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Research Article

Effect of intranasal administration of *Citrullus lanatus* var. citroides (wild watermelon) juice on influenza virus–infected mice

Kae Yoshioka[1], Emiko Nagai[2], Ryosuke Morimoto[1,†], Ayaka Nakashima[3], Kengo Suzuki[3], Ritsuko Koketsu[4], Yoshinobu Okuno[4], Yoshihisa Nakano[5], Katsuhiko Yoshizawa[1], Yuji Isegawa[1,5,*]

[1]Department of Food Sciences and Nutrition, Mukogawa Women's University, Nishinomiya, Hyogo 663-8558, Japan

[2]Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Av. Bronx NY 10461, USA

[3]Euglena, Co., Ltd, Minato-ku, Tokyo 108-0014, Japan

[4]Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan

[5]Department of Applied Biological Chemistry, Graduate School of Agriculture, Osaka Metropolitan University, Sakai, Osaka 599-8531, Japan

[*]**Corresponding author:** Yuji Isegawa, Department of Applied Biological Chemistry, Graduate School of Agriculture, Osaka Metropolitan University, Gakuen-cho 1-1, Naka-ku, Sakai, Osaka 599-8531, Japan

[†]Present address: Department of Health and Nutrition, Faculty of Human Life Science, Shikoku University Tokushima 771-1192, Japan

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Abstract

The anti-influenza effects of *Citrullus lanatus* var. citroides (wild watermelon) juice (WWMJ) have been demonstrated by *in vitro* studies. However, *in vivo* studies investigating its anti-influenza activity remain limited. This study elucidated the mechanism of nasal WWMJ administration on prolonging the lifespan of virus-infected mice. BALB/c mice were infected with influenza A/PR/8/34 (H1N1) virus, and their survival rate was observed for 2 weeks. The mice were divided into two groups: WWMJ-treated and mock-treated, and were inoculated with phosphate-buffered solution. Viral and antibody titers, lung weight, and cytokine levels were measured. Histopathological examinations of lung samples were performed. Lung inflammation was more severe and viral titers were higher in the mock-treated group than in the WWMJ-treated group. In the WWMJ-treated group, blood interleukin-6 levels were reduced and the infiltration of neutrophils and apoptotic cells into the bronchi was suppressed, leading to less severe lung inflammation and, thus, prolonged survival. Nasal WWMJ administration effectively reduced lung inflammation in virus-infected mice and extended their lifespan, highlighting the potential of WWMJ as a functional food with antiviral properties. Further research is needed on WWMJ response in human bodies to determine its efficacy, however, this study established avenues of potential clinical and nutritional applications.

Keywords: Influenza Virus; Antiviral Reagent; Wild Watermelon; Cytokine; Inflammation

Abbreviations

BSA	:	Bovine Serum Albumin
DAD	:	Diffuse Alveolar Disorder
FBS	:	Fetal Bovine Serum
H&E	:	Hematoxylin and Eosin
Iga	:	Immunoglobulin A
IFN-Γ	:	Interferon-Γ
IL-6	:	Interleukin-6
LS	:	Lavage Supernatant
MEM	:	Minimum Essential Medium
P.I.	:	Post-Infection
PBS	:	Phosphate-Buffered Saline
PFU	:	Plaque-Forming Units
TNF-A	:	Tumor Necrosis Factor-A
WWMJ	:	Wild Watermelon Juice

Introduction

Throughout human history, influenza viruses, particularly influenza A viruses, have caused numerous epidemics and pandemics, resulting in public health and economic crises [1]. Influenza virus infections can lead to complications, such as pneumonia and ischemic heart disease, significantly increasing hospitalization and mortality rates, especially among young children and geriatrics [2,3]. The fooling antiviral drugs are commonly used to treat influenza virus infections: M2 ion channel inhibitors such as amantadine and rimantadine, neuraminidase inhibitors, such as zanamivir and oseltamivir, RNA polymerase inhibitors, such as favipiravir (FPV), and endonuclease inhibitors within the RNA polymerase, such as baloxavir marboxil. [4]. However, recently, influenza A viruses resistant to neuraminidase inhibitors have been detected [5-8]. Moreover, the adverse effects of RNA polymerase inhibitors have been reported [9-11]; in clinical practice, the teratogenic risk associated with FPV use may be higher than that of other drugs in the same class [12]. Furthermore, reduced susceptibility to baloxavir has been documented [13].

