〈Original Article〉

Nattoesse inhibits the high-fat diet-induced increase in serum lipid levels in ApoE-deficient mice

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Summary Nattoesse™ is a product extracted from *Bacillus subtilis* culture medium, and is a component of natto, a traditional probiotic food in Japan. We aimed to test whether Nattoesse could improve blood lipid concentrations in ApoE-deficient mice fed a high-fat diet. Male C57BL/6J mice in the normal control group (NC) were fed standard laboratory rodent chow. C57BL/6.KOR/StmSlc-Apoe^{shl} mice were fed a high-fat diet, and were divided into two groups, the Nattoesse group (HFN) that received intragastric administration of 10 μg/g body weight Nattoesse, and high-fat control (HFC) group that received administration of sterilized distilled water. Drug administration was performed every other day for 48 days. Serum samples were collected and lipid levels, liver function, and serum sugar levels were assessed. Administration of Nattoesse significantly reduced serum lipids, improved liver function, and decreased serum sugar levels in hyperlipidemic mice. Therefore, Nattoesse improved lipid metabolism, liver function, and blood sugar concentrations in hyperlipidemic mice.

Key words: *Bacillus subtilis*, hyperlipidemia, leptin, liver function, oxidized low-density lipoprotein cholesterol

1. Introduction

Soybeans are a major crop worldwide, used globally for oil production and feed grain, and locally in Japan for processing into a variety of traditional fermented foods, such as natto and miso. Natto is produced from boiled soybeans that are fermented by addition of the bacterium *Bacillus subtilis*. Natto has been consumed as a traditional

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probiotic food in Japan for approximately 1000 years. The use of dietary supplements based on natto aid in relieving subjective symptoms of patients with essential hypertension, type 2 diabetes, and hyperlipidemia receiving medical care[1].

The ingredients of traditional fermented foods have been studied to determine their applications as probiotics in preventive medicine. Probiotic bacteria exert beneficial effects on human health through the suppression of harmful microorganisms and immunomodulatory activity. Evidence of the effects of orally administered probiotics has accumulated from preclinical and clinical studies. For example, oral administration of *Aspergillus oryzae* and *Lactobacillus sakei* modulates certain immune parameters resulting in decreased levels of allergy-specific IgE[2]. Other probiotic bacteria have been shown to have cholesterol-lowering effects mediated through interactions with bile acids[3]. Moreover, the administration of probiotics to infants alters the composition of their intestinal microbiota and reduces the risk of necrotizing enterocolitis[4].

Nattoesse™, which is marketed as a probiotic dietary supplement, is an extract obtained from *Bacillus subtilis* culture broth that contains nattokinase, isoflavone, saponin, and other active ingredients, including antioxidants[5]. Thus, Nattoesse contains many ingredients of natto[6]. In our previous study, Nattoesse was found to decrease lipid concentrations in human serum[7].

Japanese wild mice (KOR) with disruption of the *Apo E* gene, known as apolipoprotein E (ApoE)-deficient mice, have been used for the development of animal models for hyperlipidemia, arteriosclerosis, and skin xanthoma[8]. These mice have also been used as a disease model for investigating the prevention and treatment of hyperlipidemia[9], identifying the mechanisms underlying arteriosclerosis[10], and innovative studies for the development of medications and supplements[11]. The aim of this study was to examine the effects of intragastrically administered Nattoesse on blood lipid concentrations in ApoE-deficient mice fed a high-fat diet as a model of hyperlipidemia. Moreover, we determined whether Nattoesse treatment in ApoE-deficient mice fed a high-fat diet alleviated the symptoms of hyperlipidemia.

2. Materials and methods

2.1. Microorganisms

Nattoesse was provided by the OTO Corporation (Odawara, Japan); its quality was the same as that of commercially available products.

2.2. Mice

Five-week-old male C57BL/6J mice and ApoE-deficient mice (C57BL/6.KOR/StmSlc-Apoe^{shl}), which develop hyperlipidemia, were purchased from SLC (Hamamatsu, Japan). All of the mice were bred at our institution under specific pathogen-free conditions and the experiments were initiated at 6 weeks of age. The mice were maintained on a 12:12 hour light:dark cycle in an environmentally controlled animal chamber. All procedures were conducted in accordance with the Guidelines for Animal Experiments at Kitasato University and approved by the Ethical Committee at Kitasato University.

