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Original Article

Effects of Natto Extract on Endothelial Injury in a Rat Model

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Vascular endothelial damage has been found to be associated with thrombus formation, which is considered to be a risk factor for cardiovascular disease. A diet of natto leads to a low prevalence of cardiovascular disease. The aim of the present study was to investigate the effects of natto extract on vascular endothelia damage with exposure to laser irradiation. Endothelial damage both *in vitro* and *in vivo* was induced by irradiation of rose bengal using a DPSS green laser. Cell viability was determined by MTS assay, and the intimal thickening was verified by a histological approach. The antioxidant content of natto extract was determined for the free radical scavenging activity. Endothelial cells were injured in the presence of rose bengal irradiated in a dose-dependent manner. Natto extract exhibits high levels of antioxidant activity compared with purified natto kinase. Apoptosis of laser-injured endothelial cells was significantly reduced in the presence of natto extract. Both the natto extract and natto kinase suppressed intimal thickening in rats with endothelial injury. The present findings suggest that natto extract suppresses vessel thickening as a synergic effect attributed to its antioxidant and anti-apoptosis properties.

Key words: laser irradiation, natto extract, thrombolysis, thrombosis

Atherosclerosis has been considered to be a risk factor for a variety of cardiovascular diseases (CVD). Such chronic injury of blood vessels can lead to thrombotic embolism [1–5]. A great deal of effort has been expended in preventing atherosclerosis and thrombotic diseases [6, 7]. Commonly used thrombolytic agents include alteplase (t-PA or Activase), urokinase (Abbokinase), and streptokinase [8–12].

Antithrombotic agents usually activate plasminogen, which is then converted to plasmin to dissolve fibrin clots. Some thrombolytic agents are specific for fibrin without activating systemic fibrinogen, whereas non-selective ones activate systemic fibrinogen and fibrin-fibrinogen as a result of their low efficacy [13–15].

Natto extracts produced from natto, a traditional fermented soy food, are known to consist of nattokinase, which has strong fibrinolytic activity [16–20]. The *in vivo* anti-coagulant activity of nattokinase enhances blood circulation by dissolving fibrin and

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soluble fibrin monomers [21–23]. Fibrin and soluble fibrin monomers can cause hypertension and other cardiovascular diseases. Nattokinase is also thrombolytic and can activate nearly 3,000 enzymes in the body to increase the metabolic rate [24]. It directly catalyzes fibrin to fibrin degradation products and enhances urokinase activity, increasing plasminogen conversion to plasmin to enhance thrombolysis. After being absorbed into the blood from the intestinal tract, nattokinase can directly dissolve thrombus and activate pro-urokinase. Nattokinase can be taken orally and is far more stable than urokinase. Nattokinase is comparable to anticoagulants, acting as a vascular scavenger to reduce the occurrence of thrombosis [24–30]. In the present study, we determined the antioxidant capacity of natto extract and nattokinase as well as their ability to inhibit thrombus both *in vitro* and *in vivo*.

Materials and Methods

Bacterial culture. *Bacillus subtilis natto* BCRC 14714 (kindly provided by Prof. Kuo-Chen Tsai, National Taiwan Ocean University, Taiwan) was cultured in tryptic soy broth (TSB) solution and incubated for 24 h at 37℃. Bacteria at the log phase were stored in 50% glycerol at −80℃.

Preparation of natto extract. Soybeans were immersed in deionized water for 16 h and subsequently inoculated with an overnight culture of *Bacillus subtilis natto* BCRC 14714 at a 10 : 1 ratio, followed by incubation for 48 h at 37℃. After incubation, ferment was added with deionized water and incubated for 2 h at 37℃. The suspension was filtered, and the water-soluble extract was lyophilized to make a powder for storage at −80℃.

Measurement of thrombolytic activity. The thrombolytic activity of natto extract and nattokinase was determined by the fibrin plate method, as previously described [31]. The plates were punctured with holes, and nattokinase and natto extract were added to each hole. After incubation for 24 h at 37℃, the dissolved thrombus became transparent. The size of the clear zone (mm) was measured to indicate the levels of thrombolytic activity.

