Original paper

Improving Effects of Narazuke Lees on Fatty Liver of Rats Induced by High-Fat and High-Cholesterol Diets

Teruko NAKASA^{1*}, Sayuri YAMAGAMI¹, Toshiaki TANAKA², Hiroko TANAKA², Hitoshi HARIU² and Osamu OKINAKA¹

¹Department of Food Science and Nutrition, Faculty of Human Life Science, Doshisha Women's College of Liberal Arts, 602 Genbu-cho, Kamigyo-ku, Kyoto 602-0893, Japan

²Tanakachou Co. Ltd., 160 Doujisha-cho, Simogyo-ku, Kyoto 600-8421, Japan

Received November 18, 2013 ; Accepted April 18, 2014

With the purpose of using Narazuke lees as a functional foods material, its effect on fatty liver of rats fed a high-fat and high-cholesterol diet was investigated. While significant accumulation of triacylglycerol was observed in the liver of rats fed high-fat and high-cholesterol diet was observed, rats fed diet supplemented with Narazuke lees showed significantly increased fecal dry weight, as well as increased fecal excretion of total lipids and total bile acids. Further, free fatty acids, glucose and insulin concentrations in the blood were significantly decreased, and amelioration of insulin resistance was observed. In the liver, reduction of FAS activity, significant increase in CPT-1 α activity and promotion of fatty acid β -oxidation were observed. An improving effect on fatty liver was observed by the suppression of lipid accumulation. The amount of liver TBARS was significantly reduced, indicating the inhibition of NASH progression.

Keywords: Narazuke lees, fatty liver, high-fat and high-cholesterol diet, lipid metabolism

Introduction

Fatty liver refers to a state in which triacylglycerol (TG) occupies more than 5% of wet liver weight, and is classified as either alcoholic or non-alcoholic fatty liver. Non-alcoholic fatty liver disease (NAFLD) (Schaffner and Thaler, 1986) includes simple fatty liver (non-alcoholic fatty liver, NAFL) with low morbidity, whereas non-alcoholic steatohepatitis (NASH) (Ludwig *et al.*, 1980) often develops into cirrhosis and hepatocellular carcinoma. NASH is a lifestyle-related disease, characterized by fatty liver, of individuals who are non-habitual drinkers and is closely related to metabolic syndromes such as obesity, hyperlipidemia and insulin resistance (Vuppalanchi and Chalasani, 2009). At present, approximately 2 million Japanese have fatty liver, and the annual increase in patients with associated pathologies has raised considerable concern in recent years; in particular, increase in the NASH/NAFLD ratio may continue with the surge in obesity (Takaki and Yamamoto, 2010). The principle

medical treatments for fatty liver include diet and exercise therapies; and lifestyle corrections from these treatments will help to improve conditions such as obesity, diabetes, hypertension, and hyperlipidemia. However, medical therapies remain ineffective for many patients, and the condition is difficult to prevent in modern societies where stressful and irregular lifestyles prevail. Therefore, convenient functional food supplements that ameliorate fatty liver could be of significant benefit.

The suppressive effect of lactic fermented soymilk and brewing by-products on liver lipid accumulation has been reported as follows. Hirahata *et al.* (2013) reported that lactic fermented soymilk given to rats fed high-fat and high-cholesterol (Cho) diets improved lipid metabolism and lowered fatty acid synthase (FAS) expression in the liver by the promotion of sterol response element binding factor (SREBP)-2 expression. Further, Mochizuki *et al.* (2005) reported that a significant inhibitory effect was observed with 10% barley *shochu* lees supplementation in rats with orotic

*To whom correspondence should be addressed.

E-mail: tnakasa@dwc.doshisha.ac.jp

acid-induced fatty liver.

Narazuke lees used in this experiment are produced as a residue in large quantities during Narazuke manufacturing. As lees are typically discarded as byproduct, the development of an effective usage for this waste is desired. Narazuke is manufactured with a pepo in the soaking paste as raw material sake lees. This soaking paste is prepared from the plate-like cake of sake lees by the fermentation in large tank under defoaming. First, salted pepo is immersed in the soaking paste. Then, this pepo is replaced in new soaking paste several times. During this procedure, salt in pepo is diluted, and many components of soaking paste are transferred into pepo, and then, the Narazuke is completed. The duration of Narazuke production is between 1.5 to 2 years, and besides the components derived from the raw materials, it is expected that a number of ingredients are produced during fermentation.

Numerous studies have identified physiological effects of sake lees. The improving effects of sake lees on Cho metabolism was demonstrated by Ashida *et al.* (1997) using male Sprague-Dawley (SD) rats and by Mochida *et al.* (2000) using male Wistar rats. Saito *et al.* reported the isolation of six angiotensin I converting enzyme (ACE) inhibitory peptides from sake lees and sake (alcohol beverage) (Saito *et al.*, 1992), in which some peptides exhibited antihypertensive effects on spontaneously hypertensive rats (Saito *et al.*, 1994a; Saito *et al.*, 1994b). Furthermore, Saito *et al.* (1997) also reported the amnesia preventive efficacy of several prolyl endopeptidase inhibitory peptides from sake lees and sake. Izu and others have shown that sake lees suppress D-galactosamine-induced liver injury in mice (Izu *et al.*, 2006). Moreover, the natural killer (NK) cell activating effect of sake lees and sake was reported by Okuda (2003).

