Protective effects of Yinhuapinggan granule on mice with influenza viral pneumonia

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ABSTRACT

Yinhuapinggan granule (YHPG), a Chinese medicine granule on the basis of Ma-Huang-Tang (Ephedra Decoction) and the clinical experience of Professor Wan Haitong, has been shown to inhibit the growth of influenza virus in vitro. The aim of this study was to investigate the protective effects of YHPG on mice with influenza viral pneumonia and its effects on regulating related inflammatory cytokines in influenza virus A-infected mice. ICR mice were inoculated intranasally with 15 LD₅₀ viral dose of influenza virus A/PR/8/34 (H1N1) and treatments with YHPG (7.5, 15 and 30 g/kg) were orally administrated daily for 5 consecutive days after challenge, respectively. The results showed that mortality rate, lung index, lung histopathological changes, IL-6 and TNF- α in serum were significantly attenuated in the treatment of YHPG (15 and 30 g/kg) than those in the IFV control group, while the levels of IL-2 was significantly enhanced. Moreover, the RT-PCR results revealed that YHPG (15 and 30 g/kg) significantly depressed the expressions of IL-1 β , IFN- γ and TNF- α mRNA in lung tissues. Furthermore, the immunohistochemical staining results also revealed that the expression of NF- κ B p65 proteins were downregulated when treated with YHPG (15 and 30 g/kg). These results showed YHPG has protective effects on IFV-infected mice, due to its ability of alleviation of lung damage, regulation of the cytokine production via inhibiting the NF- κ B p65 activation, attenuation of systemic and pulmonary inflammatory responses.

Keywords: Yinhuapinggan granule, influenza viral pneumonia, protective effects, inflammatory responses

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1. Introduction

Influenza, an acute respiratory viral infectious disease caused by influenza virus, is one of the oldest and most common infections causing substantial morbidity and significant mortality. The recurrent outbreaks of the disease potentially brings about severe consequences for human health and global economy. The 1918-1919 Spanish influenza pandemic as the most severe pandemic outbreak was estimated to have infected one-third of the world's population and caused at least 20-40 million deaths worldwide [1, 2]. It has been reported that approximately 80% of mortality by influenza virus infection is caused by pneumonia, and the high-risk groups are the elderly and infant [3, 4]. Based on these facts, much attention has consequently been given to the development of antiviral drugs for the prevention and treatment of this disease.

To date, the M2 ion channel inhibitors and the virus neuraminidase inhibitors have been used for the treatment of influenza virus infections worldwide [5, 6]. However, the occurrence of resistant influenza A viruses and several side effects caused by these drugs have been reported [7, 8]. Therefore, to explore new anti-influenza drugs is important to overcome the limitations of existing anti-influenza virus agents. Although existent evidence tested by randomized trials is sparse and limited, traditional herbal medicines as natural substances for influenza treatment have played an

important role in the Far East countries, especially in China, Japan and Korea [9, 10]. For example, Ma-Huang-Tang (Ephedra Decoction), a famous classical formula from the Treatise on Febrile Disease of Zhang Zhongjing in the Han Dynasty, has been widely used for treating exogenous wind-cold, fever, cough and asthma [11]. It has been reported that Ma-Huang-Tang (Ephedra Decoction) shows antipyretic activity clinically [10, 12], and alleviative effects against the early phase of influenza virus infection in pharmacological research [13]. Modern clinical research has proved that modified prescriptions of Ephedra decoction work perfectly for the department of internal medicine disease such as influenza, bronchitis, asthma, respiratory infection [14]. Furthermore, in contrast to most antiviral drugs comprised of one single chemical component, traditional Chinese medicine has such advantages as multiple pathways, multitargets, and minimal side effects on the prevention and treatment of influenza, for example, Xiao-qing-long-tang [15]. Therefore, it's crucial to develop possible traditional Chinese medicine for the replacement of anti-influenza drugs.

