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Anti-pandemic influenza A (H1N1) virus potential of Xilingjiedu capsule *in vitro*

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Abstract: This study is aimed to investigate the effect of Xilingjiedu capsule (XLC), one of a preparation of traditional Chinese medicine, on influenza A (H1N1) virus as well as its preliminary mechanism. The median cell mortality (TC_{50}) to A549 cells and half effective inhibition concentration (IC_{50}) of influenza A (H1N1) virus of XLC were determined by MTT assay. Reed-Muench method was used to calculated the 50% tissue culture infective dose ($TCID_{50}$) of H1N1 virus to A549 cells. In mechanism research, the mRNA expression levels of MyD88, TLR4, TLR7 and TRAF6 and the protein expression level of MyD88 were detected by using RT-PCR and Western blot, respectively. The results suggested that XLC showed good anti influenza A (H1N1) virus activity. The antiviral mechanism of XLC was related to the Toll-like signaling pathway. It could drown regulate the mRNA expression level of MyD88 and TLR4 and the protein level of MyD88. This research provides reference for the application of XLC in anti influenza virus.

Keywords: XLC, influenza A (H1N1) virus, A549 cells, mRNA expression.

INTRODUCTION

Influenza is a highly infectious acute respiratory disease caused by influenza virus, with the characteristics of rapid transmission, high degree of infectious and serious pathogenicity. Influenza virus is a type A RNA virus belonging to the family of Orthomyxoviridae, on which there exists the major spike proteins, hemagglutinin antigen (HA) and neuraminidase antigen (NA). HA contains 16 hemagglutinin antigenic subtypes (H1-H16) and NA contains 9 neuraminidase antigenic subtypes (N1-N9), and 105 viruses produced by different combinations of HA and NA subtypes have been identified up to now (Michaelis *et al*., 2009). The H1N1 flu caused by influenza A (H1N1) virus has a qualitative change in genetic material because of the association with some gene fragments of avian influenza, swine flu and human influenza virus (Li *et al*., 2015; Smith *et al*., 2009; Kou *et al*., 2009) and its mutability and lethality are much greater than the common flu (Hamilton *et al*., 2009). Hence, it is of great realistic significance for looking for therapeutic drugs and studying the pathogenic mechanisms of the disease caused by influenza A (H1N1) virus.

In the theory of Traditional Chinese Medicine (TCM), the syndrome factors of H1N1 influenza always been known as exterior attack by wind heat such as wei phase syndrome or syndrome involving wei and qi fen simultaneously, while the other syndrome of exterior attack by wet heat are pathogenic heat in the lung and damp evil in the spleen and stomach (Wang *et al*., 2020; Shi *et al*., 2017; Chen *et al*., 2016). Most of TCM drugs for anti-influenza virus possess the effects of heat-clearing and detoxifying, and the complex mechanism is an important factor to promote the material basis of these drugs working through different signaling pathways (Zhang *et al*., 2018; Li *et al*., 2018).

As a famous TCM preparation taking the role of heat clearing and detoxicating, Xiling jiedu capsule (XLC) is composed of Lonicera japonica, Fructus forsythia, Platycodon grandiflorum and other herb medicines. With the effect of expelling wind to relieve exterior syndrome and clearing away heat and toxin, XLC is widely used in clinical for treating wind-heat common cold, fever, headache, cough, sore throats and other flu symptoms (Hu *et al*., 2013; Geng *et al*., 2000). Lonicera japonica and fructus forsythia, combined with other TCM, have been shown effective in anti influenza A (H1N1) virus (Ding *et al*., 2017; Li *et al*., 2014). As the monarch or minister herb in XLC, the two herb medicines capacitate XLC has the tremendous potential to treat influenza caused by influenza A (H1N1) virus.

Previous studies have shown that a variety of chemical ingredients such as phillyrin, orientin, luteolin-6-C-glucoside, vitexin, pulegone exist in Xi-ling-jie-du preparations (Shi *et al*., 2011; Wei *et al*., 2014; Sun *et al*., 2015) and these compounds can be used as the material basis of XLC on anti influenza A (H1N1) virus. This study focused on exploring the activity of anti-influenza A (H1N1) virus of XLC in order to provide experimental reference for its clinical application.

