Original Article

Glycyrrhizin inhibits human parainfluenza virus type 2 replication by the inhibition of genome RNA, mRNA and protein syntheses

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Summary   **The effect of glycyrrhizin on the replication of human parainfluenza virus type 2 (hPIV-2) was examined. Cell fusion induced by hPIV-2 was inhibited by glycyrrhizin, and glycyrrhizin reduced the number of viruses released from the cells. Glycyrrhizin did not change cell morphology at 1 day of culture, but caused some damage at 4 days, as determined by the effect on actin microfilaments. However, it affected the cell viability at 1 day: about 20% of the cells were not alive by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 1 day of culture. Real-time polymerase chain reaction (PCR) and PCR showed that virus genome synthesis was largely inhibited. mRNA synthesis was also inhibited by glycyrrhizin. Viral protein synthesis was largely inhibited as observed by an indirect immunofluorescence study. Multinucleated giant cell formation was studied using a recombinant green fluorescence protein (GFP)-expressing hPIV-2 without matrix protein (rhPIV-2ΔMGFP). A few single cells with fluorescence were observed, but the formation of giant cells was completely blocked. Taken together, it was shown that viral genome, mRNA and protein syntheses, including F and HN proteins, were inhibited by glycyrrhizin, and consequently multinucleated giant cell formation was not observed and the infectious virus was not detected in the culture medium.**

Keywords: *Virus replication, medicinal plant extract, recombinant green fluorescence protein expressing hPIV-2 without matrix protein*

1. Introduction

Human parainfluenza virus type 2 (hPIV-2) is a member of the genus *Rubulavirus* in the family Paramyxoviridae. hPIV-2 infects the human respiratory tract in infants and children. hPIV causes recurrent infection and there is no vaccine or anti-hPIV drug at present. It possesses a single-stranded, non-segmented, negative stranded RNA genome of 15,654 nucleotides (*1*). The genomic RNA codes 7 structural proteins, nucleoprotein (NP), V, phospho (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN) and large (L) proteins, like other

paramyxoviruses. V protein is the fifth structural protein of paramyxovirus (*2*). The gene order of hPIV-2 is 3'-(leader)-NP-V/P-M-F-HN-L-(trailer)-5' (*3*).

We have already reported some substances of large molecular weight (MW) that have inhibitory effects on hPIV-2 replication *in vitro*. Fucoidan, sulfated polysaccharides extracted from the brown alga Okinawa mozuku, inhibited hPIV-2 genome and protein syntheses, owing to the inhibition of viral adsorption to the cells (*4*). Bovine lactoferrin partially inhibited viral genome and protein expression (*5*). Legume lectins with different sugar binding specificities, concanavalin A, lens culinaris agglutinin and peanut agglutinin, also prevented hPIV-2 adsorption by binding to the specific lectin receptors (*6*).

Glycyrrhizin is extracted from the roots of *Glycyrrhiza glabra* and it has many biological and pharmacological activities, such as anti-inflammatory and anti-viral effects. Glycyrrhizin is a small molecule with MW 823. Glycyrrhizin inhibits production of pro-inflammatory cytokines (*7,8*). Anti-viral activities of glycyrrhizin have been reported previously. For instance, glycyrrhizin has inhibitory effects on H5N1 influenza A virus (*9*), hepatitis C virus (HCV) (*10,11*), rotavirus (*12*) and Kaposi's sarcoma-associated herpes virus (*13*).

In the present investigation, glycyrrhizin was tested for hPIV-2 replication. The number of viruses released from infected cells cultured with glycyrrhizin was determined. The effects of glycyrrhizin on actin microfilaments were analyzed using rhodamine phalloidin. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The effect of glycyrrhizin on viral genome synthesis was examined: viral RNA was prepared and analyzed by real-time polymerase chain reaction (PCR) and PCR. The effect of glycyrrhizin on mRNA synthesis was analyzed: cDNA was synthesized using oligo dT primer, and PCR was carried out. Viral protein syntheses were observed by indirect immunofluorescence study using monoclonal antibodies (mAbs) against NP, F and HN proteins of hPIV-2 (*14*). The effect of glycyrrhizin on hPIV-2-induced multinucleated giant cell formation was analyzed using recombinant green fluorescence protein expressing hPIV-2 without matrix protein (rhPIV-2ΔMGFP) (*6,15,16*).

