

Study on the immunomodulatory function derived from Okinawa mozuku (*Cladosiphon okamuranus* Tokida) and its basic pharmacokinetic analysis.

友利, 誠

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Study on the Immunomodulatory Function of Fucoidan Derived from
Okinawa Mozuku (*Cladosiphon okamuranus* Tokida) and Its Basic
Pharmacokinetic Analysis

オキナワモズク由来フコイダンの免疫調節機能及び体内動態に関する臨床基礎研究

2022

Laboratory of Natural Products Chemistry
Graduate School of Pharmaceutical Sciences
Kyushu University

Makoto Tomori
(友利 誠)

Contents

Introduction	1
Chapter I	9
Evaluation of the immunomodulatory effects of fucoidan derived from <i>Cladosiphon okamuranus</i> Tokida in mice	
Abstract	9
1. Introduction	10
2. Materials and Methods	12
3. Results	18
4. Discussion	27
5. Conclusion	31
Chapter II	32
Effects of ingesting fucoidan derived from <i>C. okamuranus</i> Tokida on human NK cells: A randomized, double-blind, parallel-group, placebo-controlled pilot study	
Abstract	32
1. Introduction	33
2. Materials and Methods	34
3. Results	39
4. Discussion	48
5. Conclusion	52
Chapter III	53
Are <i>Helicobacter pylori</i> infection and mozuku consumption associated with fucoidan absorption?	
Abstract	53
1. Introduction	54
2. Materials and Methods	56
3. Results	59
4. Discussion	70

5. Conclusion	74
Conclusion	75
References	78
Acknowledgments	91
Published Papers	92

Introduction

1. The history of fucoidan and edible seaweed in the world

The history of fucoidan started in 1913 with H. Kylin ¹⁾ at Uppsala University, Sweden. Fucoidan was first isolated from the brown algae *Ascophyllum nodosum*, and was named fucoidin. Subsequently, the Carbohydrate Nomenclature of the International Union of Pure and Applied Chemistry standardized the name to fucoidan, still used today.

Seaweed has been used for centuries. There are 221 types of seaweed that are used as marine resources worldwide, including 32 types of green algae, 125 types of red algae, and 64 types of brown algae ²⁾. There are 145 edible species (green algae, 28; red algae, 79; brown algae, 38). Additionally, approximately 50% of red and brown algae are utilized as raw materials to extract polysaccharides such as agar, carrageenan, and alginate. From 1994–1995, 7.6 million tons (raw equivalent) of seaweed was grown globally, 90% was grown in six countries: China, Korea, Japan, France, the United Kingdom, and Chile. Moreover, 52% of seaweeds, 74% of green algae, 22% of red algae, and 82% of brown algae are cultivated.

The brown algae “Okinawa Mozuku” (*Cladosiphon okamuranus* Tokida) is an edible seaweed that grows in the Amami Islands (Kagosima Prefecture) and the Ryukyu Islands (Okinawa Prefecture). Typical brown algae that grow in Japan include “Kombu” (*Laminaria japonica*), “Wakame” (*Undaria pinnatifida*), “Hijiki” (*Hizikia fusiforme*), “Nori” (*Porphyra tenera*), and “Mozuku” (*Nemacystus decipiens*). These species are utilized as edible species and industrial raw materials. Among them, the edible “Mozuku” in Japan are Okinawa Mozuku, Ishimozuku (*Sphaerotrichia divariate*), Futo-Mozuku (*Tinocladia crassa*), and Kuromo (*Hydrilla verticillate*). Currently, Okinawa Prefecture is the biggest producer of Okinawa Mozuku in Japan. After the Okinawa Prefectural Fisheries Research

and Extension Center established a cultivation technology in the 1970s, it was shared to several fishery cooperatives, and has since developed into the core fishery industry of the Okinawa Prefecture, with a current harvest of approximately 20,000 tons of Okinawa Mozuku.

Contrastly, fucoidan in Japan is primarily extracted from Wakame (*U. pinnatifida*), Gagome Kombu (*K. crassifolia*), and Okinawa Mozuku (*C. okamuranus*). The chemical structure depends on the type of seaweed, and the report of Kiichi Hosoda ³⁾ in 1970 confirmed the composition of Naga-kombu. In the case of Okinawa Mozuku fucoidan, Tako et al. ⁴⁾ reported the chemical analysis of mozuku-derived fucoidan in 1996. Currently, the seaweeds used to produce fucoidan in Japan are Wakame (mekabu), Gagome Kombu, and Mozuku. There are three types of fucoidan derived from Kombu ⁵⁻⁷⁾ (Figure 1): F-fucoidan, consisting primarily of sulfated fucose; U-fucoidan, containing glucuronic acid; and G-fucoidan, containing galactose. The chemical structure of fucoidan from wakame (mekabu) was reported by Lee et al. ^{8,9)} (Figure 2). The chemical structure of fucoidan derived from Okinawa mozuku was identified by Nagaoka et al. ¹⁰⁾ in 1999 (Figure 3). Additionally, chemical structure analysis using digestive enzymes derived from marine bacteria has also been performed, and predicted structures have been reported ¹¹⁾. This fucoidan has a linear backbone of 1-3-linked α -fucopyranose with 50% sulfated substitution at the four positions, and some acetylated fucose residues. This structure is simple compared with other brown algae-derived fucoidans. The determination of the chemical structure allowed quality control parameters to be set and the commercialization of fucoidan from Okinawa mozuku. Additionally, compared with other seaweeds, the very low alginic acid content of Okinawa mozuku, at approximately 0.1% (as wet weight) ¹²⁾, and the simple extraction and purification processes permitted commercial

production.

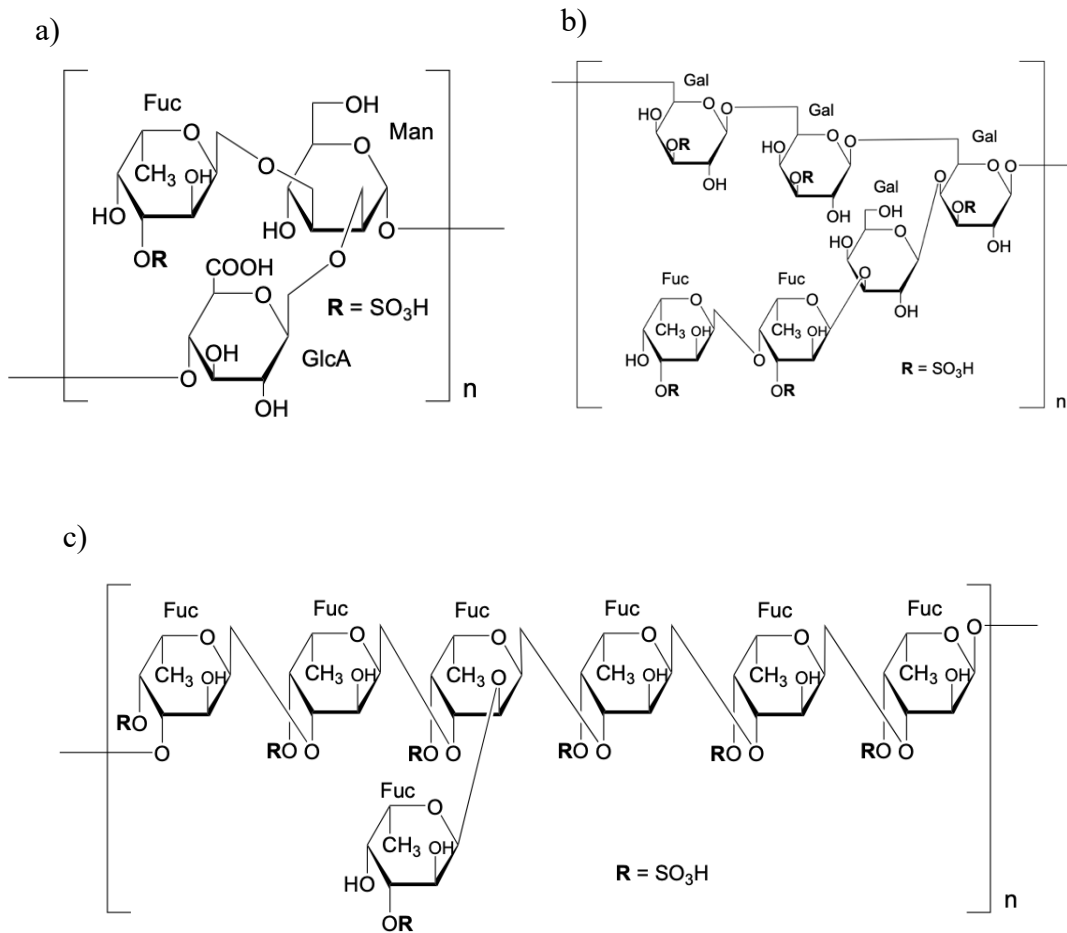


Figure 1. Structure of fucoidan derived from Gagome Kombu

a: U-fucoidan, b: G-fucoidan, c: F-fucoidan

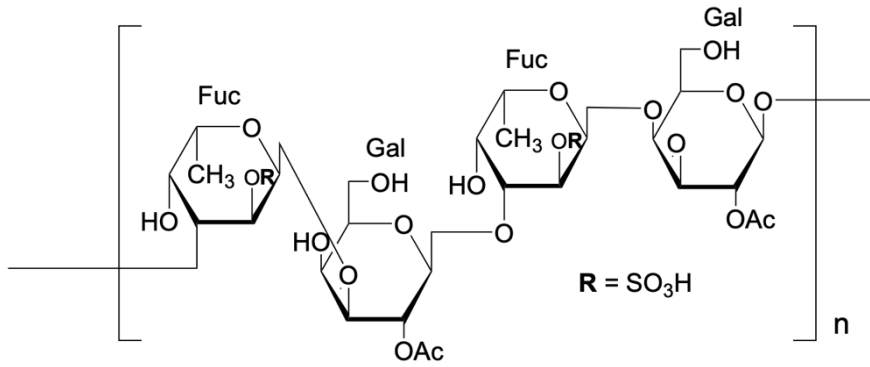


Figure 2. Structure of fucoidan derived from Wakame (Mekabu)

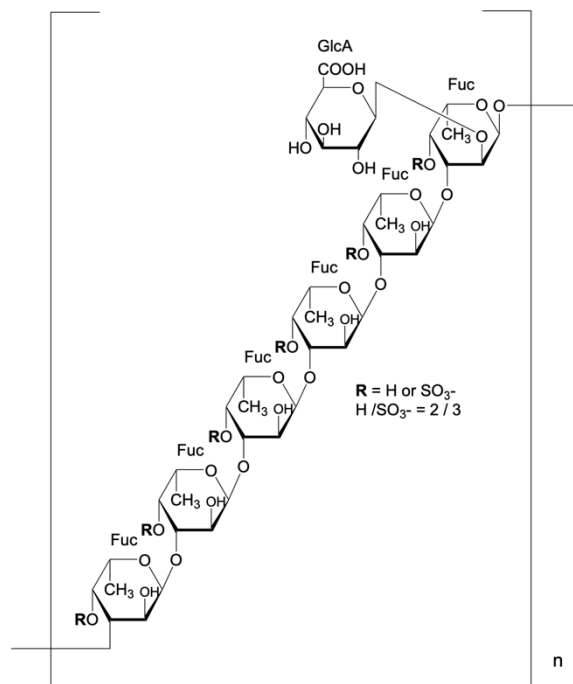


Figure 3. Structure of fucoidan derived from Okinawa Mozuku

2. Epidemiological background of the Japanese population

Currently, Japanese community is aging. The total population of Japan is decreasing, and is suggested to fall below 90 million by 2060, with the percentage of elderly people reaching 40% ¹³⁾. According to the 2020 White Paper, “*Annual Report on Aging Society*” by the Cabinet Office, the total population as at October 1, 2019, would be 126 million, and the population of 65 years of age and above would be 35 million, which is 28.4% of the total population ¹⁴⁾. Among the elderly, 17.4 million are 65–74 years of age and 18 million are 75 years of age and above, accounting for 13.8% and 14.7% of the total population, respectively, with those aged 75 and above outnumbering them. Contrastly, 15 million of the population is 0–14 years of age and 70 million of the population is 15–64 years of age, accounting for 12.1% and 59.5% of the total population, respectively, demonstrating the lower proportion of people below 15 years of age. In an aging society, the occurrence of subjective symptoms because of illness rises. According to the Comprehensive Survey of Living Conditions conducted by the Ministry of Health, Labour and Welfare in 2019, the subjective symptoms rate was 302.5 per 1000 individuals. Additionally, the number of newborns has reduced, and the number of deaths has risen since the 2019 Vital Statistics. The leading causes of death among the Japanese population are malignant neoplasms (tumors), cardiac disease, and senility, with the trachea, bronchus, lung, stomach, and pancreas being the most common sites of malignant neoplasms. Recently, allergies due to environmental improvements and food have also risen. In Japan, the Basic Law on Measures Against Allergic Diseases was passed in 2014 to tackle this challenge. Particularly, allergic diseases are increasing in children and the elderly, and the incidence of allergic rhinitis and food allergies involving anaphylaxis is rapidly increasing ¹⁵⁾. Recently, the awareness of infectious diseases spreading on a global scale, such as the new

coronavirus (SARS-CoV-2), has also dramatically increased. Given these circumstances, self-medication to manage one's own health is becoming a popular trend, leading to high expectations for functional foods.

3. Bioactivity and absorption of fucoidan derived from Okinawa mozuku (*Cladosiphon okamuranus* Tokida)

Okinawa Mozuku is an edible seaweed cultivated in the Okinawa Prefecture. This alga is a member of the genus *Cladosiphon* of the family Chordariaceae. The northern limit of the distribution is the Amami Oshima Island, Kagoshima Prefecture (29 degrees north latitude) and the southern limit is the Yaeyama Islands, Okinawa Prefecture (24 degrees north latitude). The first algological report on Okinawa mozuku, which was from the Kerama, Okinawa Prefecture was published in 1907 by Okamura ¹⁶⁾. Later, in 1942, Tokida ¹⁷⁾ reported this alga from Motobu, Okinawa Prefecture, and the scientific name was determined. Several bioactivities of fucoidan, such as anti-inflammatory ^{1,18)}, anticoagulant ^{1,18,19)}, antithrombotic ^{1,20)}, antiangiogenic ^{17,21)}, antiviral ^{1,22)}, antitumor ^{1,22,23)}, and antioxidant ^{1,24)} effects, have been reported. Contrastly, fucoidan bioactivities from Okinawa mozuku, such as antitumor ²⁵⁾, antiviral ²⁶⁾, anti-*Helicobacter pylori* ²⁷⁾, functional dyspepsia improvement ²⁸⁾, intestinal regulation ²⁹⁾, and immunomodulation ^{30,31)}, have been reported.

Generally, high molecular weight compounds are expelled from the body rather than being absorbed by the body. Fucoidan, a high molecular weight polysaccharide, was thought to be similarly unabsorbable, but mozuku-derived fucoidan is reported to be absorbed into the body ³²⁾. Additionally, a sandwich enzyme-linked immuno sorbent assay (ELISA) method using polyclonal antibodies for fucoidan detection was developed by

Tokita et al. ³²⁾, allowing highly sensitive measurement with a detection limit above 1 ng/mL. In a study, *N*-nitrosodiethylamine was administered to rats to cause hepatic fibrosis, Nakazato et al. ³³⁾ found that mozuku-derived high molecular weight fucoidan (41.4 kDa) reduced liver fibrosis compared with a standard diet and crude fucoidan (28.8 kDa). The mechanism of action was the TGF- β 1 and CXCL12 reduction, suggested to result in lipid peroxidation removal in the liver. Additionally, Nagamine et al. ³⁴⁾ detected fucoidan in the serum and liver in a study in which rats were administered *N*-butyl-*N*-hydroxynitrosamine and fucoidan, reporting that fucoidan was absorbed into the rat body. Absorption studies of fucoidan using the Caco-2 cell line showed that permeation concentration-dependently reached a maximum at one hour after fucoidan addition (1.0, 1.5, and 2.0 mg/mL), followed by a rapid decrease in permeation. These results indicate that fucoidan absorption may be mediated by transporters or pinocytosis. Tokita et al. ³⁰⁾ reported that after healthy subjects ($n = 10$) ingested 1 g fucoidan, it was detected in blood and urine at six and nine hours after ingestion, indicating that it was absorbed into the body. Additionally, Kadena et al. ³⁵⁾ conducted a fucoidan absorption study in 396 healthy adults living in Japan and measured the amount of fucoidan in urine before and after ingestion of fucoidan (0, 3, 6, and 9 h). The results showed that 385 (97.2%) of 396 subjects had detectable fucoidan in their urine. Of the 11 subjects that did not have detectable levels, three lived within the Okinawa Prefecture and eight lived outside the Okinawa Prefecture. Additionally, a significant difference was identified between those who detected fucoidan in their urine and those who did not, in terms of living factors in the Okinawa Prefecture and the habit of not eating mozuku. These studies suggest that fucoidan absorption may be affected by the dietary consumption of mozuku. Additionally, there are several reports on the anti-*H. pylori* action of mozuku-derived fucoidan; it has

unique features compared with other seaweed-derived fucoidans. It has been reported that approximately 50% of the world's population is infected with *H. pylori*³⁶). *H. pylori* infection is a risk factor for atrophic gastritis and gastric cancer, and the use of mozuku-derived fucoidan, a food product, may be improve QOL after infection with *H. pylori*.

