의학석사 학위논문

In Vitro Antiviral Activity of Ribavirin against Severe Fever with Thrombocytopenia Syndrome Virus

중증열성혈소판감소증후군 바이러스에 대한
리바비린의 항바이러스 효능에 관한 연구

2015년 2월

서울대학교 대학원

의학과 내과학 전공

이 명 진

A thesis of the Master's degree

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February 2015

Seoul National University

College of Medicine, Graduate school

Department of Internal Medicine

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지도교수 오 명 돈

이 논문을 의학석사 학위논문으로 제출함

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이명진의 의학석사 학위논문을 인준함

2015년 01월

위 원 장 _____ (인)

부위원장 _____ (인)

위 원 _____ (인)

Abstract

In Vitro Antiviral Activity of Ribavirin against Severe Fever with Thrombocytopenia Syndrome Virus

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Department of Internal Medicine

The Graduate School

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Introduction:

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease caused by a novel Bunyavirus called SFTS virus (SFTSV). No effective antiviral therapy is available yet, but ribavirin (RBV) was used for patients with SFTS. We investigated the antiviral effect of RBV against SFTSV in vitro.

Methods:

To test for cytotoxicity of RBV, Vero cells were treated with different concentrations of RBV (0 - 500 μg/mL, in serial two-fold dilutions) and analyzed by cell viability MTS assay. To determine antiviral activity of RBV against SFTSV, Vero cells were infected with SFTSV strain Gangwon/Korea/2012 at 100 tissue culture infective dose ($TCID_{50}$) per well in a 96-well plate, and RBV was added at non-cytotoxic concentrations. The antiviral activity of RBV was determined by the observation of cytopathic effects by SFTSV-infected Vero cells and the measurement of viral RNA titers in culture supernatants using one-step real-time reverse transcription - polymerase chain reaction to amplify the partial large segment of SFTSV. Statistical analysis was done by one-way ANOVA with Tukey's post hoc test.

Results:

Cytotoxicity due to RBV was not detected for RBV at concentrations ≤ 31.3 μg/mL. Viral RNAs at 24 h post-RBV treatment were reduced by administration of increasing RBV concentrations (1 - 32 μg/mL), compared with those of mock-treated cells ($P < 0.01$). RBV reduced SFTSV titers and cytopathic effects in a dose-dependent manner, with half maximal inhibitory concentrations of 3.69 to 8.72 μg/mL.

Conclusion:

Our study shows that RBV has in vitro antiviral effect against SFTSV in a dose-dependent manner. Further studies are warranted to evaluate the clinical efficacy of RBV in SFTS.

Keywords: Severe fever with thrombocytopenia syndrome, phlebovirus, ribavirin

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Introduction

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tick-borne infectious disease, which was first reported from China in 2011 (1). SFTS is caused by a novel phlebovirus called SFTS virus (SFTSV), which belongs to the family *Bunyaviridae* (1). SFTSV is known to be vectored by ticks in the Ixodidae family of the species *Haemaphysalis longicornis*, which are mainly distributed in East Asia including Korea (2).

SFTS is an acute febrile illness characterized by thrombocytopenia, leukopenia, gastrointestinal symptoms leading to multi-organ failure, which are similar to the clinical manifestations of other viral hemorrhagic fevers (3, 4). The pathogenesis of SFTS still needs to be elucidated. A recent study suggested clearing of virus-bound platelets by splenic macrophages as the mechanism for thrombocytopenia in SFTS (5). Cytokine storms are also thought to be important in determining disease severity (6). SFTS also has been reported from Japan and Korea, and the number of SFTS cases is increasing (7, 8). There is no proven antiviral treatment nor vaccine for SFTS and case-fatality rate is reported 6 - 30% (1, 7-9).

