Brain Supplement®
Home page: https://brainsupplementoff.wixsite.com/mysite/journal
(ISSN 2434-9615)

Effect of Naturido on the cognitive function improvement in healthy volunteers and subjects with mild cognitive impairment: A randomized, double-blind, parallel-group comparison study

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Abstract

Naturido is a cyclic peptide present in the extract of a fungus (Isaria japonica = Paecilomyces tenuipes) grown on silkworm pupae (Bombyx mori) that reportedly enhances astrocyte proliferation and improves spatial learning ability. This study aimed to evaluate the effects of Naturido on cognitive function in humans. We conducted a randomized, placebo-controlled, double-blind, parallel-group study of 90 healthy volunteers (33 men and 57 women, 40–70 years old, including those with mild cognitive impairment). Subjects consumed either a capsule containing 0.96 mg or 1.92 mg Naturido as the test food (provided by DKS Co. Ltd.) or a capsule not containing Naturido as the placebo. Cognitrax and eye-tracking tests were used to assess cognitive function before and 6 and 12 weeks after consumption of the test food. In the Cognitrax test, consumption of 0.96 mg Naturido significantly improved visual memory, psychomotor speed, and motor speed compared to consumption of the placebo. No adverse events attributable to the test food were observed in any participant during the study. Our results indicate that the continuous intake of Naturido improves cognitive function in healthy subjects (normal subjects and those with mild cognitive impairment).

Brain Supplement 2022; 4: 13-26

Abbreviations: acetylcholinesterase, AChE; choline acetyltransferase, ChAT; body mass index, BMI; mild cognitive impairment, MCI; mini-mental score examination, MMSE; quality of life, QOL; symbol digit coding, SDC.

Key words: Naturido, cognitive function, mild cognitive impairment, visual memory, psychomotor speed, motor speed

Received August 23, 2022; Revised received September 13, 2022; Accepted September 21, 2022

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Introduction

The number of elderly people in Japan is increasing every year, and elderly people in Japan accounted for more than 27% of the population as of 2018, heralding a super-aged society in the world1). According to the Cabinet Office's Annual Report on Aging Society, the future estimates of the number of elderly people with dementia and the prevalence of dementia in those aged 65 years and older showed that in 2012, the number of elderly people with dementia was 4,620,000 and its prevalence was about one in seven elderly people aged 65 years and older (15%) (1). However, in 2025, the prevalence is estimated to be one in five people (20%) (2). The morbidity of dementia and the decline in cognitive function with aging are major issues.

Although it has also been noticed that it maintains and improves quality of life (QOL) in the aging population, it is important to maintain a QOL that maintains cognitive function. According to the Comprehensive Survey of Living Conditions in the 2016 fiscal year, cognition has become the primary cause of long-term care (3), and early measures for dementia are desired from the viewpoint of QOL maintenance.

Alzheimer's disease and vascular dementia, which account for the majority of dementia cases, have been associated with lifestyle-related diseases (e.g., hypertension, diabetes mellitus, and dyslipidemia) (4). It has been proven that daily life management, such as keeping regular exercise habits in mind, can prevent dementia. It can also slow the progression of dementia or, in some cases, improve symptoms if the patient is aware of dementia during the mild stage of the disease and is adequately treated (5).

The main symptom of cognitive decline with aging is oblivion, but it has little effect on daily life. The condition that cannot be diagnosed as dementia is called mild cognitive impairment (MCI), and it is a condition that applies to all of the following signs (6,7).

1. Memory deficits cannot be explained solely by the effects of age or educational level
2. Complaints of forgetfulness by a person or family
3. General cognitive function within the normal range
4. Independent activities of daily living
5. No dementia

In other words, individuals who are aware of forgetfulness but have no obvious cognitive impairment other than poor memory with mild effects in daily life are considered to have MCI. Furthermore, 10–15% of people with these MCIs develop dementia every year, and this is considered a pre-stage of dementia. Not only genetic factors but also daily dietary habits are involved in cognitive decline, and the intake of specific nutritional components in the diet is also said to influence the risk of dementia (4,5).

Naturido is a cyclic peptide contained in the extract of a fungus (*Isaria japonica = Paecilomyces tenuipes*) grown on silkworm pupae (Bombyx mori) and is known to promote spatial learning ability and neuronal differentiation (8). Approximately 500 species of C. sinensis have been identified worldwide, and approximately 400 species have been identified in Japan (9). The extract of powder obtained from fruiting bodies of I. japonica artificially grown with dried and dead pupae of the silkworm Bombyx mori was prepared and orally administered to an aging-accelerated mice of the Alzheimer's disease model. It was confirmed that the gliosis generated in the CA3 area of the hippocampus almost completely disappeared, and the spatial memory was also restored (10).

When *I. japonica* powder was purified and isolated, it was confirmed that Naturido, a cyclic peptide consisting of four amino acids, is involved in cognitive improving effect (11). Naturido has been reported to proliferate astrocytes in a concentration-dependent manner8), and its anti-inflammatory effect has been demonstrated by adding Naturido to microglia8). Furthermore, when the effect of Naturido on hippocampal neurons was examined, it was found to increase the number of dendrites in neurons and promote axonal elongation. Based on these results, Naturido is expected to be an effective functional food for improving cognitive function.

Therefore, we decided to confirm and examine the effect of ingestion of Naturido derived from fungus extract (*I. japonica = Paecilomyces tenuipes*) on improving cognitive function and safety in adults with MCI and healthy adults.

Materials and Methods

Trial design and ethical considerations

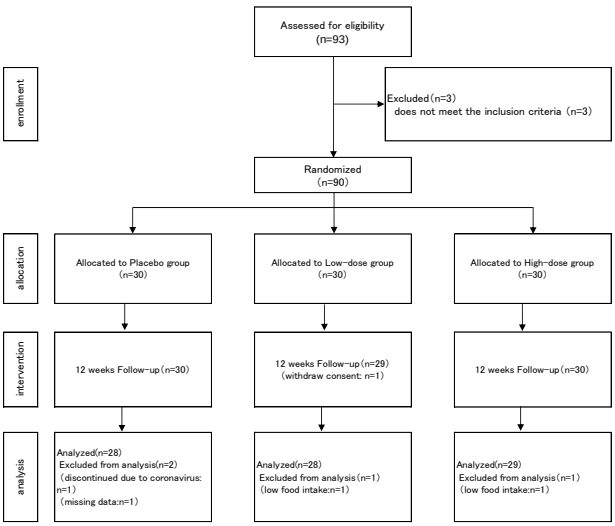
This was a randomized, placebo-controlled, double-blind, parallel-group study. This study was approved by the Ethics Review Committee of the Japan Food Evidence Society, which is organized by a third party (approval date: February 16, 2021) and conducted under the supervision of physicians at the Kanyukai Clinic in accordance with the objectives of the Ethical Guidelines for Medical and Biological Research Involving Human Subjects and the Declaration of Helsinki for Research on People.

