Ameliorating Effect of Dietary Xylitol on Human Respiratory Syncytial Virus (hRSV) Infection

Mei Ling Xu, Ga Ram Wi, Hyoung Jin Kim, and Hong-Jin Kim*

College of Pharmacy, Chung-Ang University; 84 Heukseok-ro, Dongjak-gu, Seoul 156–756, South Korea.
Received October 9, 2015; accepted January 7, 2016

Human respiratory syncytial virus (hRSV) is the most common cause of bronchiolitis and pneumonia in infants. The lack of proper prophylactics and therapeutics for controlling hRSV infection has been of great concern worldwide. Xylitol is a well-known sugar substitute and its effect against bacteria in the oral cavity is well known. However, little is known of its effect on viral infections. In this study, the effect of dietary xylitol on hRSV infection was investigated in a mouse model for the first time. Mice received xylitol for 14 d prior to virus challenge and for a further 3 d post challenge. Significantly larger reductions in lung virus titers were observed in the mice receiving xylitol than in the controls receiving phosphate-buffered saline (PBS). In addition, fewer CD3+ and CD3+CD8+ lymphocytes, whose numbers reflect inflammatory status, were recruited in the mice receiving xylitol. These results indicate that dietary xylitol can ameliorate hRSV infections and reduce inflammation-associated immune responses to hRSV infection.

Key words  human respiratory syncytial virus; xylitol; lung virus titer; immune response

Human respiratory syncytial virus (hRSV), a member of the Paramyxoviridae family, is a leading cause of lower respiratory tract infections (LRTI) that cause bronchiolitis or pneumonia in infants. Almost all children under the age of two are thought to be infected with hRSV at least once, and 20% of those infected acquire respiratory tract illness. Moreover 2–3% of the infants below one year that are infected with hRSV are ill enough to require hospitalization during their initial hRSV infection. The symptoms of hRSV infection are more severe in premature infants and those born with congenital heart disease or chronic lung disease, and the mortality rates among such hospitalized infants can be as high as 3%. Infants are more susceptible to hRSV infection and suffer persistent infections more frequently than adults. Although reinfection with hRSV occurs often throughout life, such infections are usually asymptomatic or manifest as mild flu-like symptoms in healthy adults. However hRSV causes severe respiratory illness in the elderly and in high-risk adults who have chronic heart and lung disease.

There are no commercially available vaccines or effective treatments despite the considerable medical importance of hRSV. Formalin-inactivated hRSV (FI-hRSV), live-attenuated hRSV, subunits of hRSV and DNA vectors producing hRSV antigens have been suggested as candidates for hRSV vaccines, though none of these approaches has yet been successful. Synagis, a humanized monoclonal antibody that targets hRSV F protein has been indicated for preventing hRSV infection in infants who are considered at high risk of hRSV disease. Synagis did not have any clinical benefit in children already afflicted with hRSV although it is highly effective as a prophylactic against hRSV. Ribavirin, a guanosine analog that exhibits antiviral activity against RNA and DNA viruses, is the only agent approved for treating hRSV infection. Clinical trials of ribavirin in infants had marginal effects in reducing the need for mechanical ventilator support and the duration of hospitalization following hRSV infection, whereas its anti-hRSV effect has been clearly established in vitro and in a rodent model. Thus, continuing efforts are needed to discover new agents that can prevent or treat hRSV infection.

Xylitol is a well-known sugar substitute with the formula (CHOH)₃(CH₂OH)₂. It has 40% fewer calories and a much lower glycemic index than other natural or synthetic sweeteners. In this regard, xylitol has been used as a lower-calorie alternative for sugar in diabetes patients. It has also been widely used as an ingredient of chewing gum because it is effective in preventing cavities formed by bacteria. It is well known that cavity-causing bacteria prefer to consume six-carbon sugars as energy source. The structural similarity of xylitol to six-carbon sugars causes cavity-causing bacteria to take up xylitol well although it is a non-fermentable alcohol and cannot be used as an energy source by the bacteria. Moreover, xylitol has a strong antimicrobial effect against Streptococcus pneumonia and an anti-adhesive effect against Streptococcus pneumonia and Haemophilus influenza in vitro. Because chewing 8.4 g of xylitol per day reduced the incidence of otitis media up to 50% it was proposed as a prophylactic against otitis media, and most studies have focused on its anti-bacterial actions.

