Fibrinolytic and antithrombotic effect of the protein from Bacillus subtilis (natto) by the oral administration.

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NKCP is the fragment of a part of Bacillopeptidase F from Natto bacteria (Bacillus natto), supplied as the tablet without distinct odor, bacterial cells nor vitamin K. The daily oral administration of NKCP, 28 volunteers for two weeks and 23 volunteers for several months, were examined to evaluate anti-thrombotic and fibrinolytic effect of the NKCP for humans.

The fibrinolytic effect was observed with shortening euglobulin lysis time in both trials without any significant changes in other coagulation and fibrinolytic parameters. Furthermore, we observed the statistically significant change of shoulder stiffness.

These results suggest that oral administration of NKCP has the fibrinolytic effect and the improvement of local blood flow. With further in vivo investigation, NKCP may be used as the preventing substrate from thrombotic disease.

Key Words: fibrinolysis, blood coagulation, Bacillus subtilis

Introduction

Natto, which is known as a traditional food in Japan and China, is a fermentation product of soybean using Bacillus subtilis natto and is now a common food. Because natto is high in B vitamins and contains natto bacillus-derived vitamin K, carbohydrate catabolic enzymes such as amylase, and proteases, it has been attracting attention as a functional food.[2-4)] Particularly, nattokinase, a typical protease extracted from natto, was recently found to have thrombolytic action and is expected to be effective in preventing thrombotic diseases.[5-7)]

However, because natto contains vitamin K, which interferes with anticoagulants, there is a limit to practical application.

We succeeded in removing the bacterial bodies, characteristic odor and viscosity, and most vitamin K from natto bacterial culture using a special production method and developed the food "NKCB."

Natto bacillus-produced protein (NKCP), the active ingredient of the food, shows a band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at almost the same location as the standard chicken egg white, which is 45 KDa, and has a different molecular weight from the previously reported enzyme of 20 to 30 KDa, which showed fibrinolytic activity.[8)]

The effect of oral NKCP on the blood coagulation/fibrinolysis system of humans was investigated in vivo in this study. This paper reports on interesting findings from the study.

Methods

1. Background of the study

A clinical study was conducted in humans according to the Helsinki Declaration. A preclinical study of NKCP showed that the LD50 value of acute toxicity was 5,000 or more mg/kg and the maximum ineffective dose of subacute toxicity was 1,000 or more mg/kg/day. It also showed that the antigenicity test by subcutaneous and intravenous injections and the mutagenicity test (Ames test) were both negative. No bleeding occurred in a test where 250 mg/kg NKCP was injected into the duodenum of rats. Because the doses used in these tests were high, safety problems were considered unlikely in this study.

2. Subjects

Adult volunteers who were capable of undergoing medical examination and consultation at specified medical institutions and gave prior informed consent were included in the study. Subjects who showed clear acute diseases and acute aggravation of chronic diseases, and other subjects who showed abnormal results in the tests of peripheral blood, general biochemistry and blood coagulation/fibrinolysis were excluded from the study. Exclusion of subjects by specified clinical conditions or clinically measurable parameters was not performed.

3. Study material

This study used a tablet form of NKCP produced by Daiwa Pharmaceutical Co., Ltd. One tablet contains 125 mg of NKCP.

The bacterial strain used to produce NKCP was isolated from ordinary natto. DNA sequencing showed that the strain was a common Bacillus subtilis strain. The molecular weight of NKCP was 45 KDa (Figure 1) according to the SDS-PAGE method and 34,134 Da according to the time of flight-mass spectrometry (TOF-MS). Analysis of the amino acid primary sequence of NKCP by the automated sequential Edman degradation method revealed that its main body was a fragment of Bacillopeptidase F, which is one of the proteases released by B. subtilis outside the cell at the end stage of exponential growth.[9] A basic study on NKCP showed that a physiological saline solution of NKCP has the ability to lyse an artificial thrombus and the ability to hydrolyze a synthetic substrate of plasmin. The activity of NKCP to hydrolyze S2251 (H-D-Val-Leu-Lys-pNA: Daiichi Pure Chemicals Co., Ltd.), a chromogenic substrate of plasmin of 2×10^{-3}M, was examined with 0.04 M tris (hydroxymethyl) aminomethane (pH 9.0); a 100 mg/mL solution of NKCP showed a result of 1.80×10^{-4} mol/min/L.