The subjects were paid volunteers recruited

by Tashikani-plus Co., Ltd. After receiving written and verbal explanations about the purpose, method, and expected side effects of the study, the subjects understood the study and voluntarily agreed to participate.

This study was conducted after pre-registration (UMIN ID: UMIN000043348) in UMIN-CTR operated by the Academic Hospital Medical Information Network Research Center as a registration of the clinical trial. The subject recruitment period was from March 2021 to August 2021, and the subject follow-up period was from April 2021 to November 2021.

Figure 1 Flowchart



Subjects

Upon understanding the details of this study, subjects who provided informed consent were screened and enrolled if they met the inclusion criteria and did not meet the exclusion criteria. The inclusion criteria were as follows:

1. Age ≥ 40 years and <70 years;
2. Screening test result ≥24 points on the Mini-Mental State Examination (MMSE);
3. Gender does not matter;
4. No smoking habit,
5. Diet does not contain a large amount of Naturido (C. sinensis);
6. No use of supplements or health foods;
7. No lifestyle-related diseases (such as hypertension and diabetes mellitus), rheumatism, hepatic disorder, renal disorder, or other chronic diseases;
8. No history of treatment for malignancy, heart failure, or myocardial infarction;
9. No history of allergies to foods and medicines containing a large amount of Naturido;
10. No hospital visits for treatment or medication; and
11. Full comprehension of the contents of the clinical trials and availability of written informed consent.

The exclusion criteria were as follows:

1. Pregnant or breastfeeding or willingness to become pregnant during the study period,
2. Ongoing participation in other clinical trials or clinical trials or past participation in other clinical trials or clinical trials within 3 months,
3. Inability to comply with instructions from the physician-in-charge and staff of the medical institution, and
4. Judged by the examiner to have some problems.

The required sample size was estimated based on an effect size of 0.78, power of 80%, and alpha value of 0.05 as per a previous clinical trial (12). The effect size was calculated from the mini-mental score examination (MMSE) scores of previous clinical trial using G*Power software(Ver. 3.1.9.6). Given 10% of dropout subjects, the target sample size was determined to be 30 subjects in each group and 90 subjects in total, in the three groups.

Test food

The trial was conducted as a randomized, placebo-controlled, double-blind, parallel-group study. Subjects were assigned using a random number table by personnel who were not involved in the clinical trial and were randomized to the average MMSE scores in each group. Subjects were randomly assigned to three groups, namely high-dose Naturido, low-dose Naturido, and placebo, and subjects ingested each assigned test food for 12 weeks. According to the Consumer Agency's Survey and Review Project Report on the Handling of Data on Subjects with Mild Disease in Foods with Functional Claims, dated March 26, 2019, the intake period for clinical trials in the cognitive function domain of Foods with Functional Claims is stipulated to be 12 weeks or longer. In addition, a 12-week intake period is usually used in the clinical trials of functional foods for cognitive function (13-15). Therefore, the intake period for this clinical study was set at 12 weeks. Cognitive function tests were performed at 6 weeks to confirm the midpoint results. The study period was from April 2021 to November 2021. All study-related subjects (e.g., subjects, interventionists, and assessors) were blinded, and the allocation table was not opened until the subjects included in the analysis were fixed.

Subjects were instructed to prohibit significant changes to their lifestyle, including diet, exercise, smoking, and ingestion of drugs before

participating in the study. During the study period, participants were instructed to fill out a lifestyle questionnaire if they performed excessive exercise and consumed alcohol that deviated significantly from their daily routine.

In addition, the cognitive function test was conducted in as quiet an environment as possible so that participants could perform the test accurately, while being instructed to refrain from using smartphones during the test.

Subjects performed the trial in a sitting position, and the investigator confirmed that subjects performed the test appropriately. Subjects abstained from binge eating on the previous day, consumed breakfast upon waking up on the day of examination, and were interviewed, while body measurements, blood pressure measurements, and blood and urine tests were conducted. Subsequently, a cognitive function test was performed.

Endpoint

The primary endpoint was the cognitive function test, and the evaluation methods were the Cognitrax test and Eye Tracking tests.

Cognitrax is a computer-based neurocognitive test developed as a Japanese version of the CNS Vital Signs test (16). It consists of eight tasks (tests): a verbal memory test along with a visual memory test, a finger tapping test, symbol digit coding (SDC), Stroop test, shifting attention test, continuous performance test, and four-part continuous performance test. As items from the above measurement results, the following were to be evaluated: composite memory, verbal memory, visual memory, psychomotor speed, reaction time, complex attention, cognitive flexibility, processing speed, executive function, working memory, sustained attention, simple attention, and motor speed. These can be widely evaluated for cognitive function and used to detect the cause of MCI. We performed the Cognitrax test using the same PC for all the subjects.

The eye-tracking test is a method in which eye-tracking techniques record and analyze eye-gaze movements for various task images displayed on a screen monitor for approximately 3 min, and scoring of cognitive functions are performed objectively and quantitatively (17). In the eye-tracking test, in addition to the total scores, assessments were performed on memory (four classifications), attention (two classifications), judgment, visuospatial cognition, orientation time, and orientation location as subitems of cognitive function.

The safety items include the following tests:

1) Body measurement: body weight, BMI

2) Vital signs: systolic blood pressure, diastolic blood pressure, pulse rate, and temperature

3) Hematology: white blood cell (WBC), red blood cell, hematocrit, hemoglobin, MCV, MCH, MCHC, and platelet count

4) Clinical biochemistry: AST, ALT, γ-GTP, ALP, LDH, total bilirubin, albumin, total protein, total cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, blood glucose level, HbA1c, urea nitrogen, creatinine, GFR, uric acid, sodium, potassium, and calcium

5) Urinalysis: urinary protein, urinary glucose, urobilinogen, bilirubin, ketone bodies, specific gravity, and pH

6) Physician interview and lifestyle assessment questionnaire: physician interviews were conducted at each visit to investigate the subject's physical condition, presence of adverse events, and so on.

7) Lifestyle questionnaire: records on the intake status of test foods, dietary quantity, alcohol consumption, drug/supplement intake status, and body condition

Blood tests and urinalysis were commissioned for BML and INC. The above cognitive function tests and various other tests were examined three times in total: at week 0, week 6, and week 12.