There have been few investigations of the effects of xylitol on virus-associated diseases. However, recently, a study suggested a protective effect of dietary xylitol on influenza A virus infection in a mouse model. Dietary xylitol along with the water soluble fraction of Red Ginseng (wRG) given for 5 d prior to lethal influenza A virus challenge not only enhanced survival but also reduced body weight loss. Also the xylitol and wRG regimen was shown to reduce inflammatory responses in bronchoalveolar lavage (BAL) fluid following influenza A virus infection. However, the protective effect was not seen when the mice received xylitol in combination with wRG after the influenza A virus infection or if it was administered for less than 5 d prior to virus challenge. This finding implies that the xylitol does not act directly on the influenza A virus itself but rather controls influenza A virus-associated symptoms by influencing the immune response to virus challenge. This raises the possibility that it might ameliorate the effects of other respiratory virus infections.

*To whom correspondence should be addressed. e-mail: hongjink@cau.ac.kr

© 2016 The Pharmaceutical Society of Japan
In this study, we have, for the first time, investigated the dietary effects of xylitol on hRSV infection in a mouse model.

MATERIALS AND METHODS

Ethics Five-week-old female BALB/c mice were purchased from Orient Bio (South Korea) and acclimatized for one week. All animal experiments were performed in accordance with the National Research Council’s Guide for the Care and Use of Laboratory Animals and with the Guidelines for Animal Experiments of Chung-Ang University.

Preparation and Titration of Virus Hep-2 cells were cultured in minimum essential medium (MEM)-α medium (Gibco, U.S.A.) containing 10% fetal bovine serum (FBS) (v/v; GenDEPOT, U.S.A.), 1% penicillin/streptomycin (v/v; GenDEPOT, U.S.A.) and 25 µg/mL normocin (InvivoGen, U.S.A.). Monolayers of Hep-2 cells were infected with the hRSV A2 strain at 0.1–0.5 plaque forming units (PFU) per cell and cultured for 72 h at 37°C. The infected cells were harvested with a cell scraper (Costar, Mexico) and disrupted with a Dounce homogenizer (WHEATON, U.S.A.), and the lysates were clarified by centrifugation at 352 × g for 10 min, dialyzed overnight against distilled water at 4°C. The supernatants containing hRSV were frozen quickly in 70% ethanol at −80°C until use. Virus was titrated by plaque assay as described with slight modifications.18) Mouse BAL cells were fixed with 2% glutaraldehyde (Sigma, U.S.A.) and stained with 0.75% methyl cellulose (Sigma, U.S.A.) for 7 d. The cells were collected by centrifugation and stained with peridinin chlorophyll α for the 14 d prior to virus challenge and 40 mg/kg/d ribavirin syrup for 3 d post virus challenge.28) Mice were exposed to 1×10⁶ or 5×10⁶ PFU of hRSV by intranasal instillation as well as a single intraperitoneal injection of cyclophosphamide (100 mg/kg, Sigma) 5 d prior to virus challenge to induce the immunocompromised state.39) Mock infection group received PBS for the 14 d prior to virus challenge and challenged with PBS instead of hRSV.

Determination of Lung Virus Titers Lung virus titers were determined as described previously.16) Mouse lungs were collected and disrupted in 1 mL of PBS using a Dounce homogenizer on day 4 post virus challenge. The lysates were clarified by centrifugation at 352 × g for 10 min, and the supernatants were frozen quickly in 70% ethanol at −80°C for subsequent virus titration.