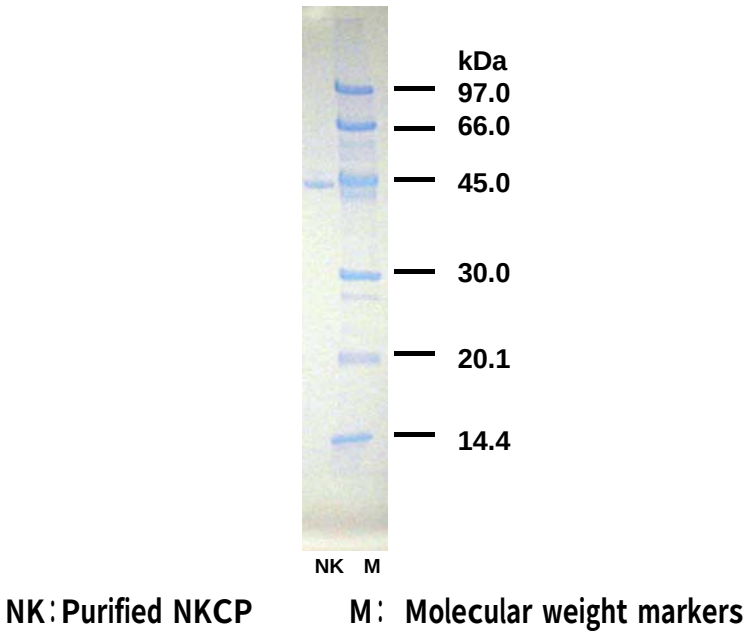

Fig. 1 Molecular weight of purified NKCP on SDS-PAGE

4. Testing methods

Subjects were provided with guidance on ingestion periods and methods, and were subjected to medical consultation and examination under the control of the medical institute. Subjects took two tablets (250 mg in terms of NKCP) daily after supper.

In Trial 1, which was aimed at examining the subacute effect of ingestion, subjects took NKCP for two consecutive weeks and clinical laboratory tests and medical examinations were performed before and after this period. For hematology, red blood cell count (RBC), hemoglobin (Hb), hematocrit (Ht), white blood cell count (WBC), and platelet count (Plt) were determined using an automatic hemocytometer, and fibrinolysis (euglobulinlysis time/ELT), tissue plasminogen activator (enzyme immunoassay/t-PA), fibrinogen (light scattering method/Fbn), fibrin decomposition product (latex agglutination turbidimetry/FDP), D-dimmer (latex near infrared turbidimetry), and activated partial thromboplastin time (light scattering method/APTT) were determined as parameters for blood coagulation/fibrinolysis activities.

In Trial 2, which was aimed at examining the effect of chronic ingestion, the parameters for the blood coagulation/fibrinolysis activities and three subjective symptoms (headache, shoulder stiffness and dizziness) during the first two months of ingestion were determined. To confirm clinical safety, total cholesterol (enzyme method/T-cho), LDL cholesterol (enzyme method/LDL-C), HDL cholesterol (enzyme method/HDL-C), triglyceride (enzyme method/TG), blood sugar (enzyme method/BS), and HbAlc (latex agglutination method) during the first three months of ingestion were determined in addition to the aforementioned hematological tests.

5. Statistical analysis

The results of clinical laboratory tests were summarized according to the time period to calculate basic statistics. Because the upper limit of measurement of ELT was 12 hours, the measurement results exceeding 12 hours were all assumed to be 12 hours. Because the lower limit of measurement of t-PA, D-dimmer and FDP was 1.5 ng/mL, 0.50 µg/mL, and 2 µg/mL, respectively, the measurement results below these limits were all assumed to be equal to the limits. The subjective symptoms of headache, shoulder stiffness and dizziness were each classed as "severe," "moderate," and "no significant symptom," and if there was a marked improvement in any of the subjective symptoms after ingestion, the symptom was classed as "marked improvement."

