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Research Article

Effect of Dietary Supplementation and Intranasal Administration of Soybean Extract on Resistance to Influenza Virus Infection in Mice

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Abstract

Soybean extract is known to have an anti-influenza virus effect on cultured cells. However, the antiviral effect of specifically the soybean component, without interference from other components in the extract, has not been determined. Moreover, studies on the antiviral effect of soybean *in vivo* are scarce. We revealed that the viral resistance of cells was dependent on the soybean component of the extract. Therefore, we aimed to examine the anti-influenza virus effect of soybean both in the form of diet and hot water extract *in vivo* in mice. Balb/c mice were fed either CE-2 (containing soybean components) or AIN-76 (containing no soybean component) diet for 1 week. Hot water soybean extract was administered intranasally to rule out the effects of other components in the feed. The mice were infected with the influenza A/PR/8/34 (H1N1) virus, and their survival rate was observed for 2 weeks. The infected mice were killed on days 0 (uninfected), 2, 3, and 4 post-infection. Subsequently, we measured the viral titer, lung weight, antibody titers, and cytokine production. Finally, we performed lung histopathology. The 50% mouse lethal dose of CE-2-fed mice was $10^{2.4}$ plaque-forming units, which was 8 times higher than that of AIN-76-fed mice. However, the survival rate of the AIN-76-fed mice treated with the soybean extract was higher than that of the control mice. Furthermore, the lungs of the mice treated with soybean extract suffered less damage, including exudate, inflammation, and bronchiole collapse, due to influenza virus infection. Soybean extract has anti-viral effects against influenza in the body of mice; it acts by suppressing inflammation caused by infection. Our results suggest that dietary habits can have beneficial effects against infectious disease.

Keywords: Influenza Virus; Antiviral Reagent; Soybean; Cytokine; Antibody

Abbreviations

ARDS	:	Acute Respiratory Distress Syndrome
BSA	:	Bovine Serum Albumin
DAD	:	Diffuse Alveolar Disorder
FBS	:	Fetal Bovine Serum
FFU	:	Focus-Forming Units
H&E	:	Hematoxylin and Eosin
IgA	:	Immunoglobulin A
IFN-γ	:	Interferon-γ
IL-6	:	Interleukin-6
LS	:	Lavage Supernatant
MEM	:	Minimum Essential Medium
MLD_{50}	:	Mouse Lethal Dose of 50%
p.i.	:	Post-Infection
PBS	:	Phosphate-Buffered Saline
PFU	:	Plaque-Forming Units
SE	:	Soybean Extract
TNF-α	:	Tumor Necrosis Factor-α

Introduction

Influenza viruses, especially influenza A viruses, have caused several human epidemics and pandemics, inflicting enormous suffering and economic losses [1]. Influenza virus infections can have added complications such as pneumonia and ischemic heart disease that considerably increase the rate of hospitalization and mortality, especially in young children and elderly individuals [2,3]. M2 ion channel inhibitors such as amantadine and rimantadine and neuraminidase inhibitors such as zanamivir and oseltamivir are used to treat influenza virus infections. Recently, there have been reports of neuraminidase inhibitor-resistant influenza A viruses [4-7]; hence, a new drug, favipiravir (FRV), for influenza virus infections was developed [8,9]. Furthermore, baloxavir marboxil, an endonuclease inhibitor, has also been developed against influenza A virus [10]. However, the adverse effects and disadvantages of these drugs have also been reported. The risk of teratogenesis with FPV use might be higher than that with other drugs in the same class in clinical practice [11]. The emergence of polymerase acidic protein variants with I38T/M/F substitutions, leading to reduced susceptibility to baloxavir, occurred in 2.2% and 9.7% of baloxavir recipients in phase 2 trial and phase 3 trials, respectively [12].

Since prehistoric times, humans have applied poultices and imbibed infusions of numerous indigenous plants. Several of these infusions include food extracts that contain biologically active substances that possess antimicrobial properties [13]. Moerman [14] compared the medicinal and food floras of the native peoples of North America and discovered an overlap of these floras by both family and taxon. That is, many medical plants also double as food and add secondary herbal compounds to diet, providing medicinal benefits with low or no adverse effects. For example, herbal teas are convenient and safer alternatives to pharmaceuticals to improve sleep quality [15]. Therefore, a library of food and food-related substance extracts would be a good source for discovering novel antimicrobial agents. The anti-influenza virus effects of food extracts have been reported both *in vivo* and *in vitro* [16-23]. Hot water extracts of some of the most popular crops worldwide, such as soybeans, adlay seeds, and naked barley seeds, inhibit the growth of influenza viruses [24]. Previously, we reported the anti-influenza effects of soybeans, and fount that one of the components of soybean extracts might suppress the virus growth by enhancing the defense ability of infected cells rather than directly inhibiting the virus [25]. At low concentrations, soybean extracts have a broad-spectrum anti-influenza virus activity without cytotoxicity *in vitro*. However, to the best of our knowledge, except one reports on tofu (fermented soybean food) extract [26], there are no reports on the effects of soybean extracts in *in vivo* models. Furthermore, although several studies have examined the antiviral effects of soybean- [24-26] and daidzein-containing [27] diets, studies focusing on the antiviral activity of the soybean component are needed.

