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Antiviral Decoction of *Isatidis Radix* (板藍根 bǎn lán gēn) Inhibited Influenza Virus Adsorption on MDCK Cells by Cytoprotective Activity

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Abstract

The aim of this study is to elucidate how the *Isatidis Radix* (板藍根 bǎn lán gēn) tonic, as an aqueous mixture of hundreds of compositions, interrupts the infection of influenza viruses to their host cells. The efficacy of the tonic was evaluated and expressed as cell proliferation rate and plaque reduction rate in Madin-Darby Canine Kidney (MDCK) cells, against 3 strains of influenza A and B viruses. This boiling water (at 100℃) extract of *Isatidis Radix* (RIE) showed antiviral activity against influenza virus A and B. The concentration for 50% inhibition of influenza virus A replication (IC_{50}) in MDCK cell was 12.6 mg/mL with a therapeutic index >8. When cells were incubated with RIE prior to virus adsorption, the numbers of viable cell were at least doubled compared to the numbers of virus control, RIE incubation after virus adsorption and RIE incubation with virus prior to adsorption, in both influenza virus A and B. Moreover, much less virus particles were spotted by scanning electron microscope (SEM) in the RIE pre-treated cells than the cells without RIE treatment. These results indicate the antiviral activity of RIE is mainly attributed to its host cell protection effect but not actions on virus or post-virus-adsorption interruption. Cell, but not virus, is more likely to be the action target of RIE.

Key words: *Isatidis Radix* (板藍根 bǎn lán gēn), Antiviral effects, Cell binding, Influenza virus, Cell protection

Introduction

Isatidis Radix (板藍根 bǎn lán gēn) is the dried roots of the plant *Isatis indigotica Fort*. or *Isatis tinctoria L.* (Fam. *Brassicaceae*), and a widely-used antiviral traditional Chinese medicine. As an officially approved medicinal material (Chinese Pharmacopoeia Commission, 2005), *Isatidis Radix* and its decoction (RIE) have been employed as a major herbal tonic in prevention and treatment against a wide range of viral infections, including seasonal flu and the deadly Severe Acute Respiratory Syndrome (SARS) (Lin et al., 2005). Regardless of the different subtypes of viruses and their constant mutations, RIE was found to be clinically effective against infections caused by various subtypes and strains of influenza viruses. The antiviral mechanism of RIE remains unclear, with no indication of the target of the antiviral action of RIE, an essential prerequisite to elucidation of the mechanism. There is, as yet, no explanation of the wide spectrum of activity against all strains of the influenza virus.

In our previous studies (Chen et al., 2006), RIE was demonstrated to prevent the influenza virus induced hemaegglutination, and remarkably change the surface

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charge of erythrocytes, measuring with capillary electrophoresis of erythrocytes (Lu et al., 2003). It implied that the anti-hemaegglutination activity of RIE might rely on the action upon erythrocytes rather than virus.

In this paper we sought to determined the antiviral activity of RIE using Madin-Darby Canine Kidney (MDCK) cells and 3 strains of influenza virus A and B. Attempts were made to identify either cell or virus the target of RIE's action, by comparison of the antiviral efficacy of RIE interference introduced before or after virus adsorption on MDCK cells.

Materials and Methods

Preparation of the aqueous extracts of *Isatidis Radix* (RIE)

Fresh roots of *Isatis indigotica* Fort. were harvested and sun-dried in Fuyang City, Anhui Province, P.R. China. Roots were dried at 20~25℃ (day), 5~10℃ (night), for 2 ~ 4 weeks. The yield of sun drying was 1 kg sun-dried roots from 2.5 kg fresh roots. The preparation of aqueous extracts of *Isatidis Radix* (RIE) was following the instructions of China Pharmacopeia (Chinese Pharmacopoeia Commission, 2005). 1400 g sun-dried roots were extracted twice with 3 L boiling water at 100℃ , for 2 h and 1 h, respectively. The solution was filtered and vacuum concentrated at 50℃ until the relative density reached 1.20. After the stock was precipitated by 60% ethanol at 4℃ , the precipitates were removed, then the suspension was vacuum concentrated at 50℃ to produce the RIE as brown powder.

Cells and virus

Continuous MDCK cells (China Center for Type Culture Collection) were maintained in DMEM (Invitrogen Corporation) with 10% calf serum (GIBCO), penicillin G (100 mg/L), streptomycin (80 mg/L), and sodium bicarbonate (3.7 g/L).

Influenza strains A/Beijing/95-262(H1N1/C_2E_3, 1:640 8/2/99) and B/Shanghai/93-1(C3C2, 1:320 9/2/99) were purchased from the National Influenza Center of China. Influenza A virus FM1 (H1N1, mouse-adapted strain) was generously provided by Fujian Centre of Disease Control (Fuzhou, Fujian, P.R.China). All viruses were propagated in the allantoic cavity of 11-day-old chick embryos at 33℃ for 48 h. Isolated virus stocks were stored in aliquots of phosphate buffered saline at -80℃ .

Determination of Cytotoxicity

Cytotoxic effects of the decoction (1-100 mg/mL) were determined by a dye uptake assay with crystal violet (0.03%, w/v) using MDCK cells (Schmidtke *et al*., 2001). Six duplicates were use for each concentration. The cell viability was presented as cell survival rate, which is calculated with *Equation 1*.

Cell survival rate

$$= (\text{mean O.D.}_{\text{sample}} / \text{mean O.D.}_{\text{control}}) \times 100\% \quad \textbf{Equation 1}$$

Determination of antiviral activities upon 3 strains of influenza virus A and B

Following the established protocol (Levi et al., 1995), MDCK cell monolayers (10^5 cells/well) in 96-well plates were washed with PBS and infected with 50 PFU (plaque forming units) of influenza virus A/Beijing/95-262, FM1, B/Shanghai/93-1, for 60 min at 33℃ in the presence or absence of RIE. The infected cells were then incubated with 200 μL maintenance medium containing RIE (1-100 mg/mL) for 3 d. Antiviral activity was determined using MTT assay (van de Loosdrecht *et al*., 1994) and plaque reduction assay (Gaush and Smith, 1968; Burleson et al., 1992). Cell control with/without the extracts and virus controls were included. Antiviral activity was calculated as a percentage of protection from virus-induced cell proliferation inhibition and cell destruction, in relation to infected cell without RIE and mock-infected control (*Equation 2&3*). The concentration for 50% inhibition of virus (IC_{50}) were calculated by Reed-Muench method (Reed and Muench, 1938).

Cells protection rate (%)

$$= (\text{O.D.}_{\text{VR}} - \text{O.D.}_{\text{V}}) / (\text{O.D.}_{\text{R}} - \text{O.D.}_{\text{V}}) \times 100 \quad \textbf{Equation 2}$$

(O.D._{VR} = infected cell with RIE; O.D._{V} = infected cell without RIE; O.D._{R} = cell with RIE)

Plaque inhibition rate (%)

$$= [(\text{control} - \text{sample}) / \text{control}] \times 100 \quad \textbf{Equation 3}$$

Determination of antiviral activities on different stages of viral replication cycle

A single concentration of RIE (50 mg/mL) was introduced to influenza virus A/Beijing/95-262, A FM1 and B/Shanghai/93-1, in three different modes: 1) cell protecting mode: cells were pre-treated with RIE at 33℃ for 180 min before virus adsorption; 2) cell