Changes in these results before and after the tests were analyzed for statistical significance. The paired t test was used in Trial 1 and Duncan's multiple comparison test was used in Trial 2. The Shirley-Williams multiple comparison test was used to examine the statistical significance of changes in subjective symptoms.

Results

1. Subacute ingestion study (Trial 1)

This study included 28 subjects (male: 11, female: 17) with an average age of 59.1 ± 12.1 years. The average body mass index of 22.7 ± 6.4 was almost equal to the reference value. The results of clinical laboratory tests are shown in Table 1. The results are presented by sex because some reference values differed according to the sex.

The average ELT value decreased significantly by 10.1% ($p < 0.01$) from 9.8 ± 2.0 hr before ingestion to 8.4 ± 1.7 hr after ingestion. There were no significant changes in the results of coagulation/fibrinolysis, peripheral blood, or blood chemistry. No adverse event was reported.

2. Chronic ingestion study (Trial 2)

This study included 23 subjects (male: 14, female: 9) with an average age of 51.7 ± 12.4 years. The average body mass index of 27.8 ± 8.3 was slightly higher than the reference value. The results of clinical laboratory tests are shown in Table 2.

Regarding the parameters for blood coagulation/fibrinolysis, the average ELT value decreased significantly ($p < 0.01$) by 8.5% from 9.0 ± 1.3 hr before ingestion to 8.1 ± 1.5 hr after one month of ingestion, and by 8.9% to 8.0 ± 1.5 hr after two months of ingestion. The average t-PA value increased significantly ($p < 0.05$) from 5.4 ± 2.6 ng/mL before ingestion to 5.8 ± 2.8 ng/mL after one month and 6.4 ± 2.2 ng/mL after two months of ingestion, and this increase was 31.0% on average. The average FDP decreased significantly ($p < 0.05$) after one month of ingestion but almost returned to the original value after two months of ingestion. No significant changes were detected in the other parameters.

No significant changes were detected in the results of peripheral blood or blood chemistry for three months of ingestion. No adverse event was reported (Table 3).

In Trial 1, some subjects reported improved headache and shoulder stiffness. The changes in the subjective symptoms of headache, shoulder stiffness and dizziness were thus examined in Trial 2 (Table 4). Before ingestion, 8 subjects reported headache and one of the 8 subjects showed severe headache. Similarly, 15 subjects reported shoulder stiffness before ingestion with 5 presenting severe symptoms. Six subjects reported dizziness before ingestion with no severe cases. Changes in these symptoms were statistically significant only in shoulder stiffness after one and two months of ingestion.

Discussion

In the coagulation/fibrinolysis system, various factors interact with each other to maintain the balance between hemostasis and the prevention of thrombosis. As known by the Virchow's triad, changes in blood flow, blood components, and vessel walls will result in the failure of the equilibrium state described above, leading to local thrombogenicity.[10, 11] A long-term sitting position, a long-term recumbent position in bed at a hospital, a surgical operation, diabetes, obesity, hyperlipidemia, varicose veins of lower extremities, and use of contraceptives and female hormones are now known to cause deep-vein thrombosis, which leads to pulmonary thromboembolism.[12-16] While pulmonary thromboembolism can cause sudden death, it lacks specific clinical conditions and is often diagnosed only after necropsy. Preventive treatments include anticoagulation therapy to prevent deep-vein thrombosis, intermittent pneumatic compression to lower limbs, and gradual stockings with elastic material. However, there is still a need for safer, easier-handling and more effective preventive measures.