2. Materials and Methods

2.1. *Glycyrrhizin*

Glycyrrhizin (Cokey Systems, Matsusaka, Japan; more than 75% pure) was dissolved at 20 mg/mL in 10 mM phosphate buffered saline, pH 7.2 (PBS). The pH of the solution was about 5, and was adjusted to 7.1-7.2 by 1 M NaOH, and it was sterilized by filtration. Glycyrrhizin was stored at – 20°C before use.

2.2. *Virus and recombinant virus*

The uses of the virus and recombinant virus were approved by the Microbiology Biosafety Committee and Recombinant DNA Biosafety Committee of Suzuka University of Medical Science, respectively.

We used hPIV-2 (Toshiba strain) that causes visible cell fusion. rhPIV-2ΔMGFP was constructed according to the method described previously (*6,15,16*), and no production of infectious virus particles without addition of M protein gene *in trans* was ascertained (*15,16*). The virus yield was about 1×10^5 TCID$_{50}$/mL.

2.3. *Cell line and cultivation of cells*

LLCMK$_2$ cells (rhesus monkey kidney cell line) were cultured in a flat-bottomed 24-well plate in 1 mL culture medium. The culture medium was minimum essential medium α (MEMα: Wako, Osaka, Japan), supplemented with 2% fetal calf serum (FCS) and 0.1 mg/mL kanamycin. The cells were cultured at 37°C in a humidified atmosphere with 5% CO$_2$. After 3 days, the cells became confluent (5×10^5 cells), and the medium was changed to MEMα with 0.5% FCS and 0.1 mg/mL kanamycin. Glycyrrhizin was added to the cells, and after about 5 min, the cells were infected with hPIV-2 (about 1×10^2 TCID$_{50}$).

2.4. *Cytopathogenic assay and MTT assay*

Cell fusion was observed under a cell culture light microscope at 4 days post infection. MTT assay was carried out according to the manufacturer's method using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Tokyo, Japan) at 1 day and at 4 days post addition of glycyrrhizin.

2.5. *RNA preparation, cDNA synthesis and real time PCR*

RNA was extracted from the cells (2×10^6 cells) cultured in a flat-bottomed 6-well plate using TRIZOL reagent (Invitrogen, CA, USA) according to the manufacturer's method at 4 days post infection. cDNA was synthesized with 1 μg RNA using Reverse Tra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) and NP gene specific primer (nucleotide number 1661-1679: 5'-CAACATTCAATGAATCAGT-3'). Real-time PCR was performed on the ABI PRISM 7700 Sequence Detection System (Life Technologies, Tokyo, Japan) using TaqMan Probe (1932-1956: 5'-FAM-AAGCACCGGATTTCTAACCCGTCCG-TAMRA-3'), forward primer (1851-1875: 5'-ACACACTCATCCAGACAAATCAAAC-3'), and reverse primer (1958-1980: 5'-TGTGGAGGTTATCTGATCACGAA-3').

2.6. *Detection of viral RNA*

cDNA was synthesized with 1 μg RNA using forward primers for NP (nucleotide number 1,081-1,100: 5'-CATGGCCAAGTACATGGCTC-3'), F (5,821-5,840: 5'-CCCTATCCCTGAATCACAAT-3') and HN (7,741-7,760: 5'-ATTTCCTGTATATGGTGGTC-3') and superscript II reverse transcriptase (Invitrogen), and PCR was carried out with forward primers for NP (nucleotide number 1,081-1,100), F (5,821-5,840) and HN (7,741-7,760), and reverse primers for NP (1,466-1,489: 5'-CC TCCGAGTATCGATTGGATTGAA-3'), F (6,661-6,681: 5'-TGTCACGAGACGTTACGGACA-3') and HN (8,481-8,500: 5'-GAACTCCCCTAAAAGAGATG-3') genes and Ex Taq (Takara, Shiga, Japan).