4. The aim of this study

In Japan, the population is aging and the birthrate is decreasing; hence, self-medication using functional foods has emerged as a popular trend. Additionally, extensive study of immune system has been triggered by the global epidemic of infectious diseases in recent years. Maintaining the innate immune system of humans can be beneficial in combating infectious diseases. Therefore, the aim of this study was to evaluate the immunomodulatory effect of mozuku fucoidan on innate immunity in animals and humans, and to elucidate the correlation between fucoidan absorption and its anti-*H. pylori* effect.

Chapter I

Evaluation of the immunomodulatory effects of fucoidan derived from *Cladosiphon okamuranus* Tokida in mice

Abstract

Okinawa mozuku (*Cladosiphon okamuranus* Tokida), an edible seaweed classified as brown algae, is a native species of the Okinawa Islands in Japan. Recently, the genomic decoding of Okinawa mozuku has been completed. Previous studies on the anti-inflammatory, antiviral, and antitumor properties of Okinawa mozuku have indicated that it impacts the modulation of cellular and humoral immunity. The aim of this study was to examine the immunoregulatory effect of fucoidan derived from *Cladosiphon okamuranus* in mice. A product containing fucoidan (purity, 88.3%; molecular weight, 49.8 kDa) was developed from *C. okamuranus* and tested for its immunoregulatory effects in mice. The experimental animals were 8-week-old female BALB/c mice to which fucoidan (0, 102.5, 205.0, 410.0, and 1025.0 mg/kg) was administered orally for six weeks. Immune cell proliferation, cytokine production, macrophage phagocytosis, and serum antibody levels were measured. The immune cell proliferation, interferon-gamma (IFN- γ), interleukin (IL)-2, macrophage phagocytes, and serum antibodies (IgM, -G, -A) were found to increase significantly, whereas IL-4, -5, and IgE decreased significantly. These results indicated that fucoidan modulated cellular and humoral immunity.

1. Introduction

Fucoidan is a generic term for several water-soluble sulfated polysaccharides present in brown algae. These compounds show several different biological properties, including anti-inflammatory^{18,37)}, anticoagulant¹⁸⁾, anti-HIV²²⁾, and antitumor^{22,25,39,40)} effects. The biological properties of fucoidan vary depending on the species of algae, molecular weight, composition, and structure. Fucoidan derived from Gagome kombu (*K. crassifolia*) has been shown to be safe in healthy volunteers⁴¹⁾ and has been reported to prevent immune function decline. Fucoidan derived from Mekabu (*U. pinnatifida*) increased helper T1 cells in BALB/c mice⁴²⁾. Okinawa mozuku (*C. okamuranus*), the raw material of fucoidan used in this study, is an edible seaweed of the Okinawa islands, Japan. The Okinawa mozuku cultivation in the Okinawa Prefecture was established by the Okinawa Prefectural Fisheries Research and Extension Center in the 1970s, and the Prefecture is currently able to provide commercial production and a stable supply of this seaweed, accounting for > 99% of domestic distribution in Japan. Recently, Nishitsuji et al.⁴³⁾ decoded the draft genome of the Okinawa mozuku S-strain. This fucoidan has a linear backbone of 1-3-linked α -fucopyranose with 50% sulfate substitution at the four positions, and some of the fucose residues are O-acetylated¹⁰⁾. This fucoidan is characterized by a simpler structure than that of fucoidan derived from other brown seaweeds, Gagome kombu and Mekabu, and shows numerous biological properties, including inhibition of the adhesion of *Helicobacter pylori*^{27,44)}, functional dyspepsia improvement^{45,46)}, antifatigue effect^{40,47)}, and improvement in bowel movement^{29,48)}. A previous study on immunity reported anti-human T-cell leukemia virus type-I (HTLV-1)^{49,50)} and antitumor^{22,25,39,40)} effects. Whereas the mechanism underlying the immunomodulatory effects of fucoidan derived from Okinawa mozuku has not been reported. Therefore, the

immunomodulatory effect was evaluated as the first outcome and the safety as the second outcome, assuming that the fucoidan products were used in daily life. In this study, it was aimed to comprehensively examine the immunomodulatory effect of fucoidan derived from *C. okamuranus* in BALB/c mice.

2. Materials and Methods

2.1. Fucoidan

In this study, a fucoidan, test food named “Mei Hai Yun[®],” which was provided by Kanwa Healthcare Ltd. (New Taipei, Taiwan) was used (Table I-1). Fucoidan derived from *C. okamuranus* was manufactured by South Product Co., Ltd. (Uruma, Japan). The compositions of this fucoidan were as follows: L-fucose content of 52.7%, uronic acid content of 18.0%, sulfate ion content of 17.6%, and mean molecular weight of 49.8 kDa (Table I-2).

Table I-1. Formula of test food

Materials	Formulation (mg/capsule)
Fucoidan	490.0
Silicon dioxide	5.0
Magnesium stearate	5.0
Amount	500.0

Table I-2. Compositions of fucoidan in test food

Compositions	Contents	Method
L-fucose (%)	52.7	Anthron sulfuric acid
Uronic acid (%)	18.0	Carbazole sulfuric acid
Sulfate ion (%)	17.6	Ion chromatography
Amount (%) (as fucoidan)	88.3	

2.2. Dosage

The experimental dose to animals was based on 205.0 mg/kg/mouse. This dose was based on 1 g/day when fucoxanthin was measured through blood and urine in humans ³²⁾. The dose to mice was set assuming that the human body weight was 60 kg, using the coefficient of human mice 12.3 as presented in the US FDA Clinical Trial Amount Guidance ⁵¹⁾. The earlier-mentioned standard dose of 205.0 mg/kg/day was defined as the Fucoxanthin Middle (FM) group. Half dose (102.5 mg/kg/day) of FM group was administered to the Fucoxanthin Low (FL) group, and two-fold dose (410.0 mg/kg/day) was administered to the Fucoxanthin Middle High (FMH) group, five-fold dose (1025.0 mg/kg/day) was administered to the Fucoxanthin High (FH) group. Sterilized water was administered to the Negative Control (NC) group. (Table I-3).

Table I-3. Experimental group and dosage

Group	Test food	Dosage (mg/kg/day)	Human conversion
NC	Sterilized water	-	-
FL		102.5	0.5 g/60kg/day
FM	Fucoxanthin	205.0	1.0 g/60kg/day
FMH	(Test food)	410.0	2.0 g/60kg/day
FH		1025.0	5.0 g/60kg/day

NC: Negative Control, FL: Fucoxanthin Low, FM: Fucoxanthin Middle, FMH: Fucoxanthin Middle High, FH: Fucoxanthin High.

2.3. Animal

Female BALB/c mice (eight weeks old) used in this study were purchased from Bio-Lasco Taiwan Co. (Taipei, Taiwan). All animals were eight weeks old at the start of the experiment and were kept in a normal environmentally controlled animal room (22°C ± 3°C, 12-h light/dark cycle) with access to pathogen-free feed and water.

2.4. Study design

In this study, the mice were randomly divided into a NC and four fucoidan groups (102.5, 205, 410, and 1025 mg/kg body weight) with ten mice per group. Fucoidan was dissolved in distilled water and administered orally at 20 mL/kg mouse body weight daily for six weeks (Figure I-1). The NC (0 mg/kg body weight) mice were treated similarly with equal volumes of distilled water orally throughout the study. All experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee of Medgaea Life Sciences (New Taipei, Taiwan).

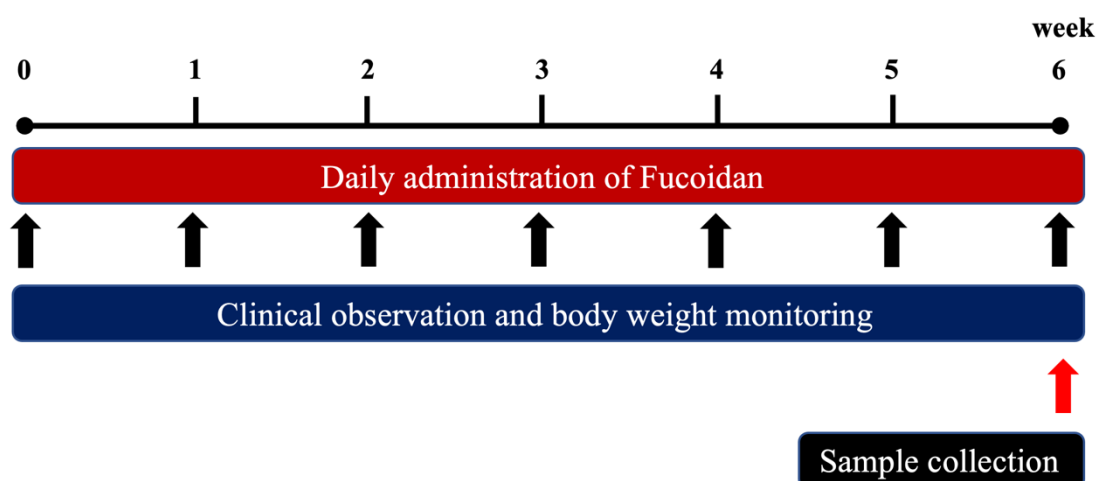


Figure I-1. Study design.

Clinical observation: general health conditions, and clinical signs of illness or discomfort.

2.5. Clinical observation and body weight monitoring

During the study period, each mouse was subjected to daily monitoring of general health conditions and clinical signs of illness or discomfort. Furthermore, each animal was weighed once a week during the study period.

2.6. Preparation of splenocytes suspension, peritoneal macrophage, and serum

At the end of the animal experiments the mice were anesthetized (Isoflurane), and their blood was collected. The blood was centrifuged at $1200 \times g$ for ten minutes to collect serum. The mice were injected with cold Hank's Balanced Salt Solution (HBSS; Sigma Aldrich, St. Louis, MO, USA) into the peritoneal cavity, and the peritoneal macrophage suspension at a density of 1×10^6 cell/mL was collected. The spleen was surgically removed and passed through nylon mesh ($75 \mu\text{m}$). Spleen cells were washed with RPMI1640 medium (Gibco, Grand Island, NY, USA), and red blood cells were removed using HBSS and centrifuged at $300 \times g$ for ten minutes to collect sediment after supernatant removal. RPMI1640 medium was added and a cell suspension was prepared at a cell density of 2×10^8 cell/mL. The splenocytes suspension was used for proliferation and cytokine secretion measurements.

2.7. Immune cells proliferation

Splenocytes were plated at a density of 4×10^5 cell/mL and stimulated with concanavalin A (5.0 $\mu\text{g/mL}$) (Con A, Sigma Aldrich, St. Louis, MO, USA) and lipopolysaccharide (10.0 $\mu\text{g/mL}$) (LPS, Sigma Aldrich, St. Louis, MO, USA). After incubation (37°C, 5% CO₂, 72 h), splenocytes were stained with a CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). After incubation (37°C, 5% CO₂, 4 h), immune cell proliferation was determined by absorption measurement at optical density (OD) of 490 nm. Results were expressed as the stimulation index (S.I). The formula for calculating S.I is shown below:

$$\text{S.I.} = \text{OD 490 nm of Con A and LPS-treated cells} / \text{OD 490 nm of untreated cells.}$$

2.8. Phagocytic activity of macrophages derived from the peritoneal cavity

Peritoneal macrophages (density of 1×10^6 cell/mL) and green fluorescent protein-labeled *E. coli* (Tunghai University, Taichung, Taiwan) were cultured (37°C, two hours) in RPMI1640 medium at *E. coli*/macrophage ratios of 25:1 and 50:1. The analytical buffer and trypan blue were added and analyzed using flow cytometry.

2.9. Cytokine secretion levels

The splenocytes suspension (density of 4×10^5 cell/mL) was stimulated by Con A and LPS. After incubation (37°C, 5% CO₂, 72 h), cell-free supernatant was collected and cytokines, including IL-4, IL-5, and IFN- γ , were measured by ELISA (eBioscience, San Diego, CA, USA). Moreover, IL-2 was determined using ELISA at 24 and 48 h after Con A and LPS stimulation.

2.10. Serum Immunoglobulin levels

Serum was collected for antibody measurements (IgM, IgG, IgA, and IgE) using ELISA (Bethyl Laboratories, Montgomery, TX, USA). The level of antibodies was calculated according to the following formula:

$$\text{ELISA unit (E.U.)} = (\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}) / (\text{OD}_{\text{negative Control}} - \text{OD}_{\text{blank}}).$$

2.11. Statistical analysis

Results were presented as the mean \pm standard deviation (SD). Statistical analysis was conducted by Williams multiple range test using Statcel version 4 software (OMS publishing, Japan). A value of $p < 0.05$ was considered statistically significant. Moreover, 95% confidence intervals (CI) for each data were calculated using the above software.

3. Results

3.1. Clinical observation and body weight monitoring

No abnormal behavior or mortality was observed during this study. As shown in Figure I-2, body weight transition was similar in all experimental groups, and fucoidan treatment did not affect mouse growth.

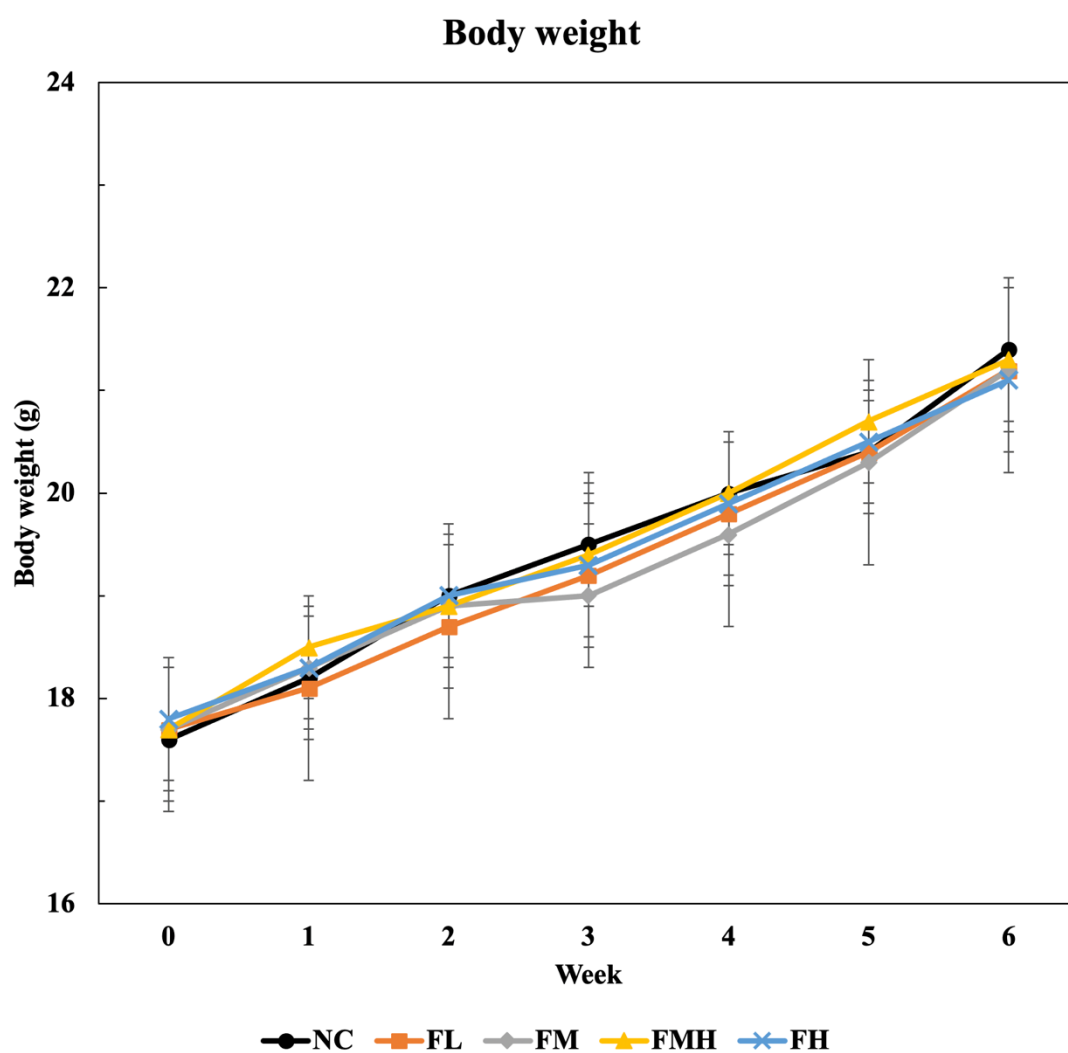


Figure I-2. Body weight transition.

All data were presented as mean \pm SD ($n = 10$ mice/group).

3.2. Immune cell proliferation

Splenocyte derived from mouse was stimulated with Con A and LPS, and the proliferative effect of fucoidan on immune cells was evaluated as a stimulation index (S.I).

As shown in Figure I-3, for Con A stimulation, the S.I in the NC group was 2.6 ± 0.3 (95%CI, 2.4–2.8), compared with 2.9 ± 0.3 (95%CI, 2.7–3.1, $p < 0.05$) in the FL group, 3.2 ± 0.2 (95%CI, 3.0–3.3, $p < 0.01$) in the FM group, and 3.4 ± 0.3 (95%CI, 3.2–3.6, $p < 0.01$) in the FMH group. The FH group was 3.5 ± 0.4 (95%CI, 3.3–3.8, $p < 0.01$), and S.I was significantly increased in all treatment groups.

