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# A Confirmatory Study of Nattokinase-Containing Food on Blood Coagulation and the Fibrinolytic System

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## Abstract

Nattokinase has been shown to have fibrinolytic effects. Here, we re-examine this therapeutic capacity using Nattokinase as a food additive. A total of 45 male and female participants consumed 219 mg/day of NSK-SD fermented soybean extract (equivalent to 4,000 FU) or one of two different placebos once a day for 8 weeks. Subjects had physiological parameters representative of fibrinolytic activity assessed by a pre-test, after 4 weeks, and after 8 weeks. Results indicated that the diet incorporating Nattokinase did not have a significant impact compared to placebo. However, when limited to participants with a BMI above average (25-28) at the pre-test, total PAI-1 significantly decreased in the experimental group after 8 weeks when compared to the P1 placebo group. Additionally, Nattokinase as a food additive resulted in no adverse health effects.

***Keywords:*** *Nattokinase; Fibrinolytic; Dietary Supplement; Food Addictive; Platelet Aggregation*

## Abbreviations

kDa: Kilodalton; t-PA: Tissue Plasminogen Activator; PAI-1: Plasminogen Activator Inhibitor 1; CVD: Cardiovascular Disease; NK: Experimental Food Group; P1: Placebo Group 1; P2: Placebo Group 2; PIC: Plasmin-α-2 Plasmin Inhibitor Complex; ECLT: Euglobulin Clot Lysis Time; FU/g: Fibrinolytic Units Per Gram; BMI: Body Mass Index

## Introduction

Cardiovascular disease ranks among the leading causes of mortality globally. Conditions such as acute myocardial infarction, ischemic heart disease, and stroke may result from the obstruction of blood flow. These blockages can arise from various factors, but removing or preventing the clot is crucial for managing and preventing thrombolytic conditions. This is typically achieved through pharmacological or surgical interventions, though these strategies may not address an existing thrombus [1].

This management approach is logical, considering that normal circulatory system function is maintained through a delicate hemostatic balance between fibrin formation and degradation. This homeostatic balance facilitates clotting when necessary: platelet aggregation occurs upon injury to reduce blood flow, coagulation follows through a series of reactions to prevent excessive bleeding, and fibrinolysis eventually removes the clot once healing is complete, restoring healthy blood circulation. This balance is usually maintained by plasmin,

which can dissolve clots without incident. However, when fibrin accumulates beyond plasmin's capacity to restore balance, a thrombus forms, potentially blocking blood flow and oxygen supply, leading to tissue damage and thrombolytic diseases such as acute myocardial infarction and stroke [2]. Therefore, an intervention that can both prevent clot formation by targeting platelet aggregation and dissolve an existing thrombus by enhancing fibrinolytic activity, without the side effects often associated with pharmacological interventions, would be highly beneficial.

Fermented soybean, also known as natto, contains natural phytochemicals with biological activity in animals and humans. The most prevalent phytochemical is Nattokinase. Nattokinase is a serine proteinase composed of 275 amino acids and has a molecular weight of 27.7 kDa [3]. It has been documented to exhibit fibrinolytic activity [4]. It is efficiently absorbed along the intestinal tract with high bioavailability and is considered stable under varying pH and temperature conditions.

*In vitro* studies have consistently demonstrated the potent fibrinolytic activity of Nattokinase [3,5,6]. Research in rodents has shown that Nattokinase is four times more effective than plasmin in dissolving thrombi (blood clots) [4,6]. An *in vivo* study reported positive thrombolytic effects of Nattokinase in a carrageenan-induced rat model of thrombosis. Additionally, a human study involving healthy Japanese subjects found that oral Nattokinase supplementation enhanced thrombolysis and increased anti-coagulation factors [7]. A subsequent, more comprehensive human study, which included healthy subjects, individuals with cardiovascular disease risk factors, and those on dialysis, re-ported that oral Nattokinase supplementation significantly decreased levels of fibrinogen, factor VII, and factor VIII, suggesting its potential utility in cardiovascular disease management [8].

Nattokinase is a serine protease distinct from other known kinases. It is primarily derived from Bacillus subtilis, the bacterium used in the production of "natto," a traditional Japanese food. Natto has been consumed in Japan since approximately 300 BC and is renowned for its numerous health benefits, including a reduced risk of cardiovascular disease mortality, increased lifespan, anti-aging effects, lower rates of chronic diseases, thrombolytic effects, antibacterial action, and antioxidant properties [9].