As several medicinal plants also serve as food, adding secondary herbal compounds to the typical diet provides therapeutic effects with few or no side effects [14,15]. The anti-influenza effects of food extracts have been confirmed by *in vivo* and *in vitro* studies [16-25]. For instance, in our previous studies [26,27], we found that extracts from *Citrullus lanatus* var. citroides (wild watermelon [WWM]) may suppress viral replication by enhancing the defense capabilities of infected cells. Additionally, we previously investigated the anti-influenza properties of the flavonoid-based components of WWM juice (WWMJ), especially phytoestrogens [27,28]. In particular, 8-prenylnaringenin inhibits influenza viruses from entering cells [27] by rapidly entering and accumulating intracellularly [28]. Although the aforementioned *in vitro* studies highlight the antiviral potential of WWMJ, *in vivo* evidence supporting the anti-influenza effects of WWMJ remains insufficient. Therefore, this study determined the inhibitory effect of WWMJ on influenza virus in infected mice and elucidate its mechanism of action. Thus, this study provided insight into the therapeutic effects of WWMJ as a food supplement.

Materials and Methods

Cells and viruses

Madin–Darby Canine Kidney cells were grown in Eagle's minimum essential medium (MEM; Sigma-Aldrich, St. Louis, MO, USA) containing 7% fetal bovine serum (FBS; Biowest Biotechnology, Nuaillé, France). For cell infection, the influenza A virus H1N1 (A/PR/8/34) was diluted in FBS-free MEM supplemented with 0.4% bovine serum albumin (BSA, Fraction V; Sigma-Aldrich) and added to the cells at a multiplicity of infection 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 the influenza virus A/PR/8/34(H1N1) was derived from serial passages of pulmonary homogenates from infected naive mice. The viral stocks were stored at −80 ˚C.

WWMJ preparation

WWMJ (Euglena, Tokyo, Japan) was treated at 80 ˚C for 30 min. Proteins were removed from the WWMJ sample to determine the antiviral activity of lectins.

Focus-forming assay

Focus formation was achieved by employing the method described by Morimoto, et al. [26] Each viral dilution was selected to obtain approximately 30 focus-forming units per well. Antiviral activity was expressed as the reciprocal of the highest dilution that reduced the number of foci to ≤ 50% of the control value.

Ethics statement

This study was approved by the Animal Experiment Committee of the Hyogo College of Medicine, Nishinomiya, Hyogo, Japan (approval number: 15-007). Additionally, the study protocol on

animal handling complied with 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).

Mice feeding and virus infection

For each group, 20 six-week-old female BALB/c mice (20-22 g) were obtained from Charles River Laboratories International (Wilmington, MA, USA). The mice were divided into two groups and housed under appropriate conditions in a laminar flow hood at the Animal Experiment Facility of the Hyogo College of Medicine under suitable conditions for a 1-week acclimatization period and 2-week experimental period (12:12 h light/dark cycle, 25 ˚C temperature, and 50% relative humidity). The mice were housed in plastic cages (in groups of four or five) with paper bedding (Paper Clean; SLC, Hamamatsu, Japan) and fed an AIN-76 purified mouse diet (CLEA Japan, Tokyo, Japan) for 1 week. After 1 week, the 20 seven-week-old BALB/c female mice were further divided into two groups, with 10 mice each. The mouse-adapted strain of the A/PR/8/34 influenza virus [20 µL each; 75 plaque-forming unit (PFU)/mouse] was administered intranasally through one nostril to all mice except controls.

Evaluation of the anti-influenza effects of WWMJ on influenza virus–infected BALB/c mouse

On day 6, in section 2.5, the experimental group of 10 mice was intraperitoneally injected with triple anesthesia (medetomidine hydrochloride, midazolam, and butorphanol tartrate; 0.2 mL/mouse). Approximately 20 µL of WWMJ (20 mg/kg/d) was inoculated into one nostril of each mouse in the treatment group, and an equal volume of Phosphate-Buffered Saline (PBS) was administered to the mock-treated group. The median lethal dose (50%) of the used virus lot was $10^{1.5}$ PFU in the mice fed with AIN-76 [29]. On day 7, each mouse was intraperitoneally injected with the triple anesthesia, and 20 µL of virus solution (75 PFU/mouse) was inoculated into one nostril of each mouse [29]. After 1 h, the treatment group was inoculated with WWMJ, while the mock-treated group was inoculated with PBS. The WWMJ- and mock-treated groups were inoculated with WWMJ and PBS, respectively, for 3 d. Changes in survival rates were monitored for 14 d after virus infection.

Collection of plasma, nasal cavity lavage fluid, and lung samples

To prevent blood coagulation, 0.2 mL of 1,000 U/mL heparin solution was intraperitoneally administered to each mouse and left for 30 min. After 30 min, 0.3 mL of triple anesthesia (medetomidine, midazolam, and butorphanol) was intraperitoneally administered to each mouse. First, the femoral vein was cut, blood was collected, and plasma was collected by centrifuging the blood samples at 800 × *g* for 15 min at 4 ˚C. The lungs were removed from the trachea and weighed. Some lung tissue was mixed with 5 mL of MEM (-) for homogenization. After centrifugation at 1,000 × g for 20 min at 4 ˚C, the supernatant was collected and used as a sample. The remaining lung tissue was fixed with neutral buffered formalin solution to facilitate the preparation of lung specimens. PBS (-) (500 µL) was injected using a syringe, and the nasal cavity lavage fluid was collected, centrifuged at 800 × *g* for 15 min at 4 ˚C, and the Lavage Supernatant (LS) was used as a sample.

Measurement of lung virus titer

The LS samples were serially diluted four-fold in 10 dilution steps in 1.5 mL Eppendorf centrifuge tubes. Thereafter, focus formation by each LS dilution was evaluated following the same method in focus-forming assay.

Measurement of immunoglobulin G (IgG) levels in the blood and IgA levels in the nasal cavity lavage fluid

The levels of IgA and IgG were measured using the Mouse IgA ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX, USA) and mouse IgG ELIZA Quantitation Set (Bethyl Laboratories), respectively, following the manufacturer's instructions.

Measurement of blood cytokines

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

Histopathological examination of the lungs

A neutral buffered formalin solution was infused through the trachea to inflate the alveoli; then, the lungs were removed from the trachea. The lungs were soaked in a neutral buffered formalin solution, washed with water, immersed in 70% ethanol solution for 30 min, set in an automatic embedding device, and dehydrated with ethanol. Afterwards, the ethanol was replaced with xylene. Subsequently, the lungs were embedded in paraffin to prepare blocks, which were sliced into 2-µm-thick sections using a microtome. The sections were attached to a glass slide, dried, and deparaffinized using xylene and 100% alcohol. Subsequently, the nucleus and cytoplasm were stained with hematoxylin and eosin solutions, respectively (H&E slides). A mounting medium was dropped onto the edge of the slide, and the slide was covered with a cover glass and dried at approximately 25 ˚C for 1 d. The H&E-stained slides were scanned and converted into whole-slide images using Nanozoomer (Hamamatsu Photonics, Shizuoka, Japan), and the images were observed using the NDP.view2 image viewing software. The histopathological terminology used for describing non-neoplastic lung lesions was in accordance with

the International Harmonization Nomenclature and Diagnostic Criteria for Lesions in Rats and Mice Project [24]. The infiltration severity of mononuclear cells and neutrophils as well as necrosis of alveoli and bronchiolar epithelial cells were assessed using a four-point scale: 1 (minimal), 2 (mild), 3 (moderate), and 4 (marked). Histopathological examinations were conducted by the pathologists certified by the International Academy of Toxicologic Pathology.

Apoptotic cells were stained using the ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, Burlington, MA, USA) according to the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining protocol.

Statistical analyses

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

Results

Effects of WWMJ treatment on the survival rate and lung inflammation of virus-infected mice

The survival rate of the virus-infected mice is shown in Figure 1. The mock-treated mice began to die on day 5 post-infection (p.i.), whereas the WWMJ-treated mice began to die on day 7 p.i. The significant difference in survival rate between the two groups ($p = 0.02$) confirmed the life-prolonging effect of intranasal WWMJ administration.

The photographs of the lungs removed at the autopsy are shown in Figure 2. On days 2-4 p.i., the area occupied by dark-red foci expanded in the lung samples from the mock-treated mice; in contrast, the extent of the area decreased in in the lung samples from the WWMJ-treated mice.

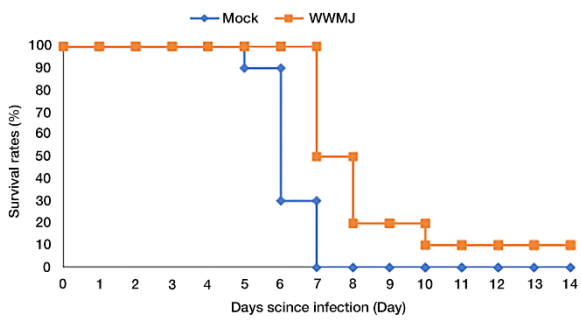

Figure 1: Effect of wild watermelon juice (WWMJ) nasal administration on the survival rate of influenza virus–infected mice.

Survival rates of WWMJ- and mock-treated mice. Both groups contained 10 mice each. Twenty microliters of WWMJ or Phosphate Buffered Solution (PBS) were inoculated via the nasal cavity of the WWMJ- and mock-treated mice for 5 d [i.e., 1 d before virus infection up to day 3 post-infection (p.i.)]. All mice were inoculated with 75 plaque-forming units (PFU) of the same influenza virus. *$p < 0.05$

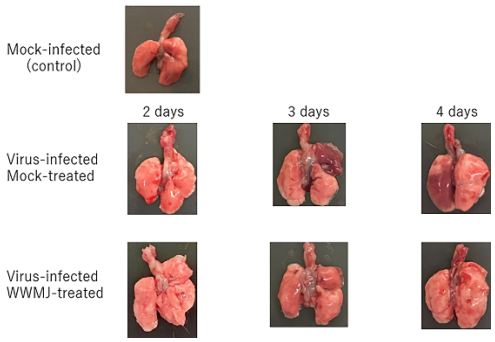

Figure 2: Effect of wild watermelon juice (WWMJ) nasal administration on the lungs of influenza virus–infected mice.

Lung samples were obtained on days 2, 3, and 4 post-infection (p.i.). Photographs of control (mock-infected) mice on day 4 p.i. Photographs of the lungs of influenza virus–infected mock-treated (phosphate buffered saline–treated) mice and WWMJ-treated mice. Red arrows and circled areas indicate lung inflammation.

Effect of WWMJ treatment on the influenza virus level in virus-infected mice

The weight of the lungs excised at the time of autopsy is shown in Figure 3A. The weight of the lung samples from the mock-treated mice increased as the duration of post-infection increased. In contrast, the increment in the lung weight of the WWMJ-treated mice was lower than of the mock-treated mice. A significant difference in lung weight was observed between the two groups on day 4 p.i. The viral titer per gram of lung tissue is shown in Figure 3B. The viral titer in the lung tissue obtained from the mock-treated mice increased and peaked on day 3 p.i. then decreased on day 4 p.i.. Meanwhile, viral titer obtained from the WWMJ-treated mice, exhibited a low rate of increase. Specifically, the viral titer in the lung tissue from the WWMJ-treated mice was significantly lower than that in the lung tissue from the mock-treated mice on days 3 and 4 p.i.

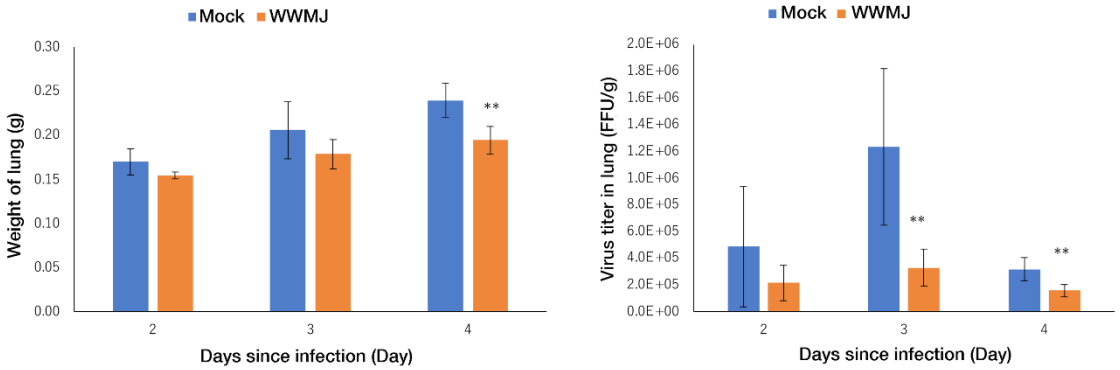

Figure 3: Effect of wild watermelon juice (WWMJ) nasal administration on lung weight and virus titer in influenza virus–infected mice.

Pathological changes in the lungs obtained on days 3 and 4 post-infection (p.i.) in WWMJ-treated and mock-treated virus-infected mice. A: Lung weight. Lung samples from WWMJ- or mock-treated (phosphate buffered saline–treated) mice were obtained on days 2, 3, and 4 p.i. The lungs were homogenized in 5 mL of minimum essential medium using a Teflon homogenizer. The homogenates were centrifuged, and the supernatant was used as lung viral suspension. The details are presented in the Materials and Methods section. Five mice from each treatment group were used on days 2, 3, and 4 p.i. $^{**}p < 0.01$

Effect of WWMJ treatment on total IgA and IgG levels in virus-infected mice

The levels of influenza-specific IgA and IgG in the nasal washings and blood samples, respectively, are shown in Figure 4. No significant difference between the two groups on days 2–4 p.i. Antibody titers did not increase in the WWMJ-treated mice.

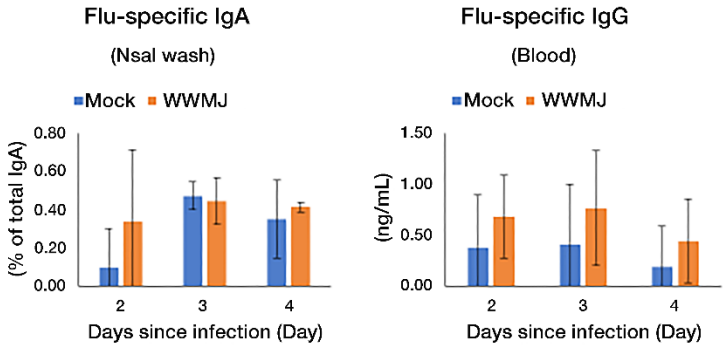

Figure 4: Effect of wild watermelon juice (WWMJ) nasal administration on the levels of influenza virus–specific IgA in accessory sinus mucus and IgG in the blood of virus-infected mice.

The lungs were sampled from virus-inoculated mice under the same conditions (Figure 3). Severe apoptotic bronchopneumonia was observed in the lungs of influenza-infected mice on days 3 and 4 p.i. The degree of lesions was lower in the WWMJ-treated group.

Antibody titers were measured using nasal washes, and the supernatant was obtained by centrifuging the blood. Five mice were used on days 2-4 p.i.

Effect of WWMJ treatment on cytokine production in virus-infected mice

The blood levels of IL-6, TNF-α, and IFN-γ are shown in Figure 5. No significant differences between the two groups in terms of cytokine levels except for IL-6 levels on days 2 ($p = 0.06$) and 4 p.i. ($p = 0.05$). IL-6 activity was suppressed in the WWMJ-treated group. The IL-4 levels in both groups were below the detection limit.

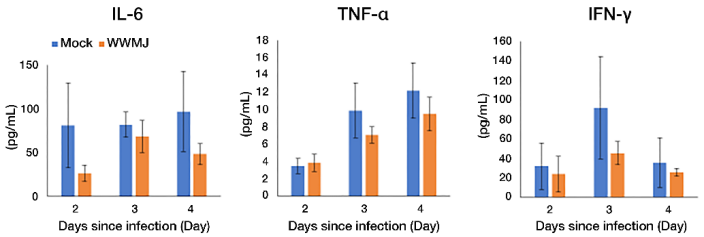

Figure 5: Levels of cytokines in virus-infected mice treated with wild watermelon juice (WWMJ) and phosphate-buffered solution (mock).

Blood was collected after lung removal from virus-inoculated mice under the same conditions as in Figure 3. Cytokine levels were measured in the supernatant obtained by centrifuging blood using a Quantikine ELISA kit. Blood IL-6 levels in WWMJ- and mock-treated mice. INF-g level in the blood. TNF-α level in the blood. Five mice from each treatment group were used on days 2, 3, and 4 p.i.

Effect of WWMJ treatment on the lungs of virus-infected mice

H&E staining (Figure 6) revealed the loss and necrosis of bronchiolar and alveolar epithelial cells in the lung tissue from the mock-treated mice (day 3 p.i.), which had progressed by day 4 p.i. Meanwhile, the thickening of bronchiolar epithelial cells was observed in the lung tissue from the WWMJ-treated mice on days 3 and 4 p.i. However, the loss and necrosis were not apparent compared with the lung tissue from the mock-treated mice, which exhibited necrotizing bronchopneumonia. Moreover, we observed in the lung tissue of mock-treated mice the infiltration of mononuclear cells, such as neutrophils, bleeding into the alveoli, and collapse of the alveolar structure (Figure 6). Additionally, 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 mononuclear cells into the bronchiolar lumen (black arrow) and bleeding into the alveoli (red arrow) were prominent, and the alveolar structure completely collapsed. Conversely, the lung tissue from the WWMJ-treated mice only exhibited minor collapse of the alveolar structure (Figure 6). In addition, the severity of mononuclear cell and neutrophils infiltration as well as necrosis of alveoli and bronchiolar epithelial cells on day 3 was 1, 1, and 1 on average. Influenza virus immunostaining (Figure 7) showed that the number of influenza virus–positive cells increased from the bronchiolar epithelium to the alveoli as the infection progressed on days 3 and 4 p.i., indicating the spread of the infection. Influenza virus–positive cells were more numerous in the lung tissue of the mock-treated mice than in that of the WWMJ-treated mice (Figure 7). Neutrophil immunostaining revealed that several cells were neutrophil-positive in the areas where mononuclear cell infiltration was observed in the lung tissue from the mock-treated mice (Figure 8). In addition, TUNEL positive images were observed in bronchiolar and alveolar epithelial cells (Figure 9), which were more prominent in the lung tissue from the mock-treated mice than in that of WWMJ-treated mice.

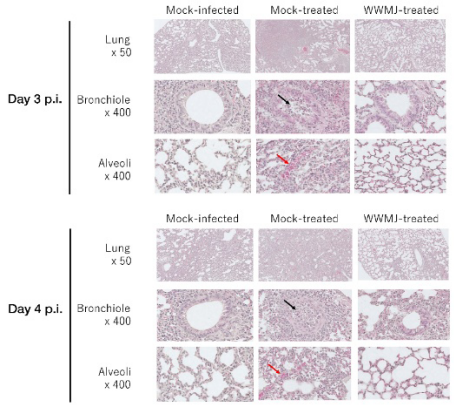

Figure 6: Hematoxylin–eosin staining of the lungs obtained on days 3 and 4 post-infection (p.i.) from mock-infected and virus-infected mice treated with wild watermelon juice (WWMJ) and phosphate-buffered solution (mock).

The observation magnification and structural names are shown on the left side of the image. Mononuclear cell infiltration (black arrow) and hemorrhage in the alveoli (red arrow) were observed.

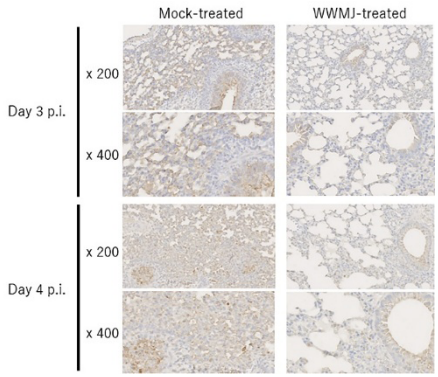

Figure 7: Influenza virus staining of the lungs obtained on days 3 and 4 post-infection (p.i.) from virus-infected mice treated with wild watermelon juice (WWMJ) and phosphate-buffered solution (mock).

Staining was performed using an anti-influenza virus polyclonal antibody (1:500) as the primary antibody and 3,3′-diaminobenzidine for color development. Magnified images are shown on left. Brown cells were observed in the bronchiolar epithelium and alveoli.

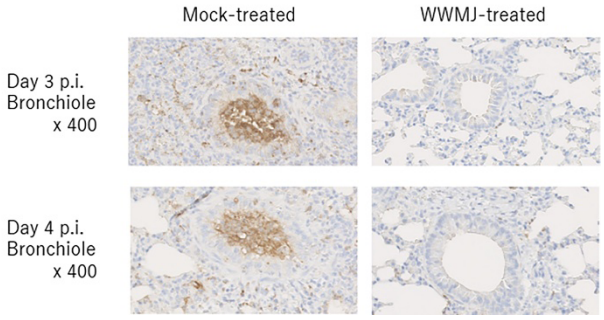

Figure 8: Neutrophil staining of the lungs obtained on days 3 and 4 post-infection (p.i.) from virus-infected mice treated with wild watermelon juice (WWMJ) and phosphate-buffered solution (mock).

Magnified images are shown on left. Staining was performed using an anti-neutrophil antibody (1:100; Abcam) as the primary antibody and 3,3′-diaminobenzidine for color development. Neutrophil infiltration (brown area) was observed mainly in bronchiolar epithelial cells.

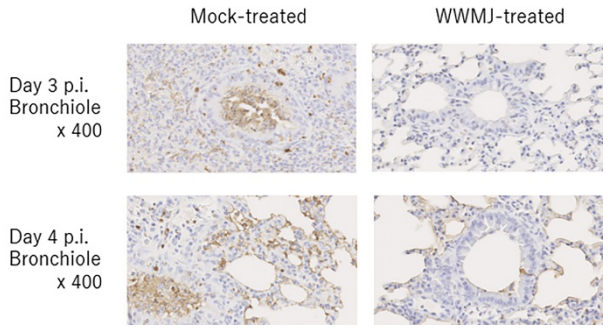

Figure 9: TUNEL staining of the lungs obtained days 3 and 4 post-infection (p.i.) from virus-infected mice treated with wild watermelon juice (WWMJ) and phosphate-buffered solution (mock).

Magnified images are shown on left. Apoptotic cells were stained using the ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, Burlington, MA, USA) according to the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining protocol. TUNEL-positive cells (brown areas) were observed in the bronchial and alveolar epithelia.

Discussion

For the administration of WWMJ, the intranasal route was selected. This is because the intranasal administration of soybean extract conducted by Nakayama et al. [29] suggested not only local effects but also systemic effects. Therefore, systemic effects were also anticipated for the administration of WWMJ. Compared with the mock-treated mice, the survival period of the WWMJ-treated virus-infected mice was prolonged. However, accelerated weight recovery was not observed, probably due to the extremely high viral load, resulting in a very high mortality rate. Nonetheless, the anti-influenza effects of intranasal WWMJ administration were confirmed, thereby necessitating further analysis (i.e., histopathological examination of the lung tissue from virus-infected mice) to determine its mechanism of action.

Regarding the direct inhibition of viral replication in the WWMJ-treated virus-infected mice, the significantly lower viral titer in the lung tissue from the WWMJ-treated mice than that in the lung tissue from the mock-treated mice as well as reduced proliferation of infected cells on days 3 and 4 p.i.. This suggested that the

influenza virus might have replicated rapidly in the lungs of mice on days 2–3 p.i., and the intranasal administration of WWMJ during this window suppressed rapid viral proliferation, leading to prolonged survival.

Moreover, regarding secondary reductions in inflammation, the lung samples from the mock-treated mice were heavier and had more numerous areas of inflammation than the lung samples from the WWMJ-treated mice. This may be due to the lungs of the mock-treated mice developing a condition resembling pulmonary edema, characterized by an advanced stage of infection and alveolar infiltration by neutrophils.

Furthermore, significant desquamation and necrosis of bronchiolar and alveolar epithelial cells as well as infiltration of neutrophils and apoptotic cells were observed in the lung samples from the mock-treated group. The severity of inflammation in the lung samples from the WWMJ-treated group was lesser than that in the lung samples from the mock-treated group. Thus, intranasal WWMJ administration may suppress influenza virus-induced lung inflammation.

However, the levels of influenza-specific IgA and IgG in the nasal washings and blood samples, respectively, did not significantly differ between the mock- and WWMJ-treated groups, probably because intranasal WWMJ administration may not have affected antibody production, or the infection was in its early stages before antibodies were produced. Research on antibody responses in the nasal mucosa of influenza-infected mice has reported that IgA produced by antibody-producing cells begins to increase from day 5 p.i. [30]. However, in the present study, the mice began to die from day 5 p.i., indicating that WWMJ administration does not affect the antibody production capacity of the host.

Production of Reactive Oxygen Species (ROS), particularly superoxide, is an important host defense mechanism for killing invading pathogens. However, excessive superoxide levels can exacerbate lung injury caused by influenza A virus infection [31]. WWMJ significantly suppresses ROS production; hence, it may not only prevent the development of acute lung injury but also the exacerbation of lung disease [31]. Citrulline and pinoresinol, which are present in WWMJ, possibly reduce intracellular ROS accumulation and inflammatory cytokines, controlling neutrophil recruitment and preventing acute lung injury [32]. One proposed mechanism for this activation is the nuclear factor erythroid 2-related factor 2 signaling pathway, exerting antioxidant effects against oxidative stress [33,34]. Therefore, antioxidants in WWMJ may also be involved in the suppression of WWMJ-induced lung disease in influenza-infected mice.

Excluding IL-6 levels, which tended to decrease in WWMJ-treated mice, no significant differences were detected in the blood cytokine levels across the groups. IL-6 is an important inflammatory cytokine in the initial phase of influenza virus clearance [35]. However, it is highly elevated in the blood and is overactivated in patients with severe influenza. The IL-6 levels in patients with severe influenza and pneumonia correlate with the frequency of intensive care unit admissions [36]. IL-6 stimulates the differentiation of naive T cells into Th17 cells, which are involved in neutrophils-mediated tissue inflammation. In this study, the WWMJ-treated mice exhibited reduced blood IL-6 levels and neutrophil infiltration, suggesting that the differentiation of naive T cells into Th17 cells may have been inhibited, potentially decreasing neutrophil-mediated tissue inflammation.

Furthermore, the level of IL-4, a cytokine necessary for the differentiation of naive T cells into Th2 cells, was below the detection limit in all mice. Th2 cells primarily stimulate B cells to produce antibodies, thereby enhancing humoral immunity. The absence of detectable IL-4 in the blood and lack of increased serum IgG levels indicate that intranasal WWMJ administration may not activate humoral immunity.

The inhibitory effect of WWMJ on the induction of inflammatory cytokines by Particulate Matter 2.5 (PM2.5) [32] was much stronger than that of influenza virus infection (Figure 5). Therefore, the anti-inflammatory effect of WWMJ alone cannot explain the survival benefits observed in infected individuals.

In summary, intranasal administration of WWMJ exhibits antiviral activity against influenza *in vivo*. WWMJ contributes to prolonging survival by directly inhibiting viral replication at the site of influenza virus infection and reducing infection inflammation.

While this study could not eliminate the possibility of immunomodulatory effects, it has demonstrated the antiviral effects of WWMJ in mice, which had previously been demonstrated only in cultured cells. It also suggests a mechanism for the antiviral action of WWMJ in host cells. Additionally, the possibility of mucosal immunity via the intranasal route cannot be eliminated, and further research is needed on the specific differences between intranasal and oral administration of WWMJ. In the future, WWMJ may be recognized as a supplement or functional food with antiviral properties. This study contributes to elucidating the mechanisms of traditional complementary medicine using WWMJ. If human consumption of WWMJ alleviates symptoms of influenza virus infection, this could open avenues of clinical and nutritional applications.

Conclusions

Intranasal WWMJ administration to influenza-infected mice suppressed viral replication in the lungs, reduced lung inflammation, and prolonged the survival period of influenza-infected mice. WWMJ directly suppressed viral replication at the site of influenza virus infection, thereby reducing inflammation. The study provides

promising preliminary evidence and a basis for future research rather than evidence of efficacy in humans. Thus, further research is necessary in the metabolic and biochemical differences of the antiviral effects of WWMJ nasal and oral administration. In addition to epidemiological research to determine whether WWMJ intake can alleviate influenza infection in humans.

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Data Availability

All data relevant to this study are included in the article, and are available from the corresponding author upon request.

Authors' contributions

All authors contributed to the conception and design of this study. Emiko Nagai, Ritsuko Koketsu and Yoshinobu Okuno: Methodology. Kae Yoshioka, Miyu Nakayama, and Ryosuke Morimoto: Investigation, Formal analysis and data curation. Ayaka Nakashima and Kengo Suzuki provided materials (WWMJ). Yoshihisa Nakano: Supervision. Katsuhiko Yoshizawa: Investigation and Writing of the original draft. Yuji Isegawa: Conceptualization, Funding acquisition, project administration, writing the original draft, writing the review, and editing. All authors have read and commented on previous versions of the manuscript and approved the final manuscript, which was edited by Yuji Isegawa.

Ethics approval and consent to participate

This study was approved by the Animal Experiment Committee of the Hyogo College of Medicine, Nishinomiya, Hyogo, Japan (approval number: 15–007).

Patient consent for publication

Not applicable.

Conflict of interest

Ayaka Nakashima and Kengo Suzuki are employees of Euglena Co. Ltd. The other authors declare no conflicts of interest.

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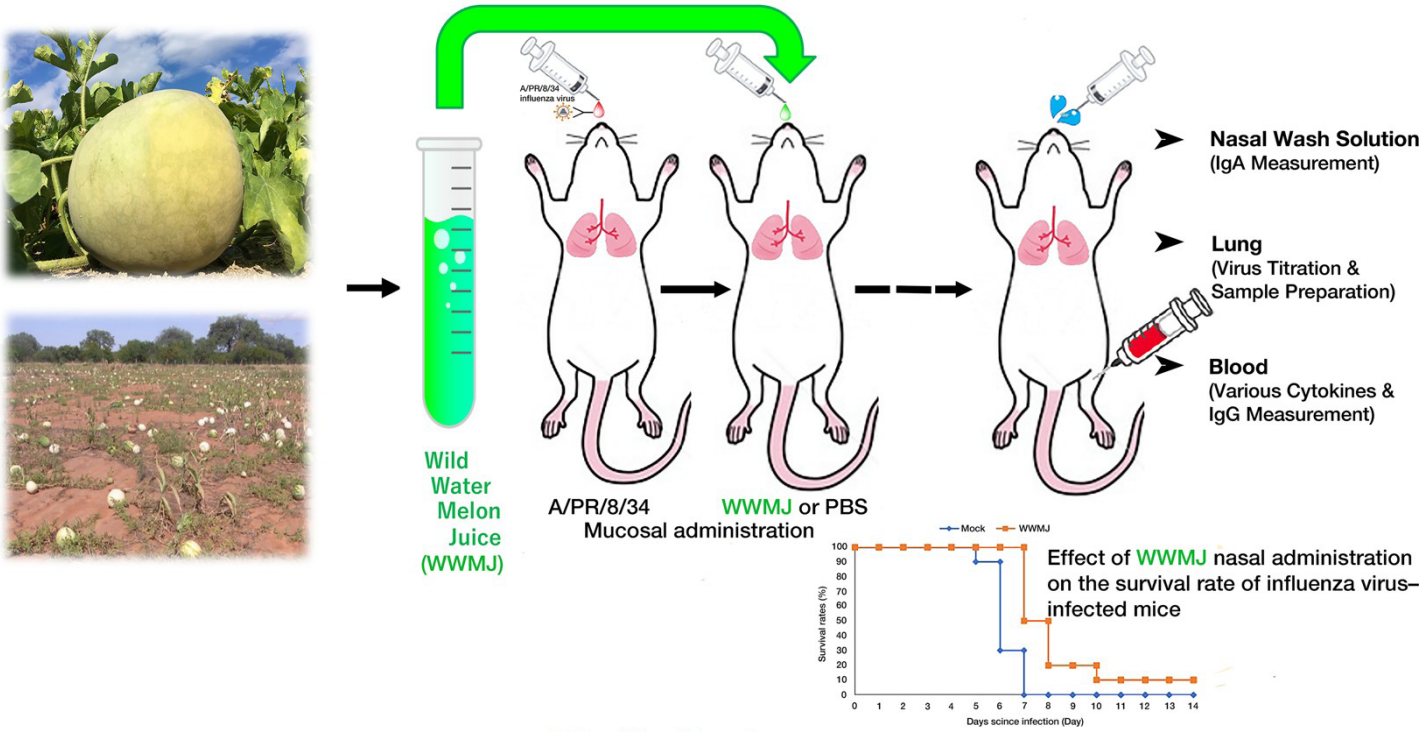

Effect of intranasal administration of *Citrullus lanatus* var. citroides (wild watermelon) juice on influenza virus–infected mice