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Effects of sulforaphane glucosinolates from broccoli seed extract on the immune system of healthy Japanese adults

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ABSTRACT

Background: The Purpose of this study was to investigate the effects of sulforaphane glucosinolate (SGS) from broccoli seed extracts on immune function and common cold symptoms in healthy Japanese adults.

Methods: This randomized, placebo-controlled, double-blind, parallel-group comparison study was conducted on Japanese adults who had worse health-related quality of life on the SF-8 and were more likely to catch colds from October 22, 2020 to April 2, 2021.
Individuals who agreed to participate in the study were randomly assigned to either the SGS 100 mg/day group or the placebo group (n = 33 each). The intervention lasted 8 weeks. The outcomes of this study were the cumulative and the maximum number of days for which common cold symptoms persisted during the intervention period per subject (primary), and the frequency with which subjects experienced these symptoms per group (secondary). Additionally, the incidence rate of subjects infected with the influenza virus, immune indices, and SF-8 were assessed.

Results: Based on a per protocol set, 64 subjects (33 in the SGS group and 31 in the placebo group) were analyzed. After the 8-week interventional period, the cumulative number of days for which common cold symptoms per subject was significantly lower for the SGS group than the placebo group (12.1 ± 13.5 for the SGS group and 20.2 ± 18.2 for the placebo group).

The cumulative number of days of common cold symptoms per group was also significantly lower in the SGS group than in the placebo group (400 days in the SGS group and 626 days in the placebo group).

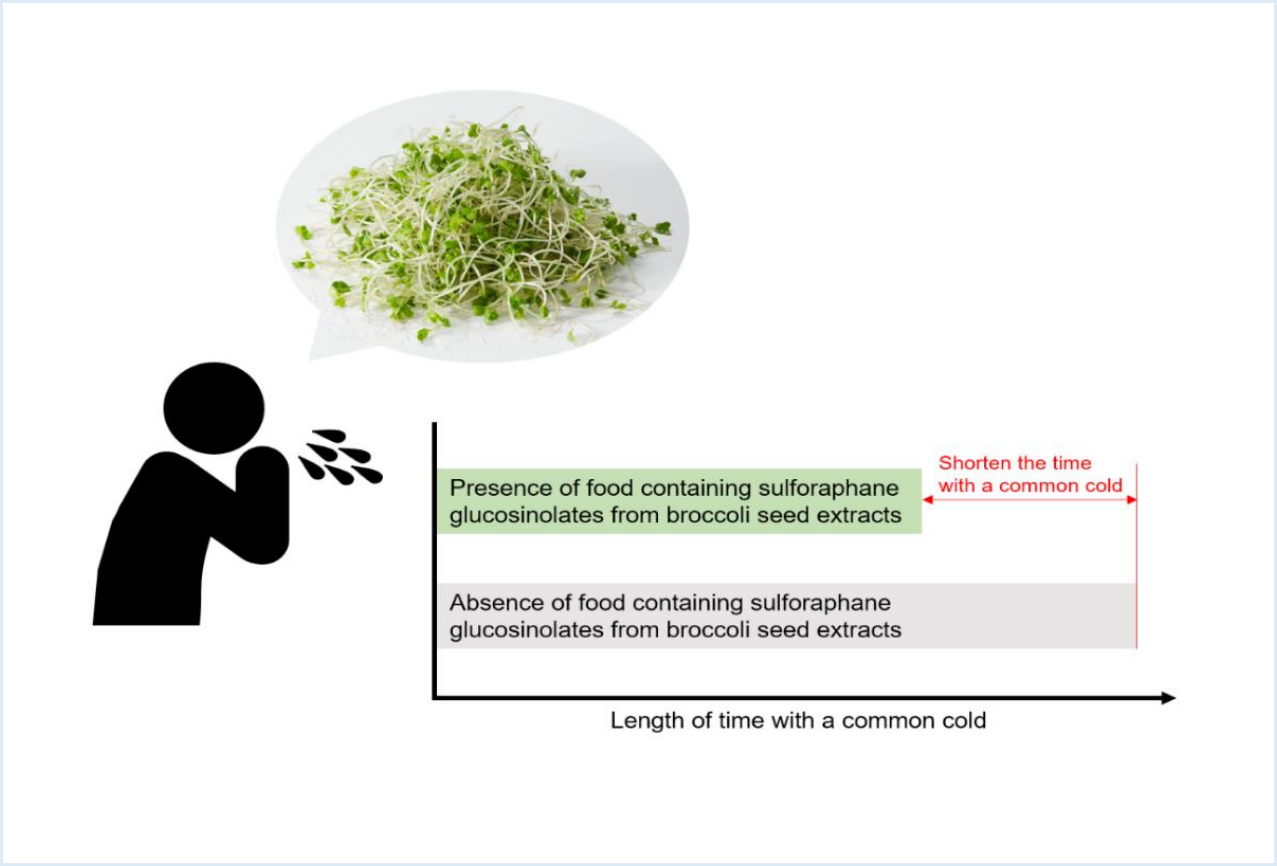
No changes attributable to the SGS intervention were observed in the other outcomes. There were no adverse events due to food ingestion.

Conclusion: A period of 8 weeks of SGS intake was shown to alleviate the onset of common cold symptoms in healthy Japanese adults who were prone to catching colds and had a low subjective sense of wellness.

Keywords: Broccoli, sulforaphane, glucoraphanin, cold symptoms, immunity

Trial registration: UMIN-CTR: UMIN000042195.

Foundation: Murakami Farm Co., Ltd.