Statistical analysis

As the statistical method for the primary endpoint, between-group comparisons for the Cognitrax test were evaluated using Dunnett's multiple comparisons test. Paired t-tests were performed for within-group comparisons of pre-intake values. Intergroup comparisons on the eye-tracking test were evaluated using the Tukey-Kramer multiple comparisons test. The Student's t-test was used to compare the safety endpoints between the groups. The Cognitrax test is a package of cognitive tests. Other trials using the Cognitrax test assess the effects on cognitive function as an independent endpoint without adjusting for multiplicity (13,18,19) . In this study, we referred to other clinical trials and did not adjust for the multiplicity of each cognitive function in the Cognitrax test.

For each test value, the subject's background is expressed as mean ± standard deviation, and the efficacy evaluation and blood test results are expressed as mean ± standard error.

The two-sided significance level was set at 5%. IBM SPSS Statistics (Ver. 28.0.1.1, IBM Japan) was used for statistical analysis.

Results

Subject characteristics

Ninety-three subjects who provided written informed consent were assessed for eligibility; three were excluded, and 90 were included. The 90 individuals were divided into three groups (high-dose Naturido group, 30 subjects; low-dose Naturido group, 30 subjects; placebo group, 30 subjects).

One of the subjects withdrew consent and discontinued the study. Figure 1 shows the flowchart of the subject inclusion process. The 90 participants included in the study were randomized according to the permuted block method. The test food was blinded and given to the participants using the identification number assigned to the food.

The characteristics of the subjects in the high-dose Naturido, low-dose Naturido, and placebo groups are presented in Table 1. The high-dose Naturido group comprised 11 male and 19 female participants, the low-dose Naturido comprised had 13 male and 17 female participants, and the placebo group comprised 9 male and 21 female participants.

For subject background data, there were no significant differences between the groups prior to intake in terms of height, weight, BMI, systolic blood pressure, diastolic blood pressure, pulse rate, temperature, or MMSE scores. In addition, there were two subjects for whom no examinations could be performed.

One subject was diagnosed with coronavirus disease 2019 (COVID-19) and could not be tested at 12 weeks (placebo group). One subject had data loss because the cognitive function test at 12 weeks could not be performed properly (placebo group).

Thirty subjects in the high-dose Naturido group (not excluded), 29 subjects in the low-dose Naturido group (one subject excluded), and 28 subjects in the placebo group (two subjects excluded) were included in the eye tracking test. In addition, there were two subjects in whom the intake rate of the test food was less than 80% of the standard, and 77% (high-dose Naturido group) and 77.9% (low-dose Naturido group) of the test food were consumed throughout the study, so the above two subjects were excluded from the analysis of the Cognitrax test as an example of protocol deviation.

Excluding the subjects who deviated from the protocol, 29 patients (excluding one subject) in the high-dose Naturido group, 28 subjects (excluding two subjects) in the low-dose Naturido group, and 28 subjects (excluding two subjects) in the placebo group were included in the Cognitrax test analysis.

For safety data, all subjects who consumed the test foods at one time (excluding subjects who withdrew consent) were assessed as intention-to-treat cases.

Table 1) Subject background

Item	Unit	Placebo group (n=30)	Low-dose group* (n=29)	High-dose group* (n=30)
Sex(Men/Women)	-	11 / 19	13 / 17	9 / 21
Age	-	48.6 ± 7.6	48.5 ± 6.1	48.6 ± 6.2
Height	cm	163.7 ± 8.9	164.8 ± 8.9	162.9 ± 7.6
Weight	kg	60.8 ± 10.5	62.4 ± 12.5	60.4 ± 11.6
BMI	kg/m2	22.6 ± 2.6	22.8 ± 3.1	22.7 ± 4.1
SBP	mmHg	111.5 ± 12.7	111.3 ± 13.9	115.7 ± 13.7
DBP	mmHg	66.7 ± 9.0	67.1 ± 9.7	70.2 ± 10.5
Pulse rate	bpm	72.0 ± 12.7	72.1 ± 8.7	73.4 ± 10.6
Body temperature	℃	36.2 ± 0.3	36.3 ± 0.3	36.2 ± 0.4
MMSE	Score	26.5 ± 1.5	26.5 ± 1.3	26.4 ± 1.6

Mean ± SD

*No significant difference compared to placebo group in all items

Results of cognitive assessment

Cognitrax test: Table 2 shows the results of the Cognitrax test from weeks 0 to 12 in the Naturido and placebo groups. The Cognitrax test showed significant differences between the low-dose Naturido and placebo groups in executive function, cognitive flexibility, and reaction time at week 0. In addition, there was a significant difference in the reaction time between the high-dose Naturido and placebo groups. There were no significant differences between the groups for the other items.

Significant differences between Naturido and placebo groups at week 12 are as follows:

・High-dose Naturido group: reaction time ($p<0.01$)

・Low-dose Naturido group: visual memory ($p<0.05$), psychomotor speed ($p<0.01$), reaction time ($p<0.01$), and motor speed ($p<0.01$)

Significant differences between the Naturido and placebo groups at week 6 are as follows:

・High-dose Naturido group: cognitive flexibility ($p<0.05$), executive function ($p<0.05$)

・Low-dose Naturido group: psychomotor speed ($p<0.05$), reaction time ($p<0.05$), cognitive flexibility ($p<0.05$), processing speed ($p<0.05$), executive function ($p<0.01$), and motor speed ($p<0.01$)

Significant within-group differences before and after intake at 12 weeks were as follows:

• High-dose Naturido group: composite memory ($p<0.05$), verbal memory ($p<0.05$), psychomotor speed ($p<0.01$), and processing speed ($p<0.01$)

・Low-dose Naturido group: composite memory ($p<0.05$), visual memory ($p<0.05$), and processing speed ($p<0.01$)

・Placebo group: composite memory ($p<0.01$), verbal

memory ($p<0.01$), and processing speed ($p<0.01$)

Significant within-group differences before and after intake at week 6 and before intake are as follows:

- High-dose Naturido group: psychomotor speed ($p<0.01$), cognitive flexibility ($p<0.05$), executive function ($p<0.05$), and motor speed ($p<0.05$).
- Low-dose Naturido group: reaction time ($p<0.05$), cognitive flexibility ($p<0.05$), processing speed ($p<0.01$), and executive function ($p<0.05$)
- Placebo group: verbal memory ($p<0.01$)

According to the Consumer Agency's Survey and Review Project Report on the Handling of Data on Subjects with Mild Disease in Foods with Functional Claims, dated March 26, 2019, subjects in the cognitive function domain of Foods with Functional Claims are healthy persons (healthy persons and persons with MCI). As a rule, individuals aged 40 years or older should be included in the study.

