematoxylin and eosin (H&E). The pathologically. Pathological grading was performed according to the relative degree of lung injury and inflammatory infiltration[26]. The scoring was on a scale from 0 to 4: 0, lung tissue structure is normal, no inflammatory cell infiltration; 1, mild structural abnormalities of lung tissue with or without inflammatory cell infiltration; 2, moderate abnormality of lung tissue structure, no inflammatory cell infiltration; 3, moderate abnormality of lung tissue structure with inflammatory cell infiltration; 4, severe abnormality of lung tissue structure with or without inflammatory cell infiltration.

All operations and experiments were performed in accordance with the principles of the National Guidelines for the Nursing.

2.10. Data analysis

All experiments were performed at least thrice. SPSS 26.0 software was used to analyze all data (IBM Corp., NY, USA). The data are expressed as the mean ± SD. One-way ANOVA analysis of variance was used to test the differences between groups. Histograms were drawn using GraphPad Prism 9.4.0 software (San Diego, CA, USA).

3. Results

3.1. AS-IV increases the cell viability of influenza-infected A549 cells but has no inhibitory effect on the influenza virus infection

Fig. 1A shows the chemical structure of AS-IV. Fig. 1B-C shows the toxicity of A549 cells treated with AS-IV for 72h. The results showed that AS-IV had no obvious toxicity to cells when the concentration reached 125μM, but when the concentration reached 250μM, AS-IV formed many drug crystals, and the cell viability was significantly decreased (P <0.01). Therefore, concentrations below 125μM were used for subsequent experiments.

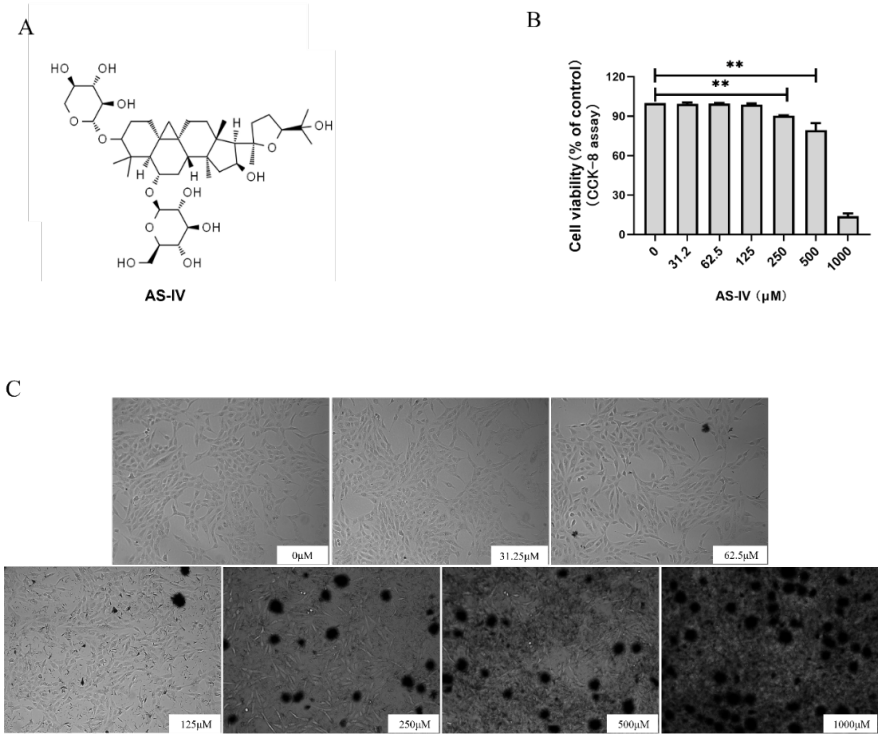


Fig 1. The cytotoxicity effects of AS-IV on the A549 cells. (A) Chemical structure of AS-IV. (B) CCK8 reagent was used to detect A549 cell viability. Cell viability was defined as the percentage of the 0μM group. (C) The growth of A549 cells was observed under the microscope. AS-IV, Astragaloside IV. *P < 0.05, ** P < 0.01.

