Journal of Ethnopharmacology 275 (2021) 114063

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Antiviral effect of fufang yinhua jiedu (FFYH) granules against influenza A virus through regulating the inflammatory responses by TLR7/MyD88 signaling pathway

Yuqian Zhang [a,b,1], Ronghua Wang [a,1], Weiqing Shi [c,1], Zhihui Zheng [a,1], Xiaoquan Wang [d], Cheng Li [b], Shuofeng Zhang [b], Pinghu Zhang [a,b,d,*]

[a] Institute of Translational Medicine, Medical College, Yangzhou University, Yangzhou, 225009, China
[b] Jiangsu Key Laboratory of Integrated Traditional Chinese and Western Medicine for Prevention and Treatment of Senile Diseases, Medical College, Yangzhou University, Yangzhou, 225009, China
[c] Jiangsu Provincial Center for Disease Control and Prevention, Nanjing, 210009, China
[d] College of Veterinary Medicine & Jiangsu Provincial Key Laboratory of Human Zoonosis, Yangzhou University, Yangzhou, 225009, China

ARTICLE INFO

Keywords:
Influenza A virus
Antivirals
Fufang-yinhua-jiedu granules
TLR7/MyD88
Acute lung injury

ABSTRACT

Ethnopharmacological relevance: Fufang-Yinhua-Jiedu Granules (FFYH) optimized from a Yin-Qiao-San, as traditional Chinese medicine (TCM), was used to treat influenza and upper respiratory tract infection and was recommended for the prevention and treatment of SARS in 2003 and current COVID-19 in Anhui Province in 2020.
Aim of study: In the clinical studies, FFYH was very effective for the treatment of influenza, but the mechanism of action against influenza A virus remains unclear. In the present study, we investigated the antiviral effect of FFYH against influenza A virus *in vitro* and *vivo*. Moreover, the potential mechanism of FFYH against influenza A virus *in vivo* was investigated for the first time.
Materials and methods: CPE inhibition assay and HA assay were used to evaluate the *in vitro* antiviral effects of FFYH against influenza A virus H1N1, H3N2, H5N1, H7N9 and H9N2. Mice were used to evaluate the antiviral effect of FFYH *in vivo* with ribavirin and lianhuaqingwen as positive controls. RT-PCR was used to quantify the mRNA transcription of TNF-α, IL-6, IFN-γ, IP10, and IL-1β mRNA. ELISA was used to examine the expression of inflammatory factors such as TNF-α, IL-6, IFN-γ, IP10, and IL-1β in sera. The blood parameters were analyzed with auto hematology analyzer. Moreover, the potential mechanism of FFYH against influenza A virus *in vivo* was also investigated.
Results: FFYH showed a broad-spectrum of antiviral activity against H1N1, H3N2, H5N1, H7N9, and H9N2 influenza A viruses. Furthermore, FFYH dose-dependently increased the survival rate, significantly prolonged the median survival time of mice, and markedly reduced lung injury caused by influenza A virus. Also, FFYH significantly improve the sick signs, food taken, weight loss, blood parameters, lung index, and lung pathological changes. Moreover, FFYH could markedly inhibit the inflammatory cytokine expression of TNF-α, IL-6, IFN-γ, IP10, IL-10, and IL-1β mRNA or protein via inhibition of the TLR7/MyD88/NF-κB signaling pathway *in vivo*.
Conclusion: FFYH not only showed a broad-spectrum of anti-influenza virus activity *in vitro*, but also exhibited a significant protective effect against lethal influenza virus infection *in vivo*. Furthermore, our results indicated that the *in vivo* antiviral effect of FFYH against influenza virus may be attributed to suppressing the expression of inflammatory cytokines via regulating the TLR7/MyD88/NF-κB signaling pathway. These findings provide evidence for the clinical treatment of influenza A virus infection with FFYH.

Abbreviations: FFYH, fufang-yinhua-jiedu granules; TCM, Traditional Chinese medicine; SARS, Severe Acute Respiratory Syndrome; COVID-19, Coronavirus virus disease-19; NA, neuraminidase; LH, lianhuaqingwen; IAV, nfluenza A virus; ARDS, Acute respiratory distress syndrome; MODS, multiple organ dysfunction syndromes; ALI, acute lung injury; DMEM, Dulbecco's modified Eagle's medium; HPLC, high-performance liquid chromatography; MDCK, Madin-Darby canine kidney; $TCID_{50}$, 50% tissue culture infective dose; LD_{50}, 50% lethal dose; CC_{50}, 50% cytotoxic concentration; CPE, cytopathic effect; HA, hemagglutination test (HA) assay; FM1, A/FM/1/47; PR8, A/PR/8/34; WSN, A/WSN/33.

* Corresponding author. Institute of Translational Medicine, Medical College, Yangzhou University, Yangzhou, 225009, China.
E-mail addresses: zhangpinghu@163.com, zhangpinghu@yzu.edu.cn (P. Zhang).
[1] These authors equally contributed to this work.

https://doi.org/10.1016/j.jep.2021.114063
Received 31 December 2020; Received in revised form 13 March 2021; Accepted 18 March 2021
Available online 1 April 2021

1. Introduction

Influenza A virus (IAV) is a universal human respiratory pathogen, which can result in worldwide pandemic outbreaks. For example, severe seasonal flu epidemic causes significant morbidity and mortality in each year (Davis et al., 2015). Based on the difference of envelope glycoproteins of hemagglutinin (HA) and neuraminidase (NA), IAVs are further divided into multiple subtypes, such as H1N1, H3N2, H5N1, and H7N9 (Park and Ryu, 2018). It is worth noting that the newly emerging cross-species transmission of avian influenza viruses such as H5N1 and H7N9 resulted in severe pathogenic respiratory diseases and high mortality in patients, which seriously threatening human health (To et al., 2013; Watanabe et al., 2014). An emerging of researches has demonstrated that IAVs are not only causing direct lung injury by viral replication, but also could lead to indirect damage triggered by IAV-induced excessive inflammatory response (Fukuyama and Kawaoka, 2011; Lobo et al., 2019; Peiris et al., 2009; Short et al., 2014). The pathogenesis of H5N1 and H7N9 virus indicated that these influenza viruses infection could stimulate host immune cells to produce a large number of proinflammatory cytokines, which resulting in cytokine storms and further causing more severe acute lung injury, such as acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndromes (MODS) (Peiris et al., 2009; To et al., 2013; Yang et al., 2017).

Due to easily antigen drift and mutations of IAVs, antiviral therapy is currently the most effective way to treat influenza infection. Thus far, NA inhibitors (oseltamivir, zanamivir, etc.), M2 ion channel blockers (amantadine, rimantadine, etc.) and RNA polymerase inhibitors (ribavirin, favipiravir, etc.) have been approved by the United States Food and Drug Administration for treating influenza virus infection (Dunning et al., 2014; Ludwig et al., 2014). Although these antiviral drugs can effectively inhibit viral replication, the high variability of IAVs quickly causes the development of drug resistance and subsequently leads to treatment failure (Dunning et al., 2014). For example, due to the drug resistance of amantadine and rimantadine, most of M2 ion channel inhibitors have been withdrawn from the market (Abdelwhab et al., 2017). Furthermore, the resistance of neuraminidase inhibitors to IAVs has been gradually reported (Michael, 2011). Notably, neuraminidase inhibitors are only useful in preventive administration (48 h post-infection). However, they are ineffective against acute lung injury (ALI) and ARDS caused by a highly pathogenic influenza virus infection, such as H5N1 and H7N9 (Dunning et al., 2014). Therefore, due to the above deficiencies of antiviral drugs targeting viral proteins, the development of new anti-influenza virus drugs is urgently needed.

Traditional Chinese medicine (TCM) has been used to treat influenza for more than 5000 years in China. An emerging of studies has demonstrated that TCM not only can inhibit viral replication by interfering with multiple steps of virus replication, but also prevent acute lung injury caused by cytokines storm through regulating inflammatory responses (Ma et al., 2018, 2020; Peng et al., 2016; Rong et al., 2016; Shi et al., 2020; Wu et al., 2016). Furthermore, the characteristics of multi-components and multi-targets pharmacodynamic advantages of TCM enable it to improve symptoms such as fever, cough, and respiratory failure and reduce pulmonary edema. For example, Yin-Qiao-San as a classic prescription for clinical treatment of influenza has been used in China for nearly 600 years. Recently, Yin-Qiao-San not only inhibited the replication of influenza A virus, but also suppressed excessive inflammatory responses caused by influenza A virus (Law et al., 2017). Fufang-Yinhua-Jiedu granule (FFYH, originally called yinhua-jiedu-granules) as Chinese patent medicine (Fig. 1), which is optimized from Yin-Qiao-San, has been used to treat influenza in clinical for more than ten years (Jiang et al., 2003; Lu et al., 2003; Xi and Xi, 2003). Hence, FFYH was recommended for prevention and treatment of SARS in 2003 and the current COVID-19 in Anhui Province in 2020. It is composed of *Artemisia annua* L., *Lonicerae japonica* Thunb., *Schizonepeta tenuifolia* (Benth.) Briq., *Mentha haplocalyx* Briq., *Chrysanthemum indicum* L., *Isatidis tinctorial* L., *Forsythia suspensa* (Thunb.) Vahl., *Commelina communis* L., *Peucedanum praeruptorum* Dunn, and *Glycine* max (L.) Merr. (Table 1). Clinical researches indicated that FFYH showed an excellent therapeutic effect on influenza and upper respiratory tract infection (Jiang et al., 2003; Lu et al., 2003). Preclinical data also indicated that FFYH exhibited a broad spectrum of pharmacological properties, such as antibacterial, antiviral, anti-inflammatory, and immunomodulatory

Table 1
Composition of Fufang-Yinhua-Jiedu granule.

Pharmaceutical name	*Botanical plant name*	Family	Weight (g)	Used part
Artemisiae Annuae Herba	*Artemisia annua* L.	Asteraceae	4.5	Aerial part
Lonicerae Flos	*Lonicerae japonica* Thunb.	Caprifoliaceae	3.6	Flower bud
Schizonepetae Herba	*Schizonepeta tenuifolia* (Benth.) Briq.	Labiatae	3	Aerial part
Menthae Haplocalycis Herba	*Mentha haplocalyx* Briq.	Labiatae	0.9	Aerial part
Chrysanthemi Indici Flos	*Chrysanthemum indicum* L.	Asteraceae	4.5	Flower head
Isatidis Folium	*Isatidis tinctorial* L.	Brassicaceae	4.5	Leaf
Forsythiae Fructus	*Forsythia suspensa* (Thunb).Vahl	Oleaceae	3	Fruit
Commelinae Herba	*Commelina communis* L.	Commelinaceae	6	Aerial part
Sojae Semen Praeparatum	*Glycine* max (L.) Merr.	Leguminosae	3	Fruit
Peucedani radix	*Peucedanum praeruptorum* Dunn	Apiaceae	3	Radix

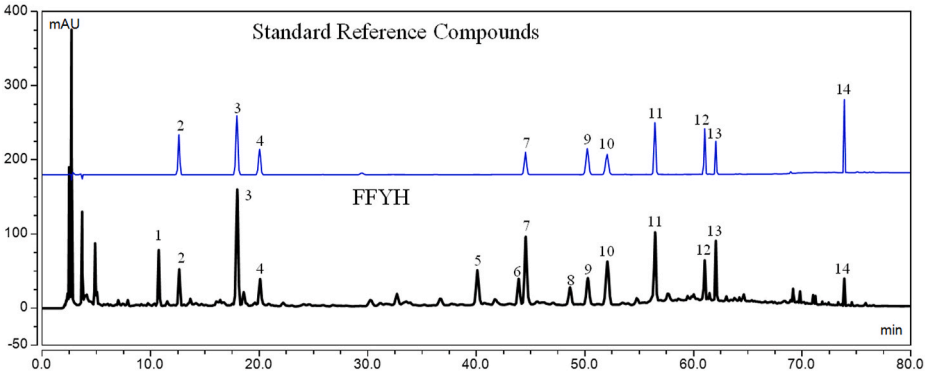

Fig. 1. HPLC chromatograms of ten bioactive constituents in FFYH at 230 nm. 2. neochlorogenic acid, 3. chlorogenic acid, 4. cryptochlorogenic acid, 7. forsythoside A, 9. isochlorogenic acid B, 10. isochlorogenic acid A, 11. 4,5-dicaffeoylquinic acid, 12. forsythoside, 13. buddleoside, 14. praeruptorin A.

activities. However, the antiviral activity of FFYH against influenza virus remains unclear. In this present study, the anti-influenza virus and anti-inflammatory effect of FFYH *in vitro* and *in vivo* were for the first time investigated. Our results indicated that FFYH could directly inhibit the replication of seasonal influenza A virus (H1N1 and H3N2) and avian influenza A virus (H5N1, H7N9 and H9N2) and showed a good protective effect against cell pathogenic effect induced by influenza A viruses. Furthermore, FFYH markedly increased the survival rate, significantly prolonged the median survival time of mice, and reduced lung injury by suppressing the inflammatory cytokine expression via inhibition of the TLR7/MyD88 signaling pathway. These findings provide evidence for the clinical treatment of influenza A virus infection with FFYH in the future.