Flow Cytometry The proportions of CD3⁺, CD3⁺CD8⁺, and CD3⁺CD4⁺ lymphocytes were analyzed according to the previous reports with modification.17) Mouse BAL cells were collected at days 7, 9 and 11 post virus challenge and prepared in MEM-α medium. Red blood cells were lysed in Tris–HCl, 140 ms NH₄Cl, pH 7.2, and the remaining cells were collected by centrifugation and stained with peridinin chlorophyll (Percp)-efluor 710 labeled anti-CD3e antibody (eBioscience, U.S.A.) alone, (Percp)-efluor 710 labeled anti-CD3e antibody together with allopseudocyanin (APC) labeled anti-CD8 antibody (eBioscience) or (Percp)-efluor 710 labeled anti-CD3e antibody together with APC labeled anti-CD4 antibody (eBioscience) for 30 min at 4°C. Cells were sorted by flow cytometry in a FACSCalibur (BD Bioscience, U.S.A.). Five thousand cells were sorted by flow cytometry and lymphocytes were gated from the forward and side scatter. The proportions of CD3⁺, CD3⁺CD8⁺ and CD3⁺CD4⁺ lymphocytes were analyzed with Flowing Software 2.5 (www. flowingsoftware.com).

Statistical Analysis The statistical significance of differences between groups was determined by two-tailed Student’s t-tests. p Values less than 0.05 or 0.01 were considered statis-

### Table 1. Treatment Regimens Used in the Present Study

<table>
<thead>
<tr>
<th>No.</th>
<th>Mouse group</th>
<th>Prior to virus challenge (for 14 d)</th>
<th>Post virus challenge (for 3 d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBS</td>
<td>200 µL/d</td>
<td>200 µL/d</td>
</tr>
<tr>
<td>2</td>
<td>Ribavirin</td>
<td>200 µL/d (PBS)</td>
<td>40 mg/kg/d</td>
</tr>
<tr>
<td>3</td>
<td>Xylitol 3.3</td>
<td>3.3 mg/kg/d</td>
<td>3.3 mg/kg/d</td>
</tr>
<tr>
<td>4</td>
<td>Xylitol 10</td>
<td>10 mg/kg/d</td>
<td>10 mg/kg/d</td>
</tr>
<tr>
<td>5</td>
<td>Xylitol 20</td>
<td>20 mg/kg/d</td>
<td>20 mg/kg/d</td>
</tr>
<tr>
<td>6</td>
<td>Xylitol 33</td>
<td>33 mg/kg/d</td>
<td>33 mg/kg/d</td>
</tr>
<tr>
<td>7</td>
<td>wRG 25</td>
<td>25 µg/kg/d</td>
<td>25 µg/kg/d</td>
</tr>
<tr>
<td>8</td>
<td>wRG 250</td>
<td>250 µg/kg/d</td>
<td>250 µg/kg/d</td>
</tr>
<tr>
<td>9</td>
<td>wRG 2500</td>
<td>2500 µg/kg/d</td>
<td>2500 µg/kg/d</td>
</tr>
<tr>
<td>10</td>
<td>Xylitol 3.3 + wRG 25</td>
<td>3.3 mg/kg/d + 25 µg/kg/d</td>
<td>3.3 mg/kg/d + 25 µg/kg/d</td>
</tr>
<tr>
<td>11</td>
<td>Xylitol 3.3 + wRG 250</td>
<td>3.3 mg/kg/d + 250 µg/kg/d</td>
<td>3.3 mg/kg/d + 250 µg/kg/d</td>
</tr>
<tr>
<td>12</td>
<td>Xylitol 3.3 + wRG 2500</td>
<td>3.3 mg/kg/d + 2500 µg/kg/d</td>
<td>3.3 mg/kg/d + 2500 µg/kg/d</td>
</tr>
</tbody>
</table>

Mice received PBS, xylitol and/or wRG orally for 14 d prior to virus challenge and for 3 d post virus challenge.
RESULTS

Evaluation of in Vitro Activity of Xylitol against hRSV
To investigate the direct effect of xylitol on hRSV, hRSV were treated with different concentrations of xylitol (0.05–25 mg/mL) and numbers of infectious virus were measured by plaque assay. As shown in Fig. 1, 0.78 mg/mL xylitol caused a 50% reduction in virus titer, and complete inhibition was achieved by 3.13 mg/mL xylitol. This result indicates that xylitol has a direct inhibitory effect on hRSV.