A549 cells infected with the influenza virus (A/ PR/ 8/ 34 at 100 PFU) were treated with 50,75 and 100μM AS-IV. After influenza virus infection, the cells showed swelling, rupture, and dissolution, and the cell density was significantly lower than that of the NC group, AS-IV can significantly reduce the A549 cell death caused by viral infection (Fig. 2).

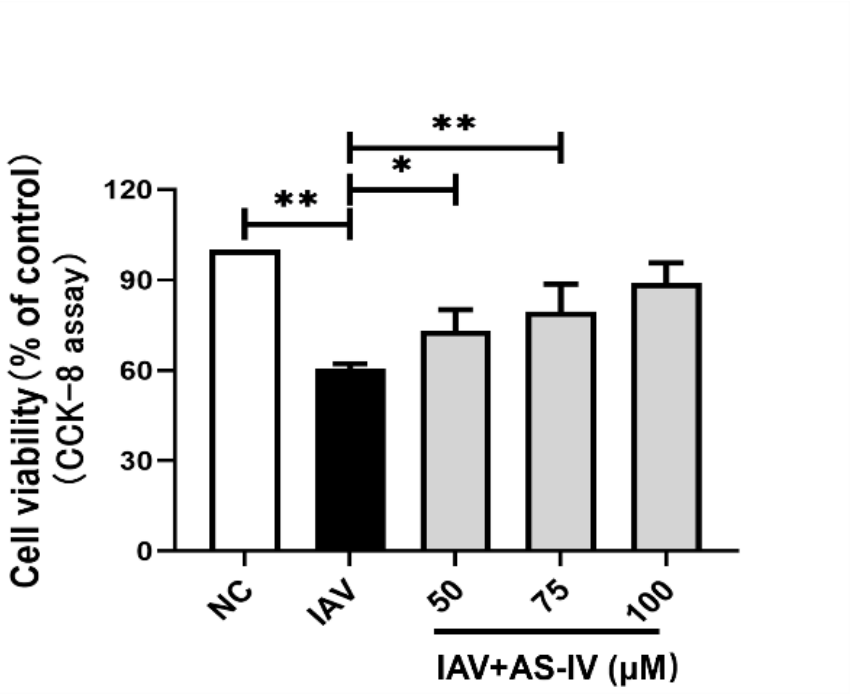
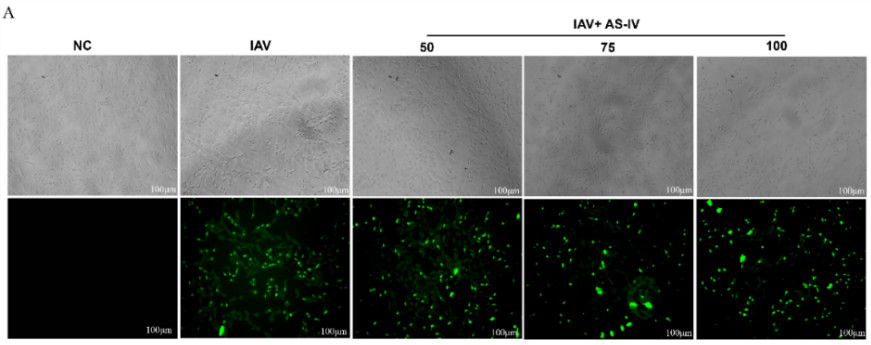


Fig 2. Protective effect of AS-IV on A549 cells infected with influenza virus. CCK8 reagent was used to detect A549 cell viability. Cell viability was defined as the percentage of the NC group. IAV, influenza A virus; NC, negative control group. * $P < 0.05$, ** $P < 0.01$.

To further evaluate the anti-influenza virus effect of AS-IV, we used the immunofluorescence method to evaluate the efficacy of AS-IV on IAV. As shown in Fig. 3A, AS-IV had no obvious inhibitory effect on the infection of the influenza virus. AS-IV also had no inhibitory effect on IAV replication (Fig. 3B-C). In addition, we also observed that AS-IV had no obvious effect on delaying the nuclear envelope shuttle transport of NP protein (Fig. 3D).