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MATERIALS AND METHODS

Cell and virus

Human lung adenocarcinoma cell line (A549) was kindly provided by professor Qi Wang of the school of public health, Peking University (Beijing, China). The influenza A1/Qian/166/85(H1N1) virus (pdm/2009) was purchased from Chinese Center for Disease Control and Prevention and maintained at a cold storage condition of -80ºC. This strain of virus was propagated in the 9-day-old embryonated chicken eggs for 3 passages by allantoic cavity inoculation, then the allantoic fluid was harvested and filtered using a 0.22μm cellulose acetate membrane. The hemagglutination titer was measured at 2^{-7}.

Drugs and reagents

The positive control drugs, Oseltamivir phosphate (OP) and Lianhua qingwen granules (LHG) were purchased from Bide Pharmaceutical Technology Co., Ltd (Shanghai, China) and Beijing Yiling pharmaceutical CO., Ltd. (Beijing, China), respectively. XLC was supplied by Shandong Hongjitang Pharmaceutical Co., Ltd. (Jinan, China).

Roswell Park Memorial Institute (RPMI)-1640 medium, Fetal bovine serum (FBS), Penicillin-streptomycin solution, phosphate buffered saline (PBS) and Trizol reagent were purchased from Gibco™ Life Technologies Inc. (Grand Island, USA). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich Co., St. Louis, (MO, USA); CCK-8 kit, Ripa lysis buffer and BCA protein assay kit were purchased from Beyotime biotechology Co. (Shanghai, China), DEPC-H_2O was bought from Gen-star Kangrun Co. (Beijing, China). EtOH, $CHCl_3$, isopropanol, and absolute alcohol were purchased from Beijing Chemical Works (Beijing, China).

The complete RPMI-1640 medium used for culturing A549 cells was supplemented with 100U/mL penicillin-streptomycin and 10% (*v*/*v*) FBS; The maintenance RPMI-1640 medium was added with 100U/ml penicillin-streptomycin and 2% (*v*/*v*) FBS.

Drug preparation

OP was dissolved into stock solution with DMSO for further use. Powder of 100 g LHG and XLC were extracted with 80% EtOH (500mL × 3 times for 1h each time), respectively. The combined extracts were enriched with rotary evaporation in a vacuum condition and lyophilized (The receiving rates of LHQW and XLJD were 29.47% and 34.53%, respectively). The stock solutions of LHQW and XLJD were prepared using a certain quality of the dry extract with DMSO as dissolving reagent.

Cytotoxicity assay

The toxicities of the two positive drugs and XLC to cell were evaluated using CCK-8 assay kit. Firstly, the A549 cells were maintained in complete RPMI-1640 medium at 37ºC in a humid atmosphere containing of 5% CO_2. After being passaged three times at a ratio of 1:3, the cells were seed in 96-well plates at a density of 1.5×10^4 cells/well and incubated for 24h. The tested drugs were double diluted into a series of concentrations from 2000μg/mL to 7.81μg/mL and added to stimulate cells. A control group was necessary in the meantime. After being incubated for 48h, 10μL of CCK-8 reagent was added to each well and kept for 3h at 37ºC. The absorbance value was measured at 570 nm by a Thermo Multiskan MK3 micro plate reader (Thermo-Labsystem, Franklin, MA, USA).

The $TCID_{50}$ of H1N1 influenza virus

The suspension of influenza A (H1N1) virus was diluted to a series of concentration with the maintenance RPMI-1640 medium and successively inoculated into a 96-well plate covered with monolayer cells with an initial seeding density of 1.5×10^4 cells/well. In addition to a normal control group, each well was added 100μL different concentrations of influenza virus H1N1 and each concentration was repeated three times. The microscopic morphology and cytopathic level of the cells was observed after being stimulated by virus for 48h at a condition of 37ºC and 5% CO_2. The well with a cytopathic rate greater than 50% was used to calculate the 50% tissue culture infective dose ($TCID_{50}$) by using Reed-Muench methods.