As studies on the *in vivo* effects of soybean extracts on influenza viruses are lacking, we aimed to demonstrate the inhibitory effect and mechanism of action of soybean extract on influenza virus in mice in this study.

Materials and Methods

Cells and viruses

Madin–Darby Canine Kidney cells were grown in Eagle's minimum essential medium (MEM; Sigma-Aldrich, Inc., St. Louis, MO, USA) containing 7% fetal bovine serum (FBS; Biowest Biotechnology Company, Nuaillé, Pays De La Loire, France). The influenza A virus H1N1 (A/PR/8/34) was used in the study. For cell infection, the virus was diluted in serum-free MEM supplemented with 0.4% bovine serum albumin (BSA, fraction V; Sigma-Aldrich, and added to the cells at a multiplicity of infection (MOI) of 0.001 for 1 h at 37 °C. The medium was then replaced with FBS-free Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% BSA and acetyl-trypsin (1 μg/

ml; Sigma-Aldrich) for the remaining infection period.

A mouse-adapted strain of influenza virus A/PR/8/34(H1N1) was derived by serial passages of pulmonary homogenates from the infected mice in naive mice. The viral stocks were stored at −80 °C.

Hot water extracts of soybeans

Roasted soybean flour was used in the study. To prepare hot water extracts of the flour, 2.2 g (soybeans) of flour was extracted with 50 ml of hot water (80 °C) for 50 min using a hot water bath. The extract was filtered through a No. 2 filter paper and then a Millex GV membrane (pore size 0.22 mm; Millipore, Billerica, MA, USA) and stored at -30 °C before use. The antiviral activities of the extracts were stable for at least 2 months under these conditions.

Focus-forming assay to evaluate virucidal activities

Focus formation was achieved using the method of Morimoto et al [28]. Each viral dilution was selected to obtain a final count of approximately 30 Focus-Forming Units (FFU) per well. The antiviral activity is expressed as the reciprocal of the highest dilution that reduced the number of foci to 50% or less of the control value.

Approval for animal testing

This study was performed with the approval of the Animal Experiment Committee of Hyogo College of Medicine, Nishinomiya, Hyogo, Japan (approval number: 15–007) and complied with the ordinance of the Regulation for Enforcement of the Act on Welfare and Management of Animals related to the care and management of experimental animals.

Mice feeding and infection with the virus

Five-week-old female BALB/c mice (20–22 g) were obtained from Charles River Laboratories International, Inc., USA. The mice were broadly divided into two groups and were maintained in a laminar flow hood at the Animal Experiment Facility of the Hyogo College of Medicine under suitable conditions (12 h light/dark cycle, 25 °C, relative humidity of 50%). One group was fed an AIN-76 purified mouse diet (containing no soybean component; Table I) (CLEA Japan, Inc., Japan), whereas the other group was fed a CE-2 diet (a general feed containing soybean components; Table 1 and Table 2 for 1 week. The major difference between AIN-76 and CE-2 is the nitrogen-free extract contained in CE-2. In addition, secondary metabolites such as soybean polyphenols are contained in the nitrogen-free extract. The mice were housed in groups of four or five in plastic cages with paper bedding (Paper Clean, SLC, Hamamatsu, Japan). After 1 week, the 60 six-week-old BALB/c female mice in the AIN-76 fed group were further divided into 6 groups of 10 mice, and the A/PR/8/34 influenza virus (20 µl each: 10, 25, 50, 75, 100 and 800 PFU/mouse), a mouse-adapted strain, was administered through one nostril of the mice. Similarly, the 40 CE-2 fed mice were further divided into 4 groups of 10 mice, and the influenza virus (20 µl each: 75, 800, 1000, and 1200 PFU/mouse) was administered.

AIN-76		CE-2	
Component	content rate (%)	Source	Component
Casein	20	Protein	Soybean meal, fishmeal, yeast
DL-Methionine	0.3		
Cornstarch	15	Carbohydrate	Wheat, corn, milo
Sucrose	50		
Corn oil	5	Lipid	Germ, soybean oil
Cellulose powder	5	Fiber	Wheat bran, rice bran, alfalfa meal
Mineral mixture	3.5	Mineral	
Vitamin mixture	1	Vitamin	
Choline bitartrate	0.2		

Table 1: Components of mouse diet.