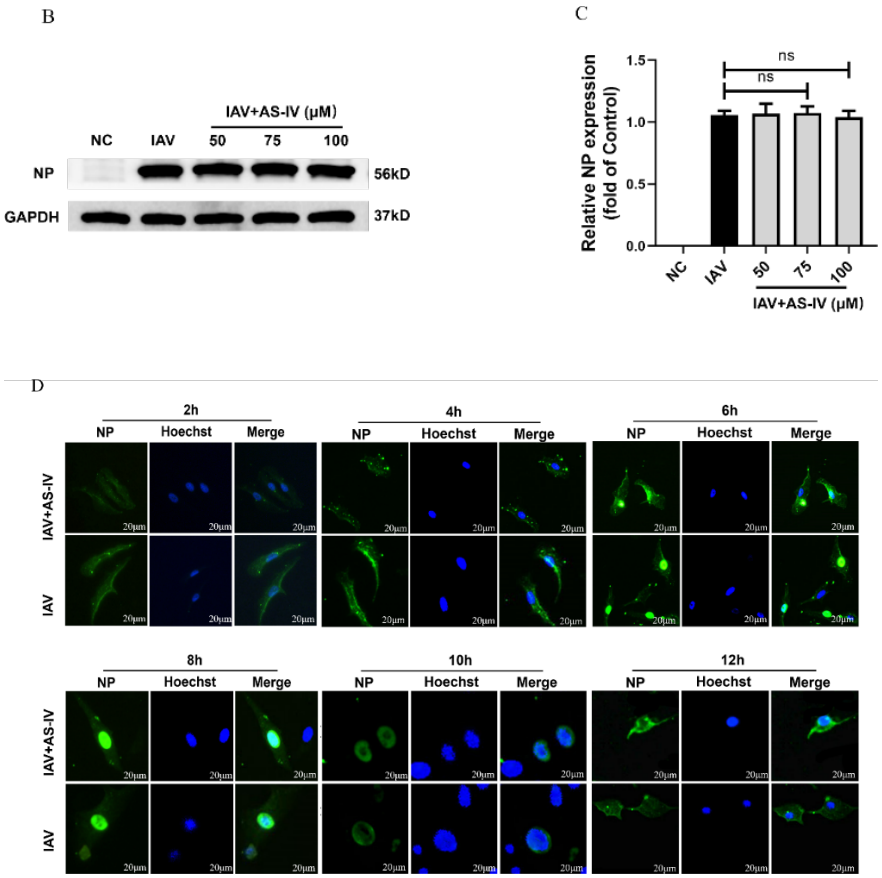


Fig 3. Effects of AS-IV on influenza virus in A549 cells. (A) A549 cells infected with IAV were treated with 50,75, and 100μM AS-IV, and the infection rate of IAV in A549 cells in each group was observed after 16h, with a scale bar, of 100 μ m. (B, C) Western blot detected NP protein expression in A549 cells treated with AS-IV after influenza virus infection. (D)100μM AS-IV treated A549 cells infected with IAV, and immunofluorescence was performed at different time points (2h, 4h, 6h, 8h, 10h, 12h) to observe the effect of AS-IV on nuclear membrane shuttle transport of IAV NP protein in A549 cells, with scale bar,20 μ m. The values are expressed as the mean ± SD. of three independent experiments. ns, no significant difference. * $P < 0.05$, ** $P < 0.01$.

3.2. AS-IV down-regulates ROS induced by influenza virus infection

To detect the antioxidant effects of AS-IV, intracellular TAC, SOD, GPX, CAT, MDA, and ROS levels were measured. We confirmed that the levels of intracellular TAC, SOD, GPX, and CAT were significantly reduced after influenza virus infection, while AS-IV treatment restored the inhibition of TAC, SOD, GPX, and CAT by influenza virus infection (Fig. 4A-D). The results of MDA detection showed that MDA concentration in the IAV group was increased compared with the NC group, while MDA level in AS-IV-treated cells was down-regulated compared with the IAV group (Fig. 4E). As shown in flow cytometry analysis, the ROS levels were dramatically elevated after influenza virus infection, while the levels of ROS for AS-IV groups were rescued after treatment (Fig. 4F).