2. Materials and methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) (SH30022.01) and 0.25% trypsin (SH40003.01) were obtained from Hyclone (Shanghai, China). Fetal bovine serum (1531294) was purchased from Biological Industries (Israel). HiScript II 1st Strand cDNA Synthesis Kit (R222) and AceQ Universal SYBR qPCR Master Mix (Q511) were purchased from Vazyme (Nanjing, China). Primary antibodies against TLR7 (AF0300), MyD88 (AF7524), TRAF3 (AF8220), TRAF6 (AF8223), IRF7 (AF2140) and ACTB (AA128) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). ELISA kit for examining TNF-α (EK0529), IL-6 (EK0411), IFN-γ(EK0375), IP-10 (EK0736) and IL-1β (EK0394) were obtained from Boster (Wuhan, China).

FFYH (20190301) was provided by Yifan Pharmaceutical Co., Ltd (Hangzhou, China). 100 mg/ml of FFYH was prepared with sterilized ultra-pure water as a stock solution for further study and stored at −20 °C. The stock solution was further diluted with DMEM (2% fetal bovine serum, 2 μg/ml TPCK-treated trypsin, 100U/ml penicillin and streptomycin) to get 2 mg/ml of FFYH dilution, which was sterilized by using a 0.22 μm syringe filter and then done double gradient dilution to get 6–8 testing concentrations. To further confirm the components of FFYH, the main components of FFYH were analyzed by high-performance liquid chromatography (HPLC). Lianhua Qingwen Capsule (LH, A1812085) produced by Shijiazhuang Yiling Pharmaceutical Co., Ltd (Shijiazhuang, China) was purchased from Baixingyuan Pharmacy of Yangzhou and was prepared as the same as above FFYH. Oseltamivir phosphate (S2597) was purchased from Selleck Chemicals Co., Ltd (Shanghai, China) and dissolved in dimethyl sulfoxide (DMSO) to get 40 mg/ml stock solution. Ribavirin (RBV) injection (100 mg/ml, 19022581) was purchased from Jiangsu Lianshui Pharmaceutical Co., Ltd (Lianshui, China) and diluted with DMEM. Working concentrations of oseltamivir phosphate and ribavirin were obtained by dilution with DMEM according to the above FFYH preparation method.

2.2. Virus and cells

Madin-Darby canine kidney (MDCK) and A549 cell lines were purchased from China Center for Type Culture Collection (CCTCC) and respectively cultured DMEM or 1640 medium with 10% fetal bovine serum, 100U/ml penicillin and streptomycin, respectively. Seasonal influenza A virus, H1N1 (A/WSN/1/33, A/PR/8/34, A/FM/1/47) and H3N2 (A/YZ/201/2010) strains and avian influenza virus A/Duck/Jiangsu/Sheyang/2004 (H5N1), A/Chicken/Jiangsu/CZJTC4/2013 (H7N9) and A/Chicken/Shangdong/SKD1/2011 (H9N2) were provided by Key Lab. of Livestock and Poultry Infectious Diseases of the Ministry of Agriculture of Yangzhou University. The 50% tissue culture infective dose ($TCID_{50}$) of the virus in MDCK cells and the 50% lethal dose (LD_{50}) of the virus in ICR mice were determined by the Reed-Muench method. Virus stocks were collected and stored at −80 °C. All experiments were performed in BSL-2 and ABSL-3 biosafety laboratory at Yangzhou University.

2.3. Cell viability assay

The 50% cytotoxic concentration of samples was determined by MTT assay in MDCK and A549 cells. Briefly, MDCK cells or A549 cells (2×10^4 cells/well) were seeded into 96-well plates overnight and were incubated with drugs for 72 h. After treatment, 20 μl of MTT (5 mg/ml) were added into each well for incubation 2 h. After then remove the medium, 200 μl of DMSO was added into each well and the absorbance in each well was measured at 570 nm with a microplate reader (BioTek, USA). The 50% cytotoxic concentration (CC_{50}) of samples was calculated as previously reported (Zhang et al., 2017).

2.4. In vitro Antiviral effects of FFYH

To investigate the antiviral effect of FFYH against the influenza virus, cytopathic effect (CPE) and hemagglutination test (HA) assay were used as previously described (Killian et al., 2020). Briefly, MDCK cells were digested into single cells by trypsin and seeded into 96-well plate for overnight (2×10^4 cells/well) and then were infected with 10 or 100 $TCID_{50}$ viruses for 1 h. After infection, the plates were washed with phosphate-buffered saline (PBS) two times to remove the free virus and were incubated with fresh medium containing various concentrations of drugs for 72 h at 35 °C under 5% CO_2. The cytopathic effect was observed every day. The inhibition effect of drugs on virus replication was detected by HA assay according to WHO guidelines for hemagglutination test. Plates were fixed with 100 μl of a 10% formaldehyde solution. After removing the solution, the dried plates were stained with a 0.1% (w/v) crystal violet staining for 30 min at room temperature to determine the protective effect of drugs on CPE caused by virus. The minimum dilution without obvious toxicity was taken as the maximum non-toxic concentration (CC_0) of drugs, and 50% cytotoxic concentration (CC_{50}) was calculated according to the Reed-Muench method (Ma et al., 2020).

2.5. In vivo Antiviral effects of FFYH against influenzas virus

ICR female mice weighing 14–15 g purchased from Comparative Medical Center for Yangzhou University were housed in IVC on a 12 h light/dark cycle and maintained at 22 ± 2 °C. The Ethics Committee approved all animal experiments at Yangzhou University (202010002) and humane care for animals was compiled with the guidelines of Jiangsu laboratory animal welfareLaboratory. The daily dosage of testing drugs for mice was transmitted from the clinical dosage of an adult human (60 kg).

To monitor the *in vivo* protective effect of FFYH against influenza A virus, mice were anesthetized with ether inhalation, and inoculated intranasally with 30 μl of viral suspension containing 5 LD_{50} of influenza virus (A/FM/1/47, H1N1, mouse-adapted) or normal saline. After 2 h of infection, the infected mice were orally administered FFYH (1.0 or 0.5 g/kg/day, dissolved in sterile water), LH (1.0 g/kg/day), ribavirin (80 mg/kg/day), or saline daily for 6 days. All mice were observed daily for 15 days. The sick signs, food consumption, and mortality in each group were recorded daily. The protective effects were evaluated by the survival time and the reduction of mortality.

Furthermore, to study the anti-inflammatory effect of FFYH *in vivo*, mice were intranasally inoculated with 30 μL of viral suspension containing 5 LD_{50} of virus and were orally administrated with FFYH (1.0 or 0.5 g/kg/day), LH (1.0 g/kg/day), ribavirin (80 mg/kg/day), or saline daily for 6 days. Mice were sacrificed on the sixth day of post-infection, blood were collected and counted with auto hematology analyzer (BC-2800Vet, Mindray, China) and the lung tissues were harvested and weighed. The lung index (the ratio of the lung weight to the body weight) was recorded. Every lung tissue of each group was divided into two parts: one was fixed in 10% phosphate-buffered formalin for the

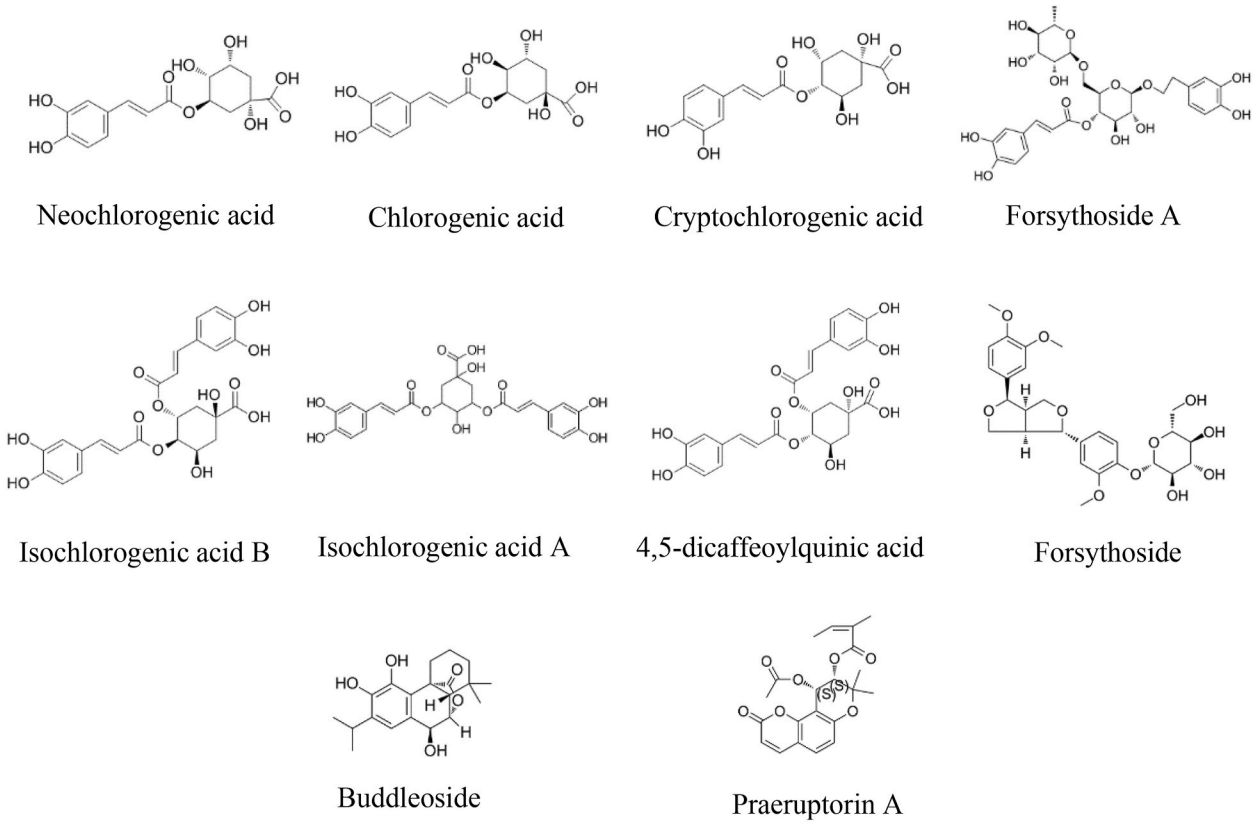

Fig. 2. Chemical structures of ten active constituents in FFYH.

hematoxylin and eosin (H&E) staining, and the other was used to test the virus and the expression of the inflammatory cytokines by RT-qPCR. The histopathological scores of the lung were evaluated as previously described (Ma et al., 2020).