In this clinical trial, only healthy subjects aged 40 years or older and those with MCI, excluding those suffering from diseases such as dementia and Alzheimer's, were recruited and complied with the above notification. As for the criteria for determining MCI, an MMSE score of 24–27 was used with reference to "Cognitive Function Evaluation Method and Diagnosis of Dementia" by The Japan Geriatrics Society (20,21). Therefore, it was concluded that subjects in this clinical study were appropriate subjects for trials on Foods with Functional Claims.

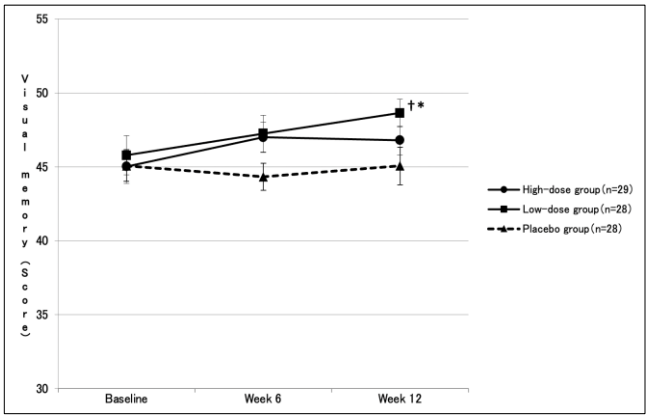


Figure 2) Cognitrax test: Visual memory expressed as Mean ± SE. Significant difference between the groups compared to placebo group (*$p<0.05$), or within the group compared to baseline (†$p<0.05$).

Eye-tracking Test: As with the Cognitrax test, cognitive function was assessed with the eye-tracking test for healthy subjects aged 40 years and older and those with MCIs.

The eye-tracking test showed no significant difference in the Naturido groups compared to the placebo group in terms of the overall score.

We also did not identify significant differences in the results in the Naturido groups compared to the placebo group in memory (four classifications), attention (two classifications), judgment, visuospatial cognition, orientation time, or orientation location as sub-items of cognitive function (data not shown).

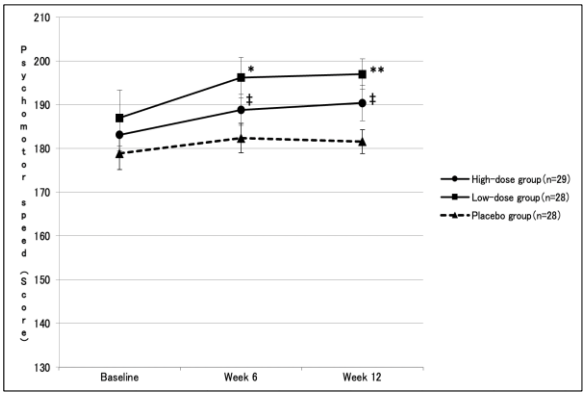


Figure 3) Cognitrax test: Psychomotor speed expressed as Mean ± SE. Significant difference between the groups compared to placebo group (*$p<0.05$, **$p<0.01$), or within the group compared to baseline (†$p<0.05$, ‡$p<0.01$).

Safety: Adverse events were observed in one subject. One subject (placebo group) was diagnosed with COVID-19 at week 8 after intake of the test food, so no efficacy evaluation was performed at week 12, and the subject was withdrawn from the study. The event was confirmed not to be related to the test food because it was not due to consumption of Naturido, and the infection with the virus causing the infectious disease was clearly an accidental event. No adverse events were identified in any other subject.

Among laboratory tests, data on the mean and standard error of each parameter for blood tests, differences before and after intake, and between groups are summarized in Table 3. At week 0, there were significant differences in creatinine, GFR, and urea nitrogen levels between the low-dose Naturido and placebo groups. There were no significant differences between the groups for the other items.

Significant differences between the Naturido and placebo groups at the end of weeks 6 and 12 were as follows:

Table 2) Cognitrax test

Item		Baseline		Week 6		Week 12	
		Mean ± SE		Mean ± SE		Mean ± SE	
Composite memory	High-dose group(n=29)	97.53 ± 2.32		101.23 ± 1.68		102.67 ± 1.58	†
	Low-dose group(n=29)	97.93 ± 2.19		100.86 ± 2.53		102.75 ± 1.51	†
	Placebo group(n=28)	94.75 ± 1.87		98.10 ± 1.50		99.71 ± 1.87	‡
Verbal memory	High-dose group(n=29)	52.50 ± 1.44		54.23 ± 0.87		55.87 ± 0.77	†
	Low-dose group(n=29)	52.14 ± 1.21		53.61 ± 1.40		54.11 ± 0.87	
	Placebo group(n=28)	49.68 ± 1.17		53.77 ± 0.88	‡	54.64 ± 0.89	‡
Visual memory	High-dose group(n=29)	45.03 ± 1.15		47.00 ± 1.02		46.80 ± 0.99	
	Low-dose group(n=29)	45.79 ± 1.33		47.25 ± 1.24		48.64 ± 0.94	†*
	Placebo group(n=28)	45.07 ± 1.04		44.33 ± 0.91		45.07 ± 1.27	
Psychomotor speed	High-dose group(n=29)	183.10 ± 3.71		188.80 ± 3.57	‡	190.37 ± 4.03	‡
	Low-dose group(n=29)	186.93 ± 6.34		196.18 ± 4.59	*	196.96 ± 3.53	**
	Placebo group(n=28)	178.82 ± 3.71		182.33 ± 3.39		181.54 ± 2.73	
Reaction time	High-dose group(n=29)	650.40 ± 12.40	*	661.70 ± 14.05		637.57 ± 10.95	**
	Low-dose group(n=29)	649.57 ± 10.35	*	629.64 ± 11.47	†*	633.29 ± 11.81	**
	Placebo group(n=28)	705.29 ± 23.79		685.40 ± 19.79		693.54 ± 18.71	
Complex attention	High-dose group(n=29)	6.27 ± 1.45		5.73 ± 0.94		6.63 ± 1.88	
	Low-dose group(n=29)	5.39 ± 0.66		5.33 ± 0.94		7.96 ± 1.94	
	Placebo group(n=28)	8.32 ± 1.90		7.07 ± 0.98		7.07 ± 1.34	
Cognitive flexibility	High-dose group(n=29)	45.73 ± 1.63		48.57 ± 1.65	†*	48.20 ± 2.20	
	Low-dose group(n=29)	46.36 ± 1.70	*	49.21 ± 1.85	†*	46.18 ± 3.63	
	Placebo group(n=28)	40.14 ± 2.12		42.87 ± 1.93		41.79 ± 3.33	
Processing speed	High-dose group(n=29)	60.00 ± 1.71		62.13 ± 1.61		64.83 ± 1.88	‡
	Low-dose group(n=29)	58.89 ± 2.15		62.46 ± 1.82	‡*	64.43 ± 2.19	‡
	Placebo group(n=28)	55.04 ± 1.32		57.20 ± 1.70		58.71 ± 1.78	‡
Executive function	High-dose group(n=29)	46.97 ± 1.59		49.67 ± 1.65	†*	49.20 ± 2.15	
	Low-dose group(n=29)	47.64 ± 1.65	*	50.79 ± 1.64	†**	47.46 ± 3.63	
	Placebo group(n=28)	41.79 ± 1.99		44.23 ± 1.92		43.00 ± 3.35	
Working memory	High-dose group(n=29)	11.59 ± 0.67		11.70 ± 0.57		12.47 ± 0.61	
	Low-dose group(n=29)	11.44 ± 0.76		10.70 ± 1.13		12.21 ± 0.52	
	Placebo group(n=28)	9.50 ± 1.46		11.17 ± 0.54		11.79 ± 0.68	
Sustained attention	High-dose group(n=29)	32.48 ± 0.84		32.83 ± 0.68		33.43 ± 0.82	
	Low-dose group(n=29)	33.00 ± 0.76		30.37 ± 1.51		33.29 ± 0.63	
	Placebo group(n=28)	29.25 ± 2.20		30.97 ± 0.99		31.66 ± 0.94	
Simple attention	High-dose group(n=29)	38.23 ± 1.32		38.47 ± 0.89		37.80 ± 1.42	
	Low-dose group(n=29)	39.68 ± 0.13		39.56 ± 0.18		38.14 ± 1.39	
	Placebo group(n=28)	37.93 ± 1.41		38.87 ± 0.39		38.72 ± 0.50	
Motor speed	High-dose group(n=29)	122.13 ± 3.24		125.47 ± 3.01	†	124.23 ± 3.13	
	Low-dose group(n=29)	126.68 ± 5.23		132.36 ± 3.71	**	131.07 ± 2.58	**
	Placebo group(n=28)	122.61 ± 3.27		123.27 ± 2.61		120.69 ± 2.00	