Nutrient components	Nutrient components (%)
Moisture	8.7
Crude protein	26.46
Crude fat	5
Crude fiber	4.04
Crude ash	8.6
Nitrogen free extract	46.2

Table 2: Mean values from analyses in fiscal 2021 of CE-2 diet. This data is modified the data in the catalog of CLEA Japan, Inc.

Evaluating the effect of soybean extracts on influenza virus in BALB/c mouse

Five-week-old BALB/c female mice were maintained in a laminar flow hood at the Animal Experiment Facility and acclimatized to feed AIN-76 for 1 week. Body weight was measured every morning during the study. The mice were randomly divided into two groups (10 per group) on day 1. On day 6, the mice were anesthetized via an intraperitoneal injection of triple anesthesia (medetomidine hydrochloride, midazolam, and butorphanol tartrate) (0.2 ml/mouse), and one group (test group) was inoculated with 20 µl of soybean extract (SE: 20 mg/kg/d) into one nostril. The mock-treated group was administered an equal volume of phosphate-buffered saline (PBS). The MLD_{50} of the first virus lot shown in Table 3 was $10^{1.5}$ PFU in the mice fed AIN-67 and $10^{2.4}$ PFU in the mice fed CE-2. Second virus lot shown in Figure 1 was less pathogenic than those shown in Table 3, and the MLD_{50} was $10^{2.0}$ PFU in the mice fed AIN-67. Therefore, in the subsequent experiments, $10^{2.0}$ PFU was inoculated. On day 7, the mice were anesthetized by intraperitoneal injection of the triple anesthesia and infected with 100 PFU influenza virus by inoculating 20 µl of virus solution into one nostril. After 1 h, SE was inoculated into the test group mice and PBS was inoculated into the mock-treated group mice. The SE-administered and mock-treated mice were inoculated with SE and PBS, respectively, for 3 d. Survival was monitored for 14 d after virus infection. The changes in body weight were monitored for 14 d from the day of infection. The mice were maintained in accordance with the ordinance of the Regulation for Enforcement of the Act on Welfare and Management of Animals related to the care and management of experimental animals (Ordinance, 2016).

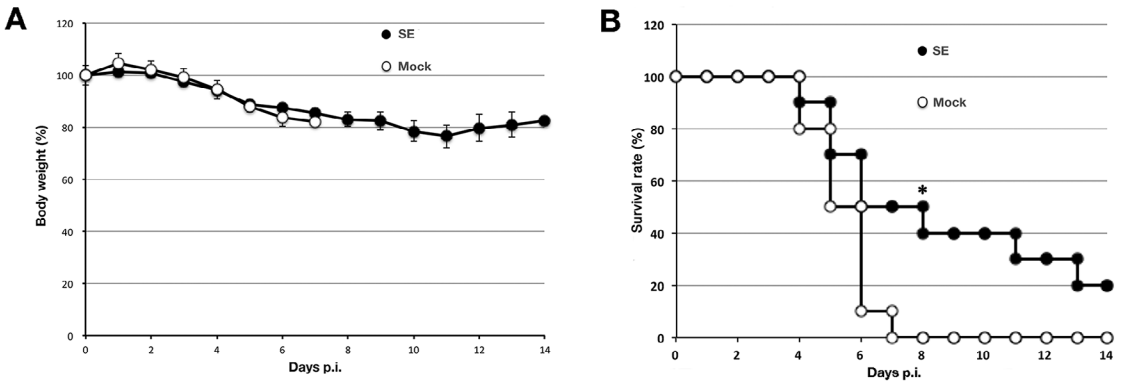

Figure 1: Effect of nasal administration of SE on the prevention of death in influenza virus-infected mice.

Survival rate of mice treated with SE via the nasal cavity and mock-treated mice. Twenty microliters of SE or PBS was inoculated, via the nasal cavity, for 5 d from 1 d before virus infection to 3 d after virus infection. All mice were inoculated with 100 PFU of the second lot influenza virus in Table 3. A: Body weights of the SE-administered and control mice. B: Survival rates of mice in the SE-administered group and the mock-treated group. Both groups contained 10 mice each. *: $p<0.05$

	Feed	
	CE-2	AIN-67
MLD_{50} (PFU/mouse)	$10^{2.4}$	$10^{1.5}$

Table 3: Mouse lethal dose 50 (MLD50) of mouse-adapted influenza virus A/PR/8/34 in the mice fed AIN-67 and CE-2.

These MLD50 values were obtained by the first viral lot, and the pathogenicity of the second lot was weaker, with an MLD50 was of 102.0 in mice fed AIN-67.

