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Anti-rotavirus effects by combination therapy of stevioside and *Sophora flavescens* extract

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ABSTRACT

Anti-rotaviral activities of *Sophora flavescens* extract (SFE) and stevioside (SV) from *Stevia rebaudiana* Bertoni either singly or in various combinations were examined *in vitro* and *in vivo* using a porcine rotavirus G5[P7] strain. Combination of SFE and SV inhibited *in vitro* virus replication more efficiently than each single treatment. In the piglet model, SV had no effect on rotavirus enteritis, whereas SFE improved but did not completely cure rotaviral enteritis. Interestingly, combination therapy of SFE and SV alleviated diarrhea, and markedly improved small intestinal lesion score and fecal virus shedding. Acute toxicity tests including the piglet lethal dose 50, and body weight, organ weight and pathological changes for the combination therapy did not show any adverse effect on the piglets. These preliminary data suggest that the combination therapy of SV and SFE is a potential curative medication for rotaviral diarrhea in pigs. Determination of the efficacy of this combination therapy in other species including humans needs to be addressed in the future.

1. Introduction

Rotaviruses are the single most important etiologic agent of severe diarrheal disease in young children, and a frequent cause of hospitalization both in developed and developing countries (Estes and Kapikian, 2007). In developing countries, rotavirus is recognized as one of the major infectious diseases of the gastrointestinal tract that kills over 600,000 people per year, usually children under 5 years of age (Parashar et al., 2006). Moreover, rotaviruses cause huge economic losses in the livestock industry and are a potential source of heterologous rotavirus infections in humans (Chingwaru et al., 2011; Estes and Kapikian, 2007; Kim et al., 2011).

Currently, monovalent and pentavalent human vaccines have been approved in the United States and other countries (Anderson, 2008). The contribution of these vaccines in the prevention of rotaviral disease has been considerably important. However, limitations exist in the capability of the vaccines to inhibit subsequent infection with other rotavirus serotypes, as well as the high cost of production (Zhou et al., 2010). Moreover, these vaccines could cause vaccine-derived transmission of rotaviruses from immunocompromised contact between vaccinated and unvaccinated groups (Baek et al., 2010). Therefore, inexpensive and effective drugs are necessary as additional approaches to control this disease, particularly in developing countries. Researchers have been investigating the effects of natural compounds found in food and herbal products that have antiviral properties (Lipson et al., 2007).

Several compounds and plant extracts have been reported as effective for inhibition of rotavirus infections *in vitro* (Ebina et al., 1990; Kiefel et al., 1996; Takahashi et al., 2002) and *in vivo* (Mukoyama et al., 1991a; Newburg et al., 1998; Takahashi et al., 2002), indicating that they may be ideal candidates for use as preventive and ther-

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apeutic drugs against rotavirus infections. In traditional Chinese medicine, the dry root of *Sophora flavescens* (*S. flavescens*) is one of the most widely used medicinal herbs in a variety of herbal formulations to treat a range of diseases, such as various virus infections, cancer, cardiac arrhythmia, ulcer, inflammatory disorder, fever, and skin diseases (Jin et al., 2010; Lin et al., 2011; Liu et al., 2011; Miao et al., 2001). Recently, our *in vitro* study showed that norkurarinol, a flavanone isolated from the root of *S. flavescens*, inhibited rotavirus replication by modulating Toll-like receptor (TLR 3) signaling and the production of pro-inflammatory cytokines (Oh et al., 2011). Stevioside (SV) is the main sweet component derived from the leaves of *Stevia rebaudiana* Bertoni, which is commonly found in South America and more recently worldwide (Guens et al., 2003). This has been used for the treatment of hyperglycemia, diabetes, cancer, and hypertension (Brahmachari et al., 2011; Lailerd et al., 2004), and has shown inhibitory effect against human rotavirus *in vitro* (Takahashi et al., 2001). However, little is known about the *in vitro* and *in vivo* anti-rotavirus activities of *S. flavescens* and SV, either singly or in combination. Therefore, this study aimed to evaluate the anti-rotaviral activities and toxicity of *S. flavescens* extract (SFE) and SV, either singly or in various combinations *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cell and virus

Fetal rhesus monkey kidney (MA-104) cells were used to culture a porcine group A rotavirus K85 (G5P[7]) strain isolated from fecal sample of a diarrheic piglet (Kim et al., 2010). Virus titer was determined by cell culture immunofluorescence assay using a monoclonal antibody against VP6 protein of OSU porcine strain and was expressed as fluorescence focus units per milliliter (FFU/ml).

2.2. Preparation of the SFE and SV

The plant material used in this study, *S. flavescens* root, was collected in Hamyang, Republic of Korea, and identified by Professor Myong Gi Chung of Gyeongsang National University (Kim et al., 2006). A voucher specimen of this raw material is deposited at Herbarium of Gyeongsang National University (GNU) (Kim et al., 2006). The roots of *S. flavescens* (3 kg) were air-dried, chopped and extracted three times with methanol (2 × 18 L) for a week at room temperature. The insoluble precipitate was removed by filtration and the solution was concentrated under in vacuum to yield a dark brown gum (89 g) (Jeong et al., 2008; Kim et al., 2006). A 1000 mg SFE/ml stock solution was made by dissolving it in absolute methanol. From the stock solution, 100 mg SFE/ml, 200 mg SFE/ml, and 400 mg SFE/ml were made by diluting the stock solution with autoclaved distilled deionized water (DDW). Synergistic effecter, SV (purity >95%) was purchased from Daepyung Co. (Republic of Korea). The material was prepared by dissolving it in DDW. The HPLC profile of SFE and SV are presented as Supplementary Fig. S1 in the online version at doi:10.1016/j.rvsc.2014.03.011.