Narazuke soaking paste is manufactured by further maturation of sake lees. Consequently, the contents of Narazuke lees, such as dietary fiber and minerals (Ca, Mg, and Mn), are increased compared with those of sake lees, potentially enhancing its physiological benefits. Since Narazuke lees are high in salt, desalting was performed prior to use in experiments. Narazuke lees were suspended in water and filtered, and the residuals were used in experiments. Most of the mineral components were removed by the filtration operation. However, the dietary fiber in the desalted Narazuke lees was almost unchanged in terms of weight, and a significant amount of protein remained; thus, the effects of these components are anticipated. Therefore, Narazuke lees are potential useful medical materials compared to sake lees.

The present study aimed to assess the application of Narazuke lees as a functional food. The effects of Narazuke lees on fatty liver were assessed using male SD rats fed a high-fat and high-Cho diet.

Materials and Methods

Materials Narazuke lees (NL) were obtained from Tanakachou Co., Ltd., and desalted prior to use in experiments. In brief, 5 volumes of water were added to Narazuke lees and stirred

for 1 h. The suspended Narazuke lees was collected by filtration using filter paper (Whatman No. 2), and used as desalted material. The desalted Narazuke lees contained 74.3% moisture (oven drying at 105°C), 9.2% crude protein (Kjeldahl decomposition method, AOAC 984.13, protein convention factor of 6.25), 8.2% dietary fiber (95% insoluble; Enzymatic-Gravimetric method, AOAC 991.43), and 1.8% salt (digital salt meter ES-421).

Experimental diets The diet constituents are shown in Table 1. The control diet (C) contained 20% protein and 5% corn oil. The high-fat and high-Cho diet (HF) contained an additional 1% corn oil, 9% lard, 0.5% Cho, and 0.125% sodium cholate. In addition to the constituents of the HF diet, the NL diet contained 8% desalted Narazuke lees; the protein and salt concentrations were adjusted accordingly. As Narazuke lees contain 74.3% moisture, an addition of 8% was 2.06 g as dry weight. The NaCl-free AIN93G mineral mix was purchased from Oriental Yeast Co., Ltd. Energy per 100 g was 386.3, 408.8, and 383.8 kcal in the C, HF, and NL diets, respectively.

Laboratory animals and feeding conditions Male 4-week-old Slc:SD (SPF) rats (body weight, 70–90 g) were purchased from Shimizu Experimental Materials Co., Ltd. (Kyoto, Japan). After acclimation feeding for 8 days with C diet, rats were divided into three groups of equal mean weights: six rats in C group (143.7 ± 2.0 g), seven in HF group (144.6 ± 2.5 g), and seven in NL group (143.9 ± 1.0 g). Rats were fed for 22 days in individual stainless steel cages in a temperature- ($22^\circ\text{C} \pm 2^\circ\text{C}$) and humidity- ($55\% \pm 10\%$) controlled room with a 12-h light/12-h dark cycle (light period, 8:00–20:00). Food and tap water were provided *ad libitum*, and body and ingested food weights were measured every other day during the feeding period. Rat feces were collected for 48 h from the 19th day of feeding, weighed, freeze-dried, and then stored at -80°C until analyses. After fasting for 8 h, the anesthetic drug somnopenyl was intraperitoneally injected, and anesthetized rats were sacrificed by collecting blood from the abdominal aorta. The liver, perirenal adipose tissue, epididymal adipose tissue, mesenteric adipose tissue, and cecum were immediately removed and weighed. After heparin processing, plasma was collected from blood samples by centrifugal separation at 12,000 rpm for 15 min and stored at -80°C until subsequent analyses. Livers were perfused in 5 mM Tris-HCl buffer (pH 7.2) containing 0.25 M sucrose and 1 mM EDTA-2Na to remove any remaining blood in the capillaries and were then finely chopped. Chopped liver samples were immediately separated into mitochondrial and cytosolic fractions using a centrifugal cell fractionation method, and mitochondrial carnitine palmitoyltransferase-1 α (CPT-1 α), and cytosolic glucose 6-phosphate dehydrogenase (G6PDH) and FAS activities were determined. The remaining livers were stored at -80°C until subsequent analyses. Animal experiments were performed according to the standards of animal feeding, safekeeping, and experimentation of the Doshisha Women's College of Liberal Arts Animal Experiment Committee.

Plasma and hepatic lipid measurements Plasma lipid contents, including TG, Cho, phospholipids (PL), and free fatty acids (FFA)

Table 1. Composition of experimental diets (weight %)

Ingredients	C	HF	NL
Casein	20.0	20.0	19.268
L-Cystine	0.3	0.3	0.3
Corn starch : α -Corn starch (3:1)	45.3286	39.7036	32.5796
Sucrose	20.0	20.0	20.0
Corn oil	5.0	1.0	1.0
Lard		9.0	9.0
Cellulose	3.0	3.0	3.0
Mineral mixture (NaCl -free) ^{*1}	5.0	5.0	5.0
NaCl	0.37	0.37	0.226
Vitamin mixture (AIN93)	1.0	1.0	1.0
<i>t</i> -Butylhydroquinone	0.0014	0.0014	0.0014
Cholesterol	—	0.5	0.5
Sodium cholate	—	0.125	0.125
Desalted Narazuke lees ^{*2}	—	—	8.0

C: Control diet, HF: high-fat/high-cholesterol diet, NL: desalted Narazuke lees supplemented diet.