Sampling of plasma, nasal cavity lavage fluid, and lung

To prevent blood coagulation, 0.2 ml of 1000 U/ml heparin solution was intraperitoneally administered per mice and left for 30 min. After 30 min, 0.3 ml of triple anesthesia (medetomidine, midazolam, and butorphanol) was intraperitoneally administered. First, the femoral vein was cut, blood was collected, and plasma was collected by centrifuging the blood at 3000 rpm for 15 min at 4 °C. The lungs were removed, weighed, and then 5 ml of MEM (-) was added for homogenization. After centrifugation at 2500 rpm for 20 min at 4 °C, the supernatant was collected and used as a sample. In addition, the lungs were removed from the trachea and fixed with neutral buffered formalin solution to prepare a lung specimen. PBS (-) (500 µl) was injected using a syringe, and the nasal cavity lavage fluid was collected, centrifuged at 3000 rpm for 15 min at 4 °C, and the lavage supernatant (LS) was used as a sample.

Histopathological examination of the lung

The mice lungs were removed from the trachea, and a neutral buffered formalin solution was injected through the trachea to inflate the alveoli. The lungs soaked in neutral buffered formalin solution were washed with water, immersed in 70% ethanol solution for 30 min, set in an automatic embedding device, and then dehydrated with ethanol and replaced in xylene. After replacement, the lungs were hardened with paraffin to prepare paraffin blocks. The paraffin blocks were sliced to a thickness of 2 µm using a microtome, attached to a glass slide, and dried. Deparaffinization was performed with xylene and 100% alcohol. Subsequently, the nucleus was stained with hematoxylin solution, and the cytoplasm was stained with eosin solution (H&E slides). A mounting medium was dropped on the edge of the slide, and the slide was covered with a cover glass and dried at room temperature for a day. The H&E slides were scanned and converted into whole slide images using a Nanozoomer (Hamamatsu Photonics, Japan), and the images were observed using NDP.view2 image viewing software. The histopathological terminology for non-neoplastic lung lesions is in accordance with the International Harmonization Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice Project [23]. The severity of infiltration of mononuclear cells and neutrophils, necrosis of alveoli, and necrosis of bronchiolar epithelial cells were graded on a four-point scale of 1. minimal, 2. mild, 3. moderate, and 4. marked. Histopathological examinations were conducted by pathologists certified by the International Academy of Toxicologic Pathology.

Measurement of blood IgG and nasal cavity lavage fluid IgA

The level of immunoglobulin A (IgA) and IgG was measured using the Mouse IgA ELISA Quantitation Set (Bethyl Laboratories, Inc., Montgomery, TX, USA) and Mouse IgG ELIZA Quantitation Set (Bethyl Laboratories, Inc.) following the manufacturer-recommended protocols.

Measurement of blood cytokines

The level of cytokines in the blood was measured using the tumor necrosis factor- α (TNF-α), interleukin-6 (IL-6), IL-4, IL-12, and interferon-γ (IFN-γ) measurement kit (R&D Systems, Minneapolis, Minnesota, USA) following the manufacturer's instructions.

Lung virus titer measurement

The LSs were serially diluted four-fold in 10 diluted steps in 1.5 ml Eppendorf centrifuge tubes. Thereafter, focus formation by each LS dilution was evaluated following the method described by Morimoto *et al* [28]. The antiviral activity is expressed as the reciprocal of the highest dilution that reduced the number of foci to 50% or less of the control value.

Statistical analyses

Statistical analyses were performed using the unpaired *t*-test and analysis of variance with the Tamhane test using SPSS (version 24.0; SPSS, Inc., Chicago, IL, USA). The amount of virus in the time-of-addition assay and the antiviral assay were analyzed using Student's *t*-test in Excel Toukei (version 6.0; Esumi, Tokyo, Japan). The survival rate of the mock- and virus-infected mice was analyzed using the Kaplan–Meier method and log-rank test in Excel Toukei Statcel 3 (OMS, Saitama, Japan). Values are presented as mean ± standard deviation. Statistical significance was set at $p < 0.05$.

Results

Survival rate of CE-2- and AIN-76-fed mice upon influenza virus infection

The survival rate of CE-2-fed mice after influenza infection, and the viral load indicating that the MLD_{50} was approximately $10^{2.4}$ plaque-forming units (PFU)/animal (Table 3**)**. The survival rate of AIN-76-fed mice after influenza infection, and the viral load indicating that the MLD_{50} was approximately $10^{1.5}$ PFU/animal (Table 3). The MLD_{50} value of the virus in the CE-2-fed mice was approximately 8 times higher compared to that in the AIN-76-fed mice.

Effect of SE administration on the survival rate of virus-infected mice

We treated the mice with approximately 20 mg/kg/d SE (SE-administered group) or PBS (mock-treated group) and evaluated their survival rates. The body weight of the SE-administered and mock-treated mice was not significantly different (Figure 1A). Both SE-administered and mock-treated groups started dying simultaneously after influenza virus infection, but the SE-administered group had a significantly longer survival time than

the mock-treated group (Figure 1B**)**.

