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Ginsenoside-Rb2 and 20(S)-ginsenoside-Rg3 from Korean red ginseng prevent rotavirus infection in newborn mice

Hui Yang¹,§, Kwang-Hoon Oh²,§, Hyun Jin Kim¹, Young Ho Cho³, and Yung Choon Yoo¹,*

¹Department of Microbiology, College of Medicine, Konyang University, Daejeon 35365, Korea
²Department of Physical Education, College of Education, Kongju National University, Kongju 32588, Korea
³Department of Pharmaceutics & Biotechnology, College of Medical Engineering, Konyang University, Daejeon 35365, Korea

Running title: Protection against rotavirus infection by ginsenoside-Rb2

§ These authors contributed equally to this work.

*Corresponding author
Yung Choon Yoo
E-mail: yc_yoo@konyang.ac.kr
Tel: +82-42-600-6495, Fax: +82-42-600-6495
Abstract

It is well known that Korean red ginseng has various biological activities. However, there is little knowledge about antiviral activity of Korean red ginseng and its ginsenosides. In this study, we addressed whether oral administration of ginsenoside-Rb2 and -Rg3 were able to protect against rotavirus (RV) infection. Protective effect of ginsenosides against RV infection was examined using an \textit{in vivo} experiment model in which newborn mice (10-day-old) were inoculated perorally (p.o.) with $1.5 \times 10^6$ plaque-forming units (PFU)/mouse of RV strain SA11. When various dosages of ginsenoside-Rb2 (25 to 250 mg/kg) were administered 3, 2 or 1 days before virus challenge, treatment with this ginsenoside at the dosage of 75 mg/kg 3 days before virus infection most effectively reduced RV-induced diarrhea. In addition, consecutive administration of ginsenoside-Rb2 (75 mg/kg) 3, 2, and 1 day before virus infection was more effective than single administration on day -3. The consecutive administration of ginsenoside-Rb2 also reduced virus titers in the bowels of RV-infected mice. In an experiment to compare the protective activity between ginsenoside-Rb2 and its two hydrolytic products [20(S)- and 20(R)-ginsenoside-Rg3], 20(S)-ginsenoside-Rg3, but not 20(R)-ginsenoside-Rg3, prevented RV infection. These results suggest that ginsenoside-Rb2 and its hydrolytic product, 20(S)-ginsenoside-Rg3, are promising candidate as an antiviral reagent to protect against RV infection.

Key words: Panax ginseng, Ginsenoside-Rb2, Ginsenoside-Rg3, Rotavirus, Diarrhea
Introduction

Rotaviruses (RVs) are important causative source of viral gastroenteritis in the immature hosts of various animal species such as human infants and young children <5 years of age [1]. Since RV brings out 114 million cases of diarrhea annually, RV-induced acute diarrhea is regarded as a significant health problem in the world [1]. Actually this virus causes 24 million hospital visits and 2.4 million hospitalization every year [1]. In addition, RV gives rise to more than 527,000 deaths annually mainly in developing countries [2]. Therefore, the morbidity and mortality caused by RV infection emphasize importance of its prophylaxis and cure. The present methods to treat RV-associated diarrhea consist mainly of symptomatic therapies, i.e. oral rehydration to prevent dehydration. Although, at present, two attenuated live vaccines were shown to elicit an anti-RV effect in humans, they are neither effective enough to prevent severe diarrhea in some cases nor globally distributed [3]. Therefore, the development of an effective approach to prevent RV-induced severe diarrhea is urgently needed.

Saponins are chemically belonged to glycosides with a triterpenoid, steroidal aglycone or sapogenin, and they possess diverse biological functions such as immunomodulatory effects and adjuvant activities [4-8]. In addition, it was found that oral administration of saponins augmented antigen-specific immune responses orally as well as parenterally [8,9], and their immunomodulating activity might be due to potentiation of mucosal immunity.