^{*1}Mineral mixture (%): 35.7% CaCO₃, 19.6% KH₂PO₄, 7.078% K₃C₆H₅O₇ · H₂O, 4.66% K₂SO₄, 2.4% MgO, 0.606% FeC₆H₅O₇ · 5H₂O, 0.165% ZnCO₃, 0.063% MnCO₃, 0.0324% CuCO₃ · Cu(OH)₂ · H₂O, 0.001% KIO₃, 0.001025% Na₂SeO₃, 0.000795% (NH₄)₆Mo₇O₂₄ · 4H₂O, 0.145% Na₂SiO₃ · 9H₂O, 0.0275% CrK(SO₄)₂ · 12H₂O, 0.00174% LiCl, 0.00815% H₃BO₃, 0.00635% NaF, 0.00306% NiCO₃ · 2Ni(OH)₂ · 4H₂O, 0.00066% NH₄VO₃, 29.50032% sucrose. ^{*2}Desalted Narazuke lees contained 5.9 g moisture.

levels, were determined by colorimetric assay methods using the commercial Triglyceride E-Test Wako Kit, Cholesterol E-Test Wako Kit, Phospholipid C Test Wako Kit, and NEFA Test Wako Kit (Wako Pure Chemical Industries, Ltd.), respectively. Total lipids (TL) content was extracted from frozen livers according to the method of Folch *et al.* (1957) and dried under reduced pressure and N₂ gas flow; the dried fats were weighed relative to liver sample weights. A portion of the dried fats was then dissolved in 10% TritonX-100 containing isopropyl alcohol, and TG, Cho, PL, and FFA levels were determined using the above-mentioned commercial kits.

Measurements of plasma glucose and insulin levels Plasma glucose levels were assayed using the Glucose C II Test Wako Kit, and insulin levels were assayed using the Rat Insulin ELISA Kit H type (Shibayagi Industries, Ltd., Tokyo, Japan).

Determination of hepatic thiobarbituric acid reactive substances (TBARS) Hepatic TBARS was assayed as described by Kosugi *et al.* (1992) with modifications of the Ohkawa method, and the red dye was quantified using a molecular extinction coefficient of 156,000 M⁻¹·cm⁻¹.

Determination of hepatic FAS Since fatty acid biosynthesis occurs in the cytosol, FAS activity was assayed in the cytosolic liver fraction. FAS activity was assayed using the method reported by Nepokroeff *et al.* (1975) with minor modifications. In brief, FAS activity was assayed by monitoring the decrease in concentrations of coenzyme NADPH using a molar extinction coefficient of 6,220 M⁻¹·cm⁻¹ and malonyl-CoA as a substrate.

Determination of hepatic G6PDH Hepatic G6PDH activity

was assayed in the crude enzyme solutions used to measure FAS activity, as described by Davidson *et al.* (1963). In brief, increases in the G6PDH product NADPH were spectrophotometrically monitored, as in FAS assays.

Determination of hepatic CPT-Ia CPT-Ia is the rate-limiting enzyme of fatty acid β -oxidation and is localized to the inner mitochondrial membrane. Mitochondrial fractions from liver extracts were suspended in 5 mM Tris-HCl buffer solution (pH 7.2) and used as enzyme solutions. CPT-Ia activity was assayed as described by Markwell *et al.* (1973). In brief, palmitoyl-CoA was used as a substrate, and CPT-Ia activity was indicated by the release of free CoA from the substrate. After reaction with 5,5'-dithiobis (2-nitrobenzoate) at 30°C, the yellow pigment formed was measured at 412 nm using a molar extinction coefficient of 13,600 M⁻¹·cm⁻¹.

Measurement of protein contents in enzyme solutions Protein concentrations of FAS and CPT-Ia enzyme solutions were determined using the Lowry method (Lowry *et al.*, 1951) with bovine serum albumin as a standard.

Determination of fecal total lipids and total bile acids After extraction from dry feces using ethanol and chloroform methanol (1:1), total lipids (TL) were weighed, and total bile acids were measured using the Total Bile Acid Test Wako Kit.

Statistical analysis Data were expressed as mean $\pm$ standard error (SE). Significant differences between diet groups were identified by one-way analysis of variance (ANOVA) and Tukey's multiple comparison test using IBM SPSS Statistics 21. Differences were considered significant at $p < 0.05$.

Table 2. Final body weights, food intake, and tissue weights of rats fed experimental diets for 22 days

	Groups		
	C (n = 6)	HF (n = 7)	NL (n = 7)
Final body weight (g)	288.3 ± 6.1	302.8 ± 8.0	286.3 ± 6.0
Body weight gain (g/22 days)	144.6 ± 4.7	157.5 ± 6.9	142.4 ± 5.5
Food intake (g/22 days)	392.3 ± 11.2	379.9 ± 11.5	384.1 ± 9.5
Energy intake (kcal/22 days) ^{*1}	1515.3 ± 43.4	1553.1 ± 47.0	1474.1 ± 36.5
Cholesterol intake (g/22 days) ^{*1}	0.00 ± 0.00	1.90 ± 0.06	1.92 ± 0.05
Liver weight (g/100 g BW)	3.69 ± 0.12 ^a	4.76 ± 0.12 ^b	4.91 ± 0.06 ^b
Adipose tissue weight (g/100 g BW)			
Perirenal	1.70 ± 0.14	1.98 ± 0.12	1.88 ± 0.14
Epididymal	1.80 ± 0.17	1.72 ± 0.08	1.86 ± 0.11
Mesenteric	1.00 ± 0.03	1.03 ± 0.05	0.99 ± 0.03
Cecal weight (g/100 g BW)	0.75 ± 0.03	0.78 ± 0.03	0.87 ± 0.04

C: Control diet, HF: high-fat/high-cholesterol diet, NL: desalted Narazuke lees supplemented diet. Values are presented as means ± SE. Values in the same line with different superscript letters are significantly different, $p < 0.05$. ^{*1}The amounts of energy intake and cholesterol intake were calculated using diet composition and food intake.