The Effect of Dietary Xylitol on hRSV Infection in Mice
The ameliorating effect of xylitol on hRSV infection in mice was evaluated by measuring lung virus titers on day 4 post hRSV challenge, at which time lung virus titers are thought to peak.20) Mice received PBS, ribavirin or xylitol as detailed in Materials and Methods and Table 1 and were challenged with \(1 \times 10^6\) PFU of hRSV. Ribavirin is a broad specificity inhibitor of RNA virus multiplication approved for the treatment of hRSV infection, effective in reducing lung hRSV titers in mice.8,19) As shown in Fig. 2, the xylitol regimens significantly reduced lung virus titers and the best effect was obtained in the 3.3 mg/kg/d xylitol group.

The Effects of Dietary Xylitol in Combination with wRG on hRSV Infection
The effects of dietary xylitol, wRG and mixtures of xylitol and wRGs (Table 1) on hRSV infection were evaluated on day 4 post hRSV challenge. Red Ginseng (RG) is a well-known herbal medicine with anticancer, anti-allergy, anti-inflammatory and anti-fatigue effects.21–23) A recent clinical study confirmed its protective effect on acute respiratory tract infections,24) and oral administration of RG extract prior to influenza A virus infection was shown to increase survival in a mouse model.25) Not only dietary RG extract but also components of the extract such as polysaccharides and saponin have been reported to increase protection against influenza A virus infection.26) Moreover, dietary wRG along with xylitol appeared to have a greater protective effect against influenza virus infection than either component alone.27) Hence the dietary effects of wRG and mixtures of xylitol and wRG were also investigated. As shown in Fig. 3, reductions of lung virus titers were observed in the wRG25 (\(p=0.06\)) and wRG250 (\(p<0.01\)) groups. However, wRG2500 appeared to be ineffective. Moreover, mixtures of xylitol with wRG (xylitol 3.3+wRG 250 and xylitol 3.3+wRG 2500) were more effective in reducing lung virus titers than either component alone. However, xylitol 3.3 +wRG25 group showed slightly enhanced lung virus titers, compared to xylitol 3.3, wRG 25 or wRG 250.

Time-Dependent Evaluation of T Lymphocytes Recruitment in Mice Receiving Xylitol Following hRSV Infection
The recruitment of T lymphocyte in the BAL, which is a site of infection, were compared between groups following hRSV challenge. Mice received PBS, ribavirin, and different dosages of xylitol for 14d, and then were intranasally challenged with \(1 \times 10^6\) PFU of hRSV. The details of the mouse experiment are shown in Table 1. Lung virus titers were determined on day 4 post virus challenge. The center line of the box represents the median, and the top (Q3) and bottom (Q1), the 75th and 25th percentiles, respectively; the top and bottom whiskers represent outliers, and the numbers in parenthesis are median values. PBS, \(n=5\); ribavirin, \(n=5\); xylitol 3.3, \(n=5\); xylitol 10, \(n=5\); xylitol 20, \(n=5\); xylitol 33, \(n=5\). **\(p<0.01\). ** indicates statistical significance when compared to PBS group.
infection. Respiratory tract virus infection induces inflammatory responses mediated in most cases by CD3⁺ lymphocytes, and excessive activation of recruited T lymphocytes disrupts the cytokine balance, which eventually causes bronchiolitis.²⁷,²⁸) T lymphocytes are elevated in mouse lungs between 6 and 12 d post hRSV challenge and peak levels are observed between 7 and 9 d.²⁰) To investigate time-dependent recruitments of T lymphocytes (CD3⁺ lymphocytes) and subsets of those (CD3⁺CD8⁺ and CD3⁺CD4⁺ lymphocytes), BAL fluids in mice receiving PBS, xylitol and ribavirin were collected at days 7, 9 and 11 post hRSV (5 × 10⁶ PFU) challenge and analyzed by flow cytometry. As shown in Fig. 4, the recruited numbers of CD3⁺, CD3⁺CD8⁺ and CD3⁺CD4⁺ lymphocytes in BAL fluid peaked at day 9 post virus challenges. Mice of xylitol 3.3 group and ribavirin group showed significantly reduced recruitments of CD3⁺ lymphocytes and its subsets in BAL, while in case of xylitol 3.3+wRG 2500, n=7; xylitol 3.3+wRG 2500, n=7; wRG 2500, n=7. * p<0.05, ** p<0.01. * and ** indicate statistical significances when compared to PBS group.