2.7. Detection of mRNA

cDNA was synthesized with 1 μg RNA using oligo dT primer (5'-TTTTTTTTTTTTTTTTTTTTTT-3') and superscript II reverse transcriptase (Invitrogen), and PCR was carried out with forward primers for NP (nucleotide number 1,081-1,100), F (5,821-5,840) and HN (7,741-7,760), and reverse primers for NP (1,466-1,489), F (6,661-6,681) and HN (8,481-8,500) genes and Ex Taq (Takara).

2.8. Immunofluorescence study

Actin was detected using rhodamine phalloidin (Invitrogen) at 1 day and 4 days of cultivation with or without glycyrrhizin. All the following procedures were carried out at 37°C, except for the microscopic observation. The cells were fixed with 3.7% formaldehyde solution in PBS for 10 min, washed with PBS, were further incubated with 0.1% Triton X-100 in PBS for 3 min, and observed under a fluorescence microscope (Olympus, Tokyo, Japan).

To detect virus proteins in the infected cells, the cells were fixed with 3.7% formaldehyde solution in PBS at room temperature for 10 min at 4 days of cultivation. After washing with PBS, the cells were further incubated with 0.1% Triton X-100 in PBS at room temperature for 3 min, washed with PBS, and incubated with mouse mAbs against NP, F and HN proteins of hPIV-2 (*14*) at room temperature for 30 min. After washing with PBS, the cells were incubated with Alexa 488 conjugated secondary antibody to mouse IgGs (Invitrogen) at room temperature for 30 min, and observed under a fluorescence microscope.

2.9. Observation of multinucleated giant cell

The cells were added with glycyrrhizin and after about 5 min they were infected with rhPIV-2ΔMGFP (1×10^4 TCID$_{50}$). After 4 days, the cells were washed with PBS and fixed with 1.2% formaldehyde solution in PBS at room temperature for 10 min, and observed under a fluorescence microscope.

3. Results

3.1. Titration of virus released from the infected cells

The titers of virus released from the cells cultured with and without glycyrrhizin at 4 days post infection were determined. Glycyrrhizin inhibited dose-dependently the virus release from the cells (Figure 1). Without glycyrrhizin, the virus titer was about $(1.9 \pm 0.7) \times 10^6$ TCID$_{50}$/mL (mean ± SEM of 3 independent experiments), and with glycyrrhizin (3 mg/mL), it was less than 10 TCID$_{50}$/mL (results of 3 independent experiments). Glycyrrhizin almost completely inhibited the virus replication and the release of virus from the cells.

In the following experiments, 3 mg/mL of glycyrrhizin was added to the cell culture.

3.2. The effect of glycyrrhizin on actin microfilaments and cell viability

Glycyrrhizin was added to the cells, and actin microfilaments were observed under a fluorescence microscope at 1 day and 4 days of cultivation. Figures 2A and B show actin microfilaments at 1 day and 4 days, respectively. As shown in Figure 2, glycyrrhizin did not disrupt actin microfilaments at 1 day of culture (Figure 2C), but caused some damage at 4 days (Figure 2D). The results indicated that glycyrrhizin did not cause severe morphological change in the cells.

Cell viability was determined by MTT assay at 1 day and 4 days. Figure 3 shows that glycyrrhizin caused some effect on the cell viability. About 20% of the cells were not alive after the addition of glycyrrhizin at 1 day.

3.3. Viral genome RNA and mRNA syntheses

RNA was prepared from the infected cells at 4 days post infection, and the viral genome RNA was analyzed
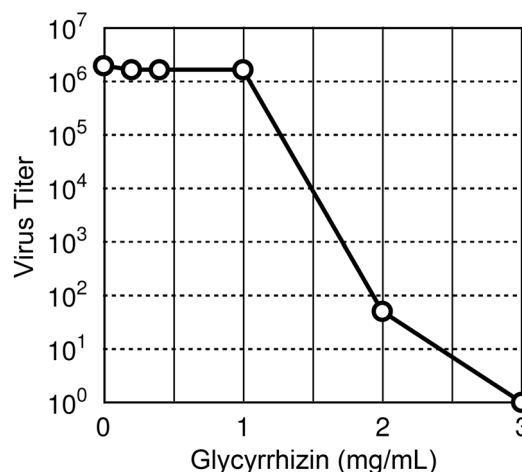

Figure 1. The effect of glycyrrhizin on the virus released from the cells. Glycyrrhizin inhibited the virus release in a dose-dependent manner.

by real-time PCR. The relative copy number in the glycyrrhizin-treated infected cells was about 0.033 compared with the copy number of the infected cells.