Natto extract has been reported to affect the blood fibrinolysis system in humans. Volunteers who took oral nattokinase for eight consecutive days showed a slight increase in fibrinolytic activity. Results suggest that this is attributable to enhanced endogenous fibrinolytic activity.[5-6] However, because natto contains high concentrations of vitamin K and living natto bacilli, and natto bacilli surviving in the intestines produce additional vitamin K, interactions with potassium warfarin and other drugs are a concern.[17] NKCP was extracted and purified from the culture of natto bacillus by a special method, and is a fragment of Bacillopeptidase F, which contains almost no vitamin K.[9] Because NKCP was found to have the ability to hydrolyze a synthetic chromogenic substrate of plasmin in a basic study, NKCP, like plasmin, appears to be a substrate-specific serine protease. Detailed enzymatic activity is being studied.

The present clinical study examined the changes in blood coagulation/fibrinolysis when NKCP was administered at 250 mg/day. The average ELT value decreased 10.1% in Trial 1, and decreased 8.5% one month after ingestion and 8.9% two months after ingestion in Trial 2. Euglobulin is thought to contain fibrinogen, plasminogen, and plasminogen activator in blood but contain almost no anti-plasmin substances. ELT is a method for determining fibrinolysis, and shortening of ELT proves enhancement of fibrinolytic activity if there is little change in fibrinogen, as in this case. Results of the study showed that there was a significant increase in fibrinolytic activity after two weeks of dosing with NKCP. We supposed that because the subjects included in the study were not suspected of having thrombosis and were lacking in fibrin, an increase in fibrinolytic activity was not accompanied with increases in FDP or D-dimmer.

It is unclear whether the NKCP's fibrinolytic activity is dependent on t-PA or plasmin because there were differences in t-PA changes between Trials 1 and 2. These clinical phenomena require further studying.

Changes in the subjective symptoms showed that while more than half the subjects had shoulder stiffness before ingestion, there were statistically significant improvements in the entire distribution range of symptoms after two months of ingestion. Effectiveness for shoulder stiffness may be due to improved local circulation in muscles and surrounding tissues.[18)] While an increase in blood flow associated with the enhancement of fibrinolytic activity is suspected to be the reason, there is a need for further in vivo studies on the relation between the changes in blood flow or viscosity and subjective symptoms.

Conclusion

The food "NKCP," which contains a protein produced by Bacillus subtilis natto, is a tablet-type food from which the peculiar odor, bacterial bodies and vitamin K were removed. Adult subjects taking NKCP showed a significant increase in fibrinolytic activity in both subacute and chronic studies. An improvement in shoulder stiffness was observed in the chronic study, suggesting an improvement in local circulation. While further studies are required to clarify NKCP's effect on the enhancement of fibrinolytic activity, its potential in preventing thrombosis was suggested.

Table 1 Change in fibrinolytic and thrombic parameters at week 2 compared to baseline in volunteers treated with NKCP(Trial 1)

Parameter	Group	Normal range	n	Baseline	Week 2	P value[1)]
ELT[2)]	Total	6 - 12 hrs.	28	9.8 ± 2.0	8.4 ± 1.7	0.0089**
	Male		11	9.9 ± 2.5	8.7 ± 2.1	0.1792
	Female		17	9.7 ± 1.7	8.2 ± 1.3	0.0288*
t-PA[3)]	Total	≦ 10 ng/mL	28	7.4 ± 2.6	7.4 ± 3.3	0.8120
	Male		11	8.9 ± 3.1	9.1 ± 4.3	0.6884
	Female		17	6.4 ± 1.7	6.3 ± 1.9	0.9049
APTT	Total	25 - 40 sec	28	31.6±2.7	31.4±2.6	0.4287
	Male		11	32.7 ± 2.8	32.0 ± 2.6	0.2520
	Female		17	30.9 ± 2.5	30.9 ± 2.6	0.9647
D-dimmer[4)]	Total	< 10 μg/mL	28	0.63 ± 0.31	0.62 ± 0.24	0.8413
	Male		11	0.56 ± 0.18	0.59 ± 0.15	0.5333
	Female		17	0.67 ± 0.38	0.65 ± 0.29	0.5263
Fbn	Total	150 – 400	28	281 ± 66	272 ± 72	0.3481
	Male		11	253 ± 53	262 ± 96	0.5998
	Female	mg/dL	17	298 ± 69	278 ± 53	0.0481*
FDP[5)]	Total	≦ 4 μg/mL	28	2.3 ± 0.4	2.1 ± 0.4	0.1845
	Male		11	2.3 ± 0.5	2.2 ± 0.4	0.3409
	Female		17	2.2 ± 0.4	2.1 ± 0.3	0.3322
Ht	Total		28	40.0 ± 3.8	39.2 ± 3.9	0.0685
	Male	39 – 52 %	11	42.0 ± 4.4	41.9 ± 3.4	0.9140
	Female	35 – 48 %	17	38.8 ± 2.9	37.4 ± 3.1	0.0040**

