OPEM

Oriental Pharmacy and Experimental Medicine 2003 **3(2)**, 106-110

Antiviral activity of Herba Patrinea (a Chinese medicinal herb) against respiratory syncytial virus *in vitro*

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SUMMARY

Respiratory syncytial virus (RSV) has long been considered an important cause of severe lower respiratory tract infection in infants and young children throughout the world. Unfortunately, no effective treatment of RSV exists. Therefore, New agents are needed to reduce the impact of RSV. We have studied the anti-viral effect of traditional Chinese midicinal herbs for over ten years and find Herba Patrinea (a Chinese medicinal herb) has the anti-RSV effect in vitro. In this study, the Herba Patrinea was extracted with hot water, condensed and sterilized. The cytotoxicity of the aqueous extract was tested by adding the diluted extract directly to HeLa cells and its effect on anti-RSV was estimated by the CPEI assay. As a result, the median cytotoxic concentration (CC_{50}) of Herba Patrinea was 32 mg/ml by morphological observation, the median effective concentration (50% effective concentration, EC_{50}) of the Herba Patrinea against replication of the Long strain of RSV in HeLa cells were 1.25 mg/ml. The selectivity index ($SI=CC_{50}/EC_{50}$) is 25.6. Moreover, Herba Patrinea gave a dose-dependent response in inhibiting RSV. In time of addition experiment, Herba Patrinea inhibited replication of RSV in HeLa cells when it was added at 0h, 2h, and 4h after virus infection. In summary, the results of this study suggest Herba Patrinea may be a novel anti-RSV drug and it is worthy of further studying.

Key words: Herba Patrinea; Respiratory syncytial virus; Anti-viral effect

INTRODUCTION

Respiratory syncytial virus (RSV) has long been considered an important cause of severe lower respiratory tract disease in infants and young children throughout the world (Chanock and Finberg, 1957; Gardner, 1973; Glezen and Denny, 1973; Kim and Arroyo, 1973; Martin *et al.*, 1978; Medical research council subcommittee on respiratory syncytial virus vaccines, 1978). Over the last decade, it has also become clear that RSV is also an important pathogen in other age groups including infant and young child, those with compromised cardiac, immune, and respiratory systems of any age, and the elderly (MacDonald *et al.*, 1982; Hall *et al.*, 1986; Groothuis, 1988; Agius *et al.*, 1990; Flemingand and Cross, 1993; Falsey *et al.*, 1995; Michael *et al.*, 2002). Unfortunately, no effective treatment of RSV exists (Martin *et al.*, 2000). Although Ribavirin is the only drug registered for the treatment of children with RSV-induced bronchiolitis and has been licensed by the Food and Drug Administration, its anti-RSV effect is not determined (Hruska *et al.*, 1982). Additional problems of Ribavirin include high costs of the drug, difficulties with administration and the potential teratogenic and carcinogenic effects (Hebert *et al.*, 1990, Kazufumi *et al.*, 2000). Therefore, new agents are needed to reduce the impact of RSV. We have studied the anti-viral effect of traditional Chinese medicinal herbs for over ten years (Liu Mifeng *et al.*, 2001) and find Herba Patrinea (a Chinese medicinal herb) has the anti-RSV effect *in vitro*. Now it is reported as followed.

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MATERIALS AND METHODS

Viruses and cells

Respiratory syncytial virus (RSV) (long strains) was kindly provided by institution of Chinese preventive sciences. HeLa cells were used as the host for RSV. The cells were grown in the Modified Eagles medium (MEM) with 5% fetal calf serum (FCS) (Institution of Harbin Veterinary medicine).

Preparation of the Herba Patrinea extract

Herba Patrine (1000 g) were locally purchased in Harbin and washed five times with tap water and three with distilled water. Then the herbs were soaked with 7000 ml distilled water for 12 hours and extracted at 100°C for 90 min. The extract was clarified with cotton cloth and decanted to a clear container. The remainder was extracted one more time. The pooled extract was condensed at 100°C to 1.0 g/ml. The aqueous extract was sterilized at 100°C for 30 min.

