Brief Report

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Evaluation of the innate immune-stimulating activity of amazake using a silkworm muscle contraction assay

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Summary

We evaluated the innate immune-stimulating activity of amazake using a silkworm muscle contraction assay. Sake cake, a raw material used to make amazake, had high innate immunity-stimulating activity, whereas rice malt, another raw material used to make amazake, did not, even after fermentation. These results suggest that the silkworm muscle contraction assay is a useful tool to screen foods with high innate immune-stimulating activity and that amazake made from sake cake has immunomodulatory potential.

Keywords: Amazake, sake cake, sake lees, rice malt, silkworm, innate immunity

1. Introduction

The immune system is comprised of two elements: acquired immunity and innate immunity. The innate immune system, which mediates rapid responses to challenges using germline-encoded components, is a biological defense system against pathogens. The complement system, natural killer (NK) cells, and macrophages play important roles in innate immune defense. The innate immune system cooperates with the acquired immune system, which is developed through responses to antigens in order to protect the body. The innate immune capabilities of modern humans are declining due to stress, and environmental and dietary changes.

The development of a system to evaluate innate immunity is important for research on the prevention and treatment of disease. The evaluation of cells *in vitro* has limited use because the systems do not reflect the complexity of innate immunity. The evaluation of immunity in animals is undesirable from both cost and ethics perspectives. Therefore, we focused on the silkworm as a model organism. The silkworm is an invertebrate that has no antibody formation organs. Therefore, in the silkworm, responses to pathogens depend on the innate immune system. The mechanisms of innate immunity are highly conserved from invertebrates to vertebrates, which makes the silkworm suitable for the evaluation of the innate immune system. We previously reported the use a basic silkworm-based research model for drug discovery and the investigation of new immunostimulators (*1-3*).

Amazake is a traditional, sweet, Japanese beverage that originated from "Amanotamuzake", which was mentioned in the Chronicles of Japan from 720. Rice malt (koji) amazake is made from glycosylated starch by the action of amylase in rice malt. Sake cake amazake is made using sake cake, which contains fermented yeast and various nutrients, and sugar. Amazake can also be made using both rice malt and sake cake. Generally, amazake contains healthy ingredients, such as sugars, amino acids, and vitamin B, and it is sometimes called a "drinking drip". In Japan, amazake is thought beverage to prevent summer weariness and winter colds. Amazake contains many bioactive substances, including the fermentation products of rice malt fungi and yeasts obtained from rice malt and sake cakes. Amazake reportedly has antihypertensive effects (*4*) and tyrosinase-inhibiting activity (*5*), while there are few scientific reports on its effects on immunity.

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In this study, we used a silkworm muscle contraction assay to evaluate the immunomodulatory activity of the amazake ingredients sake cake and rice malt. We also examined the effects of glycosylation on the activity of rice malt.

2. Materials and Methods

2.1. *Sample preparation*

Sake cake and rice malt were obtained from Morinaga & Co., Ltd. (Tokyo, Japan); they were frozen at − 30°C and freeze-dried (200 g) overnight with an Eyela FDU-2110 freeze dryer (Tokyo Rikakikai, Tokyo, Japan). The samples were ground in a mortar, and dissolved in physiological saline (Otsuka Pharmaceutical, Tokyo, Japan) at 6.3 mg sake cake/mL and 3.2 mg rice malt/mL. For rice malt glycosylation, the rice malt was dissolved in water (2-fold dilution, w/v) and glycosylated by general method.

2.2. *Silkworm muscle contraction assay*

We measured the effects of various samples on silkworm muscle contraction activity, as previously reported (*6*). Briefly, samples (50 μL) were injected into the body fluid of silkworms with a 1-mL syringe *via* a 27-gauge needle (Terumo, Tokyo, Japan). The muscle contraction value (C) was calculated by measuring the maximum length of each silkworm before (x cm) and after (y cm) the injection, such that $C = (x - y)/x$. Physiological saline was used as a negative control, and 0.2 mL air was used as a positive control (C: 0.43 ± 0.03). The concentration that caused C = 0.15 (one unit) was calculated using a dose-response curve.

3. Results and Discussion

The compositions of sake cake and rice malt are presented in Table 1. The moisture content of sake cake was higher than that of rice malt. The levels of glucose and sugars with a high degree of polymerization were lower in sake cake than in rice malt. The results of amazake-induced muscle contraction activity are summarized in Table 2. Sake cake showed four times higher immunity-stimulating activity than green tea, which is a traditional beverage that is well known in Japan for its health effects. Rice malt did not show immunity-stimulating activity, even when glycosylated. The immunomodulatory activity of sake cake was not affected by rice malt.

Polysaccharides have been reported to have immunity-stimulating activity based on silkworm assays (*7*). Our results indicated that the carbohydrates in amazake did not contribute to its immunity-stimulating activity, whereas sake cake appeared to have contributed to its activity. Okuda *et al*. reported that sake cake has natural immunity-stimulating activity, based on NK cell activity (unpublished data). Therefore, the evidence suggests that sake cake amazake may stimulate innate immunity.

Table 1. Compositions of sake cake and rice malt

g/100g	Sake cake	Rice malt
Moisture	54.5	15.5
Fat	1.5	0.5
Protein	9.5	6.9
Ash	0.3	0.3
Glucose	6.7	28.4
Maltose	2.8	2.1
Maltotriose	1.5	< 0.1
Dietary fiber	2.8	1.3
Alcohol	2.8	1.3

Table 2. Immunity-stimulating activity of sake cake, rice malt, glycosylated rice malt

Sample	Relative activity (units/mg)
sake cake	25 ± 5
rice malt	< 6.3
glycosylated rice malt	< 6.3
sake cake + glycosylated rice malt	17 ± 3
green tea	6 (*1*)

Values were mean ± standard deviation. One unit of activity was defined as the activity required to decrease the length of silkworm muscle specimens by 15% (*6*).

In larval hemocytes, immunologic stimulants can induce the production of reactive oxygen species, followed by the activation of serine proteases, thereby mediating the paralytic peptide processing reaction and leading to defense responses in the silkworm (*6*). In humans, NK cell activity is commonly assessed as a measure of natural immune activation. Broccoli extract was shown to have immunity-stimulating activity in the silkworm and to cause NK cell activation in humans (unpublished data). NK cell activity is lower in the elderly (*8*) and is also related to the risk of cancer (*9*). Therefore, natural and safe foods that stimulate NK cell activity would be beneficial, and further studies on sake cake amazake are needed to verify its NK cell-related activity.

The most well-known immunity-stimulating food is yogurt containing *Lactobacillus* species. *Lactobacillus* species improve the gut environment and stimulates NK cell activity in humans (*10-13*). Although we did not directly compare the efficacy of *Lactobacillus* species and amazake, lactic acid bacteria reportedly have high immunity-stimulating activity (250-460 units/mg) in the same silkworm muscle contraction assay (*14*). The amount of sake cake ingested at one time is about fifty times higher than that of lactic acid bacteria. Considering the levels ingested, sake cake is expected to exert high immunomodulatory activity. Kawamoto *et al*. reported that sake cake fermented with lactic acid bacteria prevents allergic rhinitis in mice (*15*); thus,

combining these health-promoting components is an attractive strategy.

In conclusion, the silkworm muscle contraction assay is an easy method to evaluate the innate immunity-stimulating activities of foods, and amazake made from sake cake is one of the expected immunity-stimulating foods. Studies in human systems will be required to confirm and extend our findings.

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