Similarly, for LPS stimulation, S.I in the NC group was 2.1 ± 0.1 (95%CI, 2.0–2.2, $p < 0.01$), FL group was 2.4 ± 0.2 (95%CI, 2.3–2.5, $p < 0.01$), FM group was 2.5 ± 0.2 (95%CI, 2.3–2.6, $p < 0.01$), FMH group was 2.7 ± 0.3 (95%CI, 2.5–2.9, $p < 0.01$), and FH group was 2.8 ± 0.2 (95%CI, 2.7–3.0, $p < 0.01$), S.I significantly increased.

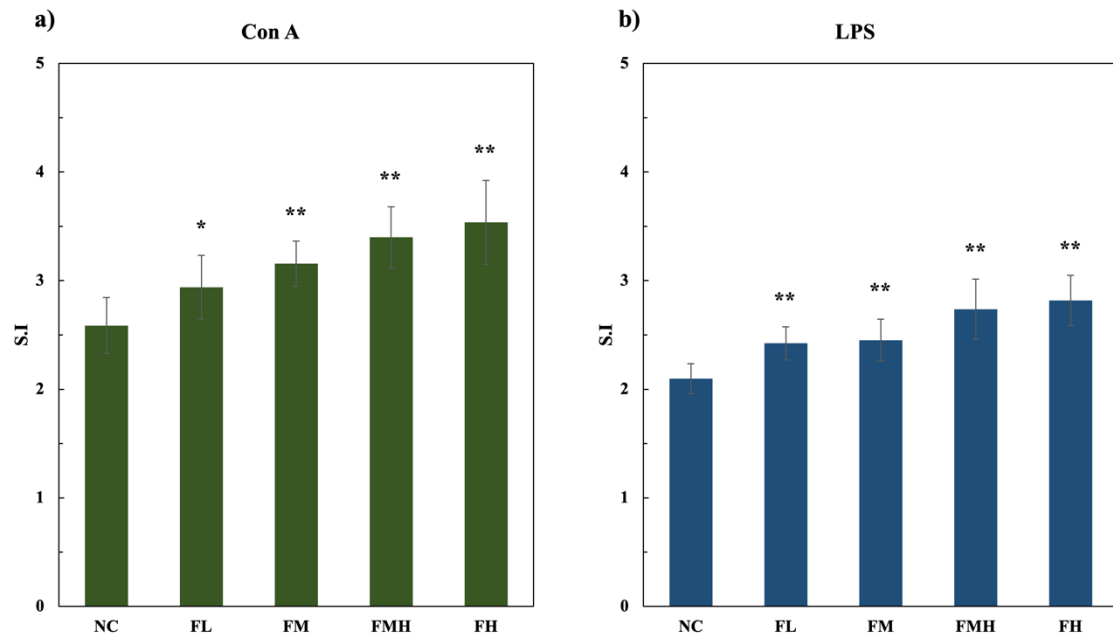


Figure I-3. Immune cell proliferation effect.

All data were presented as mean \pm SD ($n = 10$ mice/group).

a) Immune cell proliferation stimulated by Con A. b) Immune cell proliferation stimulated by LPS.

Significant different compared to Negative Control (NC) ($*p < 0.05$, $**p < 0.01$, Williams' multiple test).

3.3. Phagocytic activity of macrophage

The phagocytic activity was measured by macrophages as effector cells (E) and *E. coli* as target cells (T), with an E/T ratio of 25 or 50 (Figure I-4). At E/T Ratio of 25, the NC group phagocytosis activity was $6.0 \pm 2.1\%$ (95%CI, 4.5–7.6), while that of the FMH group was $11.1 \pm 4.2\%$ (95%CI, 8.1–14.1, $p < 0.01$) and that of the FH group was $15.4 \pm 1.9\%$ (95%CI, 14.0–16.7, $p < 0.01$). Moreover, at E/T Ratio of 50, compared with the NC group was $10.9 \pm 3.4\%$ (95%CI, 8.5–13.3), the FM group was $15.6 \pm 5.0\%$ (95%CI, 12.0–19.2, $p < 0.05$), the FMH group was $18.8 \pm 3.2\%$ (95%CI, 16.4–21.0, $p < 0.01$), and the FH group was $23.3 \pm 3.7\%$ (95%CI, 20.6–25.9, $p < 0.01$), indicating a significant increase.

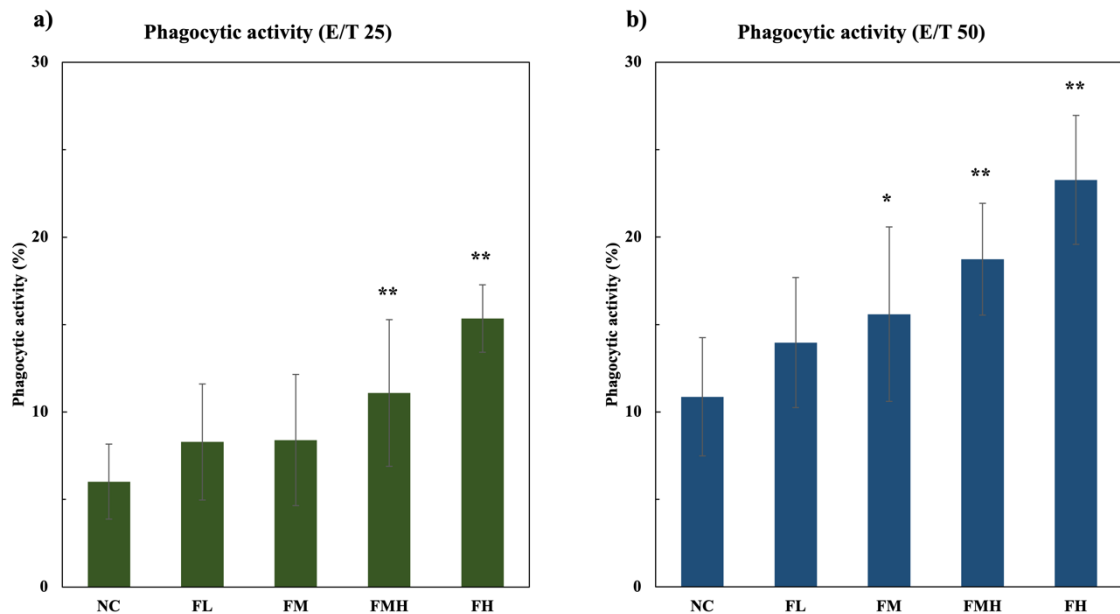


Figure I-4. Phagocytic activity of macrophage

All data were presented as mean \pm SD ($n = 10$ mice/group).

a) E/T ratio of 25. b) E/T ratio of 50.

Significant different compared to Negative Control (NC) ($*p < 0.05$, $**p < 0.01$, Williams' multiple test).

3.4. Cytokine secretion level

The serum cytokine levels in the assay are shown in Figure I-5 (Con A stimulation) and Figure I-6 (LPS stimulation). The inflammatory cytokine TNF- α was not significantly altered in all treatment groups in Con A and LPS stimulation compared with the NC group. IFN- γ , a main proinflammatory cytokine that modulates cell-mediated immunity, appeared to increase with Con A stimulation, but no significant difference was observed compared with the NC group. Contrastly, IFN- γ in LPS stimulation was significantly increased in all treatment groups, including the lowest dose of 5.1 ± 0.4 ng/mL (95%CI, 4.2–6.1, $p < 0.05$) in the FL group, compared to 0.4 ± 0.9 ng/mL (95%CI, 3.3–4.6) in the NC group. In IL-2 stimulated by Con A, the FMH group was 3.2 ± 0.5 ng/mL (95%CI, 2.8–3.6, $p < 0.05$) and the FH group was 3.2 ± 0.5 ng/mL (95%CI, 2.9–3.6, $p < 0.05$), which were significantly higher than those in the NC group (2.6 ± 0.5 ng/mL [95%CI, 2.2–3.0]). Moreover, LPS-stimulated IL-2 increased significantly in the FMH group (39.9 ± 5.6 pg/mL [95%CI, 35.9–43.9, $p < 0.05$]) and FH group (41.9 ± 10.1 pg/mL [95%CI, 34.7–49.1, $p < 0.05$]) compared with the NC group (34.1 ± 5.6 pg/mL [95%CI, 30.1–38.1]).

The cytokine IL-4 linked with humoral immunity was significantly reduced in the FH group (31.2 ± 8.8 pg/mL [95%CI, 24.9–37.4, $p < 0.01$]) when stimulated with Con A compared to the NC group (51.9 ± 11.2 pg/mL [95%CI, 43.9–59.9]). Moreover, IL-4 in LPS stimulation was significantly reduced in all treatment groups as compared with NC group (14.3 ± 2.8 pg/mL [95%CI, 12.3–16.3]). IL-5 stimulated by Con A was significantly reduced in all treatment groups compared with NC group (121.9 ± 41.3 pg/mL [95%CI, 92.4–151.5]). Similarly, LPS-stimulated IL-5 was significantly reduced in all treatment groups compared to the NC group (10.0 ± 1.8 pg/mL [95%CI, 8.7–11.2]). IL-10 was not

significantly altered in Con A and LPS stimulation in all treatment groups.

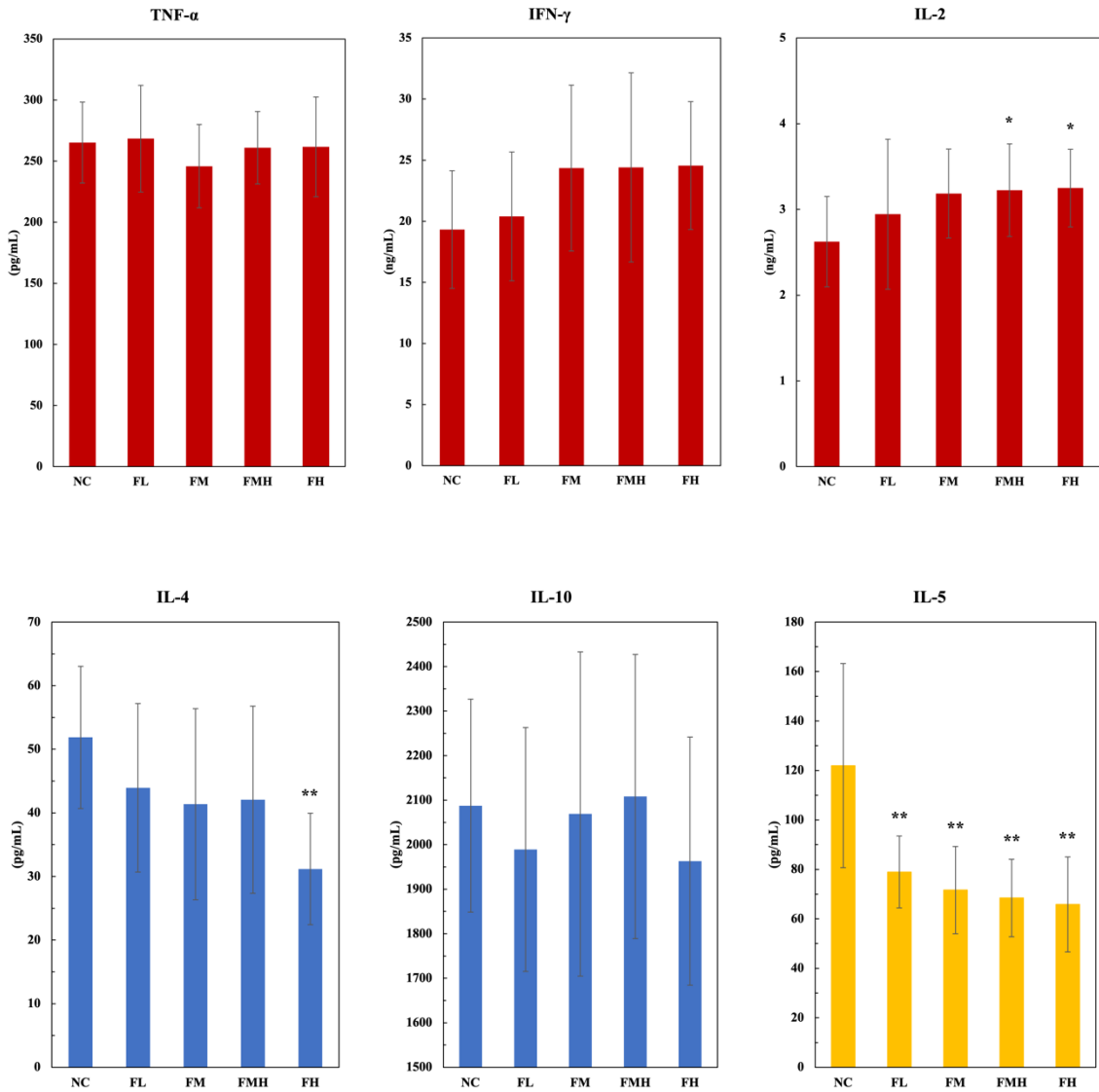


Figure I-5. Comparison of cytokines stimulated by Con A

All data were presented as mean \pm SD ($n = 10$ mice/group).

Significant different compared to Negative Control (NC) (* $p < 0.05$, ** $p < 0.01$, Williams' multiple test).

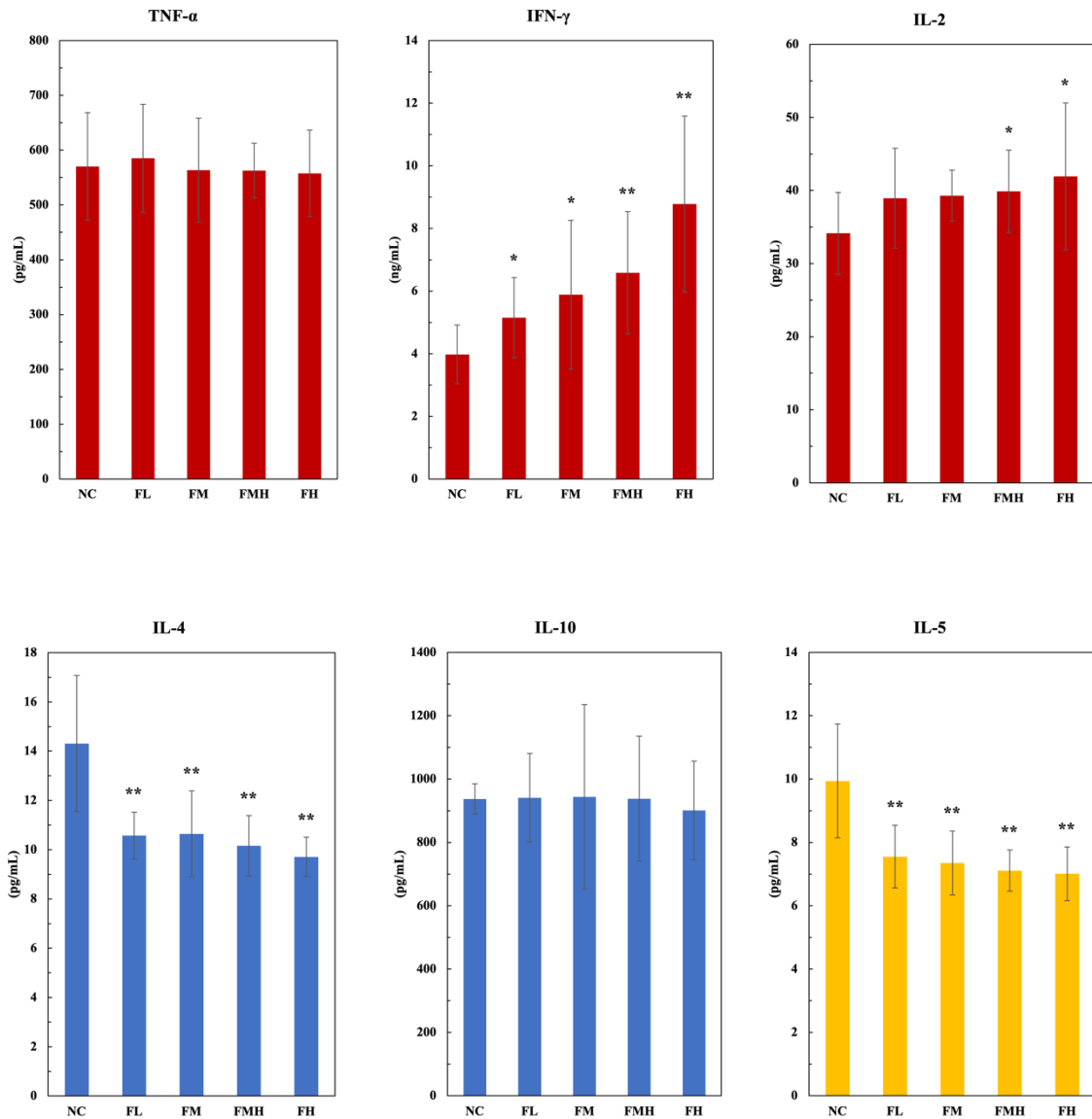


Figure I-6. Comparison of cytokines stimulated by LPS

All data were presented as mean \pm SD ($n = 10$ mice/group).