Measurement of nattokinase activity. Nattokinase activity was determined as described by the Bio Science Laboratory Co., Tokyo, Japan, on the basis of the conversion of fibrinogen to fibrin with thrombin. The optical density of tyrosine was determined by UV/VIS spectrophotometer (O.D. 275 nm), and an increase in optical density of 0.01/minute was used as one enzyme unit (U). The calculated formula for fibrinolytic enzyme activity (FU) is

$$(\mathrm{FU/mL}) = [(\mathrm{Ar} - \mathrm{Ac})/(0.01 \times 60 \times 0.1)] \times \mathrm{D}$$

where Ar is the absorbance for the experimental group at 275 nm, Ac is the absorbance for the control group at 275 nm, 60 is the time in minutes in the water bath at 37℃, 0.1 represents the testing liquid (mL), and D is the dilution factor.

Antioxidant activity assay. The antioxidant activity of natto extract and nattokinase was determined based on the scavenging capacity of α, α-diphenyl-β-picryhydrazyl (DPPH) free radicals. A 100-μl sample and 100 μl of a 0.1 mM DPPH (stock 1 mM)-methanol solution were mixed evenly and kept still for 30 min. A spectrophotometer was used to determine the absorbance at 517 nm. One hundred microliters of methanol was used in the control group. A lower value of absorbance corresponds to a stronger scavenging capacity of DPPH free radicals. The scavenging effect (SE) was calculated as

SE = [1 − (absorbance of sample at 517 nm/absorbance of the blank group at 517 nm)] × 100

A positive result was obtained if the molecular weight (MW) of ascorbic acid was observed (176.12). The MW of DPPH is 394.32.

The superoxide anion scavenging capacity was determined as previously described [32, 33]. After incubation, the mixture was measured for absorbance at 560 nm. A lower absorbance value indicated a stronger superoxide anion scavenging capacity. SE was calculated as

SE = [1 − (absorbance of sample at 562 nm/absorbance of the blank group at 562 nm)] × 100.

Ferrous ion chelation was performed as previously reported [34]. Absorbance at 562 nm was recorded. A lower absorbance value corresponded to a higher ferrous ion chelating capacity (CC) according to the following equation:

CC = [1 − (absorbance of sample at 562 nm/absorbance of the blank group at 562 nm)] × 100

Results were considered positive if the MW of EDTA (292.24) was found.

In vitro laser irradiation of endothelial cells. Mouse endothelial cells CRL-2181 were cultured in DMEM

supplemented with 10% FBS in a 48-well plate (5×10^4 cells/0.5 ml/well) at 37℃ in a humidified atmosphere of 5% CO_2. The laser source was a DPSS green laser (GE Healthcare, USA), wavelength 532 nm, 4–10 mW. Prior to irradiation, the culture media for monolayer endothelial cells was refreshed. The endothelial monolayer was covered with rose bengal dye (0.125 mg/ml), irradiated at EC 6 for 6 min, and incubated at 37℃ for 2 days. The cells were then counted.

Cell viability assay. Endothelial cells were treated with dye and irradiated by laser. Tetrazolium [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium] (MTS) was added in a ratio of 5 : 1 (culture media: MTS). After incubation at 37℃ in the dark for 1 h, absorbance was read at 490 nm. After the absorbance values of the experimental group and the control group were compared, cell viability was calculated.

Real-time PCR. A QIAGEN RNeasy Mini kit was used to extract total RNA according to the manufacturer's instructions. The quality and quantity of RNA were determined with absorbance at 260 nm and 280 nm using a spectrophotometer. Consecutive dilution was used to test the previously synthesized cDNA to find the most suitable concentration for a real-time polymerase chain reaction (PCR). One hundred-fold dilution was the optimal concentration. After the 100-fold dilution, 5 μl cDNA was added to 12.5 μl iQ™ SYBR® Green Supermix, along with 0.5 μl of primer (10 μM IL-6-F for IL-6-R; 10 μM TNF-α-F for TNF-α-R; 10 μM IL-1β-F for IL-1β-R; 10 μM GAPDH-F for GAPDH-R; 10 μM MCP-F for MCP-R; and 10 μM ICAM-1-F for ICAM-1-R) and sterilized Q-H_2O to equal a total volume of 25 μl. The reaction mixture was placed in the PCR reaction chamber (I Cycler iQ® Multicolor Real-Time PCR Detection System, BIO-RAD) at the most suitable annealing temperature for each primer, and real-time PCR (RT-PCR) was performed to analyze the gene expression of inflammatory factors and adhesion molecules. The calculated formulas were as follows:

