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Effects of *Bacillus subtilis* Var. Natto Products on Capillary Blood Flow in Healthy Subjects with Peripheral Coldness: A Double-Blind, Placebo-Controlled, Randomized Parallel Study

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Abstract

Purpose: NKCP®, a natto-derived dietary food supplement whose main component is bacillopeptidase F produced by *Bacillus subtilis* var. natto, has antithrombotic, fibrinolytic, and pressure-lowering effects, and also is suggested to improve peripheral coldness. However, existing data are based on subjective evaluations with no scientific basis about the effects on peripheral coldness. Therefore, we aimed to investigate the effectiveness of NKCP® for peripheral coldness by measuring changes in blood flow using a laser doppler rheometer and biochemical indices. Patients and Methods: This was a double-blind, randomized, controlled study of individuals aged 30 - 70 years who complained of subjective symptoms of cold hands and feet. They were randomly divided into the NKCP® group and the placebo group to receive NKCP® 250 mg once daily and dextrin 250 mg as placebo once daily, respectively. The experiment lasted 8 weeks, with an intervention period of 4 weeks and a washout period of 4 weeks. Results: One-month intake of NKCP® significantly increased blood flow rate for 1 min between 4 and 5 minutes after the end of cold loading compared to that before feeding ($p = 0.038$). Also, analysis of the 5-minute blood flow rate before and after 4 weeks of feeding showed a significant improvement in the NKCP® group ($p = 0.007$), although there was no significant difference in the placebo group ($p = 0.215$). Furthermore, the 5-minute blood flow at 4 weeks after the end of feeding was significantly improved compared to that before feeding in the NKCP® group ($p = 0.049$). Therefore, the effect continued for at least 1 month after discontinuation of administration. Conclusions: It is possible that NKCP® intake effectively improves blood flow in subjects with peripheral coldness. Therefore, continuous intake of NKCP® is expected to reduce the symptoms of peripheral coldness. In the future, it needs to investigate whether the effect of increasing blood flow after ingestion of NKCP® is effective in improving the symp-

toms of peripheral coldness.

Keywords

Peripheral Coldness, Blood Flow, *Bacillus subtilis*, Functional Foods

1. Introduction

Natto, a fermented soybean product, has been consumed as traditional food in Japan and China. Natto is rich in several nutrients such as vitamin B, vitamin K, fibers, amino acid, and several proteases. Natto intake was reported to associate with a decrease risk of mortality from ischemic stroke [1]. In 1987, Sumi *et al.* [2] discovered that natto can dissolve artificial fibrin, and they named the extracted enzyme as nattokinase. Nattokinase is a phosphoenzyme by name, but it is a serine protease that develops an action similar to plasmin in the fibrinolysis system. The activation of the fibrinolysis system reduces blood viscosity and increases plaque dissolution, preventing cardiovascular [3] and cerebrovascular diseases [4].

NKCP® is a trademark of Daiwa Pharmaceutical Corp., from fermentation of the *Bacillus subtilis* subspecies Subtilis 168 strain, supplied as a powder without cell bodies and vitamin K [5]. The biochemical analysis indicated that the main component of NKCP® was identified as a 34-kilodalton fragment of bacillopeptidase F, as extracellular serine protease secreted by *B. subtilis* after the cell growth phase [5] [6]. Concerning the effects of NKCP® on blood clotting and fibrinolysis, the anticoagulant effect of NKCP was reported to be more than 100-fold greater than nattokinase, and the specific plasmin-like activity of NKCP was reported to be about 2.5-fold higher than that of nattokinase [7]. Omura *et al.* [8] [9] also reported fibrinolytic and anti-thrombotic effect of NKCP®. Furthermore, when the effect of improving blood fluidity with NKCP® was examined, the reduction of the total blood passage time was obtained, suggesting that NKCP also has the effect of suppressing platelet aggregation and thrombus formation [10]. In addition, natto contains high doses of vitamin K, which causes drug interactions with warfarin potassium, but NKCP® contains very little vitamin K, so patients taking warfarin do not have any problems. According to the study by Hitosugi *et al.* [11], NKCP® improved the blood flow and subsequently relieved low back pain, shoulder stiffness, and coldness of the extremities. Recently, Sunagawa *et al.* [12] also reported that NKCP® significantly improved the score of visual analogue scale for neck and shoulder stiffness and pain, reduced muscle stiffness of the neck, and increased the skin surface temperature of neck and shoulder, compared to before ingestion with no adverse effects.