BACKGROUND

Several observational and interventional studies have demonstrated that broccoli, a cruciferous plant, exhibits a variety of health benefits, including antioxidant, anti-inflammatory, anti-metabolic syndrome, neuroprotective, and hepatoprotective effects [1,2]. Sulforaphane (SFN), which is found in the form of sulforaphane glucosinolate (SGS) in broccoli as its

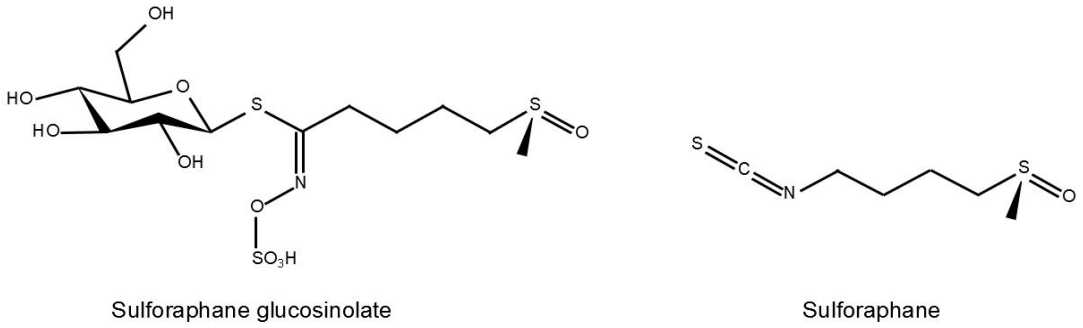
ecological precursor (Figure 1), is considered to be the bioactive substance responsible for these health effects [3]. SGS is abundantly present in broccoli sprouts and is known to form SFN through hydrolysis, which is catalyzed by contact with myrosinase contained in plant tissues during the destruction of plant tissues by eating [1]. In addition, SGS is metabolized into SFN by human intestinal bacteria [4] and metabolized again via the mercapturic acid pathway [5–7], which is thought to exhibit various health functions [1].

The first report confirming the health-related benefits of SFN showed that SFN may activate the cellular defense system [8]. Subsequently, research studies in *in vitro* and *in vivo* have demonstrated that SFN may be effective in the prevention or treatment of pancreatic cancer [9], breast cancer [10], lymphoma [10], and leukemia [11]. In addition, current literature has reported the effects of SFN on cytokines involved in immunity, including aging and neurodegeneration prevention [12] and protection against gastric ulcers [13].

The mechanism of action of SFN on the immune function involves the inhibition of inflammatory cytokines production in monocytes and macrophages and activation of antioxidant enzymes through nuclear factor-erythroid 2-related factor 2 (Nrf2) regulation, resulting in anti-inflammatory effects [5]. For dendritic cells, which function at the beginning of the immune response and are involved in triggering both innate and adaptive immunity, *in vitro* studies using human dendritic cells and cytokine cocktails have confirmed that SFN promotes T-cell activation through the expression of co-stimulatory CD80, CD83, and inhibitory B7-H1 molecules on dendritic cells and regulation of the Janus family of tyrosine kinases (JAK)/ signal transducer and activator of transcription 3 (STAT3) and microRNA signaling [14]. In human studies, the efficacy of SFN has been observed in individuals with allergic asthma [15,16], allergic rhinitis [17], and abnormal liver function [18].

Furthermore, the World Health Organization (WHO) [19] reported that by 2030, one in six people worldwide will be aged 60 or older, and between 2015 and 2050, the percentage of the world population over age 60 will almost double from 12% to 22%. Moreover, aging is accompanied by a significant remodeling of the immune system, referred to as immune aging, and an increase in systemic inflammation, referred to as inflammatory aging, both of which contribute to an increased risk of developing chronic diseases in old age [20]. Therefore, the maintenance of healthy immune function is an important initiative from the perspective of disease prevention, especially with the aging of the global population. Moreover, it has been observed that the higher frequency of colds, more severe cold symptoms, greater incidence of sleep disorders, and poorer overall health is associated with productivity, absenteeism, and daily life [21]. Thus, it is essential to reduce the incidence of colds from the perspective of labor productivity.

SGS-containing food from broccoli is expected to ameliorate cold-like symptoms by modulating immune function. However, to the best of our knowledge, few studies have examined its efficacy in healthy Japanese subjects, and the evidence for its effectiveness is weak. Hence, in the study, we examined the effects of SGS-containing food consumption for 8 weeks on the immune function of healthy Japanese adults who were prone to colds and had a low subjective sense of wellness at the time of screening and pre-consumption examinations (Scr).



Sulforaphane glucosinolate Sulforaphane

Figure 1. Chemical structure of sulforaphane and its precursor sulforaphane glucosinolate.

METHODS

Study design: The study design was a randomized, placebo-controlled, double-blind, parallel-group study, and this study was conducted under full consideration of medical ethics in accordance with the Declaration of Helsinki and the ethical guidelines for medical research involving human subjects. The study protocol was approved by the Ethics Committee of Takara Clinic, Seishinkai Medical Corporation (approval date October 16, 2020, approval number 2010-04281-0011-1C-TC).

Study participants: The main inclusion and exclusion criteria were as follows.

Inclusion criteria

(I) Healthy Japanese adults;

(II) Individuals who self-identify as being prone to catching colds; and

(III) Individuals had a relatively low general health perception score according to the Health Survey (SF-8) in Scr data of the participants.

Exclusion criteria

(I) Individuals undergoing treatment for a history of malignancy, heart failure, or myocardial infarction;

(II) Individuals with a pacemaker or implantable cardioverter-defibrillator;

(III) Individuals undergoing treatment for arrhythmia, hepatic disorder, renal disorder, cerebrovascular disorder, rheumatism, diabetes mellitus, dyslipidemia, hypertension, or other chronic diseases, autoimmune disease, hay fever, and respiratory disease;

(IV) Individuals using immunosuppressive drugs such as steroids;

(V) Individuals vaccinated against infectious diseases such as influenza (Specifically, vaccines against seasonal influenza, mumps, yellow fever, hepatitis A, hepatitis B, tetanus, rabies, polio, Japanese encephalitis, measles, rubella, and meningococcal disease;

(VI) Individuals with a history of strenuous exercise habits;

(VII) Individuals who regularly take foods for specified health uses, foods with functional claims, or other foods/beverages with possible functionality;

(VIII) Individuals who regularly take broccoli sprouts;

(IX) Individuals who regularly take medicines and food supplements;

(X) Individuals who are allergic to medicines and/or the test food related products

(XI) Individuals who were pregnant, lactating, or intending to become pregnant during the study period;

(XII) Individuals who participated in another clinical trial at least 3 months prior to the date of consent, or were planning plan to participate in another clinical trial during the study period; and

(XIII) Individuals who were judged to be inappropriate as subjects for this study by the principal physician.

