Effect of Nattokinase on the Blood Flow Improvement in Healthy Subjects

-A Randomized, Placebo-controlled, Double-blind, Cross-over Study –

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ABSTRACT

Objective Nattokinase is a thrombolytic enzyme contained in natto, which is reported that it acts on endothelial cells and enhances the production of t-PA that is used for treatment such as myocardial infarction. The purpose of this study is to evaluate the blood flow improving effect of nattokinase on peripheral blood vessels.

Methods We conducted a randomized, placebo-controlled, double-blind, crossover study for fifteen healthy volunteers (7 males and 8 females, aged from 30 to 48 years). Subjects consumed either soft capsules that contain 2000 FU of nattokinase (NSK-SD : 110 mg) as test food, or the soft capsules that contain dextrin instead of nattokinase as placebo, together with 100 mL of water. The peripheral blood flow of middle fingers of both hands, the back of the left hand, the back of the right hand and insteps of both feet were measured before and 40, 80, 120, 180 minutes after consumption of the test food.

Result The consumption of nattokinase significantly improved the change in peripheral blood flow in the middle fingers of both hands compared to the placebo. The change in peripheral blood flow in the middle fingers 180 minutes after ingestion of the test food group was significantly higher than in the placebo group ($P=0.026$), and the rate of change from 0 minute in the test food group was significantly higher than that of the placebo group at 120,180 minutes after ingestion ($P=0.036$, $P=0.016$). No adverse events were observed in all subjects during the test period.

Conclusion These results indicate that soft capsules containing nattokinase improve the peripheral blood flow of healthy adults.

(Jpn Pharmacol Ther 2018 ; 46 : 1739-48)

KEY WORDS Nattokinase, Peripheral blood flow, Hypertension

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Introduction

Neoplasm malignant (cancer), ischemic heart disease, cerebrovascular diseases account for major causes of death of Japanese people in recent years. According to ''Population Survey Report'' issued 2016, the mortality rate of cardiovascular diseases such as heart disease and cerebrovascular disease is 26.0 %, and it follows that of Neoplasm malignant (28.5 %)[1]. Furthermore, looking at medical expenditure by type of disease, cardiovascular disease occupies the most, and the expenditure is 891.8 billion yen (19.9%)[2]. Besides, according to World Health Organization (WHO), cardiovascular diseases account for approximately 30 % of all death in the world[3]. In modern society, the decrease in energy consumption brought by motorization and desk jobs, etc. and westernization of the daily diet caused the dramatical increase of hypertension, diabetes, obesity, and hyperlipidemia, etc. Consequently, the incidence of arteriosclerotic disease (ex. ischemic heart disease, cerebral infarction, arteriosclerosis obliterans) and thrombotic disease has increased. According to the basic policy of ''thorough critical prevention and aggravation of major lifestyle-related disease'' of Kenko Nippon 21 (2nd ver.) developed by the Ministry of Health, Labour and Welfare, prevention of cardiovascular disease should be promoted as one of important policies[4].

Nattokinase is a thrombolytic enzyme found in natto. It works on endothelial cells and promotes tissue plasminogen activators (t-PA) used for the treatment of myocardial infarction[5]. *Bacillus subtilis natto* is a strain that the safety is observed in the world, and nattokinase is a thrombolytic enzyme made by the strain. Regarding the molecular structure, it is a polypeptide structure that is composed of single-stranded 275 amino acid residues[6].

The fluidity of blood is determined by a lot of factors, for instance, blood coagulability, hematocrit, erythrocyte deformability, platelet aggregation, leukocyte adhesion, and fibrinolysis. However, it is reported that nattokinase not only degrades plasminogen activator inhibitor-1 (PAI-1) inhibiting t-PA and fibrinogen[7][8], but also significantly inhibits platelet aggregation *in vitro* research which rabbit platelet is used[9]. Furthermore, it is reported that *in vivo* research that rats take nattokinase found it has effects on thrombolytic activity and improving blood flow[10].