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxyadmide, RBV) is a synthetic nucleoside analogue, which exhibits broad spectrum antiviral activity against a variety of DNA and RNA viruses (10). The antiviral mechanisms of RBV are not fully elucidated and vary for different viruses. Several mechanisms of antiviral action suggested for RBV include; 1)

inhibition of inosine monophosphate dehydrogenase leading to reduction in intracellular guanosine triphosphate, 2) mutagenesis via incorporation of RBV into viral RNA genomes, 3) direct inhibition of RNA polymerase, 4) immunomodulation by enhancing type-1 cytokine responses (10-13). RBV is known to have antiviral activity for other bunyavirus infections, including Crimean-Congo hemorrhagic fever, Rift Valley fever, and hemorrhagic fever with renal syndrome (14-16). It was also used for patients with SFTS (9, 17).

However, little is known about the antiviral activity of RBV against SFTSV (9, 17). Considering the clinical significance of SFTS as an emerging life-threatening disease, it is important to establish specific treatment for it. Therefore, we evaluated the antiviral activity of RBV against SFTS replication in vitro.

Methods

Cells

Vero cells (African green monkey kidney cell lines, KCLB no.10081) purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea) were used in this study. The cells were maintained and propagated in Roswell Park Memorial Institute (RPMI) 1640 medium (Welgene, Daegu, Korea), supplemented with 2% heat-inactivated fetal bovine serum (Welgene) and 1% penicillin - streptomycin (Gibco, Carlsbad, CA, USA). Cells were incubated at 37˚C in a humidified, 5% CO_2 chamber.

Virus

The viral stocks derived from the SFTSV strain Gangwon/Korea/2012 were used throughout the experiments (8). To determine the infectivity of the viral stocks, Vero cells (1 x 10^5 cells/mL) were infected with serial 10-fold dilutions of viral stocks (10^{-1} to 10^{-8} dilutions) and incubated at 37˚C in a humidified, 5% CO_2 atmosphere for 48 h. Five replicates were performed per dilution. Then the cytopathic effect (CPE) was observed and the 50% tissue culture infective dose ($TCID_{50}$) was calculated, as previously described by Reed and Muench (18).

Compounds

RBV was obtained from Sigma-Aldrich (St. Louis, MO, USA) in the form of powder. The compound was dissolved in phosphate buffered saline to a stock solution of 10 mg/mL and then diluted in RPMI 1640 medium to each working concentration before use.

Cytotoxicity assay

Vero cells (1 x 10^5 cells/mL) were seeded in 96-well tissue culture plates for 100 µL per well and incubated overnight. Then, 50 µL of culture medium was removed and RBV was added to each well at increasing concentrations (0 – 1000 µg/mL, in serial two-fold dilutions) in 50 µL of RPMI medium and incubated for 24, 48 and 72 h, respectively. Four wells per RBV concentration were performed. The MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay was performed following the manufacture's instruction (Promega, Madison, WI, USA). In brief, at 24, 48 and 72 h post-RBV treatment, 20 µL of MTS assay reagent was added to each well and the cells were incubated for another 4 h, at 37˚C in 5% CO_2. The optical density of wells was measured at 490 nm with Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Antiviral assay

Confluent monolayers of Vero cells (1 x 10^5 cells/mL) in 96-well tissue culture plates (100 µL/well) were used for antiviral assay. After the removal of culture medium in each well, the cells were infected with 100 $TCID_{50}$ of SFTSV (100 µL/well). After 1 h of viral adsorption, the inoculum was removed and the monolayers of infected cells were washed with phosphate buffered saline for three times to remove unbound virus. Then 100 µL of RBV-containing culture medium at concentrations ranging from 0 to 32 µg/mL was added to the infected cells. Four wells per concentration of RBV were tested. At 12, 24, and 48 h post-RBV treatment, the CPE by SFTSV-infected Vero cells was observed under light microscopy and the culture supernatants from the wells corresponding to each RBV concentration were collected for quantification of SFTSV RNA loads.