Table 3. Lipids, glucose, and insulin contents in abdominal aortic blood plasma of rats fed experimental diets for 22 days

	Groups		
	C (n = 6)	HF (n = 7)	NL (n = 7)
Cholesterol (mg/dl)	55.4 ± 3.1	76.0 ± 8.1	73.2 ± 6.2
Triacylglycerol (mg/dl)	120.4 ± 13.6	85.8 ± 8.1	87.5 ± 7.8
Phospholipids (mg/dl)	123.5 ± 7.0 ^a	97.1 ± 2.8 ^b	124.8 ± 3.3 ^a
Free fatty acids (mEq/dl)	0.88 ± 0.11 ^a	0.80 ± 0.07 ^{a,b}	0.58 ± 0.07 ^b
Glucose (mg/dl)	122.8 ± 6.2 ^a	175.3 ± 13.3 ^b	136.0 ± 11.3 ^a
Insulin (ng/ml)	4.0 ± 0.4 ^a	8.0 ± 1.2 ^b	5.7 ± 0.6 ^{a,b}

C: Control diet, HF: high-fat/high-cholesterol diet, NL: desalted Narazuke lees supplemented diet. Values are presented as means ± SE. Values in the same line with different superscript letters are significantly different, $p < 0.05$.

Results

Body, tissue weights and food intake Body weights and food intake did not differ between the groups on the final day of the feeding period (Table 2). However, body weights tended to be lower and food intake tended to be higher in the NL group than in the HF group. Accordingly, the ratio of body weight gain to total food intake was significantly lower in the NL group ($37.0 \pm 0.8\%$) than in the HF group ($41.4 \pm 0.6\%$) and was similar to that in the C group ($36.8 \pm 0.4\%$). Energy and Cho intakes were calculated using diet composition and food intake. In the NL group, Cho intake was slightly higher and energy intake was slightly lower than in the HF group. Liver weights were significantly higher in the HF and NL groups than in the C group. Adipose tissue (perirenal and epididymal) weights in the HF and NL groups were higher than that in the C group. In addition, mesenteric adipose tissue weights in the HF group tended to be slightly higher, while those

weights in the NL group tended to be slightly lower. In addition, cecal weights in the HF group were slightly higher than those in the C group, and cecal weights in the NL group were higher than those in the HF group. Measurements of cecal content pH were: C group: 7.6 ± 0.1 , HF group: 7.4 ± 0.2 , NL group: 7.1 ± 0.1 .

Plasma lipid levels As shown in Table 3, plasma Cho levels in the HF group were higher than those in the C group and slightly higher than those in the NL group. TG levels were lower in both the HF and NL groups compared to the C group. PL levels in the HF group were significantly lower than those in the C and NL groups. FFA levels were lower in the NL group than in the C and HF groups, and were significantly different from the C group.

Plasma glucose and insulin levels As shown in Table 3, plasma glucose and insulin levels in the HF group were significantly higher than those in the C group. Plasma insulin levels were lower in the NL group than in the HF group, and plasma glucose levels were significantly lowered by NL supplementation.

Table 4. Concentrations of hepatic lipids in rats fed experimental diets for 22 days

	Groups		
	C (n = 6)	HF (n = 7)	NL (n = 7)
Total lipids (mg/g)	51.6 ± 1.0 ^a	163.9 ± 4.5 ^b	75.5 ± 9.9 ^a
Triacylglycerol (mg/g)	16.9 ± 1.2 ^a	82.0 ± 2.5 ^b	46.3 ± 3.5 ^c
Cholesterol (mg/g)	4.8 ± 0.1 ^a	52.9 ± 1.9 ^b	27.6 ± 3.6 ^c
Phospholipids (mg/g)	22.1 ± 0.2 ^a	29.7 ± 0.8 ^b	20.9 ± 1.1
Free fatty acids (μEq/g)	2.3 ± 0.2 ^a	11.5 ± 0.4 ^b	5.6 ± 0.5 ^c
TBARS (nmol/g)	183.8 ± 15.4 ^{a,b}	241.1 ± 32.5 ^a	131.9 ± 6.2 ^b

C: Control diet, HF: high-fat/high-cholesterol diet, NL: desalted Narazuke lees supplemented diet. TBARS: thiobarbituric acid reactive substances. Values are presented as means ± SE. Values in the same line with different superscript letters are significantly different, $p < 0.05$.

Table 5. Hepatic fatty acid synthase, glucose-6-phosphate dehydrogenase, and carnitine palmitoyltransferase-Ia activities in rats fed experimental diets for 22 days

	Groups		
	C (n = 6)	HF (n = 7)	NL (n = 7)
FAS activity ^{*1} (unit/mg protein)	9.2 ± 0.5 ^a	7.2 ± 0.4 ^{a,b}	5.8 ± 0.6 ^b
G6PDH activity ^{*2} (unit/mg protein)	173.4 ± 19.1 ^a	86.2 ± 2.8 ^b	78.2 ± 4.2 ^b
CPT-Ia activity ^{*3} (unit/mg protein)	16.9 ± 0.9 ^a	13.1 ± 0.7 ^b	18.0 ± 0.7 ^a

C: Control diet, HF: high-fat/high-cholesterol diet, NL: desalted Narazuke lees supplemented diet. FAS: fatty acid synthase, G6PDH: glucose-6-phosphate dehydrogenase, CPT-Ia: carnitine palmitoyltransferase-Ia. ^{*1}One unit of FAS was expressed as the amount of enzyme required to metabolize 1 nmol of NADPH/min at 30°C. ^{*2}One unit of G6PDH was expressed as the amount of enzyme required to metabolize 1 nmol of NADPH/min at 30°C. ^{*3}One unit of CPT-Ia was expressed as the amount of enzyme required to produce 1 nmol of CoA/min at 30°C. Values are presented as means ± SE. Values in the same line with different superscript letters are significantly different, $p < 0.05$.