Significant difference between the groups compared to placebo group(*p<0.05、**p<0.01)
Significant difference within the group compared to baseline(†p<0.05、‡p<0.01)

・Low-dose Naturido group: creatinine (p<0.05) and GFR (p<0.05)

Significant within-group differences before and after the 12-week intake period were as follows:

・High-dose Naturido group: albumin (p<0.05), ALT (p<0.05), and Na (p<0.05)
・Low-dose Naturido group: urea nitrogen (p<0.01)
・Placebo group: MCHC (p<0.05)

Significant within-group differences before and after intake at week 6 and before intake were as follows:

・High-dose Naturido group: hemoglobin (p<0.05), hematocrit (p<0.05), total protein (p<0.05), albumin (p<0.05), total cholesterol (p<0.01), and HDL cholesterol (p<0.01)
・Low-dose Naturido group: WBC (p<0.05), total protein (p<0.05), albumin (p<0.05), urea nitrogen

($p<0.05$), total cholesterol ($p<0.05$), HDL cholesterol ($p<0.01$), and HbA1c ($p<0.05$)

・Placebo group: MCV ($p<0.01$)

As mentioned above, although there were significant differences between groups before and after and in multiple test items, the mean values in all test items were within the normal range, no adverse events were identified, and the changes were transient and slight. Therefore, this was not considered a safety issue.

No specific changes were observed in the high-dose Naturido and low-dose Naturido groups, and no dose-related trends were identified. Urinalysis showed no significant differences between groups for all items, and no abnormal changes were observed in any subject.

Based on the above, no adverse events attributable to the test food were observed in any subject, and no adverse effects due to the test food were observed in the results of the general laboratory tests. Therefore, the safety of the test food was not a problem.

Discussion

In this study, the Cognitrax test demonstrated that the low-dose Naturido group exhibited a significant improvement in visual memory, psychomotor speed, and motor speed, compared with the placebo group at week 12 (Table 2 and Figure 2-3).

Although there were no significant differences in visual memory, psychomotor speed, or motor speed in the Cognitrax test between the high-dose Naturido and placebo groups, the high-dose Naturido group showed a significant improvement trend compared with the placebo group in the scores of the above three items. In the high-dose Naturido group, the visual memory score increased compared to that before intake at 6 and 12 weeks, while the placebo group showed no change in score from that before intake. As for the psychomotor speed, values in the placebo group were not significantly different from that before intake, but the high-dose Naturido group showed significant improvement before and after intake at both 6 and 12 weeks. The motor speed of the high-dose Naturido group showed a significant improvement before and after intake at 6 weeks, and the score was higher at 12 weeks than before intake; however, the placebo group showed a decrease in scores at 12 weeks.

From these results, it is considered that the effect of Naturido was observed in visual memory, psychomotor speed, and motor speed in the same way as in the low-dose Naturido group although no significant difference was observed in the high-dose Naturido group compared to the placebo group.

Significant differences were observed between the low-dose Naturido and placebo groups. One reason for the lack of significant differences between the high-dose Naturido and placebo groups may be that the low-dose Naturido group was the optimal dose for the expression of these functions. The length of axons and dendrites increased only in the low-concentration group in an in vitro study in which Naturido was added to cultured hippocampal neurons. The permeability of the blood-brain barrier must be considered in order to discuss the concentration of Naturido in the brain blood when Naturido is ingested; however, one reason that the low-dose Naturido group showed a higher effect on cognitive function than the high-dose Naturido group was that the low-dose group had a cerebral blood concentration suitable for axon and dendrite outgrowth of nerve cells. The detailed mechanism of action of Naturido and the relationship between neuronal activation and Naturido concentration in brain blood should be further investigated.

However, the results of this clinical trial suggest that low-dose Naturido may have a greater effect in terms of improving visual memory, cognitive function speed, and motor speed functions.

The *I. japonica* powder produced by the silkworm contained in the test food has been shown to increase the level of acetylcholine in the cerebrospinal fluid in clinical trials on subjects with Alzheimer's disease (22). In in vivo studies with aging-induced mice, the effect of memory retention was observed in the context of learning-memory test, and the recovery of spatial memory was observed with the spatial learning test (10). In addition, the above effects on cognitive function have been confirmed to be due to the cyclic peptide Naturido contained in the I. japonica powder produced by the silkworm (23). In an in vitro study, Naturido has been reported to promote astrocyte growth, significantly increasing the mRNA levels of nerve growth factor (NGF) and non-acronymic neuropeptide (VGF) in astrocytes and significantly increasing the dendritic length, number of hippocampal neurons, and length of axons8). Further, it has been shown to improve spatial learning performance in a Morris water maze study in which aging-induced mice were orally challenged with Naturido (8).