Quantification of viral RNA loads

The viral titers in the culture supernatants were measured by one-step real-time reverse transcription (RT) – polymerase chain reaction (PCR) with amplification of the partial large segment of SFTSV. For each RBV-treated sample, RNA was extracted using QIAamp Viral RNA Kit (QIAGEN, Hilden, Germany), as described by the manufacturer. One-step, real-time RT-PCR was performed using GoTaq Probe 1-step RT-qPCR system (Promega, Madison,

WI, USA). PCR primers and probes of each segment were modified from the previous study (19), to detect Korea/Gangwon/2012 strain. Real-time PCR cycling was performed on ABI 7500 (Applied Biosystems, Foster City, CA, USA) as follows; after reverse transcription at 45˚C for 15 minutes, 1 cycle at 95˚C for 10 minutes and 40 cycles at 95˚C for 15 seconds and 60˚C for 60 seconds. The cutoff cycle threshold (Ct) value for positive sample was set at 35 cycles. The standard curve was generated from RNAs of 10-fold dilutions of SFTSV (from 10^4 to 10^{-2} $TCID_{50}$/mL). Viral RNA titers in the samples were calculated from the comparison of Ct values with the standard curve using the ABI 7500 software version 2.0.5 (Applied Biosystems).

Data analysis

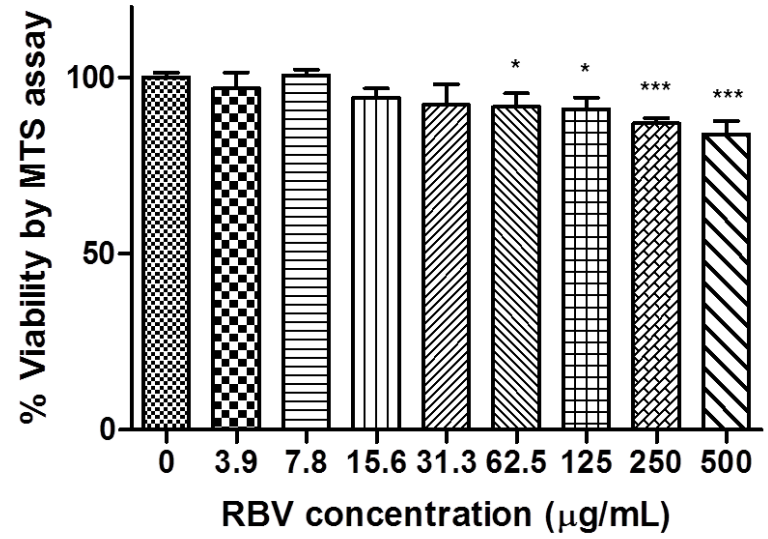
The viability of RBV-treated cells was shown as the percentage of mean absorbance value of each sample to those of mock-treated cells. The viral loads were shown as $TCID_{50}$/mL in response to increasing RBV concentrations. We analyzed the cytotoxicity and antiviral effect of RBV using one-way ANOVA with Tukey's post hoc test. The concentration-response curve was generated using non-linear regression analysis. Half maximal inhibitory concentration (IC_{50}) was calculated and fitted by a sigmoidal dose-response algorithm. All the analyses were performed with GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA).