In this way, it has been confirmed that nattokinase has effects on thrombolytic activity and improving blood flow in both *in vivo* and *in vitro*. However, because there are few reports for the human clinical trials and data for influence on healthy individuals, the human clinical trials for healthy adult individuals were held to investigate nattokinase's effect on blood flow.

I. Subjects and Methods

1. Subjects

A screening test was held for subjects who understood the content of the trial and gave their consent in writing. Then, 15 adult men and women who satisfied a selection criterion (men and women from the age of 30 to 49 at the time of acquisition of consent) and are not inconsistent with exclusion criteria below (①~⑦).

Exclusion Criteria:

① Those who regularly use ''Natural Super Kinase II'' or nutritional supplement drinks, etc.
② Those who use medicine with effects on improvement of blood flow and recovery from fatigue (ex. Warfarin, vitamin preparations, etc.) and dietary supplements.
③ Those who have diabetes, brain dysfunction, peripheral vascular disease, severe diseases in their digestive organs, pancreas, lever, kidney, etc.
④ Those who are participating in any other clinical trials at the beginning of the trial.
⑤ Those who have food allergy.
⑥ Those who are ineligible to the participation of the trial in terms of their questionnaire responses.
⑦ Those who are judged as ineligible to the participation of the trial by the investigator for any other reasons.

This trial was approved by the Ethical Review Board of Shoukoukai Clinic organized by the third party (approval date: October 28, 2015). Then, this trial was held under the supervision of doctors in compliance with ''Ethical Guidelines for Epidemiology Research'' and ''Helsinki Declaration''. Also, the subjects were paid-volunteer publicly-offered by TTC co., ltd. After the investigator explains to the subjects about the objective, method, anticipated side effects, etc., they understood the content of the trial and gave their voluntary consent in writing.

2. Materials and Test Diets

As the test preparation, ''Natural Super Kinase II'' (manufactured by Japan Bio Science Laboratory co., ltd.), soft capsules that contain nattokinase equivalent to 2,000FU per 3 capsules were used. ''Natural Super Kinase II'' has been sold since 2001 and contains 110mg Fermented Soybean Extract NSK-SD per 3 capsules. Also, Fibrin-degrading enzyme activity (FU) was used for the measurement of nattokinase. FU is a value of the amount of the acid-soluble low-molecular products of fibrin based on the absorbance of the sample at 275 nm. This assay method is standardized as the quality specification standard by Japan Health and

Nutrition Food Association[11]. Also, soft capsules, containing dextrin instead of Fermented Soybean Extract NSK-SD, were used. both test diet and placebo capsules have the same size (diameter: 10.9mm, minor axis: 7.1mm), and are manufactured to be unidentifiable in terms of appearance and odor. The analytical value of nutrition fact for nattokinase and placebo is shown in Table 1. The subjects took capsules with a cup of water (approx. 100mL).

Table 1 Nutrient Composition

	Nattokinase Ingredient amount (The amount of intake / day)	Placebo Ingredient amount (The amount of intake / day)
Soybean oil	400mg	400mg
Glycerol esters of fatty acids	45mg	45mg
Beeswax	45mg	45mg
Soybean lecithin	30mg	30mg
Fermented Soybean Extract	110mg	--
Dextrin	--	110mg
Nattokinase	2,000FU	--

3. Test Method

The study design is a randomized, placebo-controlled, double-blind, cross-over study (Figure 1). Therefore, subjects were randomly assigned by taking into consideration of their age and sex according to personnel irrelevant to the trial so that order effects do not occur. During the first administration period, Group A took nattokinase capsules, and Group B took placebos. After the two-week Washout period, Group A took the placebo and Group B took nattokinase during the second administration period. The trial was held from December 2015 to February 2016. Furthermore, all those participating in the trial (subjects, intervening people, assessor, etc.) were subject to blinding, and the assignment table was sealed until subjects to analysis populations were fixed. They were instructed not to radically change their lifestyle from before the trial (e.g. meal, exercise, smoking, medication, etc.) and keep a record on their lifestyle questionnaire if they deviate their lifestyle for excessive exercise, drinking alcohol, etc. Besides, as a restriction, they were advised to eat nothing two hours before the blood flow measurement and be relaxed by sitting on a chair in a room controlled for the temperature of 20℃ ~ 25℃. Regarding the blood flow measurement, a laser Doppler blood flow imaging system was used. (Preiscan PIM II : PERIMED). Their center points were recorded at the time of the first measurement, and all the following