Hepatic lipid contents As shown in Table 4, TL levels in the HF group were approximately 3.2 times higher than those in the C group, whereas the NL group levels were less than half those of the HF group. TG levels in the HF group were approximately 4.9 times higher than those in the C group, whereas the levels in the NL group were approximately 55% of those in the HF group. Cho levels in the NL group showed a similar tendency and were approximately 50% of levels in the HF group. PL levels were comparable in the C and NL groups but were approximately 1.3 times higher in the HF group. FFA levels in the NL group were approximately 50% of those in the HF group, which were approximately five times higher compared with the levels in the C group.

Hepatic TBARS levels As shown in Table 4, TBARS levels in the HF group were significantly higher than in the C group, and those in the NL group were markedly lower than in the C group.

Hepatic FAS, G6PDH, and CPT-Ia activities As shown in the Table 5, cytosolic FAS and G6PDH and mitochondrial CPT-Ia

activities in the HF group were significantly lower than those in the C group. However, cytosolic FAS and G6PDH activities were even lower in the NL group, and mitochondrial CPT-Ia activity was significantly higher than that in the HF group.

Fecal excretion of total lipids and total bile acids As shown in Table 6, fecal weights did not differ between the HF and C groups on the final day of feeding but were significantly higher in the NL group. Fecal total lipids excretion was significantly higher in the HF group than in the C group, and was significantly higher in the NL group than in the HF and C groups. Moreover, total bile acids excretion was seven times higher in the HF group than in the C group, and was significantly higher in the NL group than in the HF and C groups.

Discussion

In the present study, in an effort to use Narazuke lees, produced in large quantities as a byproduct, as a component in animal diets or as a functional food, its effect on HF diet-induced fatty liver was

Table 6. Fecal total lipids and total bile acids in rats fed experimental diets for 22 days

	Groups		
	C (n = 6)	HF (n = 7)	NL (n = 7)
Dry weight (g/day)	1.18 ± 0.05 ^a	1.23 ± 0.04 ^a	1.44 ± 0.06 ^b
Total lipids (mg/day)	107.1 ± 8.7 ^a	178.2 ± 8.8 ^b	281.6 ± 20.5 ^c
Total bile acids (μmol/day)	6.5 ± 0.6 ^a	45.6 ± 1.9 ^b	51.3 ± 1.5 ^c

C: Control diet, HF: high-fat/ high-cholesterol diet, NL: desalted Narazuke lees supplemented diet. Values are presented as means ± SE. Values in the same line with different superscript letters are significantly different, $p < 0.05$.

examined in rats. The total amount of food ingested and the final body weight did not differ significantly between the C, HF, and NL groups (Table 2), indicating Narazuke lees did not significantly affect these parameters. High liver weights were observed in the HF and NL groups compared to the C group, approximately 1.4 times that of the C group (Table 2). The levels of hepatic total lipids, TG and Cho in the HF and NL groups were higher than those in the C group; in particular, the values of TG and Cho in the HF group were notably higher, and fatty liver (TG and Cho occupied 13.5% of liver weight) was confirmed (Table 4). Liu *et al.* (1995) showed that the addition of Cho to diet promotes TG synthesis and lipid accumulation in the liver. Chiang *et al.* (1998) also reported that the dietary addition of Cho and cholate increased Cho levels in the blood and liver. Since Cho and cholate-added diets were used in this study, TG and Cho liver accumulation was thought to be promoted. In contrast, in the NL group, there was no difference in liver weight compared to the HF group; however, the liver showed 7.6% TG and Cho, which was about half that of the HF group. Further, visual improvement of fatty liver was observed.

Moreover, while large variation in plasma lipid concentration was observed, and significant differences were not observed for the HF group, an increase in Cho and decrease in TG levels were noted. This tendency was also observed for the NL group, and the addition of Narazuke lees did not have a plasma Cho suppressant effect (Table 3). It is known that liver dysfunction induced by excess fat accumulation results in down-regulation of lipoprotein secretion capacity and decreased blood TG levels (Liu *et al.*, 1995; Wang *et al.*, 2011). We observed remarkable lipid accumulation in the liver and decreased plasma TG concentrations in this experiment.

Mochida *et al.* (2000) reported that sake lees powder administration increased appendix contents and lowered the pH, as well as promoted fecal excretion of neutral and acidic sterols in rats fed a HF diet. Although significant differences were not observed in this experiment, the NL group showed the highest cecal weight (Table 2) and lowest cecal contents pH. Since cecal contents pH decreased with Narazuke lees supplementation, intestinal bacterial metabolism would be affected and acidic metabolic products would be increased. Kumagai *et al.* (2006) demonstrated that rice protein

contains indigestible protein (resistant protein) that exhibits dietary fiber-like physiology activities. According to the research of Watanabe (2010) on Profiber, which contained concentrated and powdered components of low digestible dietary fiber and resistant protein contained in sake lees, mesenteric, perirenal, and epididymal fat weights were decreased in obesity-induced rats; moreover, fecal weight and total fat excretion were significantly increased. The daily fecal weight of the NL group was significantly increased compared to the HF group. In addition, lipid and total bile acids excretions of the HF group were also significantly higher than the C group; further, the NL group showed high values as compared to the HF group: 1.6-fold greater TL excretion and 1.1-fold greater total bile acids excretion (Table 6). Hepatic fat accumulation in the NL group was lower than in the HF group, which might be attributable to the inhibition of lipid absorption. The Narazuke lees contained 31.9% dietary fiber on a dry weight basis, and approximately 95% of that was insoluble. In general, insoluble dietary fiber is difficult to digest and inaccessible to fermentation by general intestinal bacteria, and is therefore excreted almost unaltered. Thus, it appears in this study that the fecal weight increased as a result of the large water-holding property. This indicates that the insoluble dietary fibers exerted a large influence by adsorbing lipids from the diets and promoting excretion; thus, rats administered Narazuke lees showed increased fecal amounts and subsequently reduced hepatic TG accumulation. Also, it was thought that the decreased hepatic Cho was due to accelerated excretion of total bile acids and an accompanying increase in catabolism to bile acids from Cho. The effects of the resistant protein and dietary fiber of Narazuke lees are currently under study.