Target gene (Ct) − internal control (Ct) = ΔCt

Test gene ΔCt − control ΔCt = ΔΔCt

Gene expression = 2 − (ΔΔCt)

Inhibitory effect on hyperplasia of carotid arteries in rats. Wistar-Kyoto (WKY) rats aged 10–13 weeks were purchased from the National Laboratory Animal Center (Taipei, Taiwan). The rats were raised in the animal room in our laboratory (temperature: 24 ± 1℃, humidity: 50 ± 10%) under a 12–14 h light cycle with free access to food and water. The laser used was a DPSS green laser, wavelength 532 nm, 4–10 mW. Induction of thrombosis was carried out as follows. Rats were anaesthetized by Zoletil 50 (Virbac S.A, Paris, France) and fixed on a surgical plate. The maxillary incisors and limbs were fixed with hemp and the skin near the femur incised. The femoral vein was carefully exposed, and a catheter was inserted into the left femoral vein. Next, the neck skin was incised and the junction of the internal and external carotid arteries was identified. A DPSS Green Laser was used to irradiate the middle of the carotid arteries for 10 min, and rose Bengal dye (60 mg/kg/c.c) was injected into the femoral vein to induce thrombosis. After irradiation, the femoral and neck skins were sutured and the rats were returned to the animal room. After surgery, rats were fed with 400 mg/ml natto extract or 40 mg/ml nattokinase daily. After 28 d, the rats were sacrificed and 0.5-mm sections of the carotid arteries were removed and snap-frozen at −80℃. After 24 h, 25 carotid artery sections 20 m thick were prepared with a microtome. H&E staining was used to dye Sections 1, 5, 10, 15, 20, and 25, and the vessel thickness was analyzed using Image-pro plus 6.0 software. The thickness (mm) of the coronal vessel sections is expressed as the mean ± SEM. Immunofluorescent staining was used to observe endothelial hyperplasia in vessel walls after surgery. Carotid artery samples were snap-frozen for 24 h as previously described. Next, 25 tissue sections 20 m thick were prepared with a microtome. Tissue sections 4, 8, 13, 18, and 23 were treated with methanol for 5 min, then washed with PBS three times and blocked with 1% BSA/PBS for 30 min at room temperature. The primary antibodies, including anti-SMC actin and anti-vWF, were added at 37℃ for 2 h, and the secondary antibody was added at room temperature for 1 h. Finally, the sections were sealed with fluoro-gel.

Statistical analysis. All study results are expressed as the mean ± SEM. The student's t-test was used for analysis. All statistical analyses were conducted using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA), and statistical significance was set at $p < 0.05$.

Results

Thrombolytic activities. The fibrin plate method was utilized to determine the thrombolytic activity of natto extract. Crude natto extract had a relatively poor ability to prevent fibrin formation. The fibrinolytic enzyme (FE) activity of lyophilized natto extract was 31 ± 0.7 U/mL using purified nattokinase as a reference (Fig. 1).

Antioxidant activities. The antioxidant capacity of natto extract was evaluated on the basis of its scavenging activity in comparison with purified nattokinase. Regarding free radical scavenging, natto extract and nattokinase were found to exert 30% and 20%, respectively of the scavenging ability of potent scavengers of free radicals such as ascorbic acid. In scavenging the superoxide anion, the natto extract was quite good (> 50%) but nattokinase was poor (17.4%). Interestingly, compared to EDTA as a reference, natto extract had a 60% chelating effect on ferrous ions, whereas nattokinase showed no activity (Table 1).