Effect of SE administration on the lungs of the virus-infected mice

(Figure 2) shows an image of the lungs removed from the SE-administered and mock-treated (PBS-treated) mice. The site and extent of inflammation are indicated using arrows. On days 2 and 3 post-infection (p.i.), inflammation was observed in certain places in both SE-administered and mock-treated groups, but no significant difference was observed. However, on day 4 p.i., there was increased inflammation and overall lung enlargement in the mock-treated group than in the SE-administered group.

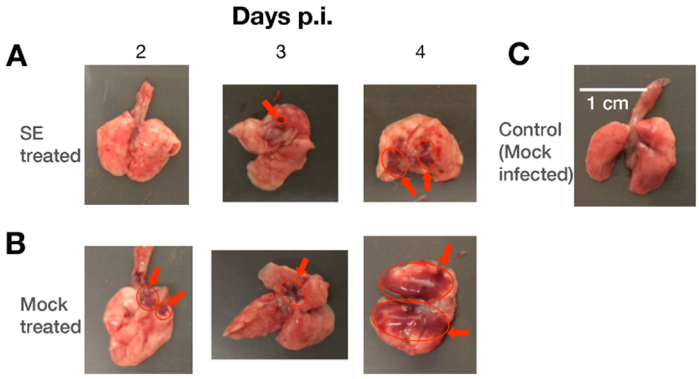

Figure 2: Effect of nasal administration of SE on the lung in influenza virus-infected mice.

Lung samples were obtained from days 2, 3, and 4 p.i. A: Photo of the lungs of SE-treated mice. B: mock-treated (PBS-treated) mice. C: Control (mock-infected) mice on day 4 p.i. The red arrows and circled areas show the inflammation in the lungs. Control mice were mock infected, which is the same as being PBS-treated, and mock-treated (PBS-treated). A photograph of the lung used in the experiment in Figure 3 was taken.

(Figure 3A) demonstrates the weight of the lungs. There was no difference between the groups on days 2 and 3 p.i., but the lung weight of the mock-treated (PBS-treated) group significantly increased compared with that of the SE-administered group on day 4 p.i. (p<0.05).

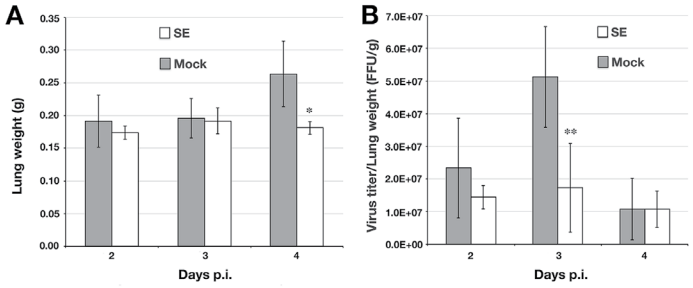

Figure 3: Effect of nasal administration of SE on lung against lung weight and virus titer in influenza virus-infected mice.

A: Lung weight. Lung samples from SE-administered or mock-treated (PBS-treated) mice were obtained from days 2, 3, and 4 p.i. B: virus titer. The lungs were homogenized using a Teflon homogenizer with 5 mL of MEM. The homogenates were centrifuged, and the supernatants were used as the virus suspension of the lungs. The details are shown in the Materials and Methods. Both treatment groups used 5 mice each on days 2, 3 and 4 p.i., respectively. *: p<0.05, **: p<0.01

The virus titer per 1 g of the lung is shown in (Figure 3B). On day 3 p.i., the SE-administered group showed significantly lower viral titers than the mock-treated group (p<0.01). In addition, the virus titer of the mock-treated group increased from day 2 to day 3, whereas there was no considerable change in the SE-administered group.

The H&E staining of the lungs is shown in (Figure 4). In the lungs of influenza virus-infected mice, the infiltration of mononuclear cells and neutrophils and shedding and necrosis of bronchiolar epithelial cells, which cause apoptotic bronchopneumonia, were observed. In the mock-treated group, the severity of infiltration of mononuclear cells and neutrophils, necrosis of alveoli, and necrosis of bronchiolar epithelial cells on Day 3 was 3, 3, and 4 on average. The influx of neutrophils into the bronchiolar lumen (black arrow), bleeding into the alveoli (white arrow), and cell death due to apoptosis (red arrow) were prominent, and the alveolar structure completely collapsed. Although similar findings were observed in the SE-administered group, the severity of infiltration of mononuclear cells and neutrophils, necrosis of alveoli, and necrosis of bronchiolar epithelial cells on Day 3 was 1, 1, and 1 on average. The degree of damage to the bronchiolar epithelium and alveoli was low.

