Antiviral activity of Chongkukjang extracts against influenza A virus 
in vitro and in vivo

Bai Wei a, *, Se-Yeoun Cha a, *, Min Kang a, Young Jin Kim b, Chang-Won Cho b, Young Kyung Rhee b, Hee-Do Hong b, Hyung-Kwan Jang a, *

a Departments of Infectious Diseases and Avian Diseases, College of Veterinary Medicine and Korea Zoonosis Research Institute, Chonbuk National University, Iksan, Jeollabuk-do, South Korea
b Korea Food Research Institute, Songnam, Kyongki-do, South Korea

ARTICLE INFO

Article history:
Available online 5 May 2015

Keywords:
antiviral effect
Chongkukjang extracts
influenza virus
neuraminidase inhibitory activity

ABSTRACT

Background: Chongkukjang is a traditional Korean fermented product prepared from soybeans and reported to have multiple biological functions, including antidiabetic, antiinflammatory, and neuroprotective effects. Influenza is a respiratory disease caused by influenza viruses and continues to be a worldwide threat with a high potential to cause pandemics. Besides vaccination, only two classes of drugs are available for antiviral treatment against these pathogens.

Methods: We tested the inhibitory activity of an ethyl acetate extract from Chongkukjang toward influenza A virus neuraminidase.

Results: All 10 compounds extracted from Chongkukjang showed neuraminidase inhibitory activity. Extracts A3 and A8, with high neuraminidase content, had the best inhibitory activities. The in vivo antiviral virus activities of the ethyl acetate, A3, and A8 extracts as well as commercially available genistein were evaluated using H1N1 (A/NWS/33) to test mice survivability after virus challenge. The Chongkukjang extracts did not reduce mortality, but the A3 and A8 extracts delayed the median time to death after influenza A virus infection of mice.

Conclusion: Our results suggest that the Chongkukjang extracts may have potential as a therapeutic agent to treat influenza virus infection.

Copyright © 2015, Korea Food Research Institute, Published by Elsevier. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

Chongkukjang is a fermented product manufactured by short-term fermentation of soybean using Bacillus subtilis that contains many microorganisms and bioactive compounds that are absent from unfermented soybeans (Fig. 1) [1–3]. Flavonoid glycosides are converted into aglycones by hydrolysis during fermentation of Chongkukjang, and many of the proteins are degraded into small peptides and amino acids [4]. Koreans have been consuming Chongkukjang for hundreds of years, and most believe that Chongkukjang consists of proteins and minerals that promote the generation and growth of human cells and strengthen the immune system [5,6]. Chongkukjang has multiple functions, including antidiabetic, antiinflammatory, and neuroprotective effects [5,7].

Influenza viruses are respiratory pathogens that affect humans and are responsible for substantial morbidity, mortality, and decreased productivity worldwide [8]. Influenza viruses contain two glycoproteins on their surface, hemagglutinin (HA) and neuraminidase (NA), which recognize sialic acid in host–cell glycoconjugates. HA binds to terminal sialic acid groups on cell surface glycoconjugates, leading to attachment and subsequent penetration of virus into cells, and NA exhibits enzymatic activity that removes sialic acid from glycoconjugates, facilitating the release of progeny virions from infected cells and preventing the aggregation of progeny virions [9]. Neuraminidase inhibitors (NAIs) act by binding to the active site of the viral NA enzyme and preventing release and spread of progeny virions from infected cells during the replication cycle [10]. NAIs block the neuraminidase active site and prevent release and spread of new virions [11]. NA was chosen as a suitable drug target because it plays a major role in the propagation of influenza viruses, and the amino acid residues of the active site
with an equal volume (50 and 0.3 mg/mL bovine serum albumin) were prepared, mixed and stored at −80°C until use. Viral HA units were determined in 96-well microtiter plates using 1% chicken red blood cells.

2. Materials and methods

2.1. Viruses

The influenza virus used in this study (Influenza A/NWS/33; Influenza A/chicken/Korea/MS96/96) was propagated in the allantoic sac of 10-day-old specific pathogen-free embryonated eggs. These virus stocks were frozen at −80°C until use. Viral HA units were determined in 96-well microtiter plates using 1% chicken red blood cells.