Ginsenosides, saponin preparations isolated from Panax ginseng are classified into two
groups of saponins according to chemical structure: dammarane-type having 20(S)-
protopanaxadiol/triol skeleton and oleanolic acid type having oleanolic acid skeleton.
Many investigators have reported that ginsenosides possess a diversity of biological
activities including anti-cancer [10-13] and immuno-potentiating [7-9, 14] effect.
However, only a little knowledge of the protective effect by ginsenosides against viral
infection has been reported. Recently, Kim JY et al. reported that oral administration of
Korean red ginseng protected against influenza A (H1N1) virus [15]. Also, Yoo DG et al.
demonstrated protective activity of Korean red ginseng extract against two types of
influenza viruses, H1N2 and H3N2, in animal models [16]. Furthermore, Lee MH et al.
clearly showed that Korean red ginseng extract and ginsenosides have anti-viral activity
to protect against murine norovirus and feline calicivirus as surrogates for human
norovirus [17].
Ginsenoside-Rb2, a dammarane-type saponin, is known to have a variety of biological
activities, especially immunomodulating activity to regulate the proliferation of
lymphocytes [14]. Besides, this ginsenoside was shown to possess inhibitory effect on
metabolic syndromes such as diabetes and hyperlipidemia in mice [18]. Previously we
suggested that treatment of ginsenoside-Rb2 inhibited lung metastasis of B16-BL6
melanoma cells and angiogenesis produced by tumor cells in mice [11]. We also reported
that oral administration of ginsenoside-Rb2 protected the host against the lethal
respiratory infection of Haemagglutinating virus of Japan (HVJ) which caused a severe
acute respiratory infection in mice [19]. These findings led us to a possibility that oral
administration of ginsenoside-Rb2 can augment the host resistance to prevent RV-induced
gastrointestinal infections.

In this study, we examined the application of ginsenoside-Rb2 as a mucosal immunostimulant to enhance non-specific resistance against RV infection in an infection model using immature mice. And we compared the protective activity between ginsenoside-Rb2 and its hydrolytic products such as 20(S)- and 20(R)-ginsenoside-Rg3.
Materials and Methods

Reagents

Ginsenosides used in this study were kindly provided by the Korean Ginseng Corporation (Daejeon, Korea). All ginsenosides were isolated from the roots of 6-year old *Panax ginseng* C. A. Meyer in Korea as described previously [19], and the purity of them was above 99.9% as estimated by high performance liquid chromatography [20]. The chemical structures of these ginsenosides are shown in Fig.1. Each ginsenoside was suspended in phosphate-buffered saline (PBS) before use.

Animals

Specific pathogen-free pregnant BALB/c mice were purchased from Raon Bio Ltd. (Yongin, Korea). Mice were housed in plastic cages in vinylfilm isolators. All mice had water and pelleted diets *ad libitum*. All animal experiments were carried out according to the Laboratory Animal Control Guidelines of IACUC of Konyang University (Approval No. P-16-01-A-01).

Virus and Cell Line

Rotavirus strain SA11 (RV-SA11) was kindly supplied by Dr. J. Arikawa in Hokkaido University in Japan. MA-104 cells, a cell line derived from fetal rhesus monkey, were cultivated in Eagle’s minimum essential medium (EMEM) supplemented with 5 mM glutamine, 0.1% sodium bicarbonate, 50 µg/ml of gentamicin, 3 µg/ml of amphotericin
B and 10% fetal calf serum (FCS). RV-SA11 was replicated in MA-104 cells as described previously [21]. The titer of virus stocks used in this study was $2.5 \times 10^8$ plaque-forming units (PFU)/ml.

**Protection against RV Infection**

The liters of 10-day-old BALB/c newborn mice were inoculated p.o. with various doses of RV, $1.5 \times 10^6$ to $1.5 \times 10^7$ PFU/50 µl/mouse. Mice were fasted for 4 hr before virus infection. Ginsenosides were administered p.o. on the indicated days prior to virus infection. A clinical score for RV-induced diarrhea was measured by the severity of diarrhea per mouse every 24 hr after virus infection as described previously [21]. The scores were determined by the following criteria: point 2; serious, point 1; moderate, point 0; cured. And the diarrhea score of each group was determined by a calculation of ‘(the number of mice under serious diarrhea) $\times$ 2 + (the number of mice under moderate diarrhea) $\times$ 1 / total number of mice’. Total severity of diarrhea was expressed as a cumulative number of diarrhea score obtained from the whole observation period.

