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## EXPERIMENTAL STUDY

## Antiviral activity of Jinchai capsule against influenza virus

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## Abstract

**OBJECTIVE:** To evaluate the effect on influenza virus of Jinchai, a capsule made of Traditional Chinese Medicine.**METHODS:** Madin-darby canine kidney (MDCK) cells were infected with the FM1 strain of influenza virus A (subtype H1N1) *in vitro*. They were used to explore how Jinchai affected cell adsorption, cell membrane fusion, transcription and replication of the influenza virus. Hemagglutinin (HA) protein, intracellular pH, and influenza virus protein acid (PA) polymerase subunit were detected with confocal microscopy and real-time fluorescent quantitative polymerase chain reaction.**RESULTS:** Jinchai significantly reduced the expression of HA and PA polymerase subunit mRNA in infected MDCK cells. Jinchai also significantly decreased intracellular pH in infected cells.**CONCLUSIONS:** Jinchai had strong anti-influenza

activity against the influenza virus. It weakened the ability of the influenza virus to adsorb to cell wall and fuse with cell membranes in the early infection stage, and inhibited the transcription and replication of the virus.

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**Key words:** Antiviral agents; Influenza A virus; Jinchai capsule

## INTRODUCTION

Jinchai, made of Traditional Chinese Medicine, consists of Jinyinhua (*Flos lonicerae*), Chaihu (*Radix bupleuri*), Huangqin (*Radix astragali*) and Dangshen (*Radix codonopsis*). As the most important component in the capsule, *Lonicera japonica* contains chlorogenic acid, isochlorogenic acid and hyperin, and therefore plays an implicit role as an anti-influenza antiviral agent.<sup>1</sup> The active ingredients of *scutellaria* are flavonoids,  $\beta$ -sitosterol, benzoic acid and baicalinase. A study reported that *scutellaria* had effect on inhibiting the propagation of the influenza virus,<sup>2</sup> and a decoction of *bupleurum* and *scutellaria* could protect cells against the virus.<sup>3</sup> The main active constituents of *Dangshen* (*Radix codonopsis*) include multiple trace elements such as potassium, copper, chromium and lithium, multiple amino acids and 6 polysaccharides. It could enhance the immunity and resilience in the experimental mice.<sup>4</sup> Serum chemistry analysis revealed that the main active constituents of Jinchai were baicalin and chlorogenic acid.<sup>5</sup> Our early study showed that Jinchai had a role in inhibiting viruses, including coronavirus causing SARS, parainfluenza, respiratory syncytial, herpes simplex types I and II, the 3,7-type adenovirus and others.<sup>6</sup> We used influenza FM1 and PR8 strains to infect normal mice and immunocompromised mice, and

then Jinchai was administered to them. It was found that Jinchai had significant effect on lung inflammation in mice with normal immune function.<sup>7,8</sup>

An influenza virus enters a host cell through HA's adsorption to the host cell surface, and the transcription and replication of the virus are accomplished by using the enzymes, raw materials and energy of the host cell. After cells are infected, degeneration, necrosis and shedding occur. In the study of assessing the antiviral activity of Jinchai, the process of the virus adsorption, membrane fusion, transcription and replication were investigated, and HA, influenza virus PA polymerase subunit and intracellular pH were detected.

## MATERIALS AND METHODS

### Materials

Madin-darby canine kidney (MDCK) cells were obtained from the cell center of Peking Union Medical College and subcultured in the biological safety laboratory. Jinchai, a pilot product, was produced by the Preparation center of the China-Japanese Friendship Hospital (lot no. 20090922, clinical approval document No. 2007 L 05135). A stock solution of 32 mg/mL was prepared in sterile deionized water and filter-sterilized before the experiment. The maximum nontoxic concentration was 2 mg/mL.

The FM1 strain of influenza virus (H1N1 subtype) was purchased from Chinese Center for Disease Control and Prevention. A routine subpassage in embryonated eggs was performed in the biological safety laboratory. Strains were frozen at -80°C.