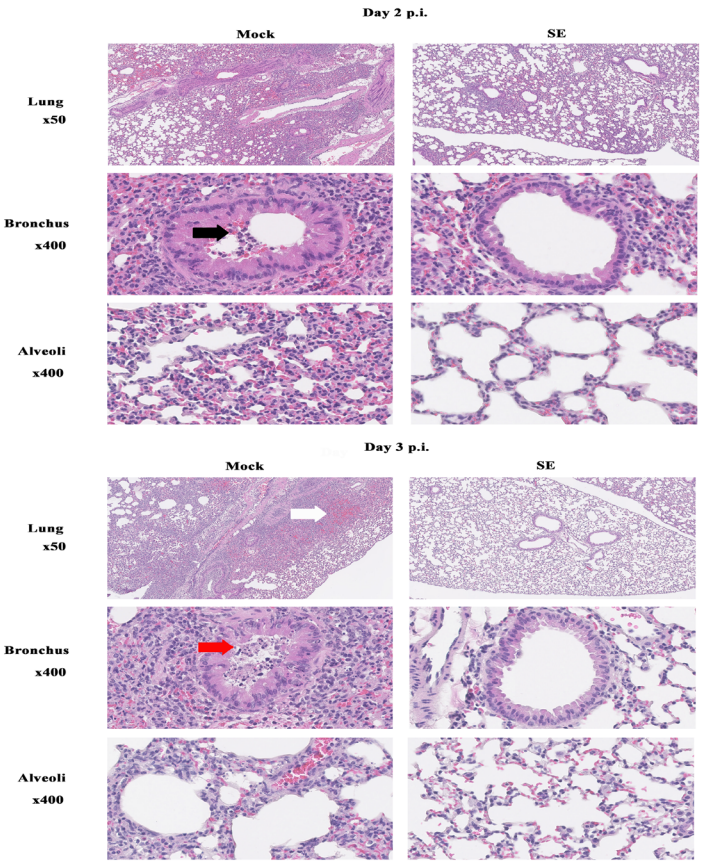

Figure 4: Pathological changes in the lungs obtained from days 2 and 3 p.i. of SE-administered and mock-treated virus-infected mice.

The lungs were sampled from the mice that were virus-inoculated under the same conditions as shown in Figure 3. In the lungs of influenza-infected mice, severe apoptotic bronchopneumonia was observed on days 2 and 3. In the SE-administered group, the degree of lesions tended to be lower. Both treatment groups used 5 mice each on days 2 and 3 p.i., respectively.

Effect of SE administration on cytokine production in influenza virus-infected mice

Figure 5A–D shows the blood IL-6, TNF-α, IFN-γ, and IL-12 levels. No significant difference was observed in the cytokines between the groups. However, IL-6 was reduced on day 4 p.i. in the SE-administered group than in the mock-treated group (p = 0.06). In addition, the IL-4 level was below the detection limit in both groups.

Effect of SE administration on total IgA and IgG antibodies in influenza virus-infected mice

(Figure 5E) and (Figure 5F) illustrates the abundance of total IgA and total IgG in the nasal cavity lavage fluid and blood, respectively. There was no significant difference between the test and mock-treated groups on days 3 and 4 p.i. Furthermore, there was no increase in antibody titer due to SE administration.

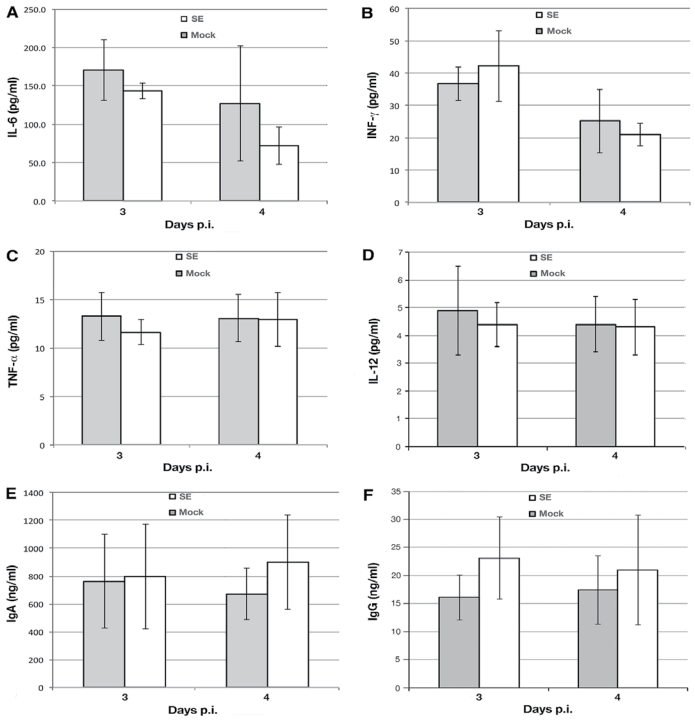

Figure 5: Levels of cytokines and IgG in the blood and IgA in accessory sinus mucus of virus-infected mice treated with SE and mock.