**Isolation of RV from the Bowels**

The bowels harvested from RV-infected mice were homogenized by a glass homogenizer in 1 ml of EMEM. After centrifugation, the supernatants were massed up to 5 ml with EMEM, and stored at -80°C. Virus titer of the bowel homogenates was measured by plaque formation test using MA-104 cells [21]. Briefly, the monolayer of MA-105 cells were incubated with 1000-fold diluted homogenates (0.5 ml/well) in 6-well tissue culture
plates for 1 hr at 37°C. After washing with EMEM, the cells were overlaid with an overlay medium (2.5 ml/well) consisting of 0.7% purified agar (Agarose; SEAKEM ME, FMC bio Products, ME, USA) and 0.0001% trypsin in EMEM, and incubated for 5 days at 37°C in 5% CO₂. Thereafter, the cells were treated with a second overlay medium (2 ml/well) containing 0.7% purified agar and 0.005% neutral red for 48 hr, and the plaques formed in each well were counted.

Statistics

Statistical significance was determined by Student’s two-tailed t test.
Results and Discussion

In Vivo Titration of RV-SA11 in Newborn Mice

To establish an animal model, 10-day-old newborn mice were inoculated p.o. with various doses of RV-SA11, and the severity of diarrhea was calculated. Prominent symptoms of diarrhea were observed in all of RV-infected mice during the infection period of 2-5 days (Table 1). And the severity of diarrhea was dependent upon the titer of RV challenged. Based on the results of Table 1, we carried out the following experiments using an infection model in which 10-day-old newborn mice were inoculated with $1.5 \times 10^6$ PFU/mouse of RV-SA11.

Protective Effect of Ginsenoside-Rb2 on RV Infection

Although many investigators have tried to find effective substances available for potentiating the protective effect of the host on mucosal pathogens, there are few scientific evidence of beneficial stimulants that can enhance host resistance against mucosal infections [21-23]. Saponin preparations from red ginseng have been widely recognized to possess immunomodulating activity to enhance cytokine production from immune cells, natural killer (NK) cell activity, and cellular immune responses, even though the precise mechanisms associated with their biological activities is unclear [22-25]. In a series of previous studies, we clearly demonstrated that oral administration of this ginsenoside enhanced nonspecific resistance against tumor cells, inhibited lung metastasis of B16-BL6 melanoma cells [13] and prevented the lethal infection of HVJ
Here, we addressed a possibility that oral administration of ginsenoside-Rb2 enhances the resistance of immature host against RV that causes severe gastrointestinal disease in the bowels.

In order to examine the protective effect of ginsenoside-Rb2 on RV infection, newborn mice were treated orally with 75 mg/kg of this ginsenoside 3, 2 or 1 day before RV infection, and the severity of diarrhea of RV-infected mice was calculated. As seen in Table 2, all mice treated with ginsenoside-Rb2 protected against RV infection regardless of the timing of administration, and the highest activity was observed in mice treated 3 days before RV infection. In addition, in an experiment in which various doses of ginsenoside-Rb2 ranging from 25 to 250 mg/kg were administered to mice 3 days before RV infection, ginsenoside-Rb2 at the dose of 75 mg/kg elicited higher protective activity compared to either of the dose of 25 to 250 mg/kg (Table 3). This implied that protective effect of ginsenoside-Rb2 against RV infection was not dose-dependent. Of particular significance was the finding that ginsenoside-Rb2 (75 mg/kg) administered consecutively for 3 days before infection was more active to protect against RV infection than that administered one time 3 days before virus infection (Table 3). These data indicate that oral administration of ginsenoside-Rb2 is active to prevent RV infection, and the optimal administration condition for its prophylactic effect on RV infection was multiple administration at the dose of 75 mg/kg for 3 days before virus infection in newborn mice.