Reagents included 10% fetal bovine serum (Hao Yang Technology, Tianjin, China), high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, New York, USA), an RNA extraction kit (Invitrogen, Shanghai, China), chloroform (Beijing Chemical Plant, China), diethylpyrocarbonate (DEPC; Sigma, New York, USA), a One Step SYBR RT-PCR Kit (Takara, Shanghai, China), the pH sensitive C-SNARF-4 fluorescent probe (Molecular Probes, USA), primers of GAPDH and FM1-HA (Invitrogen, Shanghai, China), isopropanol (Huadong Reagent Plant, Tianjin, China) and ethanol (Beijing Chemical Plant, China). Instruments included the Applied Biosystems 7500 Real-Time PCR System (ABI, Foster, USA); and a FV1000 Confocal Laser Scanning Microscope. Six-well plates and 35 mm dishes were purchased from Met-Tech Industries in the United States. Krebs-Henseleit (KH) solution was prepared with 117 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl<sub>2</sub>, 1.2 mmol/L MgCl<sub>2</sub>, 10 mmol/L HEPES, 1.2 mmol/L KH<sub>2</sub> PO<sub>4</sub> and 11.1 mmol/L glucose.

### Methods

Inhibiting the influenza virus adsorption: Monolayer MDCK cells were incubated for 1 h with different con-

centrations of Jinchai in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. After discarding the supernatant, the cells were washed with Phosphate Buffer Solution (PBS). MDCK cells were then infected with FM1 and incubated for 1 h at 37°C with 5% CO<sub>2</sub>. The supernatant was discarded and the cells washed with PBS. After digestion with digestive enzyme (Gibco), cells were centrifuged and the pellet collected for real-time quantitative PCR.

To extract the total cellular mRNA, MDCK cells (a density of 5×10<sup>5</sup> cells/mL) were incubated in one milliliter TRIzol for 20 min at room temperature. The suspension was then centrifuged at 13 400 ×g for 10 min at 4°C. The supernatant was mixed gently with 0.2 mL chloroform for 15 s and incubated at room temperature for 2-3 min. After centrifugation again at 13 400 ×g for 15 min at 4°C, the supernatant was mixed with 0.5 mL isopropanol and incubated at room temperature for 30 min. The precipitate was collected after a third centrifugation at 13 400 ×g for 10 min at 4°C and washed with one milliliter 75% ethanol, then centrifuged again at 10 400 ×g for 5 min at 4°C. The RNA precipitate was dried for 5-10 min, and then dissolved with 15 μL DEPC water, and immediately stored at -80°C.

Before the one-step real-time fluorescent RT-PCR reaction was performed, samples were diluted 20 times with DEPC water. The 20 μL total reaction volume included 2 μL of the RNA sample, 16.4 μL of One Step SYBR GREEN, and 0.8 μL (10 pMol) of each primer. Duplicate PCR reactions were tested using the following amplification protocol: 95°C for 10 s followed by 40 cycles at 95°C for 5 s and at 60°C for 34 s. After the reaction, a melting curve analysis was performed to identify the specificity of PCR products. The cycle threshold (Ct) value of each sample was analyzed by Sequence Detection System software.

The A/FM/1/47 sequence was downloaded from the national center for biotechnology (NCBI)-GenBank. Sequence analysis and primer design were performed by using primer premier 5.0 software. The melting temperature value of primers, GC content and the stability of the 3' end were determined according to optimized primer principles.

Upstream and downstream primers of influenza HA virus gene sequence were 5'- GACCAATCCTGT-CACCTCTGAC-3' and 5'-GGGCATTTGGA-CAAACGTCTACG-3', respectively. Upstream and downstream primers of the housekeeping gene GAPDH were 5'- GGTGAAGGTCCGGTGTGAACG -3' and 5'- CTCGCTCCTGGAAGATGGTG -3', respectively.