Visual memory as determined by the Cognitrax test is assessed by the visual memory test and evaluated for immediate and delayed memory, by checking how many various shapes can be memorized and recognized. It involves the ability to memorize photographs, illustrations, figures, and symbols; recall

Table 3) Blood test

Item (Unit)		Baseline Mean ± SE		Week 6 Mean ± SE		Week 12 Mean ± SE	
WBC (×102/μL)	High-dose group(n=30)	5677.67 ± 240.29		5701.00 ± 265.75		5785.67 ± 228.83	
	Low-dose group(n=29)	5694.48 ± 229.05		5135.52 ± 241.14	†	5393.45 ± 269.13	
	Placebo group(n=30)	5541.33 ± 239.42		5471.33 ± 199.38		5347.00 ± 230.07	
RBC (×104/μL)	High-dose group(n=30)	442.93 ± 9.24		438.13 ± 8.94		439.87 ± 7.84	
	Low-dose group(n=29)	448.55 ± 7.46		445.21 ± 7.08		443.59 ± 7.58	
	Placebo group(n=30)	441.53 ± 8.17		442.83 ± 7.08		440.47 ± 6.81	
Hemoglobin (g/dL)	High-dose group(n=30)	13.02 ± 0.35		12.85 ± 0.34	†	12.92 ± 0.30	
	Low-dose group(n=29)	13.48 ± 0.30		13.40 ± 0.29		13.32 ± 0.30	
	Placebo group(n=30)	13.37 ± 0.33		13.41 ± 0.31		13.39 ± 0.28	
Haematocrit (%)	High-dose group(n=30)	39.16 ± 0.85		38.68 ± 0.83	†	38.77 ± 0.68	
	Low-dose group(n=29)	40.32 ± 0.83		39.86 ± 0.75		39.69 ± 0.78	
	Placebo group(n=30)	39.99 ± 0.76		39.78 ± 0.73		39.66 ± 0.69	
MCV (fL)	High-dose group(n=30)	88.67 ± 1.32		88.50 ± 1.34		88.43 ± 1.28	
	Low-dose group(n=29)	89.83 ± 0.94		89.52 ± 0.94		89.48 ± 0.85	
	Placebo group(n=30)	90.77 ± 1.15		89.80 ± 1.07	ǂ	90.17 ± 1.12	
MCH (pg)	High-dose group(n=30)	29.43 ± 0.58		29.37 ± 0.60		29.45 ± 0.57	
	Low-dose group(n=29)	30.04 ± 0.42		30.10 ± 0.40		30.00 ± 0.37	
	Placebo group(n=30)	30.28 ± 0.48		30.24 ± 0.44		30.40 ± 0.44	
MCHC (%)	High-dose group(n=30)	33.15 ± 0.28		33.12 ± 0.25		33.24 ± 0.26	
	Low-dose group(n=29)	33.41 ± 0.20		33.59 ± 0.18		33.54 ± 0.18	
	Placebo group(n=30)	33.32 ± 0.25		33.61 ± 0.24		33.70 ± 0.19	†
Platelet count (×104/μL)	High-dose group(n=30)	27.10 ± 1.19		26.95 ± 1.13		26.30 ± 1.08	
	Low-dose group(n=29)	26.22 ± 0.83		25.16 ± 0.97		25.89 ± 1.00	
	Placebo group(n=30)	25.45 ± 0.81		25.16 ± 0.86		24.83 ± 0.84	
Total protein (g/dL)	High-dose group(n=30)	7.16 ± 0.05		7.04 ± 0.05	†	7.07 ± 0.06	
	Low-dose group(n=29)	7.20 ± 0.09		7.06 ± 0.07	†	7.14 ± 0.08	
	Placebo group(n=30)	7.15 ± 0.07		7.11 ± 0.06		7.13 ± 0.07	
Albumin (g/dL)	High-dose group(n=30)	4.41 ± 0.04		4.33 ± 0.04	†	4.33 ± 0.05	†
	Low-dose group(n=29)	4.41 ± 0.05		4.33 ± 0.04	†	4.36 ± 0.05	
	Placebo group(n=30)	4.40 ± 0.04		4.36 ± 0.05		4.36 ± 0.04	
AST (U/L)	High-dose group(n=30)	21.43 ± 1.65		21.90 ± 1.51		21.70 ± 1.44	
	Low-dose group(n=29)	23.10 ± 2.50		24.66 ± 4.66		25.90 ± 4.55	
	Placebo group(n=30)	21.47 ± 1.40		23.73 ± 3.04		22.43 ± 2.11	
ALT (U/L)	High-dose group(n=30)	20.80 ± 3.67		24.17 ± 2.56		24.37 ± 2.58	†
	Low-dose group(n=29)	25.10 ± 5.27		25.34 ± 4.61		26.03 ± 5.22	
	Placebo group(n=30)	20.43 ± 2.20		20.47 ± 1.80		20.00 ± 1.94	
LDH (U/L)	High-dose group(n=30)	163.57 ± 4.84		165.77 ± 5.63		163.97 ± 5.10	
	Low-dose group(n=29)	164.14 ± 3.99		169.10 ± 7.05		171.69 ± 7.06	
	Placebo group(n=30)	163.03 ± 3.99		167.23 ± 5.56		165.00 ± 4.50	
ALP (U/L)	High-dose group(n=30)	62.83 ± 2.89		64.10 ± 3.45		65.77 ± 3.20	
	Low-dose group(n=29)	65.97 ± 2.31		64.97 ± 2.62		65.93 ± 2.18	
	Placebo group(n=30)	62.03 ± 2.52		62.73 ± 2.92		63.27 ± 2.78	
γ-GTP (U/L)	High-dose group(n=30)	28.90 ± 5.49		26.03 ± 3.52		28.67 ± 4.69	
	Low-dose group(n=29)	43.62 ± 9.82		37.17 ± 7.52		37.55 ± 8.03	
	Placebo group(n=30)	44.03 ± 8.79		43.17 ± 8.14		39.83 ± 8.45	
Total bilirubin (mg/dL)	High-dose group(n=30)	0.60 ± 0.04		0.54 ± 0.03		0.57 ± 0.04	
	Low-dose group(n=29)	0.61 ± 0.04		0.60 ± 0.03		0.59 ± 0.04	
	Placebo group(n=30)	0.72 ± 0.09		0.69 ± 0.07		0.64 ± 0.05	
Creatinine (mg/dL)	High-dose group(n=30)	0.67 ± 0.02		0.66 ± 0.02		0.66 ± 0.02	
	Low-dose group(n=29)	0.79 ± 0.04	*	0.78 ± 0.04	*	0.78 ± 0.04	*
	Placebo group(n=30)	0.67 ± 0.02		0.69 ± 0.02		0.67 ± 0.02	
GFR (mL/min/1.73m²)	High-dose group(n=30)	83.37 ± 2.85		83.97 ± 2.80		84.20 ± 2.74	
	Low-dose group(n=29)	74.03 ± 2.82	*	75.03 ± 2.87	*	74.52 ± 2.86	*
	Placebo group(n=30)	82.87 ± 1.98		81.60 ± 2.11		83.97 ± 2.40	
Urea nitrogen (mg/dL)	High-dose group(n=30)	12.88 ± 0.50		13.51 ± 0.59		11.86 ± 0.47	
	Low-dose group(n=29)	15.52 ± 0.85	**	14.08 ± 0.78	†	13.51 ± 0.77	ǂ
	Placebo group(n=30)	12.58 ± 0.60		13.23 ± 0.63		12.85 ± 0.67	
Uric acid (mg/dL)	High-dose group(n=30)	5.13 ± 0.22		5.13 ± 0.24		5.23 ± 0.25	
	Low-dose group(n=29)	5.77 ± 0.38		5.55 ± 0.35		5.67 ± 0.36	
	Placebo group(n=30)	4.98 ± 0.29		5.11 ± 0.24		4.99 ± 0.23	
Total cholesterol (mg/dL)	High-dose group(n=30)	205.57 ± 6.70		196.47 ± 5.66	ǂ	201.83 ± 6.67	
	Low-dose group(n=29)	209.48 ± 7.16		200.93 ± 5.81	†	204.34 ± 6.47	
	Placebo group(n=30)	210.50 ± 6.78		210.37 ± 6.97		208.93 ± 7.90	
HDL cholesterol (mg/dL)	High-dose group(n=30)	69.77 ± 2.75		65.03 ± 2.50	ǂ	67.63 ± 2.78	
	Low-dose group(n=29)	66.76 ± 2.77		61.97 ± 2.63	ǂ	64.45 ± 2.81	
	Placebo group(n=30)	69.33 ± 3.27		69.53 ± 3.02		69.23 ± 3.52	
LDL cholesterol (mg/dL)	High-dose group(n=30)	113.57 ± 6.27		109.40 ± 6.36		113.57 ± 6.19	
	Low-dose group(n=29)	114.66 ± 6.92		112.17 ± 6.17		113.59 ± 6.39	
	Placebo group(n=30)	117.73 ± 5.99		115.27 ± 5.86		115.33 ± 6.07	
Triglyceride(TG) (mg/dL)	High-dose group(n=30)	101.17 ± 8.42		115.87 ± 16.16		103.23 ± 9.38	
	Low-dose group(n=29)	131.14 ± 24.43		134.83 ± 19.70		134.34 ± 24.46	
	Placebo group(n=30)	108.83 ± 17.50		139.57 ± 27.63		124.70 ± 18.72	
Na (mEq/L)	High-dose group(n=30)	140.37 ± 0.34		140.30 ± 0.28		141.03 ± 0.26	†
	Low-dose group(n=29)	140.69 ± 0.29		140.86 ± 0.33		140.86 ± 0.28	
	Placebo group(n=30)	140.27 ± 0.42		140.53 ± 0.37		140.87 ± 0.31	
K (mEq/L)	High-dose group(n=30)	4.10 ± 0.05		4.05 ± 0.05		4.04 ± 0.06	
	Low-dose group(n=29)	4.07 ± 0.04		4.00 ± 0.04		4.04 ± 0.04	
	Placebo group(n=30)	4.02 ± 0.05		4.01 ± 0.03		4.03 ± 0.04	
Ca (mg/dL)	High-dose group(n=30)	9.23 ± 0.04		9.20 ± 0.04		9.30 ± 0.05	
	Low-dose group(n=29)	9.24 ± 0.06		9.17 ± 0.07		9.20 ± 0.07	
	Placebo group(n=30)	9.25 ± 0.05		9.15 ± 0.05		9.19 ± 0.05	
Blood glucose level (mg/dL)	High-dose group(n=30)	91.40 ± 2.55		92.20 ± 1.99		91.33 ± 1.64	
	Low-dose group(n=29)	89.34 ± 2.48		94.83 ± 3.16		90.59 ± 2.79	
	Placebo group(n=30)	94.13 ± 3.51		99.90 ± 8.58		95.63 ± 3.88	
HbA1c (%)	High-dose group(n=30)	5.28 ± 0.05		5.33 ± 0.05		5.26 ± 0.06	
	Low-dose group(n=29)	5.25 ± 0.05		5.31 ± 0.04	†	5.27 ± 0.05	
	Placebo group(n=30)	5.41 ± 0.15		5.38 ± 0.13		5.39 ± 0.13	