Viral genome RNAs of NP, F and HN were analyzed using hPIV-2 specific primers by PCR (Figure 4). The number of base pairs between forward and reverse primers of NP, F and HN genes was about 400, 860 and 760, respectively. Lane 1 (NP), 2 (F) and 3 (HN) were non-infected cells, and no visible bands were observed. In the virus-infected cells, NP (lane 4),

F (lane 5) and HN (lane 6) genes were clearly detected. In the glycyrrhizin-treated infected cells, NP (lane 7), F (lane 8) and HN (lane 9) gene syntheses were almost completely inhibited.

In the following experiment, mRNA synthesis was analyzed. cDNA was synthesized using oligo dT primer, and PCR was carried out using hPIV-2 specific primers. Figure 5 shows that in non-infected cells, no bands were detected, and NP (lane 4), F (lane 5) and HN (lane 6) mRNAs were detected in the virus-infected cells.
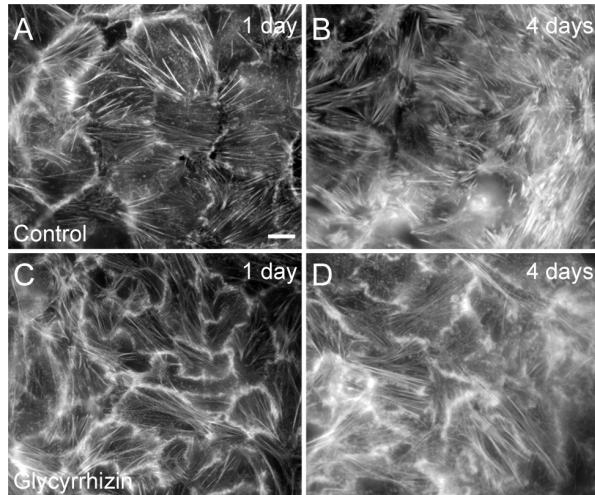

Figure 2. The effect of glycyrrhizin on actin microfilaments at 1 day and 4 days. The cells were cultured with and without glycyrrhizin for 1 day and 4 days, and actin microfilaments were stained with rhodamine phalloidin. Figure 2 shows actin microfilaments of LLCMK$_2$ cells cultured without glycyrrhizin for 1 day **(A)** and 4 days **(B)**, and those of the cells cultured with glycyrrhizin for 1 day **(C)** and 4 days **(D)**. (bar: 50 μm). Glycyrrhizin did not cause severe morphological change in the cells.
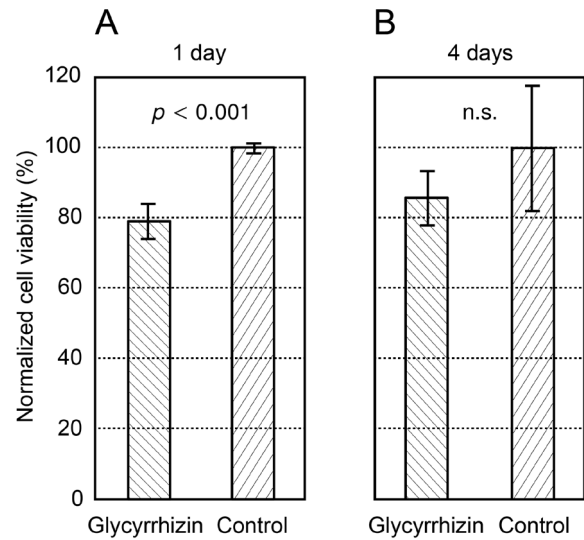