Blood was collected after lung removal from the mice that were virus-inoculated under the same conditions as in Figure 3. A: IL-6 level in the blood of SE-administered and mock-treated mice. B: INF-γ level in the blood. C: TNF-α level in the blood. D: IL-12 level in the blood. E: IgA level in accessory sinus mucus of SE-administered and mock-treated mice, which were days 3 and 4 p.i. F: IgG level in blood of SE-administered and mock-treated mice. Both treatment groups used 5 mice each on days 3 and 4 p.i., respectively.

Discussion

In this study, we studied the differences in susceptibility to influenza virus depending on diet in mice. AIN-76 (without

the soybean component), a standard purified feed for nutritional research using mice and rats also known as the AIN Rodent Diet, was formulated by the National Institute of Health and the American Institute of Nutrition (AIN) in 1977. The CE-2 diet comprises soybeans as a source of protein and lipid. The major difference in composition between AIN-76 and CE-2 is the nitrogen-free extract contained in CE-2. In addition, secondary metabolites such as soybean polyphenols are contained in nitrogen-free extract. The MLD_{50} value of the virus in the CE-2-fed mice was approximately 8 times higher compared to that in the AIN-76-fed mice. This result suggests that soybean may have antiviral properties *in vivo*. There is evidence that adding food ingredients to mice feed results in changes in the immune response to influenza infection [22,29]. Therefore, oral ingestion of a sample containing soybean components may have increased the protective function against the influenza virus. However, as CE-2 contains germs, bran, and other substances that have been reported to have anti-inflammatory and anti-stress effects [30,31], both soybean and gramineous plants such as barley and adlay may be responsible for the anti-influenza virus effects of the CE-2 feed [25], although the possibility of immunomodulation cannot be ruled out. As it cannot be asserted that the high antiviral activity and/or immunomodulation of CE-2 is caused only by the soybean component, we investigated the effect of specifically the soybean component on enhancing the antiviral activity and/or immunomodulation in mice.

In an effort to study the antiviral properties, specifically of the soybean component in the diet, soybean hot water extract was intranasally administered to influenza-virus infected mice, and the *in vivo* antiviral activity of soybean was examined. We observed that soybean-administered mice had an extended survival period after influenza virus infection (Figure 2). Weight loss was used to evaluate the severity of influenza virus infection. However, SE administration did not affect weight loss. In this experiment, the mice were inoculated with a lethal dose of virus, which prevented the reduction of weight loss. As the bronchioles were clogged by neutrophils and apoptotic cells on day 3 p.i. and large amounts of infiltrates accumulated inside the lungs on day 4 p.i. in the mock-treated mice, it was likely that the mice died before substantial weight loss. In SE-administered mice, the SE was administered for only 3 d; therefore, its effects on weight loss might not be apparent, and a significant reduction in weight loss was not observed. As we observed an antiviral effect of SE administered intranasally, we decided to dissect the mice and investigate the antiviral mechanism of SE (Figure 3 and Figure 5).

At the time of dissection, red, inflamed sites were observed in the lungs of both virus-infected SE-administered and mock-treated groups but not in the lungs of uninfected mice. There was no significant difference between the groups on days 2 and 3 p.i. However, the lung condition on day 4 p.i. worsened in the mock-treated group (Figure 3), with several red inflamed areas and lung enlargement, as observed with the naked eyes. Furthermore, the lung weight was significantly higher in the mock-treated group than in the SE-administered group on day 4 p.i. (Figure 4A). Hence, the lungs of the mock-treated group might have had an advanced inflammation due to influenza virus infection and were in a state of pulmonary edema, which was averted upon SE administration.

Furthermore, the virus titer per 1 g of the lung was significantly lower in the SE-administered group on day 3 p.i. than in the mock-treated group (Figure 4B). The virus titers increased from days 2 to 3 p.i. in the mock-treated group. However, in the SE-administered group, there was no significant change in the virus titer from days 2 to 4 p.i. This result suggests that the influenza virus proliferates rapidly in the lungs of mice from days 2 to 3 p.i., which is suppressed by the administration of SE, resulting in prolonged survival of mice.