measurements were held in the same position so that the value does not differ from person to person. They sat down on a chair and took the measurement. Also, the put their hand on a desk in case that blood flow of their hands was measured.

Figure 1 Flow Chart of the Trial

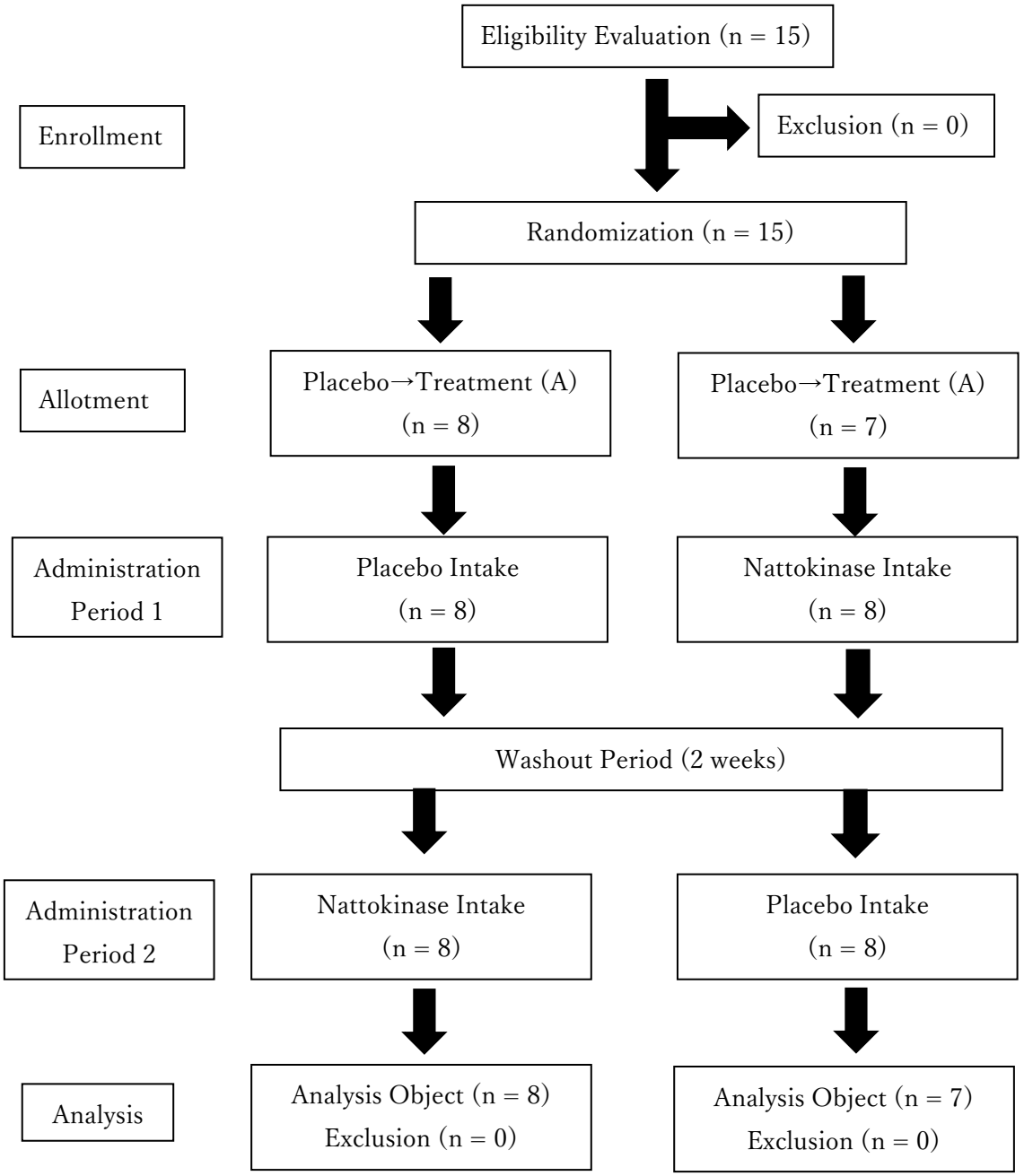


Table 2 Subject Background

Item	Unit	Placebo (n = 15)	Treatment (n= 15)
Sex (M/F)		7/8	7/8
Age		39.1±6.6	39.1±6.6
Height	cm	163.71±6.85	163.71±6.85
Body Weight	kg	61.77±10.97	61.99±10.72
BMI	kg/㎡	22.97±3.31	23.05±3.23
Systolic BP	mmHg	117.2±11.68	123.1±15.8
Diastolic BP	mmHg	75±8.76	77.9±13.56
Pulse Rate	bpm	75.5±8.37	76.5±9.8

4. Test Method

Primary endpoints are the amount of blood flow measured by the laser Doppler blood flow imaging system (measurement sites: right and left middle finger, the dorsum of the light and left hand, and the dorsum of the right and left foot). The unit is the number of blood cell counts (n) × the velocity of blood flow (v) = the amount of blood flow (V).

5. Statistical Analysis

Regarding the amount of change in blood flow, it was evaluated by Student's t-test for the inter-group comparison and by paired t-test for the within-group comparison to before the administration period. Values of subject backgrounds are presented in mean ±standard deviation, and all the results of the amount of blood flow and the amount-of-change in blood flow were presented in mean±standard error. The significance level was 5% (two-sided), and StatMate V (ATOMS Co.) was used as statistical analysis software. Furthermore, no adjustment was held for multiple testing at multiple items and time points.

II. Results

1. Subject Background

15 subjects with consent took the screening test and were divided into 2 groups (Group A: n=8, Group B: n=7). All the subjects completed prescribed experimental schedules without conflicting exclusion criteria of the analysis set. Their background is shown in **Table 2**. There is no significant difference between groups in any item. Then, concerning the amount of change in blood flow of each body part from the pre-administration period to 180 minutes after the administration, a test for carry-over