Values are represented as mean ± SD.

1) By paired t-test, * $P < 0.05$, ** $P < 0.01$.

2) Upper limit of measurement(ULM) is 12 hrs .

3) Lower limit of measurement(LLM) is 1.5 ng/mL .

4) LLM is 0.50 μg/mL.

5) LLM is 2 μg/mL.

Table 2 Change in fibrinolytic and thrombic parameters compared to baseline over time in volunteers treated with NKCP(Trial 2)

Parameter	Group	Normal range	n	Baseline	Month 1	Month 2
ELT[2)]	Total	6 - 12 hrs.	23	9.0±1.3	8.1±1.5**	8.0±1.5**
	Male		14	9.2±1.6	8.2±1.6**	8.4±1.3**
	Female		9	8.6±0.7	8.0±1.6	7.4±1.6*
t-PA[3)]	Total	≦ 10 ng/mL	23	5.4±2.6	5.8±2.8	6.4±2.2*
	Male		14	6.2±2.2	6.8±2.8	7.4±2.0*
	Female		9	4.2±2.7	4.3±2.3	4.4±1.2
APTT	Total	25 - 40 sec.	23	29.9±2.9	30.4±3.5	29.5±3.8
	Male		14	30.2±3.4	30.8±4.1	30.0±4.2
	Female		9	29.4±2.1	29.8±2.2	28.5±2.6
D-dimmer[4)]	Total	< 10 μg/mL	23	0.59±0.28	0.56±0.21	0.57±0.24
	Male		14	0.50±0.00	0.52±0.08	0.52±0.05
	Female		9	0.72±0.42	0.62±0.33	0.68±0.40
Fbn	Total	150 – 400	23	249±33	251±42	239±32
	Male		14	250±30	253±47	237±32
	Female	mg/dL	9	247±39	248±34	242±33
FDP[5)]	Total	≦ 4 μg/mL	23	3.0±0.7	2.0±0.6*	3.0±0.7
	Male		14	2.0±0.6	2.0±0.6	2.0±0.7
	Female		9	3.0±0.8	2.0±0.4**	3.0±0.6
Ht	Total		23	39.9±3.8	40.3±3.9	40.5±4.1
	Male	39 – 52 %	14	42.2±2.9	42.5±3.2	42.7±3.1
	Female	35 – 48 %	9	36.3±1.6	36.7±1.5	36.0±1.3

Values are represented as mean ± SD.

1) By Duncan's multiple test, * $P < 0.05$, ** $P < 0.01$.

2) Upper limit of measurement(ULM) is 12 hrs .

3) Lower limit of measurement(LLM) is 1.5 ng/mL .

4) LLM is 0.50 μg/mL.

5) LLM is 2 μg/mL.