Yinhuapinggan granule (YHPG), whose major ingredients are based upon classical Ephedra decoction formula, is composed of *Puerariae Lobatae Radix*, *Flos Lonicerae Japonicae*, *Polygoni Cuspidati Rhizoma*, *Ephedrae Herba*, *Armeniacae Semen Amarum* and *Glycyrrhizae Radix* with a ratio of 4:4:4:2:2:1. As an improved formula, YHPG has the good effect of relieving cough, diffusing the lung and releasing exterior, clearing heat and resolving toxins [16, 17]. Furthermore, YHPG has been proved to inhibit the growth of influenza virus in vitro [18] and to inhibit the influenza virus replication, alleviate the damage of the lung, ameliorate inflammatory responses and adjust for deviant immunologic function of infected mice [19, 20]. Nevertheless, there is no reports clarified

the protective effects of YHPG on influenza viral pneumonia in mice. However, several ingredients of YHPG and their constituents have been reported to have antiviral effects on several viruses [10, 21-32]. Therefore, we hypothesize that YHPG can be effective against influenza virus in vivo. In this study, an intranasal influenza virus infection in ICR mouse was utilized as the model to evaluate the protective effects of YHPG and its effects on regulating related inflammatory cytokines.

2. Materials and Methods

2.1. Plant materials and reagents

As shown in Table 1, *Puerariae Lobatae Radix* (batch number: 20131001), *Flos Lonicerae Japonicae* (batch number: 20131101), *Polygoni Cuspidati Rhizoma* (batch number: 20130801), *Herba Ephedrae* (batch number: 20130801), *Armeniacae Semen Amarum* (batch number: 20130801), *Glycyrrhizae Radix* (batch number: 20131002) were purchased from Hangzhou Huadong Chinese Herbal Medicine Co., Ltd. (Hangzhou, China). These six herbs were identified by Prof. Shengwu Huang, College of Pharmaceutical Science, Zhejiang Chinese Medical University, where voucher specimen was deposited. The crude slices of these drugs conformed to quality standards in Chinese Pharmacopoeia (2010 edition).

Standards of L-ephedrine, D-pseudoephedrine (from *Herba Ephedrae*), glycyrrhizic acid (from *Glycyrrhizae Radix*), amygdalin (from *Armeniacae Semen Amarum*), chlorogenic acid (from *Flos Lonicerae Japonicae*), puerarin (from *Puerariae Lobatae Radix*), polygonin and emodin (from *Polygoni Cuspidati Rhizoma*) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Moroxydine was purchased from Lishui Zhenan pharmaceutical Co. Ltd., (Zhejiang, China). Rabbit-anti-mouse polyclonal antibody for

NF- κ B p65 were purchased from Santa Cruz Biotechnology (California, USA). ELISA kits for IL-2, IL-6 and TNF- α were purchased from the Sigma-Aldrich (St. Louis, USA).

2.2. Preparation of YHPG

YHPG granules (batch number: 20141101) were produced and supplied by Hangzhou Huadong Medicine Group Kangrun Pharmaceutical Co., Ltd. (Hangzhou, China). Briefly, the powdered *Puerariae Lobatae Radix* (1000 g) and *Flos Lonicerae Japonicae* (1000 g) were respectively immersed in 10 L 70% ethanol for 12 h and extracted using a heating reflux method at 85 °C for twice (1 h each time). The two collected extractions were mixed, filtered and concentrated under reduced pressure to relative density of 1.14 (50~60 °C) as the solution A. The powdered *Polygoni Cuspidati Rhizoma* (1000 g) was immersed in 10 L 80% ethanol for 12 h and extracted using a heating reflux method at 85 °C for twice (1 h each time). Then the extraction was mixed, filtered and concentrated under reduced pressure to relative density of 1.13 (45~50 °C) as the solution B. After *Herba Ephedrae* (500 g) and *Glycyrrhizae Radix* (250 g) were soaked in 12.5 L water for 2 h, the mixture was decocted with the powdered *Armeniacae Semen Amarum* (500 g) for three times (1 h each time). The decoction was filtered and concentrated to relative density of 1.15 (50~60 °C) as the solution C. Subsequently, the solution A was mixed with the solution B and C. After spray drying, the drying powder was thoroughly blent with cornstarch (150 g) then made into granules, sterilized and encapsulated into bags (6 g per bag).

