

The Antithrombotic and Fibrinolytic Effect of Natto in Hypercholesterolemia Rats

– Research Note –

Kum-Ju Park[†], Jung Il Kang, Tae-Seok Kim, and Ik-Hyun Yeo

The Food and Culture Institute, Pulmuone Holdings, Seoul 120-600, Korea

Abstract

Antithrombotic and fibrinolytic activity of natto was evaluated on platelet aggregation *in vitro* and *in vivo*. Natto showed inhibitory effects on platelet aggregation induced by adenosine 5'diphosphate (ADP) and collagen. Orally administered natto also showed fibrinolytic activity in hypercholesterolemia rats. Normal levels of natto, when administered for four weeks, shortened euglobulin clot lysis time (ECLT) and prolonged partial thromboplastin time (PATT) significantly compared to non-treated group. In addition, the natto treatment decreased total cholesterol in serum. These results showed that intake of normal levels of natto can elicit antithrombotic and fibrinolytic effects, suggesting its consumption may improve blood circulation.

Key words: natto, antithrombotic effect, fibrinolytic effect, hypercholesterolemia rats.

INTRODUCTION

Natto is a traditional Japanese food made from fermented soy and is well known as a healthy and nutritious meal or snack. More recently, natto has been recognized in Korea as a healthy food choice. Natto is produced through the fermentation of soy with *Bacillus subtilis natto*. During the fermentation process, the extracellular enzymes from the bacteria act on the soybeans to produce mucilage, which contains nattokinase that has been purified by Japanese scientists (1-3). Due to the ability of nattokinase to lyse fibrinogen in the blood, it has been promoted in the alternative medicine community as a clot-buster and blood thinner (4). In addition, nattokinase has been suggested as a substitute for typical thrombolytic agents such as urokinase or streptokinase. However, since the purification of nattokinase requires additional processes, a lack of economical merit exists compared to typical thrombolytic agents. Therefore, nattokinase is not readily accessible to the general population.

On the other hand, many other peptides and amino acids are also produced during the natto fermentation process. As reported in previous literature, scientists have identified several components of natto that mimic nattokinase effects (5,6). Therefore, if sufficient medicinal effects can be obtained in the acceptable consumption range of natto, then natto can be consumed by itself. To clarify this hypothesis, we examined both the antithrombotic and antiplatelet effects of natto in hypercholesterolemia rats given normal dietary levels of natto powder.

MATERIALS AND METHODS

Materials

Natto was purchased from a local market (Seoul, Korea) and was manufactured in Korea. Natto was manufactured using the same procedure method which fermented steamed soybean with *bacillus subtilis natto* (1,2). To make a proper feeding type, natto was lyophilized and ground into a powder. Adenosine 5'-diphosphate (ADP) and collagen were purchased from the Chrono-log Corporation (Chicago, IL, USA) and aspirin from the Kun-wha Pharmaceutical Company (Seoul, Korea). All chemicals used and purchased were of analytical grade, and solubilized in distilled water or saline.

Animals

The animals used in this study were four to six weeks old Sprague-Dawley male rats purchased from the Koatech animal company (Pyungtack, Korea). The animals were acclimated under controlled conditions (temperature, $22 \pm 2^\circ\text{C}$; relative humidity, $50 \pm 5\%$; light/dark cycle, 12 hr/12 hr) and had free access to a commercial pellet diet and drinking water one week prior to the initiation of the experiment. Experiments performed complied with the rulings of the Animal Protection Act and approved by the Ethical Committee of the Chemon Co., Ltd.

Preparation of rat platelets

Blood was harvested through an abdominal aortic puncture from normal six weeks old rats. Blood samples were mixed with a 3.8% (w/v) sodium citrate solution (9:1) and centrifuged at $260 \times g$, 20°C for 10 minutes

[†]Corresponding author. E-mail: kjparka@pulmuone.co.kr
Phone: +82-2-3277-8558, Fax: +82-2-6499-0128

to obtain platelet-rich plasma (PRP). The platelet count was determined using a cell counter (ADVIA 2120 SIMENS, München, Germany). All *in vitro* aggregation studies were carried out with PRP having platelet counts between 2.5 and 3.0×10^8 /mL of plasma. All experiments were performed within two hours of PRP preparation.