Significant different compared to Negative Control (NC) (* $p < 0.05$, ** $p < 0.01$, Williams' multiple test).

3.5. Antibody production

The antibody levels in mouse serum were measured (Figure I-7). The IgM levels in the NC group was 655.2 ± 52.3 $\mu\text{g/mL}$ (95%CI, 617.8–692.6), whereas that in the FMH group (742.0 ± 73.6 $\mu\text{g/mL}$ [95%CI, 689.4–794.7, $p < 0.01$]) and FH group (891.5 ± 62.0 $\mu\text{g/mL}$ [95%CI, 847.1–935.9, $p < 0.01$]) were increased significantly. The IgG levels in the NC group was 216.8 ± 14.9 $\mu\text{g/mL}$ (95%CI, 206.2–227.5), whereas that in the FM group was 235.9 ± 10.0 $\mu\text{g/mL}$ (95%CI, 228.7–243.1, $p < 0.05$), that in the FMH group was 244.7 ± 11.4 $\mu\text{g/mL}$ (95%CI, 236.5–252.8, $p < 0.01$), and that in the FH group was 275.3 ± 38.8 $\mu\text{g/mL}$ (95%CI, 247.6–303.1, $p < 0.01$), which were increased significantly. The IgA levels were increased significantly in all treatment groups compared to the NC group (450.8 ± 38.1 $\mu\text{g/mL}$ [95%CI, 423.6–478.1]). Contrastly, the IgE levels in the NC group was 1.7 ± 0.5 $\mu\text{g/mL}$ (95%CI, 1.4–2.0), whereas a significant decrease was observed in all fucoidan treatment groups.

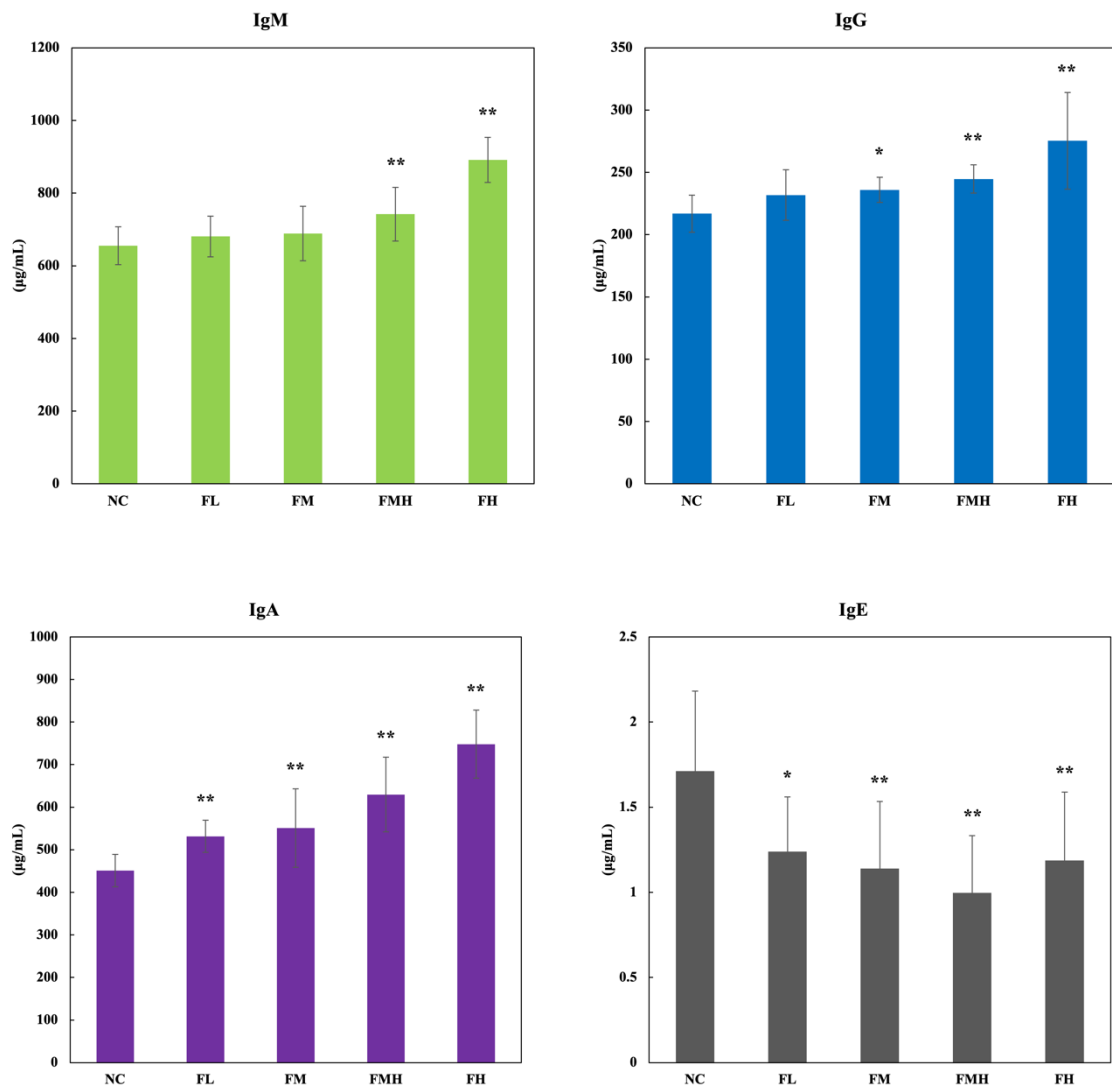


Figure I-7. Antibody levels

All data were presented as mean \pm SD ($n = 10$ mice/group).

Significant different compared to Negative Control (NC) (* $p < 0.05$, ** $p < 0.01$, Williams' multiple test).

4. Discussion

The fucoidan bioactivity is affected by its chemical structure and molecular weight. Moreover, the chemical structure and molecular weight affect absorption and physiological activity in the body ^{32,52}). Previously, it was reported that fucoidan is involved in immune activities, such as those of macrophages, NK cells, and cytokines ^{1,53}). Fucoidan derived from Okinawa mozuku is absorbed by animals and humans ^{34,35,54}). In this study, fucoidan was shown to be active in splenocyte cells, and macrophages in female BALB/c mice. For cytokines, IL-2 and IFN- γ were increased, whereas IL-4 and IL-5 were decreased. Serum antibodies IgM, IgG, and IgA increased, but IgE decreased. Cytokines affect splenocyte and macrophage activities. The immune cells in the spleen are T and B cells. In this study, splenic immune cells were increased in the fucoidan treatment groups. Con A propagates the differentiation of T cells, and LPS is a mitogen that promotes the differentiation of B cells. Fucoidan has been shown to activate T and B cells growth present in the spleen. Shimizu et al. ⁵⁵) found that different molecular weights of fucoidan had different effects on T cells and NK cells growth, which were derived from the spleen; they found that there was a greater effect on high molecular weight (2×10^5 – 3×10^5) fucoidan than on low molecular weight (1×10^3 – 9×10^3) fucoidan. The fucoidan used in this study also had high molecular weight and yielded similar results. Jang et al. ⁵⁶) reported that they observed a proliferative effect on mouse splenocytes using high molecular weight fucoidan (130 kDa). In this study, IL-2 and IFN- γ production were increased in the fucoidan treatment groups. These results indicate that macrophage phagocytes activity was stimulated. The activity of macrophages also has another mechanism. Doi et al. ⁵⁷) showed that macrophages attach to several negatively charged molecules and reported that they were involved in biological defense mechanisms and processing

mechanisms. The serum antibodies IgM, IgG, and IgA were significantly increased, whereas IgE was reduced significantly. B cells are involved in antibody secretion, and IL-4, IL-21, TGF- β , and IFN- γ are affected by their activity. For fucoidan derived from Mekabu (*U. pinnatifida*) used by Takai et al.⁵⁸), a fucoidan with a molecular weight of ≥ 2 kDa increased IgM, IgG, and IgA, with IgE reported to be below the detection limit. These test results indicate that a class switch of B cells induced by IFN- γ was involved in the production enhancement of IgM, IgG, and IgA. Contrastly, IL-4 and IL-5, having involvements in humoral immunity, were decreased in the fucoidan treatment group. These results show that fucoidan can adjust the balance between cellular and humoral immunity (Table I-4, Figure I-8).

Table I-4. Immunomodulatory effect after treatment of fucoidan derived from *C. okamuranus*

Test items		Results
Immune cell proliferation	Con A, LPS	Enhanced ↑
	TNF- α	Unchanged
Cytokine production	IL-2, IFN- γ	Enhanced ↑
	IL-4, IL-5	Suppressed ↓
	IL-10	Unchanged
Macrophage phagocytosis		Enhanced ↑
Antibody production	IgM, IgG, IgA	Enhanced ↑
	IgE	Suppressed ↓

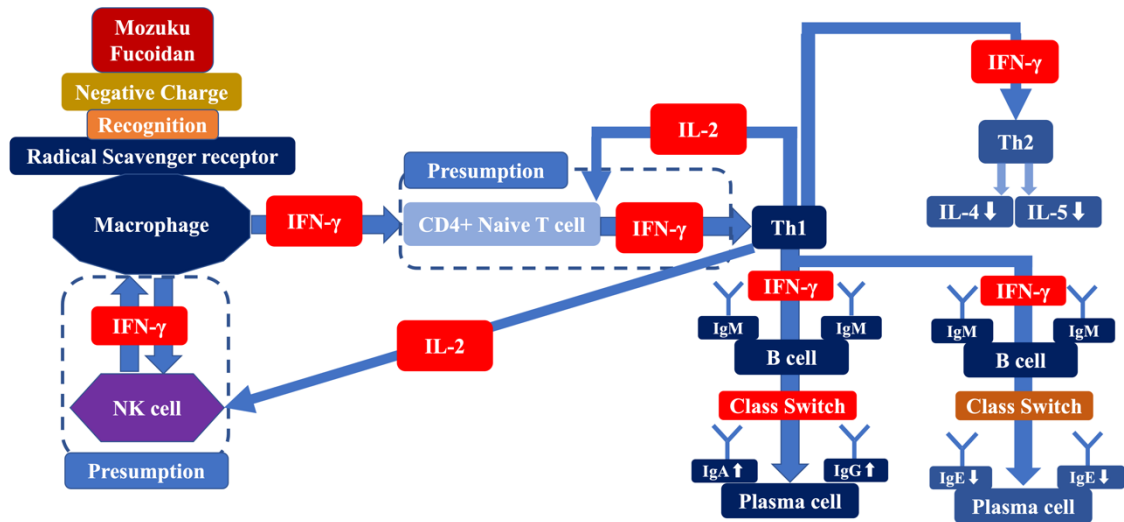


Figure I-8. Correlation of several cytokines produced by T and B cells in mozuku-derived fucoidan treatment.

Macrophage scavenger receptors recognize the sulfate group of mozuku-derived fucoidan. Activated macrophages produce IFN- γ and induce helper T cell (Th1) differentiation. Th1 cells produce IFN- γ and induce B-cell class switching. Conversely, IFN- γ produced by Th1 cells decreases IL-4 and IL-5 production in Th2 cells and also suppresses IgE production.

5. Conclusion

The immunomodulatory effects of fucoidan derived from *C. okamuranus* in mice was evaluated. Fucoidan was shown to stimulate immune cell proliferation, macrophage phagocytes, and cell-mediated immunity. These results indicate that fucoidan derived from *C. okamuranus* modulates natural immunity in mice.

Chapter II

Effects of ingesting fucoidan derived from *C. okamuranus* Tokida on human NK cells: A randomized, double-blind, parallel-group, placebo-controlled pilot study

Abstract

The aim of this study is to evaluate the effects of ingesting fucoidan derived from Okinawa mozuku (*C. okamuranus*) on natural killer (NK) cell activity and to assess its safety in healthy Japanese subjects via a randomized, double-blind, parallel-group, placebo-controlled pilot study. Subjects were divided randomly into two groups—a placebo group (ingesting citric acid, sucralose, and caramel beverages; $n = 20$; 45.5 ± 7.8 years [mean \pm standard deviation]) and a fucoidan group (3.0 g/day from beverages; $n = 20$; 47.0 ± 7.6 years). After 12 weeks, blood, biochemical, and immunological tests were conducted. Clinically adverse events were not observed in any of the tests during this study. Moreover, adverse events due to the test food were not observed. In the immunological tests, NK cell activity was significantly enhanced at eight weeks in the fucoidan group, compared to before ingestion (0 weeks). Furthermore, a significantly enhanced NK cell activity was observed in male subjects at eight weeks, compared with the placebo group. These results confirm that fucoidan derived from *C. okamuranus* increases NK cell activity and suggest that it is a safe food material.

1. Introduction

Fucoidan is a general term for the sulfated polysaccharides contained in brown algae, the chemical structure of which differs depending on the seaweed species ¹⁾. Previous studies on fucoidan were reported on animal and human trials, whereas studies have been performed on tumor bearing animal models and subjects with specific disease trends ^{22,23,50,59,60}). In Chapter I, it was reported that fucoidan derived from *C. okamuranasu* activates mouse immunity. In the results, fucoidan derived from *C. okamuranus* improved immune cell proliferation, macrophage phagocytosis, and antibody production in mice. On the basis of the results, an immunological evaluation study for fucoidan derived from *C. okamuranus* in the healthy Japanese subjects was planned.

In this pilot study, a clinical trial using a placebo-controlled, randomized, double-blind, parallel-group comparison method were performed. The primary outcome was to evaluate the effects of ingesting fucoidan derived from *C. okamuranus* on NK cells derived from healthy subjects. The secondary outcome was to assess the safety of fucoidan consumption in healthy subjects under the observation of a medical doctor. Summarily, adverse events due to the test food were not observed and fucoidan derived from *C. okamuranus* promoted NK cell activity, particularly in male subjects.

2. Methods and Materials

2.1. Materials

A beverage containing 1.5 g/50 mL fucoidan derived from *C. okamuranus* (South Product, Uruma, Japan) was utilized as a test food for the fucoidan treatment group; citric acid and sucralose were added to the raw materials (which included 51.3% L-fucose, 18.8% sulfate ions, 14.4% uronic acid; mean molecular weight: 73.4 kDa). For the placebo group, citric acid and sucralose were blended in a beverage with caramel, which was used to ensure that the appearance of the placebo beverage did not differ from that of the fucoidan beverage.

2.2. Subjects

This study was conducted according to the Declaration of Helsinki. The study implementation plan, subject diary, and consent form were approved by the ethics review committee of Nihonbashi Cardiology Clinic, Tokyo, Japan (UMIN000043804), who also gave final approval to conduct the study. Furthermore, the study was conducted under the supervision of a medical doctor at the Shinagawa Season Terrace Healthcare Clinic, Tokyo, Japan. The subjects were healthy male and female between the ages of 20 and 65 years old; each subject provided written consent after being given an adequate explanation of the study. The target number of participants was set as the number needed for statistical analysis. Subjects were provided by KSO Corporation (Tokyo, Japan) and were assigned by block randomization to ensure that independent variables, e.g., age, gender, salivary IgA, etc., did not differ significantly (Figure II-1).

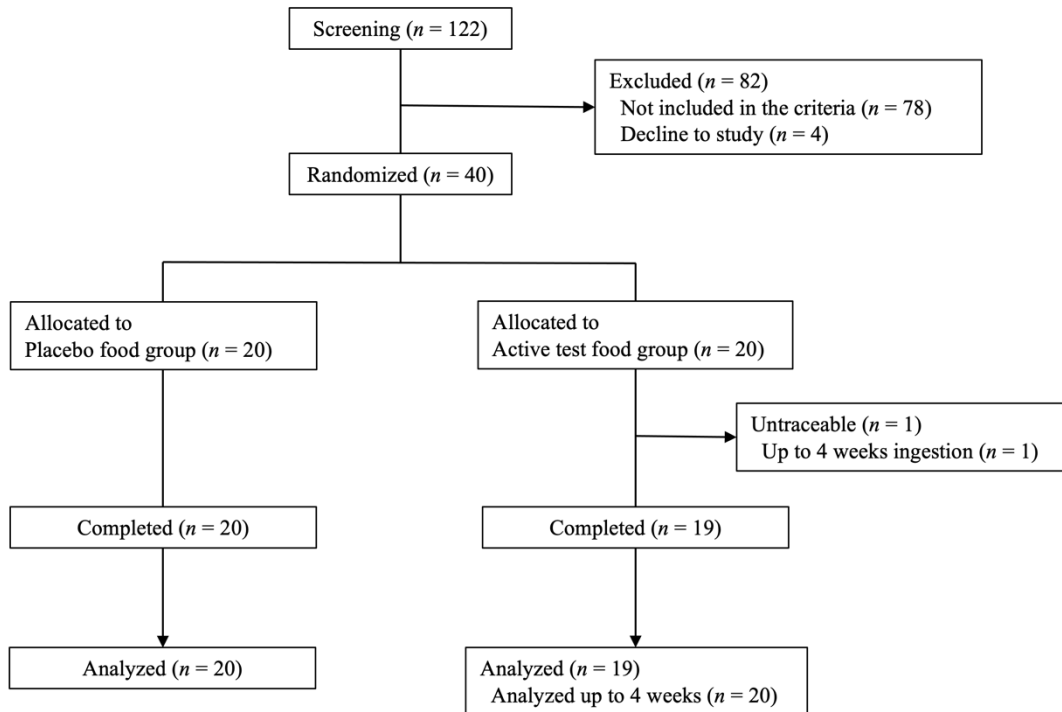


Figure II-1. Flow diagram of the clinical study procedure. Value in parentheses shows the number of participants (*n*).