Figure 3. Cell viability determined by MTT assay at 1 day and 4 days. The cells were cultured with and without glycyrrhizin for 1 day **(A)** and 4 days **(B)**. Glycyrrhizin caused a slight effect on the cell viability at 1 day **(A)**.
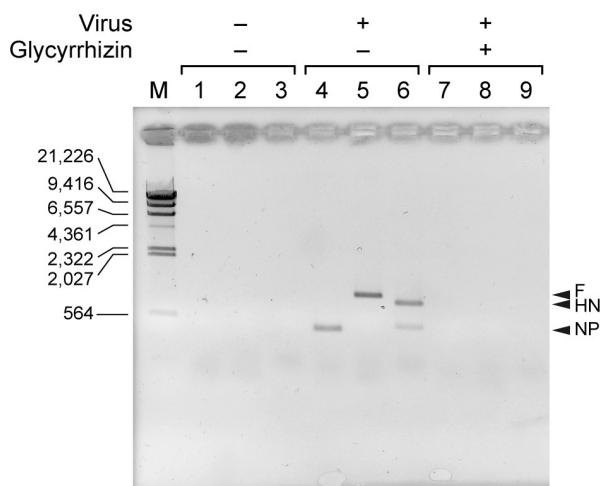

Figure 4. Viral genome RNA synthesis was inhibited by glycyrrhizin. Lane M: marker (base pair), lanes 1 (NP), 2 (F) and 3 (HN): non-infected cells, lanes 4 (NP), 5 (F) and 6 (HN): hPIV-2 infected cells, lanes 7 (NP), 8 (F) and 9 (HN): hPIV-2 infected cells cultured with glycyrrhizin. No visible bands were observed in lanes, 7, 8 and 9. The results were the same as those of real-time PCR, indicating that glycyrrhizin inhibited viral genome synthesis.
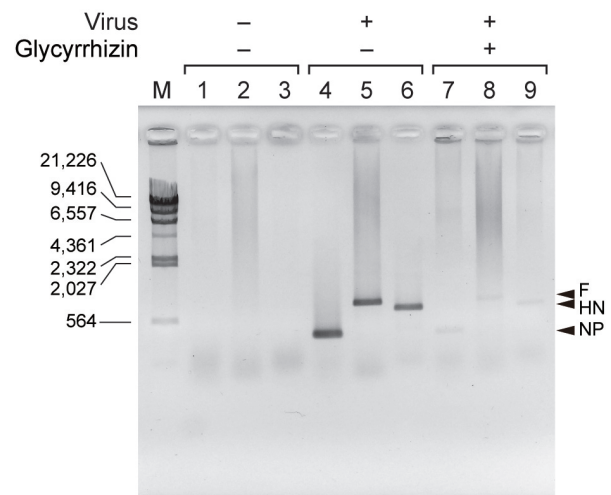