Although cold or numbness in the extremities, commonly referred to as peripheral coldness, is not classified as a disease, it markedly lowers an individual's quality of life. Peripheral cold sensitivity is predominant in women aged over 40

years. The cold sensation is primarily caused by narrowing of the arterioles of the hands and toes, causing poor circulation [13]. Chinese herbs [14] and vitamin E [15] [16] [17] have been used to improve circulation at the extremities. In addition, functional foods are also sometimes used to improve symptoms. However, the effects of these measures have only been evaluated subjectively, and scientific evidence on their effectiveness remains scarce.

Therefore, this study aimed to investigate the effect of NKCP® on peripheral circulation. Towards this goal, we measured peripheral circulation in healthy subjects with peripheral coldness before and after NKCP® ingestion by measuring the microcirculatory dynamics of the fingertip using a laser Doppler blood flow meter.

2. Materials and Methods

According to the description by the report Omura K *et al.* [5], NKCP® was obtained as follows; Using *B. subtilis* subsp. Subtilis 168 strain, the organisms were cultured in fine granulated soy beans and glucose medium for two days at 42˚C, a refined protein layer was obtained as a supernatant from the medium by centrifugation and ultrafiltration of 10,000 mol weight mesh. NKCP® was provided as powder by decompressed dehydration of the supernatant.

NKCP® and placebo were obtained from Daiwa Pharmaceutical Co., Ltd. NKCP® contained >30 μg of bacillopeptidase F as a major component in a 250 mg/tablet. Meanwhile, in the placebo drug, becillopeptidase F was replaced with the same volume of dextrin in one tablet.

2.1. Study Design and Subjects

This was a double-blind, randomized, and controlled study of healthy subjects with complaints of peripheral coldness sensation. The participants were invited between February 2018 and January 2019 at Kanagawa Science Park Clinic, a third-party medical institution. The study plan was carried out in accordance with the Declaration of Helsinki and UMIN Clinical Trial Register (UMIN000031114). The candidates who were applicable under the diagnosis at the clinic were then judged to be eligible. The inclusion criteria were 1) age between 30 years and 70 years at the time of consent to participate; 2) complaint of subjective symptoms such as cold hands and feet; 3) ability to consent verbally and in writing; and 4) fit to participate as deemed by the investigators. The exclusion criteria were a) current drug treatment influencing blood coagulation; b) history of and treatment for cardiovascular or cerebrovascular diseases; c) diagnosis of intractable peripheral circulatory disease, such as Raynaud's disease; d) with conditions affecting cognition; e) allergic reaction to foods, food components, or pharmaceutical products; f) current smoking status; g) with plans for lifestyle changes, such as night work, long-term trip, or job transfer; h) consumption of health-promoting foods or supplements in the past 3 months; i) participation in other clinical trials that concluded in less than 3 months prior to

the present study; and j) pregnancy, nursing, or possibly pregnant.

Eligible candidates were given an oral explanation of the study protocol and a written consent statement and consent form. The number of cases was calculated according to the report of Sunagawa *et al.* [12], with an effect size of 0.5, a detection power of 0.7, and a significance level of 0.05. The first 30 individuals who submitted their consent form were included.

2.2. Randomization and Trial Protocol

The patients were assigned numbers and equally randomly divided into two groups as the NKCP® group and the placebo group. The NKCP® group (n = 15, 3 males, 12 females) received NKCP® 250 mg once daily before bedtime, while the placebo group (n = 15, 3 males, 12 females) received dextrin 250 mg dextrin once daily before bedtime. (**Table 1**) The experiment lasted 8 weeks, with an intervention period of 4 weeks and a washout period of 4 weeks. The implementation schedule is shown in **Figure 1**. There were no restrictions on daily activities during the study period.

Table 1. Age of participants in NKCP® group (administration of 250 mg of NKCP once a day before bedtime) and the Placebo group (administration of 250 mg of placebo once a day before bedtime).

	Placebo	NKCP®	p-Value
n (Male/Female)	15 (3/12)	14 (2/12)	-
Age	45.5 ± 1.8	44.7 ± 2.1	0.77

Data are expressed as mean ± SE.