Measurement of platelet aggregation

Aggregation was monitored using a dual channel Lumu Aggregometer (Model 400 Chronolog Corporation, Chicago, IL, USA) with 0.4 mL aliquots of PRP (7). The final volume was brought to 0.5 mL with the test drug dissolved in normal saline or the appropriate vehicle known to be devoid of any effects on aggregation. Platelet aggregation was induced with ADP and collagen agonists. The concentration dependent anti-aggregatory effect was studied by pretreatment of PRP with the test materials for one minute followed by addition of the agonists. The resulting aggregation was recorded for eight minutes by the change in light transmission as a function of time. To investigate the impact of natto on the resulting aggregation, a natto-extracted powder was prepared. In brief, natto was dissolved into distilled water for 12 hr at 4°C. Next, the supernatant was separated and purified through filtration using filter paper (Whatman No. 2) and the supernatant was lyophilized (Il-shin Lab Co. Ltd., Yangju, Korea)

Induction of hypercholesterolemia

Animals with an average body weight of 80~100 g were induced to a hypercholesterolemia state by being fed a high-cholesterol diet purchased from G-Bio Co., Ltd. (Gwacheon, Korea) for four weeks (8,9). As shown in Table 1, the high-cholesterol diet consisted of AIN-76, 1% cholesterol, and 0.5% cholic acid. Hypercholesterolemia was confirmed by measuring the levels of serum cholesterol after four weeks.

Experimental design

The hypercholesterolemia rats were divided into five

Table 1. Ingredient composition of the high cholesterol diet (AIN 76 modified)

Ingredient	g/kg
Casein	200
DL-Methionine	3
Cornstarch	150
Sucrose	485
Cellulose	50
Corn oil	50
Salt mix #200000	35
Vitamin mix #300050	10
Choline bitartrate	2
Cholesterol	1
Colic acid	0.5

experimental groups containing 10 rats per group. Group 1 (G1): normal rats treated with vehicle only, G2: hypercholesterolemia rats treated with vehicle only, G3: hypercholesterolemia rats treated with 750 mg natto powder/head/day for four weeks, G4: hypercholesterolemia rats treated with 1,500 mg natto powder/head/day for four weeks, G5: hypercholesterolemia rats treated with aspirin (100 mg/kg/day) for four weeks. The vehicle or natto powder was administered to the rats using a gastric force-feeding needle. Body weight and dietary intake were measured each week throughout the study. Serum lipid levels and antithrombotic effects were measured every two weeks and the ECLT was measured on the day of sacrifice. During the experiment period we observed the general symptoms. After sacrifice, we anatomized and then confirmed the disorders.

In vitro anticoagulation assay

Measurement of the plasma activated partial thromboplastin time (APTT), and prothrombin time (PT) were performed using an Automated Coagulation Laboratory 100 Instrument and ACL-assay reagent kit (Instrumentation Laboratory, Milano, Italy).

Measurement of euglobulin clot lysis time (ECLT)

ECLT was measured using the modified Smith method (10). After separating out the serum, a 0.35 mL aliquot of serum was added to 6.3 mL of 0.017% acetic acid and incubated at 4°C for 20 minutes. Next, the euglobulin fraction was obtained by centrifugation (4°, 3,000 × g, 10 minutes). The resulting euglobulin fraction was mixed with 55 µL of re-suspension buffer and distributed to a 96 well plate. After 50 µL of a 25 mM CaCl₂ solution was added to the wells, the change in absorption at 405 nm was recorded every five minutes for 12 hr.

Statistical analysis

All of the data are expressed as the mean ± standard error of mean (n=number of experiments). Analysis of variance (ANOVA) was performed using SPSS software (SPSS Inc., Chicago, IL, USA). A Student's t-test or parametric multiple comparison procedure was used to determine significant differences between the groups, and p<0.05 was considered as statistically significant.