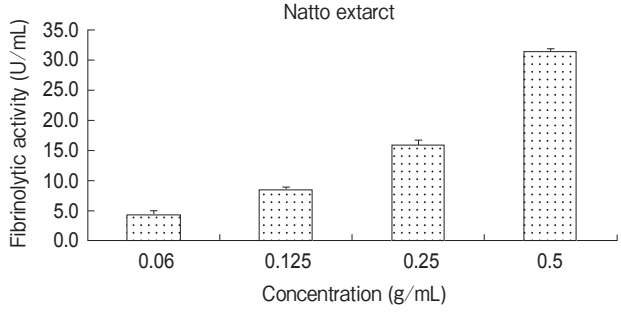

Fig. 1 Determination of the fibrinolytic activity of natto extracts. Purified nattokinase with activity of 3,000 U/mg was used as a reference.

Table 1 Effects of natto extracts and nattokinase to scavenge DPPH free radicals and superoxide anion radicals, and to chelate ferrous iron

	DPPH (%)	O^{2-} (%)	Fe^{2+} (%)
nattokinase (0.06 mg/ml)	20 ± 3.1	17.4 ± 6.7	0
natto-extracts (0.5 mg/ml)	30.1 ± 2.5	54.9 ± 2.2	60.2 ± 0.8

*Ascorbic acid (0.5 mM): 82.4 ± 0.2%, Gallic acid (5 mM): 36.7 ± 4%, EDTA (1 mM): 68.6 ± 0.01%.

Effects of natto extract on the viability of endothelial cells. Prior to the experiment, the cytotoxicity of rose bengal dye was evaluated using the MTS assay to optimize the best conditions for the subsequent experiments. Rose bengal dye showed toxicity to endothelial cells at concentrations exceeding 0.125 mg/ml. Nevertheless, the viability of endothelial cells was significantly reduced by laser irradiation in the presence of rose bengal (Fig. 2A). In Fig. 2B, it can be seen that the decrease in cell viability was significant at 6, 8, and 10 min with rates of 50%, 62%, and 82%, respectively. Nattokinase exhibited toxicity to endothelial cells at concentrations > 0.06 mg/ml (Fig. 2C). Natto extract and nattokinase were added separately to treat and prevent thrombus in endothelial cells, and cell viability was measured. As shown in Fig. 3, purified nattokinase did not significantly affect the viability of photochemically injured endothelial cells. In the treatment group, the viability of injured endothelial cells was increased up to 80%.

The reponses of endothelial cells to treatment with rose bengal dye and DPSS laser irradiation with or without nattokinase and natto extract were investigated. Expressions of inflammatory cytokines such as interleukin-6 (IL-6), IL-1β and TNF-α, intercellular adhesion molecule-1 (ICAM-1), and MCP were determined. Regarding inflammatory cytokines, the levels of IL-6, IL-1β, and TNF-α were significantly decreased in the nattokinase and natto extract groups compared to those of the controls ($p < 0.05$) (Fig. 4).

Anti-hyperplasia effect of natto extract. The anti-hyperplasia effect was determined using a rat model in which hyperplasia of carotid arteries was induced by a combination of dye and laser irradiation. As shown in Fig. 5, hyperplasia of the carotid arteries was reduced in the rats fed with nattokinase and natto extract compared with the control group. The difference in the range of hyperplasia of carotid arteries between control and treated groups was significant ($p < 0.05$) (Fig. 6).

Discussion

Sumi *et al.* have used natto and natto extract to study artificial thrombus (fibrin) and have found that thrombus is dissolved by the addition of natto, which is indicative of thrombolytic activity. We therefore used a fibrin plate test to compare thrombus inhibition