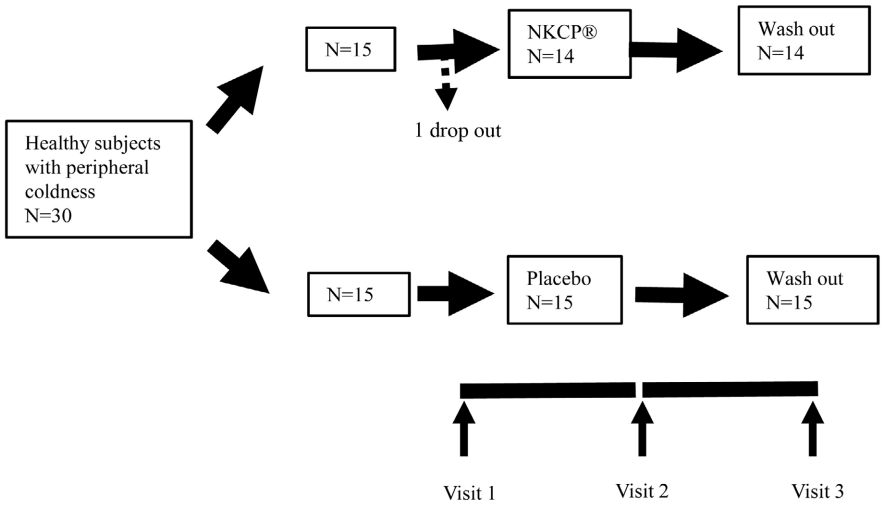


Figure 1. Patient inclusion flowchart. Thirty participants were randomly allocated into the NKCP® group (n = 15, 3 men and 12 women) to receive 250 mg NKCP® once a day before bedtime and into the placebo group (n = 15, 3 men and 12 women) to receive 250 mg placebo once a day before bedtime. One man from the control group was excluded because of compliance violations. Data were collected before and after the intervention period (visit 1 and visit 2, respectively) as well as after the washout period (visit 3).

2.3. Assessments

Data were obtained using a laser doppler rheometer (ALF21; Advance, Tokyo, Japan).

The subject waited in a sitting position for at least 30 minutes in an environment of a temperature of 23˚C and a humidity of 40%, and then a laser doppler probe was attached to the middle finger of the dominant hand of each subject, and blood flow was measured for 10 min. After confirming the stability of blood flow, the hand was immersed in ice-cold water for 60 sec. Next, the probe was remounted, and blood flow was measured for 20 min. The cold load test was conducted the day before the start of the test (Visit 1: V1), 4 weeks after the start of feeding (Visit 2: V2), and 4 weeks after the end of feeding (8 weeks after the start of the test (Visit 3: V3).

Blood biochemical assessments were outsourced to a company specializing in clinical testing. Data on biochemical indices related to blood flow, including activated partial thromboplastin time (APTT), total plasminogen activator inhibitor (PAI)-1, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and triglycerides, were also obtained in V1, V2 and V3. In addition, participants kept a daily journal of their food, drug, or supplement intake; alcohol consumption; and comments throughout the experimental period.

2.4. Statistical Analysis

Blood flow at the fingertip was measured using laser Doppler and expressed as mL/100g tissue/min. Given that the fingertip blood flow has large diurnal and seasonal fluctuations, and no standard value has been established, the average of the blood flow for 10 minutes before the cold load was set as 100%. Then, the rate of the blood flow obtained after the load was shown as a blood flow percentage (%). Data were expressed as the mean ± standard error (SE). Comparisons between groups were performed using one way ANOVA. Comparison within same group was performed using the paired t-test. All statistical analyses were performed using JMP Statistical Analysis Software (SAS Institute Japan Co.), and $p < 0.05$ was considered significant.

3. Results

3.1. Subject Characteristics

Of the 30 healthy subjects with peripheral cold sensation enrolled in this study, one placebo was excluded from the study due to non-compliance (Figure 1).

3.1.1. Primary Endpoint

Changes in blood flow after cold loading.

Before starting eating (V1).

The 1-min blood flow rates was significantly decreased after the cold load in both the NKCP® group (−42.36% vs. −9.6%, $p = 0.0002$) and the placebo group

(−35.21% vs. −9.78%, p = 0.0014) (Figure 2), but there were no significant differences in blood flow between the two groups (p = 0.606). The blood flow rate for 1 minute between 4 and 5 minutes after the end of loading was −43.03% ± 10.93% in the NKCP® group and −9.75% ± 1.157% in the placebo group, with no significant difference between the two groups (p = 0.05, Figure 2).

Meanwhile, the blood flow rates for 5 minutes at rest before loading and for 5 minutes after the end of loading were −41.0% ± 9.89% in the NKCP® group and −20.45% ± 9.53% in the placebo group, with no significant difference between them (p = 0.145) (Figure 3).