The detailed criteria were as per the pre-registered UMIN-CTR (UMIN000042195).

Subjects were recruited through the Go-toroku website (https://www.go106.jp/), which is operated by Orthomedico Inc. Written informed consent was obtained from all participants at the office of Orthomedico Inc. None of the study participants were affiliated with the company that presided over or funded the study. The Seishinkai Takara Clinic (Shinagawa-ku, Tokyo) was responsible for obtaining data and assessing individuals' health status, and all examinations were conducted at the Minamimachi Clinic (Nerima-ku, Tokyo), a co-operating medical institution.

In addition, the following were compliance requirements in this study:

(I) Consume the test food in the prescribed dosage and administration;

(II) Consume the test food at a minimum of 80% intake rate;

(III) Avoid binge eating and drinking and make no changes to their previous lifestyle from the date of consent to the final examination (after 8-week intervention);

(IV) Avoid alcohol consumption and excessive physical activity the day before each test until the end of the test on the day of the examination;

(V) No eating or drinking for 6 hours prior to the blood collection;

(VI) On the day of the examination, toothpaste and mouthwash should not be used for 2 hours prior to the examination until the saliva collection is completed;

(VII) If any change in physical condition occurs during the examination period, the sponsoring clinical research organization (CRO) should be notified immediately and instructions for further action should be sought;

(VIII) During the study period, foods for specified health uses, foods with functional claims, or other foods/beverages with possible functionality should be avoided as much as possible.

Intervention: The ingredient composition of the test food per daily dose (2000 mg) is shown in Table 1. Subjects received food containing broccoli seed extract with SGS 100 mg/day once daily (SGS group), or a food without broccoli seed extract (placebo group). The establishment of the intake dose was based on a report by Yagishita *et al*. [1] They summarized the intake of the previous clinical trials of SGS administration and found that 25–800 µmol (median 190 µmol) [10.9–350 mg, median 83 mg] of SGS per day was effective [1]. We also sell broccoli sprouts containing high concentrations of SGS, with 214 mg of SGS per 50 g [22]. The evidence in this study is intended to be used for data on broccoli sprouts that are eventually sold. Consumers rarely eat the full 50 g of broccoli sprouts in a day and often eat half the amount, 25 g (107 mg as SGS). Therefore, we set the dose of broccoli sprouts to 100 mg of SGS/day in this study. The intervention period was 8 weeks. All test foods were in powder form, and at the time of ethics review prior to the beginning of the study, it was confirmed that the test and placebo foods were indistinguishable by color, odor, and flavor

Table 1. Contents of test foods (2000 mg/day).

Component	Foods containing SGS	Placebo food
Foods containing broccoli seed extract contain SGS	485 mg (SGS 100 mg)	0 mg (0 mg)
Maltodextrin	1485 mg	1970 mg
Silicon dioxide	30 mg	30 mg

Assessment items: Common cold symptoms were recorded in a daily logbook, and other efficacy and safety outcomes were performed at Scr, 4-week post-test (4wks), and 8-week post-test (8wks).

Study subjects were asked to record their daily living conditions, including consumption of the test foods and changes in their physical conditions, in a daily logbook. Study subjects were also asked to record any side effects and adverse events experienced in a logbook and to report this event as soon as they were aware of the contract research organization. In the event of an adverse event, the investigator was to immediately take necessary and appropriate steps to determine whether the study participant could continue the study and whether the emergency key could be opened. The aim of the investigator was to evaluate and provide a written report with respect to the association between the adverse events and the test foods. When a subject took either prescription or over-the-counter drugs, the CRO was asked to report the reason for the drug selection, the type of drug used, the date of use, the amount used, etc. In addition, questionnaires and a dietary survey using the Calorie and Nutrition Diary (CAND) [22] were conducted on each examination day to confirm the health status of the study subjects. Study subjects were excluded from the study if they withdrew their consent or in case of a deviation from the protocol.

(I) A survey of common cold symptoms [23]*:* Subjects were instructed to record the presence or absence of common cold symptoms in their daily records during the intervention period. “Whole body malaise,” “chilliness,” “feverishness,” “fatigue,” “sneezing,” “nasal discharge,” “blocked nose,” “throat pain,” “cough,” “joint pain,” and “muscle pain” were among the symptoms of common cold. Subjects were required to respond to each question on "with symptoms” or “without symptoms". In this study, common cold symptoms were defined as the onset of at least one of the above symptoms. The cumulative number of days for which common cold symptoms persisted during the intervention period per subject, was set as the primary outcome. Moreover, the maximum number of days for which common cold symptoms persisted in the subjects during the intervention period, and the frequency with which subjects experienced them were assessed as secondary outcomes. These survey items were based on Kuwabara et al. [23].

(II) SF8 Health Survey [24]*:* Health-related quality of life was assessed using the SF-8 (standard version). The survey items included the physical component summary score (PCS), mental component summary score (MCS), physical functioning, role physical, bodily pain, general health, vitality, social functioning, role emotional, and mental health [24]. According to the procedure manual of the SF8 Health Survey method [24], after recoding the choices and processing missing values for each question, raw subscale scores were calculated and converted to subscale scores, and then Norm-based Scoring scores and summary scores were calculated. Scr, 4wks, and 8wks

served as the assessment timepoints, and the secondary outcomes were defined by the difference between the measured values and Scr at 4wks and 8wks.