2.3. High performance liquid chromatography (HPLC) analysis of YHPG

HPLC analysis was performed by using an Agilent 1200 HPLC system, including a G1322A on-line degasser, a G1311A quaternary pump, a G1316A column oven, a G1329A autosampler, a

G1315B photodiode array detector, controlled by Agilent Rev. B.04.01 chemstation.

Chromatographic separation was performed on Agilent Eclipse XDB-C18 column ($4.6 \times 250 \text{ mm}^2$, $5 \mu\text{m}$) at 35°C . The photodiode array detector was set at 210 nm . The mobile phase was a mixture of water containing 0.1% phosphoric acid (A) and acetonitrile (B). The linear gradients between the time points were as follows: 0-10 min at 3-9% B, 10-25 min at 9-9% B, 25-40 min at 9-20% B, 40-65 min at 20-55% B, 65-75 min at 55-70% B, 75-80 min at 70-3% B. The flow rate was maintained at 1.0 mL/min and the injection volume was $10 \mu\text{L}$.

2.4. Virus

Mouse-adapted influenza virus A/PR/8/34 (H1N1) was donated by Professor Yi-yu Lu from the Zhejiang Center for Disease Control and Prevention, China. The virus was grown in allantoic cavity of 9-days-old embryonated egg for 48 h at 37°C . The allantoic fluid was harvested and centrifuged at $1000 \times g$ for 20 min, and then the resulting supernatant, with haemagglutination titer 1024, was stored in small portions at -80°C . Reed–Muench method was used to determine the LD_{50} of virus in mice ($\text{LD}_{50}=10^{-3.5}/0.02 \text{ mL}$).

2.5. Animals and preparation of influenza virus infection model

Male special pathogen free ICR mice, weighing $14\sim 15 \text{ g}$, were obtained from Animal Experimental Center, Zhejiang Chinese Medical University (Laboratory animal certificate: scxk 2008-0016). Animals were housed plastic cages in an air-conditioned room at $23 \pm 2^\circ\text{C}$ with a relative humidity of $55 \pm 10\%$ under a 12 h light/dark cycle, fed a standard laboratory diet and given water ad libitum. Animal experiments were carried out in accordance with local guidelines for the care of laboratory animals of Animal Experimental Center, Zhejiang Chinese Medical University,

and were approved by the ethics committee for research on laboratory animal use of the institution.

Mice were randomly divided into six groups (10 mice each group): Normal control group (Normal-C), IFV-infected control group (IFV-C), Moroxydine treated group, low, moderate and high dose of YHPG treated group. Mice were intranasally inoculated with 15 LD₅₀ doses of influenza virus in a volume of 20 µl sterile saline except normal control group. After 2 hours of inoculation, YHPG (7.5, 15, and 30 g/kg) or moroxydine (0.25 g/kg) was orally administered to experimental groups daily for 5 days, while mice in the normal and IFV-infected control group were treated with normal saline at the same intervals. The low dose of YHPG for mouse study was equivalent to the human dosage in clinical practice, while the moderate and high dose of YHPG were twice and four times of the human clinical dosage, respectively.