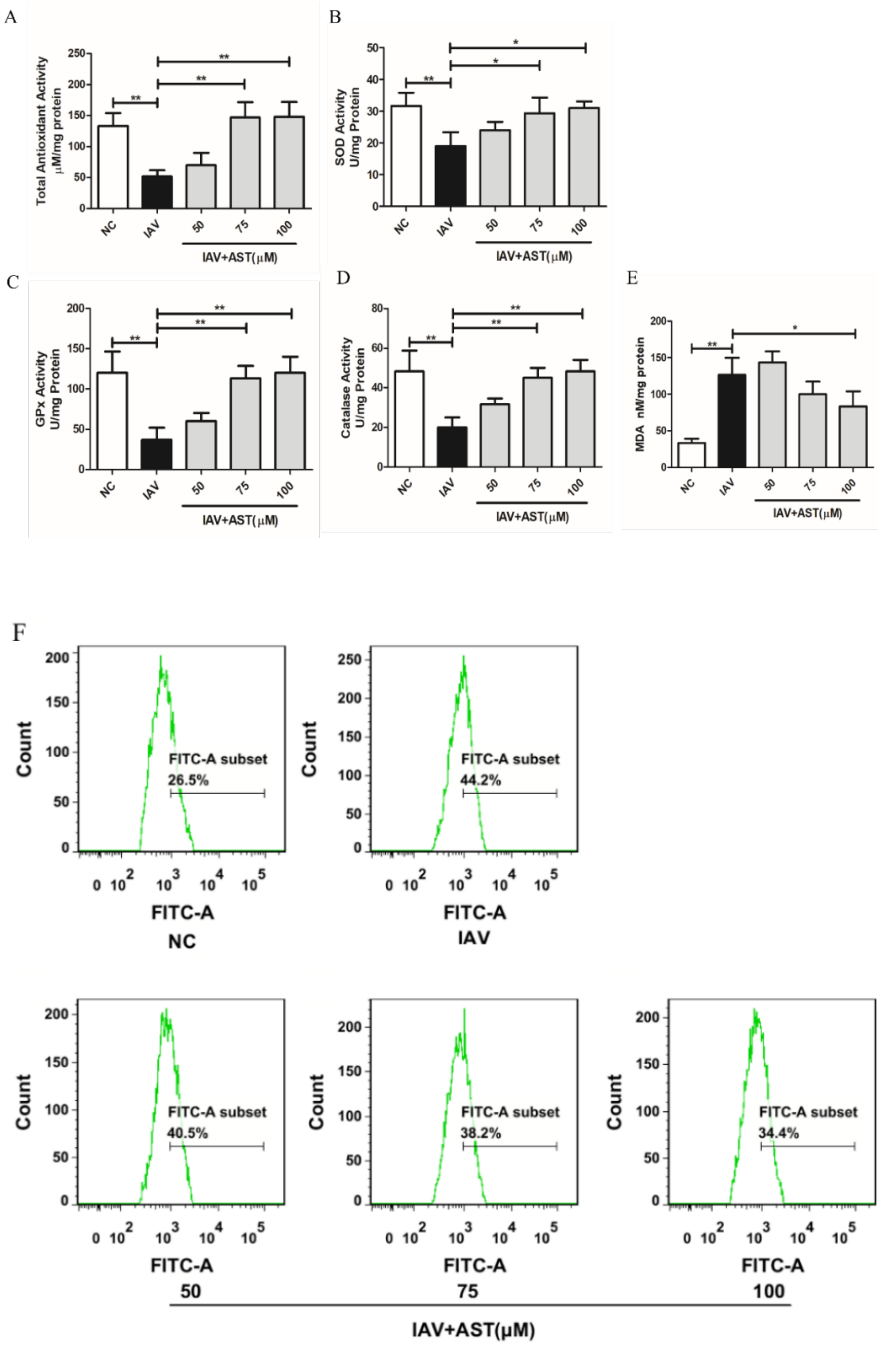


Fig 4. Antioxidative effect of AS-IV. (A) Effects of AS-IV on TAC, SOD, GPX, CAT, and MDA in A549 cells infected with influenza virus. (B) Effects of AS-IV on ROS in A549 cells infected with influenza virus. The values are expressed as the mean ± SD. of three independent experiments. * $P < 0.05$, ** $P < 0.01$.