RESULTS

Effect of natto on total cholesterol in hypercholesterolemia rat serum

Throughout the study, the body weight of the rats in each group increased; however, there were no significant differences in measured body weights between normal and hypercholesterolemia rats (Table 2). In addition, the

Table 2. Effect of four-week treatment with natto powder on the body weight of hypercholesterolemia rats (Unit: g)

Group	Body weight				
	Week 0	Week 1	Week 2	Week 3	Week 4
G1	302.90 ± 7.50	334.88 ± 9.57	353.87 ± 10.28	367.73 ± 10.48	386.75 ± 10.84
G2	303.08 ± 10.21	337.54 ± 12.54	356.91 ± 14.12	371.24 ± 15.41	387.46 ± 15.62
G3	307.26 ± 9.41	339.64 ± 13.27	359.03 ± 14.81	374.42 ± 16.36	391.62 ± 17.71
G4	303.13 ± 9.31	336.11 ± 10.05	355.18 ± 10.66	369.76 ± 13.35	388.27 ± 14.28
G5	298.26 ± 6.84	332.86 ± 7.10	350.12 ± 8.47	366.41 ± 8.17	386.30 ± 7.78

Values are expressed as mean ± SD (n=10). Normal diet group with vehicle (G1) and four high cholesterol diet groups: high cholesterol diet with vehicle (G2), high cholesterol diet with 750, 1,500 mg/head/day natto powder (G3, G4) and high cholesterol diet with 100 mg/kg/day aspirin.

dietary intake or observable differences of the high cholesterol fed group were not significantly higher or comparable to the normal group seen (data not shown). However, serum levels of total cholesterol allotted between the normal and hypercholesterolemia rats after four weeks with the high cholesterol diet were significantly different ($p < 0.01$) (Table 3). In contrast, there were no differences observed among the hypercholesterolemia groups (G2~G5). After four weeks of treatment, the differences in total cholesterol between G2 (non treatment group) and G4 (high dose treatment group) rats were significant ($p < 0.01$).

Evaluation of platelet aggregation

The natto extracted powder was tested for its potential antiplatelet activity in normal rat platelet-rich plasma against ADP and collagen induced platelet aggregation (Fig. 1). The observed inhibitory effect of the natto powder on ADP and collagen induced platelet aggregation were 53.85% and 53.03% at 2 mg/mL concentrations, respectively, demonstrating that natto has the potential to effectively prevent platelet aggregation *in vivo*.

Evaluation of antithrombotic effects

The PT and APTT were measured every two weeks for one month (Table 4). In the case of PT, no differences between the G1 (normal) and G2 (hypercholesterolemia) rats were observed during the experimental period. However, the PT of the natto powder treatment groups

(G3, G4) was prolonged this period of time. The PT of G3 and G4 increased from 14.78 ± 0.37 to 17.79 ± 0.20 and 15.16 ± 0.51 to 17.56 ± 0.32 , respectively, throughout the four weeks. These differences were significant compared to non-treated hypercholesterolemia rats ($p < 0.01$). The APTT was not altered as a result of treatment with the natto powder and aspirin in this study, although the only difference observed was between G1 and G2~G5 rats. Natto powder treatment shortened the ECLT in hypercholesterolemia rats (G4) significantly from 0 to 4.33 mins compared to G2 (untreated hypercholesterolemia rats) ($p < 0.01$ or $p < 0.05$) (Fig. 2).