Results

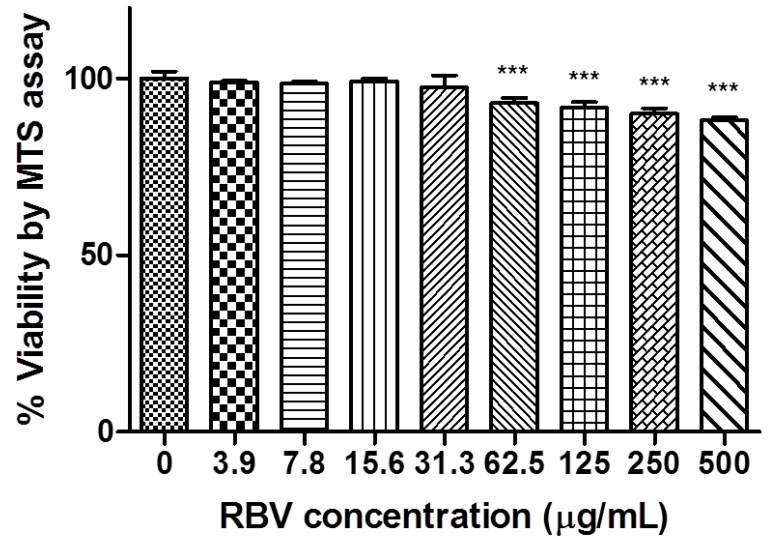
Cytotoxicity of ribavirin

No significant cytotoxicity was detected for RBV at concentrations ≤ 31.3 μg/mL at 24, 48 and 72 h post-RBV treatment (Figure 1). Cellular viability decreased at RBV concentrations ≥ 62.5 μg/mL at 24 and 48 h post-RBV treatment ($P < 0.05$, $P < 0.001$). At 72 h post-RBV treatment, a cytotoxic effect was observed at higher concentrations (125 - 500 μg/mL, $P < 0.001$). Although cytotoxicity of some degree was identified at high RBV concentrations, the actual decrease of viable cells was less than 20% compared to those of mock-treated cells even at the highest concentration of tested RBV. Based on these results, we performed the antiviral assays at non-cytotoxic RBV concentrations ranging from 0 to 32 μg/mL.

(A)


% Viability by MTS assay
100
50
0
*
*
***
***
0
3.9
7.8
15.6
31.3
62.5
125
250
500
RBV concentration (μg/mL)

(B)


% Viability by MTS assay
100
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RBV concentration (μg/mL)

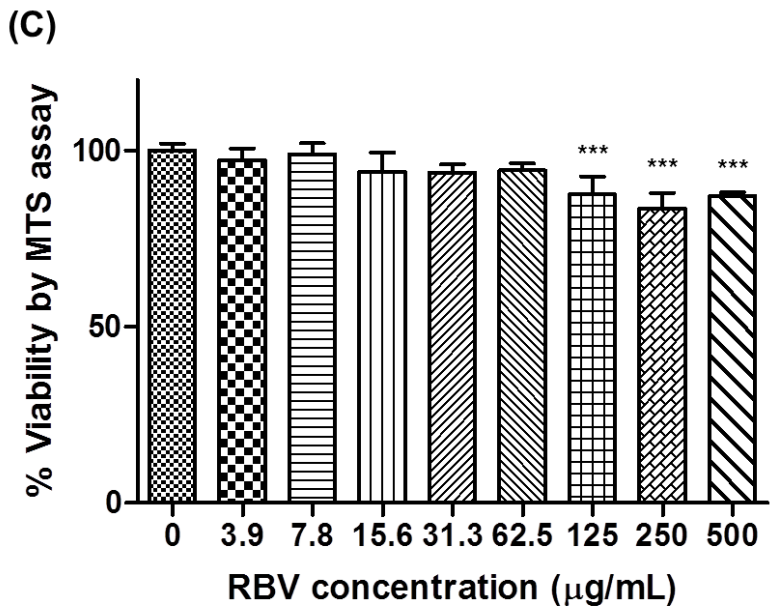

Figure 1. Effect of RBV on cell viability of Vero cells. To determine cytotoxicity of increasing RBV concentrations, MTS assay was done at 24, 48 and 72 h post-RBV treatment for uninfected Vero cells, as described in Methods. (A) 24 h, (B) 48 h, (C) 72 h post-RBV treatment. The data represent the mean ± standard error of mean (S.E.M.) and are expressed as the percentage of untreated controls. One-way ANOVA with Tukey's post hoc test (GraphPad Prism 5, San Diego, CA, USA) was used for the data analysis. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, as compared to RBV-untreated Vero cells.