Cytotoxicity

The cytotoxic effect of the aqueous extract on HeLa cells was tested. The sterilized aqueous extract was diluted in culture medium (MEM with 2% (v/v) heat-inactivated calf serum) before added directly to HeLa cells. The culture were incubated at 35°C in a CO_2 incubator, morphological changes resulting from aqueous extract were observed under an inverted light microscopy. Morphological change scale (MCS) was graded to 0-4 with 4 being defined as complete cytotoxicity. The 50% cytotoxic concentration (CC_{50}) in HeLa cells was calculated using Reed-Muench method (Reed, 1938) using the means of the MCS at each concentration of aqueous extract. In this assay the cytotoxic effect of Ribavirin was also observed.

Virus titration in HeLa cells

Monolayers of HeLa cells grown on 96-well culture plates were infected with various concentrations of respiratory syncytial virus and incubated for 2 hours at 35°C in a CO_2 incubator. The virus inoculum was removed from the cultures and the culture medium (MEM with 2% (v/v) heat-inactivated calf serum) was added to the plates. The plates were incubated at 35°C in a CO_2 incubator for 72 h and the virus-induced cytopathic (CPE) was observed under an inverted light microscope (CPE score: score 0=0%CPE, score1=1%-25% CPE, score 2=25%-50% CPE, score 3=50%-75% CPE, score 4=75%-100%CPE). Four wells were set at each concentration and four wells were added with maintenance medium as uninfected, untreated control. The 50% tissue culture infectious dose ($TCID_{50}$) was calculated by the Reed-Muench method using the means of the CPE scores at each virus concentration.

CPE inhibition assay

Monolayers of HeLa cells grown on 96-well culture plates were infected with 50 $TCID_{50}$ RSV. After incubation for 2 h at 35°C in a CO_2 incubator, the virus inoculum was removed from the cultures and the extract was diluted in culture medium (MEM with 2% (v/v) heat-inactivated calf serum) before added to the plate. The plates were incubated at 35°C in a CO_2 incubator for 72 h and the virus-induced cytopathic (CPE) was observed under an inverted light microscope. Ribavirin was used as positive control and the plates overlaid with culture medium without the extract were used as virus control. The median effective concentration (50% effective concentration, EC_{50}) of the Herba Patrinea against replication of the Long strain of RSV in HeLa cells was calculated using Reed-Muench method. A selectivity index (SI) was also calculated for each compound using the fomula: $SI=CC_{50}/EC_{50}$.

Time of addition experiment

HeLa cells were infected with virus and incubated 4°C for 1h to allow viral absorption. Virus inoculum was then removed and cultures were washed three times with pre-chilled medium prior to incubation with culture medium at 35°C in a CO_2 incubator. At 0, 2, 4, 6 and 8 h post-virus infection, the extract and Ribavirin were added to duplicate cultures. The plates were incubated at 35°C in a CO_2 incubator for 72 h and the CPE was observed.

RESULTS

Cytotoxicity

The cytotoxicity of the extract was tested in HeLa cells. The median cytotoxic concentration (CC_{50}) of

Table 1. The inhibitory effects of Herba Patrinea and Ribavirin on RSV replication

Drugs	Dose	CPE score	Average CPE score	CPE inhibition	Total		CPE inhibitory rate (%)
					CPE	CPE inhibition	
Herba Patrinea (mg/ml)	5	1 0 1 1	0.75	3.25	14.50	3.25	81.69
	2.5	1 1 2 1	1.25	2.75	11.25	6.00	65.22
	1.25	1 2 1 2	1.50	2.50	8.50	8.50	50.00
	0.625	2 1 1 2	1.50	2.50	6.00	14.50	29.27
	0.3125	3 2 2 3	2.50	1.50	3.50	18.00	16.28
	0.156	2 3 3 2	2.50	1.50	2.00	20.00	9.09
	0.078	3 4 3 4	3.50	0.50	0.50	20.50	2.38
Ribarivin (μg/ml)	10	0 0 0 0	0.00	4.00	13.50	0.00	100.00
	5	1 0 1 0	0.50	3.50	9.50	0.50	95.00
	2.5	1 2 2 1	1.50	2.50	6.00	2.00	75.00
	1.25	1 3 2 3	2.25	1.75	3.50	4.25	45.16
	0.625	3 2 3 3	2.75	1.25	1.75	7.00	20.00
	0.3125	4 4 3 3	3.50	0.50	0.50	10.50	4.54
Cell control		0 0 0 0	0	4.00			
Viral control		4 4 3 4	3.75	0.25			