(III) Immunity test: An immunity test was conducted at the Institute for Health and Life Science (Tokyo, Japan). Seven immune subscores, including (1) the number of T cells, (2) ratio of $CD4^+/CD8^+$ T cells, (3) number of naive T cells, (4) ratio of naive/memory T cells, (5) number of B cells, (6) number of NK cells, and (7) number of $CD8^+CD28^+$ T cells, were measured on a scale of one to three points according to the reference values. The total of these scores was used as the immunological vigor score (the upper limit was 21 points), which was subsequently converted into immunological grades on a five-point scale [25–28]. In addition, the T lymphocyte age calculated based on the negative correlation found between the number of $CD8^+CD28^+$ T cells and age [26,27] was assessed. Scr, 4wks, and 8wks served as the assessment timepoints, and the secondary outcomes were defined by the difference between the measured values and Scr at 4wks and 8wks.

(IV) Saliva analysis: Saliva samples were collected from each study subject at the medical institution. The salivary secretory immunoglobulin A (salivary IgA) was evaluated and the analysis was performed by the LSI Medience Corporation (Chiyoda-ku, Tokyo). Scr, 4wks, and 8wks served as the assessment timepoints, and the secondary outcomes were defined by the difference between the measured values and Scr at 4wks and 8wks.

(V) Incidence rate of study subjects infected with influenza virus: During the study period, the number of study subjects with influenza virus infection noted in their adverse event reports was counted, and the incidence rate was determined in each group (secondary outcome). The definition of influenza determination was based on the total number of cases in which study participants had been to the hospital themselves and were diagnosed with influenza by physicians.

(VI) Safety evaluation: Body weight, body mass index (BMI), body fat percentage, body temperature, systolic blood pressure, diastolic blood pressure, and pulse rate were measured by a physical examination. Height was measured at the time of the briefing only.

Urinalyses included measurements of protein, glucose, urobilinogen, bilirubin, ketone bodies, pH, and occult blood, and each urinalysis item was measured by the LSI Medience Corporation according to the usual method.

Hematological examination items included white blood cell count, red blood cell count, hemoglobin, hematocrit level, platelet count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and white blood cell picture (neutrophil percentage and count, lymphocyte percentage and count, monocyte percentage and count, eosinophil percentage and count, basophil percentage and count). Blood biochemistry examination items included alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transpeptidase, alkaline phosphatase, lactate dehydrogenase, leucine aminopeptidase, total bilirubin, direct bilirubin, indirect bilirubin, cholinesterase, total protein, urea nitrogen, creatinine, uric acid, creatine kinase, calcium, serum amylase, total cholesterol, high - density cholesterol, low - density cholesterol-cholesterol, triglyceride, glycoalbumin, serum iron, sodium, potassium, chlor, inorganic phosphorus, glucose, hemoglobin A1c (NGSP), and non-specific IgE. Each blood test item was measured by the LSI Medience Corporation according to the usual method.

The number of adverse reactions and adverse events was counted, and the incidence rate was determined in each group.

Sample size: To our knowledge, no previous study has demonstrated the cumulative number of days for which common cold symptoms persisted in the subjects during the intervention period as a primary outcome in humans consuming SGS-containing foods for 8 weeks. Consequently, we assumed a large difference between the SGS and placebo groups with respect to the cumulative number of days for which common cold symptoms persisted during the intervention period, and we calculated the sample size using an effect size of d = 0.80 based on Cohen's suggestion [29]. Statistical significance level (α), and statistical power ($1-\beta$) were set as 0.05 and 0.80, respectively, then 52 subjects (26 in each group) were calculated as the required number of subjects. Furthermore, the target number of subjects was set at 60 (30 in each group) to maximize statistical power ($1-\beta$), providing a statistical power of 0.86. In addition, the number of subjects was set to 66 (33 in each group) in anticipation of dropouts and non-compliance with the protocol during the study period.

Selection, randomization and blinding: Of the 91 subjects who provided informed consent, 66 subjects deemed suitable for participation in this study based on the inclusion and exclusion criteria were selected by the physician. The test foods were provided to the CRO by the sponsor. The individual in charge of shipping provided the code of the test foods to an allocation controller who was not directly involved in the studies after confirming that the test foods were undistinguishable and after entering and verifying the data at Scr. Subjects were assigned to each of the two groups by the allocation manager at the same day after the number of enrolled subjects reached the target number of subjects in the protocol. The allocation was performed according to a computer-generated randomization list using this allocation controller. The allocation adjustment factors were sex, age, and general health of SF-8 at Scr. Subjects were equally but randomly assigned to either the SGS or placebo group (n = 33 per group). Only the person in charge of shipping had access to the allocation table with the coded test foods, and the test foods were sent to each subject according to that table. After the test foods were shipped, the allocation table was kept at a secure location until the subjects for analysis and statistical analysis methods were fixed. The sponsor, principal physician, subphysician, entire CRO staff (i.e., director of the trial, director of trial conduction, individual in charge of monitoring, director and staff of statistical analysis, and individual in charge of shipping), medical institution staff, ethics committee members, contract laboratory, and others related to this study were not aware of the group assignments. The allocation controller locked the allocation table until the key-opening day.