Figure 5. Viral mRNA synthesis was largely inhibited by glycyrrhizin. cDNA was synthesized using oligo dT primer and PCR was carried out using NP, F and HN specific primers. Lane M: marker (base pair), lanes 1 (NP), 2 (F) and 3 (HN): non-infected cells, lanes 4 (NP), 5 (F) and 6 (HN): hPIV-2 infected cells, lanes 7 (NP), 8 (F) and 9 (HN): hPIV-2 infected cells cultured with glycyrrhizin. In lane 7, 8 and 9, slight bands were seen. Glycyrrhizin largely inhibited mRNA synthesis.
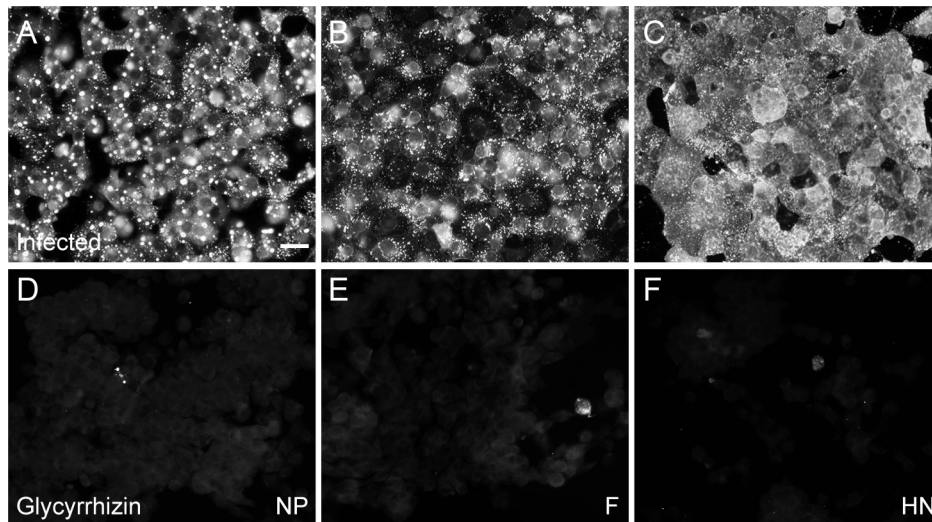
Figure 6. Glycyrrhizin inhibited the expression of NP, F and HN proteins of hPIV-2. The expression of NP **(A)**, F **(B)** and HN **(C)** proteins of hPIV-2 infected cells. A small number of cells had fluorescence, indicating that glycyrrhizin largely inhibited the expression of NP **(D)**, F **(E)** and HN **(F)** proteins. (bar: 50 μm).

But mRNAs were only slightly seen in the glycyrrhizin-treated infected cells (NP: lane 7, F: lane 8 and HN: lane 9). These results show that glycyrrhizin largely inhibited the transcription of NP, F and HN genes.

3.4. *Viral protein synthesis*

Viral protein synthesis was analyzed by indirect immunofluorescence study using mAbs against hPIV-2. Glycyrrhizin was added to the cells, and they were infected with hPIV-2. At 4 days post infection, the cells were fixed, stained with the mAbs and secondary antibody. Figures 6A, B and C show the NP, F and HN protein expression in hPIV-2 infected cells, respectively. Non-infected cells had no fluorescence of the proteins (data not shown). In hPIV-2 infected cells, NP, F and HN proteins were observed in almost all the cells: NP protein was observed in many strong fluorescent dots mainly in the cytoplasm; F and HN proteins were in small dots in the cytoplasm and on the cell surface. In glycyrrhizin-treated infected cells, a few cells had fluorescence, indicating that the syntheses of NP (Figure 6D), F (Figure 6E), and HN (Figure 6F) proteins were almost completely inhibited by glycyrrhizin. As shown in Figure 5, mRNA was only slightly detected in glycyrrhizin-treated infected cells. Therefore, it was suggested that the protein synthesis inhibition by glycyrrhizin was caused by the inhibition of transcription.

3.5. *The effect of glycyrrhizin on multinucleated giant cell formation*

The above results showed that glycyrrhizin inhibited viral genome RNA synthesis and consequently inhibited mRNA and protein syntheses. In the following experiment, we determined the effect of glycyrrhizin on
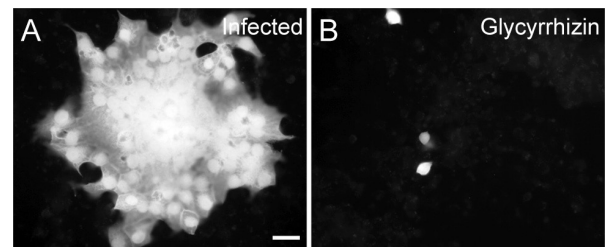

Figure 7. The effect of glycyrrhizin on rhPIV-2ΔMGFP replication. The cells were infected with rhPIV-2ΔMGFP and cultured for 4 days **(A)**, the rhPIV-2ΔMGFP infected cells were cultured with glycyrrhizin for 4 days **(B)**. Figure 7B shows that there are a few fluorescent cells with single nucleus, but there were no multinucleated giant cells, indicating that cell-cell spreading of hPIV-2 was inhibited due to insufficient expression of F and HN proteins. (bar: 50 μm).

the formation of multinucleated giant cells using rhPIV-2ΔMGFP (Figure 7). Figure 7A is rhPIV-2ΔMGFP infected cells at 4 days and there were multinucleated giant cells with strong fluorescence. In non-infected cells, there were no giant cells or fluorescent cells (data not shown). Figure 7B shows that a few fluorescent cells with single nucleus were observed in the infected cells cultured with glycyrrhizin. The results indicate that glycyrrhizin blocked giant cell formation by the inhibition of F and HN protein expressions.