DISCUSSION

Cardiovascular disease is one of the most common and serious chronic diseases among the Korean population, increasing in incidence with age. According to statistical reports conducted by the Korean government, the ratio of Korea's elderly population, over 65 years old, has risen to 10.9% in 2010, with an estimated increase to 15.7% in 2020 (Statistics Korea 2010). Therefore, therapeutic strategies to prevent the incidence of cardiovascular disease are essential. The most basic level of prevention for cardiovascular disease is dietary, taking in foods that have a fibrinolytic function. Some foods are well established in containing fibrinolytic enzymes,

Table 3. Serum biochemical values in high cholesterol diet induced hypercholesterolemia rats (Unit: mg/dL)

Group	Total cholesterol			Triglyceride
	Week 0	Week 2	Week 4	Week 4
G1	104.30 ± 11.01**	97.80 ± 7.71**	94.20 ± 8.63**	73.30 ± 7.79
G2	359.40 ± 45.02###	280.30 ± 37.49###	280.90 ± 32.16###	61.40 ± 9.88
G3	357.80 ± 42.28###	222.30 ± 28.99###	224.50 ± 26.89###	58.70 ± 7.34
G4	355.80 ± 39.08###	216.90 ± 20.98###	181.00 ± 14.85###	70.30 ± 11.81
G5	357.60 ± 39.32###	294.40 ± 53.88###	255.30 ± 37.29###	61.40 ± 7.00

Data are expressed as mean ± SE. The results were statistically analyzed by Student's t-test.

significantly different from G1, $p < 0.01$, ** significantly different from G2, $p < 0.01$.

G1: Normal control (distilled water, n=10), G2: Vehicle control (distilled water, n=10), G3: Test article (natto dried powder 750 mg/head/day, n=10), G4: Test article (natto dried powder 1,500 mg/head/day, n=10), G5: Positive control (aspirin 100 mg/kg/day, n=10).

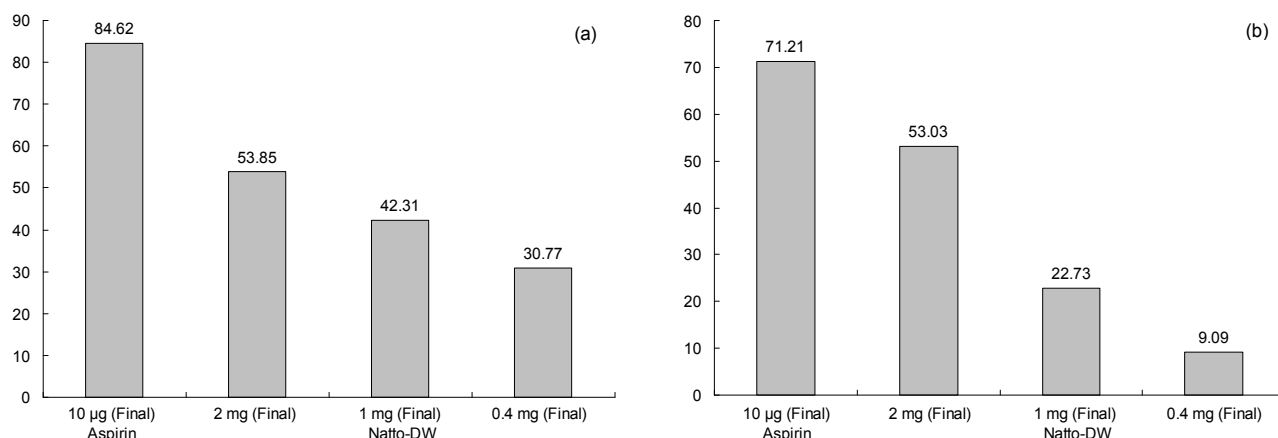


Fig. 1. The antiplatelet effect of natto in normal rat platelet-rich plasma against ADP and collagen induced platelet aggregation. The inhibitory effects of natto water extract on adenosine 5'-diphosphate (ADP) and collagen induced normal rat platelet aggregation. (a) ADP induced aggregation, (b) collagen induced aggregation.