2.6. RT-qPCR analysis of the expression of inflammatory cytokines

Total RNA was extracted using RNA extraction reagent (R401) (Vazyme, China) according to the manufacturer's instructions. cDNA synthesis was performed with HiScript II Q RT Supermix (R222) (Vazyme, China) according to the manufacturer's instructions. qPCR was conducted with AceQ Universal SYBR qPCR Master Mix (Vazyme, China). qPCR was performed as follows: 5 min at 95 °C, followed by 42 cycles of 10s at 95 °C and 60s at 60 °C, and a melting curve step. Primers were synthesized by Genscript (Nanjing, China) and are detailed in Table 1. Relative transcript quantities were calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as a reference.

2.7. Western blot analysis

Western blot assay was performed as described previously [Zhang et al., 2017]. Briefly, cells were lysed with RIPA lysis buffer (Beyontime, China) to get total protein lysates. Protein samples were separated by 10% or 12% SDS-PAGE gels, and then transferred onto nitrocellulose (NC) membrane (0.45 μm, Millipore) using a tank transfer system (Bio-Rad). The membranes were blocked for 1 h in 5% BSA in Tris-buffered saline (BST: 20 mM Tris, 166 mM NaCl, and 0.05% Tween 20, pH 7.5) and then incubated with primary antibodies overnight at 4 °C, and finally incubated with a horseradish peroxidase-conjugated species-specific secondary antibodies at room temperature for 1 h. Bands were visualized using an enhanced chemiluminescence kit (Millipore) with a Molecular Imager SH-523 System (Hangzhou Shenhua). Quantification relative to ACTB by densitometric analysis was performed using Quality One software (Bio-Rad).

2.8. Statistical analysis

All results are expressed as the mean ± standard deviation (S.D.). For multiple groups, statistical difference was evaluated by one-way analysis of variance (ANOVA) or Student's t-test. Differences in the survival rate between groups were analyzed using the Log-rank test. *P*-values of less than or equal to 0.05 were considered statistically significant.

3. Results

3.1. HPLC profile of FFYH

The ten bioactive components of FFYH, namely, neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, isochlorogenic acid B, isochlorogenic acid A, 4,5-Dicaffeoylquinic acid, forsythoside A, forsythoside, buddleoside, and praeruptorin A were quantified using HPLC in comparison with standard reference compounds (Figs. 1 and 2). The contents of these compounds in FFYH were 1.5%, 6.4%, 1.6%, 5.3%, 1.7%, 3.3%, 2.8%, 1.0%, 2.0%, and 0.4%, respectively.

3.2. In vitro antiviral effect of FFYH against influenzas virus

The cytotoxicity of FFYH, LH, ribavirin, oseltamivir was tested by MTT assay as previously described (Zhang et al., 2017). The CC_{50} of FFYH and LH were greater than 1.5 mg/ml. No cytotoxicity was observed at 40 μg/ml of oseltamivir and 50 μg/mL of ribavirin on MDCK cells. Therefore, the antiviral effects of drugs were performed at concentrations that showed no significant cytotoxicity in MDCK cells. As shown in Table 2, FFYH treatment inhibited the replication of influenza virus H1N1 (FM1, PR8, WSN), H3N2, H5N1, H7N9, and H9N2 in a dose-dependent manner. The IC_{50} of FFYH against FM1, PR8, WSN,

Table 2
In vitro antiviral effect of FFYH against influenza A viruses.

Virus	IC_{50}[a]				
	FFYH[b]		LH[b]	Ose[b]	RBV[b]
	$100TCID_{50}$[c]	$10TCID_{50}$[d]	$10TCID_{50}$	$10TCID_{50}$	$10TCID_{50}$
H1N1/FM1	354.8	177.8	>1000	<2	<5
H1N1/PR8	707.9	354.8	>1000	<2	<5
H1N1/WSN	707.9	354.8	100.2	<2	<5
H3N2/JS/2012	707.9	354.8	>1000	<2	<5
H5N1/SY/2004	707.9	707.9	199.6	<2	<5
H7N9/JTC4/2013	707.9	354.8	>1000	<2	<5
H9N2/SKD/2013	398.2	199.6	>1000	<2	<5

[a] IC_{50}: 50% inhibitory concentrations to inhibit the replication of influenza A virus; the data is representative of three independent experiments, only showing the mean value of four replicates.
[b] μg/ml.
[c] MDCK cells were infected with $100TCID_{50}$ viral infection dose.
[d] MDCK cells were infected with $10TCID_{50}$ viral infection dose. FFYH, Fufang-Yinhua-Jiedu granule; Ose, Oseltamivir phosphate; RBV, ribavirin.

H3N2, H5N1, H7N9, and H9N2 ($100TCID_{50}$) is 354.8 μg/ml, 707.9 μg/ml, 707.9 μg/ml, 707.9 μg/ml, 707.9 μg/ml, 707.9 μg/ml and 398.2 μg/ml, respectively. The IC_{50} of FFYH against FM1, PR8, WSN, H3N2, H5N1, H7N9, and H9N2 ($10TCID_{50}$) is 177.8 μg/ml, 354.8 μg/ml, 354.8 μg/ml, 354.8 μg/ml, 354.8 μg/ml, 354.8 μg/ml and 199.6 μg/ml, respectively and the selectively index (SI) was 5.62, 2.82, 2.82, 2.82, 2.82, 2.82 and 5.0, respectively. Furthermore, FFYH exerted a good protective effect on CPE induced by the above influenza A virus. However, LH, which is commonly used in the treatment of seasonal influenza and COVID-19 (Li et al., 2020), only has a good inhibition effect on H1N1 and H5N1 but has no obvious inhibition effect on the replication of other subtypes of influenza virus. Ribavirin (50 μg/ml, 10 μg/ml and 5 μg/ml) and oseltamivir (8 μg/ml, 4 μg/ml and 2 μg/ml) could completely inhibit the replication of the above influenza virus and protect the cytopathic effect caused by various influenza A viruses.

3.3. *In vivo antiviral effect of FFYH against H1N1 influenzas virus*

To identify the antiviral effect of FFYH *in vivo*, we recorded daily the clinical signs, food intake, and weight change. During the investigation time, the clinical symptoms of the FFYH treatment group were significantly improved compared with the virus-infected group. After 2 days post-infection, the virus-infected group showed obvious clinical signs such as inactivity, ruffled fur, reduced feed intake, weight loss and respiratory distress. In contrast, FFYH administration dose-dependently improved the above illness symptoms, increased in the diet, and prevented weight loss. As shown in Fig. 3, until the 6th-day post-infection, only 40% of mice treated with high-dose of FFYH (1.0 g/kg) showed slight clinical signs. However, about 30% of the weight body of the virus-infected group was reduced compared to initial weight, and all the mice died between 8- and 12-days post-infection with 9.3 ± 1.3 days of median survival time (Table 3). Compared with the virus-infected group, FFYH treatment (1.0 g and 0.5 g/kg) significantly increased the death protective rate of the mice (77.8% and 55.6%, respectively) and prolonged the survival time (14.3 ± 1.5 and 13.5 ± 2.2 days, respectively) (Table 3). In addition, although compared with the virus-infected group, the above clinical signs, weight loss and diet-reduced of the mice treated with LH were improved, its protective effect is markedly weaker than that of the FFYH treatment group. Furthermore, the death protective

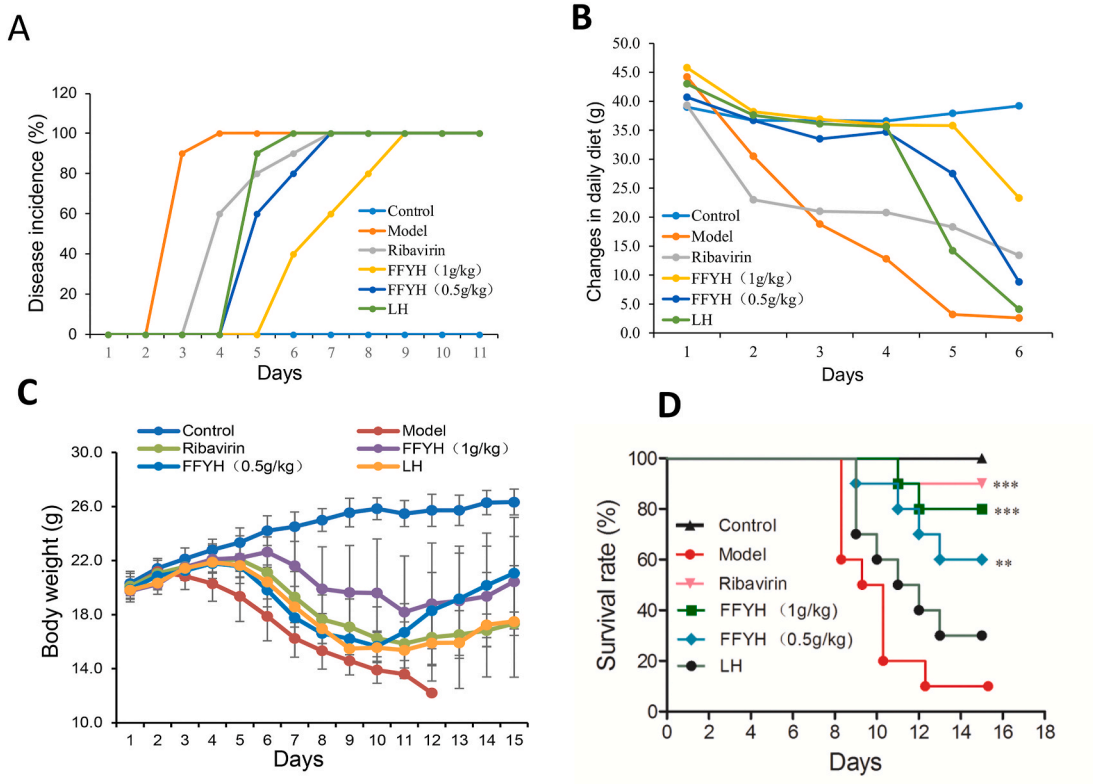

Fig. 3. The anti-influenza effect of FFYH on lethal FM1 virus infected mice. ICR mice were infected with influenza virus A/FM/1/47 (H1N1) and then were treated with orally with FFYH (1.0 g/kg or 0.5 g/kg), ribavirin (80 mg/kg), or LH (1.0 g/kg) for 6 days. All mice were observed daily for 15 days. The sick signs, food intake, and mortality in each group was recorded daily. The protective effects were evaluated by the survival time and the reduction of mortality. **$p < 0.05$, ***$p < 0.001$, when compared with the virus-infected group ($n = 10$).

Table 3
The protective effect of FFYH on lethal influenza A virus H1N1.

Group	Dose	Mice (n)	Death (n)	Death rate (%)	Death protective rate (%)	Mean survival days	Life extension rate (%)
Control	/	10	0	0	/	15.0	/
Model	/	10	9	90	/	9.6 ± 2.1	/
Ribavirin	80.0 mg/kg	10	1	10	88.9	14.6 ± 1.3***	55.6***
FFYH (H)	1.0 g/kg	10	2	20	77.8	14.3 ± 1.5***	49.0***
FFYH (L)	0.5 g/kg	10	4	40	55.6	13.5 ± 2.2**	40.6**
LH	1.0 g/kg	10	7	70	22.2	11.8 ± 2.6*	22.9*

Note: *P < 0.05, **P < 0.01, ***P < 0.001 vs Model.