4. Discussion

The present study showed that glycyrrhizin had an inhibitory effect on hPIV-2 replication in $LLCMK_2$ cells mainly by the inhibition of viral genome RNA and mRNA syntheses.

It was reported that glycyrrhizin has anti-human immunodeficiency virus activity by inhibiting virus replication by interfering with virus to cell binding (*17*). Glycyrrhizin also exhibited anti-HCV activity

by the inhibition of the expression of HCV core gene when combined with interferon (*10*). Matsumoto *et al.* (*15*) also reported that glycyrrhizin inhibited the release of infectious HCV particles due to the inhibition of phospholipase A2. Hardy *et al.* (*12*) also reported glycyrrhizin primary metabolite 18β-glycyrrhetinic acid reduced rotavirus yield by 99% when added to infected cell culture post virus adsorption, occurring at a step or steps subsequent to virus entry. In addition, glycyrrhizin inhibited respiratory viruses such as respiratory syncytial virus (RSV) mainly by preventing viral attachments (*18*), and severe acute respiratory syndrome corona virus by affecting many cellular signaling pathways, but the real mechanism is unclear (*19*). Glycyrrhizin was shown to inhibit H5N1 influenza virus replication and also caused anti-inflammatory responses, indicating that glycyrrhizin is beneficial for treatment of H5N1 virus (*9*). Wang *et al.* showed that glycyrrhizin directly inactivated coxsackievirus A16, but its inhibitory effect on enterovirus 71 was associated with an event(s) post virus entry (*20*). However, the effects of glycyrrhizin on parainfluenza virus, which causes respiratory infection, have not been reported. The present investigation is the first report on the inhibitory effect of glycyrrhizin on parainfluenza virus replication.

Glycyrrhizin did not cause morphological change of actin microfilaments at 1 day of culture but caused some damage at 4 days. Cytoskeleton was reported to have an important role in paramyxovirus replication. Actin microfilaments are important in the hPIV-3 life cycle, specifically at the level of viral transport and replication (*21*). The results indicate that the inhibitory effect of glycyrrhizin on hPIV-2 replication was in part caused by the slight disruption of actin microfilaments at 4 days. MTT assay shows that about 20% of the cells were not alive at 1st day after the addition of glycyrrhizin without virus infection. There is some discrepancy between the actin morphology and cell viability.

Almost no virus was released into the culture medium from glycyrrhizin-treated cells infected with hPIV-2. Viral genome RNA was not detected in the glycyrrhizin-treated infected cells. As the viral genome RNA and mRNA syntheses were inhibited, syntheses of viral proteins were suppressed, consequently cell-cell spreading of hPIV-2 and giant cell formation were inhibited due to insufficient expression of F and HN proteins.

Ribavirin, a drug, which is effective on both DNA and RNA viruses, also inhibits hPIV-2 replication *in vitro* (*22*). Ribavirin aerosol (20 mg/mL) was effective in the treatment of viral pneumonia caused by hPIV-3 and RSV in severe combined immunodeficiency disease of two boys (*23*). Thus, the aerosol method may be useful for the treatment of hPIV-2 by glycyrrhizin, because its optimal concentration *in vitro* is high (3 mg/mL).

In summary, the present investigation reported that glycyrrhizin, one of the important components of herbal medicines, inhibited hPIV-2 replication in LLCMK$_2$ cells by the inhibition of viral genome RNA, mRNA and protein syntheses, resulting in the inhibition of giant cell formation and viral release from the infected cells into the culture medium. Herbal medicines containing glycyrrhizin or glycyrrhizin may become useful drugs for the treatment of viruses that infect the respiratory tract, such as hPIV and RSV.

Acknowledgements

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