2.3. Study Design

The controller assigned subjects to two groups—the fucoidan and placebo groups—in a randomized, double-blind, parallel-group, placebo-controlled study (Figure II-2). Both groups ingested two bottles of samples daily. In a previous study ³²⁾, the amount of fucoidan derived from *C. okamuranus* detected in human blood and urine was 1 g/day. Moreover, Abe et al. ⁶¹⁾ administered fucoidan at a dose of 4.05 g/day, which was confirmed to be safe for human consumption; therefore, the dose in this study was set to 3 g/day. This study was conducted between 17 July and 26 December 2016.

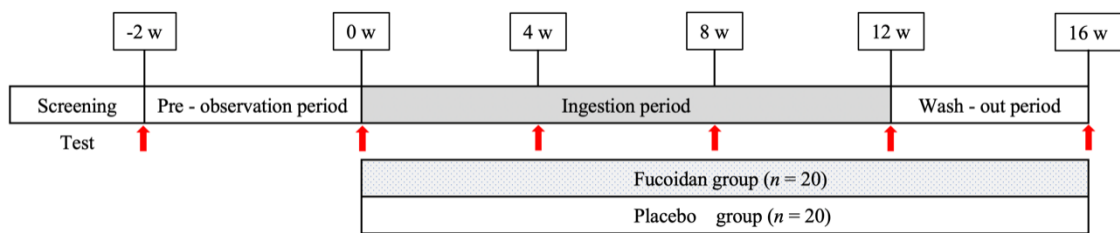


Figure II-2. Overall study design

w: week; red arrows: testing of subjects; n: number of subjects.

2.4. NK cell activity

NK cell activity was measured in the K562 cell line (Dainippon Pharmaceutical, Japan) labeled with ^{51}Cr using a cytotoxicity test. The NK cell activity assay utilized subject blood on the first day of ingestion (week 0) and after 4, 8, 12, and 16 weeks. Blood samples were collected into heparinized tubes. After centrifugation of the blood samples using a lymphocyte separation medium, interface mononuclear cells were collected and suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). The samples were centrifuged again, and mononuclear cells were collected and mixed with ^{51}Cr -labeled target cells (K562) at a ratio of 50:1. The cell mixture was cultured at 37°C and 5% CO_2 for four hours. The ^{51}Cr released from the target cells by NK cell cytotoxicity was measured using a gamma counter (ARC370, Hitachi Aloka Medical, Mitaka, Japan).

The percentage of cytotoxicity was calculated as follows:

$$\text{cytotoxicity (\%)} = (\text{experimental } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}) / (\text{maximal } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}) \times 100.$$

2.5. Interferon (IFN)-gamma and interleukin (IL)-2 concentration in the blood

IFN- γ (Human IFN-gamma Quantikine ELISA Kit, R&D Systems, Minneapolis, MN, USA) and IL-2 (Human IL-2 Quantikine ELISA Kit, R&D Systems, USA) concentrations in the plasma of subjects were measured using sandwich ELISA on the first day of ingestion (week 0) and after 4, 8, 12, and 16 weeks.

2.6. Blood, biochemical tests and safety assessment

Blood tests (white blood cells, red blood cells, hemoglobin, hematocrit, and platelets) and biochemical tests (aspartate aminotransferase, alanine aminotransferase, LDH, T-bil, alkaline phosphatase, γ -glutamyl transpeptidase, creatine kinase, fasting blood sugar, HbA1c, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglyceride, total protein, albumin, urea nitrogen, CRE, uric acid, sodium, chlorine, potassium, calcium, inorganic phosphorus, magnesium, and iron) were measured on the first day of ingestion (week 0) and 4, 8, 12, and 16 weeks later. Furthermore, during the same time, a medical doctor interviewed and examined the subjects as a safety evaluation.

2.7. Statistical analysis

Data were presented as means \pm standard deviation (SD). Data were analyzed using Dunnett's multiple test within same group and Welch's t-test between different group. $P < 0.05$ was considered statistically significant. Statcel version 4 software (OMS Publishing, Japan) was used to conduct all statistical analyses. Moreover, 95% confidence intervals (CI) for each data were calculated using the above software.

3. Results

3.1. Subject background and test food ingestion ratio

The background of the 39 subjects that participated in this study is shown in Table II-3. In the fucoidan group, the mean age (\pm SD) of male and female subjects was 44.5 ± 8.0 and 46.5 ± 7.0 years, respectively; in the placebo group, these respective mean ages were 46.7 ± 6.5 years and 45.7 ± 7.1 years. The ingestion ratio of the test food was 100.0%, 98.8%, 96.4%, 95.8%, and 86.9% in 33, 2, 2, 1, and 1 subject(s), respectively. The study began with 20 subjects in the fucoidan and placebo groups; however, one subject in the fucoidan group was not present on the examination day after eight weeks and could not be contacted. Thus, in the fucoidan group, statistical analysis was performed with 20 subjects within four weeks and 19 subjects after eight weeks (Figure II-1).

Table II-1. Background of subjects in the placebo and fucoidan groups in this study

Parameter	Placebo	Fucoidan	<i>p</i> -value
Subjects (<i>n</i>)	20	20	1.00
Male (<i>n</i>)	10	10	1.00
Female (<i>n</i>)	10	10	1.00
Age (years)	45.5 ± 7.8	47.0 ± 7.6	0.55
Salivary IgA (mg/dL)	12.0 ± 8.3	11.9 ± 8.4	0.96
Body weight (kg)	59.1 ± 9.5	57.6 ± 9.8	0.63
Body mass index (kg/m ²)	21.7 ± 2.3	21.0 ± 2.6	0.39

Age, salivary IgA, body weight, and body mass index data were presented as mean \pm SD (*n* = 20 subjects per group). *n*: number of subjects; *p*: *p*-value (placebo vs. fucoidan).

3.2. NK cell activity

The activity data of NK cells was evaluated by converting the increase rate after ingestion of fucoidan ingestion (0 w) to basal value (100%) before ingestion of fucoidan. In the fucoidan group, a significant increase in NK cell activity ($124.9 \pm 41.8\%$, [95%CI, 104.8–145.1%]) was observed at eight weeks after ingestion when compared with NK cell activity at week 0 (Figure II-3a). In male subjects in the fucoidan group, a significant increase in NK cell activity ($133.8 \pm 43.4\%$, [95%CI, 93.5–174.2%]) was observed at eight weeks, relative to activity at eight weeks in the placebo group, after fucoidan ingestion (Figure II-3b). Contrastly, there was no significant impact of fucoidan on NK cell activity in female subjects (Figure II-3c).

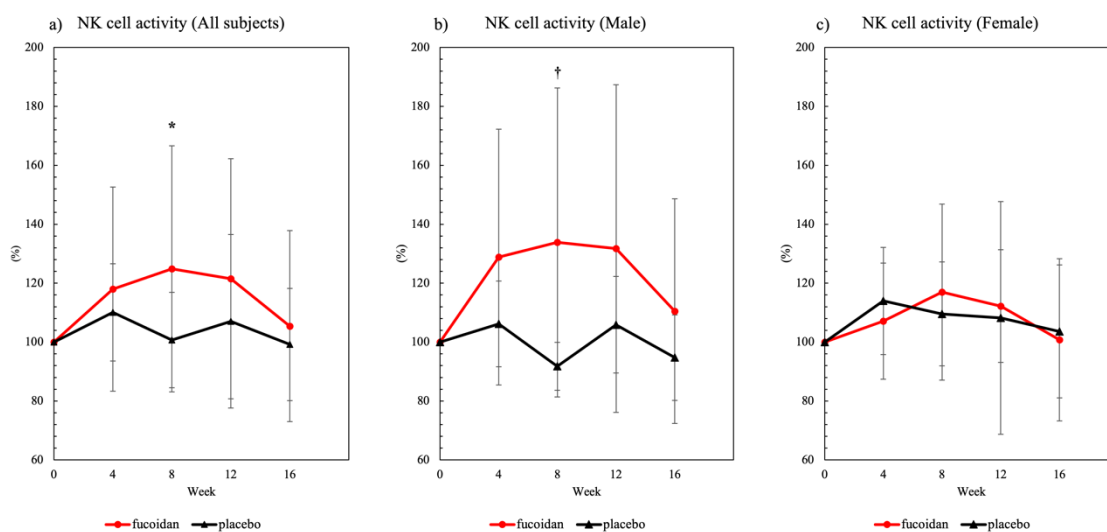


Figure II-3. Natural killer (NK) cell activity

NK cell activity was expressed as relative to basal value (100%). All data were presented as mean \pm SD ($n = 20$ subjects per group). NK cell activity in a) all subjects, b) males, and c) females.

*Significant difference compared with week 0 within the fucoidan group ($p < 0.05$, Dunnett's multiple test). †Significant difference between the placebo and fucoidan groups within the same week ($p < 0.05$, t-test).

3.3. Interferon-gamma (IFN- γ) and interleukin-2 (IL-2) levels in the blood

In tests of plasma IFN- γ levels, the number of fucoidan group subjects in which IFN- γ was detected (0, 4, and 8 weeks: four cases per week; 12 weeks: six cases) during the ingestion period tended to increase, whereas the number of placebo group subjects in which IFN- γ was detected (0 weeks: five cases; 4 weeks: five cases; 8 weeks: three cases; and 12 weeks: four cases) tended to decrease (Table II-2). Contrastly, the IFN- γ levels decreased after the washout period.

In test of plasma IL-2 levels were below the detection limit in all subjects during the test period.

Table II-2. number of subjects detected in IFN- γ and IL-2

Group	0 w	4 w	8 w	12 w	16 w
	IFN- γ				
Fucoidan	4	4	4	6	3
Placebo	5	6	3	4	3
	IL-2				
Fucoidan	0	0	0	0	0
Placebo	0	0	0	0	0

Between-group comparison of subjects in which IFN- γ and IL-2 levels were detected in the blood.

Detection limit: IFN- γ >1.56 pg/mL; IL-2 >15.6 pg/mL.

3.4. Safety assessment

3.4.1. Blood and biochemical test

There were no significant differences in white blood cell count, hemoglobin, platelet count, or hematocrit between the placebo and fucoidan groups during the study period (Table II-3). The results of the biochemical tests showed no abnormalities (Table II-4). In the fucoidan and placebo group, compared with determinations at the same week, significant changes were observed at 12 and 16 weeks for magnesium (Mg), eight weeks for iron (Fe).

Table II-3. Blood test results

Group	0 w	4 w	8 w	12 w	16 w
	WBCs (/μL)				
Fucoidan	5720 ± 1233	5080 ± 1307	5179 ± 1280	5168 ± 1433	5589 ± 1610
Placebo	5950 ± 1674	5535 ± 911	5660 ± 985	5520 ± 1282	5685 ± 1132
	RBCs (×10 ⁴ /μL)				
Fucoidan	453.6 ± 41.4	458.2 ± 37.5	453.3 ± 36.4	456.8 ± 40.2	463.6 ± 37.4
Placebo	459.8 ± 28.3	462.7 ± 30.3	462.7 ± 28.9	472.1 ± 33.0	465.3 ± 36.2
	Hb (g/dL)				
Fucoidan	13.7 ± 1.6	13.8 ± 1.5	13.7 ± 1.7	13.6 ± 1.7	13.8 ± 1.7
Placebo	14.2 ± 0.9	14.2 ± 1.0	14.2 ± 0.8	14.5 ± 1.1	14.2 ± 1.2
	Ht (%)				
Fucoidan	42.7 ± 4.7	42.9 ± 4.2	42.3 ± 4.2	42.5 ± 4.4	43.2 ± 4.7
Placebo	43.1 ± 2.2	43.7 ± 3.0	43.6 ± 2.1	44.7 ± 2.8	43.8 ± 3.2
	Plt (×10 ⁴ /μL)				
Fucoidan	26.2 ± 5.4	26.5 ± 5.9	26.1 ± 4.6	28.4 ± 9.4	27.4 ± 4.8
Placebo	27.3 ± 3.5	27.4 ± 4.0	27.5 ± 4.2	27.9 ± 4.4	28.3 ± 4.3

All data represent means ± SD (*n* = 20 subjects per group). WBCs: White Blood Cells; RBCs: Red Blood Cells; Hb: Hemoglobin; Ht: Hematocrit; Plt: Platelets; w: week.

Table II-4. Biochemical test results

Group	0 w	4 w	8 w	12 w	16 w
	AST (U/L)				
Fucoidan	22.4 ± 11.2	23.9 ± 17.6	21.2 ± 11.2	21.2 ± 8.8	22.7 ± 8.9
Placebo	20.2 ± 5.9	21.3 ± 6.0	20.9 ± 7.1	21.8 ± 6.7	22.2 ± 4.8
	ALT (U/L)				
Fucoidan	17.9 ± 10.7	19.6 ± 17.1	16.9 ± 10.8	16.5 ± 10.0	18.7 ± 10.9
Placebo	17.7 ± 9.6	18.3 ± 10.2	17.7 ± 7.9	20.9 ± 11.9	19.5 ± 9.3
	LDH (U/L)				
Fucoidan	177.5 ± 25.5	180.5 ± 28.0	176.6 ± 25.4	178.8 ± 28.6	177.3 ± 20.9
Placebo	171.1 ± 21.6	176.0 ± 28.5	179.8 ± 29.8	173.0 ± 24.9	170.8 ± 25.1
	T-bil (mg/dL)				
Fucoidan	0.9 ± 0.3	0.9 ± 0.3	0.7 ± 0.2	0.8 ± 0.3	0.8 ± 0.3
Placebo	0.9 ± 0.4	0.8 ± 0.4	0.8 ± 0.3	0.8 ± 0.3	0.7 ± 0.2
	ALP (U/L)				
Fucoidan	183.6 ± 51.5	180.7 ± 46.8	190.1 ± 53.4	189.9 ± 53.5	198.1 ± 55.1
Placebo	172.4 ± 49.3	174.1 ± 47.1	175.6 ± 47.0	179.0 ± 52.5	182.6 ± 61.8
	γ-GTP (U/L)				
Fucoidan	41.2 ± 57.4	37.7 ± 46.6	34.4 ± 37.1	34.6 ± 31.9	32.5 ± 29.7
Placebo	22.6 ± 15.5	23.4 ± 16.7	25.1 ± 17.3	27.1 ± 24.8	25.2 ± 18.1
	CK (U/L)				
Fucoidan	119.3 ± 67.2	133.9 ± 109.8	114.2 ± 47.5	122.3 ± 74.6	141.8 ± 112.0
Placebo	122.7 ± 66.0	118.6 ± 66.5	136.3 ± 92.0	126.9 ± 88.1	114.7 ± 57.3
	FBS (mg/dL)				
Fucoidan	83.1 ± 8.3	82.4 ± 9.7	81.3 ± 7.4	79.1 ± 8.2	82.6 ± 12.5
Placebo	82.8 ± 8.0	82.5 ± 7.0	82.7 ± 7.2	82.3 ± 7.8	81.0 ± 6.1
	HbA1c (%)				
Fucoidan	5.4 ± 0.3	5.5 ± 0.2	5.4 ± 0.3	5.4 ± 0.3	5.4 ± 0.2
Placebo	5.4 ± 0.3	5.5 ± 0.3	5.4 ± 0.3	5.4 ± 0.2	5.4 ± 0.3

Table II-4. Biochemical test result (Continued)

Group	0 w	4 w	8 w	12 w	16 w
	TC (mg/dL)				
Fucoidan	200.7 ± 32.0	203.2 ± 29.7	201.7 ± 26.7	202.6 ± 26.6	213.9 ± 33.1
Placebo	203.2 ± 29.8	205.3 ± 30.1	212.3 ± 33.9	215.7 ± 37.1	213.1 ± 30.8
	LDL-C (mg/dL)				
Fucoidan	111.1 ± 32.0	111.1 ± 31.8	109.8 ± 31.9	106.8 ± 32.4	115.5 ± 34.5
Placebo	117.6 ± 26.7	119.2 ± 26.0	125.6 ± 29.9	126.9 ± 33.7	124.6 ± 31.6
	HDL-C (mg/dL)				
Fucoidan	69.8 ± 16.2	72.1 ± 17.2	68.8 ± 16.1	72.0 ± 20.1	76.4 ± 21.5
Placebo	64.7 ± 13.1	64.7 ± 15.6	67.4 ± 13.5	66.4 ± 15.4	66.0 ± 15.7
	TG (mg/dL)				
Fucoidan	81.9 ± 32.0	81.3 ± 48.8	93.8 ± 48.8	95.2 ± 92.4	97.0 ± 105.7
Placebo	98.0 ± 92.3	86.4 ± 47.7	84.8 ± 39.9	88.8 ± 39.8	96.8 ± 44.2
	TP (g/dL)				
Fucoidan	7.2 ± 0.4	7.2 ± 0.3	7.1 ± 0.4	7.3 ± 0.3	7.3 ± 0.3
Placebo	7.3 ± 0.4	7.4 ± 0.4	7.3 ± 0.4	7.4 ± 0.4	7.4 ± 0.3
	Alb (g/dL)				
Fucoidan	4.5 ± 0.3	4.5 ± 0.2	4.4 ± 0.2	4.5 ± 0.2	4.5 ± 0.2
Placebo	4.5 ± 0.3	4.5 ± 0.2	4.5 ± 0.2	4.5 ± 0.2	4.5 ± 0.2
	UN (mg/dL)				
Fucoidan	12.4 ± 4.4	12.2 ± 3.7	12.2 ± 2.9	13.1 ± 3.9	13.6 ± 3.1
Placebo	13.1 ± 3.5	13.0 ± 3.9	12.2 ± 3.7	12.2 ± 3.7	14.3 ± 4.7
	CRE (mg/dL)				
Fucoidan	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Placebo	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
	UA (mg/dL)				
Fucoidan	5.3 ± 1.3	5.3 ± 1.6	5.1 ± 1.6	5.2 ± 1.6	5.2 ± 1.6
Placebo	5.1 ± 1.3	5.2 ± 1.3	5.2 ± 1.3	5.3 ± 1.2	5.2 ± 1.6