2.2. Fluorometric neuraminidase activity assay

A fluorescence-based NA inhibition assay was used to determine the sensitivity of the viruses to NAIs. The assay is based on the release of a 4-methylumbelliferone fluorescent product from the 2-(4-methylumbelliferyl)-a-d-N-acetyleneuraminic acid (MUNANA) substrate (Sigma, St. Louis, MO, USA) as a measure of NA activity. The NA activity assay was carried out to determine the dilution of each virus to be used in the subsequent NA inhibition assay. Two-fold dilutions of virus in assay buffer (32.5 mM MES [2-(N-morpholino)ethanesulfonic acid], pH 6.5, 4 mM CaCl2 with 0.1% NP-40, and 0.3 mg/mL bovine serum albumin) were prepared, mixed with an equal volume (50 μL) of MUNANA substrate (0.3 mM), and incubated at 37°C for 60 minutes. The reaction was terminated by adding 100 μL stop solution (0.14 M NaOH in 83% ethanol). The fluorometric quantification of 4-methylumbelliferone was determined using a VICTOR X4 Multilabel Plate Reader (PerkinElmer Inc., Waltham, MA, USA) at an excitation wavelength of 360 nm and an emission wavelength of 448 nm. The appropriate concentration of virus for use in the NA inhibition assay was determined by selecting a dilution of virus in the linear portion of the enzyme activity curve. Zanamivir was obtained from Sigma and used as a positive control.

This assay was performed in duplicate for each isolate, and the half-maximal inhibitory concentration (IC50) value for each virus and for each antiviral agent was determined. The IC50 is the antiviral agent concentration that inhibits 50% of neuraminidase activity.

2.3. Isolation of an active component from Chongkukjang

Commercial Chongkukjang samples were obtained from Sunchang MoonOkrae Food (Sunchang, Korea), and the extracts prepared by the Korea Food Research Institute. Briefly, Chongkukjang was extracted with ethanol, and the liquid was removed by evaporation. The residue was suspended and successively partitioned in fractions of ethyl acetate, butanol, and water. The total crude extract was dissolved in CH2Cl2/H2O (95:5), and the ethyl acetate fraction was subjected to silica open-column chromatography. The mobile phase solvent was dichloromethane/ethanol diluted as 95:5, 90:10, 80:20, and 50:50. All 10 subfractions were combined (A1–A10) based on thin-layer chromatography analysis.

2.4. Animal model and experimental design

Female (weight, 17–19 g) 6-week-old specific pathogen-free BALB/c mice (Daejeon, Chungnam, Korea) were used. The mice were quarantined 24 hours prior to use. The AIN-93M diet was provided as feed throughout the experiment, as recommended by the American Institute of Nutrition [15]. All animals received humane care in compliance with the National Association of Laboratory Animal Care and were fed in isolation (Three-Shine, Daejeon, Korea).

To investigate the safety of the Chongkukjang extract in mice, the mice were administered the ethyl acetate, A3, and A8 fractions, as well as genistein (0.4 g/kg/d of body weight). Phosphate-buffered saline was given to the normal control group. The mice were divided into groups of 10 each, and the treatments were carried out for 14 days. Clinical signs were recorded daily, and the body weight was measured on Days 0, 7, 10, and 14. To investigate the protective activity of the Chongkukjang extract against lethal influenza virus in vivo, the mice were divided into groups of 10 each. The inoculated mice received the ethyl acetate, A3, and A8 extracts as well as genistein (0.2 and 0.4 g/kg/d), respectively. The treatments were administered daily via gavages for 6 days before challenge and 14 days after influenza A virus challenge. Studies with mice were performed as described previously, and phosphate-buffered saline was used as the negative control. The mice were anesthetized with diethyl ether and exposed to 0.1 mL virus by intranasal instillation. A viral challenge dose of 107.0 50% embryo infectious dose (EID50)/0.1 mL (A/NWS/33), which was approximately 80–90% of the lethal dose, was used. Mice were observed daily for 14 days after infection. The protective effect was estimated by preventing death and prolonging the median time to death (MTD).

2.5. Data analysis

Data are expressed as the mean ± standard deviation of at least three independent experiments. Body weight was analyzed with one-way analysis of variance [16]. Statistical analyses were
performed using the SPSS software (SPSS Inc., Chicago, IL, USA). A p value <0.05 was considered statistically significant.