Antiviral assay

Briefly, 96-well plates seeded with 1.5×10^4 cells/well were cultivated in an incubator for 24h at 37ºC and 5% CO_2. After being washed with PBS, virus diluent at 150 folds of $TCID_{50}$ was added into the plates. A series of diluted concentrations of drugs with a maximum non-toxic concentration were added successively 6h later. Normal and virus control group were set up simultaneously. Further maintained in an incubator for 24h, the micropathological changes of the cells were observed. Cytopathic effect (CPE) were represented by 0%~25% (+), 25%~50% (++), 50%~75% (+++) and 75% ~100 % (++++). The results were recorded when CPE in the viral control group were ++++. The cells were continuously cultivated 24h and 10μL of CCK-8 reagent was added to each well and kept for 3h at 37ºC. The cytopathic effect inhibition rate (ER) was calculated with the absorbance value measured at 570 nm according to the following formula: $ER= (A_{test}-A_{model})/(A_{control}-A_{model})\Diamond100\%$. This experiment was verified for three times.

qPCR assay

Toll-like receptor signaling pathway, a classical signal pathway of infectious mechanism of influenza virus and immunity responsion in *vivo,* was chosen to study the antiviral mechanism of XLC. In brief, the A549 cells were seed in 12-well plates with density of 1.5×10^4 cells/well.

After incubation for 24h, virus diluent at 150 folds of $TCID_{50}$ was added in and then two different concentrations of drugs were added 6 hours later. The cells were continuously cultivated for 48 h and the total RNA was extracted from the cells with Trizol reagent. Complementary DNA (cDNA) was obtained from 1 μg of total RNA using a q-RT super mix (Bimake Co., Ltd., Houston, SD, USA) after determining the concentration. The amplification of cDNA was performed using SYBR green qPCR master mix (Bimake Co., Ltd., Houston, USA) on a Quantitative RT-PCR system (Agilent Technologies, Stratagene, Inc., Santa Clara, USA). The primers were listed in table 1 and the PCR based cycle method was set as: Step 1, 50ºC for 2min, 95ºC for 10 min; Step 2, 40 cycles of 95ºC for 30s, 55ºC for 1 min, and 72ºC for 1min and step 3, 95ºC for 1min, 55ºC for 30s and 95ºC for 30 s.

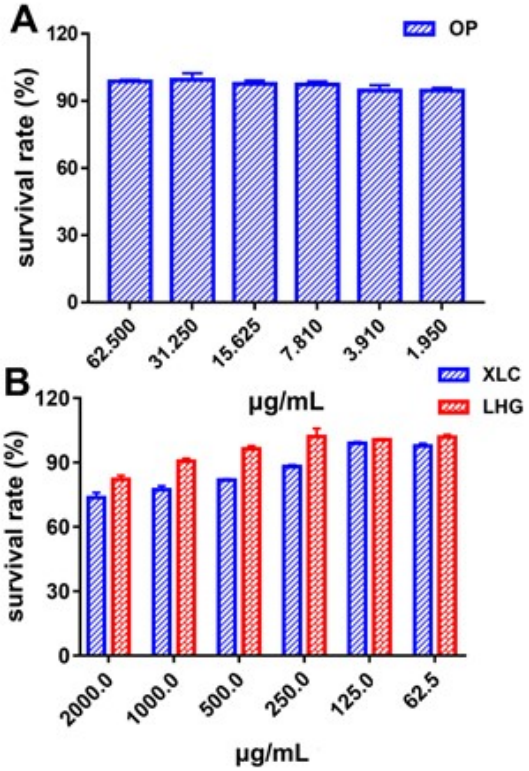

Fig. 1: Survival rate of A549 cells after being treated with OP, XLC and LHG

Western blot

The total proteins were extracted from A549 cells that were seeded in 6-well plates and treated with H1N1 virus and the three drugs using Ripa lysis buffer containing protease and phosphatase inhibitor cocktail. After being centrifuged at 13,000 rpm for 10min, the protein concentration was measured with a BCA protein assay kit. The protein expression levels of MyD88 and TRAF6 were measured according to the Wes Training Kit (PS-T001) by using Wes^{TM} automatic protein expression analysis system (Protein Simple Co., Ltd., Santa Clara, California, USA).