In previous study, we reported that the preventive effect of ginsenoside-Rb2 on HVJ virus was strictly dependent upon the dose and administration frequency, and, however, multiple administration of ginsenoside-Rb2 at the dosage of 75 mg/kg was not active to
prevent HVJ infection in adult mice [19]. This discrepancy in protective activity of
ginsenoside-Rb2 against two different types of viruses, HVJ and RV, may result from the
characters of virus infected and the maturity of the host.

**Effect of 20(S)- and 20(R)-Ginsenoside-Rg3 on RV Infection**

Two types of epimeric ginsenosides, 20(R)- and 20(S)-ginsenoside-Rg3, are generated
from hydrolysis of ginsenoside-Rb2 [11]. Our previous study showed that both types of
ginsenoside-Rg3 suppressed lung metastasis of B16-BL6 tumor cells, and their antitumor
activity was almost same with that of ginsenoside-Rb2 [11]. However, in protective effect
against virus infection, oral administration of 20(S)-ginsenoside-Rg3, but not 20(R)-
ginsenoside-Rg3, showed a significant protection against HVJ [19]. Since multiple
administration of ginsenoside-Rb2 effectively prevented RV infection (Table 3), we next
addressed whether consecutive administration of its hydrolytic products, 20(R)- and
20(S)-ginsenoside-Rg3, could elicit protective effect on RV infection in newborn mice.
In oral administration 3, 2, and 1 days before virus infection, 20(S)-ginsenoside-Rg3 (75
mg/kg) significantly protected against RV infection, although its inhibition of total
diarrhea score was lower than that of ginsenoside-Rb2 (Table 4). However, 20(R)-
ginsenoside-Rg3 in an epimeric structure of 20(S)-ginsenoside-Rg3 was not active. These
results strongly suggest that protective effect on RV infection by ginsenosides
administered orally varies from the chemical structures of these ginsenosides.

**Growth Inhibition of RV by Ginsenoside Administration in the Bowels**
We performed an experiment to compare the growth of RV in the bowels between untreated and ginsenoside-treated mice. Since RV is known to cause an acute diarrhea symptom in the intestines at the early time after virus exposure [1,19], we isolated RV from the bowels in the early time after infection. As shown in Fig.2, mice inoculated with RV showed the maximal virus titer (about $6.5 \times 10^4$ PFU/mouse) 18 hr after infection, and decreased thereafter. Consecutive oral administration of ginsenoside-Rb2 (75 mg/kg) 3, 2, and 1 days before infection significantly reduced the growth of RV in the bowels in RV-infected mice. Similarly, multiple administration of 20(S)-ginsenoside-Rg3, a hydrolytic product of ginsenoside-Rb2, at the dose of 75 mg/kg also significantly inhibited the growth of RV in the bowels, even though its inhibitory effect was lower than that of ginsenoside-Rb2. However, 20(R)-ginsenoside-Rg3, an epimeric type of 20(S)-ginsenoside-Rg3, had no effect to inhibit the growth of RV in the bowels. Collectively, these results indicate that oral administration of ginsenoside-Rb2 and 20(S)-ginsenoside-Rg3 significantly protected against RV infection via reduction of virus titer in the bowels, and also suggest that, in comparison of protective activity between ginsenoside-Rb2 and its hydrolytic products, ginsenoside-Rb2 was most effective.

In the present study, we demonstrated that ginsenoside-Rb2 and its hydrolytic product, 20(S)-ginsenoside-Rg3, are potent mucosal stimulants to potentiate nonspecific resistance against severe infection of RV in newborn mice, and that their protective effect against RV was associated with inhibition of virus growth in the bowels at the early period of RV infection. Further study to examine the mechanisms involved in activation of mucosal immune systems in the guts by ginsenoside-Rb2, and the relationship between
chemical structures and biological activities of ginsenoside-Rb2 and its hydrolytic products is now on the way.