2.6. Survival rate

Mice were randomly divided into six groups (10 mice each group): Normal control group (Normal-C), IFV-infected control group (IFV-C), Moroxydine treated group, low, moderate and high dose of YHPG treated group. Mice were intranasally inoculated with 15 LD₅₀ doses of influenza virus in a volume of 20 µl sterile saline except normal control group. After 2 hours of inoculation, YHPG (7.5, 15, and 30 g/kg) or moroxydine (0.25 g/kg) was orally administered to experimental groups daily for 7 days, while mice in the normal and IFV-infected control group were treated with normal saline at the same intervals. Ten mice from each group was observed daily for 15 d after IFV infection. In addition, clinical signs and time of death were recorded daily.

2.7. Biochemical analysis

On day 6 (5 d after infection), ten mice in each group were sacrificed to calculate lung index.

Then the lung were harvested and divided into two parts (the right lung for histopathology and immunohistochemical analysis, the left lung for RT-PCR). Blood samples were collected and centrifuged at 3000 g for 20 min to obtain serum. The levels of IL-2, IL-6 and TNF- α in serum were determined by ELISA. The lung index and inhibition rate of lung index were used as an indicator of pneumonia [33], according to the following equation:

$$\text{Lung index} = \text{Wet weight (g)} / \text{Body weight (g)} \times 100 \%$$

$$\text{Inhibition rate of lung index} = (\text{the average lung index of IFV-infected group} - \text{the average lung index of experimental group}) / \text{the average lung index of IFV-infected group} \times 100 \%$$

2.8. Real-Time PCR Analysis

RT-PCR was used to assess the levels of IL-1 β , IFN- γ and TNF- α mRNA in lung tissues. The left lungs were respectively homogenized and centrifuged at 4000 rpm for 10 min at 4 °C. Total RNA in the supernatant were extracted with RNA Qiagen reagent (QIAGEN, Germany), dissolved in DEPC-H₂O (30 μ L). The total RNA was reverse transcribed with PrimeScriptTM RT Master Mix Kit (TaKaRa, Japan). The primers were synthesized according to the literature [34] and PCR primer used are described in Table 2. PCR reactions were carried out in 20 μ L final volume which contained 10 μ mol/L each primer (0.8 μ L), 1 μ L template DNA, 2 $\times$ SYBR Green (10 μ L), and RNase-free H₂O (7.4 μ L). The reaction conditions were as follows: 95 °C 2 min, followed by 95 °C 15 s, 55 °C 35 s for 40 cycles using a QuanStudio 12K Flex Real time PCR (ABI, USA). The relative quantification of PCR products was performed according to the 2^{- $\Delta\Delta$ Ct} method [35]. The fold change in target gene cDNA relative to the GAPDH internal control was determined using the following formula:

$$\text{Fold change} = 2^{-\Delta\Delta\text{CT}}, \text{ where } \Delta\Delta\text{Ct} = (\text{Ct}_{\text{target gene}} - \text{Ct}_{\text{GAPDH}}) - (\text{Ct}_{\text{control}} - \text{Ct}_{\text{GAPDH}}).$$

2.9. Histopathology of lungs

The right lung lobes were washed with normal saline, fixed with 10% formaldehyde solution. Tissue sections were then embedded in low-melting point paraffin, cut at 3 μ m thickness, and stained with hematoxylin and eosin (H&E). Slides of lungs were observed in a double-blinded fashion under light microscopy.

2.10. Detection of NF- κ B p65 expression

For the immunohistochemical analysis, the 3 μ m thick lung sections were treated with primary antibody against NF- κ B p65 (rabbit-anti-mouse, 1:100) at room temperature for 60 min. The lung sections were washed 3 times in PBS for 5 min each and then incubated with secondary antiserum (EnVision Two-Step kit, Denmark) for 40 min at room temperature. Subsequently, the lung sections were washed 3 times with PBS and then incubated with diaminobenzidine (DAB) and Hematoxylin staining solution (Harris) for 2-3 min, dehydrated using graded alcohols and xylene. Finally, the brown reaction product was observed at 200 amplification times of light microscope. The positive cells showed yellow or brown articles or clumps in the cytoplasm and nucleus. Furthermore, the integrated optical density (IOD) of NF- κ B p65 were measured by averaging 5 microscopic fields (200 magnification) with use of Image-ProPlus 6.0 software (Media Cybernetics, USA).