STATISTICAL ANALYSIS

All of the relevant results were expressed as Mean ± SD. The IC_{50} was calculated using GraphPad Prism 7.0 (Graph Pad Software Co. Ltd., USA). The therapeutic index (TI) was calculated based on the formula of TI= TC_{50} / IC_{50}.

RESULTS

Cytotoxicity

The toxicity of OP, LHG and XLC to cells were screened according to the description in "Cytotoxicity assay". As shown in fig. 1, the survival rates of A549 cells were close to 100% in the concentration range of 1.95~62.5μg/mL of OP, which means OP has little cytotoxicity to the cells with TC_{50} of 528.6μg/mL. The cytotoxicities of XLC and LHG were also compared. Within the set detection range, the TC_{50} of LHG and XLC were 7198μg/mL and 5450μg/mL, respectively. The cell survival rate in LHG group was a little higher than that of XLC group, but there was no significant difference between the two groups.

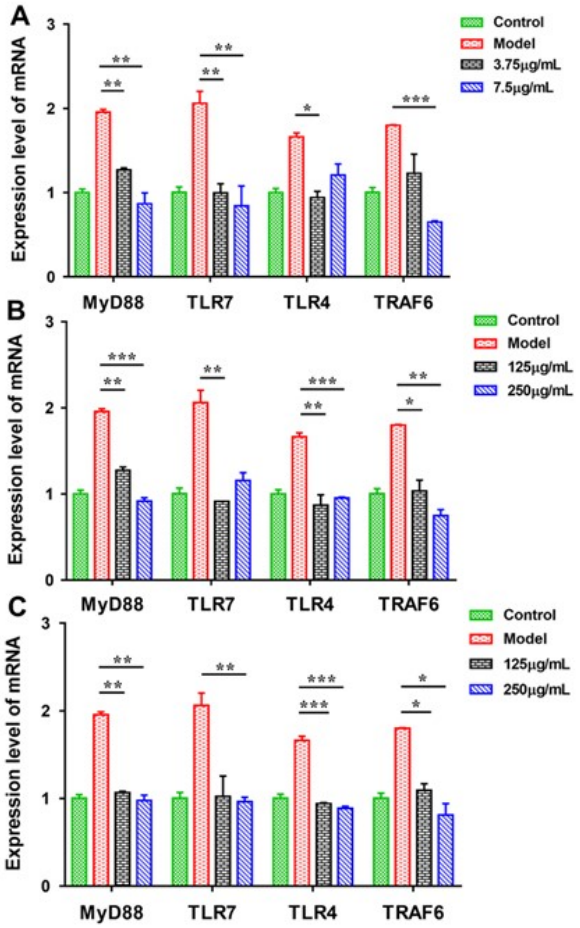

Fig. 2: The mRNA expression of Toll-like signaling pathway genes of A549 cells effected by OP (**A**), LHG (**B**) and XLC (**C**). P-values were calculated using multiple t-test, **P*<0.05; ***P*<0.01; ****P*<0.001.

The $TCID_{50}$

The pathological changes of A549 cells were observed by microscope after addition of the influenza virus diluent. The $TCID_{50}$ of H1N1 influenza virus to A549 cells was calculated using Reed-Muench method and the final value was $10^{-3.23}$/0.1mL.