Table 4. PT and APTT levels in high fat diet induced hypercholesterolemia rats (Unit: sec)

Group	PT			APTT		
	Week 0	Week 2	Week 4	Week 0	Week 2	Week 4
G1	15.46 ± 0.76	15.89 ± 0.57	16.51 ± 0.37	16.39 ± 1.09	17.63 ± 0.68	18.42 ± 0.25*
G2	14.94 ± 0.46	15.59 ± 0.34	16.01 ± 0.37	14.65 ± 0.83	17.18 ± 0.54	17.35 ± 0.49#
G3	14.78 ± 0.37	16.88 ± 0.31***	17.79 ± 0.20***	14.12 ± 0.86	16.03 ± 0.87	17.10 ± 0.42#
G4	15.16 ± 0.51	16.84 ± 0.41***	17.56 ± 0.32***	14.91 ± 0.60	16.25 ± 0.58	16.97 ± 0.55#
G5	15.63 ± 0.46	16.79 ± 0.46***	16.83 ± 0.31*	14.92 ± 0.66	17.70 ± 0.36	16.95 ± 0.64#

Data are expressed as mean ± SE. The results were statistically analyzed by Student's t-test.

#significantly different from G1, p<0.05, ##significantly different from G1, p<0.01.

*significantly different from G2, p<0.05, **significantly different from G2, p<0.01.

G1: Normal control (distilled water, n=10), G2: Vehicle control (distilled water, n=10), G3: Test article (natto dried powder 750 mg/head/day, n=10), G4: Test article (natto dried powder 1,500 mg/head/day, n=10), G5: Positive control (aspirin 100 mg/kg/day, n=10).

such as natto and *chongkukjang*, leading scientists to purify the therapeutic activity (11). However, these materials have not been translated to the clinic due to the expense and decreased effects in humans as compared to animal models. Therefore, further research is needed to investigate the impact of the potential for therapeutic activity with dietary intake. Natto is one example and representative of foods which possess a fibrinolytic effect. In addition, natto can be consumed raw without processing or heating, which may impact the efficacy of the fibrinolytic effect.

To ascertain whether one can obtain health benefits through an ordinary amount of natto, hyperlipidemic rats were fed with natto powder as much as normal dietary amounts, which were based on the total body surface area (BSA). The common amount of natto within products is about 50 g/pack. According to the BSA calculation (12), a human consuming 50 g natto per day is comparable to 3.58 g/day in a mouse model. The 3.58 g of natto is converted to about 1.5 g of dry natto after lyophilization. In a previous study, Kim et al. also investigated the hypolipidemia effects of spice added natto supplementation in hypercholesterolemia rats (13). How-

ever, this study was limited to show the effects of natto sufficiently since the fed amounts were too high compared to ordinary amounts. Therefore, we investigated

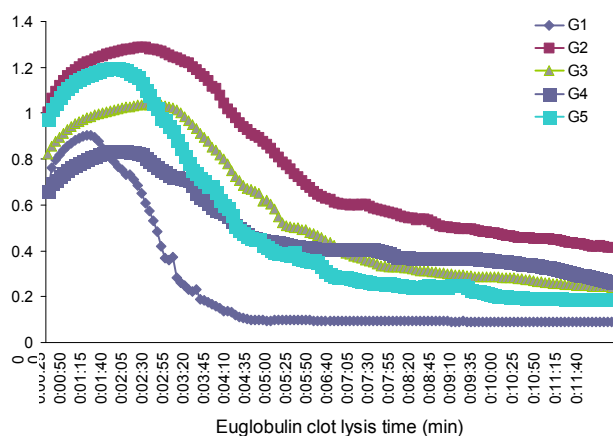


Fig. 2. Euglobulin clot lysis time of high cholesterol diet induced hypercholesterolemia rats. Data are expressed as mean. The results were statistically analyzed by Student's t-test. G1: Normal control (distilled water, n=10), G2: Vehicle control (distilled water, n=10), G3: Test article (natto dried powder 750 mg/head/day, n=10), G4: Test article (natto dried powder 1,500 mg/head/day, n=10), G5: Positive control (aspirin 100 mg/kg/day, n=10).

the antithrombotic and antiplatelet effects of natto in hypercholesterolemia rats by feeding ordinary amounts for four weeks. Our results demonstrate that the dietary natto increased the PT and ECLT significantly and inhibited coagulation of platelets.