3.1.2. 4 Weeks after the Start of Feeding (V2)

The 1-minute blood flow rate before and after the cold loading were −4.07% ± 2.17% in the NKCP® group and −20.48% ± 13.86% in the placebo group, with no significant differences between the time points (p = 0.103 and p = 0.393, respectively, Figure 2). However, blood flow was markedly decreased in the placebo group, although there was no significant difference between the two groups (p =

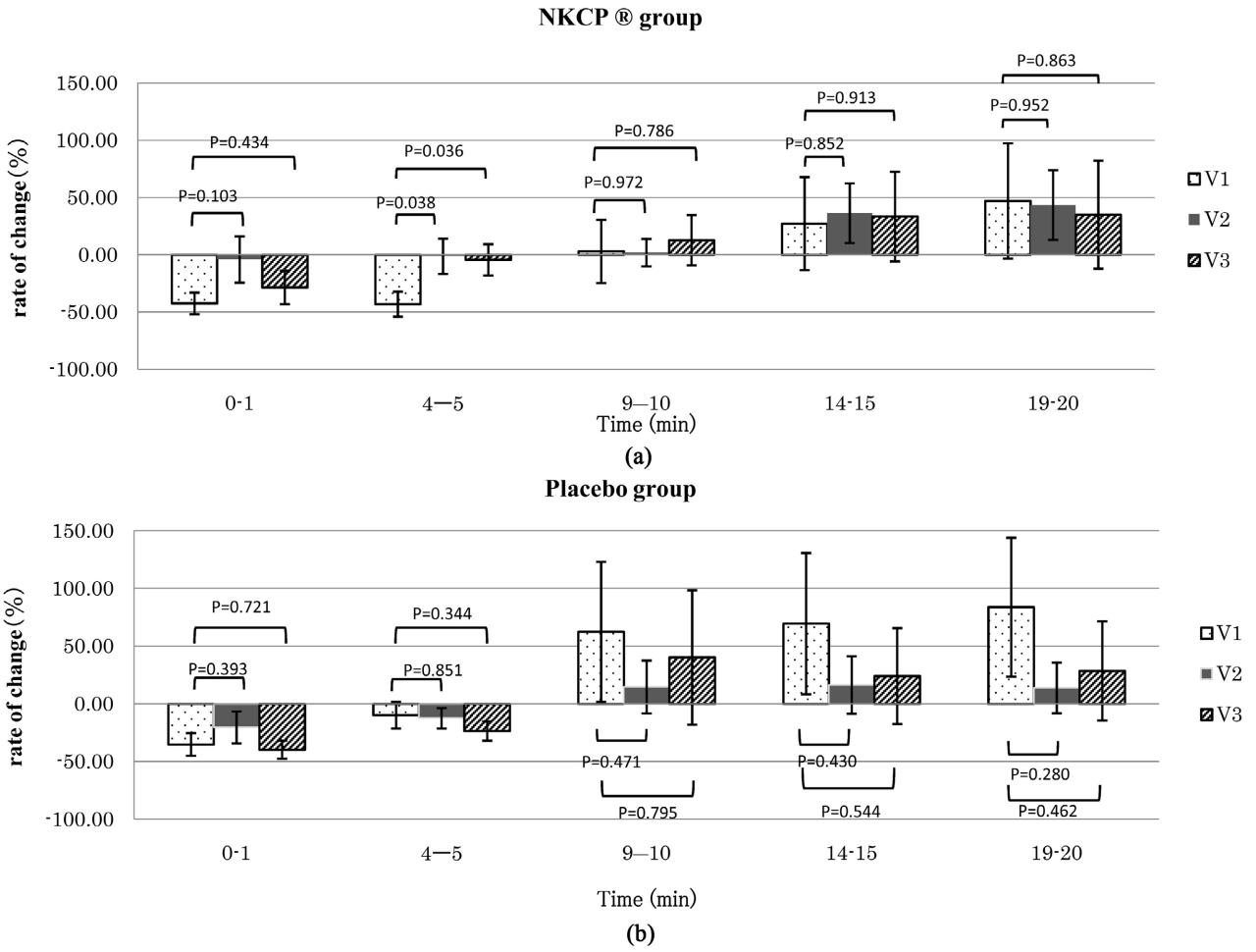
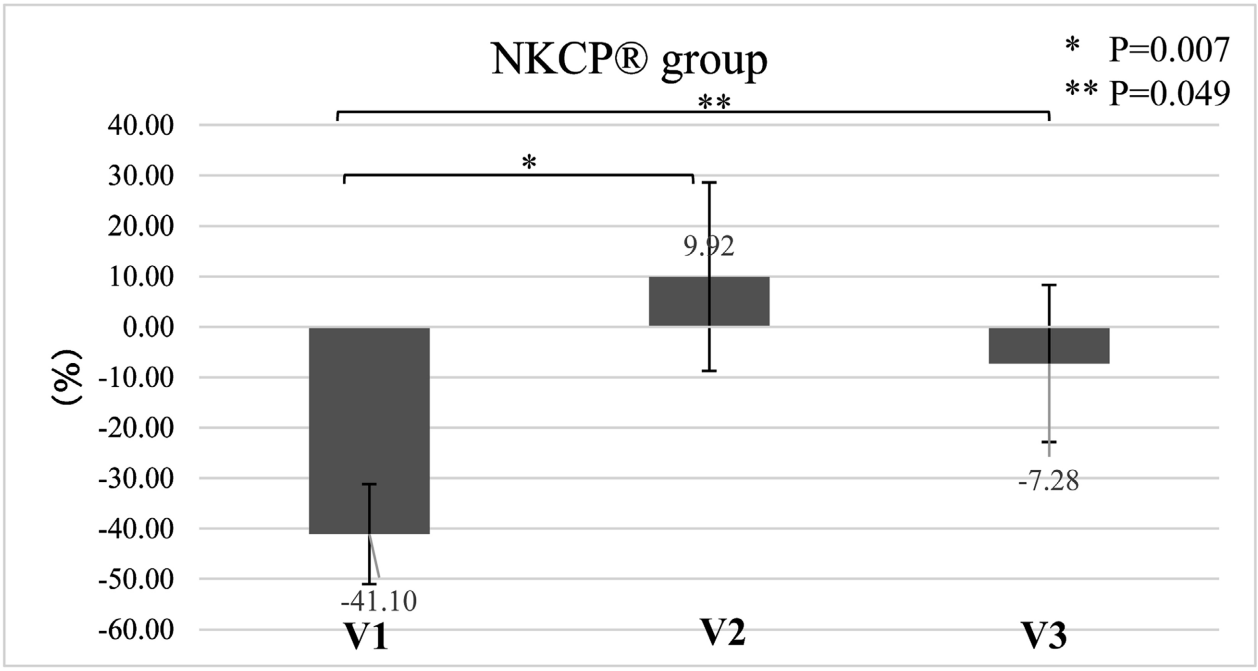
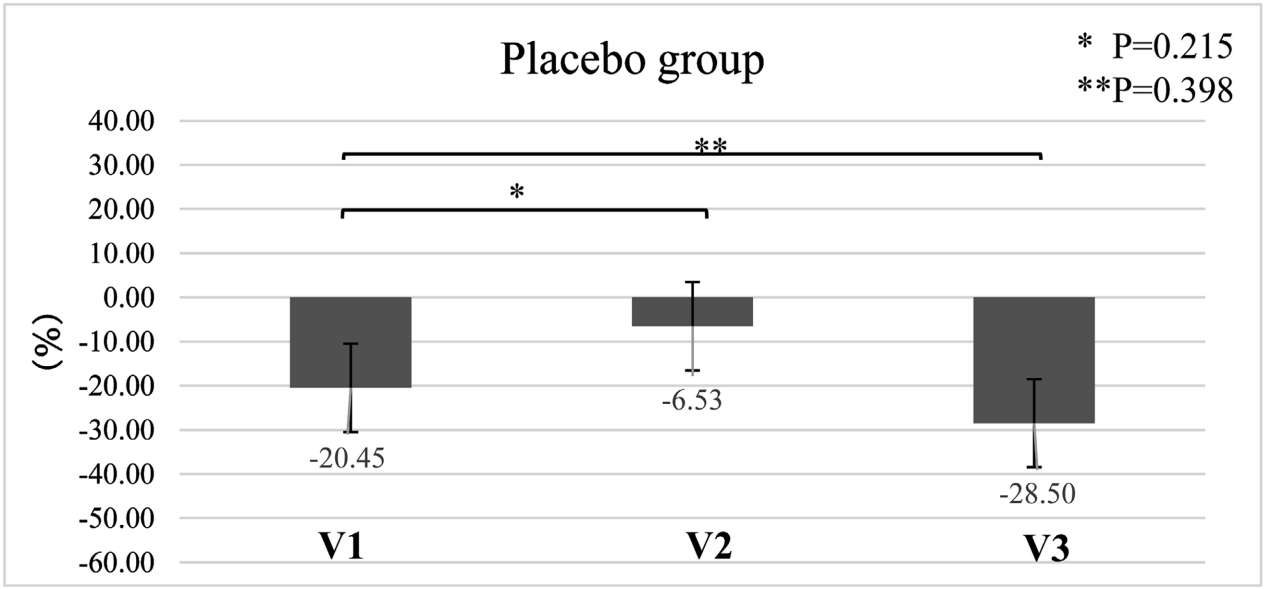