Drugs on viral cytopathic effects

When A549 cells were infected with H1N1 virus, morphology of the cells would change in different degrees under the influence of H1N1 virus. CPE and ER often used as two important indicators to evaluate the intensity of virus infection on cells (Wei *et al*., 2018; Liu *et al*., 2016). From table 2 we can get information that OP had the protective effect on pathological change of A549 cell. The cells had almost no obvious lesions and the survival rate was 95.53% when the concentration of OP was 50μg/mL. With the decrease of the concentration, CPE became larger and ER values went down gradually, which showed that these two parameters were concentration dependent.

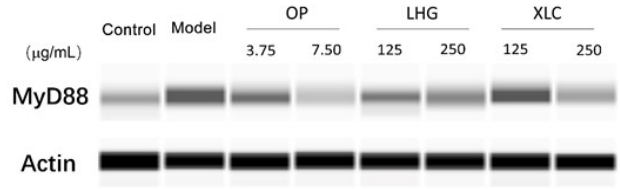

Fig. 3: The MyD88 protein expression level of A549 cells regulated by OP, LHG and XLC

As shown in table 3, the protective effect of LHG on CPE was slightly higher than that of XLC, but there was no significant difference between the two TCM preparations. Within the range of 500~250μg/mL, the survival rate of both groups was obviously higher than that of the virus-infected group. Furthermore, no significant difference of change occurred in cell morphology in both of these two groups, and the ER values were 73.85±0.77% and 69.45±1.07%, respectively. The cell appearance was changed from fusi form to long-narrow or other irregular shapes as the concentration of XLC and LHG decreased gradually and the degree of cell fragmentation and refractive index increased more and more obviously, which showed a certain concentration dependence in these two groups.

Antiviral activity

According to the MTT results, the half inhibitory concentration (IC_{50}) and TI of OP, XLC and LHG were calculated by using Graph Pad Prism 7.0 software. It could be seen from table 4 that XLC and LHG showed a similar anti-H1N1 influenza virus effect. The IC_{50} of XLC was 232.65±5.30μg/mL which was a little higher than that of LHG, but there was no significant difference between these two drugs. In the meantime, XLC was safety and effective in against H1N1 virus invasion and infection because of a greater TI value more than 20.

qPCR assay

XLC was proved to have a good inhibitory effect on influenza A (H1N1) virus. In order to further study its related mechanism, the main signal transduction pathway of influenza was preliminarily selected as the research basis. MyD88, TLR7, TLR4 and TRAF6 on this pathway were taken as the research object (Wei *et al*., 2018) and PCR was used to study the mRNA expression of these factors regulated by XLC, LHG and OP. fig. 2A gave us information that OP had a certain regulatory effect on MyD88, TLR7, TLR4 and TRAF6, especially down-regulated the mRNA expression of MyD88, TLR7 and TRAF6 with concentration-dependent manner. LHG and XLC also down regulated the mRNA expression of these cytokines to a certain extent (fig. 2B and C), especially on MyD88 and TLR4, but the regulation effect on TLR7 and TRAF6 was slightly weaker than that of OP. Compared XLC and LHG groups, it could be seen that the mRNA expression level of TLR4 regulated by XLC with a certain concentration correlation was similar with that by LHG, It means that the XLC mainly through regulating of TLR4, MyD88 and other conduction factors on the toll-like receptor signaling pathway to achieve the purpose of anti-influenza A (H1N1) virus.

Western blot

The MyD88 protein was down regulated by XLC and the two positive control drugs with a concentration-dependent manner (fig. 3). The protein expression level of MyD88 could be down regulated more significantly by XLC than LHG. Combined with the results of qPCR, XLC could induce the down-regulation of TLR4 and TLR7, thereby to regulate the expression level of MyD88 serially, a key transduction protein in the toll-like receptor signaling pathway. Once the expression level of MyD88 protein was inhibited, it is possible to hold back the inhibitor of nuclear factor kappa-B kinase (IKK) through the MyD88-dependent pathway, and then prevent the release of pro-inflammatory cytokines that could mediate the body's inflammatory response. Thus, XLC could achieve the purpose of resisting or weakening the invasion of H1N1 influenza virus in the body.