Inhibition of cytopathic effect by ribavirin

The CPE of SFTSV-infected Vero cells was observed microscopically at 12, 24 and 48 h post-RBV treatment to evaluate antiviral activity of RBV against SFTSV. At 12 h post-RBV treatment, no significant morphological change of Vero cells was observed. At 24 h post-RBV treatment, CPE formation was reduced by RBV treatment in a dose-dependent manner. At 48 h post-RBV treatment, RBV clearly inhibited CPE at concentrations of 16 μg/mL or above (Figure 2).

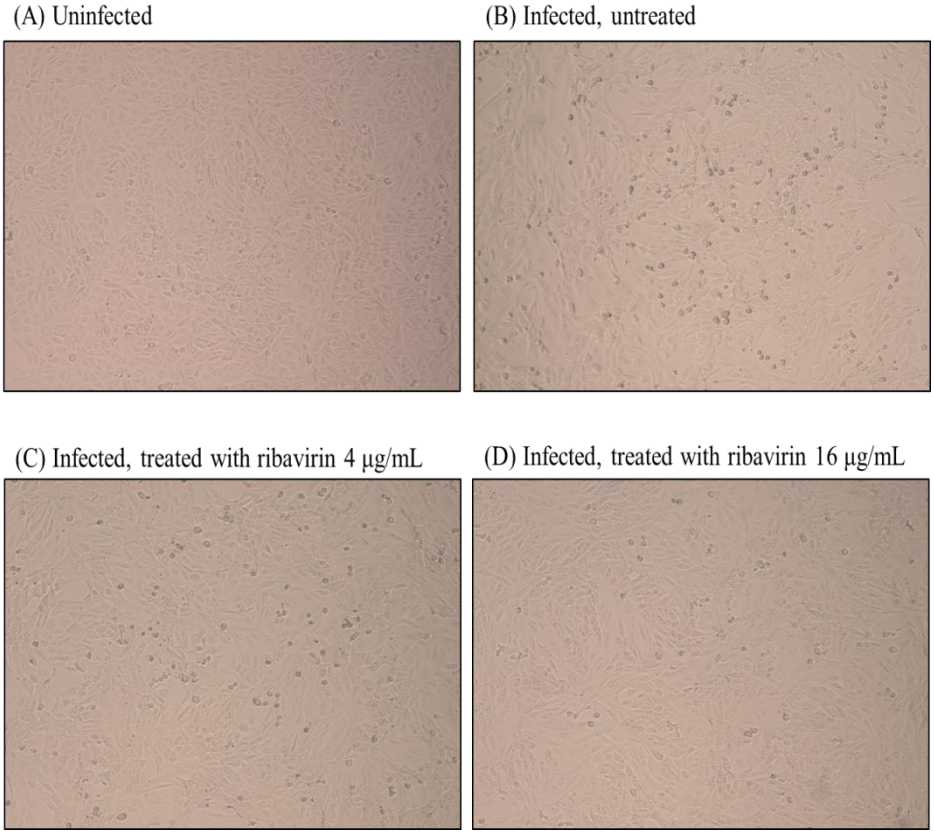

Figure 2. **Inhibition of cytopathic effect of SFTSV-infected Vero cells by RBV treatment.** The CPE of SFTSV-infected cells was observed under light microscopy at 48 h post-RBV treatment. (A) Uninfected Vero cell controls, (B) SFTSV-infected Vero cells without RBV treatment, (C) SFTSV-infected Vero cells treated with 4 μg/mL of RBV, (D) SFTSV-infected Vero cells treated with 16 μg/mL of RBV. Light microscopy images were captured at 100x magnification.

Inhibition of SFTSV replication in Vero cells by ribavirin

The measurement of viral RNA titers in culture supernatants using one- step real-time RT-PCR was done for evaluation of the antiviral activity of RBV against SFTSV. Treatment of SFTSV-infected Vero cells with RBV showed a dose-dependent inhibitory effect on viral RNA replication (Figure 3). At 12 h post-RBV treatment, RBV concentrations $\geq$ 8 μg/mL showed a significant decrease in viral titers ($P < 0.001$). A dose-dependent ($r^2 > 0.9$) decrease of viral RNA in culture supernatants was identified at 24 h post-RBV treatment, at RBV concentrations from 1 to 32 μg/mL, compared with those of mock-treated cells ($P < 0.01$). The inhibition of viral replication persisted for 48 h at RBV concentrations $\geq$ 16 μg/mL ($P < 0.01$).