The inhibition of fatty liver development by brewing by-products such as sake lees or *shochu* lees has been reported. Mochizuki *et al.* (2005) reported that administration of 10% barley *shochu* lees powder improved orotic acid-induced fatty liver in rats. In contrast, the addition of 10% rice or potato *shochu* lees, sake lees, or soybean lees, showed little fatty liver improvement. Moreover, Nohara *et al.* (2010) reported that in Cho and cholate diet-induced fatty liver, supplementation with Awamori lees significantly reduced Cho in the serum and liver by the 5% added

diets, while the 50% added diet significantly reduced Cho and TG, and fatty liver was suppressed completely. The increased fecal and total bile acids excretion was thought to be due to the undigestible protein and dietary fiber contained in Awamori lees. Notably, experiments using Awamori lees and barley *shochu* lees showed fatty liver inhibiting effects with the dietary addition of 10% and 20% dry powders. In this study, 8% Narazuke lees was incorporated; and with a moisture content of 74.3%, this translates to about 2% on a dry weight basis. As insufficient amounts of dietary fiber were contained in the NL diet, the observed effects might be due to factors other than dietary fiber.

In addition to the excess lipids and carbohydrate derived from the diets, it was thought that the liver fat deposition was due to the increased influx of fatty acids from adipose tissues, promoting fat synthesis, inhibiting fatty acid β -oxidation, and suppressing the secretion of apoprotein. The formation of fatty liver due to metabolic disorders is known to be based on insulin resistance induced by visceral fat accumulation (Hamaguchi *et al.*, 2005), and appears to involve insulin receptor substrate (IRS) as the cause of insulin resistance (Taniguchi *et al.*, 2006). Liver insulin action is the promotion of fat synthesis through the IRS-1 pathway and glucose production suppression through the IRS-2 pathway. The IRS-1 pathway is required for the induction of SREBP-1c expression, which plays a central role in fat synthesis (Matsumoto *et al.*, 2002). Under insulin resistance-associated hyperinsulinemia, IRS-2 expression is selectively suppressed (Shimomura *et al.*, 2000) and the IRS-1-SREBP-1c pathway is selectively activated; fat synthesis is promoted and fat accumulation in the liver is enhanced (Tobe *et al.*, 2001; Brown and Goldstein, 2008). In our study, the mesenteric adipose tissue weight, a visceral adipose tissue, did not significantly differ between the C and HF groups, while plasma glucose and insulin concentrations of the HF groups were significantly increased, and showed insulin resistance (Table 3). According to these facts, hormone sensitive lipase in the adipose tissue was enhanced, fatty acids produced by fat decomposition were transported to the liver, fat synthesis in the liver was increased, and fatty liver due to significant TG accumulation was observed. In our study, the NL group had significantly lower plasma glucose and insulin concentrations than the HF group, and insulin resistance was improved. FAS activities, an enzyme of the fatty acid synthesis system in the liver, and G6PDH, related to the supply of NADPH, a cofactor during fatty acid synthesis, were lower in the HF group than the C group, and showed lower values in the NL group than the HF group, and significant differences were observed compared to the C group (Table 5). The results of our experiment on liver fat accumulation are consistent with the results of Hirahata *et al.* (2013), in which lactate fermented soymilk was administered to rats fed a high-fat and high-Cho diet and the expression of lipid metabolism-related genes in the liver was assessed. FAS activity was low in the high-fat and high-Cho diet group, and was further reduced upon

administration of fermented soymilk, resulting in the suppression of lipid accumulation in the liver.

Further, Kadowaki *et al.* (2006) reported on the effect of adiponectin on insulin sensitivity, and showed reduced liver TG by enhancement of fatty acid β -oxidation via activation of the transcription factor PPAR α in the liver. Therefore, insulin resistance was associated with enlarged adipose cells, the activity of CPT-1a in connection with β -oxidation in the liver was suppressed, leading to hepatic fat accumulation. We demonstrated that the enzymatic activity of the fatty acid synthesis system did not showed high values in the HF group compared to the C group, and since CPT-1a activity showed a significantly low value, the inhibition of fatty acid degradation was considered an important factor in fat accumulation. In contrast, in the NL group, CPT-1a activity increased significantly compared to the HF group, and was significantly higher in the C group, indicating acceleration of fatty acid combustion in the liver and suppression of fatty liver. Results from this study are consistent with the results described in the following two reports dealing with fatty liver suppression. Zhang *et al.* (2013) reported that in HF diet-induced nonalcoholic fatty liver of rats, flavonoids derived from *Rosa laevigata* Michx fruit significantly reduced glucose and insulin concentrations in the blood, alleviated insulin resistance by the suppression of fatty acid synthetic pathway expression in the liver, and inhibited lipid accumulation in the liver by promoting fatty acid β -oxidation. Alberdi *et al.* (2013) reported on the increase of hepatic fat accumulation in rats fed commercially available obesity diets (4.6 kcal/g). Results indicated that although resveratrol administration did not change FAS and G6PDH liver activities, it reduced acetyl-CoA carboxylase activity, increased CPT-1a and acetyl-CoA oxidase activities, promoted fatty acid oxidation, and fat accumulation in the liver was subsequently reduced by improved lipid synthesis. When simple fatty liver is complicated by inflammatory cytokines and oxidative stress, inflammation occurs in the liver and NASH symptoms develop. It is known that reduced serum adiponectin levels not only induce insulin resistance but lower liver fatty acid metabolism, and are furthermore involved in the pathogenesis of NASH / NAFLD by promoting inflammation.