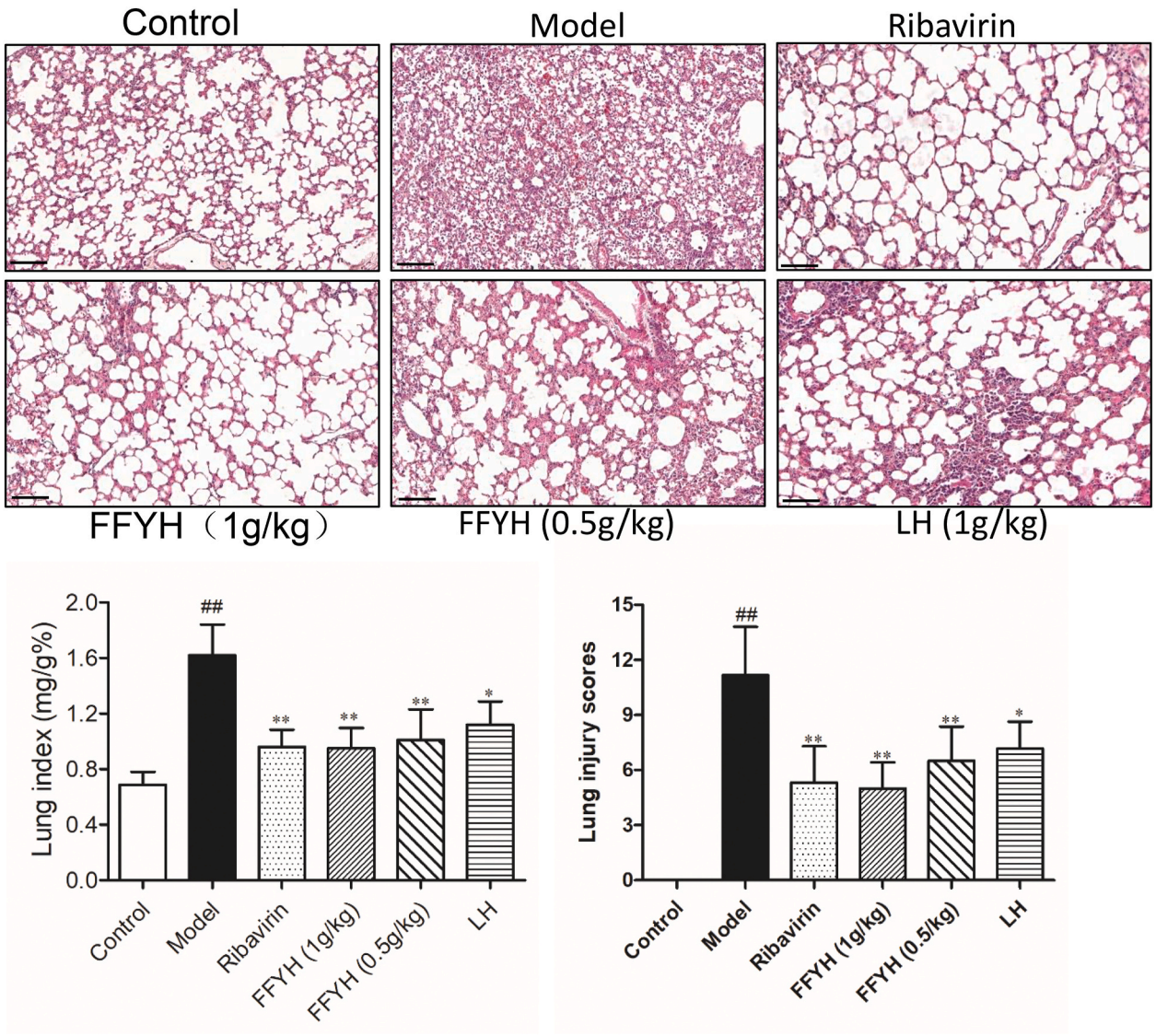

Fig. 4. The protective effect of FFYH on lung injury *in vivo*. ICR mice were infected with influenza virus A/FM/1/47 (H1N1) and then were treated with orally with water, FFYH (1.0 g/kg or 0.5 g/kg), ribavirin (80 mg/kg), or LH (1.0 g/kg) (10 mice per group) for 6 days. And then the mice were sacrificed and the lung tissues were harvested and weighed. The lung index was recorded. Representative histological changes stained by hematoxylin and eosin in lung were photographed (200x); scale bar: 20 μm. The histopathological scores of lungs in each group were evaluated as previously described. *p < 0.05, **p < 0.01, when compared with the virus-infected group; ##p < 0.01, when compared with the normal group (n = 10).

rate (22.2%) and life extension rate (11.8 ± 2.6 days) of the LH treatment group were significantly lower than those of the FFYH treatment group (Table 3). Notably, ribavirin treatment significantly increased the death protective rate of the mice (88.9%) and markedly extended the survival time of the mice (14.6 ± 1.3), while it didn't affect preventing weight loss and reducing the disease incidence, which is weaker than that of FFYH treatment (Table 3).

3.4. Protective effect of FFYH on acute lung injury induced by influenza virus

As shown in Fig. 4, the lung index of the virus-infected group was 1.62 ± 0.22, which was markedly higher than that of the control group (0.69 ± 0.09, p < 0.01). However, compared with the virus-infected group, the lung index of the mice with treatment with FFYH, LH, or

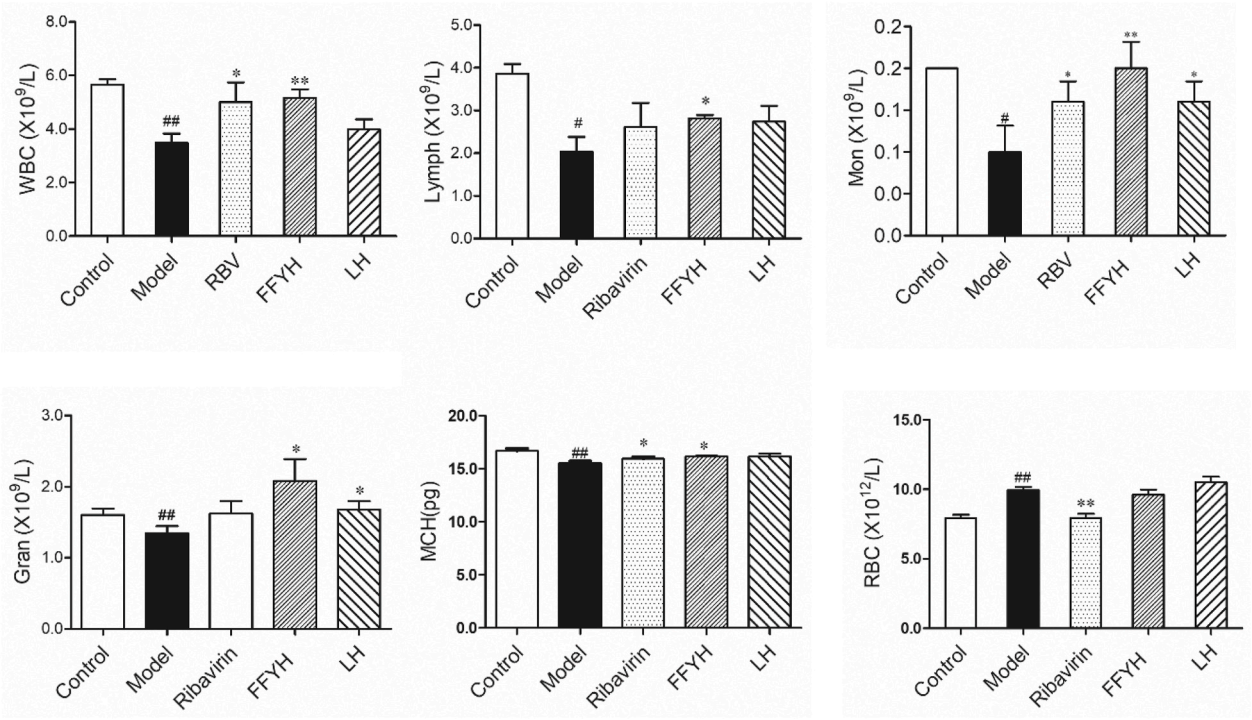

Fig. 5. Effect of FFYH treatment on blood routine caused by influenza A virus. Blood samples of mice (n = 6) were collected on days 5 post-infection. 100 μl of blood from each mouse was analyzed with auto hematology analyzer (BC-2800Vet). The absolute number of white blood cells, red blood cells, lymphocytes, monocytes, neutrophils, as well as hemoglobin mean red blood cell hemoglobin $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$ virus infection group & normal group; $^{*}p < 0.05$ or $^{**}p < 0.01$ treatment group & virus infection group. White blood cells (WBC), lymphocytes (lymph), monocytes (mon), neutrophils (Gran), red blood cells (RBC), and mean red blood cell hemoglobin (MCH).

ribavirin was significantly improved ($p < 0.01$). The lung index of the mice-treated with FFYH (1.0 g/kg and 0.5 g/kg), LH or ribavirin were 1.01 ± 0.20, 1.01 ± 0.22, 1.12 ± 0.17, and 0.96 ± 0.12, respectively. Moreover, the inhibition effect of FFYH (1.0 g/kg and 0.5 g/kg) on the lung index is more potent than that of LH treatment. To further investigate whether the FFYH could enhance the protective effect on acute lung injury, we next examined the lung histological changes of mice. As shown in Fig. 4, severe lung damage with a diffuse swelling, severe cell necrosis, alveolar cavity collapse, alveolar thickening, severe infiltration of large inflammatory cells, and hemorrhage were observed in the virus-infected model group. The lung injury evaluated by pathology scores was significantly improved in the FFYH-treated group, when compared with the virus-infected group. Notably, the protective effect of FFYH high dose (1.0 g/kg) is better than that of the low dose group, which is similar to that of LH treatment. Also, the pathological changes in ribavirin-treated mice were identical to that of the FFYH high-dose group. These results indicated that FFYH has apparent protective effects on acute lung injury caused by influenza A virus infection.

3.5. Improvement effect of FFYH on blood parameters caused by influenza A virus

Blood parameters are important indicators of inflammation caused by influenza A virus. To investigate the improvement effect of FFYH on blood parameters, we compared blood parameters of mice treated with FFYH, LH, ribavirin, normal and virus-infected group. Blood samples (n = 6) on days 5 p.i. were collected and analyzed for white blood cells, lymphocytes, monocytes, granulocytes, red blood cells, hemoglobin and platelet counts (Fig. 5). Compared with normal control group, levels of lymphocytes, monocytes, granulocytes and total white blood cells were markedly decreased at days 5 p.i., while FFYH treatment could significantly prevent the decrease of total white blood cells, lymphocytes, monocytes, and granulocytes, which is stronger than that of ribavirin. On the other hand, a significant drop of hemoglobin and an apparent increase of red blood cells were observed in virus-infected group. Compared with the virus-infected group, FFYH treatment could markedly prevent the decrease of hemoglobin. Except for the above parameters, we didn't observe any significant change in other blood parameters between virus-infected group and treatment group with FFYH, Ribavirin, and LH.