Many of the health benefits associated with natto consumption are directly attributed to the Nattokinase protein. Nattokinase is known for its ability to dissolve fibrin clots and enhance tissue plasminogen activator (t-PA) function, which in turn dissolves plasminogen activator inhibitor 1 (PAI-1) and activates prourokinase. PAI-1 and t-PA are known predictive indicators for cardiovascular disease (CVD) in at-risk populations such as those with metabolic syndrome or those who have suffered prior CVD events. Nattokinase, with a molecular weight of approximately 28 kDa, is absorbed in the intestines while retaining its fibrinolytic activity. Therefore, Nattokinase, even when orally ingested, appears to be a viable candidate as a therapeutic or preventative measure for CVD and arteriosclerotic diseases by promoting fibrinolytic activity [10].

Despite its benefits, natto has a slimy texture and pungent odor, which may be a barrier to accessibility for many people. This is significant because many health benefits of natto are observed from daily consumption. To overcome this, NSK-SD offers a more palatable alternative. NSK-SD is a milky white powder derived from Bacillus subtilis natto culture extract. It is free of *Bacillus subtilis*, Vitamin K2, and its characteristic odor, while still maintaining a high Nattokinase content. Commercially available natto pro-vides a fibrinolytic activity of 4,000 FU per 100 grams, whereas NSK-SD has a fibrinolytic activity of 20,000 FU/g. It is an ideal food ingredient due to its lack of odor and high fibrinolytic activity even at low intake levels. This study aimed to evaluate the fibrinolytic effect of an experimental food containing Nattokinase in the form of NSK-SD.

## Materials and Methods

### Participants

This was a randomized, double-blind, placebo-controlled study design with two different placebo groups. Men and women between the ages of 40 and 65 with a BMI of 23 to 28 who applied for the study and met inclusion criteria were selected for the study. The exclusion criteria included those who took medicine or supplements that could affect fibrinolytic activity, regularly consume natto or Nattokinase, suffered from diabetes, liver diseases, kidney diseases, or heart diseases, had risk of developing an allergic response to the experimental food, would be pregnant and/or breastfeeding during the duration of the experiment, were participating in another clinical study, were undergoing medical treatment, or were assessed to be otherwise unsuitable for the study by the doctor carrying out the physiological assessments. Participants were asked to maintain their normal lifestyle, such that diet, exercise, and smoking habits remained the same.

### Test food

There were three different types of food used in this study. The experimental group received the food with 219 mg Nattokinase (active) NSK-SD (Nattokinase + Dextrin + Soybean lecithin + Beeswax + Soybean Oil + Glycerin Fatty Acid Ester), Placebo 1 (dextrin + Soybean lecithin + Beeswax + Soybean Oil + Glycerin Fatty Acid Ester), placebo 2 (soybean oil) (See table 1). Participants took 3 capsules of test food per day approximately 30 minutes after dinner for 8 weeks.

| Ingredients | Active | Placebo 1 | Placebo 2 |
|---|---|---|---|
| NSK-D | 219 mg (4,000 FU Nattokinase) | None | None |
| Dextrin | None | 219 mg | None |
| Soybean lecithin | 30 mg | 30 mg | None |
| Beeswax | 36 mg | 36 mg | None |
| Soybean Oil | 309 mg | 309 mg | 594 mg |
| Glycerin Fatty Acid Ester | 36 mg | 36 mg | 36 mg |

***Table 1:*** *Test food.*

### Observation and testing

A pre-test was performed to assess if participants met inclusion criteria, and to get a baseline reading of fibrinolytic activity. Along with the pre-test, participants were also given a journal to keep track of their daily routine and to record their meals and adherence to their assigned test food. Participants were re-evaluated at 4 weeks and 8 weeks. Participants were asked to refrain from drinking alcohol for 3 days prior to the test, and to refrain from eating after midnight the day before the test. Participants were also instructed not to rush or run to their appointments. To assess efficacy of the experimental food on fibrinolytic activity, levels of plasminogen activator inhib-itor-1 (PAI-1), plasmin-α-2 plasmin inhibitor complex (PIC), tissue plasminogen activator (t-PA), and euglobulin clot lysis time (ECLT) were measured. The Dunnett method was used to compare amount of change between placebo 1 and 2 to assess whether dextrin, soybean lecithin, and beeswax had any effect on fibrinolytic activity. Additionally, the Dunnett method was used to assess whether NSK-SD influenced fibrinolytic activity by comparing the active and placebo 1. A one sample t-test was used to assess within groups changes from baseline to 4 and 8 weeks. Participants that completed the test would be excluded if they were not reliable in their journaling schedule adherence, if they missed their scheduled appointment at the hospital, if they dropped out willingly, met exclusion criteria previously, or if their fibrinolytic test results were different by an excessive margin making them an outlier.