The relative changes in gene expression were calculated using the relative quantitative method (2<sup>-ΔΔCt</sup>). The ΔCt was calculated by subtracting the Ct value of the GAPDH reference from the Ct values of the gene expression of interest (sample): ΔCt<sub>sample</sub> = Ct<sub>sample</sub> - Ct<sub>GAPDH</sub>.

The  $2^{-\Delta\Delta C_t}$  value represents the relative fold changes between the calibrator and every treatment group gene expression. Changes in the relative content were equal to:  $2^{-\Delta\Delta C_t} \times 100\%$ .

### ***Inhibiting the cell membrane fusion of influenza virus***

**Fluorescent probe dilution:** the concentrated solution of fluorescent probe from the manufacturer was diluted to a one millimolar stock solution with dimethyl sulfoxide (DMSO) and stored in the dark at  $-70^\circ\text{C}$ . The working solution was diluted to 10 mMol/L with KH, to be used as an indicator of pH value.

**Determination of fluorescent probe stability:** MDCK cells at the log phase of growth were digested with trypsin-ethylenediaminetetraacetic acid (EDTA), made into a single-cell suspension, and seeded into pre-sterilized dishes at a density of  $5 \times 10^5$  cells/mL. After dishes were covered by a monolayer of cells, the culture solution was discarded, the cells washed three times with KH, then one milliliter of 10 mMol Snarf-4/AM fluorescent probe was added. Cells were incubated in the dark at  $37^\circ\text{C}$  for 60 min and washed three times with KH. Dynamic changes in fluorescence intensity in the Snarf-4/AM-labeled cells at 5 min were observed by confocal laser microscopy.

**FM1 infection and administration of Jinchai:** MDCK cells were treated according to the process specified in 2), but after being treated with the fluorescent probe and washed with KH, cells were infected with one milliliter of prepared 100TCID<sub>50</sub> FM1. Simultaneously, 1 or 2 mg/mL of the Jinchai dilutions were added to the infected cells. All experimental groups included control group, FM1 control group and Jinchai-treated groups (with different doses).

**Measurement by confocal laser microscopy:** cells were observed with an inverted phase contrast microscope at an excitation wavelength of 488 nm and dual emission wavelengths 580 and 640 nm. A one-way scan was performed at 8  $\mu\text{s}$ /pixel, at a zoom ratio of 1 : 1.6. The fluorescence intensity under the selected view was detected by scanning once every 6 s with a total scan time of 2000 s. The intracellular pH value was represented as the fluorescent intensity ratio (640 nm/580 nm). A decrease in the ratio indicated that the intracellular pH value increased, i.e., an increase in alkalinity. Conversely, an increase in the ratio indicated an increase in acidity.

Data were collected with the Olympus FV1000, and analyzed with LSM 510 software.

Monolayers of cells were cultured in 6-well plates. After 1.5 mL of the influenza strain FM1 was added to every well, cells were incubated for one hour at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$ . After washed with PBS, Jinchai dilutions (1 or 2 mg/mL) were added. Plates were then incubated at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$ . Both control group and virus-infected groups were included. Every treatment group consisted of 6 identical wells and the experiment was repeated 3 times. After 48 or 72 h of incubation,

cells were harvested and total cellular RNA was extracted. Real-time quantitative RT-PCR was carried out as mentioned above.

The sequences of the upstream and downstream primers of the polymerase PA subunit gene were 5'-TATTG-GCAAAGTTCGGTAT-3' and 5'-CAGGTTGTCCCTA-AGAGC-3', respectively. Upstream and downstream primers of the housekeeping gene GAPDH were: 5'-GGTGAAGGTCGGTGTGAACG-3' and 5'-CTC-GCTCCTGGAAGATGGTG-3', respectively.

Data analysis: one way ANOVA was performed to detect difference between groups.