2.11. Statistical analysis

All statistical analyses were performed using SPSS 19.0 and all results were presented as mean $\pm$ S.D. The significance of differences among multiple group comparisons were performed using one-way analysis of variance (ANOVA). Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. HPLC profile of YHPG

As compared with standard reference compounds, eight effective constituents of YHPG, including L-ephedrine, D-pseudoephedrine, chlorogenic acid, amygdalin, puerarin, polygonin, glycyrrhizic acid and emodin, were analyzed by HPLC (Fig. 1-2). The contents of these compounds in YHPG were observed as 0.73 mg/g, 0.407 mg/g, 1.92 mg/g, 6.37 mg/g, 4.35 mg/g, 1.79 mg/g, 0.692 mg/g, 0.329 mg/g, respectively.

3.2. Efficacies of YHPG in IFV-induced mice

The experimental mice were monitored for 2 weeks, and almost all of the mice in the IFV control group died within 11 days after infection and none of the mice died in the normal control group (Fig. 3). Mice in the IFV group presented clinical signs such as inactive, ruffled fur, hunched posture, lack of appetite and respiratory distress. Although the similar clinical features were observed in the treated group, the signs were better than that in the IFV control group. Furthermore, compared with IFV control group, treatment with YHPG (15 and 30 g/kg) or moroxydine significantly reduced mortality, prolonged life span and increased the survival time of infected mice ($P<0.01$ and $P<0.05$) (Fig. 3).

3.3. Effects of YHPG on influenza viral pneumonia

Lung index and inhibition rate of lung index were calculated to assess influenza viral pneumonia. As shown in Fig. 3, lung index was significantly higher in the IFV-infected group than in the normal control group after influenza virus infection ($P<0.01$). Treatment with YHPG (15 and 30 g/kg) or moroxydine significantly decreased lung index ($P<0.01$), and increased the inhibition rate

of lung index (Table 3). However, YHPG (7.5 g/kg) had no significant effect on lung index. These results indicate that YHPG (15 and 30 g/kg) can inhibit pneumonia induced by influenza virus.

3.4. Effect of YHPG Treatment on IFV-induced lung pathological changes

Lung pathological changes from treated and untreated mice with IFV infection were shown (Fig. 4A-F). The mice in the control group showed no obvious histological changes (Fig. 4A). In the IFV-infected group, the lungs of mice showed the presence of necrotic bronchial and bronchiolar epithelium, hemorrhage, thickening of the alveolar and marked infiltration of inflammatory cells (Fig. 4B). Compared with the IFV control group, the IFV-induced lung inflammation was significantly reduced through treatment with YHPG (15 and 30 g/kg) or moroxydine (0.25 g/kg) (Fig. 4C and Fig. 4E-F). However, treatment with YHPG (7.5 g/kg) was as similar in histological changes as the IFV control group (Fig. 4D). This results indicates that treatment of YHPG (15 and 30 g/kg) can ameliorate the lung damage of in IFV-infected mice.

3.5. Effect of YHPG on cytokines production in IFV-infected mice

To investigate the effects of YHPG on the regulation of cytokine productions, the levels of three inflammatory cytokines were measured from the mice serum. As shown in Fig. 5, IL-6 and TNF- α levels in serum were significantly increased after IFV infection, whereas the IL-2 level was significantly decreased compared with the normal control group. Oral administration of 7.5 g/kg to 30 g/kg YHPG significantly decreased the production of IL-6 and TNF- α in serum and administration of 15 g/kg to 30 g/kg YHPG increased the production of serum IL-2 compared with the IFV control group.