Herba patrinea was 32 mg/ml by morphological observation. TC_0 of the extract is 20 mg/ml. No significant cytotoxicity of Ribavirin was observed against HeLa cells by Morphological observation, even at the highest concentration of the Ribavirin examined (500 μg/ml).

Virus titration in HeLa cells

The $TCID_{50}$ of respiratory syncytial virus in HeLa cells was $10^{-7.7}$.

CPE inhibition assay

As it shows in Table 1, the inhibitory effect of Herba Patrinea and Ribavirin on RSV replication was analyzed using KruskalWallis method. As a result, the distribution of CPE scores in those concentrations ranging from 5 to 0.625 mg/ml of Herba Patrinea were significant different with respect to that of the virus control by Kruskal-Wallis test ($P<0.01$); the distribution of CPE scores in those concentrations ranging from 10 to 2.5 μg/ml of Ribavirin were significant different with respect to that of the virus control by Kruskal-Wallis test ($P<0.01$).

The median effective concentration (50% effective concentration, EC_{50}) of the Herba Patrinea against replication of the Long strain of RSV in HeLa cells was estimated as 1.25 mg/ml, and selectivity index (SI) was 25.6. The EC_{50} and SI of the Ribavirin were estimated as 1.43 μg/ml and more than 349, respectively.

Dose-depended Inhibition on RSV by Herba Patrinea and Ribavirin

In order to observe the dose-effect relation, the correlation analysis was done between the logarithmic concentrations and CPE inhibitory ratios for RSV. As it is showed in Fig. 1 and Fig. 2, the correlation coefficient (r) for Herba Patrinea is 0.9849 (P=0.0001), while r is for Ribavirin is 0.9859 (P=0.0003). There existed significant correlations between the logarithmic concentrations and CPE inhibitory ratios for Herba Patrinea and Ribavirin.

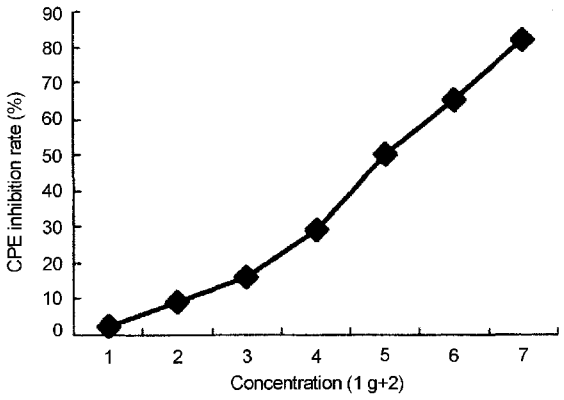

Fig. 1. Dose-depended inhibition on RSV by Herba Patrinea.

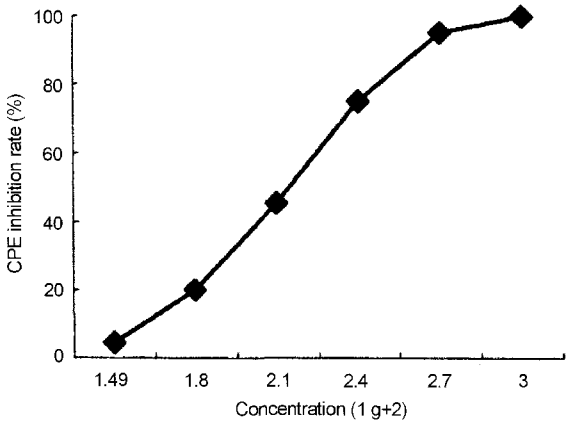

Fig. 2. Dose-depended inhibition on RSV by Ribavirin.