effects was held. As a result, there is no significant difference in order-effect (left and right middle fingers: P=0.86, back of right hands: P=0.86, back of left hands: P=0.87, back of left and right feet: P=0.72) and period effects (left and right middle fingers: P=0.38, back of right hands: P=0.31, back of left hands: P=0.27, back of left and right feet: P=0.31), and it was determined that this trial can be promptly evaluated by cross-over method.

2. Result: The Amount of Blood Flow

Table 3 and **Table 4** shows the test results of changes in blood flow from the pre-administration period to 180 minutes after the administration of test foods or placebo.

To begin with, regarding the amount of change in blood flow of left and right fingers, the amount of blood flow gradually increased in both the treatment group and the placebo group. Therefore, compared to the pre-administration, significant differences were observed in the treatment group at 80min, 120min, and 180min after the administration (P=0.002, P<0.001, P<0.001). However, no significant differences were observed in the placebo group (P=0.38, P=0.37, P=0.35). in the inter-group comparison of the amount of change in blood flow of left and right fingers, an increase was observed in that of Treatment group at 120 min after the administration (P=0.076), and there is a significant increase in that of treatment group at 180 min (P=0.026), compared to that of Placebo group (**Figure 2A**). Furthermore, regarding the rate of change in the blood flow, a significant increase was observed at 120 min and 180 min, compared to that of the Placebo group (P=0.036, P=0.016) (**Figure 2B**). On the other hand, Regarding the amount of change in blood flow of the back of the right hands, significant increases were observed at 40min, 80min, 120min, and 180min in both the Treatment and the Placebo group, however, there were no significant differences between groups (**Figure 3A**). Regarding the amount of change in blood flow of the back of the right hands, significant increases were observed at 40min, 80min, 120min, and 180min in both the Treatment and Placebo group, however, there were no significant differences between groups (**Figure 3B**). Regarding the amount of change in blood flow of the back of the left and right feet, significant increases were observed at 40min, 80min, 120min, and 180min in both Treatment and Placebo group, however, there were no significant differences between groups (**Figure 4**)