## **RESULTS**

After normal cells were treated with Jinchai, the fluorescence intensity ratio was  $(2.26 \pm 0.11)$ . There was no significant difference when compared with control group ( $P > 0.05$ , see Figure 1). The expression of HA mRNA increased significantly in the control group, while expressions decreased in Jinchai-treated groups. There were significant differences between the Jinchai-treated groups at both concentrations (1 and 2 mg/mL) and the control group ( $P < 0.01$ ), and inhibition rates of the two Jinchai-treated groups were 99.92% and 99.93%, respectively.

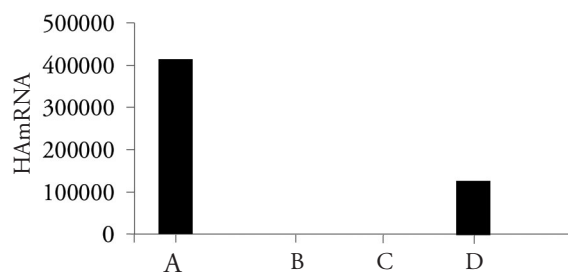


Figure 1 Expression of HA mRNA in every group

A: control group; B: large dose group; C: medium dose group; D: small dose group. HA: hemagglutinin; FM1: Influenza A1 virus. An asterisk indicates a significant difference from FM1 infected cells at  $P < 0.05$ . Double asterisk indicates a significant difference from FM1 infected cells at  $P < 0.01$  ( $n = 3$ ,  $\bar{x} \pm SD$ ).

Intracellular fluorescence intensity was  $4.70 \pm 0.27$  after MDCK cells were infected with the influenza virus. Compared with the control group, there was a significant difference ( $P < 0.01$ , see Table 1). Their fluorescence intensity was  $2.89 \pm 0.05$  after the infected cells were treated with Jinchai. Compared with the FM1 group, this was a significant difference ( $P < 0.01$ ).

PA mRNA expression was significantly higher in infected cells than that in the control group ( $P < 0.01$ , see Figure 2). PA expression decreased in both Jinchai-treated groups, and there was a significant difference between the treatment groups and the control group ( $P < 0.01$ ). The 2 mg/mL Jin Chai-treated group showed significant greater inhibitory effect than that of the 1 mg/mL Jin Chai-treated group. Inhibition rates were 95.72% and 98.88%, respectively.



Table 1 Fluorescence intensity ratio in MDCK cells

Group	Fluorescence intensity ratio
Control	2.10±0.19
Jinchai	2.26±0.11
FM1	4.70±0.27 <sup>a</sup>
Jinchai + FM1	2.89±0.05 <sup>b</sup>

Notes: Control: control group; Jinchai: jinchai treated group; FM1: FM1 group; Jinchai+FM1: Jinchai+FM1 treated group. FM1: Influenza A1 virus. Compared to control group, <sup>a</sup>*P*<0.01; compared to FM1 group, <sup>b</sup>*P*<0.01.

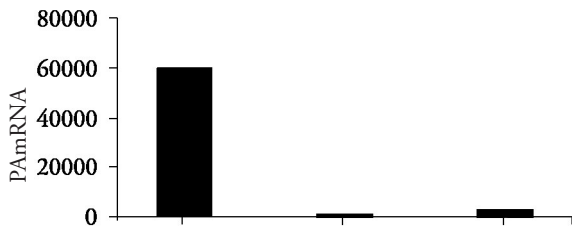


Figure 2 Expression of PA polymerase subunit mRNA  
PA: protein acid; FM1: Influenza A1 virus. An asterisk indicates a significant difference from FM1 infected cells at *P*<0.05. Double asterisk indicates a significant difference from FM1 infected cells at *P*<0.01 (*n*=3,  $\bar{x} \pm SD$ ).