The report of Zhang *et al.* (2013) is of interest in that fruit flavonoids were responsible for the active oxygen-scavenging system, increased SOD and GSH-Px activities, GSH content, and reduced MDA, resulting in the suppression of NASH progression. While the current study did not involve experiments exploring the fibrosis of liver tissue, liver TBARS were significantly increased in the HF group. The TBARS level was shown to increase according to the amount of fat used as a substrate (the amount of fatty acid). The TBARS level increased with increasing TG level in the HF group; furthermore, even with minimal changes in TG levels, alterations in TBARS level were observed, with individual samples exhibiting accelerated peroxidation. With an extended feeding

period, NASH appeared to develop. In the NL group, the low TG value was associated with low TBARS value; and individual differences were small, and oxidation was suppressed. The TG content of the C group was lower than the NL group, but the TBARS level was higher than the NL group. As corn oil was used as the dietary fat source in the C group, the results were thought to be due to the intake of unsaturated fatty acids, which is easily oxidized. In the future, we plan to measure the enzyme activities of the active oxygen scavenging system.

From the above experimental results, supplementation with Narazuke lees suppressed the absorption of fat, promoted the excretion of fat, decreased the amount ingested, reduced insulin resistance, enhanced fatty acid decomposition, inhibited fat accumulation in the liver, and ameliorated fatty liver. Thus, Narazuke lees exhibit functional properties for the prevention of fatty liver development in rats. We also considered that the observed functional properties were derived not only from resistant proteins within the raw material of the sake lees but from components (such as melanoidins) generated during long-term fermentation. The active ingredient is not thought to be a single component, but multiple components that act compositely to exert a specific function.

Acknowledgements The authors would like to thank Ms. R. Takenaka, Ms. C. Hayashi, Ms. C. Hirooka and Ms. Y. Matusima for their technical support.

References

- Alberdi, G., Rodriguez, V.M., Macarulla, M.T., Miranda, J., Churrua, I., and Portillo, M.P. (2013). Hepatic lipid metabolic pathways modified by resveratrol in rats fed an obesogenic diet. *Nutrition*, **29**, 562-567.
- Ashida, Y., Saito, Y., Kawato, A., Suginami, K., and Imayasu, S. (1997). Effects of dietary sakecake on cholesterol metabolism in rats. *Nippon Nogeikagaku Kaishi*, **71**, 137-143.
- Brown, M.S. and Goldstein, J.L. (2008). Selective versus total insulin resistance: A pathogenic paradox. *Cell Metab.*, **7**, 95-96.
- Chiang, M.T., Chen, Y.C., and Huang, A.L. (1998). Plasma lipoprotein cholesterol levels in rats fed a diet enriched in cholesterol and cholic acid. *Int. J. Vitam. Nutr. Res.*, **68**, 328-334.
- Davidson, R.G., Ntowsky, H.M., and Childs, B. (1963). Demonstration of two populations of cells in the human female heterozygous for glucose-6-phosphate dehydrogenase variants. *Proc. Natl. Acad. Sci. USA.*, **50**, 481-485.
- Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.*, **226**, 497-509.
- Hamaguchi, M., Kojima, T., Takeda, N., Nakagawa, T., Taniguchi, H., Fujii, K., Omatsu, T., Nakajima, T., Sarui, H., Shimazaki, M., Kato, T., Okuda, J., and Ida, K. (2005). The metabolic syndrome as a predictor of nonalcoholic fatty liver disease. *Ann. Intern. Med.*, **143**, 722-728.
- Hirahata, R., Kobayashi, M., Egusa, S., Sakakibara, R., and Fukuda, M. (2013). Lactic fermented soymilk improves hepatic lipid metabolism in rats fed a high fat and high cholesterol diet. *Nippon Shokuhin Kagaku Kogaku Kaishi*, **60**, 72-79.
- Izu, H., Goto, K., and Iefuji, H. (2006). Effect of sake cakes on D-galactosamine-induced liver injury in mice. *J. Brew. Soc. Japan.*, **101**, 896-899.
- Kadowaki, T., Yamauchi, T., Kubota, N., Hara, K., Ueki, K., and Tobe, K. (2006). Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *J. Clin. Invest.*, **116**, 1784-1792.
- Kosugi, H., Kojima, T., Yamaki, S., and Kikugawa, K. (1992). Interpretation of the thiobarbituric acid reactivity of rat liver and brain homogenates in the presence of ferric ion and ethylenediaminetetraacetic acid. *Anal. Biochem.*, **202**, 249-255.
- Kumagai, T., Kawamura, H., Fuse, T., Watanabe, T., Saito, Y., Masumura, T., Watanabe, R., and Kadowaki, M. (2006). Production of rice protein by alkaline extraction improves its digestibility. *J. Nutri. Sci. Vitaminol.*, **52**, 467-472.
- Liu, C.H., Huang, M., and Huang, P.C. (1995). Sources of triacylglycerol accumulation in livers of rats fed a cholesterol-supplemented diet. *Lipids*, **30**, 527-531.
- Lowry, O.H., Rosenbrough, N.J., Farr, A.J., and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275.
- Ludwig, J., Viggiano, T.R., McGill, D.B., and Oh, B.J. (1980). Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin. Proc.*, **55**, 434-438.
- Markwell, M.A.K., McGroarty, E.J., Bieber, L.L., and Tolbert, N.E. (1973). The Subcellular distribution of carnitineacyltransferases in mammalian liver and kidney. *J. Biol. Chem.*, **248**, 3426-3432.
- Matsumoto, M., Ogawa, W., Teshigawara, K., Inoue, H., Miyake, K., Sakaue, H., and Kasuga, M. (2002). Role of the insulin receptor substrate 1 and phosphatidylinositol 3-kinase signaling pathway in insulin-induced expression of sterol regulatory element binding protein 1c and glucokinase genes in rat hepatocytes. *Diabetes*, **51**, 1672-1680.
- Mochida, K., Kuribayashi, T., Saito, K., and Sugawara, M. (2000). Production of sake lees powder and its effect on sterol metabolism of rat. *Nippon Shokuhin Kagaku Kogaku Kaishi*, **47**, 78-84.
- Mochizuki, S., Miyamoto, A., Hagiwara, M., Yasumatsu, N., Wakabayashi, R., Hokazono, H., Takeshima, N., and Omori, T. (2005). Effects of several by-products of alcoholic beverage on the fatty liver in rats fed orotic acid. *Res. Bull. Fac. Educ. & Welf. Sci., Oita Univ.*, **27**, 197-206.
- Nepokroeff, C.M., Lakshmanan, M.R., and Porter, J.W. (1975). Fatty acid synthase from rat liver. *Methods Enzymol.*, **35**, 37-44.
- Nohara, T., Uechi, S., Ogura, G., Hayashi, K., and Sunagawa, K. (2010). Effect of Awamori lees on growth, serum and liver lipid concentration in rats fed a high cholesterol diet. *Nippon Shokuhin Kagaku Kogaku Kaishi*, **57**, 93-99.
- Okuda, H. (2003). Elucidation of biologically active substances in sake and sake lees. *J. Brew. Soc. Japan.*, **98**, 750-755.
- Saito, Y., Nakamura, K., Kawato, A., and Imayasu, S. (1992). Angiotensin I converting enzyme inhibitors in sake and its by-products. *Nippon*