Statistical method: All statistical analyses in this study were two-sided, and the significance level was set at 5%. Data analyses were performed using Windows SPSS, v.23.0 (IBM Japan, Ltd., Tokyo, Japan). Subjects who markedly deviated from the protocol were excluded from the analysis population (Specifically, we excluded the following participants: (i) participants in which less than 80% of the test foods were consumed; (ii) participants in which the reliability of the study results was significantly undermined by missing diary records; (iii) participants in which it became clear after inclusion that the exclusion criteria were met; (iv) participants in which it became clear that compliance with the study protocol had been violated during the study period; (v) participants in which the study results were significantly affected by food or drug intake during the study period; (vi) participants in

which the study results were significantly affected by different living habits or activities during the study period; (vii) participants who consumed food or medicines that may have significantly affected the study results during the study period; (viii) participants who engaged in activities significantly different from their lifestyle at the time of study enrollment, and (ix) participants who had other clear reasons to be excluded from the study). A primary outcome-oriented analysis was conducted, and multiplicity in multiple items and time points occurring in secondary outcomes were not considered. The subjects' baseline data were demographically aggregated by the enrolled and analyzed subjects. The subjects' sex for each group were compared using chi-square tests, and the subjects' age, height, and non-specific IgE for each group were compared using Student's *t* -tests.

The measured values of the primary and secondary outcomes are shown as mean, standard deviation (SD), group differences, and standard error (SE). Group differences were presented as 95% confidence interval. A group comparison between the SGS and the placebo groups in a survey on common cold symptoms was performed using a student's *t*-test. The cumulative number of days for which common cold symptoms persisted per group and subjects infected with the influenza virus during the study period were expressed as the number and percentage of the cumulative number of study days for each group, differences percentage between groups, and their 95% CI. These were calculated and compared between groups using the chi-square test.

Other efficacy endpoints are presented as mean ± SD. Scr was compared between groups using the student's *t*-test. Measurements at 4wks and 8wks were compared between groups in a linear mixed model with time point, group, interaction between time point and group, interaction between baseline values and time point, and study participants as the factors and baseline values as a covariate. In contrast, changes from Scr (Δ4wks, Δ8wks) were compared between groups by time point in a linear mixed model with time point, group, interaction between time point and group, and study participants as the factors. Scr was presented as group differences in means and their SE and 95% CI, and post-intervention data were presented as group differences in estimated marginal mean (EMM) and their SEs and 95% CI.

For adverse reactions and events, the number of cases and incidence rate per group, the difference in incidence rate, and its 95% CI were calculated and compared between groups using the chi-square test. Physical measurements except for height, physical examination, and peripheral blood tests were compared between groups at each time point using the Student's *t*-test. Urinalysis and blood tests were coded "1" if they were within the reference values and "0" if they were outside the reference values, and the number of cases in each group was tabulated. In addition, the principal physician or subphysician assessed the safety endpoints on an individual basis to confirm that there were no medically problematic changes associated with the intake of the test foods.

RESULTS

Analysis set: Subjects were recruited between October 22 and December 12, 2020, and the study period was from October 22, 2020 to April 2, 2021.

Two subjects in the placebo group were excluded from the analysis due to the uncertainty of whether the intervention was successfully followed (those two subjects do not have data after randomization). Therefore, the per protocol set used for the efficacy

assessment and the safety analysis population used for the safety assessment both included 64 subjects (33 in the SGS group and 31 in the placebo group) (Figure 1 and Table 2). Although the analysis of this study was the per protocol set, we thought that the intention to treat principle was preserved because the excluded subjects were those with missing post-randomization data.

The baseline data for the study subjects is shown in Table 2. There were no significant differences between the two groups. No study subjects were infected with the influenza virus during the study period.

Survey of common cold symptoms: The results of the cumulative number and maximum number of days for which common cold symptoms persisted, defined as the onset of at least one of the symptoms ("whole body malaise," "chilliness," "feverishness," "fatigue," "sneezing," "nasal discharge," "blocked nose," "throat pain," "cough," "joint pain," and "muscle pain") during the intervention period per subject and group, are shown in Tables 3 and 4, respectively. The results of the cumulative number and maximum number of days for which each common cold symptom persisted during the intervention period per subject and group are shown in Appendix 1.

As shown in Table 3, the cumulative number of days for which common cold symptoms persisted during the intervention period per subject was significantly lower in the SGS group than that in the placebo group (12.1 ± 13.5 in the SGS group and 20.2 ± 18.2 days in the placebo group; between-group difference, −8.1 days; 95% CI, −16.0 to −0.1; P = 0.047). Furthermore, the maximum number of days was marginally lower in the SGS group (4.6 ± 7.3 days in the SGS group and 8.5 ± 11.5 days in the placebo group; between-group difference, −3.9 days; 95% CI, −8.7 to 0.9; P = 0.106; Table 3). Pertaining to the group analysis, the cumulative number of days per group was significantly lower in the SGS group than in the placebo group (400 days (21.5%) in the SGS group and 626 days (35.9%) in the placebo group, P < 0.001; Table 4).

Although there were no significant differences between the two groups for each common cold symptom, whole body malaise, chilliness, feverishness, and fatigue in the SGS group showed a lower mean cumulative number and maximum number of days for which common cold symptoms persisted than in the placebo group (Appendix 1).

Table 2. Background characteristics of the subjects.

Item (unit)	SGS group (n = 33)	Placebo group (n = 31)	P value
Gender	Men: 15 (45.5%)	Men: 15 (48.4%)	1.000
	Women: 18 (54.5%)	Women: 16 (51.6%)	
Age (years)	47.8 ± 11.2	49.0 ± 10.2	0.652
Height (cm)	163.5 ± 7.2	165.3 ± 7.9	0.335
Non-specific IgE (IU/mL)	193.8 ± 282.6	176.5 ± 302.2	0.813

Values of sex are shown as the number of cases and percentage of subjects in each group. In other items, values are shown as mean and standard deviation (SD).