3.6. Expression of IL-1 β , IFN- γ and TNF- α in lung tissues by Real-Time PCR

According to Fig. 6., in IFV control group, the expression of IFN- γ mRNA was significantly downregulated, while the expression of IL-1 β and TNF- α mRNA were markedly upregulated compared with normal control group ($P<0.01$). In the treatment group of YHPG (15 and 30 g/kg), the expression of IFN- γ mRNA was significantly upregulated, while the expression of IL-1 β and TNF- α mRNA were markedly downregulated compared with IFV-infected group ($P<0.01$).

3.7. Effect of YHPG on NF- κ B p65 expression

The expressions of NF- κ B p65 proteins in the lung tissues were delineated and quantified (Fig. 7 and Fig. 8). We measured the IOD of NF- κ B p65 and calculated the mean values and SDs. Compared with normal control group, the IOD of NF- κ B p65 significantly increased in the IFV-infected group ($P<0.01$). In the YHPG (15 and 30 g/kg) treated mice, the IOD of NF- κ B p65 was significantly lower than that in the IFV-infected group ($P<0.05$ and $P<0.01$). However, no significant difference was observed between the YHPG (7.5 g/kg) group and IFV-infected group.

4. Discussion

In this study, the effects of YHPG on mice infected by H1N1 virus were investigated. Our present study indicated that YHPG (15 and 30 g/kg) significantly reduced mortality, prolonged life span and increased the survival time of infected mice. The clinical signs were alleviated in the YHPG-treated group. To evaluate the severity of pneumonia, lung index and pathological changes in lung tissues were used in our experiment. A number of studies have shown that lung index, which is an indicator of the severity of pneumonia, has an evident increase in the early phase of influenza virus infection [36-38]. Our research showed that remarkable decreases in the lung indexes were observed in YHPG (15 and 30g/kg) group. In addition, excessive immune response, associating with

pathological changes in lung tissues, is believed to be an evidence for influenza-mediated death [39, 40]. The pathological analysis demonstrated that there were obviously necrotic bronchial, thickening of the alveolar and marked infiltration of inflammatory cells in the IFV-infected group, which was in accordance with precious studies [41]. When mice were treated with YHPG (15 and 30 g/kg), the histological changes in lung tissues were significantly reduced. These results indicated that treatment with YHPG could effectively alleviate the lung lesions and inflammation of mice infected with influenza virus.

The immune system plays a crucial role in regulating the host response to control inflammation and prevent excessive injury [42]. After infection, exaggerated inflammatory responses, which in turn resulted in the overproduction of inflammatory cytokines and chemokines, were likely to generate pneumonia [43, 44]. Therefore, there is a strong correlation between cytokines and pneumonia. IL-1 is identified as a primary target for therapeutic intervention in the pathogenesis and treatment of inflammatory diseases [45]. TNF- α , one of the most prominent inflammatory cytokines in influenza A virus infection, has a significant role in priming epithelial cells for higher cytokine and chemokine production [46]. In addition, TNF- α level is directly related with the severity of histologic lung lesions after infection [47]. At the same time, patients with pneumonia was also detected to have high level of pro-inflammatory cytokine IL-6, which was demonstrated to have a positive correlation with lung inflammation [48, 49]. IL-2 is a T-helper 1 (Th1) cytokine that stimulates IFN- γ secretion, which is crucial for viral clearance [50]. IFN- γ , which is produced by T cells and natural killer cells, protects infected mice from death at the early stage of influenza virus infection and ameliorates the severity of inflammation and lung damage [51, 52]. Moreover, the