3. Results

3.1. Inhibitory effect of the Chongkukjang extracts on influenza virus in vitro

The standard soybean isoflavones, such as daidzin, daidzein, genistin, genistein, glycitin, and glycitein (Sigma), were applied to the fluorometric NA activity assay, and genistin showed the best NA inhibitory effect compared with that of the five other standard soybean isoflavones (data not shown). Thus, genistein was used to control the extract composition of the Chongkukjang eluent at each step.

NA is one of the most important targets to screen antinfluenza virus A drugs. We first investigated whether the Chongkukjang fractions exerted antiviral action. The ethyl acetate, butanol, and water fractions were measured using the fluorometric NA activity assay. The ethyl acetate fraction showed the best NA inhibitory effect compared with that of butanol and water (data not shown). Thus, the ethyl acetate fraction was used for further purification. The Chongkukjang extracts were dose-dependently effective against influenza A virus, and the calculated IC50 values of the extracts against the influenza virus are shown in Table 1. The A3 extract showed the best NA inhibitory effect against influenza A virus. The A8 extract also showed a high inhibitory effect. The Chongkukjang extract seemed to be more effective against A/NWS/33 (H1N1) than against A/chicken/Korea/MS96/9 (H9N2). Genistein showed similar effects against A/NWS/33 (H1N1) and A/chicken/Korea/MS96/96 (H9N2).

3.2. Inhibitory effect of the Chongkukjang extracts on the influenza virus in vivo

We next tested whether the Chongkukjang extract, which inhibited NA activity, could relieve a viral infection in vivo. First, we assessed the safety of the Chongkukjang extract in mice. Animals treated with the Chongkukjang extract maintained a relatively steady weight, and mean body weights of the treated mice, except the A8 group, were higher than those of the control group, but no significant differences were observed throughout the study (Table 2). No clinical symptoms were observed throughout the study.

To investigate the protective effect of the Chongkukjang extract in vivo, groups of mice were inoculated with 10^6 EID50/0.1 mL A/NWS/33 (H1N1). All of the mice that received the ethyl acetate, A3, and A8 extracts as well as genistein (0.4 g/kg/d) died within the experimental period (data not shown). The results for mice that received a dose of 0.2 g/kg/d are shown in Fig. 2. About 10% of the mice from the positive control group survived until the end of the experiment. All challenge groups, except the genistein group (0.2 g/kg) and the A3 group (0.2 g/kg), died by 8 days after infection. The MTD of the mice treated with the A3 and A8 (0.2 g/kg) extracts was 5 and 6 days, which was longer than that of the viral control group (3 days). The survival rate in the A3 group (0.2 g/kg) at 14 days post infection was 20%, which was higher than that of the viral control group (10%).

4. Discussion

There is an urgent medical need to develop new strategies against influenza virus infection. At the moment antiviral drugs are the only known defense against the disease, as no vaccine is available, which is similar to the early phase of a pandemic or when the prediction for the seasonal influenza vaccine composition fails. In 2010, the World Health Organization suggested the development of novel and effective treatment strategies for influenza virus including natural products [17]. This prompted us to investigate the antiviral activity of traditional food extracts against influenza virus infection. The goal of this study was to determine whether Chongkukjang extracts could function as an antiviral agent against influenza A virus, because Chongkukjang has a strong record in the literature for its beneficial effects on human health [12,18]. Our results suggest for the first time that the Chongkukjang extracts were active against the influenza A virus.

Influenza virus NA is thought to be required to secrete newly synthesized virus from infected cells and to aid in the movement of the virus. NA can be administered relatively late in the viral infection, including natural products [17]. This prompted us to investigate the antiviral activity of traditional food extracts against influenza virus infection. The goal of this study was to determine whether Chongkukjang extracts could function as an antiviral agent against influenza A virus, because Chongkukjang has a strong record in the literature for its beneficial effects on human health [12,18]. Our results suggest for the first time that the Chongkukjang extracts were active against the influenza A virus.