2.3. Intragastric administration of Nattoesse to mice fed a high-fat diet

C57BL/6J mice and ApoE-deficient mice were fed standard laboratory rodent chow (CRF-1; Oriental Yeast, Tokyo, Japan) and allowed to acclimate for 1 week. Male C57BL/6J mice (n = 6 per group) as the normal control (NC) group without hyperlipidemia was fed standard laboratory rodent chow. ApoE-deficient mice fed a high-fat diet (high-fat diet 32; CLEA Japan, Inc., Tokyo, Japan) were divided into two groups (high-fat control [HFC] and high-fat diet with Nattoesse treatment [HFN]; n = 6 per group) and were distributed such that the average weight of the mice in each group did not differ. Intragastric administration of 10 μg/g body weight Nattoesse dissolved in sterilized distilled water was performed in ApoE-deficient mice fed a high-fat diet beginning on day 0, with administration every other day until day 48. The amount of Nattoesse administered was determined based on the measurement of body weight once a week. All animals were fasted

for 24 h from day 49 and anesthetized with isoflurane on day 50. Blood was then collected from the heart. Finally, all mice were sacrificed using isoflurane. Blood samples were centrifuged at 17,360 × *g* at 4°C for 10 min. The supernatants were then collected as serum samples and stored at -80 °C until analysis. The experiments were performed with six mice per group.

2.4. Body weight measurement

To observe the effects of Nattoesse administration on weight gain, the body weight of each animal was measured once per week until day 50.

2.5. Serum measurements of liver function

To examine the effects of Nattoesse on liver failure caused by hyperlipidemia in mice, liver function was measured by assessing the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LD), leucine aminopeptidase (LAP), γ-glutamyl transpeptidase (γGT), cholinesterase (ChE), total protein (TP), albumin (ALB), total bilirubin (T-Bil), direct bilirubin (D-Bil), indirect-bilirubin (I-Bil), and total bile acid (TBA). All measurements were performed by Oriental Yeast (Tokyo, Japan). The ALT, AST, ALP, LD, and γ-GT levels were measured using methods described by the Japan Society of Clinical Chemistry. The LAP level was measured using methods described by the German Society of Clinical Chemistry. The ChE level was measured by the enzyme method, and the TP level was measured by the Biuret method. The ALB level was measured using the bromocresol green method, and T-Bil, D-Bil, and I-Bil levels were measured by the enzyme method. The TBA level was measured by the enzyme cycling method. These measurements were performed using a Hitachi 7180 type autoanalyzer (Hitachi High-technologies Corporation, Tokyo, Japan).

2.6. Measurement of lipid levels in serum

To analyze the effects of Nattoesse on serum lipid levels in mice, the serum levels of total cholesterol (T-CHO), triglycerides (TGs), free cholesterol (F-CHO), cholesteryl esters (E-CHO), low-density lipoprotein (LDL-C), and high-density lipoprotein (HDL-C) were determined. These measurements were performed by Oriental Yeast. The T-CHO, TG, and F-CHO levels were measured by the enzyme method. The E-CHO level was determined by subtracting the F-CHO level from the T-CHO level. The LDL-C and HDL-C levels were measured by direct methods. These measurements were performed using a Hitachi 7180 type autoanalyzer. The serum leptin level (Shibayagi, Gunma, Japan) and oxidized LDL (oxLDL; USCN Life Science Inc., Wuhan, China) were determined using commercially available enzyme-linked immunosorbent assay kits in accordance with the manufacturer's instructions.

2.7. Measurement of blood glucose (BS) levels

The effects of Nattoesse on glucose levels in mouse serum were determined by measurement of the BS level in the serum by Oriental Yeast. The BS level was measured using the hexokinase-glucose-6-phosphate dehydrogenase method with a Hitachi 7180 type autoanalyzer.

2.8. Statistical analysis

All data are shown as the mean ± standard deviation (SD). Multiple comparisons between groups were made using Tukey-Kramer HDS tests in the JMP software (version 11.2.0). $p < 0.05$ was considered significant.

3. Results

3.1. Changes in body weight

The average body weight of animals in the HFC group on day 50 was significantly higher than that in the NC group ($p < 0.01$). Co-administration of Nattoesse reduced the body weight on day 50 as compared to the HFC group ($p < 0.01$).