Fig. 3. Lung Virus Titors of Mice Receiving Xylitol, wRG and Xylitol Plus wRG Following hRSV Challenge

Mice received PBS, ribavirin, xylitol, wRG or xylitol in combination with wRG, and were then intranasally challenged with 1×10⁶ PFU of hRSV. Lung virus titors were determined on day 4 post virus challenge. The center line of the box represents the median, and the top (Q3) and bottom (Q1), the 75th and 25th percentiles, respectively; the top and bottom whiskers represent outliers, and the numbers in parenthesis are median values. PBS, n=7; ribavirin, n=7; xylitol 3.3, n=7; xylitol 3.3+wRG 25, n=7; xylitol 3.3+wRG 250, n=7; xylitol 3.3+wRG 2500, n=7; wRG 25, n=7; wRG 250, n=7; wRG 2500, n=7. * p<0.05, ** p<0.01. * and ** indicate statistical significances when compared to PBS group.

DISCUSSION

In the present study, the in vitro effects of xylitol on hRSV and the effects of dietary xylitol on hRSV infections in mice were investigated, and the recruitment of CD3⁺ lymphocytes and its subsets in BAL were assessed post hRSV challenge. The results indicate that xylitol not only has a direct inhibitory effect on hRSV but also reduces the severity of hRSV infections in mice.

An almost complete loss of hRSV activity was observed when the virus was treated with a concentration of 3.13 mg/mL of xylitol, while no reduction was observed at 0.2 mg/mL or less than 0.2 mg/mL, as shown in Fig. 1. Xylitol is one of four isomers of 1,2,3,4,5-pentapentanol. Interestingly, two types of different isomers of 1,2,3,4,5-pentapentanol, L-arabitol and adonitol, were confirmed to exert the inhibitory effect against hRSV in vitro (data not shown). Therefore, it seems that certain chemical structure of 1,2,3,4,5-pentapentanol may share a function for inhibiting hRSV activity. In addition, it was investigated which replication stage of hRSV is targeted by xylitol (Supplementary material 2). Xylitol did not exert the inhibitory effect when that was treated post hRSV infection (Supplementary material 2B) while that exerts the effect when that was treated with hRSV prior to virus infection (Supplementary material 2A). Therefore, these results indicate that xylitol targets the process prior to cell internalization of hRSV. Meanwhile in vivo experiments showed that 3.3 mg/
kg/d of dietary xylitol significantly reduced lung virus titers after hRSV challenge. The total blood volume of mice is less than 2.5 mL. The mice of the xylitol 3.3 group received 66 µg of xylitol per day, considering the body weight of the mice to be 20 g. Therefore, the maximum concentration of xylitol in the bloodstream must have been about 0.026 mg/mL, assuming that the xylitol (3.3 mg/kg/d–66 µg/dose) was totally absorbed (2.5 mL). This concentration in the blood is far less than the minimum concentration (0.2 mg/mL) needed to inhibit hRSV directly (Fig. 1). Clearly the in vivo ameliorating effects of xylitol are due to improved physical or immune status rather than a direct effect of the xylitol.