Acknowledgements

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Conflict of Interest

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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in vitro structure-related anti-cancer activity of ginsenosides and their derivatives.
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Figure Legends

Fig. 1 Chemical structures of ginsenoside-Rb2, 20(S)-ginsenoside-Rg3, and 20(R)-ginsenoside-Rg3.

Fig. 2 Inhibitory effect of ginsenosides on RV growth in the bowels of mice. Three BALB/c newborn mice per group were administered orally with 75 mg/kg of each ginsenoside 3, 2, and 1 days before RV infection. Virus titers in the bowels were measured by counting the number of PFU on MA-104 cells in 6-well plastic tissue culture plates as described in Materials and Methods. *p<0.05, **p<0.01, ***p<0.001, compared with non-treated (infection only) group (by Student's two-tailed t-test).
Fig. 1

Ginsenoside-Rb2
$R^1 = \text{glic} - \text{glic}$ \hspace{1cm} $R^2 = \text{CH}_3$

20(R)-ginsenoside-Rg3
$R^1 = \text{CH}_3$ \hspace{1cm} $R^2 = \text{OH}$

20(S)-ginsenoside-Rg3
$R^1 = \text{OH}$ \hspace{1cm} $R^2 = \text{CH}_3$
Table 1 Titration of RV-SA11 in newborn mice

<table>
<thead>
<tr>
<th>Doses of inoculum (PFU/mouse)</th>
<th>Duration of diarrhea (days)</th>
<th>Total diarrhea score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 × 10⁵</td>
<td>2 - 3</td>
<td>ND</td>
</tr>
<tr>
<td>1.5 × 10⁶</td>
<td>2 - 5</td>
<td>2 - 3</td>
</tr>
<tr>
<td>1.5 × 10⁷</td>
<td>2 - 5</td>
<td>2 - 4</td>
</tr>
</tbody>
</table>

Groups of five BALB/c newborn mice (10-day-old) were inoculated p.o. with the indicated doses of RV-SA11. All mice were deprived of foods for 4 hr before virus infection, being isolated from their maternal mice. a The total duration of diarrhea observed. b The partial duration of severe diarrhea showing more than 1.0 value of diarrhea score. c Accumulative diarrhea score per group during the whole observation period. d Not detected.
Table 2 Protective effect of oral administration of ginsenoside-Rb2 on RV infection in newborn mice

<table>
<thead>
<tr>
<th>Treatment of ginsenoside-Rb2</th>
<th>Duration of diarrhea (days)</th>
<th>Total diarrhea score (Inhibition %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doses</td>
<td>On day</td>
<td>Total</td>
</tr>
<tr>
<td>Infection only</td>
<td>-</td>
<td>2 – 5</td>
</tr>
<tr>
<td>75 mg/kg</td>
<td>-3</td>
<td>2 – 5</td>
</tr>
<tr>
<td>-2</td>
<td></td>
<td>2 – 5</td>
</tr>
<tr>
<td>-1</td>
<td></td>
<td>2 – 4</td>
</tr>
</tbody>
</table>

Groups of five BALB/c newborn mice were inoculated p.o. with RV-SA11 (1.5×10^6 PFU/mouse) and administered p.o. with 75 mg/kg of ginsenoside-Rb2 on the indicated days before virus infection.
Table 3 Effect of multiple oral administration and dose-dependent activity of ginsenoside-Rb2 on RV infection in newborn mice

<table>
<thead>
<tr>
<th>Treatment of ginsenoside-Rb2</th>
<th>Duration of diarrhea (days)</th>
<th>Total diarrhea score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doses</td>
<td>On day</td>
<td>Total</td>
</tr>
<tr>
<td>Doses</td>
<td>On day</td>
<td>Total</td>
</tr>
<tr>
<td>---</td>
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<tr>
<td>---</td>
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<td>---</td>
</tr>
</tbody>
</table>