3.3. AS-IV inhibits the activation of the NLRP3/Caspase-1 signaling pathway induced by influenza infection

Former studies have shown that increased ROS can activate NLRP3 inflammasome-mediated cell damage[10],

so we examined the expression levels of factors associated with the NLRP3/Caspase-1 signaling pathway in each group of cells. Our results showed that NLRP3 levels increased after influenza virus infection, but NLRP3 mRNA and protein levels decreased in a dose-dependent manner after AS-IV treatment (Fig. 5A, E-F). Moreover, compared with the IAV group, the activation of Caspase-1 in A549 cells after AS-IV treatment and the secretion of cytokines IL-18 and IL-1β were decreased (Fig. 5B-E, G-J).

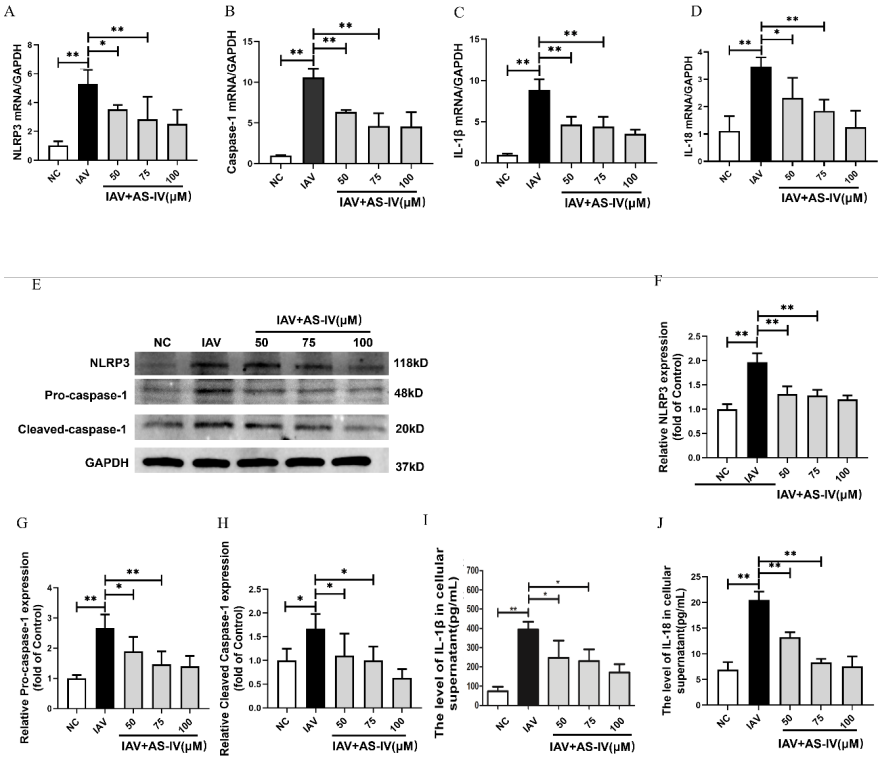


Fig 5. Effect of AS-IV therapy on the NLRP3 inflammasome pathway in A549 cells infected with influenza virus. (A-D) Real-time PCR analysis was used to analyze the mRNA levels of NLRP3, caspase-1, IL-1β, and IL-18 in different groups. (E-H) The protein expression levels of NLRP3 inflammasome, pro-caspase-1, and cleaved caspase-1 in each group were detected by western blot. (I-J) ELISA was used to detect the expression levels of inflammatory cytokines IL-18 and IL-1β in each group. The values are expressed as the mean ± SD. of three independent experiments. * $P< 0.05$, ** $P < 0.01$.

3.4. AS-IV attenuates lung inflammatory lesions in mice caused by poly (I: C)

The above results led us to further investigate whether AS-IV can inhibit viral pneumonia induced by viral RNA mimics poly (I: C) *in vivo* . The results showed that compared with the NC group, the lung tissue structure of mice in the poly (I: C) group was seriously abnormal, with a large number of alveolar epithelial cells proliferating and alveolar walls thickening, accompanied by a large number of inflammatory cells infiltrating, and pulmonary scores and lung indexes were significantly increased (Fig. 6A-C). Compared with the poly (I: C) group, the increase of lung index in the AS-IV treatment group was reduced by 11.7-19.8%, and AS-IV significantly alleviated the lung tissue structure destruction and inflammatory cell infiltration caused by poly (I: C) infection (Fig. 6A-C).