Sumi et al. have reported that orally fed nattokinase caused a two-fold increase in the amounts of tissue type plasminogen activator (t-PA) and fibrin degradation products in the plasma (2). Therefore, the antithrombotic and fibrinolytic effects of natto powder in this study must contain not only the nattokinase effect but also other compounds in natto (4). This study confirms that the normal dietary intake of natto also offers health benefits to potentially prevent cardiovascular disease. Therefore, we conclude that dietary natto has potential therapeutic benefits.

REFERENCES

1. Sumi H, Hamada H, Tsushima K, Mihara H. 1987. A novel fibrinolytic enzyme (nattokinase) in the vegetable cheese natto: a typical and popular soybean food Japanese diet. *Experientia* 43: 1110-1111.
2. Sumi H, Hamada H, Nakanishi K, Hiratani H. 1990. Enhancement of the fibrinolytic activity in plasma by oral administration of nattokinase. *Acta Haematol* 84: 139-143
3. Fujita M, Nomura K, Hong K, Ito Y, Asada A, Nishimuro S. 1993. Purification and characterization of a strong fibrinolytic enzyme (nattokinase) in the vegetable cheese natto, a popular soybean fermented food in Japan. *Biochem Biophys Res Commun* 197: 1340-1343.
4. Noh KH, Park CM, Jang JH, Shin JH, Cho MK, Kim JO, Song YS. 2009. Effects of nattokinase fibrinol supplementation on fibrinolysis and atherogenesis. *J Life Science* 19: 289-208.
5. Omura K, Kaketani K, Maeda H, Hitosugi M. 2004. Fibrinolytic and anti-thrombotic effect of the protein from *Bacillus subtilis* (natto) by the oral administration. *J Jpn Soc Biorheol* 18: 44-51.
6. Omura K, Hitosugi M, Zhu X, Ikeda M, Maeda H, Tokudome S. 2005. A newly derived protein from *Bacillus subtilis* natto with both antithrombotic and fibrinolytic effects. *J Pharmacol Sci* 99: 247-251.
7. Shah BH, Siddiqui A, Qureshi KA, Khan M, Rafi S, Ujan VA, Yaqub Y, Rasheed H, Saeed SA. 1999. Co-activation of Gi and Gq proteins exerts synergistic effect on human platelet aggregation through activation of phospholipase C and Ca²⁺ signaling. *Exp Mol Med* 31: 42-46.
8. Lee CK, Shin JS, Kim BS, Cho IH, Kim YS, Lee EB. 2007. Antithrombotic effect by oral administration of novel proteinase fraction from earthworm *Eisenia andrei* on venous thrombosis model in rats. *Arch Pharm Res* 30: 475-480.
9. Jang HS, Rhee SJ, Woo MH, Cho SH. 2007. Anti-thrombogenic and anti-inflammatory effects of solvent fractions from leaves of *Zanthoxylum schinifolium* (Sancho Namu) in rats fed high fat diet. *Korean J Nutr* 40: 606-615.
10. Smith AA, Jacobson LJ, Miller BI, Hathaway WE, Manco-Johnson MJ. 2003. A new euglobulin clot lysis assay for global fibrinolysis. *Thromb Res* 112: 329-337.
11. Yan JL, Kim HS, Hong JH, Song YS. 2006. Purification and characteristics of fibrinolytic enzymes from *Chongkukjang*. *J Food Sci Nutr* 11: 127-132.
12. Shannon RS, Minakshi N, Nihal A. 2007. Dose translation from animal to human studies revisited. *FASEB J* 22: 659-661.
13. Kim BN, Kim JD, Ham SS, Choi YS, Lee SS. 1995. Effect of spice added natto supplementation on the lipid metabolism in rats. *J Korean Soc Food Nutr* 24: 121-126.

(Received December 7, 2011; Accepted February 27, 2012)