Table II-4. Biochemical test result (Continued)

Group	0 w	4 w	8 w	12 w	16 w
	Na (mEq/L)				
Fucoidan	140.1 ± 1.8	140.0 ± 1.6	140.4 ± 2.1	140.8 ± 1.8	140.2 ± 1.1
Placebo	140.4 ± 1.1	140.3 ± 1.5	141.0 ± 1.5	141.0 ± 1.8	140.7 ± 1.1
	Cl (mEq/L)				
Fucoidan	103.0 ± 1.6	103.1 ± 2.2	103.9 ± 1.9	103.7 ± 2.1	103.3 ± 1.9
Placebo	103.3 ± 1.3	103.7 ± 1.6	103.9 ± 1.6	103.6 ± 2.0	104.1 ± 1.8
	K (mEq/L)				
Fucoidan	4.3 ± 0.3	4.2 ± 0.3	4.4 ± 0.2	4.3 ± 0.3	4.4 ± 0.2
Placebo	4.2 ± 0.2	4.3 ± 0.4	4.3 ± 0.3	4.2 ± 0.3	4.3 ± 0.2
	Ca (mg/dL)				
Fucoidan	9.6 ± 0.3	9.6 ± 0.3	9.4 ± 0.3	9.6 ± 0.4	9.6 ± 0.3
Placebo	9.7 ± 0.3	9.7 ± 0.3	9.5 ± 0.3	9.6 ± 0.3	9.6 ± 0.3
	IP (mg/dL)				
Fucoidan	3.6 ± 0.5	3.6 ± 0.5	3.6 ± 0.4	3.8 ± 0.5	3.6 ± 0.5
Placebo	3.7 ± 0.4	3.7 ± 0.5	3.6 ± 0.5	3.6 ± 0.4	3.6 ± 0.6
	Mg (mg/dL)				
Fucoidan	2.1 ± 0.1	2.2 ± 0.2	2.1 ± 0.1	2.2 ± 0.1 [†]	2.1 ± 0.1 [†]
Placebo	2.2 ± 0.1	2.2 ± 0.2	2.1 ± 0.1	2.1 ± 0.1 [†]	2.2 ± 0.1 [†]
	Fe (µg/dL)				
Fucoidan	117.1 ± 49.0	104.7 ± 35.0	112.9 ± 37.7 [†]	108.7 ± 51.6	98.9 ± 52.6
Placebo	99.9 ± 43.5	95.9 ± 45.2	86.4 ± 32.5 [†]	103.2 ± 48.4	116.6 ± 37.4

All data were represented mean ± SD ($n = 20$ subjects per group). [†]Significant difference between the placebo and fucoidan groups within the same week ($p < 0.05$, t-test). AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; T-bill: Total bilirubin; ALP: Alkaline phosphatase; γ -GTP: γ -Glutamyl transpeptidase; CK: Creatine kinase; FBS: Fasting blood sugar; HbA1c: Hemoglobin A1c; TC: Total cholesterol; LDL-C: Low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; TG: Triglyceride; TP: Total protein; Alb: Albumin; UN: Urea

nitrogen; CRE: Creatinine; UA: Uric acid; Na: Sodium; Cl: Chlorine; K: potassium; Ca: Calcium; IP:

Inorganic phosphorus; Fe: Iron; w: week.

3.4.2. Evaluation of adverse events

To evaluate the test food safety, adverse events during the ingestion period were recorded. Across both groups, 28 adverse events were noted during the ingestion period. Whereas following interviews and medical examinations by a doctor, it was concluded that severe adverse events had not occurred, and causal relationships with the test food did not exist. Moreover, subjects recovered from all adverse events without further problems (Table II-5).

Table II-5. List of adverse events during the ingestion period

Group	Adverse Events	Case	Treatment	Outcome	Causality	Study
Fucoidan	Cold	7	3	7	unrelated	continue
	Pharyngitis	2	1	2		
	Neck strain	1	1	1		
	Tooth cavity	1	1	1		
Placebo	Cold	10	9	10	unrelated	continue
	Pharyngitis and cough	1	0	1		
	Abdominal pain	1	0	1		
	Automatic nerves disorder	1	1	1	probably unrelated	
	Sprain	1	1	1	unrelated	
	Back pain	1	1	1		
	Fatigue	1	1	1		
Gum disease	1	1	1			

Numbers represent the number of subjects.

4. Discussion

4.1. NK cell activity

The raw materials for fucoidan, i.e., edible seaweeds such as Kombu, Wakame, and Mozuku, have a long history of consumption as food in Japan. In this study, fucoidan derived from mozuku (*C. okamuranus*) was used as a test food. In previous studies, fucoidan has been reported to have effects on immunity in animals^{42,62} and humans^{30,63}. In this study, healthy Japanese subjects were randomly assigned to fucoidan and placebo groups, and peripheral-blood-derived NK cell activity was measured after daily oral ingestion of fucoidan (3 g) for 12 weeks. Results showed that NK cell activity was significantly improved in the fucoidan group after ingestion of fucoidan; particularly, this activity was significantly improved in male subjects but not in female subjects. Normal cells have MHC class I receptors on their surface that inhibit the activity of NK cells. However, cancer cells and virus-infected cells have reduced or insufficient MHC class I expression, whereby the activation signal for NK cells is increased; this is characteristic of attacking and removing cancer and virus-infected cells. In this study, however, healthy subjects were tested; hence, conditions related to cancer and virus-infected cells are not important. Typically, NK cells activation requires cytokines secretion, such as IFN- γ , IL-2, and IL-12, by macrophages and helper T cells. In this study, IFN- γ in the blood appeared to increase periodically in fucoidan group subjects, whereas IL-2 levels did not change significantly in any subject in either group during the ingestion and washout periods. Previously, Takahashi et al.⁶⁴ conducted an oral administration study of fucoidan in subjects with advanced cancer and reported that the secretion of IL-1 β , IL-6, and TNF- α was significantly decreased two weeks after ingestion. Furthermore, Ohnogi et al.⁴¹ reported that healthy subjects (one male and 14 female) who ingested fucoidan derived from Gagome

kombu (*Kjellmaniella crassifolia*) for four weeks showed a significant suppression in the reduction of IFN- γ and IL-2 secretion. In a previous study in which fucoidan was administered to mice, NK cell activation significantly promoted IFN- γ secretion ⁶⁵). Similarly, Murayama et al. ⁵³) reported increased IFN- γ secretion in mice administered fucoidan, suggesting that the mechanism of action was by helper Th1 cell enhancement.

Previous studies have shown that macrophages are involved in NK cells activation by fucoidan. As mentioned in the Chapter I, it was shown that fucoidan derived from *C. okamuranus* significantly improves IL-2 and IFN- γ levels in mice and macrophage phagocytosis ⁶²). Additional flows, other than those of cytokines, are involved in macrophage activation, e.g., radical scavenger receptors are involved. These receptors widely identify negatively charged macromolecules such as low-density lipoproteins, lipopolysaccharides, and lipoteichoic acid ^{57,66}). Since fucoidan is a negatively charged polymer to which a sulfate group is bound, it is suggested that fucoidan activity is promoted via the radical scavenger receptor of macrophages. Furthermore, Miyazaki et al. ⁶⁷) reported that fucoidan binds to the plasma membrane of macrophages to increase the production of nitric oxide and TNF- α . This indicates that the reaction is mediated by various pattern recognition receptors, such as Dectin-1, on the cell surface rather than through macrophage phagocytosis. Thus, it can be suggested that cytokines such as IFN- γ , which are produced by macrophages, are involved in NK cells activation by fucoidan. Overall, no significant change in IFN- γ levels was observed in this study. However, the cause of the seemingly fucoidan-mediated time-dependent increase in subjects with IFN- γ in the blood requires further investigation.

Typically, innate and adaptive immunity tends to be higher in women than in men, whereas NK cells number in men is higher than that in women ⁶⁸). Specifically, the normal

range of NK cell activity is higher in adult males (postpuberty/adulthood) whereas becomes higher in females at old age ⁶⁹⁾. Furthermore, hormones, genes, environment, age, etc. are also linked to gender-specific differences in immune responses ⁷⁰⁾. The mean age of the subjects in the fucoidan ingestion group in this study was 47.0 ± 7.6 years, which is the period in which male NK cell activity is high. Moreover, Nagamine et al. ³⁰⁾ showed a fucoidan administration study in cancer survivors and reported that the NK cell activity of older male subjects (mean age: 73.9 ± 4.9 ; $n = 11$) was significantly higher than that of female subjects (mean age: 59.0 ± 7.7 ; $n = 4$). In a previous *ex vivo* study using ovariectomized rats ⁷¹⁾, it was reported that fucoidan-treated NK cells had increased tumoricidal activity, but fucoidan-free standard diet-treated NK cells did not. This result indicates that fucoidan supplementation causes NK cell activity, modulating immunity induced by estrogen deficiency. Thus, female immune modulation is affected by organs such as the ovaries, and fucoidan may act on postmenopausal immunoregulation. On the basis of these results, fucoidan obtained from *C. okamuranus* tends to improve NK cells activity in males. The underlying mechanism of this effect is unfortunately unknown and requires further investigation. In the future, a preference to increase the number of subjects and investigate the immunomodulatory effect of fucoidan derived from *C. okamuranus* would be welcomed.

4.2. Safety Assessment

In this study, fucoidan did not induce severe adverse events when ingested at 3g daily for 12 weeks. Furthermore, abnormalities were not confirmed in blood and biochemical tests. Additionally, blood tests showed a significant change in magnesium and iron, whereas it was considered to be within normal range and therefore posed no issue. Although adverse events were occasionally observed during the study period, all were diagnosis were unrelated to fucoidan oral ingestion as confirmed by the medical doctor, and patients recovered and continued with this study. These results are consistent with mozuku, the raw material of fucoidan, being a type of seaweed that is usually eaten in Japan and considered highly safe as a food item. Similar to these results, Abe et al. ⁶¹⁾ reported blood and urinalysis test results indicating that ingestion of 4g fucoidan derived *C. okamuranus* daily for two weeks was safe. Moreover, no abnormalities was previously reported in blood and biochemical tests conducted on healthy Japanese subjects following ingestion of 2g fucoidan derived from *C. okamuranus* daily for four weeks ⁷²⁾. High intake of mozuku-derived fucoidan has been reported to induce diarrhea ⁵⁰⁾. However, fucoidan intake in appropriate amounts can relieve defecation ²⁹⁾. Diarrhea was not reported by the fucoidan treatment group of this study.

5. Conclusion

In conclusion, it has shown that fucoidan derived from *C. okamuranus* is safe as a food item and that it improves NK cell activity, especially in males. In future research, investigating the effects of fucoidan on dendritic cells, macrophages, another cytokine and sex difference to elucidate the immunomodulatory effect of these sulfated polysaccharides is required.

Chapter III

Are *Helicobacter pylori* infection and mozuku consumption associated with fucoidan absorption?

Abstract

The associations of *Helicobacter pylori* and mozuku consumption with fucoidan absorption was performed. Overall, 259 healthy Japanese subjects consumed 3 g fucoidan, and their urine samples were collected to measure fucoidan values and *H. pylori* titers before and 3, 6, and 9 h after fucoidan ingestion. Compared to the basal levels (3.7 ± 3.4 ng/mL), the urinary fucoidan values significantly increased 3, 6, and 9 h (15.3 ± 18.8 , 24.4 ± 35.1 , and 24.2 ± 35.2 ng/mL), respectively, after fucoidan ingestion. The basal fucoidan levels were significantly lower in *H. pylori*-negative subjects who rarely consumed mozuku than in those who regularly consumed it. Regarding the Δ Max fucoidan value (highest value- basal value) in *H. pylori*-positive subjects who consumed mozuku at least once monthly, those aged ≥ 40 years showed significantly lower values than < 40 years old. Among subjects ≥ 40 years old who regularly consumed mozuku, the Δ Max fucoidan value was significantly lower in *H. pylori*-positive subjects than in *H. pylori*-negative ones. In *H. pylori*-positive subjects who consumed mozuku at least once monthly, basal fucoidan values showed positive correlations with *H. pylori* titers and Δ Max fucoidan values in subjects < 40 years old. No correlations were found in *H. pylori*-positive subjects who consumed mozuku once every 2–3 months or less. Thus, fucoidan absorption is related to *H. pylori* infection and mozuku consumption frequency.

1. Introduction

Fucoidan is a complex sulfated polysaccharide mostly found in brown marine algae. Fucoidan shows a broad spectrum of biological activities, including anti-inflammatory, immunomodulatory, antioxidant, antitumor, and anti-infection effects^{18,73–76}. Many investigators reported a potential role of fucoidan derived from *C. okamuranus* (Okinawa mozuku) as an anti-*Helicobacter pylori* (*H. pylori*) agent on the basis of its ability to disrupt the adhesion of the microbe to the gastric epithelium *in vivo* and *in vitro*^{27,44,77,78}. The inhibitory effect of fucoidan derived from *C. okamuranus* on *H. pylori* was shown *in vitro* by Shibata et al.⁴⁴. Their study demonstrated that the *H. pylori* binding to human gastric cell lines was restricted more by fucoidan derived from *C. okamuranus* than by fucoidan obtained from *Fucus*. Moreover, fucoidan restricted both Lewis-b and sulfatide-mediated attachment of *H. pylori* to gastric cells. They concluded that the *Cladosiphon* fucoidan inhibitory effect on the binding of *H. pylori* and gastric cells might be induced by the coating with this component of the bacterial surface. However, no bacteriostatic or bactericidal activity was noted against *H. pylori* for any fucoidan preparation⁷⁸.

Fucoidan obtained from *C. okamuranus* is reported to be absorbed across the intestinal tract via energy-dependent processes and pinocytosis^{79–81}. In healthy Japanese participants, fucoidan was detected in most urine following oral administration³⁵. Since the fucoidan absorption rate through the small intestine was highly variable among the subjects, several factors were suggested to influence its absorption. For example, the consumption of Okinawa mozuku (*C. okamuranus*), a brown seaweed including fucoidan, is an essential factor associated with fucoidan absorption. In reference to a previous report by Hehemann et al.⁸², it was speculated that the gastrointestinal microbiota influences fucoidan absorption.

H. pylori is a gram-negative, spiral-shaped, microaerophilic bacterium. It colonizes the entire gastric mucosa in approximately half of the world's human population, and a poor socioeconomic condition is a significant risk factor for infection^{83–86}). *H. pylori* induces peptic ulcers and atrophic gastritis, and it is associated with primary gastric B-cell lymphoma (MALT lymphoma) and gastric adenocarcinoma. Thus, the host immune system cannot clear the *H. pylori* infection, which persists without treatment.

Many previous studies focused on modifying the gastric environment induced by *H. pylori* infection. For example, *H. pylori* infection can lead to the deficiency of vitamins, such as vitamin C, vitamin A, α -tocopherol, vitamin B₁₂, and folic acid, and essential minerals^{87–89}). Additionally, gastric *H. pylori* infection affects local and distant microbial populations and host responses.

Since fucoidan can bind *H. pylori* and disrupt its attachment to the gastric epithelium^{27,44,77}), *H. pylori* infection is believed to affect fucoidan absorption. In this study, the effects of *H. pylori* infection on the absorption of fucoidan derived from *C. okamuranus* in healthy Japanese volunteers was conducted. Although fucoidan absorption is deficient in humans, the fucoidan levels after oral administration are approximately ten times higher in urine than in serum³²). Thus, urinary fucoidan levels were measured before and after the oral administration of fucoidan obtained from *C. okamuranus*.

2. Methods and Materials

2.1. Subjects

Pamphlets describing the purpose, methods, and exclusion items of the research titled “The reference of *H. pylori* infection to absorption of mozuku fucoidan” was published on the website and volunteer participants were recruited. Two hundred sixty-two healthy Japanese submitted applications between April 2014 and June 2016. They completed a questionnaire assessing gender, age, and mozuku consumption. The 259 healthy Japanese volunteers who completed questionnaires were enrolled and urine samples were collected as planned. Subjects were divided into five age groups: 20–29, 30–39, 40–49, 50–59, and ≥ 60 years old.