The range of IC_{50} of RBV was between 3.69 and 8.72 μg/mL, as determined from viral titers calculated from the culture supernatants collected at 24 and 48 h post-RBV treatment (Figure 4).

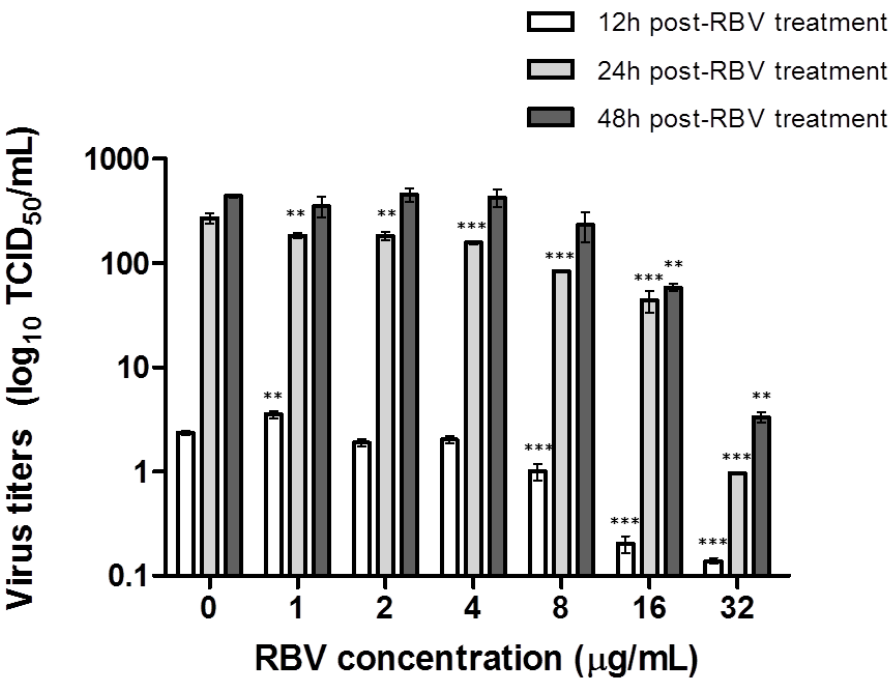

Figure 3. In vitro inhibitory effect of RBV on SFTSV replication in Vero cells, assessed by real time RT-PCR. After infection with 100 $TCID_{50}$ of SFTSV for 1 h, cells were treated with RBV in increasing concentrations. Culture supernatants were obtained at different times and assayed for viral RNA titer. One-way ANOVA with Tukey's post hoc test (GraphPad Prism 5) was used for the data analysis. The data represents the mean ± standard error of mean (S.E.M.). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, as compared to mock-treated cells (indicated as 0 μg/mL of RBV).