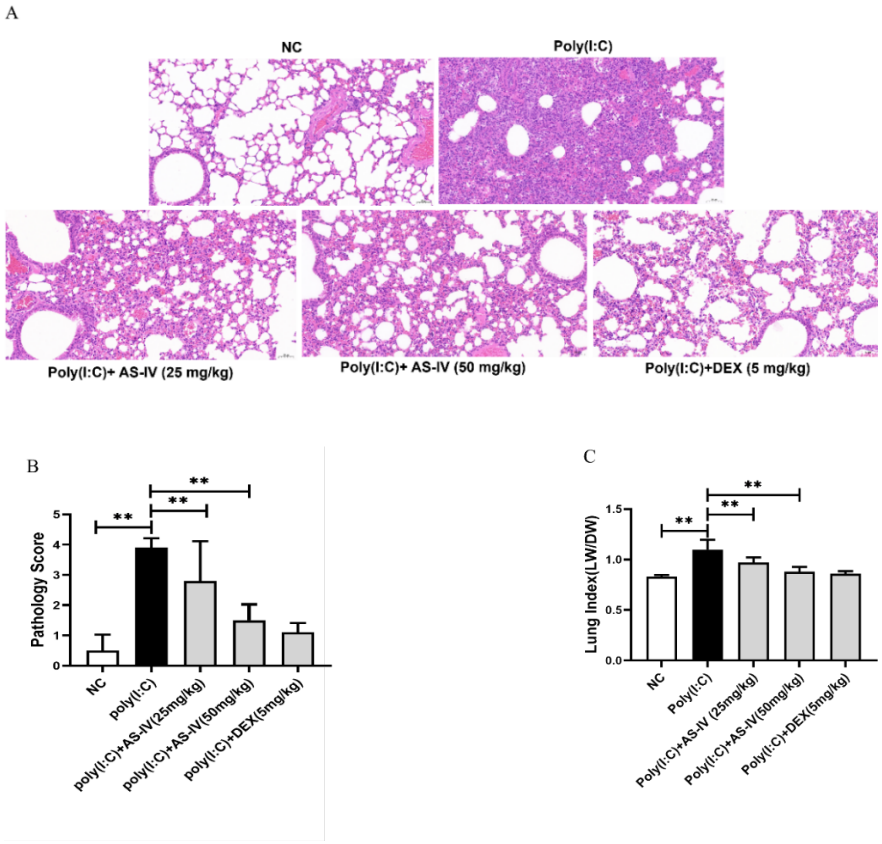


Fig 6. Effects of AS-IV treatment on poly (I: C)-induced lung inflammatory lesions in mice. Mice infected with poly (I: C) were treated with the specified concentration of AS-IV (25, 50 mg/kg /d), and the mice were sacrificed after 6 days of treatment with AS-IV to evaluate the protective effect of AS-IV on mice. (A) H&E staining of lung tissue. (B) Pathological scores of lung tissue in mice of each group. (C) Lung index for mice sacrificed at the day 6. All data are expressed as mean ± SD, (n= 10 per group). * $P < 0.05$, ** $P < 0.01$.

4. Discussion

Influenza virus infection has been a great challenge to the global health system, causing a large number of deaths and economic losses worldwide every year [3]. The initial host response to influenza virus invasion is acute inflammation, one which is characterized by the activation of inflammatory cytokines or chemokines, leading to the recruitment of inflammatory cells [27]. A moderate immune response will help clear the virus, while excessive immune stimulation can cause tissue and organ damage, and a large number of inflammatory cytokines will even overflow into the circulatory system, causing a systemic cytokine storm, resulting in multiple organ dysfunction [28,29]. This serious consequence urgently requires the development of drugs to reduce lung damage caused by influenza virus, and the secondary development and application of existing drugs seems to be more advantageous.

Traditional Chinese herbs have been used to prevent and treat viral infections for hundreds of years and are popular around the world due to their good tolerance. AS-IV is a natural compound with multi-target therapeutic properties extracted from *Radix Astragali* , which has a good antiviral effect on hepatitis B virus [30], Coxsackie virus, [31,32] and dengue virus [33], etc. Its antiviral function has also been widely verified in respiratory diseases [25,34,35]. Although Zhang et al. found that AS-IV reduces the level of IL-1β in influenza virus infection, the specific mechanism of action of AS-IV in influenza virus infection remains unclear. Here,

we further investigated the effect of AS-IV on influenza virus infection and its underlying mechanism which may contribute to the application of AS-IV and its analogues in the therapy of severe viral pneumonia.