2.3. Evaluating in vitro anti-rotaviral activities of SFE, SV and various combinations of SFE and SV

The *in vitro* antiviral assays used in this study have been previously described (Kim et al., 2012). Simultaneous treatment assays were performed to evaluate if the activity resulted from inactivation of infectious virions. Various concentrations of each candidate or mixture of both candidates (0.2, 0.4, 0.8, 1.6, 3.1, 6.2, 12.5, 25 mg SV/ml; 1.5, 3.1, 6.2, 12.5, 25, 50, 100 μg SFE/ml; 2 mg SV/ml (fixed) plus 3.1, 6.2, 12.5, 25, 50, 100, 200 μg SFE/ml) were mixed with equal volume of porcine rotavirus K85 strain with 0.01 multiplicity of infection (MOI) and incubated at 4 °C for 1 h. Virus titer was determined by inoculating serial 10-fold dilutions of porcine rotavirus K85 strain into African rhesus monkey kidney (MA-104) cell monolayer grown in 96-well-plates and then immunofluorescence assay using monoclonal antibody against rotavirus VP6 protein. Virus titer was calculated by Reed–Muench method (Reed and Muench, 1938) as fluorescent focus units (FFU) per milliliter. Each mixture of virus and either single or various combinations of candidates was transferred into MA-104 cell monolayer grown in 96-well-plate (1×10^5 cells/well) and incubated for 1 h with occasional rocking. As a control, MA-104 cell monolayer grown in 96-well-plate were neither mock-inoculated nor mock-treated. After removing the inoculums or medium from treated or control plates, Eagle's minimal essential medium (EMEM) containing 1 μg/ml trypsin was added to each well. The plates were incubated for 72 h at 37 °C under 5% CO_2 atmosphere until the cells in the infected, untreated control showed complete cytopathic effect (CPE) under light microscopy. At this time point, it could be easily evaluated how anti-rotaviral drug candidates reduced rotavirus-induced CPE in comparison with that in infected but untreated wells. Each candidate and mixture was assayed in triplicate in MA-104 monolayer cells. The 50% effective concentration (EC_{50}) was estimated by regression analysis.

Post-treatment assay used in this study were performed as previously described (Kim et al., 2012). Ninety-six-well plate monolayered with MA-104 cells (1×10^5 cells/well) was inoculated with or without porcine rotavirus K85 strain at 0.01 MOI and incubated for 1 h with occasional rocking. The inoculums were replaced by EMEM containing 1 μg/ml trypsin and various concentrations of drug candidates as mentioned above. The cells were then incubated for 72 h at 37 °C until cells in the infected, untreated control well showed complete viral CPE by light microscopy. Plates were incubated for 2 h at 37 °C in the absence of light after adding 0.034% (w/v) neutral red solution. The neutral red solution was discarded and cells were washed with PBS (pH 7.4), and destaining solution (1% glacial acetic acid, 49% H_2O and 50% ethanol) was added. The plates were incubated at room temperature for 15 min and the absorbance of each plate was read at 540 nm using a microplate reader. Regression analysis was used to create a standard curve and to estimate the EC_{50} of each extracts. Each candidate and mixture was assayed in triplicate (Kim et al., 2012).

2.4. Experimental design for in vivo evaluation of anti-rotaviral effects of SFE and SV in colostrum-deprived piglets

2.4.1. Animal

Colostrum-deprived piglets were obtained from sows by hysterectomy and maintained in isolator units as previously described (Gonzalez et al., 2010). Piglets were fed every 6 h with a diet consisting of sterilized commercialized milk (Sprayfo®, Sloten B.V., Antwerpenweg, Netherlands). At 3-day-old, piglets were orally inoculated with 3 ml of the K85 (G5P[7]) strain containing a virus titer of 5×10^5 FFU/ml. Mock-inoculated and mock-treated piglets were given 3 ml of alpha minimum essential medium (α-MEM) orally. Once diarrhea was observed, piglets were treated orally with various concentrations of antiviral drug candidates, either singly or in combination. All animal experiments were approved by the Chonnam National Animal Care Committee and the local ethical board (CNU IACUC-YB-2009-15).

2.4.2. Treatment of colostrum-deprived piglets with SV

To evaluate the anti-rotaviral effects of SV, 15 piglets were randomly divided into 5 groups (Table 2): 1) mock-inoculated and mock-treated, 2) virus-inoculated and mock-treated, 3) virus-inoculated and drug-treated with 1 g SV, 4) virus-inoculated and drug-treated with 2 g SV, and 5) virus-inoculated and drug-treated with 3 g SV.