3.6. Inhibition effect of FFYH on the expression of inflammatory factors

It is commonly accepted that pro-inflammatory cytokines are significantly increased during severe influenza virus infections, the "cytokine storm" has been hypothesized to play an essential cause of ARDS and mortality (Damjanovic et al., 2012; Fukuyama and Kawaoka, 2011; Lobo et al., 2019; Short et al., 2014). Therefore, we firstly examined the levels of the proinflammatory factors caused by the influenza virus. Results showed that levels of TNF-α, IL-6, IFN-γ, IP10, and IL-1β mRNA in the virus-infected group were significantly increased ($p < 0.01$), when compared with the control group as shown in Fig. 6. Notably, similar to positive control ribavirin, FFYH treatment markedly suppressed the expression of TNF-α, IL-6, IFN-γ, IP10, and IL-1β mRNA, compared with the virus-infected group and LH treatment. Moreover, the inhibitory effect of FFYH treatment on the proinflammatory factors TNFα and IP-10 was significantly stronger than that of the ribavirin treatment group. However, the inhibitory effect of FFYH treatment on cytokines with antiviral effects such as IFN-γ, and IL-1β is slightly weaker than that of the ribavirin group. Surprisingly, except for the slight inhibition of the expression of TNF-α, LH treatment did not show an obvious inhibitory effect on other cytokines. To further verify the anti-inflammatory effect of FFYH *in vivo*, the levels of some inflammatory cytokines in mouse sera after 5 day of post-infection were measured by ELISA. As shown in Fig. 7, compared with the infected group, the cytokines such as TNF-a, IL-6, IFN-a, IL-1β, and IP-10 in the FFYH

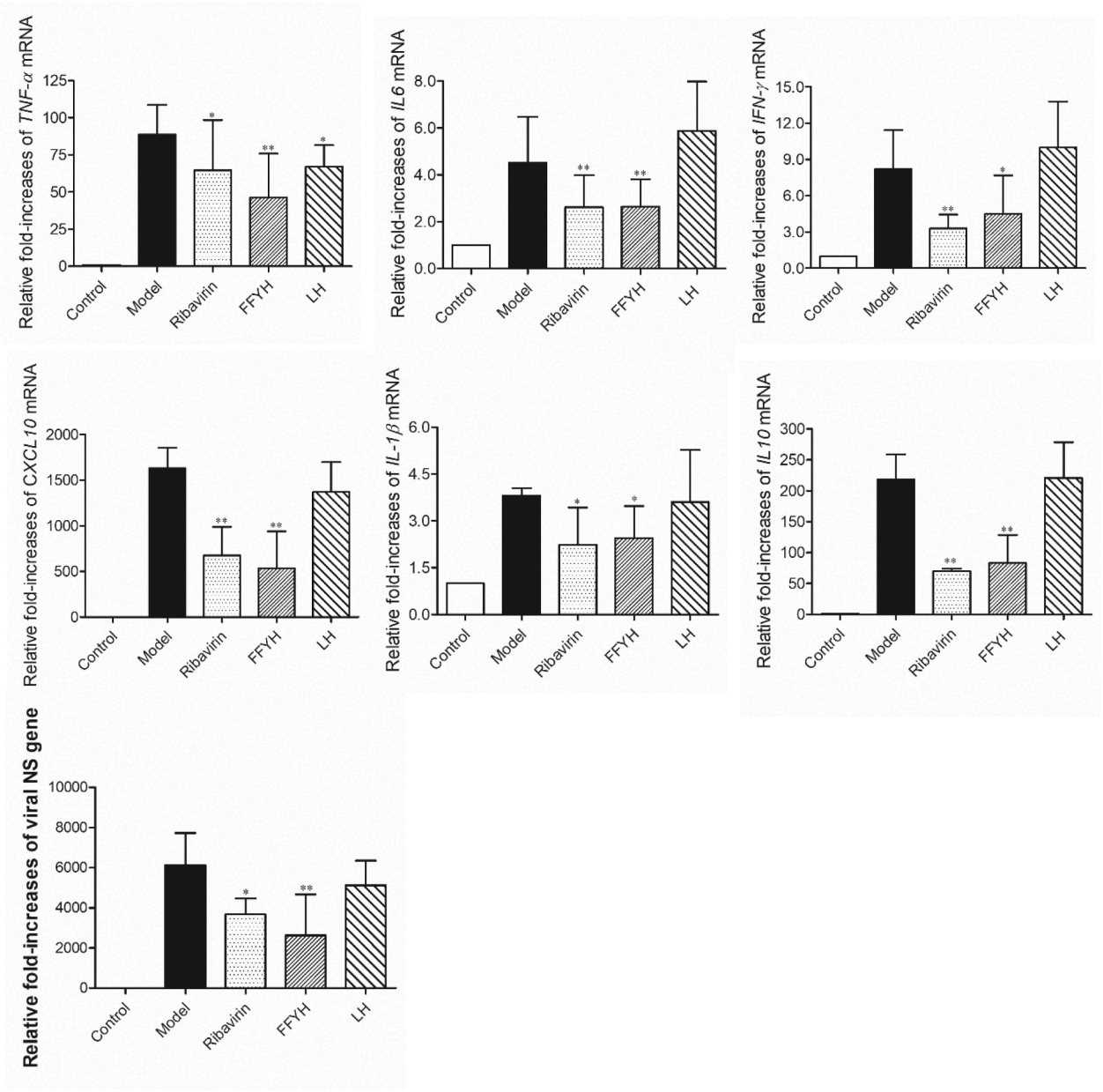

Fig. 6. Effect of FFYH on the expression of cytokines, TNF-α, IL-6, IFN-γ, IP10, IL-10, and IL-1β mRNA in lung tissues of mice-infected with influenza a virus (H1N1). Furthermore, relative expression level of viral RNA was also quantified by qPCR. Relative transcript quantities were calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as a reference. *p < 0.05, *p < 0.01, compared with the virus-infected group (n = 6).

treatment group were significantly reduced (p < 0.05 or 0.01). In addition, the positive drug ribavirin treatment can also markedly reduce the expression of these above inflammatory factors, while LH has no obvious anti-inflammatory effect except on TNF-a. Taken together, these results suggest that FFYH treatment can effectively suppress the expression of pro-inflammatory factors induced by the influenza virus.

3.7. Inhibitory effect of FFYH on the TLR7/MyD88 signaling pathway

Toll-like receptors (TLRs) play a critical role in innate immune responses to pathogen infections such as bacteria, viruses (Arora et al., 2019; Elshabrawy et al., 2017). To further clarify the underlying anti-inflammatory mechanisms of FFYH, we examined the mRNA transcription of TLR7, MyD88, IRF7, IRAK4, TRAF6 , and TRAF3. As shown in Fig. 8, compared with the control group, the mRNA transcript levels of TLR7, MyD88, IRF7, IRAK4, TRAF6 , and TRAF3 were markedly increased in the virus-infected group. At the same time, FFHY significantly suppressed the expression of TLR7, MyD88, IRF7, IRAK4, TRAF6, and TRAF3 mRNA, although no apparent effect on the transcript of TLR3 and TLR4 mRNA (data not shown). Similar to FFYH, ribavirin treatment could markedly reduce the expression of TLR7, MyD88, IRF7, IRAK4 mRNA, but it does not affect the transcript of TRAF3 mRNA. To confirm the above effects of FFYH against influenza A virus, we used A549 lung adenocarcinoma as a cell model to further investigate the anti-inflammatory mechanism of FFYH by immunoblotting. As shown in Fig. 9, compared with the virus-infected group, the expression levels of TLR7, MyD88, IRF7, TRAF6 and TRAF3 proteins in the FFYH treatment group were markedly decreased. These results indicated that FFYH might effectively inhibit the expression of proinflammatory factors via inhibiting TLR7-MyD88 signaling pathway.

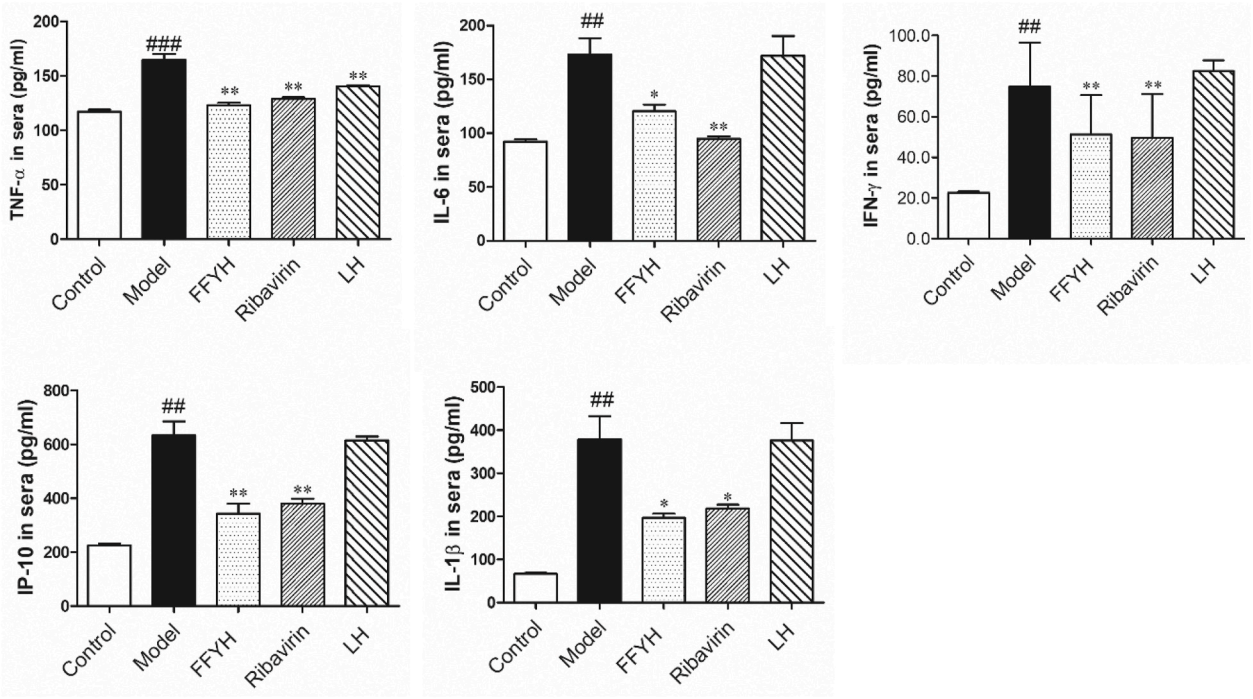

Fig. 7. *In vivo* inhibitory effect of FFYH on the inflammatory factors, TNF-α, IL-6, IFN-γ, IP10, and IL-1β caused by influenza a virus (H1N1). The concentration of inflammatory factors in mice sera were quantified with ELISA assays. $^{\#\#}p < 0.05$, $^{\#\#\#}p < 0.01$, compared with normal control group; $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the virus-infected group (n = 6).