3.2. Liver function in mice consuming a high-fat diet

To determine whether the liver function in ApoE-deficient mice decreased, we compared the liver function measurements between the HFC and

NC groups. The serum levels of ALT, AST, ALP, LD, LAP, ChE, TP, ALB, T-Bil, and D-Bil in animals in the HFC group were significantly higher than those in the NC group ($p < 0.01$). Additionally, repeated administration of Nattoesse significantly reduced the levels of serum LD ($p < 0.05$), ALT, LAP, ChE, and ALB ($p < 0.01$) compared with those in animals in the HFC group. Importantly, the levels of LD, T-Bil ($p < 0.05$), ALT, AST, LAP, ChE, TP, and D-Bil ($p < 0.01$) also differed significantly between animals in the HFN and NC groups (Table 1).

Table 1. Body weight, serum lipid levels, liver function, and sugar levels in mice fed a high-fat or standard diet, and mice treated with or without Nattoesse.

Measurement	Group data			Statistical analysis		
	NC	HFN	HFC	HFC vs. NC	HFN vs. HFC	HFN vs. NC
Body weight (g)	27.96 ±2.03	37.3 ± 2.60	42.52 ± 1.86	$p < 0.01$	$p < 0.01$	$p < 0.01$
ALT (IU/L)	23.3 ± 1.6	174.2 ± 35.2	314.0 ± 113.6	$p < 0.01$	$p < 0.01$	$p < 0.01$
AST (IU/L)	100.5 ± 47.6	493.5 ± 203.8	618.5 ± 216.8	$p < 0.01$	NS	$p < 0.01$
ALP (IU/L)	315.5 ± 30.3	411.7 ± 115.4	516.2 ± 62.0	$p < 0.01$	NS	NS
LD (IU/L)	830.7 ± 71.0	1471.3 ± 300.5	2174.3 ± 616.4	$p < 0.01$	$p < 0.05$	$p < 0.05$
LAP (IU/L)	41.5 ± 2.1	74.7 ± 7.8	108.0 ± 15.0	$p < 0.01$	$p < 0.01$	$p < 0.01$
r-GT (IU/L)	ND	ND	ND	-	-	-
ChE (IU/L)	30.2 ± 1.8	56 ± 4.6	64.7 ± 3.5	$p < 0.01$	$p < 0.01$	$p < 0.01$
TP (g/dL)	5.2 ± 0.1	6.2 ± 0.6	6.7 ± 0.4	$p < 0.01$	NS	$p < 0.01$
ALB (g/dL)	3.5 ± 0.1	3.4 ± 0.2	3.8 ± 0.1	$p < 0.01$	$p < 0.01$	NS
T-Bil (mg/dL)	0.05 ± 0.02	1.14 ± 0.03	0.11 ± 0.05	$p < 0.01$	NS	$p < 0.05$
D-Bil (mg/dL)	0 ± 0.01	0.04 ± 0.02	0.05 ± 0.02	$p < 0.01$	NS	$p < 0.01$
I-Bil (mg/dL)	0.05 ± 0.01	0.09 ± 0.04	0.06 ± 0.04	NS	NS	NS
TBA (μmol/L)	6.2 ± 4.8	29.7 ± 43.1	40.7 ± 88.9	NS	NS	NS
BS (mg/dL)	128.7 ± 21.47	164.7 ± 23.27	221.7 ± 49.29	$p < 0.01$	$p <0.05$	NS
T-CHO (mg/dL)	71.8 ± 4.79	554.5 ± 131.6	770.7 ± 223.5	$p < 0.01$	NS	$p < 0.01$
TG (mg/dL)	18.7 ± 7.92	100.2 ± 24.9	137.8 ± 17.6	$p < 0.01$	$p < 0.01$	$p < 0.01$
F-CHO (mg/dL)	12.3 ± 1.37	144.7 ± 24.7	204.2 ± 57.9	$p < 0.01$	$p < 0.05$	$p < 0.01$
E-CHO (mg/dL)	59.5 ± 3.89	409.8 ± 107.8	566.5 ± 167.6	$p < 0.01$	NS	$p < 0.01$
E-CHO/T-CHO (%)	82.8 ± 1.47	73.3 ± 2.9	73.3 ± 2.3	$p < 0.01$	NS	$p < 0.01$
LDL-C (mg/dL)	5.8 ± 0.75	117.7 ± 23.6	167 ± 43.3	$p < 0.01$	$p < 0.05$	$p < 0.01$
HDL-C (mg/dL)	48.5 ± 3.56	22.7 ± 7.8	28.0 ± 7.5	$p < 0.01$	NS	$p < 0.01$
Leptin (pg/mL)	ND	277.8 ± 139.7	433.3 ± 90.6	$p < 0.01$	$p < 0.05$	$p < 0.01$
oxLDL (pg/mL)	49.6 ± 6.5	71.3 ± 13.8	128.1 ± 19.5	$p < 0.01$	$p < 0.01$	$p < 0.05$