Figure 2. Recovery rate of blood flow at 0 - 1 min, 4 - 5 min, 9 - 10 min, 14 - 15 min, and 19 - 20 min after cold stress before and after the intervention period (visit 1 and visit 2, respectively) and after the washout period (visit 3). (a) NKCP® group. (b) Placebo group.



(a)



(b)

Figure 3. Recovery rate of blood flow at 5 min after cold stress before and after the intervention period (visit 1 and visit 2, respectively) and after the washout period (visit 3). (a) NKCP® group. (b) Placebo group.

0.509).

The 1-minute blood flow rate between 4 and 5 minutes after the end of loading was −1.20% ± 15.53% in the NKCP® group and −12.51 ± 8.80 in the placebo group, with no significant difference between the two groups (p = 0.533) (**Figure 2**). Next, the change in blood flow rate for 1 minute after the end of the cold loading was compared between before eating (V1) and after eating (V2). We

found a significant increase in blood flow between V1 and V2 in the NKCP® group ($p = 0.038$). However, no significant difference was found in the placebo group ($p = 0.851$) (Figure 2).

The 5-minute blood flow rates before and after the end of loading were 9.92% ± 18.68% in the NKCP® group and −6.53% ± 11.40% in the placebo group, with no significant difference between the two groups ($p = 0.460$). However, analysis of the 5-minute blood flow rate before and after feeding (comparison between V1 and V2) showed it was significantly improved in the NKCP® group ($p = 0.007$). Meanwhile, there was no significant difference in the placebo group ($p = 0.215$) (Figure 3).

3.1.3. 4 Weeks after the End of Feeding (V3)

In both the NKCP® group and the placebo group, the blood flow at the fingertips was significantly lower after cold loading than that before loading. No significant difference in blood flow after cold loading was observed between the two groups ($p = 0.215$).

The 1-minute blood flow rates before and after the end of the cold loading were −28.48% ± 14.54% in the NKCP® group and −39.74 ± 7.85% in the placebo group. A strong decrease in blood flow was observed in the placebo group, but there was no significant difference between the two groups ($p = 0.503$) (Figure 2).

The 1-minute blood flow rate between 4 and 5 minutes after the end of loading was −4.22% ± 13.61% in the NKCP® group and −23.56% ± 8.44% in the placebo group, with no significant difference between the two groups ($p = 0.24$). The change in blood flow for 1 minute after the end of the cold loading was compared between before eating (V1) and after eating (V3), and no significant difference between V1 and V3 was found in both groups ($p = 0.434$ and $p = 0.721$, Figure 2). However, the 1-minute blood flow rate between 4 and 5 minutes after the end of the cold loading showed a significant increase in blood flow between V1 and V3 in the NKCP® group ($p = 0.036$). However, there was no significant difference in the placebo group ($p = 0.344$) (Figure 2).

The 5-minute blood flow rates before and after loading were −7.28% ± 15.60% in the NKCP® group and −28.50% ± 8.04% in the placebo group, with no significant difference between the two groups ($p = 0.241$). However, the 5-minute blood flow in each group before and 4 weeks after the end of feeding (comparison between V1 and V3) was significantly improved in the NKCP® group ($p = 0.049$). Meanwhile, there was no significant difference in the placebo group ($p = 0.398$) (Figure 3).

3.2. Secondary Endpoint

Table 2 shows the changes in blood biochemical indicators of the NKCP® group and the placebo group. None of the groups showed changes exceeding the standard values in the coagulation system test and serum biochemical lipid test.

Table 2. Blood biochemical indies of NKCP® group (administration of 250 mg of NKCP® once a Day before bedtime) and Placebo group (administration of 250 mg of dextrin once a day before bedtime) before and after the intervention period (Visit 1 and Visit 2, respectively) as well as after the washout period (Visit 3).