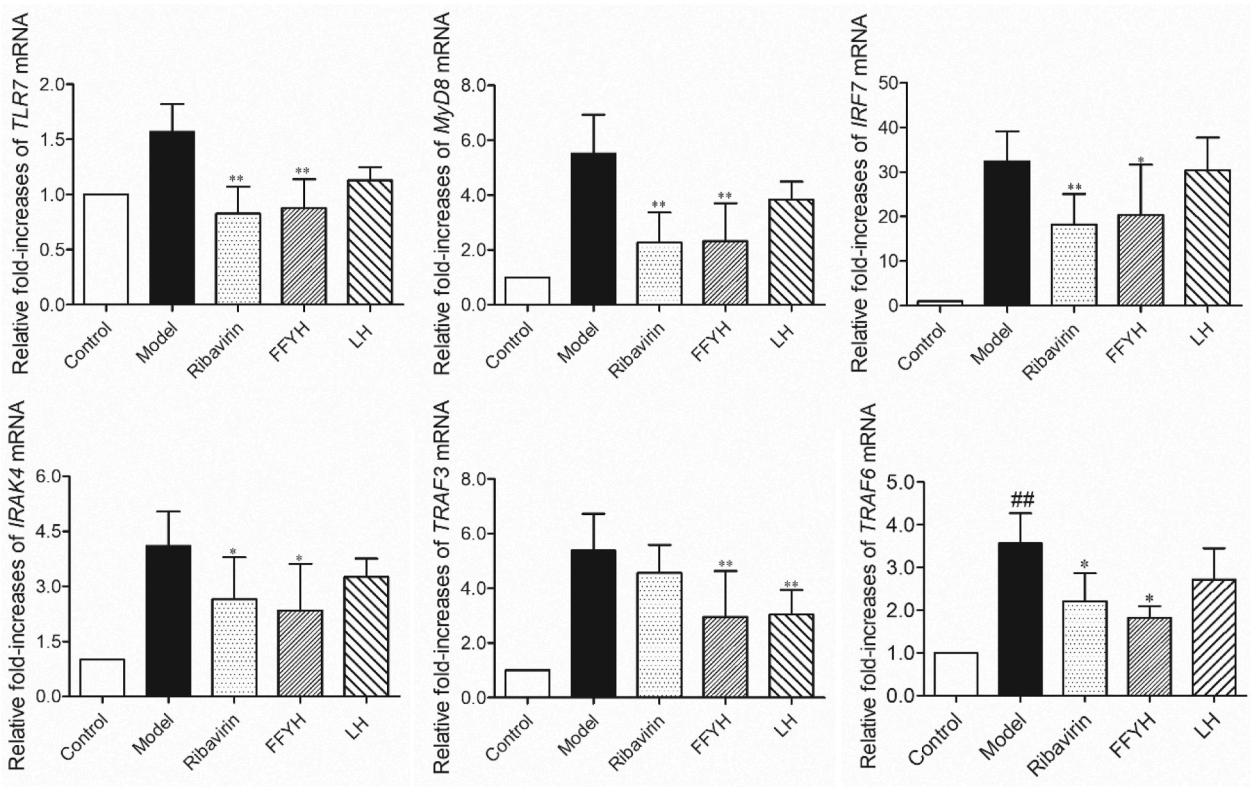

Fig. 8. FFYH suppressed the inflammatory responses induced by influenza a virus through regulating the TLR7/MyD88 signaling pathway. Relative expression levels of TLR7, MyD88, IRF7, IRAK4, TRAF3 and TRAF6 mRNA in lung tissues in each group. Relative transcript quantities were calculated using the $2^{-\Delta\Delta Ct}$ method with GAPDH as a reference. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with the virus-infected group (n = 6).

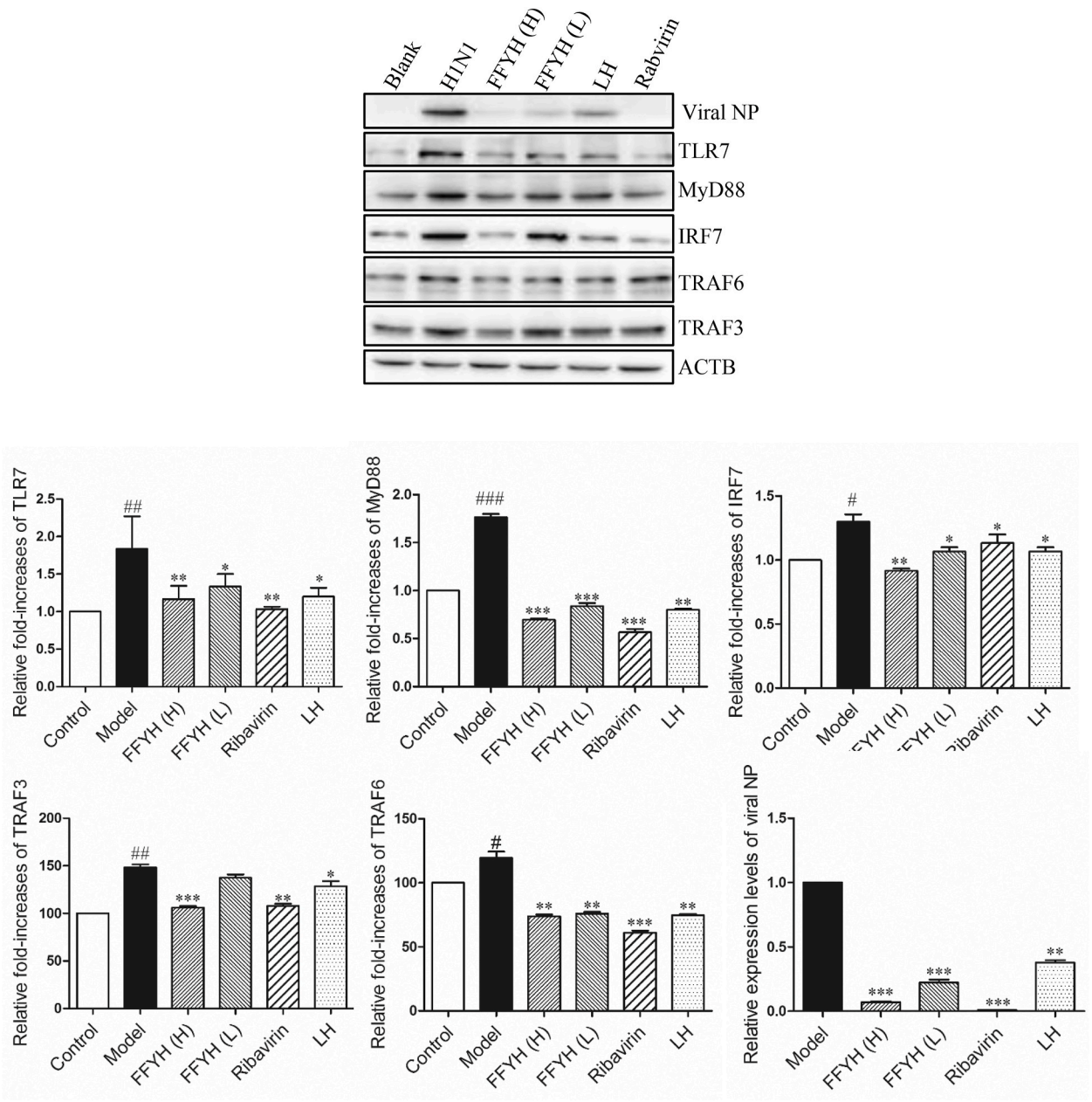

Fig. 9. The inhibitory effect of FFYH on the TLR7/MyD88 signaling pathway. After infection with influenza A virus (H1N1), A549 cells were treated with FFYH, Ribavirin, and LH for 24 h, and the expression levels of viral NP, TLR7, MyD88, IRF7, TRAF3 and TRAF6 were examined with immunoblotting. Relative expression levels were quantified with using quality one software (Bio-Rad). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ or $^{\#\#\#}p < 0.001$, compared with normal control group; $^{*}p < 0.05$, $^{**}p < 0.01$ or $^{***}p < 0.001$, compared with the virus-infected group.

4. Discussion

Influenza A virus such as severe seasonal H1N1, H5N1 and H7N9 are highly infectious and important causes of mortality and morbidity (Short et al., 2014; To et al., 2013; Watanabe et al., 2014). Known antiviral therapies with such as oseltamivir, zanamivir, amantadine could improve outcomes in patients infected by H1N1, H5N1 and H7N9 influenza viruses (Dunning et al., 2014). Despite antivirals, significant number of deaths occur (e.g., 50% or 39% mortality in patients infected with influenza H5N1 or H7N9, respectively) (Yang et al., 2017). Therefore, antiviral therapy may not be an effective strategy by itself to minimize morbidity and mortality from severe influenza infection. Notably, high levels of pro-inflammatory cytokines have been reported in patients with severe H1N1, H5N1, and H7N9 virus infection (Fukuyama and Kawaoka, 2011; Lobo et al., 2019; Short et al., 2014). The researchers proposed that the pathogenesis of severe influenza infection is associated with the uncontrolled pro-inflammatory cytokines (Damjanovic et al., 2012; Fukuyama and Kawaoka, 2011; Peiris et al., 2009; To et al., 2013). Therefore, anti-inflammatory therapies through regulating immune responses may provide an effective method to reduce morbidity and mortality during an influenza pandemic (Peiris et al., 2009). Traditional Chinese medicine has been used to treat infectious diseases for more than 5000 years and showed a good therapeutic effect in the clinic, such as Yin-Qiao-San and Xiao-Qing-Long Decoction (Law et al., 2017; Chang et al., 2013). Furthermore, due to the characteristics of multi-components and multi-pharmaceutical effects of TCMs, they not only could have an antiviral effect and anti-inflammatory, but also could improve patients fever, respiratory failure and other adverse symptoms (Dunning et al., 2014; Ma et al., 2018, 2020; Rong et al., 2016; Shi et al., 2020; Yin et al., 2017). FFYH as

a TCM remedy has been used to treat common colds, influenza, and upper respiratory tract infection with symptoms such as fever, headache, nasal congestion for more than 10 years (Jiang et al., 2003; Lu et al., 2003; Xi and Xi, 2003). Phase II clinical trial with randomized double-blind and parallel controls indicated that the total effective rate of FFYH in treating upper respiratory tract infections with the wind-heat syndrome was 90.48%. Furthermore, preclinical pharmacological data have demonstrated that FFYH might reduce the lung edema caused by influenza A virus and has anti-inflammatory, fever-reducing, and analgesic effect. In this study, we found that FFYH *in vitro* could significantly exhibit a broad-spectrum of antiviral activity against H1N1, H3N2, H5N1, H7N9, and H9N2 influenza A viruses. Furthermore, the antiviral activity of FFYH *in vivo* was further confirmed in mice infected with the H1N1 virus. Our results showed that FFYH dose-dependently reduced the mortality and extended the survival time of the infected mice. Moreover, the clinical signs, pulmonary edema, and pathological scores were markedly alleviated, and virus titers were significantly decreased. These results suggested that FFYH possessed potential anti-influenza virus activity. Furthermore, we demonstrated for the first time that FFYH could significantly suppress the expression of inflammatory cytokines such as TNF-α, IL-6, IP-10, IFN-γ, IL-10, and IL-1β via TLR7-MyD88-IRF7 signaling pathway in the infected mice.

An emerging of studies has demonstrated that excessive inflammatory responses resulting in cytokine storm play a critical role in the development of viral pneumonia induced by influenza virus infection (Fukuyama and Kawaoka, 2011; Peiris et al., 2009; Short et al., 2014). Thus, regulating the uncontrolled inflammatory responses may be an effective therapy for viral pneumonia. Several proinflammatory cytokines including TNF-α, IL-6, IP-10, IFN-γ, and IL-1β have been reported to play a crucial role in influenza viral pneumonia (Dunning et al., 2014; Fukuyama and Kawaoka, 2011; Madera and Libraty, 2013; Peiris et al., 2009; Short et al., 2014; Yang et al., 2017). For example, excessive expression of TNF-a and IL-6 in sera or lung during the early stage of influenza A virus infection is highly associated with disease severity (Daniela et al., 2011). Recently, clinical researches have revealed that excessive expression of IL-6 in sera may also be related with the severity of novel coronaviral pneumonia (COVID-19) caused by SARS-CoV-2, suggesting that it may be a potential therapeutic target for viral pneumonia (Liu et al., 2020; Svitek et al., 2008). Furthermore, IL-1β may be one of the critical inflammatory factors resulting in acute lung injury and ARDS (Fukuyama and Kawaoka, 2011; Niu et al., 2019). In addition, IFN-γ is a major inflammatory factor leading to acute lung injury and ARDS caused by severe influenza virus infection (Arora et al., 2019; Liu et al., 2018). Moreover, the chemokines CXCL10 (IP 10) as a critical effector induced by IFN-γ plays a crucial role in acute lung injury and ARDS caused by virus or bacteria due to its effect of recruiting or activating neutrophils (Hayney et al., 2017; Liu et al., 2011; Sidahmed et al., 2012). Therefore, anti-inflammatory therapy may contribute to reducing acute lung injury or ARDS caused by influenza virus infection. Here, our results indicated that FFYH treatment markedly inhibits the expression of TNF-α, IL-6, IP-10, IFN-γ, and IL-1β, suggested that inhibition of "cytokines storm" might be the potential mechanism of FFYH against influenza viral pneumonia.