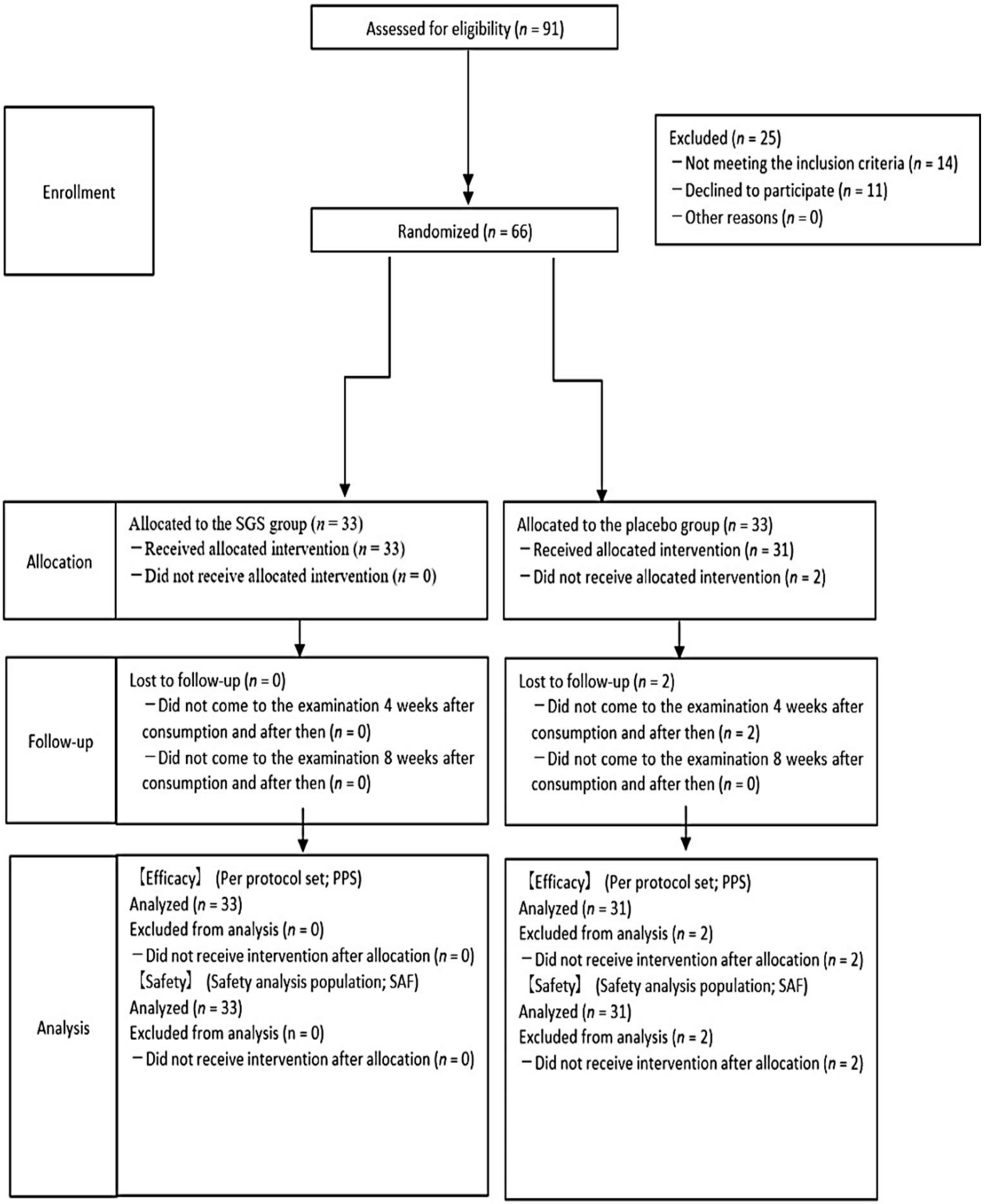


Figure 2. Flowchart of subjects in this study.
n: number of subjects

Table 3. Cumulative and maximum number of days for which common cold symptoms persisted during the intervention period per subject.

	SGS group (*n* = 33)		Placebo group (*n* = 31)		Group comparison			
	Mean	SD	Mean	SD	Difference	SE	95%CI	*P* value
The cumulative number of days (day)	12.1	± 13.5	20.2	± 18.2	−8.1	4.0	−16 to −0.1	0.047
The maximum number of days (day)	4.6	± 7.3	8.5	± 11.5	−3.9	2.4	−8.7 to 0.9	0.106

Values are shown as mean ± standard deviation (SD), group differences, and standard error (SE) and 95% confidence interval (95% CI) for group differences.

Table 4. Cumulative number of days for which common cold symptoms persisted during the intervention period per group.

	SGS group (n = 33)	Placebo group (n = 31)	Group comparison		
			Group differences of ratios (%)	95%CI	*P* value
Cumulative number of days (incidence rate)	400 (21.5%)	626 (35.9%)	−14.4	−17.3 to −11.5	*P* < 0.001

Values are shown as mean ± standard deviation (SD), group differences, and standard error (SE) and 95% confidence interval (95% CI) for group differences. The cumulative number of days indicates the number of days each study subject in the group exhibited common cold symptoms, and the incidence rate indicates the percentage of the cumulative number of days per sum of the days of the 8-week intervention period in each group.

Immunity test, SF-8, and saliva analysis: The results of the immunity test, SF-8, and saliva analyses are shown in Table 5 and Appendixes 2–4. In the immunity test, there were no significant differences in the immunity scores or grades between the two groups (Appendix 2). However, the $CD4^+/CD8^+$ T-cell ratio score at 4wks (SGS group, 2.1 ± 0.8; placebo group, 1.7 ± 0.7; between-group difference, 0.3; 95% CI, 0.0 to 0.5, *P* = 0.044) and the measured and the change in $CD8^+CD28^+$ T-cell count score at 8wks were higher in the SGS group (measured values: SGS group, 2.3 ± 0.5; placebo group, 2.1 ± 0.4; between-group difference, 0.2; 95% CI, 0.0 to 0.4; *P* = 0.041/ change values: SGS group, 0.1 ± 0.5; placebo group, -0.1 ± 0.4; between-group difference, 0.3; 95% CI, 0.0 to 0.5; *P* = 0.029) (Table 5). The other items in the immunity test, SF-8, and saliva analysis showed significant differences between the two groups after the intervention, but these differences were not related to SGS-containing foods (Appendixes 2–4).