The frequency of mozuku consumption was divided into five groups: approximately 1–3 times weekly, approximately once every two weeks, approximately once monthly, approximately once every 2–3 months, and rarely (Table III-1).

This study was conducted according to the Declaration of Helsinki. The Ethics Committee approved the protocol of the study of South Product Co., LTD (Uruma, Japan). (UMIN000039117). Following an explanation of the study and its aim, all subjects gave informed consent.

2.2. Oral administration of fucoidan beverage and collection of urine samples

All subjects avoided seaweed and fucoidan supplementation on the day before the test and on the test day to prevent the effects of diet. Subjects orally ingested two fucoidan beverages (1500 mg/bottle) at 9:00 am in the morning. Urine samples were collected four times; before (0) and 3, 6, and 9 h after fucoidan ingestion. A parcel delivery service collected urine samples. In this study, the subjects orally ingested 3 g fucoidan derived from *C. okamuranus*. South Product Co., Ltd prepared the test beverage.

2.3. Assay for fucoidan levels in urine samples

Urinary fucoidan levels were assayed using a sandwich ELISA method developed by the School of health sciences, Faculty of Medicine, Gunma University ³²). The reproducibility of the fucoidan ELISA method was as follows; The intra- and interassay CVs for serum, plasma, and urine, using high and low concentrations of fucoidan, were in the range of 1.5%–13.4%. The detection limit concentration of this ELISA was <1 ng/mL.

2.4. Assay for anti-*H. pylori* antibody titers in urine samples

Until use, single-void urine samples were obtained and stored at 2°C–8°C. Urinary IgG antibodies to *H. pylori* were measured using a urine-based ELISA kit (URINELISA®, Otsuka Pharmaceutical Co., Ltd) that uses a VacA- and CagA-positive *H. pylori* strain isolated from a Japanese patient with gastritis as the antigen source. This ELISA-based test result was considered positive when a cutoff index of 1.0 (optical density = 0.218) or greater was derived after measurement of the optical density according to the manufacturer's instructions ^{90–92}).

The Δ Max fucoidan value was calculated as the highest level of urinary fucoidan following fucoidan ingestion—the basal value (before ingestion). If the basal fucoidan level was higher than that after fucoidan ingestion, then the Δ Max fucoidan value was recorded as 0.

2.5. Statistical analysis

Urinary fucoidan values after fucoidan ingestion were analyzed using a two-way analysis of variance (ANOVA) or one-way ANOVA, followed by Tukey's test for multiple comparisons. SAS version 9.4 (Statistical Analysis Software 9.4, SAS Institute Inc., Cary, NC, USA) was used to conduct statistical analyses.

The Mann–Whitney U-test was used to analyze between-group differences. Statistical correlations were analyzed using Spearman's rank correlation coefficient. Moreover, multiple regression analysis was conducted with *H. pylori* infection as the dependent variable, while age and mozuku consumption as the independent variables. The results were expressed as the hazard ratio and 95% confidence interval (CI). Data were expressed as the mean \pm standard deviation (SD). $P < 0.05$ indicated a statistically significant difference.

3. Results

3.1. Prevalence of *H. pylori* infection according to the frequency of mozuku consumption and age

The importance of mozuku consumption and age to *H. pylori* infection is shown in Table III-1. Regarding age, *H. pylori* infection was detected in 60.0, 58.7, 61.9, 77.8, and 88.5% of subjects aged 20–29, 30–39, 40–49, 50–59, and ≥ 60 years old, respectively. According to logistic regression analysis, age was a significant risk factor for *H. pylori* infection. The risk of infection was significantly higher in subjects ≥ 40 years old than in those < 40 years old. Mozuku consumption was not a significant risk factor for *H. pylori* infection (Table II-2).

Table III-1. *H. pylori* infection according to the frequency of mozuku consumption and age

Age group	1-3 times weekly		Once every 2 weeks		Once monthly		Once every 2-3 months		hardly eat	
	<i>H. pylori</i>		<i>H. pylori</i>		<i>H. pylori</i>		<i>H. pylori</i>		<i>H. pylori</i>	
	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)
20's (n = 50)	2	5	6	7	5	5	4	5	3	8
30's (n = 75)	2	3	3	10	6	11	10	11	9	10
40's (n = 63)	3	4	2	10	6	11	10	7	3	7
50's (n = 45)	2	6	2	7	2	11	3	4	1	7
≥ 60 's (n = 26)	0	6	0	2	2	7	1	5	0	3

20's: 20–29 years old, 30's: 30–39 years old, 40's: 40–49 years old, ≥ 60 's: over 60 years old.

n = number of subjects.

Table III-2. Relevance of age and mozuku consumption to *H. pylori* infection: logistic regression analysis

	Odds Ratio	95%CI
Habit of eating mozuku	1.12	0.89-1.42
Age		
40y.o.<	1.00	
\geq 40y.o.	1.70	1.01-2.85

y.o.: years old.

3.2. Urinary fucoidan values before and after fucoidan ingestion

In all subjects, the urinary fucoidan values significantly increased 3, 6, and 9h after fucoidan beverage ingestion compared to the basal values (Table III-3). The urinary fucoidan values were significantly higher at six and nine hour than those at three hour. A significant difference was not observed in the urinary fucoidan values between the group that regularly consumed mozuku and the group that rarely consumed mozuku.

Table III-3. Time course of urinary fucoidan

	0	3 h	6 h	9 h
	ng/mL			
Subjects (<i>n</i> = 259)	3.7 ± 3.4	15.3 ± 18.8 ^a	24.4 ± 35.1 ^{a,b}	24.2 ± 35.2 ^{a,b}

All data were presented as mean ± SD.

Different letters indicate a significant difference as follows:

a: Compare to basal value ($p < 0.01$).

b: Compare to the fucoidan values at 3 h ($p < 0.01$).

n = number of subjects.

3.3. Basal levels (before ingestion) of urinary fucoidan

Among subjects who rarely consumed mozuku, the basal fucoidan levels were significantly lower in the *H. pylori*-negative group than in the *H. pylori*-positive group. Among *H. pylori*-negative subjects, the basal fucoidan levels were significantly lower in those who rarely consumed mozuku than those who consumed mozuku 1–3 times weekly, once monthly, or once every 2–3 months. Therefore, the basal fucoidan levels were not affected by the frequency of mozuku consumption or age in *H. pylori*-positive subjects (Table III-4).

Table III-4. Basal fucoidan levels according to the frequency of mozuku consumption in *H. pylori*-negative and *H. pylori*-positive subjects

Habit of eating mozuku	<i>H. pylori</i> (-)	<i>H. pylori</i> (+)	<i>P</i> -value <i>H. pylori</i> (-) vs <i>H. pylori</i> (+)
1-3 times weekly	4.1 ± 1.3 (<i>n</i> = 9) ^a	3.2 ± 3.0 (<i>n</i> = 24)	0.29
Once every 2 weeks	2.7 ± 3.2 (<i>n</i> = 14)	3.2 ± 2.6 (<i>n</i> = 35)	0.58
Once monthly	3.1 ± 3.0 (<i>n</i> = 21) ^b	2.8 ± 2.8 (<i>n</i> = 44)	0.69
Once every 2-3 months	3.3 ± 3.3 (<i>n</i> = 28) ^c	4.3 ± 5.7 (<i>n</i> = 33)	0.40
hardly eat	1.4 ± 1.5 (<i>n</i> = 16)	3.3 ± 3.4 (<i>n</i> = 35)	0.01

All data were presented as mean ± SD.

Different letters indicate a significant difference as follows:

a: Compare to *H. pylori*-negative subjects who hardly ate mozuku (*p* = 0.01).

b: Compare to *H. pylori*-negative subjects who hardly ate mozuku (*p* = 0.03).

c: Compare to *H. pylori*-negative subjects who hardly ate mozuku (*p* = 0.03).

n = number of subjects.

3.4. Relationship between *H. pylori* titers and basal fucoidan levels

Among *H. pylori*-positive subjects, a significant positive correlation existed between *H. pylori* titers and basal fucoidan levels in subjects <40 years old who consumed mozuku at least once monthly. A significant correlation between *H. pylori* titers and basal fucoidan levels was not found in subjects aged ≥ 40 years irrespective of the frequency of mozuku consumption (Figure III-1).

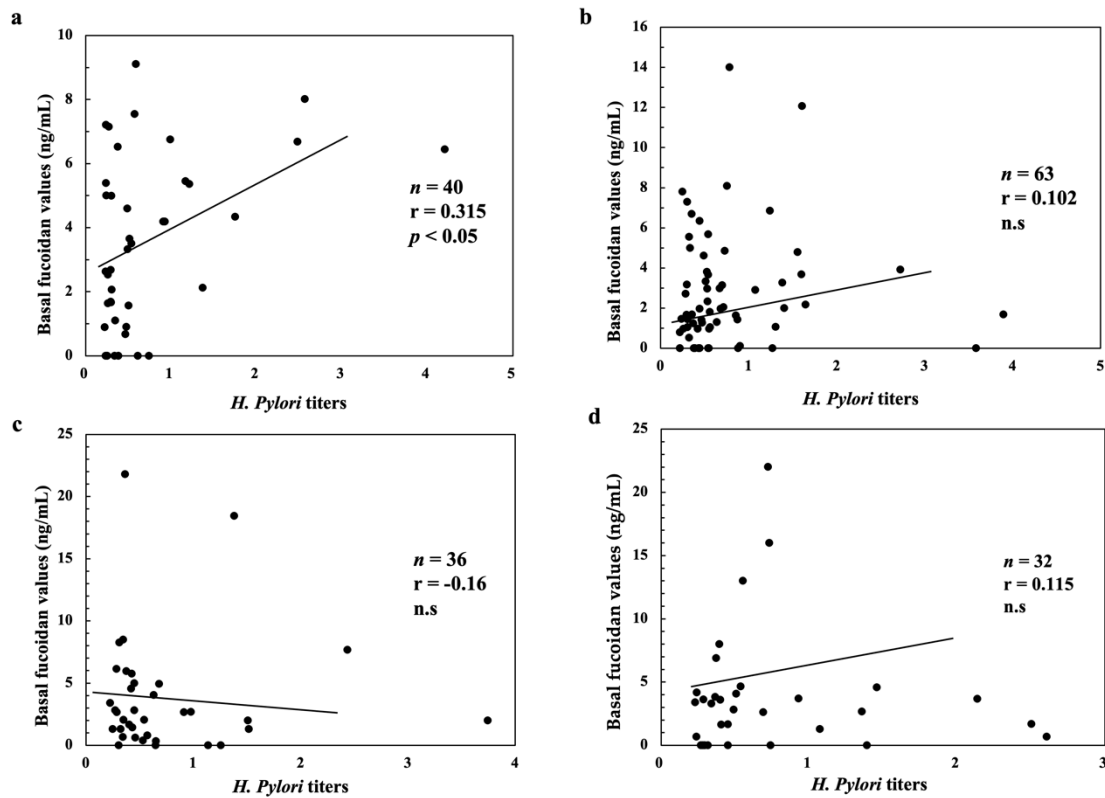


Figure III-1. Relationship between *H. pylori* titers and basal fucoidan levels according to the frequency of mozuku consumption and age among *H. pylori*-positive subjects.

a: Subjects aged <40 years who ate mozuku at least once a month. b: Subjects aged ≥ 40 years who ate mozuku at least once a month. c: Subjects aged <40 years who ate mozuku once every 2–3 months or less. d: Subjects aged ≥ 40 years who ate mozuku once every 2–3 months or less.

n = number of subjects, r = correlation coefficient, n.s = not significant.

3.5. Maximum absorption of fucoidan (Δ Max fucoidan value)

Urinary fucoidan was detected in 252 of 259 subjects following a single 3g oral dose. The Δ Max fucoidan values showed a wide distribution, ranging from 0 to 273.6 ng/mL. Among the subjects in whom urinary fucoidan was not detected, three rarely consumed mozuku, one consumed mozuku once every 2–3 months, and three ate mozuku once monthly.

Table III-5 shows the relevance of *H. pylori* infection and age to the Δ Max fucoidan values. The Δ Max fucoidan values in all subjects were similar between *H. pylori*-positive and *H. pylori*-negative subjects. Compared with the values in *H. pylori*-negative subjects, the Δ Max fucoidan values of *H. pylori*-positive subjects tended to be higher in subjects in their 20's and 30's and lower in those in their 40's and 50's (Table III-6). The subjects were divided into two age groups (<40 and \geq 40 years) to determine relevance of age to fucoidan absorption.

Δ Max fucoidan values were significantly lower in subjects aged \geq 40 years than in younger subjects among *H. pylori*-positive subjects. No effect of age on Δ Max fucoidan values was observed among *H. pylori*-negative subjects. No significant difference in Δ Max fucoidan values was found according to the presence of *H. pylori* infection in either age group.

Table III-5. Comparison of Δ Max fucoidan values by age

	<i>H. pylori</i> (-)	<i>H. pylori</i> (+)	<i>H. pylori</i> (-) vs <i>H. pylori</i> (+)
Total	29.4 \pm 40.1 (<i>n</i> = 88)	24.2 \pm 37.1 (<i>n</i> = 171)	<i>P</i> = 0.300
40 y.o. <	26.4 \pm 38.8 (<i>n</i> = 52)	35.3 \pm 47.6 (<i>n</i> = 76)	<i>P</i> = 0.323
\geq 40 y.o.	33.8 \pm 59.8 (<i>n</i> = 36)	21.9 \pm 35.3 (<i>n</i> = 95) ^a	<i>P</i> = 0.135

All data were presented as mean \pm SD.

a: There is a significant difference (*p* < 0.01) compared to *H. pylori*-positive subjects aged <40 years.

n = number of subjects, y.o.: years old; *P* = *p*-value.

Table III-6. Comparison of Δ Max fucoidan by age group

Subject	20's	30's	40's	50's	\geq 60's
<i>H. pylori</i> (-)	27.1 \pm 28.7 (<i>n</i> = 21)	33.2 \pm 43.9 (<i>n</i> = 31)	40.5 \pm 51.6 (<i>n</i> = 23)	26.9 \pm 17.2 (<i>n</i> = 10)	34.8 \pm 22.5 (<i>n</i> = 3)
<i>H. pylori</i> (+)	31.6 \pm 31.4 (<i>n</i> = 30)	41.9 \pm 57.0 (<i>n</i> = 47)	20.3 \pm 30.1 (<i>n</i> = 39)	25.7 \pm 24.6 (<i>n</i> = 33)	31.1 \pm 56.0 (<i>n</i> = 22)

All data were presented as mean \pm SD.

n = number of subjects.

20's: 20–29 years old, 30's: 30–39 years old, 40's: 40–49 years old, 50's: 50–59 years old, \geq 60's: over 60 years old.

3.6. Relevance of *H. pylori* infection and mozuku consumption to fucoidan absorption

In a comparison between subjects aged ≥ 40 years and those aged < 40 years, the Δ Max fucoidan values were decreased by regular mozuku consumption in *H. pylori*-positive subjects but not in *H. pylori*-negative subjects (Table III-7). Specifically, Δ Max fucoidan values were lower in *H. pylori*-positive subjects aged ≥ 40 years who consumed mozuku at least once a month than in those who consumed mozuku less frequently. Subsequently, the subjects were divided into groups based on the frequency of mozuku consumption, and the relevance of mozuku consumption to Δ Max fucoidan values was elucidated (Table III-8).

Table III-7. Relevance of *H. pylori* infection, frequency of mozuku consumption, and age to Δ Max fucoidan values

Habit of eating mozuku	<i>H. pylori</i> (-)		<i>H. pylori</i> (+)		<i>P</i> -value <i>H. pylori</i> (+) Aged < 40 y.o. vs. <i>H. pylori</i> (+) Aged ≥ 40 y.o.
	40 y.o. $<$	≥ 40 y.o.	40 y.o. $<$	≥ 40 y.o.	
1-3 times weekly	25.0 \pm 8.2 (<i>n</i> = 4)	37.8 \pm 20.3 (<i>n</i> = 5)	20.4 \pm 16.1 (<i>n</i> = 8)	17.4 \pm 25.6 (<i>n</i> = 16)	n.s
Once every 2 weeks	24.5 \pm 25.2 (<i>n</i> = 10)	36.4 \pm 14.8 ^a (<i>n</i> = 4)	34.8 \pm 52.7 (<i>n</i> = 17)	12.9 \pm 14.7 (<i>n</i> = 18)	0.08
Once monthly	29.1 \pm 71.6 (<i>n</i> = 11)	27.7 \pm 17.8 (<i>n</i> = 10)	41.9 \pm 54.5 (<i>n</i> = 16)	18.9 \pm 21.4 (<i>n</i> = 28)	0.06
Once every 2-3 months	31.1 \pm 31.8 (<i>n</i> = 15)	39.3 \pm 67.0 (<i>n</i> = 13)	36.2 \pm 46.7 (<i>n</i> = 18)	42.5 \pm 73.1 (<i>n</i> = 15)	n.s
hardly eat	24.4 \pm 22.0 (<i>n</i> = 12)	15.8 \pm 22.9 (<i>n</i> = 4)	38.1 \pm 53.3 (<i>n</i> = 18)	22.2 \pm 17.9 (<i>n</i> = 17)	n.s

All data were presented as mean \pm SD.

a: Compared to *H. pylori*-negative subjects aged ≥ 40 years who hardly ate mozuku ($p < 0.01$).

n = number of subjects, n.s.: not significant; y.o.: years old.