Time of addition experiment

The inhibitory effect of the Herba Patrinea on RSV replication in HeLa cells was further investigated in a one-step growth study, in which the extract and Ribavirin were added at 0, 2, 4, 6 and 8 h post-virus infection. The effect was analyzed by Kruskal-Wallis method. As a result, Herba Patrinea and Ribavirin showed active effect against RSV replication when they were added at 0, 2, 4 h after virus infection ($P<0.01$) (Table 2).

DISCUSSION

Acute respiratory infectious caused by RSV are known to be most prevalent in infants and children. The mortality of RSV infection is usually low, whereas it increases to 37%-73% in infants with heart or pulmonary failure, and to 36%-45% in recipients of bone marrow transplant (MacDonald *et al.*, 1982; Englund *et al.*, 1988; Harrington *et al.*, 1992). A novel anti-RSV agent with better therapeutic efficacy and safety than Ribavirin is needed. We have studied anti-RSV drugs for over ten years. After screening many Chinese medicinal herbs using cells culture methods, we found that Herba Patrinea had the ability to inhibit the replication of respiratory syncytial virus effectively in HeLa cells. In this study, Herba Patrinea was extracted with hot water at 100°C for 90 minutes twice times and condensed at 100°C to 1.0 g/ml. The process is much like the Chinese traditional method of herb extracting and easy to operate. As a result we got 1000 ml brown aqueous extract.

In the assay of cytotoxicity, the aqueous extract was diluted in culture medium (MEM with 2% (v/v) heat-inactivated calf serum) and added to HeLa cells. TC_{50} of the extract is 32 mg/ml.

The inhibitory effect of Herba Patrinea on RSV replication was tested in HeLa cell using several assays. As the results of CPE inhibition assay, the EC_{50} and SI of Herba Patrinea are 1.25 mg/ml, 25.6, respectively, Furthermore, Herba Patrinea shows a dose-depended effect against RSV.

At 4°C RSV binds to cells but not penetrate through the cell membrane. When virus bound cells are exposed to a 35°C condition, the virus starts to penetrate the cell membrane (Hosoya *et al.*, 1991). The results of the effect of Herba Patrinea on RSV growth show that Herba Patrinea is a good inhibitor of virus penetration as well as virus replication. The cells were put at 4°C after infection with RSV for 60 minutes to allow the virus adsorption and then washed three times. The Herba Patrinea was added before the cells incubated at 35°C. We found that the virus was totally inhibited by the extract after three days of observation, which suggests that the extract of Herba Patrinea could inhibit virus penetration. In additional, when the extract was added at 2 h, 4 h, 6 h post-viral infection, it also

Table 2. Time of addition of Herba Patrinea and Ribavirin to infected cells and inhibitory effects of Herba Patrinea and Ribavirin on RSV replication

Drug	Time of addition	CPE score	Drug	Time of addition	CPE score
Herba Patrinea (2 mg/ml)	0h	0 0 0 0	Ribavirin (10 μg/ml)	0h	0 0 0 0
	2h	1 1 0 2		2h	1 1 0 1
	4h	1 1 2 2		4h	1 3 2 1
	6h	2 2 4 3		6h	2 3 3 4
	8h	4 4 3 4		8h	4 4 4 3
Viral control		4 4 4 4	Virus control		4 4 4 4
Cell control		0 0 0 0	Cell control		0 0 0 0

showed inhibitory effect on RSV replication. Since virus began replicating when incubated at 35°C, we can conclude that the extract can inhibit virus replication as well. But the mechanism is not clear yet and need further studying.

In summary, Herba Patrinea proved to be potent anti-RSV agent and it is low toxic. As a potential anti-RSV agent, it is worth further investigating.

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