## DISCUSSION

Jinchai inhibits influenza virus infection by blocking adsorption of virus to cells. HA protein on the surface of the influenza virion recognizes glycoprotein on the host cell membrane, and the virion thus adsorbs to the cell surface.<sup>9</sup> HA plays an important role in the pathogenicity of influenza viruses.<sup>10</sup> Through antigenic drift and shift, HA and NA proteins at the surface of the influenza virion enable it further bind to sialic acid receptors, fuse with the membrane, and thus induce stronger infectivity.<sup>11</sup> Therefore, inhibiting the adsorption of HA proteins to host cells is an effective way to prevent viral infection. In the study, we found that Jinchai could inhibit infection by blocking the adsorption of influenza virions to cells. There were two possible reasons. Firstly, Jinchai contained substances that were similar to the HA protein in structure, and therefore competed for the receptors on host cell surface. Secondly, some components in Jinchai had antiviral effect by directly inhibiting the binding of HA to cells.

Jinchai prevents influenza virus HA-induced membrane fusion. When influenza virus adsorbs onto the cell surface, virus particles are wrapped in the invaginated cell membrane and the virus particles enter by endocytosis. Upon viral entry, the intracellular pH value decreases from neutral to 5.0-6.0 due to the acidulation of the endoplasm. The HA molecule changes structure into a fusion peptide to enable itself to insert into the membrane and then induce membrane fusion. The nucleocapsid of influenza virus particles is subsequently released. The influenza virus requires an acidic pH for

maximal fusion. Changes in the conformation of influenza virus HA drive membrane fusion process.<sup>12-14</sup>

In our study, the results showed that the fluorescence intensity of infected MDCK cells was higher than uninfected cells, and Jinchai treatment induced a significant decrease in the fluorescence intensity of infected cells. As a result, we speculate that Jinchai prevented the acidification of the cytoplasm and subsequently conformational changes of HA, and thereby inhibited the fusion of influenza virions with cell membrane.

Jinchai attenuates transcription and replication of the influenza virus. The viral polymerase, consisting of PA, PB1, and PB2 subunits, is responsible for both transcription and replication of the viral RNA and has essential role in maintaining the life cycle of the virus. The amino-acid residues of PA and PB1 are highly conserved and their mutation rate is low,<sup>15,16</sup> so RNA polymerase is regarded as a potentially important target for antiviral drug design. It has been found that the PA subunit is involved in multiple virus activities including viral RNA polymerase transcription, endonuclease activity, protease activity and assembly of virus particles.<sup>17-19</sup> PA is synthesized in the infected cytoplasm, and then enters the nucleus.<sup>17,20-28</sup> When the gene expression of PA is reduced or disappears, polymerase synthesis and viral replication is blocked, resulting in a decreased number of virions. Dissemination to surrounding tissues and the ability to infect nearby normal cells is therefore limited.<sup>29</sup> In the study Jinchai might have an antiviral effect by inhibiting the activity of PA subunit of RNA polymerase and thus interfering with the transcription and replication of the influenza virus.

To explore the mechanism of anti-influenza activity of Jinchai, we used FM1 to infect MDCK cells and detected changes in intracellular pH value and mRNA expression of the HA gene. Results showed that Jinchai had an essential inhibitory role in the adsorption and membrane fusion of the influenza virus. Intracellular virus particles are responsible for gene transcription and the primers are its own encoding RNA-dependent polymerase complex. When there are enough virus proteins in infected cells, virus replication initiates and substantial progeny virions are generated. With the help of NA, progeny virions are released to destroy surrounding tissues and cells. In our study, we found that Jinchai decreased mRNA expression of PA and inhibited the transcription and replication of the influenza virus, reducing its ability to infect surrounding tissues.

In our previous study,<sup>30-33</sup> we found that Jinchai relieved the lung inflammation induced by influenza viruses and chemicals, and had antipyretic and immune regulatory activities. Jinchai improved membrane lipid fluidity of infected cells and prevented further infections, and simultaneously inhibited the release of the inflammatory factor nitric oxide, which reduced the degree of lung injury. In the study, we also found that Jinchai had similar antiviral activities. It inhibited the ad-

sorption and cell membrane fusion of the influenza virus in the early stage of infection, which not only exerted antiviral activities against the virus but avoided damaging the normal functions of host cells.

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