Although the Chongkukjang extracts showed less activity than zanamivir and oseltamivir, Chongkukjang extracts showed possible research directions as a potential candidate for zanamivir- and oseltamivir-resistant cases in clinical practice. This provides a good backdrop considering that after 3 years of NAi use, it has been noted that 0.35% of isolates were found to have become resistant—in particular, higher percentages of such resistance have been observed in children [20,21].

Wide variations in the IC50 for oseltamivir are observed in seasonal influenza A with or without the virus subtype [22]. Genistein has a weak NA inhibitory effect of about 20 mg/mL IC50 against the H1N1 influenza A virus [23]. Our result showed about six times higher efficiency of the IC50 against the same NA type of influenza A virus. An NA gene mutation (H274Y, H275Y in N1 numbering) has been associated with NA inhibitor susceptibility [24]. The

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Inhibitory effect of the Chongkukjang extracts against influenza A virus.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Compound</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Ethyl acetate extract</td>
</tr>
<tr>
<td>2</td>
<td>A3</td>
</tr>
<tr>
<td>3</td>
<td>A8</td>
</tr>
<tr>
<td>4</td>
<td>Genistein</td>
</tr>
</tbody>
</table>

IC50, drug concentration required to inhibit half of neuraminidase activity. *Fluorescence-based neuraminidase (NA) inhibition assay was used to determine the sensitivity of influenza A viruses to Chongkukjang extracts.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Body weights (mean ± standard deviation) of the mice in each group.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Body weight (g)</td>
</tr>
<tr>
<td>0 DPI</td>
<td>17.9 ± 0.5</td>
</tr>
<tr>
<td>7 DPI</td>
<td>19.2 ± 0.8</td>
</tr>
<tr>
<td>10 DPI</td>
<td>19.7 ± 0.5</td>
</tr>
<tr>
<td>14 DPI</td>
<td>0.3 ± 19.4</td>
</tr>
</tbody>
</table>

DPI, days post inoculation. *Safety of the Chongkukjang extract in mice administered with ethyl acetate, A3, and A8 fractions, as well as genistein (0.4 g/kg/d of body weight) for consecutive 14 days.
Chongkukjang extracts showed a better inhibitory effect against A/ NWS/33 (H1N1) than that against A/chicken/Korea/MS96/96 (H9N2). This result agrees with the finding that avian isolates have significantly higher resistance to NAIs than that of human isolates [25]. It is not apparent how accurately the IC50 values determined from the enzyme inhibition assay results reflect actual differences in inhibitor potency. Once efficacy is observed against an influenza virus, it is important to use a variety of other virus strains in follow-up evaluations to ascertain if the antiviral effect applies to all viral strains encountered in the clinic.

As the Chongkukjang extracts showed antiviral effects in vitro, a mouse study was performed to determine the in vivo effects of the Chongkukjang extracts against influenza A virus. Treatment with the A3 and A8 (0.2 g/kg) extracts produced a modest inhibitory effect on influenza A virus replication in mice. Influenza-virus-infected mice receiving the Chongkukjang extract treatment did not show reduced mortality, but the A3 and A8 extracts delayed the mice MTD after influenza A virus infection. This result shows that the Chongkukjang extract is a potential candidate to fight influenza A virus infection. The Chongkukjang-treated groups (except the A8 group) maintained a higher body weight compared with the control group, which agrees with previous reports that soybean-related products increase body weight and lower the risk of toxic side effects at a low concentration [14,26]. This extract may be a potent antiviral agent against influenza virus with no host toxicity.

We found that the A3 and A8 Chongkukjang extracts had a potential inhibitory activity against influenza viruses. This study is another example showing how plant extracts are capable of inhibiting influenza A virus infection. This study addresses the latest developments in theoretical and experimental research on the properties of NA that are and will be driving antiinfluenza drug development today and in the near future. Two further research directions can be undertaken. One would be to further characterize and identify the antiviral compounds in the extracts. Nevertheless, it is also important to determine whether only one compound, several closely related compounds, or the composite activity of several different molecules is/are responsible for the observed antiviral activity and whether the activity is selective for influenza viruses. We clearly need to focus more attention on ethnic foods, not only for nutrition but also for the functionality they provide in disease control.

**Conflicts of interest**

The authors have no conflicts of interest to declare.

**Acknowledgments**

This study was supported by research grants from the Korea Food Research Institute (E0143003528).

**References**