Table III-8. Relevance of *H. pylori* infection and age to Δ Max fucoidan values according to the frequency of mozuku consumption

Habit of eating mozuku	<i>H. pylori</i> (-)		<i>H. pylori</i> (+)	
	40 y.o <	\geq 40 y.o	40 y.o <	\geq 40 y.o
Regularly consumed mozuku ¹⁾	26.6 \pm 48.7 (n = 25)	32.1 \pm 17.6 (n = 19)	34.5 \pm 48.1 (n = 40)	16.8 \pm 20.8 ^{a,b,c} (n = 63)
Rarely ate mozuku ²⁾	28.1 \pm 27.6 (n = 27)	33.8 \pm 59.8 (n = 17)	33.9 \pm 47.7 (n = 36)	30.5 \pm 51.9 (n = 32)

All data were presented as mean \pm SD.

1) Regularly consumed mozuku: 1–3 times weekly + once every two weeks + once monthly.

2) Rarely ate mozuku: once every 2–3 months + hardly ate.

a: Compared to *H. pylori*-positive subjects aged <40 years who ate mozuku at least once monthly ($p = 0.03$).

b: Compared to *H. pylori*-positive subjects aged \geq 40 years who ate mozuku once every 2–3 months or less ($p = 0.01$).

c: Compared to *H. pylori*-negative subjects aged \geq 40 years who ate mozuku at least once monthly ($p = 0.01$).

n = number of subjects, n.s.: not significant; y.o.: years old.

Among *H. pylori*-positive subjects who ate mozuku at least once a month, Δ Max fucoidan values were significantly lower in those aged \geq 40 years than in those aged <40 years. Furthermore, among *H. pylori*-positive subjects aged \geq 40 years, the Δ Max fucoidan values were significantly lower in those who regularly consumed mozuku than in those who rarely ate mozuku. Moreover, the Δ Max fucoidan values were significantly different between *H. pylori*-positive (16.8 \pm 20.8) and *H. pylori*-negative subjects (32.1 \pm 17.6) among those who ate mozuku at least once a month. However, no difference in Δ Max fucoidan values was observed according to age or frequency of mozuku

consumption among *H. pylori*-negative subjects.

3.7. Relationship between the basal and Δ Max fucoidan values in *H. pylori*-positive subjects

Among *H. pylori*-positive subjects who regularly consumed mozuku, a significant positive correlation between the basal and Δ Max fucoidan values was found for those aged < 40 years but not those aged ≥ 40 years. There was no significant correlation between the basal and Δ Max fucoidan levels among subjects who rarely consumed mozuku (Figure III-2). Moreover, no significant correlations were found between the basal and Δ Max fucoidan levels in *H. pylori*-negative subjects regardless of the frequency of mozuku consumption.

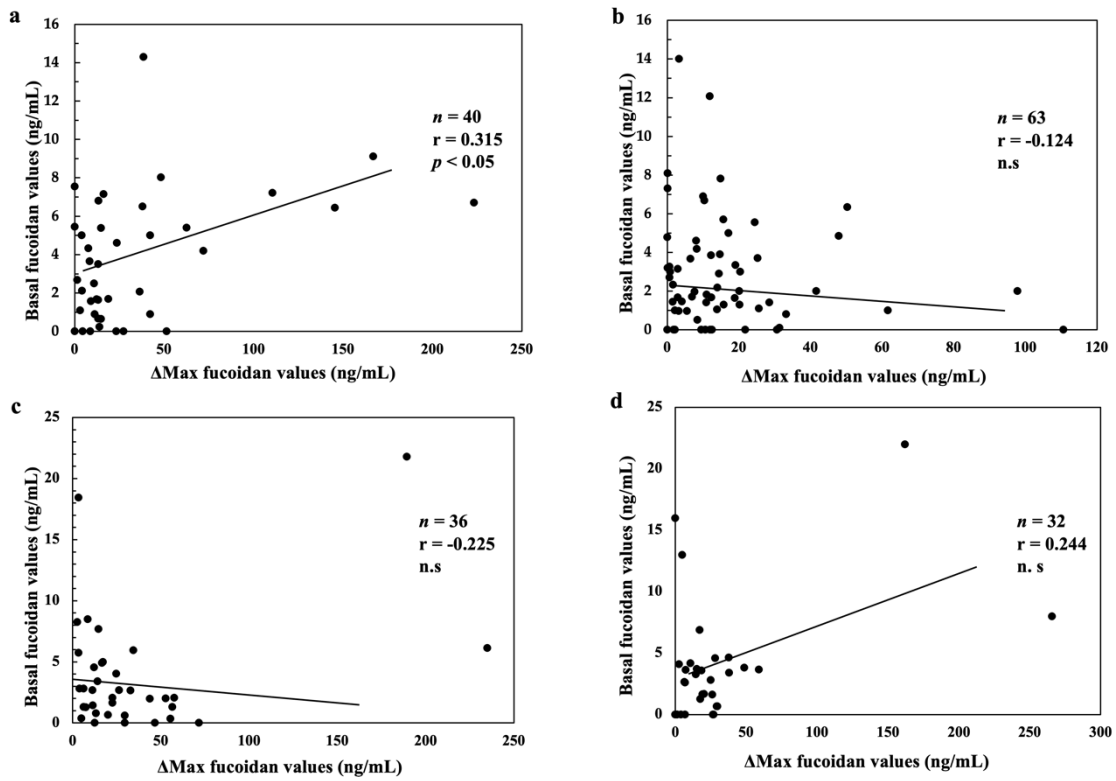


Figure III-2. Relationship between basal fucoidan values and the maximum absorption of fucoidan (Δ Max fucoidan values) according to the frequency of mozuku consumption and age in *H. pylori*-positive subjects.

a: Consumption of mozuku at least once monthly among subjects aged <40 years.

b: Consumption of mozuku at least once monthly among subjects aged ≥ 40 years.

c: Consumption of mozuku once every 2–3 months or less among subjects aged <40 years.

d: Consumption of mozuku once every 2–3 months or less among subjects aged ≥ 40 years.

n = number of subjects, n.s.: not significant

4. Discussion

This study showed an association between *H. pylori* infection and fucoidan absorption. Specifically, fucoidan absorption was significantly reduced among *H. pylori*-positive subjects aged ≥ 40 years who consumed mozuku at least once monthly, whereas no association was found among *H. pylori*-negative subjects irrespective of the frequency of mozuku consumption and age. Furthermore, fucoidan absorption was not reduced among *H. pylori*-positive subjects who consumed mozuku once every 2–3 months or less; thus, mozuku consumption affects the absorption of fucoidan. Although the precise mechanisms by which *H. pylori* infection and mozuku consumption reduce fucoidan absorption have not been determined, a few possibilities have been suggested.

As ΔMax fucoidan values were similar between *H. pylori*-negative and *H. pylori*-positive subjects among subjects aged ≥ 40 years, *H. pylori* was less likely to diminish the absorption of fucoidan in this age group directly. Excluding *H. pylori*-positive subjects aged ≥ 40 years who consumed mozuku regularly, ΔMax fucoidan values were similar between *H. pylori*-positive and *H. pylori*-negative subjects. Furthermore, the frequency of mozuku consumption among *H. pylori*-positive subjects was similar between subjects aged < 40 years and those aged ≥ 40 years; thus, the frequency of mozuku consumption is not directly associated with fucoidan absorption. Given that *H. pylori* positivity and regular mozuku consumption were associated with reduced fucoidan absorption among subjects aged ≥ 40 years but not among younger subjects, the duration of *H. pylori* infection and the frequency of mozuku appear important for fucoidan absorption.

How do frequent mozuku consumption and *H. pylori* infection disturb fucoidan absorption in subjects aged ≥ 40 years? *H. pylori* can alter the secretion and acidification functions of the stomach because it penetrates this organ. Although nutrient absorption

does not occur in the stomach, *H. pylori* infection can affect the digestion and absorption of nutrients such as vitamin B₁₂, vitamin C, vitamin A, vitamin E, and folate⁸⁷⁻⁸⁹). Shibata et al.²⁷) reported that mozuku fucoidan binds to *H. pylori* and inhibit its attachment to the gastric mucosa at pH 2.0 and 4.0, but not at pH 7.4. *H. pylori* rarely causes atrophic gastritis in young people (<40 years old), whereas *H. pylori*-induced atrophic gastritis tends to be relatively common in the elderly⁸³⁻⁸⁶). When hypochlorhydria occurs after *H. pylori*-induced atrophic gastritis, intragastric pH increases, consequently inhibiting the ability of *H. pylori* to bind fucoidan. However, fucoidan absorption was not reduced in *H. pylori*-positive subjects aged ≥ 40 years who rarely consumed mozuku, suggesting the influence of a long duration of mozuku ingestion on fucoidan absorption. Amornlerdpison et al.⁹³) reported that fucoidan present in mozuku acts as an antagonist of the H₂ receptor (similarly to cimetidine), decreasing the acidity of gastric acid and raising the pH in the stomach. In summary, *H. pylori*-induced atrophic gastritis and a long duration of mozuku ingestion may notably decrease acid secretion, consequently leading to the failure of *H. pylori* to bind fucoidan and reduces its absorption in the small intestine in subjects aged ≥ 40 years. Therefore, the possible mechanism by which *H. pylori* infection leads to reduced gastric acid secretion and fucoidan absorption has not been fully investigated to draw definite conclusions. Other mechanisms different from hypochlorhydria following *H. pylori* infections are possible.

Incidentally, significant positive correlations of the basal fucoidan levels with both *H. pylori* titers and Δ Max fucoidan values were shown in *H. pylori*-positive subjects aged <40 years who frequently consumed fucoidan. Such correlations were absent in the corresponding group of subjects aged ≥ 40 years, nor were they observed in *H. pylori*-positive subjects who rarely consumed mozuku or in *H. pylori*-negative subjects. Since the

significance of the positive correlation observed in *H. pylori*-positive subjects aged <40 years who frequently consumed fucoïdan is unclear, further research is necessary to elucidate the relevance of *H. pylori* infection and mozuku ingestion to fucoïdan absorption using a large number of subjects.

Interestingly, basal fucoïdan levels were significantly increased by *H. pylori* infection and mozuku consumption. As *H. pylori* affects the absorption of various nutrients, this stomach bacterium may be involved in basal fucoïdan absorption. Recently, “the nutrition-gut microbiome-physiology axis” has attracted substantial attention^{94–98}). Since *H. pylori* can cause drastic changes in various gastrointestinal microbiota^{99–101}), the microbe is suggested to increase basal fucoïdan levels by regulating the gastrointestinal microbiota. Basal fucoïdan levels were also remarkably higher in *H. pylori*-negative subjects who regularly consumed mozuku than in their counterparts who rarely consumed mozuku, which confirmed the previous study³⁵). It was presumed that Japanese people may have acquired digestive enzymes from mozuku because the seaweed is widely consumed within this area. Due to the limited evidence, the overall significance of *H. pylori* infection and mozuku consumption to basal fucoïdan levels is unclear.

This study had several limitations. First, subjects who received eradication therapy for *H. pylori* and underwent gastrectomy before the study were not excluded. The study also did not exclude subjects who used complementary and alternative medicines, which can affect the absorption of fucoïdan.

Secondly, a urine-based ELISA kit (URINELISA) was utilized to assay *H. pylori* infection, and the high accuracy of this test was verified by several investigators^{90,91}). A disadvantage of this test is that proteinuria can cause false-positive results; thus, urinary protein levels should be measured in future research.

Furthermore, the specificity of the fucoidan ELISA was limited. Urinary fucoidan levels were measured using a polyclonal antibody for Okinawa mozuku fucoidan, which weakly cross-reacted with fucoidan derived from *Fucus vesiculosus*³²⁾. Since the brown seaweeds of Kombu (*Laminaria japonica*) and Wakame (*Undaria pinnatifida*) are traditional foodstuffs in Japan, fucoidan included in these seaweeds may cross-react with ELISA antibody. Further studies are necessary to elucidate the effects of mozuku consumption on the digestive tract absorption of fucoidan using ELISA with a monoclonal antibody.

5. Conclusion

The present data showed that fucoidan derived from *C. okamuranus* absorption is associated with *H. pylori* infection and mozuku consumption. Fucoidan absorption in *H. pylori*-positive subjects who regularly consumed mozuku differed by age, being significantly lower in subjects aged ≥ 40 years than in their younger *H. pylori*-positive subjects. A significant positive correlation between the basal fucoidan level and Δ Max fucoidan value was found among subjects aged $40 <$ who regularly consumed mozuku but not among their older counterparts. Further studies are needed to elucidate the full mechanisms influencing fucoidan absorption.

Conclusion

In this study, I performed experiments in animals and humans intending to clarify the immunomodulatory ability of fucoidan derived from Okinawa mozuku. Fucoidan is a sulfated polysaccharide in edible seaweeds, including brown algae such as Kombu, Wakame, and Mozuku. Fucoidan derived from edible seaweed, has a long history of use as a food, and its safety is well established.

In Chapter I, I evaluated the immunomodulatory effect of mozuku fucoidan in mice. BALB/c female mice were evaluated in five groups (10 mice/group): a control group and four test groups (102.5, 205.0, 410.0, and 1025.0 mg/kg fucoidan). The results showed that fucoidan promoted the proliferation of spleen-derived immune cells, increased macrophages phagocytosis, increased interferon- γ and interleukin-2 production, and suppressed interleukin-4 and -5 production; and in the mouse immune system, increased the production of IgG, IgM, and IgA, and suppressed IgE production.

In Chapter II, I reported a clinical study in healthy human subjects between 20 and 65 years of age. The study aimed to evaluate the safety of fucoidan and the effect of fucoidan ingestion on NK cells. The study was conducted using a randomized, double-blind, parallel-group, placebo-controlled methodology in which 40 healthy subjects were randomly assigned to either the fucoidan group (ten males and ten females) or the placebo group (ten males and ten females). The study period was a total of 16 weeks, with an ingestion period of 12 weeks and a washout of four weeks; subjects in the fucoidan group ingested 3 g/day of fucoidan. The results showed significant NK cell activity in the fucoidan group after eight weeks of ingestion compared with the placebo group. Notably, after 8 weeks of ingestion in the fucoidan group, male subjects showed significantly higher NK cell activity than the placebo group. No problematic adverse events were observed during the

study period in the safety evaluation.

In Chapter III, the relationships between the presence or absence of *H. pylori* infection, the dietary consumption of mozuku, and the concentration of urinary fucoidan were investigated. The urinary fucoidan concentration and anti-*H. pylori* antibody titer were measured before and 3, 6, and 9 h after fucoidan intake in 259 healthy Japanese volunteers who consumed 3 g of fucoidan. Additionally, I also investigated the relationship between the dietary consumption of mozuku, the concentration of fucoidan in the urine, and the anti-*H. pylori* antibody titer.

The concentration of urinary fucoidan was significantly higher after fucoidan intake than before. Additionally, the comparison of Δ Max fucoidan levels by age in *H. pylori*-positive subjects showed that the levels were significantly lower in subjects >40 years of age. The following correlation with mozuku eating habits was determined:

- 1) In *H. pylori*-negative subjects, those who regularly consumed mozuku had significantly higher basal levels of urinary fucoidan than those who rarely consumed mozuku.
- 2) In *H. pylori*-positive subjects, the Δ Max fucoidan level of subjects >40 years of age who consumed mozuku at least once per month was significantly lower than that of subjects < 40 years of age.
- 3) In the group of subjects who regularly consumed mozuku, the Δ Max fucoidan level of *H. pylori*-positive subjects >40 years of age was significantly lower than that of *H. pylori*-negative subjects <40 years of age.
- 4) In *H. pylori*-positive subjects, a positive correlation was found between the basal fucoidan level in urine and the Δ Max fucoidan level in subjects who consumed mozuku at least once monthly.

Therefore, it was suggested that fucoidan absorption was related to *H. pylori* infection

and the frequency of mozuku consumption.

My findings are expected to contribute to maintaining health and improving QOL to support self-medication for everyday infectious diseases. Additionally, *H. pylori* infection and mozuku eating habits were suggested to be related to fucoidan absorption, elucidating one aspect of fucoidan metabolism. Therefore, I am sure that these novel findings will contribute to the development of nutraceuticals and pharmaceuticals in the gastrointestinal and immune fields.

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Published Papers

1. Makoto Tomori, Takeaki Nagamine, Tomofumi Miyamoto, Masahiko Iha. Evaluation of the immunomodulatory effects of fucoidan derived from *Cladosiphon okamuranus* Tokida in mice. *Mar Drugs*. 2019, 17, 547.

(Corresponding to Chapter I.)

2. Makoto Tomori, Takeaki Nagamine, Tomofumi Miyamoto, Masahiko Iha. Effects of ingesting fucoidan derived from *Cladosiphon okamuranus* Tokida on human NK cells: a randomized, double-blind, parallel-group, placebo-controlled pilot study. *Mar Drugs*. 2021, 19, 340.

(Corresponding to Chapter II.)

3. Makoto Tomori, Takeaki Nagamine, Masahiko Iha. Are *Helicobacter pylori* infection and fucoidan consumption associated with fucoidan absorption? *Mar Drugs*. 2020, 18, 235.

(Corresponding to Chapter III.)