**Experiment-1**

<table>
<thead>
<tr>
<th>Doses</th>
<th>On day</th>
<th>Total</th>
<th>Severe</th>
<th>(Inhibition %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection only</td>
<td>-</td>
<td>2 – 5</td>
<td>2 - 4</td>
<td>5.80</td>
</tr>
<tr>
<td>250 mg/kg</td>
<td>-3</td>
<td>1 – 5</td>
<td>2 - 4</td>
<td>4.80 (17.2)</td>
</tr>
<tr>
<td>75 mg/kg</td>
<td>-3</td>
<td>2 – 5</td>
<td>2 – 4</td>
<td>4.00 (31.0)</td>
</tr>
<tr>
<td>25 mg/kg</td>
<td>-3</td>
<td>2 – 5</td>
<td>2 – 4</td>
<td>4.40 (24.1)</td>
</tr>
</tbody>
</table>

**Experiment-2**

<table>
<thead>
<tr>
<th>Doses</th>
<th>On day</th>
<th>Total</th>
<th>Severe</th>
<th>(Inhibition %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection only</td>
<td>-</td>
<td>2 - 5</td>
<td>3 - 5</td>
<td>5.00</td>
</tr>
<tr>
<td>75 mg/kg</td>
<td>-3</td>
<td>2 – 5</td>
<td>2 – 4</td>
<td>3.35 (33.0)</td>
</tr>
<tr>
<td>25 mg/kg</td>
<td>-1,-2,-3</td>
<td>2 - 4</td>
<td>4</td>
<td>2.25 (54.8)</td>
</tr>
</tbody>
</table>

Groups of five BALB/c newborn mice were inoculated p.o. with RV-SA11 (1.5×10^6 PFU/mouse) and administered p.o. with the indicated doses of ginsenoside-Rb2 on the indicated days.
Table 4 Comparison of protective effect against RV infection among ginsenoside-Rb2, 20(S)- and 20(R)-ginsenoside-Rg3 in newborn mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration of diarrhea (days)</th>
<th>Total diarrhea score (Inhibition %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Severe</td>
</tr>
<tr>
<td>Infection only</td>
<td>2 - 6</td>
<td>2 - 4</td>
</tr>
<tr>
<td>Ginsenoside-Rb2</td>
<td>2 - 4</td>
<td>0</td>
</tr>
<tr>
<td>20(S)-ginsenoside-Rg3</td>
<td>2 - 5</td>
<td>3 - 4</td>
</tr>
<tr>
<td>20(R)-ginsenoside-Rg3</td>
<td>2 - 6</td>
<td>2 - 4</td>
</tr>
</tbody>
</table>

Groups of five BALB/c newborn mice were inoculated p.o. with RV-SA11 (1.5×10^6 PFU/mouse) and consecutively administered p.o. with 75 mg/kg of ginsenoside-Rb2, 20(S)- or 20(R)-ginsenoside-Rg3 3, 2, and 1 days before virus infection.
Fig. 2

![Graph showing viral titer over times after infection (hr). The graph compares different treatments: Infection only, Ginsenoside-Rb2, 20(S)-ginsenoside-Rg3, and 20(R)-ginsenoside-Rg3. The x-axis represents times after infection (hr), ranging from 3 to 72. The y-axis represents viral titer (10^3 PFU/mouse), ranging from 0 to 80.](image-url)
\[ R^1 = \text{OH} \]
\[ R^2 = \text{CH}_3 \]

\[ R^1 = \text{CH} \]
\[ R^2 = \text{OH} \]

\[ R^1 = \text{OH} \]
\[ R^2 = \text{CH}_3 \]

**Ginsenoside-Rb2**
\[ R^1 = \text{glc-glc} \quad R^2 = \text{CH}_3 \]

**20(R)-ginsenoside-Rg3**
\[ R^1 = \text{CH}_3 \quad R^2 = \text{OH} \]

**20(S)-ginsenoside-Rg3**
\[ R^1 = \text{OH} \quad R^2 = \text{CH}_3 \]
Fig. 2

Virus titers (x10^3 PFU/mouse) vs. Times after infection (hr)

- Infection only
- Ginsenoside-Rb2
- 20(S)-ginsenoside-Rg3
- 20(R)-ginsenoside-Rg3

* Indicates significant difference
** Indicates highly significant difference