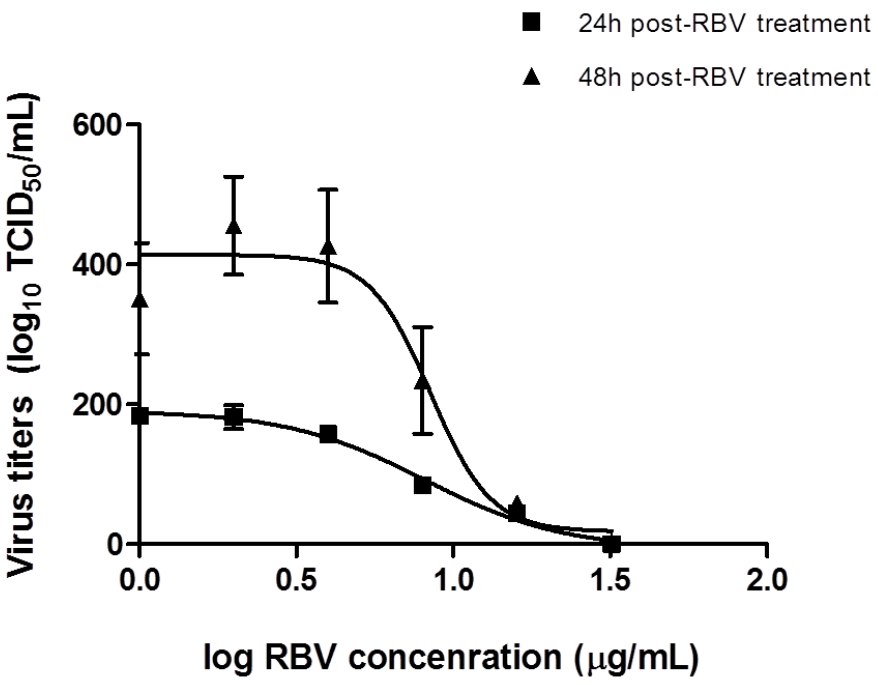

Figure 4. Non-linear regression of the concentration-dependent curves showing antiviral activity of RBV against SFTSV replication. Data are expressed as mean ± standard error of mean (S.E.M.). IC_{50} value was calculated by using GraphPad Prism 5 and fitted by a sigmoidal dose-response algorithm.

Discussion

In this study, we evaluated the antiviral activity of RBV against SFTSV in vitro, because RBV has been anecdotally used for treatment of SFTS. Despite unknown efficacy for SFTSV, RBV use was based on its broad spectrum of antiviral activity. We demonstrate that RBV inhibits the viral replication of SFTSV in Vero cells in a dose-dependent manner. Although inhibitory concentrations of RBV against SFTS replication varied according to the duration of treatment, our study documented both the reduction of viral titers and the inhibition of CPE by RBV treatment. These data suggest that RBV can be used as a potential antiviral agent for SFTS.

In the current study, we found that RBV inhibits SFTSV replication with an IC_{50} between 3.69 and 8.72 μg/mL, which is similar to that for Andes virus, but lower than those for Rift Valley fever virus and Crimean-Congo hemorrhagic fever virus (20-22). These suggest that SFTSV may be sufficiently controlled with the therapeutic doses of RBV recommended for other viral hemorrhagic fevers (4).

In contrast to our in vitro observations, there was a recent report of an observational study showing that the case-fatality ratio did not differ between the SFTS patients who received RBV and those who did not (9). In the study, however, SFTSV titers were lower in patients who received RBV than in those who did not (9). We suppose that the discrepancy between the in vitro and the clinical observations may arise from the following factors; 1) due to

the therapeutic windows, i.e. delay in starting RBV after the onset of illness, 2) due to the suboptimal dosage of RBV given to the patients with SFTS (500 mg daily), which was much lower than those used for other viral hemorrhagic fevers (2000 – 4000 mg daily), or 3) due to the complex pathogenesis of SFTS including cytokine storms, despite RBV per se has antiviral effect against SFTSV (4, 6, 9).

A recent study from Japan reported that RBV added before virus inoculation inhibited SFTSV proliferation, but RBV did not reduce viral production in pre-infected cells when added after viral inoculation (23). In contrast, our study clearly showed that treatment with RBV concentrations ≥ 8 μg/mL significantly reduced SFTSV release from infected cells when the drug was added after viral adsorption. We presume that the actual concentrations of RBV showing antiviral effects might be lower than our results considering that Vero cells are more resistant to RBV than other cell lines (24).