- Nogeikagaku Kaishi*, **66**, 1081-1087.
- Saito, Y., Ohura, S., Kawato, A., and Suginami, K. (1997). Prolyl endopeptidase inhibitors in sake and its byproducts. *J. Agric. Food Chem.*, **45**, 720-724.
- Saito, Y., Wanezaki (Nakamura), K., Kawato, A., and Imayasu, S. (1994a). Structure and activity of angiotensin I converting enzyme inhibitory peptides from sake and sake lees. *Biosci. Biotechnol. Biochem.*, **58**, 1767-1771.
- Saito, Y., Wanezaki (Nakamura), K., Kawato, A., and Imayasu, S. (1994b). Antihypertensive effects of peptide in sake and its by-products on spontaneously hypertensive rats. *Biosci. Biotechnol. Biochem.*, **58**, 812-816.
- Schaffner, F. and Thaler, H. (1986). Nonalcoholic fatty liver disease. *Prog. Liver Dis.*, **8**, 283-298.
- Shimomura, I., Mashuda, M., Hammer, R.E., Bashmakov, Y., Brown, M.S., and Goldstein, J.L. (2000). Decreased IRS-2 and increased SREBP-1c lead to mixed insulin resistance and sensitivity in livers of lipodystrophic and *ob/ob* mice. *Mol. Cell.*, **6**, 77-86.
- Takaki, A. and Yamamoto, K. (2010). Epidemiology of NASH/NAFLD. *Japanese J. Clin. Nutr.*, **116**, 617-622.
- Taniguchi, C.M., Emanuelli, B., and Kahn, C.R. (2006). Critical nodes in signaling pathways: insights into insulin action. *Nat. Rev. Mol. Cell Biol.*, **7**, 85-96.
- Tobe, K., Suzuki, R., Aoyama, M., Yamauchi, T., Kamon, J., Kubota, N., Terauchi, Y., Matsui, J., Akanuma, Y., Kimura, S., Tanaka, J., Abe, M., Ohsumi, J., Nagai, R., and Kadowaki, T. (2001). Increased expression of the sterol regulatory element-binding protein-1 gene in insulin receptor substrate-2⁺ mouse liver. *J. Biol. Chem.*, **276**, 38337-38340.
- Vuppalanchi, R. and Chalasani, N. (2009). Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis: selected practical issues in their evaluation and management. *Hepatology*, **49**, 306-317.
- Wang, X., Cao, Y., Fu, Y., Guo, G., and Zhang, X. (2011). Liver fatty acid composition in mice with or without nonalcoholic fatty liver disease. *Lipids in Health and Disease*, **10**, 234-240.
- Watanabe, T. (2010). Information of products: Profiber: function of sake lees indigestible product. *J. Japan. Council. Adv. Food Ingrid. Res.*, **13**, 19-22.
- Zhang, S., Zheng, L., Dong, D., Xu, L., Yin, L., Qi, Y., Han, X., Lin, Y., Liu, K., and Peng, J. (2013). Effects of flavonoids from *Rosa laevigata* Michx fruit against high-fat diet-induced non-alcoholic fatty liver disease in rats. *Food Chem.*, **141**, 2108-2116.