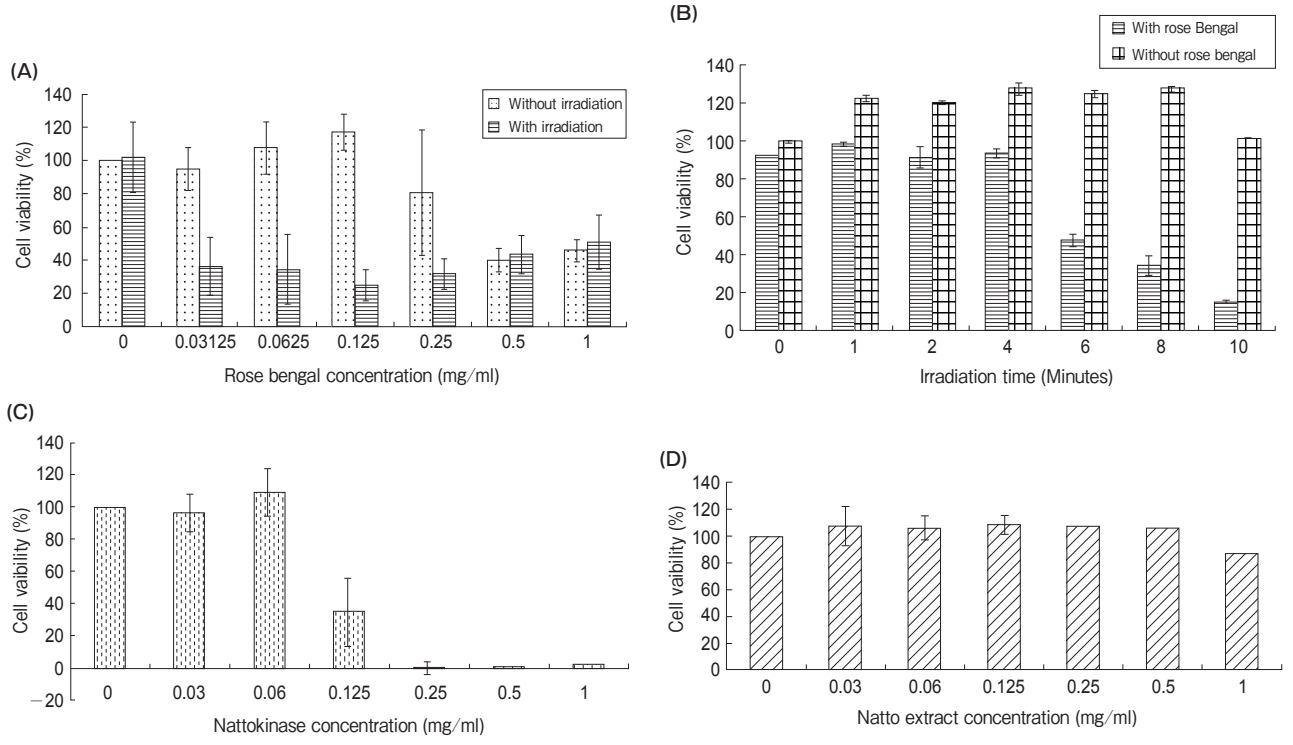

Fig. 2 Dose-dependency on (**A**) rose bengal dye incorporating green laser irradiation, (**B**) exposure time of laser irradiation with 10^{-1}mg/ml rose bengal dye, (**C**) nattokinase and (**D**) natto extracts. Endothelial cells were cultured in a 48-well plate overnight, then treated with different concentrations of dye and laser-incubated at 37℃ for 48h. Cell viability was detected by MTS assay. $^{*}p<0.05$ was considered statistically significant compared with dye and laser stimulation.

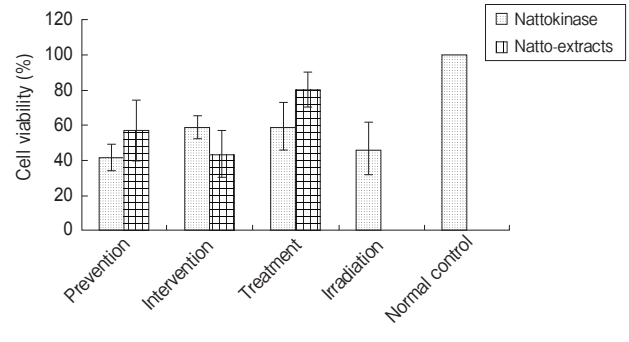

Fig. 3 Effects of nattokinase and natto extracts on the viability of photochemically injured endothelial cells. Endothelial cells were cultured in a 48-well plate overnight and exposed to red Bengal incorporating laser irradiation. Cells were treated with nattokinase and natto extract at concentrations of 0.06mg/mL and 0.5mg/mL, respectively, for both prevention and treatment. Cell viability was determined after 24h of incubation at 37℃ by MTS assay.