overproduction of cytokines are the main reason for lung tissue damage [53]. In our study, treatment with YHPG (7.5 g/kg to 30 g/kg) significantly decreased IL-6 and TNF- α levels, whereas the level of IL-2 was significantly increased at doses of 15 or 30 g/kg. In addition, our results demonstrated that the expression levels of IL-1 β and TNF- α mRNA were remarkably increased while the expression levels of IFN- γ mRNA were significantly decreased in lung tissues after infection [34]. In contrast, YHPG at doses of 15 or 30 g/kg dramatically decreased the expression levels of IL-1 β and TNF- α mRNA and increased the expression levels of IFN- γ mRNA in comparison with IFV-infected group. These results indicated that YHPG could be effective in treatment to mice with influenza viral pneumonia, which correlated with the regulation of systemic inflammatory cytokine levels. The regulating effects of YHPG on systemic inflammatory cytokine levels was beneficial to alleviate the systemic and pulmonary inflammatory responses.

It is well-known that rapid production of inflammatory cytokines is regulated by a transcription factor NF- κ B, which plays a crucial role in inducing transcription of inflammatory genes [54, 55]. The activation of NF- κ B is widely involved in inflammatory diseases [56]. In this study, immunohistochemical analysis showed that NF- κ B p65 was activated after infection. Compare with normal control group, the expressions of NF- κ B p65 in the IFV-infected group were markedly increased as reported in previous studies [33, 57]. Treatment with YHPG (15 and 30 g/kg) after infection significantly decreased the expressions of NF- κ B p65 in comparison to that observed in the IFV-infected group, suggesting that the protective effects of YHPG on IFV-infected mice were partly connected with the inhibition of the expressions of NF- κ B p65.

YHPG is a Chinese medicine granule whose major ingredients are based upon the classical

MHT formula. Our previous studies have demonstrated that YHPG has obvious antiviral, anti-inflammatory and antitussive effects in mice and rats [17-20]. The HPLC analysis clearly revealed the presence of the major constituents of YHPG (Fig. 1). From literatures, we found that the main component of *Flos Lonicerae Japonicae*, chlorogenic acid, has shown significant antiviral activity in vitro [26, 58]. Glycyrrhizin, which is an active component of *Glycyrrhizae Radix*, may protect mice infected with lethal doses of influenza virus through the induction of IFN- γ production by T cells [59], and has been reported to inhibit influenza A virus uptake into the cell [30]. Further, the main component of *Polygoni Cuspidati Rhizoma*, emodin, inhibits the replication of influenza virus H1N1 in A549 cells [32]. So, we hypothesize that chlorogenic acid, glycyrrhizin and emodin may be the active components of YHPG against influenza virus. However, additional investigations are required to identify the possible anti-influenza bioactive components in YHPG.

Conclusion

In summary, our results demonstrated that YHPG had a protective effect on IFV-infected mice, which might be associated with alleviation of lung injury, regulation of the cytokine production via inhibiting the NF- κ B p65 activation, attenuation of systemic and pulmonary inflammatory responses. These findings were useful for the further investigation of the mechanisms of YHPG and provide a new choice for the treatment of influenza virus infection.

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Table 1

Component herbs of YHPG.

Pharmaceutical name	Botanical plant name	Family	Weight (g)	Used part
Flos Lonicerae Japonicae	<i>Lonicera japonica</i> Thunb.	Caprifoliaceae	10	Flower bud
Herba Ephedrae	<i>Ephedra sinica</i> Stapf.	Ephedraceae	5	Aerial part
Puerariae Lobatae Radix	<i>Pueraria lobata</i> (Willd.) Ohwi	Lamiaceae	10	Radix
Polygoni Cuspidati Rhizoma	<i>Polygonum cuspidatum</i> Sieb. et Zucc.	Polygonaceae	10	Root and Rhizome
Armeniaca Semen Amarum	<i>Prunus armeniaca</i> L. var. <i>ansu</i> . Maxim.	Rosaceae	5	Fruit
Glycyrrhizae Radix	<i>Glycyrrhiza uralensis</i> Fischer	Leguminosae	2.5	Root and Stolon

Table 2

Primer sequences used for RT-PCR analysis.