Safety assessment: No side effects were observed during the study period; however, eight adverse events were noted in the SGS group and one in the placebo group.

However, based on the criteria established at the time of the study design, the principal physician determined that there was no causal relationship with the study food. During the intervention period, urinalysis and blood tests (hemoglobin, platelet count, MCHC, inorganic phosphorus, HbA1c, and total cholesterol) showed that there were no significant differences between the two groups. However, the principal physician assessed each item on an individual basis and determined that the variations were not medically problematic.

Table 5. Immunity test (score of $CD8^+CD28^+$T cells, score of ratio of $CD4^+/CD8^+$T cell).

Item (Unit)	Time point	SGS group (*n* = 33)		Placebo group (*n* = 31)		Group comparison			
		Mean	SD	Mean	SD	Difference	SE	95%CI	*P* value
Score of $CD8^+CD28^+$T cells	Scr	2.2	± 0.5	2.3	± 0.5	−0.1	0.1	−0.4 to 0.1	0.408
	4wks	2.0	± 0.5	2.2	± 0.5	−0.1	0.1	−0.3 to 0.1	0.443
	8wks	2.3	± 0.5	2.1	± 0.4	0.2	0.1	0.0 to 0.4	0.041
	Δ4wks	−0.1	± 0.5	−0.1	± 0.4	0.0	0.1	−0.2 to 0.2	0.829
	Δ8wks	0.1	± 0.5	−0.1	± 0.4	0.3	0.1	0.0 to 0.5	0.029
Score of ratio $CD4^+/CD8^+$T cell	Scr	2.2	± 0.8	1.9	± 0.9	0.2	0.2	−0.2 to 0.7	0.331
	4wks	2.1	± 0.8	1.7	± 0.7	0.3	0.1	0.0 to 0.5	0.044
	8wks	2.2	± 0.8	1.9	± 0.9	0.1	0.1	−0.1 to 0.4	0.392
	Δ4wks	−0.1	± 0.5	−0.3	± 0.6	0.2	0.1	−0.1 to 0.5	0.156
	Δ8wks	0.0	± 0.4	−0.1	± 0.6	0.1	0.1	−0.2 to 0.3	0.641

Values are shown as mean ± standard deviation (SD), group difference in measurements (Scr) or group difference in estimated marginal means (4wks, 8wks), and standard error (SE) and 95% confidence interval (95% CI) for group differences.

DISCUSSION

In the 80%–90% of cases with the common cold, acute respiratory infections caused by viral infections were prevalent [30]. It is well known that infection of the upper respiratory tract mucosa by common cold viruses induces various symptoms such as fever, headache, whole body malaise, nasal discharge, nasal obstruction, cough, as well as the production of various cytokines [30]. Therefore, we set the effect of the test foods on common cold symptoms (which was the primary outcome in the study) and compared the cumulative number of days of common cold symptoms such as “Whole body malaise,” “chilliness,” “feverishness,” “fatigue,” “sneezing,” “nasal discharge,” “blocked nose,” “throat pain,” “cough,” “joint pain,” and “muscle pain” during the study period. The results showed that the cumulative number of days for which common cold symptoms persisted during the intervention period per subject was significantly lower in the SGS group than in the placebo group.

SFN contributed to increased expression of the oxidative stress response Nrf2 [31], which is thought to be involved in the transcription of antiviral response genes, and Hemeoxygenase 1 (HO-1) [32], which is known to respond to viral infections by regulating

immune function under the transcriptional control of Nrf2 [31]. In a previous study of mice nosed with the influenza virus, an increase in HO-1 expression was found to suppress inflammation and enhance the survival of cells around the upper respiratory tract [33]. Several previous studies have also suggested that HO-1 indirectly promotes an antiviral state through the interaction of HO-1 with interferon (IFN) regulators and activation of the type I IFN response [34]. Furthermore, SFN has been reported to promote T -cell activation by dendritic cells through the regulating JAK/STAT3 and microRNA signaling [14]. Dendritic cells are the major mediators of immune activity and are involved in the induction of innate and acquired immune responses as early responders of the immune system [35,36]. Additionally, among T cells, $CD8^+$ T cells are responsible for the destruction of cells infected by foreign substances, such as viruses, inhibiting the spread of the infection [36]. The results of the $CD8^+CD28^+$ T-cell count score in this study also confirmed that SFN could activate T cells. Therefore, the findings of the present study suggested that SFN, which is metabolically converted in the body after SGS intake, could suppress common cold symptoms by modulating the immune systems through increased expression of HO-1 in response to viral infection and T -cell activation via dendritic cells.

Activation of Nrf2 by SFN has been found to increase the transforming growth factor (TGF)-β, IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin (IL)-2, and type I helper T cell (Th1) cytokines [37]. In a mouse model of asthma, SFN suppressed type II T helper cell (Th2) cytokines, which include IL-5, IL-10, and IL-13 [38]. Although conflicting evidence exists on immunomodulation by SFN under conditions of low glutathione concentrations and in type 2 diabetes [39–41], our study demonstrated a significant reduction in the number days for which common cold symptoms could persist during the intervention period with consumption of SGS-containing foods. Thus, we speculated that SFN is involved in Th1/Th2 balance in healthy individuals via the activation of Nrf2, increasing the concentration of Th1 cytokines and suppressing the activity of Th2 cytokines, while at the same time exhibiting both anti-inflammatory (immune tolerance) and cellular immune activation functions.