Table 3. The Amount of Blood Flow

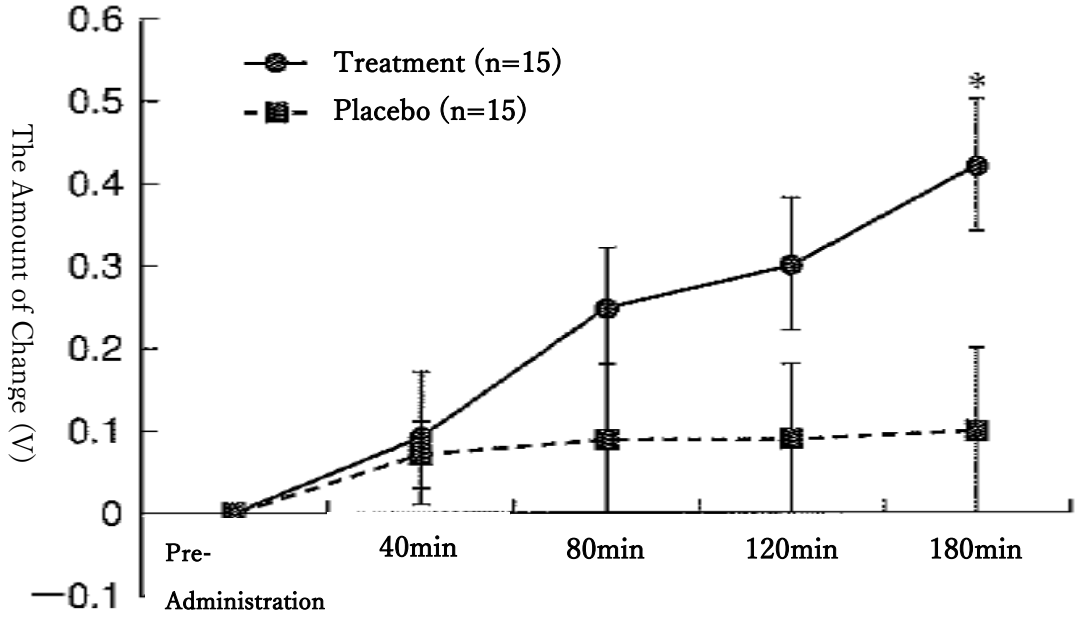
Item	Unit	Time Elapsed	Placebo (n = 15)	Treatment (n= 15)
Blood Flow (left and right middle fingers)	(V)	Pre-Administration	2.11±0.05	1.89±0.09
		40min	2.18±0.07	1.98±0.10
		80min	2.20±0.11	2.15±0.08**
		120min	2.20±0.11	2.19±0.07**
		180min	2.21±0.12	2.31±0.06**
Blood Flow (the back of right hand)	(V)	Pre-Administration	0.58±0.02	0.53±0.02
		40min	0.62±0.02*	0.60±0.02**
		80min	0.68±0.03**	0.68±0.03**
		120min	0.73±0.04**	0.76±0.05**
		180min	0.82±0.04**	0.84±0.05**
Blood Flow (the back of left hand)	(V)	Pre-Administration	0.62±0.02	0.58±0.03
		40min	0.66±0.02	0.65±0.03**
		80min	0.70±0.02*	0.71±0.03**
		120min	0.77±0.04**	0.78±0.05**
		180min	0.86±0.04**	0.89±0.06**
Blood Flow (the back of feet)	(V)	Pre-Administration	0.45±0.04	0.44±0.03
		40min	0.50±0.04**	0.50±0.02**
		80min	0.55±0.04**	0.54±0.02**
		120min	0.60±0.04**	0.58±0.02**
		180min	0.86±0.04**	0.66±0.03**