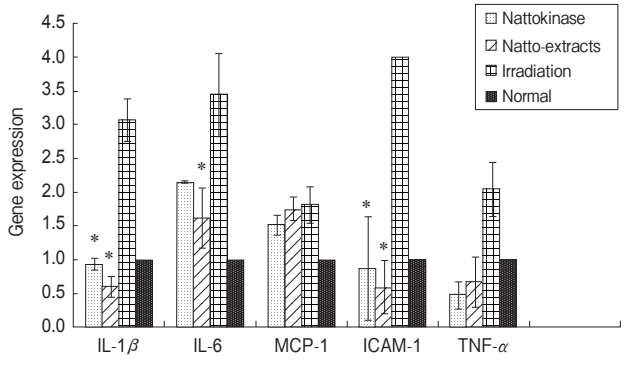

Fig. 4 Effects of nattokinase and natto extracts on the expression of pro-inflammatory cytokines in endothelial cells treated with rose bengal dye and laser irradiation. $^{*}p < 0.05$ indicates a significant difference compared with cells alone as determined by the Student's t-test.

by nattokinase or natto extract incubated at different temperatures but stirred for the same amount of time. The results showed larger clear zones for thrombus inhibition by natto extract and freeze-dried natto extract powder (stirring for 2h, at 27℃) than those previously reported [35].

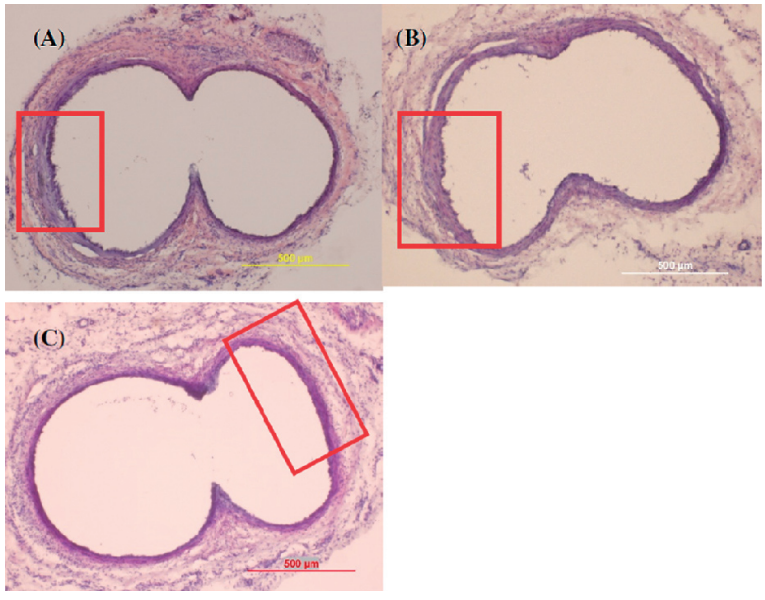

Fig. 5 Green laser irradiation for 10 min and rose bengal dye at 60 mg/kg. Rat artery after photochemically induced endothelial thickening of 28 days was observed under light photomicrographs by HE staining. (**A**) control, (**B**) nattokinase (40 mg), and (**C**) natto extracts (400 mg).

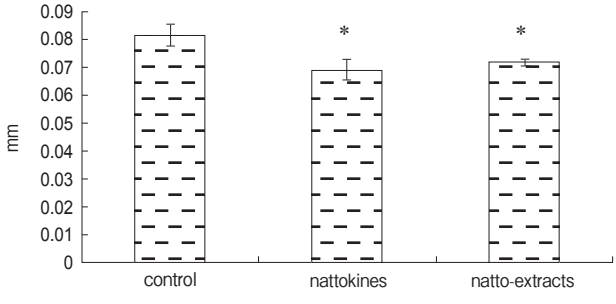

Fig. 6 Green laser irradiation for 10 min and rose bengal dye at 60 mg/kg, feed nattokinase and natto-extracts rats artery after photochemically induced endothelial thickening of 28 days was observed under light photomicrographs. $^{*}p < 0.05$ was considered statistically significant compared with control. N = 6