C57BL/6J mice fed laboratory rodent chow served as the normal control group (NC). C57BL/6.KOR/StmSlc-Apoe^{shl} mice fed a high-fat diet were the high-fat control (HFC), and those supplemented with Nattoesse (HFN) received intragastric administration of 10 μg/mg body weight Nattoesse. Nattoesse was administered every other day until Day 48. After a 24-h fast, the mice were sacrificed on Day 50. Serum levels of leptin and oxidized LDL (oxLDL) were determined by ELISA. The other measurements were performed by Oriental Yeast (Tokyo, Japan). ALT; alanine aminotransferase, AST; aspartate aminotransferase, ALP; alkaline phosphatase, LD; lactate dehydrogenase, LAP; leucine aminopeptidase, γGT; γ-glutamyl transpeptidase, ChE; cholinesterase, TP; total protein, ALB; albumin, T-Bil; total bilirubin, D-Bil; direct bilirubin, I-Bil; indirect-bilirubin, TBA; total bile acid, T-CHO; total cholesterol, TGs; triglycerides, F-CHO; free cholesterol, E-CHO; cholesteryl esters, LDL-C; low-density lipoprotein, HDL-C; high-density lipoprotein. The data are displayed as the mean ± SD (n = 6). ND; not detected, NS: not significant. Differences with $p < 0.05$ were considered significant.

3.3. Serum lipid levels in mice fed a high-fat diet

To determine the effects of Nattoesse on serum lipid levels in ApoE-deficient mice, we compared the levels of lipids among the groups of mice. Serum values of T-CHO, TG, F-CHO, E-CHO, E-CHO/T-CHO, LDL-C, HDL-C, leptin, and oxLDL were significantly higher in the HFC group than in the NC group ($p < 0.01$). Additionally, the HFN group had significantly reduced levels of serum F-CHO, LDL-C, leptin ($p < 0.05$), TG, and oxLDL ($p < 0.01$) as compared to those in animals in the HFC group. Moreover, significant differences were observed for oxLDL ($p < 0.05$), T-CHO, TG, F-CHO, E-CHO, E-CHO/F-CHO, LDL-C, HDL-C, and leptin ($p < 0.01$) between the HFN and NC groups (Table 1).

3.4. Changes in BS levels following administration of Nattoesse in mice fed a high-fat diet

Next, we examined whether blood sugar levels were altered in ApoE-deficient mice by measuring the glucose content of the serum from NC, HFC, and HFN mice. Serum glucose levels in the HFC group were significantly higher than those in the NC group ($p < 0.01$). Moreover, Nattoesse treatment significantly reduced the level of serum glucose ($p < 0.05$) compared with that in the HFC group. However, there were no significant differences between the NC and HFN groups (Table 1).

4. Discussion

In this study, we examined the effects of intragastrically administered Nattoesse on blood lipid concentrations in ApoE-deficient mice fed a high-fat diet as a model of hyperlipidemia. Our results demonstrated that Nattoesse improved lipid metabolism, liver function, and blood sugar levels in hyperlipidemic mice.

In our study, we demonstrated that Nattoesse used as a probiotic ameliorated symptoms of hyperlipidemia in ApoE-deficient mice. Dietary cholesterol in ApoE-deficient mice induces hyperlipidemia through overproduction of cholesterol and impaired plasma clearance secondary to the down-regulation of LDL-C receptor expression in the liver. Thus, blood concentrations of HDL-C, LDL-C, and TGs in ApoE-deficient mice increase relative to those in normal mice[12]. In this study, we fed ApoE-deficient mice a high-fat diet for 48 days to induce hyperlipidemia. As a result, T-CHO, TG, F-CHO, E-CHO, HDL-C, LDL-C, leptin, and oxLDL levels were higher in these mice than in normal mice. Additionally, the body weights of mice fed a high-fat diet were higher than those of mice fed a normal diet. Liver function, assessed by the measurement of serum levels of ALT, AST, LD, LAP, ChE, TP, ALB, T-Bil, and D-Bil, was worse in mice after induction of hyperlipidemia as compared to that in normal mice. Additionally, BS levels in mice increased following consumption of a high-fat diet. Consistent with these findings, intake of high-cholesterol and high-fat diets is associated with liver steatosis[13], and a high-fat diet was shown to decrease insulin secretion and increase BS levels in mice[14].