### Safety evaluation

Safety evaluations were conducted on participants that had taken the experimental food at least once. The doctor overseeing the participants' evaluation recorded any ad-verse events, the date that symptoms appeared, the date that symptoms disappeared, symptom severity, if the symptoms were considered serious or not, treatment administered, treatment outcomes, and the adverse event's relevance to the experimental food. If any adverse events did occur, treatments would be administered upon their identification.

The Study was conducted by EP Mediate Co, Ltd.

## Results

### Participants

19 males and 29 females (48 total) registered for the experiment and were divided into 3 groups; the experimental food group (NK), placebo group 1 (P1), and placebo group 2 (P2). Each group had 16 participants. Among these 48, 2 participants in the P1 group dropped out due to personal circumstances. All other participants completed the protocol. There was a total of 46 qualifying participants: 16 in the NK group (5 male, 11 female), 14 in P1 (5 male, 9 female), and 16 in P2 (7 male, 9 female). Further, the participants who's total PAI-1 exceeded the standard value (NK group: 3, P1 group: 4, P2 group: 4) were eliminated from the analysis. Participants whose -PA activation value exceeded 500% compared to the pre-test (NK group: 3, P2 group: 2) were also excluded. Finally, participants that met the exclusion criteria of excessive fibrinolytic test result change were excluded. As a result, the target participants for total PAI-1 were 13 participants in the NK group (3 male, 10 female, 10 participants in the P1 group (3 male, 7 female), and 12 participants in the P2 group (4 male, 8 female). Target participants for t-PA activation included 13 in the NK group (4 male, 9 female), 14 participants in the P1 group (5 male, 9 female), and 14 participants in the P2 group (7 male, 7 female).

### Fibrinolytic activity

Table 2 shows the mean and standard error of PIC, total PAI-1, t-PA activation, and ECLT, with figure 1 illustrating that transition over time.

| c | Foods | #participants | Pre-test (0w) | Test at 4 weeks (4w) | | Test at 8 weeks (8W) | |
|---|---|---|---|---|---|---|---|
| APTT (second) | NK | 16 | 25.93 ± 0.48 | 26.71 ± 0.55 | # | 26.84 ± 0.51 | # |
| | P1 | 14 | 25.36 ± 0.53 | 26.11 ± 0.66 | | 25.82 ± 0.67 | |
| | P2 | 16 | 26.33 ± 0.48 | 26.49 ± 0.54 | | 25.88 ± 0.61 | |
| Plasma PT time (second) | NK | 16 | 11.31 ± 0.08 | 11.28 ± 0.12 | p=0.082 | 11.60 ± 0.11 | ## |
| | P1 | 14 | 11.29 ± 0.10 | 10.91 ± 0.12 | ## | 11.31 ± 0.12 | |
| | P2 | 16 | 11.34 ± 0.17 | 11.20 ± 0.13 | | 11.39 ± 0.15 | |
| PIC (μg/mL) | NK | 16 | 0.45 ± 0.05 | 0.49 ± 0.05 | | 0.57 ± 0.07 | # |
| | P1 | 14 | 0.42 ± 0.04 | 0.52 ± 0.05 | # | 0.51 ± 0.04 | # |
| | P2 | 16 | 0.43 ± 0.04 | 0.57 ± 0.04 | ## | 0.52 ± 0.04 | |
| ECLT (hr) | NK | 16 | 9.44 ± 0.79 | 9.96 ± 0.66 | | 9.31 ± 0.77 | |
| | P1 | 14 | 8.86 ± 0.54 | 9.74 ± 0.65 | # | 10.12 ± 0.58 | # |
| | P2 | 16 | 8.88 ± 0.78 | 9.79 ± 0.55 | | 9.27 ± 0.75 | |
| Total PAI-1 (ng/mL) ※1 | NK | 13 | 23.0 ± 3.0 | 26.7 ± 3.6 | | 21.2 ± 2.9 | |
| | P1 | 10 | 18.4 ± 1.6 | 23.1 ± 3.6 | | 23.3 ± 3.8 | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | P2 | 12 | 22.8 ± 3.2 | 22.0 ± 1.7 | | 18.7 ± 2.3 | |
| t-PA activation<br>※2 | NK<br>P1 | 13<br>14 | 0.0390 ± 0.0175<br>0.0240 ± 0.0072 | 0.0248 ± 0.0124<br>0.0237 ± 0.0128 | | 0.0297 ± 0.0186<br>0.0226 ± 0.0096 | |
| | P2 | 14 | 0.0347 ± 0.0142 | 0.0161 ± 0.0026 | | 0.0512 ± 0.0167 | |