Placebo group				p-Value		
Parameter	Visit 1	Visit 2	Visit 3	Visit 1 vs. Visit 2	Visit 2 vs. Visit 3	Visit 1 vs. Visit 3
Total cholesterol (mg/dL)	207.4 ± 9.1	208.3 ± 10.0	212.7 ± 7.9	0.949	0.729	0.663
HDL cholesterol (mg/dL)	73.8 ± 4.6	72.3 ± 3.3	72.5 ± 3.9	0.972	0.929	0.911
LDL cholesterol (mg/dL)	120.0 ± 7.5	120.8 ± 8.3	121.5 ± 6.7	0.944	0.946	0.88
TG (mg/dL)	77.6 ± 8.0	61.9 ± 7.4	63.4 ± 6.9	0.162	0.886	0.189
APTT (sec)	28.5 ± 0.8	26.6 ± 0.5	25.9 ± 0.6	0.077	0.385	0.02
Total PAI-1 (ng/mL)	21.3 ± 2.4	24.0 ± 2.5	23.5 ± 2.7	0.441	0.886	0.552

Data are expressed as mean ± SE.

NKCP® group				p-Value		
Parameter	Visit 1	Visit 2	Visit 3	Visit 1 vs. Visit 2	Visit 2 vs. Visit 3	Visit 1 vs. Visit 3
Total cholesterol (mg/dL)	222.3 ± 13.6	215.5 ± 13.5	229.0 ± 11.5	0.686	0.408	0.709
HDL cholesterol (mg/dL)	69.3 ± 2.6	66.7 ± 3.2	70.5 ± 3.7	0.553	0.562	0.85
LDL cholesterol (mg/dL)	136.0 ± 14.1	129.6 ± 12.8	139.4 ± 11.6	0.738	0.576	0.856
TG (mg/dL)	80.1 ± 12.8	80.8 ± 8.9	76.6 ± 9.0	0.967	0.743	0.822
APTT (sec)	27.8 ± 1.0	26.6 ± 0.9	26.1 ± 0.9	0.351	0.732	0.206
Total PAI-1 (ng/mL)	30.2 ± 3.3	29.4 ± 3.4	30.5 ± 3.7	0.857	0.82	0.954

Data are expressed as mean ± SE.

Parameter	Visit 1	Visit 2	Visit 3
Total cholesterol (mg/dL)	0.373	0.691	0.256
HDL cholesterol (mg/dL)	0.597	0.299	0.652
LDL cholesterol (mg/dL)	0.328	0.571	0.199
TG (mg/dL)	0.868	0.117	0.257
APTT (sec)	0.615	0.964	0.852
Total PAI-1 (ng/mL)	0.04	0.213	0.136

4. Discussion

In general, blood circulation is improved by reducing blood viscosity and dilating small blood vessels, and thus, drugs that promote fibrinolysis (e.g., vasodilators) are prescribed [18] [19]. Peripheral circulatory disorders without skin

symptoms and without impaired motor function mainly present as coldness and pain at the extremities of the limbs. Although these symptoms without skin lesions are not defined as diseases, they significantly lower the individual's quality of life and activities of daily living [20]. Given that these symptoms disappear with improved blood circulation, hot compresses and calidinogenase and tocopherol, which improve peripheral circulation, are the first choice.

Disorder of peripheral circulation, regardless of the disease and its symptom, will become to induce poor nutrition and hypoxia in its surround region. Skin ulcer with arteriosclerosis obliterans (ASO) is its typical example. Therefore, it is important to keep the peripheral circulation.

In this study, we investigated the effect of NKCP®-derived bacillopeptidase F on the peripheral circulatory dynamics that affects the fibrinolytic system. Considering that the cold symptoms are basically the same as those of Raynaud's disease, the cold stress loading method reported by Kurosawa *et al.* [21] was used, and the hemodynamics were evaluated as an index. As shown in **Figure 2(a)**, the administration of NKCP® for 1 month greatly improved 1-minute blood flow immediately after cold loading, although there was no significant difference. On the other hand, this effect was significantly improved in the 1-minute measurement between 4 and 5 minutes after the end of loading ($p = 0.038$). Furthermore, one-month administration of NKCP® also significantly improved the cumulative blood flow for 5 minutes after the cold loading ($p = 0.007$). Meanwhile, no significant change in blood flow was observed in the placebo group who received dextrin.