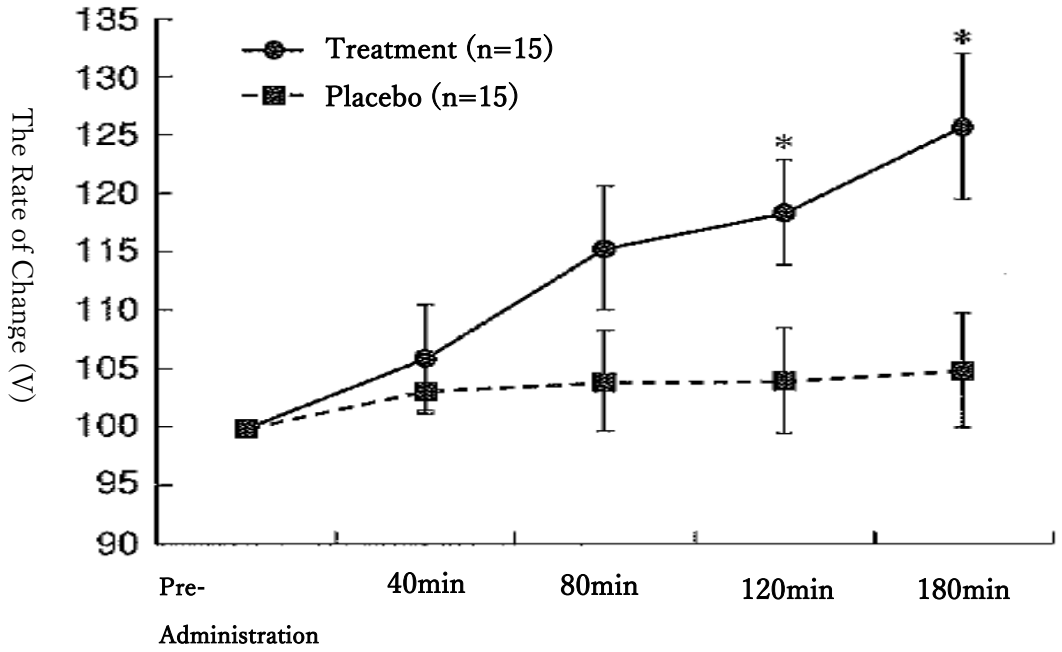
Mean±standard deviation

Significant different compared to pre-administration period (*P<0.05, **P<0.01)

Table 4. The Amount of Change in Blood Flow Compared to Pre-Administration Period

Item	Unit	Time Elapsed	Placebo (n = 15)	Treatment (n= 15)
Blood Flow (left and right middle fingers)	(V)	Pre-Administration	0.00±0.00	0.00±0.00
		40min	0.07±0.04	0.09±0.08
		80min	2.20±0.11	2.15±0.08**
		120min	2.20±0.11	2.19±0.07**
		180min	2.21±0.12	2.31±0.06**
Blood Flow (the back of right hand)	(V)	Pre-Administration	0.58±0.02	0.53±0.02
		40min	0.62±0.02*	0.60±0.02**
		80min	0.68±0.03**	0.68±0.03**
		120min	0.73±0.04**	0.76±0.05**
		180min	0.82±0.04**	0.84±0.05**
Blood Flow (the back of left hand)	(V)	Pre-Administration	0.62±0.02	0.58±0.03
		40min	0.66±0.02	0.65±0.03**
		80min	0.70±0.02*	0.71±0.03**
		120min	0.77±0.04**	0.78±0.05**
		180min	0.86±0.04**	0.89±0.06**
Blood Flow (the back of feet)	(V)	Pre-Administration	0.45±0.04	0.44±0.03
		40min	0.50±0.04**	0.50±0.02**
		80min	0.55±0.04**	0.54±0.02**
		120min	0.60±0.04**	0.58±0.02**
		180min	0.86±0.04**	0.66±0.03**