SFN has been shown to exhibit antiviral activity even before viral infection [31]. In human nasal epithelial cells, independent of the influenza infection, SFN has been found to increase the expression levels of the antiviral response genes IFN-β, retinoic acid inducible gene I (RIG-I), and MxGTPases (MxA)[31]. In general, viral infections trigger the production of type I IFN, which in turn activates the synthesis of numerous IFN-stimulated genes (ISGs)[31]. ISGs collectively induce an antiviral state both in the host and adjacent cells, thus limiting viral replication [31]. MxA is one of the ISGs that belong to a family of GTPases shown to inhibit viral replication and exhibit antiviral activity. RIG-I is also an IFN-inducible gene [31]. The IFN-β, RIG-I, and MxA genes are thought to contain Nrf2 binding sites in their promoter regions, and Nrf2 induction increases the transcription of these antiviral genes, possibly enabling SFN to exert antiviral responses and inhibit viral entry and replication prior to viral infections [31]. Therefore, in addition to immune function modulation via increased expression of HO-1, the suppression of common cold symptoms observed in this study may also be due to inhibition of viral entry and replication through SFN-mediated Nrf2-mediated antiviral action.

SFN has a potent inducer of phase II enzymes [42]. Phase II enzymes protect cells from various oxidative stresses, and the induction of these enzymes contributes to the mechanisms by which cells protect against the toxicity of reactive oxygen species and other forms of oxidative toxicity [42]. Oxidative stress is involved in the onset and exacerbation of various diseases, including

inflammation, respiratory diseases, and skin diseases [43–45], and it is believed that air pollutants and pollen cause asthma symptoms due to inflammation caused by oxidative stress in the airways [46]. A study in which healthy subjects aged ≥18 years consumed at least 100 g/day of broccoli sprout homogenate has confirmed a significant increase in the expression of phase II enzyme genes collected from the nose relatively after the intervention compared to the baseline (before ingestion), possibly via the activation of the Nrf2 transcription factor and antioxidant response elements [46]. SFN has also been suggested to suppress Nrf2-mediated reduction of IFN-γ expression when allergic dermatitis is induced in aged mice, and the reduced Nrf2 activity with aging is thought to induce oxidative stress-mediated inflammatory responses in cells of the innate immune system [37]. Furthermore, in a mouse asthma model induced with ovalbumin, SFN was found to significantly reduce airway hyperresponsiveness by upregulating the expression levels of suppressor of cytokine signaling (SOCS)-3, GATA-3, and IL-4, suggesting that SFN modulates Th2 immune responses [38]. Therefore, although “those with hay fever” and “those with a history or current history of respiratory disease” were excluded from this study, it is possible that SFN suppressed not only common cold symptoms caused by the virus but also symptoms caused by air pollutants and pollen. In the future, we expect that the effects of the test foods on allergic symptoms caused by inflammation induced by oxidative stress in the airways due to these allergens will also be examined. It would also be interesting to conduct a symptom-by-symptom analysis or test on subjects with limited symptoms, since we have not conducted an analysis using the type of common cold symptoms as a factor.

Previously, improvement in immune indices with SFN has been observed in several cancer cells [5]. Accordingly, we also expected that the present study would improve immune indices; however, it was not possible to determine the effect of SFN on immune indices among individuals prone to catching colds and a low subjective sense of wellness assessed by SF-8. However, the SF-8 summary values and subscores identified in this study were within the mean and standard deviation of a national survey conducted in Japan in 2017 [47], and the subjective sense of wellness in this study was the norm for the Japanese population. Therefore, based on the results of the previous study, we believe that SGS-containing foods can reduce the number of days that common cold symptoms persists in individuals who have a standard sense of wellness, but who feel that they are consciously prone to catching colds.

Although our analysis was based on a per protocol set, this is the first study to confirm that SGS-containing foods could reduce the cumulative number of days for which common cold symptoms persist in healthy subjects, providing new insights into the study of SFN in its immunomodulatory function. Therefore, future studies can confirm the reproducibility of this study and elaborate on the more detailed mechanisms of SGS-containing food in suppressing common cold symptoms in healthy subjects by measuring the expression levels of phase II enzyme genes and inflammatory cytokines. In addition, in investigating the impact of SGS-containing foods on the sense of wellness, it would be an interesting initiative to conduct a study among healthy individuals whose general health perception on the SF-8 is lower than that of the Japanese national norm.

CONCLUSION

This study examined the effects of an 8-week intake of SGS-containing food on the immune function of healthy Japanese adults who were prone to catching colds and had relatively low SF-8 general health. The cumulative number of days for which common cold symptoms persisted during the intervention period per subject was

significantly reduced in the SGS group compared with the placebo group, suggesting that SGS-containing food could suppress common cold symptoms. In addition, SGS-containing food was found to be safe under the conditions of the study.

List of Abbreviations: SFN: sulforaphane, SGS: sulforaphane glycosylate, Nrf2: nuclear factor-erythroid 2-related factor 2, PCS: physical component summary, MCS: mental component summary, NK cell: natural killer cell, BMI: body mass index, SD: standard deviation, EMM estimated marginal means, HO-1: Hemeoxygenase 1, IFN: interferon, TGF-β: transforming growth factor-β, GM-CSF: granulocyte-macrophage colony-stimulating factor, IL-2: interleukin-2, RIG-I: Retinoic acid -inducible gene I, MxA: MxGTPases, ISG: IFN-stimulated gene, SOCS: suppressor of cytokine signaling.

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Competing Interests: The sponsor of this study, Murakami Farm Co., Ltd., entrusted Orthomedico Inc. with conducting the Inc.. Naoyuki Kouno is a member of Murakami Farm Co., Ltd., and Naoko Suzuki is employees of Orthomedico Inc. Tsuyoshi Takara (MD), the principal investigator of this study, is a director of Medical Corporation Seishinkai, Takara Clinic, and he monitored all of the conditions of the subjects.

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