***Table 2:*** *Fibrinolytic activity.*

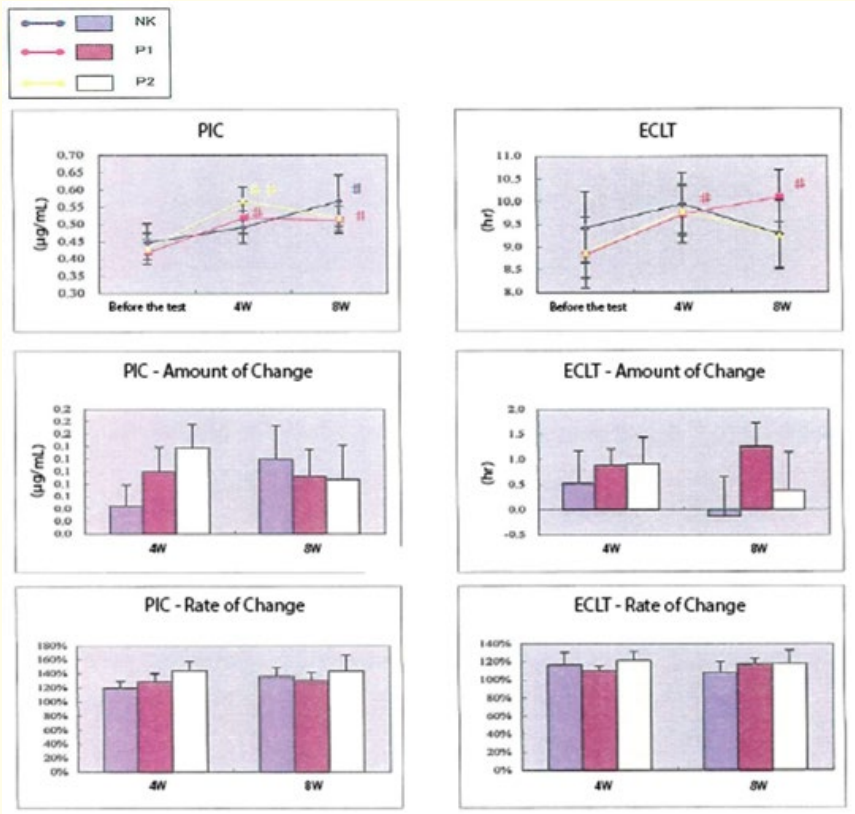


***Figure 1:*** *PIC and ECLT measured from target participants. Inner-group comparison 1 sample t-test compared to before the test #: p<0.05, ##: p<0.01. Intergroup comparison using Dunnett test, *: p<0.05, **: p<0.01.*

## PIC

There was a significant change in the NK group after 4 weeks of taking the experimental food, in the P1 group after both 4 and 8 weeks, and in the P2 group after 4 weeks when compared to baseline PIC. The changes observed in each group were minor and are considered within natural variance.

## ECLT

ECLT showed similar variability between experimental groups, with no significant change within and between groups (Figure 1).

### Total PAI-1

There was no significant difference between or within each group. However, when total PAI-1 of participants with a BMI over 25 were analyzed, the mean value of the pre-test, the value after 8 weeks ($p < 0.05$) and the amount of change ($p < 0.05$) de-creased significantly and to a greater extent than that of the placebo groups.

### t-PA activation

All groups of participants showed similar t-PA activation measurements, with no significant change within or between each group.

Each measure of fibrinolytic activity showed no significant change within or between groups, and there was no observed effect from the experimental food. However, when a threshold for participants above 25 BMI at the pre-test was set, total PAI-1 significantly decreased 8 weeks after eating the experimental food daily when compared to placebo group 1 ($p < 0.05$).