TLRs are key receptors of PRRs for recognizing pathogens such as bacteria, viruses (Arora et al., 2019; Elshabrawy et al., 2017). TLR7 is a key member of the TLR family to recognize single-stranded viral RNA (ssRNA) viruses (Arora et al., 2019; Shi et al., 2020). MyD88, as a key molecule downstream of TLR7, can activate the NF-κB signaling pathway to promote the expression of proinflammatory cytokines, such as TNF-α, IL-6, IP-10, IFN-γ, and IL-1β by recruiting IRAK and TRAF (Madera and Libraty, 2013). Therefore, in theory, activation of this signaling pathway play a protective effect on acute lung injury caused by the influenza A virus. However, excessive activation of this signaling pathway might cause detrimental inflammation (Arora et al., 2019; Madera and Libraty, 2013; Shi et al., 2020). In such cases, inhibiting the TLR7 signaling pathway contribute to reducing acute lung injury caused by a viral infection (Arora et al., 2019; Elshabrawy et al., 2017). Furthermore, we detected that FFYH treatment markedly inhibited the expression of TLR7, MyD88, IRAK4, and TRAF3 mRNA, but has no obvious effect on TLR3 and TLR4 mRNA, suggesting the anti-inflammatory effect of FFYH against viral pneumonia may be achieved through regulating the TLR7/MyD88 signaling pathway. Compared with the mice with LH treatment or infected with the virus, the expression levels of TLR7, MyD88, IRAK4, and TRAF3 mRNA in the FFYH treatment were significantly decreased, suggesting the better anti-inflammatory effect of FFYH against influenza virus. In view of the effect of TLR7/MyD88 in recognizing viral RNA to induce excessive inflammatory response, we speculate that FFYH may exert anti-inflammatory effect by inhibiting the replication of virus to reduce the production of viral RNA. However, future work is required to better understand the underlying mechanism of FFYH against influenza A virus by regulating TLR7/MyD88 signaling pathway.

Although TCM comes from the accumulation of clinical treatment experience, many TCMs have been demonstrated to be effective in preclinical and clinical studies for the treatment of influenza virus (Li et al., 2018, 2020; Ma et al., 2018, 2020; Peng et al., 2016; Shi et al., 2020; Tian et al., 2011; Wu et al., 2016; Yin et al., 2017). For example, Yin-Qiao-San, as a perfect prescription, has been used for treating common cold, fever, coughing, and other respiratory diseases for more than 600 years in China. Recent studies by Law et al. (2017) also confirmed that YQS could inhibit the replication of influenza A virus H1N1 and increased the survival rate of the mice-infected with H1N1. FFYH optimized from Yin-Qiao-San is composed of ten plants including *Artemisia annua* L., *Lonicerae japonica* Thunb., *Schizonepeta tenuifolia* (Benth.) Briq., *Mentha haplocalyx* Briq., *Chrysanthemum indicum* L., *Isatidis tinctorial* L., *Forsythia suspensa* (Thunb.) Vahl., *Commelina communis* L., *Peucedanum praeruptorum* Dunn, and *Glycine* max (L.) Merr. Most of the active components from FFYH such as chlorogenic acid, cryptochlorogenic acid, neochlorogenic acid, isochlorogenic acid A, isochlorogenic acid B, forsythoside A, and 4,5-dicaffeoylquinic acid have been reported to have antiviral activities against influenza virus (Law et al., 2017; Liau et al., 2007; Mok et al., 2014; Shi et al., 2007; Zhao et al., 2019). For example, Zhao et al. recently reported that most of the phenolic acids such as chlorogenic acid, neochlorogenic acid, isochlorogenic acid A, isochlorogenic acid B extracted from *Lonicera japonica* (Jinyinhua) showed a good inhibitory activity to neuraminidase from influenza A virus (Zhao et al., 2019). Law et al. have demonstrated that forsythoside A from *Forsythia suspens* (Thunb.) Vahi fruit not only could inhibit the replication of influenza A virus *in vitro*, but also could markedly increase the survival rate of the mice infected with H1N1 influenza a virus (Law et al., 2017). Shi et al. also demonstrated that 4, 5-dicaffeoylquinic acid, as a critical bioactive dicaffeoylquinic acid, showed better antiviral activity against influenza A virus, HSV-1, and HSV-2 (Shi et al., 2007). Furthermore, the anti-inflammatory activity of chlorogenic acid (Zhang et al., 2010), forsythoside A (Zhang et al., 2018), and 4,5-dicaffeoylquinic acid (Ryu et al., 2016) have been demonstrated *in vitro* or *in vivo*. In this study, these above major active components in FFYH were confirmed with HPLC. Although the anti-influenza virus activity of these bioactive components in FFYH needs to be further confirmed, these previous researches provide a reasonable explanation for the potential mechanism of FFYH against influenza a virus. However, to reveal the potential mechanism of FFYH against influenza A virus, other antiviral and anti-inflammatory components in FFYH need to be further isolated and confirmed in the future. Also, the detailed mechanism of FFYH against influenza A virus and acute lung injury caused by influenza virus infection need to be further explored.

5. Conclusion

In summary, our results for the first time demonstrated that FFYH can markedly inhibit the replication of influenza A virus, including

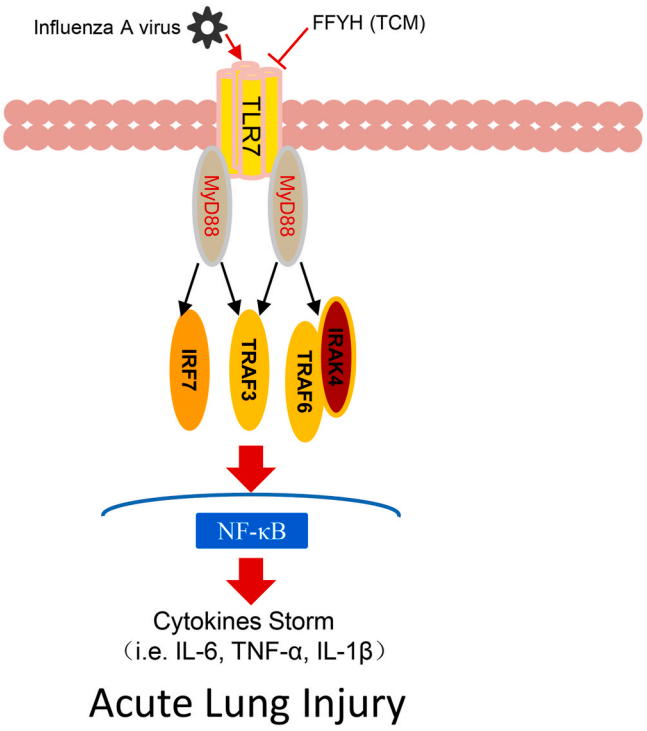

Fig. 10. Potential model of FFYH attenuating acute lung injury caused by influenza a virus through inhibiting cytokine storms via regulating the TLR7/MyD88 signaling pathway.

H1N1, H3N2, H5N1, H7N9, and H9N2 *in vitro* and significantly improve the survival rate of lethal influenza virus. The *in vivo* protective effect of FFYH against acute lung injury caused by the influenza virus may be associated with the inhibition of inflammatory responses through regulating the TLR7/MyD88 signaling pathway (Fig. 10). These findings suggested that FFYH may be an effective TCM for the clinical treatment of influenza virus infection.

Author contributions

Pinghu Zhang designed the study, analyzed the data, and wrote the manuscript. Yuqian Zhang, Ronghua Wang and Zhihui Zheng performed the experiments and collected the data. Weiqing Shi performed the pathological section and pathological lung analysis. Cheng Li and Shuofeng Zhang assist in completing animal experiments and animal anatomy. Xiaoquan Wang identified the virus strains and determined the virus titers.

Declaration of competing interest

All authors declare no conflict of interest.

Acknowledgements

We thank Mr. Mohammed Salem for improvement language. This study was supported by the Program for New Century Excellent Talents in University (Program No. NCET-12-0975), the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry and Open Project from Jiangsu Provincial Key Laboratory of Human Zoonosis (R1707), 2019 Yangzhou University Postgraduate Research and Innovation Program (XKYCX19-159).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2021.114063.