Table 3 Change in other parameters compared to baseline over time in volunteers treated with NKCP(Trial 2)

Parameter	Group	Normal range	n	Baseline	Month 1	Month 2	Month 3
RBC	Total		23	443 ± 48	446 ± 48	451 ± 51	451 ± 53
	Male	440 – 560 $\times 10^4/\mu L$	14	469±40	472±43	476±42	483±41
	Female	380 – 500 $\times 10^4/\mu L$	9	401±20	406±17	402±19	404±24
Hb	Total		23	14.2 ± 1.4	14.2 ± 1.4	14.3 ± 1.5	14.4 ± 1.6
	Male	14 – 18 g/dL	14	15.2±0.9	15.1±1.0	15.2±0.9	15.5±1.1
	Female	12 – 16 g/dL	9	12.7±0.5	12.7±0.5	12.6±0.6	12.8±0.6
Ht	Total		23	39.9±3.8	40.3±3.9	40.5±4.1	40.6 ± 4.5
	Male	39 – 52 %	14	42.2±2.9	42.5±3.2	42.7±3.1	43.5±3.3
	Female	35 – 48 %	9	36.3±1.6	36.7±1.5	36.0±1.3	36.4±1.8
Plt	Total	10 – 40 $\times 10^4/\mu L$	23	20.1 ± 6.5	20.9 ± 5.7	21.0 ± 6.4	21.4 ± 5.8
	Male		14	18.4±6.6	19.6±5.9	19.3±6.0	20.0±5.6
	Female		9	22.7±5.6	22.9±5.0	24.3±6.1	23.4±5.7
WBC	Total	39 – 98 $\times 10^2/\mu L$	23	60.7 ± 14.2	58.5 ± 11.6	56.7 ± 10.0	60.5 ± 14.3
	Male		14	61.0±17.3	59.6±13.3	56.2±11.3	59.1±16.4
	Female		9	60.3± 8.1	56.8± 8.7	57.6± 7.5	62.4±11.2
BS	Total	70 – 110 mg/dL	23	112 ± 37	121 ± 67	111 ± 38	114 ± 34
	Male		14	103±39	127±83	109±39	120±41
	Female		9	126±32	112±34	115±36	106±20
HbA1c	Total	5.0 – 8.0 %	23	4.8 ± 0.8	4.9 ± 0.9	4.8 ± 0.9	4.9 ± 0.8
	Male		14	4.9±1.0	5.0±1.1	4.9±1.1	5.0±1.0
	Female		9	4.6±0.3	4.6±0.4	4.7±0.4	4.7±0.3
TG	Total	50 -149 mg/dL	23	203 ± 189	253 ± 206	196 ± 183	176 ± 119
	Male		14	260±221	275±220	239±210	204±128
	Female		9	115±61	218±189	111±55	135±96
TC	Total	150 – 219 mg/dL	23	203 ± 35	206 ± 36	198 ± 30	207 ± 35
	Male		14	197±29	202±32	199±30	205±31
	Female		9	212±44	213±42	198±32	210±43
HDL-C	Total		23	59 ± 18	59 ± 16	57 ± 16	61 ± 17
	Male	41 - 86 mg/dL	14	50±12	50±12	49±11	52±12
	Female	41 - 96 mg/dL	9	74±15	73±11	73±15	73±16
LDL-C	Total	70 – 139 mg/dL	23	114 ± 31	109 ± 28	106 ± 24	112 ± 26
	Male		14	111±29	109±29	107±24	114±23
	Female		9	117±36	110±29	102±25	110±30

Values are represented as mean ± SD.

Table 4 Change in conditions compared to baseline over time in volunteers treated with NKCP(Trial 2)

Symptoms	Condition	Baseline	Month 1	Month 2
Headache	Severe	1	1	1
	Intermediate	7	3	4
	No significant(partially improved)	15	16	16
	Remarkable improved	—	3	2
	Shirley-Williams multiple test	—	Not significant	Not significant
Stiffness of shoulder	Severe	5	1	1
	Intermediate	10	9	10
	No significant(partially improved)	8	9	11
	Remarkable improved	—	4	1
	Shirley-Williams multiple test	—	$P < 0.05$	$P < 0.05$
Struggle	Severe	0	0	0
	Intermediate	6	4	4
	No significant(partially improved)	17	18	18
	Remarkable improved	—	1	1
	Shirley-Williams multiple test	—	Not significant	Not significant

Data are numbers of the patients.

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