Chiang *et al.* (2006) have shown the antioxidant capacity of isoflavone and Vitamin E in natto, and we therefore sought to determine the antioxidant capacity of nattokinase and natto extract. Kao (2004) has measured the free radical scavenging capacity of natto extract at < 50%, finding that the superoxide anion scavenging capacity of natto solution increases with solution concentration. The chelating capacity was reported to be 11–14 μg. The present study showed the poor free radical scavenging of nattokinase and natto extract, with the results indicating that natto itself is not a potent free radical scavenger. The superoxide anion scavenging capacity of natto extract may be > 50%, but that of nattokinase is low. Nattokinase had no chelating effect on ferrous ions, but natto extract showed a 60% chelating effect. Nattokinase as a purified protein may have a relatively low antioxidant effect.

The induced model previously described [36] was used to study apoptosis in endothelial cells after stimulation with 3 mM rose Bengal dye and 514 nm green light. Therefore, we also established a cell model for studying nattokinase and natto extract. The results showed that light is needed to trigger free radicals induced by dye, and that free radicals significantly reduce the survival rate of endothelial cells, resulting in apoptosis. Moreover, a longer irradiation time resulted in more dead cells. Cell viability assays showed that the survival rates of cells were indeed significantly increased in the treatment group (the natto extract group), demonstrating the healing ability of natto extract.

We also explored the genetic changes in endothelial cells. It has been found that IL-1β, IL-6, and TNF-α are the major cytokines involved in inflammation [37]. These 3 cytokines increase vascular permeability, causing inflammatory reactions such as

redness, swelling, fever, and pain. They continuously stimulate and activate macrophages to secrete more pro-inflammatory cytokines. Research has shown that TNF-α is the regulatory factor first induced, followed by IL-1β and IL-6, and then adhesion molecules, in infected vessels [38–40]. The adhesion molecules then stimulate endothelial cells to produce platelet-activating factor, resulting in blood coagulation and local vessel occlusion. We compared the group treated with nattokinase and natto extract with the control group. The results showed that free radicals (caused by rose Bengal dye and DPSS laser) damaged endothelial cells, stimulating them to produce platelet-activating factor, resulting in local vessel occlusion and significant inflammatory reactions. However, nattokinase and natto extract reduced these inflammatory reactions.

We demonstrated that ICAM-1 expression in photochemically injured endothelial cells is increased and reduced in the presence of nattokinase and natto extract. These results suggest that natto extract has the potential to reduce the adhesion of monocytes to endothelia. When vessel walls are injured, the endothelial permeability increases and large macromolecules easily pass through and are deposited around the vessel walls. Meanwhile, chemokines such as MCP-1 secreted by vessel walls will make monocytes adhere to the vessel walls, moving to the space under the endothelial cells and transforming into macrophages through activation and differentiation. The results regarding chemokines in the present study indicate that nattokinase and natto extract do not significantly affect MCP-1 expression.

In the present study, the hyperplasia model of the carotid artery in rats was established by dye and laser stimulation. Significant carotid artery angiogenesis in rats was seen 7, 21, and 28 days after dye and laser stimulation. The area of angiogenesis was 1.21 ± 0.16 ($\times 0.01\,mm^2$) in rats fed with high-fat food, but it was significantly decreased to 0.69 ± 0.10 ($\times 0.01\,mm^2$) in those fed with natto extract. Moreover, H&E staining showed obvious angiogenesis in the control group that was decreased in the nattokinase and natto extract groups. The vessel width in the control group was $0.081 \pm 0.003\,mm$, but was $0.068 \pm 0.003\,mm$ and $0.071 \pm 0.001\,mm$ in the nattokinase and natto extract groups, respectively. Azuma *et al.* have studied the expression of α-actin and von Willebrand factor in the muscular layer of blood vessels in rats using immunofluorescence [41]. Tissue sections from rats fed with nattokinase and natto extract showed significantly reduced hypertrophy of the vascular smooth muscles and repair of damaged cells and nuclei, indicating that nattokinase and natto extract inhibit hyperplasia of the carotid artery.

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