0.6
0.5
0.4
0.3
0.2
0.1
0
−0.1
The Amount of Change (V)
Treatment (n=15)
Placebo (n=15)
*
Pre-
Administration
40min
80min
120min
180min


135
130
125
120
115
110
105
100
95
90
The Rate of Change (V)
Treatment (n=15)
Placebo (n=15)
*
*
Pre-
Administration
40min
80min
120min
180min

3. Safety

no adverse events occurred to any of the subjects during the study period, and it was confirmed that the test foods were safe.

III. Discussion

As a result of investigating the effect of nattokinase on improving blood flow with the laser Doppler blood flow imaging system, Regarding the amount of change in blood flow of left and right middle fingers, an increase was observed in that of Treatment group at 120 min after the administration (P=0.076), and there was a significant increase in that of treatment group at 180 min (P=0.026), compared to that of the Placebo group. Furthermore, regarding the rate of change in the blood flow, a significant increase was observed at 120 min and 180 min, compared to that of the Placebo group (P=0.036, P=0.016). Regarding the amount of change in blood flow of the back of hands and feet, although significant increases were observed at most of the time period in both the Treatment and Placebo group, there were no significant differences between groups. These factors include the small amount of blood flow. Looking at the amount of blood flow of Placebo group at the pre-administration period, that of the back of hands and feet has much lower mean blood flow (0.58, 0.62, 0.45 V) than that of left and right middle fingers (2.11 V), and it can be assumed that measurement errors easily happen due to the small amount of blood flow. Regarding the measured value of the amount of blood flow, as a principle of the blood flow imaging system, when a laser light hits body tissues, the light hits red blood cells. Furthermore, the optical frequency changes due to the Doppler shift effect. On the other hand, the light which hits stationary tissues returns to the optical receiver without changing its frequency. Therefore, there is a mixture of the former and the latter. The rate of lights with changed frequency is proportional to blood cell counts, and the deviation of lights is proportional to the velocity of blood flow. From these results, it is possible to calculate the amount of blood flow by multiplying the blood cell counts by the velocity of blood flow[12].

With regard to the back of hands and feet measured, the amount of blood flow increased in Placebo groups compared to the pre-administration period. This is due to the dietary restriction 2 hours before intake and the increase of the velocity of blood flow by the intake and hydration (100mL). Consequently, the amount of blood flow increased. It is already reported that hydration significantly lowers blood viscosity[13], As a result of the hydration, there was an increase in the amount of blood flow in the Placebo group.

As mentioned before, Regarding the amount of blood flow of each body part, that of left and right middle fingers is much larger than that of the back of hands and feet. On the

other hand, that of the back of hands and feet has a third ~ fourth of that of left and right middle fingers. It is consistent with the result of previous studies relevant to cutaneous blood flow using laser doppler[14].

In this trial, the cutaneous blood flow of fingers has a larger amount of cutaneous blood flow than the back of the hands, following the bottom lip. It is suggested that AVA (arteriovenous anastomoses) in fingers influences on the blood flow[14)15)]. AVA, vacuum tubes connecting arteriolar and venules, exists in the skin of specific body parts such as the periphery of the limbs and lips[16)17)].

Regarding the mechanism of nattokinase's effect on improving blood flow, previous studies infer that the effect is due to a synergy of various physiological effects of nattokinase, for example, activating pro-urokinase, fibrinolytic activity via producing t-PA and degrading PAI-1, degrading fibrinogen, inhibiting platelet aggregation, etc.