References

Abdelwhab, E.M., Veits, J., Mettenleiter, T.C., 2017. Biological fitness and natural selection of amantadine resistant variants of avian influenza H5N1 viruses. Virus Res. 228, 109–113.
Arora, S., Ahmad, S., Irshad, R., Goyal, Y., Rafat, S., Siddiqui, N., Dev, K., Husain, M., Ali, S., Mohan, A., Syed, M.A., 2019. TLRs in pulmonary diseases. Life Sci. 233, 116671.
Chang, J.S., Yeh, C.F., Wang, K.C., Shieh, D.E., Yen, M.H., Chiang, L.C., 2013. Xiao-Qing-Long-Tang (Sho-seiryu-to) inhibited cytopathic effect of human respiratory syncytial virus in cell lines of human respiratory tract. J. Ethnopharmacol. 147 (2), 481–487.
Damjanovic, D., Small, C.L., Jeyananthan, M., McCormick, S., Xing, Z., 2012. Immunopathology in influenza virus infection: uncoupling the friend from foe. Clin. Immunol. 144 (1), 57–69.
Daniela, D., Divangahi, M., Kugathasan, K., Small, C.L., Zganiacz, A., Brown, E.G., Hogaboam, C.M., Gauldie, J., Xing, Z., 2011. Negative regulation of lung inflammation and immunopathology by TNF-a during acute influenza infection. Am. J. Pathol. 179 (6), 2963–2976.
Davis, A.S., Taubenberger, J.K., Bray, M., 2015. The use of nonhuman primates in research on seasonal, pandemic and avian influenza. Antivir. Res. 117 (24), 1893–2014.
Dunning, J., Baillie, J.K., Cao, B., Hayden, F.G., 2014. Antiviral combinations for severe influenza. Lancet Infect. Dis. 14 (12), 1259–1270.
Elshabrawy, H.A., Essani, A.E., Szekanecz, Z., Fox, D.A., Shahrara, S., 2017. TLRs, future potential therapeutic targets for RA. Autoimmun. Rev. 16 (2), 103–113.
Fukuyama, S., Kawaoka, Y., 2011. The pathogenesis of influenza virus infections: the contributions of virus and host factors. Curr. Opin. Immunol. 23 (4), 481–486.
Hayney, M.S., Henriquez, K.M., Barnet, J.H., Ewers, T., Champion, H.M., Flannery, S., Barrett, B., 2017. Serum IFN-γ-induced protein 10 (IP-10) as a biomarker for severity of acute respiratory infection in healthy adults. J. Clin. Virol. 90, 32–37.
Jiang, M., Xiong, N.N., Xi, Z.Q., Zou, J.D., 2003. Clinical trial for treating wind-heat syndrome of upper respiratory infection and influenza with with yinhua jiedu granule. Tradit. Chin. Drug Res. Clin. Pharmacol. 14 (4), 270–272.
Killian, M.L., 2020. Hemagglutination assay for influenza virus. Methods Mol. Biol. 2123, 3–10.
Law, A.H., Yang, C.L., Lau, A.S., Chan, G.C., 2017. Antiviral effect of forsythoside A from Forsythia suspensa (Thunb.) Vahl fruit against influenza A virus through reduction of viral M1 protein. J. Ethnopharmacol. 209, 236–247.
Li, Z.T., Zhao, J.L., Zhou, H.X., Li, L., Ding, Y.Y., Li, J., Zhou, B.X., Jiang, H.M., Zhong, N. S., Hu, W.H., Yang, Z.F., 2018. Cappariloside A shows antiviral and better anti-inflammatory effects against influenza virus via regulating host IFN signaling, in vitro and vivo. Life Sci. 200, 115–125.
Li, R.F., Hou, Y.L., Huang, J.C., Pan, W.Q., Ma, Q.H., Shi, Y.X., Li, C.F., Zhao, J., Jia, Z.H., Jiang, H.M., Zheng, K., Huang, S.X., Dai, J., Li, X.B., Hou, X.T., Wang, L., Zhong, N. S., Yang, Z.F., 2020. Lianhuaqingwen exerts anti-viral and anti-inflammatory activity against novel coronavirus (SARS-CoV-2). Pharmacol. Res. 156, 104761.
Liau, B.C., Jong, T.T., Lee, M.R., Chen, S.S., 2007. LC-APCI-MS method for detection and analysis of tryptanthrin, indigo, and indirubin in Daqingye and Banlangen. J. Pharmaceut. Biomed. Anal. 43 (1), 346–351.
Liu, M., Guo, S., Hibbert, J.M., Jain, V., Singh, N., Wilson, N.O., Stiles, J.K., 2011. CXCL10/IP-10 in infectious diseases pathogenesis and potential therapeutic implications. Cytokine Growth Factor Rev. 22 (3), 121–130.
Liu, B., Bao, L.L., Wang, L., Li, F.D., Wen, M.J., Li, H., Deng, Wei, Zhang, X.L., Cao, Bin, 2018. Anti-IFN-γ therapy alleviates acute lung injury induced by severe influenza A (H1N1) pdm09 infection in mice. J. Microbiol. Immunol. Infect. S1684–1182 (180), 30438–30439.
Liu, B., Li, M., Zhou, Z., Guan, X., Xiang, Y.F., 2020. Can we use interleukin-6 (IL-6) blockade for coronavirus disease 2019 (COVID-19)-induced cytokine release syndrome (CRS)? J. Autoimmun., 102452
Lobo, S.M., Watanabe, A.S.A., Salomão, M.L.M., Queiroz, F., Gandolfi, J.V., de Oliveira, N.E., Covello, L.H.S., Sacillotto, G.H., de Godoy, L.G., Simões, E.S., Frini, I. C.M., Da Silva Teixeira, R.E.R., Furlan, N.P., Dutra, K.R., Nogueira, M.L., 2019. Excess mortality is associated with influenza A (H1N1) in patients with severe acute respiratory illness. J. Clin. Virol. 116, 62–68.
Lu, J.P., Jiang, J.H., Zhang, Hui, 2003. Clinical study of yinhua jiedu granules for influenza with wind-heat type. Chin Trad Pat Med 25 (2), 126–128.
Ludwig, S., Zell, R., Schwemmle, M., Herold, S., 2014. Influenza, a One Health paradigm–novel therapeutic strategies to fight a zoonotic pathogen with pandemic potential. Int J Med Microbiol 304 (7), 894–901.
Ma, Q.H., Yu, Q.T., Xing, X.F., Liu, S.N., Shi, C.Y., Luo, J.B., 2018. San Wu huangqin decoction, a Chinese herbal formula, inhibits influenza a/PR/8/34 (H1N1) virus infection in vitro and in vivo. Viruses 10 (3), 117.
Ma, Q.H., Huang, W.B., Zhao, J., Yang, Z.F., 2020. Liu Shen Wan inhibits influenza a virus and excessive virus-induced inflammatory response via suppression of TLR4/NF-κB signaling pathway in vitro and in vivo. J. Ethnopharmacol. 252, 112584.
Madera, R.F., Libraty, D.H., 2013. The role of MyD88 signaling in heterosubtypic influenza A virus infections. Virus Res. 171 (1), 216–221.
Michael, 2011. Antivirals and resistance: influenza virus. Curr Opin Virol 1 (6), 563–573, 639.
Mok, C.K.P., Kang, S.S.R., Chan, R.W.Y., Yue, P.Y.K., Mak, N.K., Poon, L.L.M., Wong, R. N.S., Peiris, J.S.M., Chan, M.C.W., 2014. Anti-inflammatory and antiviral effects of indirubin derivatives in influenza A (H5N1) virus infected primary human peripheral blood-derived macrophages and alveolar epithelial cells. Antivir. Res. 106, 95–104.

Niu, J.L., W, S.X., Chen, M.K., Xu, K., Guo, Q.H., Lu, A.L., Zhao, L.P., Sun, B., Meng, G.X., 2019. Hyperactivation of the NLRP3 inflammasome protects mice against influenza A virus infection via IL-1β mediated neutrophil recruitment. Cytokine 120, 115–124.
Park, J.E., Ryu, Y., 2018. Transmissibility and severity of influenza virus by subtype. Infect. Genet. Evol. 65, 288–292.
Peiris, J.S., Cheung, C.Y., Leung, C.Y., Nicholls, J.M., 2009. Innate immune responses to influenza A H5N1: friend or foe? Trends Immunol. 30 (12), 574–584.
Peng, X.Q., Zhou, H.F., Lu, Y.Y., Chen, J.K., Wan, H.T., Zhang, Y.Y., 2016. Protective effects of Yinhuapinggan granule on mice with influenza viral pneumonia. Int. Immunopharm. 30, 85–93.
Rong, R., Li, R.R., Hou, Y.B., Li, J., Ding, J.X., Zhang, C.B., Yang, Y., 2016. Mahuang-Xixin-Fuzi decoction reduces the infection of influenza A virus in Kidney-Yang deficiency syndrome mice. J. Ethnopharmacol. 192, 217–224.
Ryu, H.W., Kim, K.O., Yuk, H.J., Kwon, O.K., Kim, J.H., Na, M.K., Ahn, K.S., Oh, S.R., 2016. The constituent, anti-inflammation, and human neutrophil elastase inhibitory activity of Gnaphalium affine. J Funct Foods 27, 674–684.
Shi, S.Y., Huang, K.L., Zhang, Y.P., Zhao, Y., Du, Q.Z., 2007. Purification and identification of antiviral components from Laggera pterodonta by high-speed counter-current chromatography. J Chromatogr B Analyt Technol Biomed Life Sci 859 (1), 119–124.
Shi, Y.C., Xu, H.C., Xiao, Y.K., Liu, P., Pang, P., Wu, S.Z., Deng, L., Chen, X.Y., 2020. Gegen qinlian decoction downregulates the TLR7 signalling pathway to control influenza A virus infection. Biomed. Pharmacother. 121, 109471.
Short, K.R., Kroeze, E.J.B.V., Fouchier, R.A.M., Kuiken, T., 2014. Pathogenesis of influenza-induced acute respiratory distress syndrome. Lancet Infect. Dis. 14 (1), 57–69.
Sidahmed, A.M.E., León, A.J., Bosinger, S.E., Banner, D., Danesh, A., Cameron, M.J., Kelvin, D.J., 2012. CXCL10 contributes to p38-mediated apoptosis in primary T lymphocytes in vitro. Cytokine 59 (2), 433–441.
Svitek, N., Rudd, P.A., Obojes, K., Pillet, S., von Messling, V., 2008. Severe seasonal influenza in ferrets correlates with reduced interferon and increased IL-6 induction. Virology 376 (1), 53–59.
Tian, L., Wang, Z.Y., Wu, H., Wang, S., Wang, Y., Wang, Y.Y., Xu, J.W., Wang, L.Y., Qi, F. C., Fang, M.L., Yu, M.L., Fang, X.X., 2011. Evaluation of the anti-neuraminidase activity of the traditional Chinese medicines and determination of the anti-influenza A virus effects of the neuraminidase inhibitory TCMs in vitro and in vivo. J. Ethnopharmacol. 137 (1), 534–542.
To, K.K., Chan, J.F., Chen, H., Li, L., Yuen, K.Y., 2013. The emergence of influenza A H7N9 in human beings 16 years after influenza A H5N1: a tale of two cities. Lancet Infect. Dis. 13 (9), 809–821.
Watanabe, T., Watanabe, S., Maher, E.A., Neumann, G., Kawaoka, Y., 2014. Pandemic potential of avian influenza A (H7N9) viruses. Trends Microbiol. 22 (11), 623–631.
Wu, Q.F., Zhu, W.R., Yan, Y.L., Zhang, X.X., Jiang, Y.Q., Zhang, F.L., 2016. Anti-H1N1 influenza effects and its possible mechanism of Huanglian Xiangru Decoction. J. Ethnopharmacol. 185, 282–288.
Xi, R.Z., Xi, Z.Q., 2003. Summary of phase II clinical trial of yinhua jiedu granules in treating upper respiratory tract infection with wind-heat syndrome. J Shandong Univ TCM 27 (2), 127–129.
Yang, Y., Zhong, H., Song, T., He, J., Guo, L., Tan, X., Huang, G., Kang, M., 2017. Epidemiological and clinical characteristics of humans with avian influenza A (H7N9) infection in Guangdong, China, 2013-2017. Int. J. Infect. Dis. 65, 148–155.
Yin, J., Ma, L.L., Wang, H.Q., Yan, H.Y., Hu, J., Jiang, W., Li, T.H., 2017. Chinese herbal medicine compound Yi-Zhi-Hao pellet inhibits replication of influenza virus infection through activation of heme oxygenase-1. Acta Pharm. Sin. B 7 (6), 630–637.
Zhang, X., Huang, H., Yang, T., Ye, Y., Shan, J., Yin, Z., Luo, L., 2010. Chlorogenic acid protects mice against lipopolysaccharide-induced acute lung injury. Injury 41 (7), 746–752.
Zhang, P.H., Zheng, Z.G., Ling, L., Yang, X.H., Zhang, N., Wang, X., Hu, M.Z., Xia, Y., Ma, Y.W., Yang, H.R., Wang, Y.Y., Liu, H.Q., 2017. w09, a novel autophagy enhancer, induces autophagy-dependent cell apoptosis via activation of the EGFR-mediated RAS-RAF1-MAP2K-MAPK1/3 pathway. Autophagy 13 (7), 1093–1112.
Zhang, J.L., Zhang, Y., Huang, H.L., Zhang, H.W., Lu, W., Fu, G.Q., Zhu, Y.Z., 2018. Forsythoside A inhibited S. aureus stimulated inflammatory response in primary bovine mammary epithelial cells. Microb. Pathog. 116, 158–163.
Zhao H.Q., Lai C., Zhang M.M., Zhou S.D., Liu Q., Wang D.J., Geng Y.L., Wang X., An improved 2D-HPLC-UF-ESI-TOF/MS approach for enrichment and comprehensive characterization of minor neuraminidase inhibitors from Flos Lonicerae Japonicae. J. Pharmaceut. Biomed. Anal.. 175:112758.