Significant difference between the groups compared to placebo group(*p<0.05、**p<0.01)
Significant difference within the group compared to baseline(†p<0.05、ǂp<0.01)

calendar events (16,24,25).

Psychomotor speed was calculated as the sum of the finger-tapping test and SDC test, which reflects the subject's speed of movement and information processing, visual perceptual speed, how the subject recognizes and processes the information and responds to perceptual and input information, movement speed, and fine motor adjustments. It involves the use of precision instruments such as driving a car and playing instruments (16,24,25).

Motor speed was calculated using the finger-tapping test. It evaluates how the subject recognizes and processes information and how they respond to perceptual input information, movement speed, and fine movement adjustment. Then, the moving speed, information processing speed, and visual perception speed of the subject are evaluated. Motor speed is involved in the ability to perform tasks with fingers, dexterity of the hands, and ability to manipulate objects1 (6,24,25).

The mechanism of cognitive function improvement action by Naturido is confirmed by the Cognitrax test, astrocyte proliferation action (8), neuronal differentiation facilitation action (8), acetylcholine concentration increasing action (8), and spatial memory improvement action (8,23), among others. Astrocytes play an important role in the central nervous system, have been implicated in dementia and brain aging (26), and have also been reported to affect the gene activation of NGF and VGF (26,27). NGF is a neurotrophic factor involved in promoting neuronal differentiation and maintaining function (28,29). VGF promotes hippocampal neural applications and synaptic activity (30). It has also been reported that nerve cells decrease with aging (31,32), and with aging, the memory ability and information processing speed (33), spatial cognition, and perception speed also decreases (34).