Gene	Product size	Forward (5'-3')	Reverse (5'-3')
IL-1 β	130 bp	TCAGGCAGGCAGTATCACTC	AGGATGGGCTCTTCTTCAA
IFN- γ	179 bp	AGGCCATCAGCAACAACATA	TGAGCTCATTGAATGCTTGG
TNF- α	133 bp	CCAAAGGGATGAGAAGTTCC	CTCCACTTGGTGGTTTGCTA
GAPDH	141 bp	GCAAGTTCAACGGCACAG	CGCCAGTAGACTCCACGAC

Table 3

The inhibition rate of lung index in mice treated with YHPG or moroxydine (n=10).

Groups	Dose(g/kg)	Inhibition rate of lung index(%)
Normal-C	-	-
IFV-C	-	-
Moroxydine	0.25	22.4
YHPG	7.5	7.2
YHPG	15	20.4
YHPG	30	30.3

Figure captions

Fig. 1. 2D HPLC chromatograms of eight effective constituents in YHPG at 210 nm. 1. L-ephedrine; 2. D-pseudoephedrine; 3. chlorogenic acid; 4. amygdalin; 5. puerarin; 6. polygonin; 7. glycyrrhizic acid and 8. emodin.

Fig. 2. Chemical structures of eight effective constituents in YHPG.

Fig. 3. Survival rate of YHPG treatment on IFV-infected mice (n=10 in each group). ^{##} $P<0.01$ vs. Normal-C, ^{*} $P<0.05$, ^{**} $P<0.01$ vs. IFV-C.

Fig. 4. Change of YHPG treatment on the lung index of IFV-infected mice (n=10 in each group). Lung index was significantly decreased in YHPG-treated groups as compared to the IFV control group. ^{##} $P<0.01$ vs. Normal-C, ^{**} $P<0.01$ and ^{*} $P<0.05$ vs. IFV-C.

Fig. 5. Effects of YHPG on IFV-induced lung histopathologic changes. (A) Normal control group; (B) IFV control group; (C) infected mice treated with moroxydine (0.25 g/kg); (D) infected mice treated with YHPG (7.5 g/kg); (E) infected mice treated with YHPG (15 g/kg); and (F) infected mice treated with YHPG (30 g/kg). Representative histological section of the lungs was stained by hematoxylin and eosin (magnification 100 ×).

Fig. 6. Effect of YHPG treatment on the production of (A) IL-2, (B) IL-6 and (C) TNF- α in the serum of IFV-infected mice (n=10 in each group). ^{##} $P<0.01$ vs. Normal-C, ^{*} $P<0.05$, ^{**} $P<0.01$ vs. IFV-C.

Fig. 7. Effect of YHPG treatment on the expression of IL-1 β , IFN- γ and TNF- α in lung tissues of IFV-infected mice (n=10 in each group). ^{##} $P<0.01$ vs. Normal-C, ^{*} $P<0.05$, ^{**} $P<0.01$ vs. IFV-C.

Fig. 8. Immunostaining microphotographs of NF- κ B p65 proteins in the lung tissues of IFV-infected mice (magnification 200 ×). (A) Normal control group; (B) IFV control group; (C) infected mice treated with moroxydine (0.25 g/kg); (D) infected mice treated with YHPG (7.5 g/kg); (E) infected mice treated with YHPG (15 g/kg); (F) infected mice treated with YHPG (30 g/kg).

Fig. 9. Immunohistochemical analysis of NF- κ B p65 in the lung sections (n=10 in each group). (A) Normal control group; (B) IFV control group; (C) infected mice treated with moroxydine (0.25 g/kg; (D) infected mice treated with YHPG (7.5 g/kg); (E) infected mice treated with YHPG (15 g/kg); (F) infected mice treated with YHPG(30 g/kg).

^{##} $P<0.01$ vs. Normal-C, ^{*} $P<0.05$, ^{**} $P<0.01$ vs. IFV-C.

Figure 1

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