On the other hand, it is reported that nattokinase affects lowering blood pressure. Studies report that it has an antihypertensive effect by inhibiting renin activity and ACE[19)20)]. Furthermore, it is also reported that nattokinase produces kinin[21)]. Kinin is known for the physiological effects such as antihypertensive effect, increasing capillary permeability, vasodilatory effect, etc[22)].

Hemodynamic change is closely related to hypertension, and the increases in peripheral resistance are regarded as a factor. Also, the increase of blood viscosity is a factor of hypertension because it causes peripheral resistance[23)]. Calcium channel blocker and thiazide diuretic also have an antihypertensive activity by improving the resistance[24)]. The peripheral blood flow is negatively correlated with the resistance. Thus, the more peripheral blood flow is, the less the resistance is.[25)] Regarding AVA mentioned before, a study shows a high negative correlation with changes in blood flow of AVA and change in mean blood pressure[26)]. Also, the rate of AVA accounting for blood flow in peripheral blood vessels is high[18)], and it is implied that nattokinase is related to the increase of blood flow. The increase of blood flow of AVA depends on passive vasodilatation due to inhibition of sympathetic nerve activity[15)]. As mentioned, nattokinase affects inhibiting renin activity, and a study shows a positive correlation with the renin activity (for healthy individuals and patients with essential hypertension) and sympathetic nervous systems[27)]. From these results, it is considered that nattokinase inhibits renin activity, has a restrictive effect on the sympathetic nervous systems, and is related to the increase of blood flow of AVA. In this trial, it was found that the amount of change in the blood flow of middle fingers significantly increased. This suggests that blood vessel is dilated as the amount of peripheral blood flow increases. As a result, peripheral vascular resistance and blood pressure lowered.

Recently, more and more people suffer from cardiovascular diseases including hypertension because of changing food demands, and prevention is an urgent problem. Low blood flow increases the risk of hypertension and cardiovascular diseases. Nattokinase is expected to be future prevention for these lifestyle-related diseases by improving blood flow and maintain a healthy condition.

Furthermore, the number of young women who have the sensitivity to coldness is increasing because of changes in our environment, especially cooling systems in their office. Peripheral vasoconstriction and the reduction of peripheral blood flow is considered to be causes of the sensitivity to coldness[29)30)]. As objective evaluation criteria, the amount of peripheral blood flow is measured by laser Doppler[31)32)]. They feel the sensitivity in fingertips of their hands and feet mainly. In addition, they have unidentified complaint such as shoulder muscle stiffness, dizziness, low back pain, etc[28)]. Improving the sensitivity to coldness has profound implications for their healthy life. this trial shows nattokinase's effects on improving the sensitivity of coldness by improving peripheral blood flow, and further clinical trials are now eagerly awaited.

Regarding the limitations of the trial, other endpoints except for peripheral blood flow are not evaluated. Therefore, a variety of evaluations will be needed. In this trial, no adverse events were observed, and It is considered that nattokinase is safe to humans.

Conclusion

This trial evaluated the effect of nattokinase on the blood flow improvement by consuming soft gel capsule that contains 2,000FU of nattokinase ''Natural Super Kinase II'', and a randomized, placebo-controlled, double-blind, cross-over study was conducted. Consequently, the change in peripheral blood flow in the middle fingers 180 minutes after ingestion of the test food group was significantly higher than in the placebo group, and the rate of change（right and left middle finger） from 0 minute in the test food group was significantly higher than that of the placebo group at 120,180 minutes after ingestion. These results indicate that soft gel capsules that contain 2,000FU of nattokinase ''Natural Super Kinase II'' improve the blood flow of healthy adults.

【Conflicts of Interest】

Japan Bio Science Laboratory Co., Ltd. covered the cost of the trial and provided ''Natural Super Kinase II'', and the trial was conducted. Also, Japan Bio Science Laboratory is irrelevant to the data analysis.

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The date of receipt (August 29, 2018)
The date of adoption (September 28, 2018)