ROS is a by-product of biological aerobic metabolism and a general term for a class of oxygen-containing and active substances. The balance of oxidative and antioxidant mechanisms is the key to maintaining ROS levels during physiological metabolism. Appropriate levels of intracellular ROS are necessary for signal transduction and apoptosis during cell growth, but excessive accumulation of ROS can cause oxidative stress leading to cell death [36,37]. After infecting the host, the influenza virus reduces and consumes the antioxidant oxidase activity of TAC, GPX, SOD, CAT, and other antioxidant systems, thereby increasing the ROS content in the body, leading to the occurrence of oxidative stress [38-40]. Antioxidant enzymes can terminate free radical chain reactions, and MDA is the end product of free radical chain reactions and is widely used to measure the degree of oxidation deterioration in biological systems [41]. Our results showed that compared with the NC group, influenza virus infection decreased the activities of TAC, SOD, GPX, and CAT in A549 cells, and significantly increased MDA and ROS levels, suggesting that influenza virus infection induced oxidative stress injury of A549 cells. After AS-IV treatment TAC, GPX, SOD, and CAT were significantly increased, while MDA and ROS levels were significantly decreased, suggesting that AS-IV has a strong antioxidant effect. These data indicate that AS-IV also exhibits the ability to inhibit ROS and antioxidants in influenza virus infection. Our subsequent experiments showed that the mRNA and protein levels of NLRP3 were significantly increased in A549 cells after influenza virus infection, and its downstream Caspase-1, IL-18, and IL-1β were also increased. When AS-IV inhibited intracellular ROS levels in a dose-dependent manner, intracellular NLRP3, Caspase-1, IL-18, and IL-1β levels were all reduced in a dose-dependent manner. Our data demonstrate that AS-IV may inhibit influenza virus-induced inflammation mediated by the ROS/NLRP3/Caspase-1 signaling pathway *in vitro* .

In vivo , we selected poly (I: C) to simulate viral infection in mice. Poly (I: C) is a double-stranded RNA analog that can cause lung inflammation, interstitial edema, bronchiolar epithelial hypertrophy, and lung function changes in mice, and can induce pro-inflammatory factors IL-6, TNF-α, Elevated levels of IL-1β and IL-8 and increased inflammatory cells [42,43]. Our results showed that the lung tissue structure of mice in the poly (I: C) group was severely damaged, and the pathological score and lung index were significantly increased compared with the normal control group. While in the therapy group, AS-IV significantly alleviated the destruction of lung tissue structure and decreased the pathological score and lung index of mice. These results demonstrate that AS-IV can effectively attenuate viral lung injury.

5. Conclusion

This study shows that AS-IV can reduce the acute inflammatory response induced by influenza virus *in vitro* . The potential mechanism may be that AS-IV inhibits the activation of NLRP3 inflammasome and reduces the secretion of inflammatory factors by inhibiting intracellular ROS levels after influenza virus infection. In addition, AS-IV alleviates lung tissue structural damage caused by poly (I: C) and reduces lung inflammation *in vivo* . These data promote the possibility of AS-IV in the future clinical treatment of viral pneumonia caused by influenza viruses. Our study also highlights antioxidants as one of the effective strategies to combat viral pneumonia.

CRediT authorship contribution statement

Xiaoli Huang and Yifan Zhou: resources, software, project administration, visualization, writing—original draft; Yi Li: methodology; Ting Wang: investigation; Yandong Chen: formal analysis, supervision; Yuanhong Zhou: data curation, funding acquisition; Xiaolin Zhou: Conceptualization; Qiang Liu: validation, writing—review and editing, funding acquisition. Xiaoli Huang and Yifan Zhou contributed equally to this work and share the first authorship. All authors have read and approved the manuscript.

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Ethics approval statement

The China Three Gorges University approved the animal ethics permit for this study (SCXK 2022-0061).

Data availability

Data for the results of this study are available from the corresponding author.

Declaration of competing interest

All authors declare no conflicts of interest.

ORCID

Qiang Liu, https://orcid.org/0000-0002-9719-2023.

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