DISCUSSION

Influenza is an acute respiratory infectious disease caused by influenza virus that happens regularly with the change of seasons or outburst abnormally. This disease can be spread in a short period of time with a rapid trend throughout the world, and characterized by its propagation speed, strong infectivity and severe pathogenicity. Respiratory diseases caused by influenza virus infection are usually widely affecting human health, however, the new infection caused by virus mutation brings certain difficulties and challenges for more medical treatment and drug development (Stertz *et al*., 2018; Tandel *et al*., 2018; Al Khatib *et al*., 2018).

Table 1: The primer sequences for qPCR

Primer	Forward (5′-3′)	Reverse (5′-3′)
GADPH	GTGACACCCACTCTTCCACC	GTGGTCCAGGAGGCTCTTAC
MyD88	CCGGATGGTAGTGGTTGTCT	GGCACCTGGAGAGAGACTGA
TLR7	GGTGAAGTTGGCTTCTGCTC	TGCAACTCCTTGCATACTCG
TLR4	AACTGTATCGCCTCCTCAGC	GCATCCTCTGTTCCTTCTGG
TRAF6	TTGTCTCCAGTGCCAACGTA	CCATCAATGCAGCACAGTTC

Table 2: OP on CPE and ER of A549 cells (n=3)

	(μg/mL)	CPE	ER (%)
1	50.00	+	95.53±2.36
2	25.00	+	75.30±0.75
3	12.50	++	67.35±2.46
4	6.25	++	66.21±2.46
5	3.12	+++	46.32±1.02
6	1.56	+++	21.06±2.14

Table 3: Effects of LHG and XLC on CPE and ER of H1N1 influenza virus (n=3)

No.	Concentration	CPE		ER (%)	
	(μg/mL)	LHG	XLC	LHG	XLC
1	500.00	+	+	73.85±0.77	69.45±1.07
2	250.00	++	++	52.29±0.69	47.99±0.67
3	125.00	+++	+++	33.34±0.76	33.97±0.17
4	62.50	+++	+++	29.54±1.75	22.99±3.41
5	31.25	++++	++++	18.70±0.01	14.52±0.68
6	15.63	++++	++++	11.88±0.41	11.83±1.45

Table 4: The IC_{50} and TI of XLC, LHG and OP（n=3）

Drugs	IC_{50} (μg/mL)	TI
XLC	232.65±5.30	23.49
LHG	195.85±7.71	36.75
OP	4.39±0.18	120.4

At present, there are many kinds of drugs with various curative effects on the market for the treatment of H1N1 virus. TCM drugs, as LHG, Yinqiao Powder, and Qingkailing Oral Solution having the functions of clearing heat and detoxifying, dispelling wind and dehumidifying, all exhibit good effect on anti influenza A (H1N1) virus (Duan *et al*., 2011; Dai *et al*., 2013; He *et al*., 2010). XLC is a kind of TCM drugs for treating the common influenza illness in clinical and contents many different kinds of bioactivity compounds. This Chinese patent medicine has been proved to have low cytotoxicity and high safety with good anti H1N1 virus activity of IC_{50} at 232.65±5.30μg/mL in the present study. Furthermore, the mRNA or protein expression levels of TLR4, TRAF6 and MyD88 in the toll-like receptor signaling pathway have been down regulated significantly. These results can provide experimental basis for clinical application of XLC in treating diseases caused by H1N1 influenza A virus and give evidence that XLC has great potential in treating influenza caused by other influenza subtype virus.

CONCLUSION

In this experiment, we primarily studied the inhibition of XLC on pandemic influenza A (H1N1) virus, and briefly investigated a potential mechanism for treating this influenza from a relevant signal pathway, though more detailed mechanism of XLC on influenza virus infection requires further investigation. In nowadays, covid-19, as an infectious disease caused by a novel coronavirus, is widely spreading around the world (Velavan *et al*., 2020; Ahn *et al*., 2020), LHG has been shown obvious resistance to this novel coronavirus (Li *et al*., 2020). This result may provide experimental guidance for the further screening of XLC in this field.

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