Nattoesse was intragastrically administered every other day to hyperlipidemic mice, resulting in improved serum lipid levels, body weight, and liver function as compared to those in hyperlipidemic mice without Nattoesse administration. In our previous study, when hyperlipidemic volunteers received tablets containing Nattoesse for 4 weeks, serum lipid concentrations decreased and blood cell hemorheology improved[7]. Hamsters administered *Bacillus* experienced significantly reduced serum and hepatic T-CHO, TG levels, and LDL-C/HDL-C ratios. Therefore, total antioxidant and superoxide dismutase activities increased as compared to those in the high-cholesterol group[15]. Another study provided insights into the protective effects of supplementation with 1-deoxynojirimycin isolated from *B. subtilis* against obesity-induced hepatic lipid abnormalities and mitochondrial dysfunction[16]. Thus, taken together, these findings suggest that Nattoesse controls hyperlipidemia and improves liver function.

From the published external product specification data, soybean saponin was present in Nattoesse at more than 300 mg/100 g (supplemental table 2). In a previous study, isolated soy saponin was shown to prevent dietary hypercholesterolemia in rats[17].

Table 2. Components of Nattoesse.

Analysis item	Specification
Natto-protease Ⅰ activity (Nattokinase)	>20,000 U/g
Natto-protease II activity	>5,000 pU/g
Natto-protease III activity	positive
Heavy metal (Pb)	<20 ppm
Arsenicum	<2 ppm
Vitamin K	0 μg
Soybean saponin	>300 mg/100 g
γ-aminobutyric acid (GABA)	>450 mg/100 g
Soy isoflavone	>50 mg/100 g

Nattoesse standard components were quoted from the OTO Corporation Ltd. published external product specification data. This data of Nattoesse was revised on September 2, 2014.

However, the exact mechanism of inhibition remains unclear. Thus, we assumed that the saponin in Nattoesse might be responsible for the reduction of lipid levels in the blood. Additionally, we observed an improvement in liver function, as indicated by decreased blood lipid concentrations. Accordingly, future studies are needed to identify the components of Nattoesse that have antihyperlipidemic effects and improve liver function.

In our study, blood leptin concentrations decreased following Nattoesse administration, resulting in suppression of the increase in body weight. Leptin has been reported to alleviate diabetes through its glycemic control mechanism[18]. Therefore, based on these previous studies, we hypothesize that Nattoesse suppressed the increases in leptin secretion. As a result, the increases in BS concentrations and obesity were also suppressed.

The oxygen radical scavenging activity of natto and its inhibitory effects on the oxidation of rat plasma LDL-C were previously investigated *in vitro*[19]. We found that blood oxLDL concentrations decreased following Nattoesse administration. The anti-inflammatory properties of certain soy proteins may be a possible mechanism mediating the prevention of atherosclerosis[20]. The reduction of oxLDL blood levels induced by Nattoesse administration could reduce the risk of atherosclerosis; however, further studies are needed to test this hypothesis.

In summary, we have shown that Nattoesse, an extract obtained from *B. subtilis* culture broth, has the ability to decrease serum T-CHO, TG, and LDL-C levels in ApoE-deficient mice fed a high-fat diet, and to inhibit the increase in body weight in these mice. Lipid concentrations in the blood decreased, and liver function was improved. In addition, oxLDL levels were decreased in hyperlipidemic mice. These results demonstrate that oral administration of Nattoesse could reduce serum lipid levels, suggesting that Nattoesse may prevent and/or suppress the development of hyperlipidemia in mice.

Conflicts of interest

Tsuyoshi Sugiyama is an employee of OTO Corporation. The sponsor had no control over the interpretation, writing, or publication of this work. Authors have any other conflicts of interest to declare.

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