However, the RBV concentration for inhibition of SFTSV replication in human cannot be extrapolated from our results. In chronic hepatitis C virus infection, for which RBV is used at a dosage of 800 – 1400 mg/day based on patient's body weight, the plasma concentrations of RBV at 8 weeks of treatment are reported between 2-3 μg/mL (25, 26). Higher plasma concentration of RBV (> 3.5 μg/mL) is related to the development of hemolytic anemia, which is one of the serious adverse effects of RBV (25). Whether the plasma concentrations of RBV enough to suppress SFTSV replication can be achieved within the range of permissive dosage is another

issue to be solved through further studies.

There are a few limitations in our study. First, we used only one cell line for our experiments that we could not compare the range of inhibitory concentrations of RBV on viral replication among various cell lines. The antiviral activity of RBV in distinctive cell lines is expected to be different due to the differences of intracellular phosphorylation (10). We chose Vero cells because they are known to be susceptible to SFTSV infection and commonly used for the culture of SFTSV (1, 7, 8). Second, we determined antiviral efficacy of RBV by measuring the virus titers in culture supernatants, and therefore the amount of cell-associated or intracellular SFTSV were not measured. Nevertheless, our results showed a dose-dependent inhibitory effect of RBV against SFTSV replication.

In summary, we found that RBV had a dose-dependent antiviral activity against SFTSV in vitro. Although there are remaining issues to utilize RBV for SFTS in clinical practice, including optimal dosage and dosing-intervals to effectively control the viral replication, our results are of value as this is the report to prove actual inhibitory effect of RBV against SFTSV replication. Further studies are warranted to evaluate potential use of RBV for treatment of SFTS.

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초 록

배경:

중증열성혈소판감소증후군은 중증열성혈소판감소증후군 바이러스에 의해 발생하는 신종 감염병이다. 현재까지 이에 대해 효능이 있는 것으로 입증된 항바이러스제는 없으나, 최근 일부에서는 중증열성혈소판감소증후군 환자들을 대상으로 리바비린을 사용한 바가 있다. 중증열성혈소판감소증후군 바이러스에 대한 리바비린의 항바이러스 효능에 대해 평가하기 위해 본 연구를 시행하였다.

방법:

리바비린의 세포 독성 여부를 평가하기 위해 베로 세포에 0 – 500 μg/mL 범위 내에서 2배씩 희석한 다양한 농도의 리바비린을 투여하고, MTS assay를 이용하여 세포 생존율을 평가하였다. 리바비린의 항바이러스 효능을 평가하기 위해서 중증열성혈소판감소증후군바이러스(Gangwon/Korea/2012)를 베로 세포에 100 $TCID_{50}$ 단위로 감염시키고, 세포 독성을 나타내지 않는 농도의 리바비린을

투여하였다. 중증열성혈소판감소증후군 바이러스에 대한 리바비린의 항바이러스 효과는, 바이러스에 감염된 베로 세포의 세포변성효과에 대한 관찰 및 세포 배양 상층액에서 추출한 바이러스 RNA를 역전사 중합효소 연쇄반응을 통해 증폭하여 측정한 바이러스 역가의 비교를 통해 평가하였다.

결과:

31.3 μg/mL 이하 농도의 리바비린은 베로 세포에 대한 독성을 나타내지 않았다. 리바비린 투여 24 시간 후, 리바비린의 농도가 증가함에 따라 세포 배양 상층액에서 추출한 바이러스 RNA양이 감소하는 것을 확인하였다 ($P < 0.01$).

리바비린이 중증열성혈소판감소증후군 바이러스의 증식 및 바이러스 감염으로 인한 세포변성효과를 농도 의존적으로 억제하는 효과가 있음을 확인하였다. 중증열성혈소판감소증후군 바이러스에 대한 리바비린의 중간억제농도의 범위는 3.69 – 8.72 μg/mL 였다.

결론:

중증열성혈소판감소증후군 바이러스에 대해 리바비린이 농도 의존적 항바이러스 효능을 나타냈다.

주요어: 중증열성혈소판감소증후군, 플레보바이러스, 리바비린

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