Neurogenesis in the hippocampus is related to the acquisition of memory (35). Inhibition of neurogenesis in the hippocampus by irradiation has been reported to cause a marked decrease in memory and learning abilities even in young mice (36). Neurogenesis and hippocampal differentiation are considered crucial factors affecting memory performance. As previously described, Naturido has been reported to increase the length and effect of dendrites in hippocampal neurons and length of axons during neuronal differentiation (8).

Thus, NGF and VGF are involved in neurite outgrowth, and the mechanism of improving memory learning by neurite outgrowth was considered (8,23).

The neurotransmitter acetylcholine is known to affect cognitive function (37). Choline acetyltransferase (ChAT) activity of acetylcholine synthase and acetylcholinesterase (AChE) activity of acetylcholine-degrading enzymes are decreased in patients with Alzheimer’s disease. ChAT activity in the cerebral cortex of Alzheimer's disease patients and cognitive function scores have been reported to be correlated (38,39). Pharmacological studies have shown that blockade of brain ACh neurotransmission by atropine and scopolamine inhibits learning and memory behaviors, and the choline hypothesis proposes that impairment of the acetylcholinergic nervous system is a major pathology of Alzheimer's disease (40). AChE inhibitors inhibit acetylcholine breakdown and increase acetylcholine levels in the synaptic cleft, which promotes transmission in the acetylcholine nervous system and increases acetylcholine levels in the brain, which is thought to ameliorate Alzheimer's disease symptoms such as memory impairment (41,42).

In this context, the possible mechanism by which the increasing effect of acetylcholine concentration by Naturido intake affects cognitive function and memory ability was considered.

Naturido also significantly improved in the Morris water maze test in senescence-accelerated mice8). The Morris water maze test is an assessment system related to spatial cognitive and spatial grasping abilities and visual and spatial memory abilities (43,44).

The level-increasing effects of acetylcholine in Alzheimer's disease patients and improvement of spatial learning performance in aging-induced mice have both been observed at 8 weeks after the oral intake of Naturido. These effects may be involved in this clinical study, in which cognitive improvement was not observed at week 6 but was observed at week 12.

Therefore, it is considered that Naturido intake is associated with the improvement of spatial cognition and grasping and that it leads to the improvement of visual memory ability.

Cognitive functions can be classified as fluid intelligence and crystallized intelligence (45,46). Fluid intelligence includes information-processing speed and spatial cognition. The peak has been observed in 20–24-year-olds followed by a slow decrease; this suggests that the speed of decline is accelerated from around 50 years of age (47). It has also been reported that the speed of information processing is decreased in subjects with neuronal degeneration and neurological disorders (48). The neurogenesis- and differentiation-promoting effects of Naturido may affect the speed of cognitive function (speed of information processing). In addition, visual memory has been reported to be reduced because of delayed information processing speed (49,50).

According to the Neuropsychological Pyramid at the University of New York Medical Center, memory is ranked higher than the speed of information processing (51), and memory is said to be affected by the speed of information processing. These results suggest that the effects of Naturido on cognitive function may be related to psychomotor speed and visual memory and may influence each other.

In this study, we found significant differences between the Naturido and placebo groups in visual memory, psychomotor speed, and motor speed in the Cognitrax test in healthy subjects and subjects with MCIs since their 40s. It can be considered that the effectiveness of Naturido on cognitive function (e.g., memory and information processing speed) lowered by the decrease of nerve cells and neurofibril change with aging in middle-aged and elderly people is associated with astrocyte proliferation action, nerve cell differentiation promotion action, acetylcholine concentration increase action, spatial memory improvement action, and so on.

A limitation of this study is that the effectiveness of the eye-tracking test was not confirmed. The eye-tracking test is a tool for recording and analyzing eye-tracking techniques of eye-gaze movements for various task images displayed on a screen for approximately 3 min and for evaluating them objectively and quantitatively for cognitive function (17) and autistic spectrum disorder (52). The eye tracking test is superior in detecting people with MCIs and dementia and is correlated with the MMSE and Alzheimer's Disease Assessment Scale-cognitive subscale (17). However, correlations between the Cognitrax test and other cognitive tests have not yet been identified.

Moreover, in this clinical study, it was not necessary to set up a PC in the Cognitrax test, and the test implementation circumstances of the test subjects were uniform. On the other hand, in the eye-tracking test, it was necessary to set the position of the face in advance every time the subject performed the test. However, subjects had different pre-set times for reasons such as that the position of the face not being fixed well and taing more than 15 min being taken for some subjects to set.

As noted above, the preset time for performing the test was different for each subject in the eye-tracking test, and the test environment was not uniform, suggesting that the appropriate cognitive function may not have been assessed. Further studies are required in the future.

Furthermore, the intake period in this clinical trial was 12 weeks, and the efficacy of intake over a longer period has not been confirmed. Therefore, further research is necessary in the future.

In this study, no adverse events attributable to the test food were observed and significant differences were observed in some blood tests. However, the changes in each subject were slight, within the reference values, and no adverse events related to the laboratory values were observed. In an acute toxicity study in female mice, no toxicity or mortality was observed at oral doses of 25–3000 mg/kg of *I. japonica* powder produced by the silkworm containing the same Naturido as the test food (53). In a subchronic toxicity study in adult female rats, oral administration at doses of 25 and 500 mg/kg for 28 consecutive days did not significantly affect food or water consumption, hematological parameters, or relative organ weights between the treatment and control groups (53). This study also confirmed the safety of consuming high and low doses of Naturido for 12 weeks.

Therefore, there was no problem with the safety of the test food in this study, and it is considered that Naturido can be consumed safely for a 12-week period.

Conclusion

The effect of 12-week consumption of a capsule containing 0.96–1.92 mg of Naturido on improving cognitive function was evaluated in a randomized, placebo-controlled, double-blind, parallel-group study. Twelve weeks after intake, the low-dose Naturido group (0.96 mg Naturido) showed statistically significant improvements in visual memory, psychomotor speed, and motor speed in the Cognitrax test compared to the placebo group.

The 12-week safety of Naturido at a dose of 0.96–1.92 mg daily was also confirmed. These results indicate that the intake of foods containing 0.96 mg of Naturido has a beneficial effect on cognitive function.

Acknowledgement

We would like to express gratitude to Koichi Suzuki in the effort for the discovery of Naturido and related research

Conflicts of interests

This study was conducted by DKS Co. Ltd.

by providing cost-sharing and test foods. DKS Co. Ltd. was not involved in the clinical trials or data analysis.

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