Next, to examine the effect of influenza virus infection on the lung tissue, H&E-stained specimens were prepared. In the mock-treated group, a wide inflamed area, the collapse of the alveolar structure, thickening and shedding of bronchiolar epithelial cells, and necrosis were observed in the lung. These conditions are considered to be symptoms of apoptotic bronchopneumonia. Infiltration of mononuclear cells, such as neutrophils, was also observed. Furthermore, inflammation was observed in both groups, but the damage to alveolar and bronchiolic epithelial cells was less in the SE-administered group than in the mock-treated group. Highly pathogenic influenza viruses, such as the pandemic-causing H1N1 and H5N1, may lead to acute respiratory distress syndrome (ARDS) with Diffuse Alveolar Disorder (DAD). At present, the mortality rate associated with ARDS is 60%, and no effective treatment is known. However, inhibiting DAD may lead to a reduction in ARDS-induced mortality [32]. Lung histopathological findings in patients with DAD have revealed vitreous membrane formation. In this study, we used a seasonal virus strain, and DAD was not detected because the histopathological examination of the lungs was performed on days 2 and 3 p.i., representing the early stages of influenza virus infection. If soybean administration can inhibit DAD, it will reduce the mortality rate, and thus can be a new therapeutic method for DAD.

There was no significant difference in the level of IgG in the blood and IgA in the nasal cavity lavage fluid between the mock-treated and SE-administered groups. These results can be attributed to two reasons. First, soybean administration does not affect antibody production against influenza virus infection. Second, as our study involves the early stages of infection, there was less production of antibodies. In fact, in a study of virus growth in the nasal mucosa and antibody response in nasal lavage fluid after infection in influenza-infected mice, IgA produced by antibody-producing cells began to increase from day 5 p.i. [33]. However, in this study, the mice began to die from day 5 p.i. Hence, it is more

likely that SE-administration does not affect the host's antibody-producing ability.

There was no significant difference in blood cytokine levels between the groups, but a tendency to suppress blood cytokines (IL-6) was confirmed in the SE-administered group on day 4 p.i. The earliest biological responses after influenza infection are the induction of inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, and the increase in virus titer in the airway after peaking of cytokines on days 1 to 2 p.i. [34]. As IL-6 increases upon physical and psychological stresses, it has bene suggested that its production might also increase upon influenza virus infection [35]. Because the administration of SE lowered IL-6 in the blood, various inflammatory reactions caused by IL-6 were alleviated, and as a result, the survival of the mice was prolonged. IL-6 differentiates the naive T cells into Th17 cells. Th17 cells, in contrast to Th1 and Th2 cells, are involved in tissue inflammation, leading to neutrophilia. The reduction of IL-6 in the SE-administered group, in turn, suppressed its differentiation into Th17 cells, which reduced tissue inflammation caused by neutrophils. The IL-4 level was below the detection limit in both groups. IL-4 is a cytokine required for the differentiation of naive T cells into Th2 cells. Th2 cells stimulate B cells to produce antibodies and enhance humoral immunity. As we could not detect blood IL-4 and there was no increase in blood IgG in the SE-administered group, we concluded that humoral immunity was not activated by soybean.

These results suggest that the protective function in mice against the influenza virus was activated by the administration of SE. Furthermore, the suppression of infection-induced inflammation might have led to an increase in the survival of mice. However, the effects of SE-administration on natural killer cells and killer T cells involved in cell-mediated immunity are unknown, necessitating further evaluation. Our research highlighted the induction of the influenza virus defense mechanism through the feeding and administration of soybean components to individual mice, where the active soybean components were directly administered via the nasal cavity and airway mucosa. Our study had two limitations. First, the nasal administration of soybean ingredients was performed with the mice under anesthesia. After the virus infection, repeated anesthesia treatments killed the mice, and therefore, the sedative effect could not be maintained for a long time. Second, it was difficult to discern the effects of diet and those of nasal administration on influenza infection. There are two future therapeutical research directions. First, a regimen to control the aggravation of illness can be developed using soybean extract or soybean food in combination with drugs, or improved eating habits. Second, new drug candidates can be identified by elucidating the antiviral mechanism of soybean components.

Overall, our study of the antiviral effects of food ingredients might show the antiviral effects of SE in mice, which were previously demonstrated only in cultured cells, although the possibility of immunomodulation cannot be ruled out. We also suggested the possible antiviral mechanism of SE in host cells. In addition, whether the antiviral effects are the same or affected by different feeding routes in the possibility of mucosal immunization by the nostril route warrants further study. In the future, soybean extracts and soybean foods may be recognized as supplements and functional foods with antiviral properties. This study contributed to the elucidation of the mechanisms of traditional folk medicines and herbal medicines using legumes. If soybean intake in humans can alleviate the symptoms of influenza virus infections, it may pave the way for clinical and nutritional applications.

Data Availability

All data relevant to the study are included in the article. The data analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of Interest

The authors declare no conflict of interest.

Authors' contributions

All authors contributed to the study conception and design. Details of methodology were provided by Ritsuko Koketsu and Yoshinobu Okuno. Material preparation, and data collection and analysis were performed by Miyu Nakayama, Emiko Nagai, Kae Yoshioka, Yuka Horio, and Ryosuke Morimoto. The first draft of the manuscript was written by Katsuhiko Yoshizawa and Yuji Isegawa, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript, which was edited by Yuji Isegawa.

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