Previously, some evidence of systemic changes induced by xylitol has been presented. One study suggested that dietary xylitol increased bone density in rats by improving bone metabolism: xylitol not only increased serum calcium concentrations and alkaline phosphatase activity, which are important markers of osteoblast function in osteogenesis, but also re-
duced the level of serum tartrate-resistant acid phosphatase, a marker of bone resorption. Dietary xylitol was suggested to improve growth and inflammatory performance in chickens in which inflammation was induced by lipopolysaccharide along with Sephadex: xylitol not only prevented the loss of body weight and reduction in food intake but also reduced alpha-1-acid glycoprotein and interleukin-1-like activity in plasma, which are indicators of inflammation levels in the chicken. Furthermore, dietary xylitol along with wRG significantly enhanced survival rates and reduced levels of inflammation-associated dendritic cells in BAL following influenza A virus infection. These findings indicate that dietary xylitol can improve physical function and have immunomodulatory effects.

The components of BAL such as lymphocytes and polymorphonuclear leukocytes (neutrophils and eosinophils) have been used as indicators of various allergic and inflammatory lung diseases, and the numbers and subset composition of lymphocytes in BAL are known to reflect the severity of respiratory diseases. As shown in Figs. 4A, B, mice receiving xylitol had significantly reduced proportions of CD3+ and CD3 CD8+ lymphocytes in BAL fluid at day 7 post virus challenge. Also, it appeared that the reduction trends of CD3 CD8+ lymphocytes in PBS, ribavirin and xylitol groups at day 7 post virus challenge are similar to those of lung virus titer shown in Fig. 2. Therefore, it is thought that the recruitment rate of CD3+ lymphocytes may be affected by the lung virus titer (Fig. 2). However, remarkable differences of xylitol groups were not found in the levels of CD3 CD4+ lymphocytes, compared to PBS group (Fig. 4C). Meanwhile, ribavirin group showed significantly elevated levels of CD3 CD4+ lymphocytes, compared to PBS or xylitol groups (Fig. 4C). Therefore, it is thought that the mechanism of xylitol to inhibit hRSV is different from that of ribavirin.

hRSV infection is the most frequent cause of hospitalization in infants and young children worldwide, and recurrent wheezing and asthma can occur among the children who experience hRSV infection in their early life. The global burden associated with hRSV disease is estimated at 64 million cases and 160000 deaths annually, and the total annual direct medical costs of hRSV infection-related hospitalizations were estimated at 394 million U.S. dollars for children under 5 in the U.S.A. According to 2005 statistics, 33.8 million new episodes of hRSV-associated acute lower respiratory infection occurred in those under the age of 5 and 99% of those cases occurred in developing countries. Xylitol, a five-carbon alcohol, is a sugar substitute that can be extracted from hardwood with ease on an industrial scale. It is not toxic to humans, and it is therefore categorized as a safe food additive by the U.S. Food and Drug Administration.

Nowadays, palivizumab is used to prevent hRSV infection. Ribavirin has been approved specifically against hRSV. An investigation of the market prices revealed that Synagis™ (palivizumab) is traded at a wholesale price of 1416.48 U.S. dollars for a single dose (100mg vial), and VIRAZOLE, which contains 6g of ribavirin, retails at 1500 U.S. dollars per vial. These prices of palivizumab and ribavirin are far too high for them to be used in developing countries, whereas the retail price of xylitol is considerably lower (1–5 U.S. dollars per kg). Therefore, xylitol appears to have great potential for widespread use in developing countries.

It this study, we found that dietary xylitol has a significant ameliorating effect against hRSV in a rodent model. Further studies are needed to assess the effects of xylitol against hRSV in human trials or some other rodent model such as the cotton rat, and such studies should provide new insights into the actions and effects of xylitol.

Acknowledgments We thank Prof. Hoan Jong Lee (Department of Pediatrics, College of Medicine, Seoul National University, South Korea) for providing hRSV. This research was supported by the Chung-Ang University Grant.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES