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Tofuyo (fermented soybean food) extract prolongs the survival of mice infected with influenza virus

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Abstract. Fermented soybean, known as tofuyo, is a traditional food in Okinawa (Japan). Tofuyo extract is obtained from the supernatant of fermented soybean after homogenization, filtration and centrifugation. In this study, we analyzed the *in vitro* effect of tofuyo extract on influenza virus using immunochromatography of the influenza virus nucleoprotein (NP). The results showed that tofuyo extract does not have a strong inhibitory effect on NP. This finding was confirmed by an assay for virus activity using embryonated eggs. To investigate the potential anti-influenza virus activity of tofuyo *in vivo*, 5 nude mice (BALB/cSlc-*nu*/*nu*) were administered drinking water supplemented with tofuyo extract the day prior to intranasal inoculation with influenza virus, while the control group (5 mice) was administered tofuyo extract-free water. Control group mice (40%) were dead within 14 days, while the mice that were administered water containing tofuyo extract survived. Nutrient component analysis showed that tofuyo extract contains proteins, carbohydrates, vitamins and minerals that provide nutritional value, which may help to maintain good health.

Introduction

Tofuyo (fermented soybean food) is a traditional local specialty product of Okinawa, Japan (Fig. 1), and it is believed that the nobles of Okinawa during the Ryukyu Dynasty period identified tofuyo as a nutritious food good for recovery from illness. Tofuyo is prepared by fermenting tofu in a mixture of alcoholic, sake-like beverage (known as awamori) along with rice malt and red yeast, which gives tofuyo its distinctive red color. It has been demonstrated that tofuyo contains a variety of functional components and that it has numerous properties as a healthy food, including protection of the stomach wall and mucous membranes, as well as inhibition of cholesterol synthesis (1,2).

Influenza occurs every winter and the virus is transmitted through the mucous membrane of the nose and the respiratory tract. Therefore, the aim of this study was to investigate whether or not tofuyo ingestion has an effect on influenza infection. As a result, an animal experiment was conducted to determine the effects of tofuyo extract on the influenza virus and influenza infection *in vitro* and *in vivo*; tofuyo extract nutrient components were also analyzed.

Materials and methods

Preparation of tofuyo extract. Tofuyo prepared from red and yellow kojis (2) was supplied by Benihama Co., Ltd. (Okinawa, Japan). Tofuyo was suspended in 3 volumes of distilled water, homogenized, and then mixed at room temperature for 1 h. The mixture was centrifuged at 12,000 x g for 15 min, and the resulting precipitate was removed. The supernatant was filtered, and the filtered solution was used as the tofuyo extract.

Influenza virus. Influenza virus strain PR8 [A/Puerto Rico/8/34 (H1N1)] was used in this study (3).

In vitro analysis. The culture supernatant (3.16×10^{14} $TCID_{50}$/ml) of Madin-Darby canine kidney (MDCK) cells infected with influenza virus strain PR8 was mixed with tofuyo extract and incubated. Changes in the amount of nucleoprotein (NP) of influenza virus were measured using immunochromatography (ESPLINE® Influenza A&B-N, Fujirebio, Inc., Tokyo, Japan). Similarly, changes in the sensitivity of embryonated chicken eggs to influenza virus were analyzed by injecting the above mixture into 12-day-old embryonated chicken eggs.

In vivo analysis. Influenza virus strain PR8 was inoculated into embryonated chicken eggs. Influenza virus-infected allantoic fluid was collected 48 h following incubation [a unit for hemagglutinin (HA) value of 10^9], and a 10^5-fold diluted solution of allantoic fluid was nasally inoculated into nude mice (BALB/cSlc-*nu*/*nu*) by inhalation through the nose under anesthesia. For the tofuyo extract-administered group, tofuyo extract solution, which was prepared by adding 1 ml of extract to 200 ml of water, was administered as drinking water from the day prior to inoculation. Tofuyo extract-free water was

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Key words: tofuyo, awamori, influenza, fermented food, healthy food

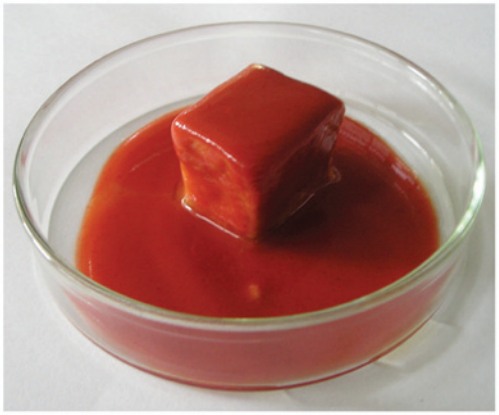

Figure 1. Tofuyo prepared from red and yellow kojis.

administered to the control group. Following inoculation, mice were observed for changes in weight against time. Lungs were then collected from the mice that died and those that survived until 14 days following inoculation. Each collected lung was homogenized to prepare a 0.5% homogenate, and the amount of NP of the influenza virus was measured by immunochromatography (ESPLINE® Influenza A&B-N). The animals were treated in accordance with the procedures approved by the Animal Experiment Committee of the University of Ryukyus (Nishihara, Japan). This experiment was permitted by the Guidelines for the Care and Use of Laboratory Animals, approved by the Animal Experiment Committee of the University of Ryukyus (permit no. 5465).

Nutrient component analysis. According to the Analytical Manual for Standard Tables of Food Composition in Japan (4) and the Tables of Food Composition (5), the content of energy, water, protein, lipid, carbohydrate, ash, sodium, potassium, calcium, magnesium, phosphorus, iron, zinc, copper, manganese, vitamins B_1, B_2 and B_6, soluble, insoluble and total dietary fibers, sodium chloride equivalent and alcohol was measured in the tofuyo extract. In addition, the content of arginine, lysine, histidine, phenylalanine, tyrosine, leucine, isoleucine, methionine, valine, alanine, glycine, proline, glutamic acid, serine, threonine, aspartic acid, tryptophan as well as cysteine and cystine was measured using amino acid autoanalysis and high-performance liquid chromatography.

Results

In vitro effect of tofuyo extract on nucleoprotein and influenza virus infection. The culture supernatant of MDCK cells infected with influenza virus strain PR8 was mixed with tofuyo extract, phosphate-buffered saline (PBS) or 8.5% ethanol at a dilution of 1:9, and incubated at 37˚C for 30 min. The amount of NP in the influenza virus was then compared by immunochromatography. As a result, incubation with 8.5% ethanol and tofuyo extract showed no major differences in the amount of NP compared to PBS (Fig. 2).

Following incubation the above samples were inoculated into the allantoic cavity of embryonated chicken eggs, and allantoic fluid was collected 48 h later. Sensitivity to the virus

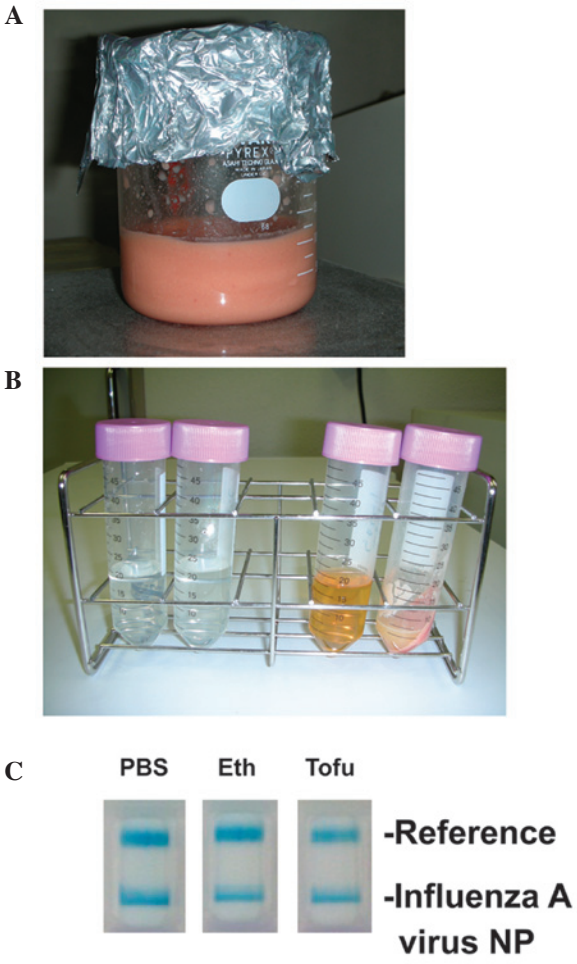

Figure 2. *In vitro* effect of tofuyo extract on nucleoprotein (NP) of influenza virus is shown. (A) Tofuyo diluted with distilled water and homogenized. (B) Left to right: phosphate-buffered saline (PBS), 8.5% ethanol, supernatant of tofuyo filtered and centrifuged, pellet of tofuyo filtered and centrifuged. (C) Cell culture medium of cells infected with influenza virus strain PR8 was mixed with the indicated samples. NP of influenza virus was detected by immunochromatography (PBS, PBS + influenza virus, 37˚C for 30 min; Eth, 8.5% ethanol + influenza virus, 37˚C for 30 min; Tofu, tofuyo extract + influenza virus, 37˚C for 30 min). The reference line verified the success of the assay. The intensity of the influenza A virus NP line indicated the quantity of influenza A virus NP. Modified from Figs. 1 and 2 in Sakudo and Sesoko (6) with the permission of the Fuji Foundation for Protein Research.

after each treatment was compared by measuring the amount of NP of the influenza virus in the allantoic fluid using immunochromatography. As a result, it was demonstrated that treatment with 8.5% ethanol reduced the activity of the influenza virus, while the tofuyo extract did not reduce the infectivity of the virus (Fig. 3). Thus, this indicated that low ethanol in the tofuyo extract did not reduce the activity of the influenza virus.

Tofuyo extract prolongs survival of mice and delays decrease in body weight of mice caused by influenza virus infection. A 10^5-diluted solution of allantoic fluid infected with influenza virus strain PR8 was nasally inoculated into nude mice. The mice were then reared for 14 days and changes in their weight were measured. In the group administered the water-containing tofuyo extract from the day prior to inoculation, the mice (5/5) survived, while in the control group where mice were administered tofuyo extract-free water, 2 mice died

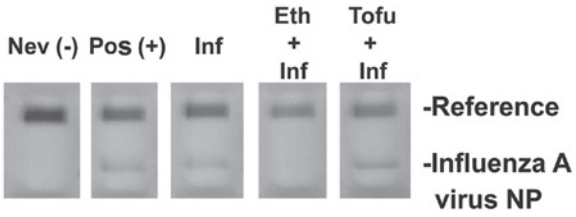

Figure 3. *In vitro* effect of tofuyo extract on influenza virus infection is shown. Cell culture medium of cells infected with influenza virus strain PR8 and treated by the indicated procedures was injected into embryonated eggs and incubated for 48 h. Allantoic fluid was collected and subjected to immunochromatography. The reference line verified the success of the assay. The intensity of influenza A virus NP line indicated the quantity of influenza A virus NP. Left to right: Nev (-), no influenza virus; Pos (+), no treated influenza virus; Inf, influenza virus incubated at 37˚C for 30 min; Eth + Inf, 8.5% ethanol + influenza virus incubated at 37˚C for 30 min; Tofu + Inf, tofuyo extract + influenza virus incubated at 37˚C for 30 min. Modified from Fig. 3 in Sakudo and Sesoko (6) with the permission of the Fuji Foundation for Protein Research.

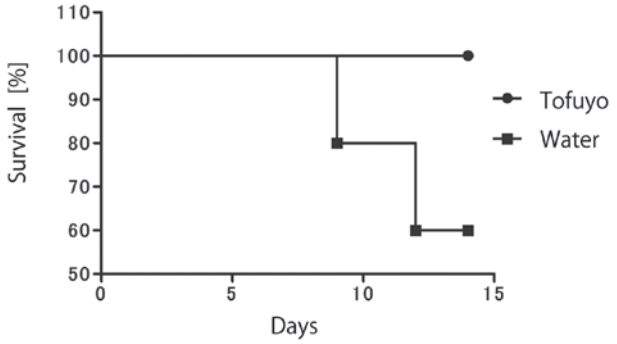

Figure 4. Tofuyo extract prolongs the survival of mice infected with influenza virus. Graph shows the survival percentage of two groups of nude mice (BALB/cSlc-*nu*/*nu*) after intranasal infection with influenza virus [A/Puerto Rico/8/34 (H1N1)]. The control group was administered tofuyo extract-free water, whereas the study group was administered water-containing tofuyo extract.

on days 9 and 12 following inoculation, and 3/5 mice survived (Fig. 4). In addition, comparing weight changes, weight loss was significantly smaller in the tofuyo extract-treated group compared to the control group ($P<0.05$, paired Student's t-test) (Fig. 5).

Tofuyo extract does not inhibit proliferation of the influenza virus in lung, but exhibits indirect protective effects against influenza virus infection. Immunochromatography of the influenza virus NP in the lungs from dead mice showed that NP of the influenza virus was expressed in the dead mice (Fig. 6), suggesting that the mice died of influenza virus infection. In addition, NP was detected in 2/5 mice of the tofuyo extract-treated group and 3/5 mice of the control group in mice sacrificed 14 days following inoculation. Therefore, tofuyo extract did not have any direct effects on the influenza virus or its production, while it had indirect protective effects against influenza virus infection.

Nutrient component and amino acid analyses exhibits health-promoting effects. Nutrient component analysis was conducted to investigate the mechanisms underlying the anti-influenza

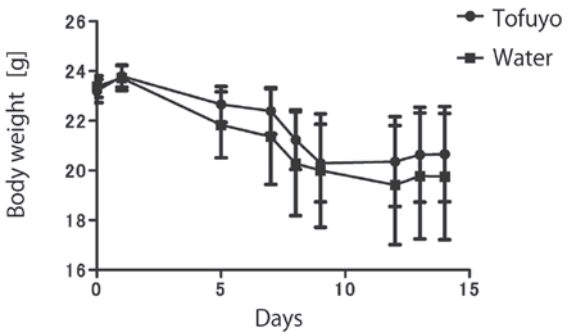

Figure 5. Tofuyo extract significantly delays the decrease in body weight caused by infection with influenza virus [A/Puerto Rico/8/34 (H1N1)] in mice ($P<0.05$, paired Student's t-test). Tofuyo, nude mice (BALB/cSlc-*nu*/*nu*) administered tofuyo extract-containing water; Water, nude mice administered tofuyo extract-free water.

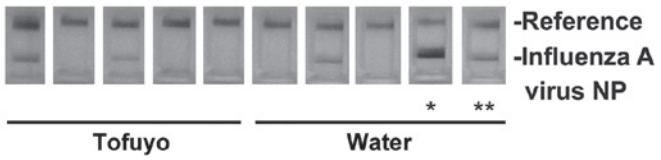

Figure 6. Tofuyo extract does not inhibit proliferation of influenza virus in lung. Lungs of nude mice (BALB/cSlc-*nu*/*nu*) infected with influenza virus [A/Puerto Rico/8/34 (H1N1)] were collected and subjected to immunochromatography. Tofuyo, mice administered tofuyo extract-containing water; Water, mice administered tofuyo extract-free water. Mice died on *day 9 and **12. The remaining mice were sacrificed on day 14.

virus effect of the tofuyo extract. The analysis indicated that 100 g of tofuyo extract contains 47 kcal of energy, 88.8 g of water, 1.0 g of protein, 0 g of lipid, 6.9 g of carbohydrate, 1.2 g of ash, 420 mg of sodium, 28 mg of potassium, 6 mg of calcium, 13 mg of magnesium, 0 mg of phosphorus, 0.1 mg of iron, 0 mg of zinc, 0.01 mg of copper, 0.01 mg of manganese, 0 mg of vitamin B_1, 0.02 mg of vitamin B_2, 0.02 mg of vitamin B_6, 0 g of soluble fiber, 0 g of insoluble fiber, 0 g of total dietary fiber, 1.1 g of sodium chloride equivalent and 2.1 g of alcohol (Table I).

In addition, the amino acid analysis indicated that 100 g of tofuyo extract contain 21 mg of arginine, 56 mg of lysine, 22 mg of histidine, 48 mg of phenylalanine, 38 mg of tyrosine, 70 mg of leucine, 45 mg of isoleucine, 11 mg of methionine, 48 mg of valine, 52 mg of alanine, 43 mg of glycine, 58 mg of proline, 213 mg of glutamic acid, 43 mg of serine, 35 mg of threonine, 101 mg of aspartic acid, 6 mg of tryptophan and 10 mg of cysteine and cystine (Table II).

Discussion

In the *in vitro* analysis, tofuyo did not show any direct inhibitory or disruptive effects against influenza virus (6). However, alleviation of influenza symptoms, including an increase in the survival rate and suppression of weight loss, was observed in the animal experiment performed in this study, suggesting that indirect protective effects of tofuyo occur in mice. Due to the fact that there was no significant difference between the tofuyo extract-treated and control groups, tofuyo extract was considered to have no inhibitory

Table I. Nutrient component analysis of tofuyo extract.

Nutrient	Value/100 g of tofuyo extract[a]	Method
Energy	47 kcal	Modified Atwater method[b]
Water	88.8 g	Reduced pressure and drying by heating method[c]
Protein	1.0 g	Modified Kjeldahl method (protein conversion factor 5.71)
Lipid	0 g	Soxhlet extraction method
Carbohydrate	6.9 g	Subtraction method[d]
Ash content	1.2 g	Direct ashing method
Sodium	420 mg	Atomic absorption method
Potassium	28 mg	Atomic absorption method
Calcium	6 mg	Atomic absorption method
Magnesium	13 mg	Atomic absorption method
Phosphorus	0 mg	Vanadic and molybdic acid absorption photometry
Iron	0.1 mg	Atomic absorption method
Zinc	0 mg	Atomic absorption method
Copper	0.01 mg	Atomic absorption method
Manganese	0.01 mg	Atomic absorption method
Vitamin B_1	0 mg	High-performance liquid chromatographic method
Vitamin B_2	0.02 mg	High-performance liquid chromatographic method
Vitamin B_6	0.02 mg	Microbial quantification method
Provide soluble fiber	0 g	Modified Prosky method
Insoluble dietary fiber	0 g	Modified Prosky method
Total amount of food fiber	0 g	Modified Prosky method
Corresponding value of salt	1.1 g	Corresponding value from sodium
Alcohol	2.1 g	Gas chromatography

[a]Zero means either <0.1 of the minimum quantity in food composition reported previously (5) or undetectable; [b]energy conversion factor was 4 (protein), 9 (lipid), 4 (carbohydrate), 2 (food fiber) and 7.1 kcal/g (alcohol); [c]water quantity was calculated by the dry weight method with subtraction of the alcohol quantity; [d]carbohydrate was calculated by subtraction of alcohol.

Table II. Amino acid analysis of tofuyo extract.

Amino acid	Value (mg/100 g tofuyo extract)	Method
Arginine	21	Automatic analysis method for amino acids
Lysine	56	Automatic analysis method for amino acids
Histidine	22	Automatic analysis method for amino acids
Phenylalanine	48	Automatic analysis method for amino acids
Tyrosine	38	Automatic analysis method for amino acids
Leucine	70	Automatic analysis method for amino acids
Isoleucine	45	Automatic analysis method for amino acids
Methionine	11	Automatic analysis method for amino acids
Valine	48	Automatic analysis method for amino acids
Alanine	52	Automatic analysis method for amino acids
Glycine	43	Automatic analysis method for amino acids
Proline	58	Automatic analysis method for amino acids
Glutamic acid	213	Automatic analysis method for amino acids
Serine	43	Automatic analysis method for amino acids
Threonine	35	Automatic analysis method for amino acids
Aspartic acid	101	Automatic analysis method for amino acids
Tryptophan	6	High-performance liquid chromatographic method
Cysteine and cystine	10	Automatic analysis method for amino acids (performic acid oxidation + hydrochloric acid hydrolysis)

effect on influenza virus infection, while it contributes to the maintenance of health following infection. This hypothesis is supported by the results of this study, indicating that tofuyo contains proteins, carbohydrates, vitamins and minerals. Consequently, tofuyo has health-promoting effects. In addition, amino acid analysis demonstrated that tofuyo is rich in glutamic and aspartic acid; therefore, umami components evidently remained in the extract.

In a previous study, tofuyo extract was found to contain di- and tri-peptides (1). In addition, short peptides from soybean extract were shown to be absorbed via the intestine and reach a higher concentration in the blood (7,8). Therefore, such short peptides in tofuyo extract may act in the blood and show anti-influenza virus effects. Furthermore, the anti-oxidative properties of tofuyo (9) may contribute to the defense against influenza virus infection-induced oxidative stress. The induction of immune functions in tofuyo extract-treated animals is an additional potential anti-influenza virus mechanism. However, to fully elucidate the anti-influenza effect of the tofuyo extract additional studies are required.

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