### Side effects

All side effects were minor 53 out of 54 adverse events were confirmed to have nothing to do with the experimental food. The 1 complaint was minor GI issue that re-solved quickly. This data supports that the experimental food is safe to eat.

## Discussion

Metabolic syndrome, a complication of obesity, characterized by high blood pressure, dyslipidemia, insulin resistance has become a growing concern. Cardiovascular disease is a very common downstream consequence of unchecked metabolic syndrome. With cardiovascular disease being the leading cause of death worldwide, there's an in-crease in interest to find innovative ways to tackle this problem [11]. The accumulation of fat and its improper internal storage is thought to be the defining characteristic of metabolic syndrome [12]. It has been reported that fat cells produce and secrete a considerable amount of adipocytokines [13]. In particular, plasminogen activator inhibitor-1 (PAI-1), which positively correlates with progression of obesity and diabetes, has garnered attention. A major role of PAI-1 is inhibiting the activity of plasminogen activator, which as its name suggests, activates plasminogen, stabilizing fibrin clots in blood [14]. The balance between PAI-1 and t-PA regulates the blood coagulation system and fibrinolytic activity. PAI-1 also affects several metabolic disorders and arteriosclerosis diseases [15]. PAI-1 may by itself be sufficient to contribute to the progression of atherosclerosis, promote fat accumulation, and negatively modulate insulin insensitivity [16-19].

By contrast, Nattokinase the enzyme derived from Natto, is a fibrinolytic enzyme. It affects fibrin clots directly by hydrolyzing fibrin and plasmin substrates alike. It also affects fibrin clots indirectly, by turning endogenous prourokinase into urokinase, de-grading PAI-1, and through upregulation of t-PA [20]. The fibrinolytic activity of Nattokinase is, importantly, retained after being orally ingested [21,22]. Commercially available Nattokinase, NSK-SD, which was used in this study for the experimental food, is made via extraction of Nattokinase from soybeans fermented with bacillus subtilis. While NSD-SD lacks Vitamin K2 found in natto, it also lacks natto's potent odor and slimy texture, making it more accessible to the general public. It contains 20,000 Fibrinolytic units per gram (FU/g) of Nattokinase activity. A pack of Natto (~100g) available on the market, by comparison, contains approximately 400 FU of Nattokinase activity. This means that NSK-SD is effective even in low dosage for administering biologically effective doses of Nattokinase. A previous study illustrated blood flow on the surface of the body after a single dose of NSK-SD (2000 FU) had a significantly more prominent effect on improved blood flow compared to that of placebo when compared to subjects of BMI greater than 23 [23]. This was the basis for this current study's interest in participants with a BMI between 23 and 28. The data thus far on safe and effective dosage for Nattokinase has been somewhat inconclusive, as benefits have been illustrated from ranges of 1000 FU/g all the way up to 14,000 FU/g, with safety in animal models being illustrated as high as 22,000 FU/g [24]. As research continues to elucidate the mechanisms and effective doses for Nattokinase for various benefits, we can continue to narrow down how to best optimize its therapeutic potency. With data currently available, this study

determined that food containing 219 mg of NSK-SD (4000 FU) for 8 weeks was appropriate to assess blood coagulation and fibrinolytic effects but the dose may need to be adjusted to be effective in people with different BMI.

When analyses were limited to participants whose BMI was over 25, the experimental group (NK group) observed a statistically significant decrease in total PAI-1 8 weeks after the oral ingestion of test food when compared to the P1 placebo group. It is known that there is a positive correlation between PAI-1 and t-PA, and a negative correlation between PAI-1 and ECLT, and this observation was only seen within the NK group in this study. There were no problems detected regarding subjective symptoms or other safety evaluation items either, reinforcing NSK-SD as a safe food additive.

## Conclusion

There were no significant differences between or within groups in fibrinolytic activity. However, when data from participants with a BMI > 25 BMI were analyzed separately, total PAI-1 significantly decreased 8 weeks after eating the experimental food daily when compared to placebo group 1 ($p<0.05$). Overall, NSK-SD is indicated to be a safe, orally administered vector for Nattokinase, with the ability to decrease PAI-1 in humans with a BMI greater than 25.

## Conflict of Interest

Mr. Shinsaku Takaoka and Mr. Kenichi Inoue are employed by Japan Bio Science Laboratory Co., Ltd. The funder of this study. Scott Miruzzi and Douglas Kalman declare no conflict of interest.

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