A double-blind study by Sunagawa *et al.* [12] has shown the efficacy of NKCP® in subjects with chief complaints of stiff shoulders and neck, with the suggested mechanism of action being regulation of the fibrinolytic system to improve blood flow. Hitosugi *et al.* [7] also reported the antithrombotic and fibrinolytic effects of NKCP®. In the present study, administration of NKCP® for 1 month had no effect on normal blood flow, but a shortened recovery time of blood flow after cold loading was observed. Specifically, we found that NKCP® can affect blood flow dynamics. This may have been the cause for its efficacy against shoulder/neck symptoms and in the coldness associated with peripheral circulatory failure.

Furthermore, it was suggested that the blood flow improving effect may continue to some extent even one month after the end of NKCP® administration. That is, no significant difference was observed in the blood flow 1 minute after the cold load, but a significant improvement in the blood flow between 4 and 5 minutes after the end of loading (**Figure 2(a)**) ($p = 0.036$) and a significant improvement in the cumulative blood flow for 5 minutes were also observed (**Figure 3(a)**) ($p = 0.049$). However, no such effect was observed with dextrin administered in the placebo group throughout the study period.

Additionally, an improvement in the cold sensory state before and after ingestion of the NKCP® or placebo was measured using the VAS method. (Score is between 0 to 10, and the larger number is weaker in coldness) In the NKCP®

group, VAS score was 4.00, 4.07 and 4.27 in V1, V2 and V3, respectively. On the other hand, in placebo group, VAS score was 4.73, 3.73 and 4.53 in V1, V2 and V3, respectively (data not shown). This result suggests that there may be a slight improvement in the subjective symptoms of coldness in the NKCP® group compared to the placebo group, although there is no significant difference.

Foods with functional claims are characterized by the specificity of their active ingredients and mild effects. The amount of NKCP® contained in food has a weak effect on the living body even if a clear pharmacological action is observed in vitro. Furthermore, the peripheral blood flow at the fingertips is greatly affected in daily life and cannot be set to a standard value. A laser Doppler blood flow meter is commonly used to continuously monitor the peripheral circulatory dynamics of a transplanted tissue (e.g., a flap) and to prevent necrosis due to ischemia. From this perspective, no significant difference could be observed between two unpaired groups because the absolute flow rate differs between individuals. However, a significant difference in a comparative study between two paired groups could be observed because the blood flow characteristics of the individual could be traced.

According to the report by Ohmura *et al.* [5] no abnormalities were found in the test results in a study of the effects of 250 mg of NKCP® daily intake for 2 consecutive weeks on the blood coagulation/fibrinolytic system. Similar to the above reports, 250 mg of NKCP® daily intake for 4 consecutive weeks in this study, APTT and Total PAI-1 were within normal range by intake of 250 mg of NKCP®. In addition, concerning the four parameters of blood lipids, no abnormal results were obtained by ingestion of NKCP® in this study [**Table 2**].

In the examination of the safety of NKCP® intake, Omura *et al.* [9] reported that LD50 of NKCP® was >5000 mg/kg/day in the acute toxicity test, and the maximum NOAEL was >1000 mg/kg/day in the subacute toxicity test. Additionally, Lampe and English [22] reported that consumption of 10 mg/kg/day nattokinase for 4 weeks was well tolerated in healthy human volunteers. Furthermore, studies on the effects of NKCP® on shoulder stiffness and low back pain [11] and the effects of NKCP® on neck and shoulder and stiffness [12] are both examined at 250 mg daily for 4 weeks. No adverse events have been reported.

This supports that NKCP® is safe as long as it is consumed as a food supplement. Based on these results, we plan to investigate the effects of long-term intake of NKCP® on systemic blood flow using thermography and according to changes in skin temperature at the fingertips.

5. Conclusion

The administration of 250 mg NKCP® once daily for 4 weeks significantly improves blood flow in healthy subjects with peripheral cold sensation. The effect continued for at least 4 weeks after the discontinuation of treatment. Therefore, continuous intake of NKCP® is expected to reduce the symptoms of peripheral coldness. In the future, it needs to investigate whether the effect of increasing blood flow after ingestion of NKCP® is effective in improving the symptoms of

peripheral coldness.

Ethics Approval and Informed Consent

This study was approved by the clinical research ethics committee of the Japan Koganebashi Sakura Clinic and was carried out in accordance with the Declaration of Helsinki. The study was registered at the UMIN Clinical Trial Register (UMIN000031114).

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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