Supplementation of a Fermented Soybean Extract Reduces Body Mass and Prevents Obesity in High Fat Diet-Induced C57BL/6J Obese Mice.

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ABSTRACT: Obesity is a growing health problem that many countries face, mostly due to the consumption of a Westernized diet. In this present study we observed the effects of a soybean extract fermented by *Bacillus subtilis* MORI (BTD-1) containing 1-deoxynojirimycin against high fat diet-induced obesity. The results obtained from this study indicated that BTD-1 reduced body weight, regulated hepatic lipid content and adipose tissue, and also affected liver antioxidant enzymes and glucose metabolism. These results suggest that administration of BTD-1 affects obesity by inhibiting hyperglycemia and free radical-mediated stress; it also reduces lipid accumulation. Therefore, BTD-1 may be potentially useful for the prevention of obesity and its related secondary complications.

Keywords: obesity, fermented soybean extracts, glucose metabolism, antioxidant enzymes, lipid accumulation

INTRODUCTION

Diets high in fats, sugars, and calories decreased physical activity and exercise can lead to metabolic and inflammatory diseases such as obesity, hypertension, diabetes, cancers, and other chronic pathologies. Obesity is the most significant risk factor for chronic liver disease, coronary heart disease, atherosclerosis, fatty liver, diabetes mellitus (DM), and hypertension (1,2). More than 80% of diabetic deaths occur in low- to middle-income countries each year (3). Over the past decade, there has been a global increase in obesity and type 2 diabetes. In 2012, it was estimated that about 1.5 million deaths were directly caused by diabetes (3). The WHO has projected that in 2030, diabetes will be the 7th leading cause of death worldwide (4). However, many plant-derived products exhibit pharmacological properties without any associated side effects (6). Recent studies on obesity in the field of food science have concentrated on the search for functional ingredients in foods or herbal extracts that could reduce body weight and body fat (7,8).

Soybean (*Glycine max* Merr) has several positive effects due to the presence of proteins, isoflavonone, and dietary fiber. Soybean has been reported to possess antiobesity (9), anti-inflammatory (10), and antidiabetic (11) properties as well as having an association with decreased adipose deposition (12). Antioxidants are considered the best treatment for DM (11). 1-Deoxynojirimycin (DNJ), a polyhydroxylated alkaloid, has been reported to be a strong intestinal α-glucosidase inhibitor (AGI) (13). DNJ is a naturally occurring azasugar, a characteristic component of mulberry (Moraceae) leaves and dietary mulberry, and it is also produced by several *Bacillus* and *Streptomyces* strains isolated from the soil (14,15). α-Glucosidase in the small intestine is potentially inhibited by DNJ, thereby binding to the active center of α-glucosidase (16). AGIs are used as therapeutic agents to prevent or treat DM worldwide (17). Fermented Korean soybean has peculiar characteristics compared to other available fermented soybean products, because during the fermentation process, isoflavonoids are converted from glycosides into aglycones. The amount of daidzein in fermented soybean increases 44-fold when compared to that of unfermented soybean (18). Above all things, AGIs were considered to be the first therapeutic targets for the treatment of diabetes. Meanwhile, the DNJ com-

pound found in mulberry and soybean extract has been reported to mimic a sugar molecule (19,20) and consequently bind to the α-glucosidase enzyme that hydrolyzes starches into sugars in the small intestine (19,21). When DNJ binds to α-glucosidase, disaccharides such as maltose and sucrose present in the small intestine cannot be processed; hence, these non-digested disaccharides pass through the small intestine instead of being absorbed into the blood (22). DNJ was also reported to function similarly to the antidiabetic drug metformin by reducing the production of excess glucose in the liver (23).

Taken together, it is worthwhile to develop safe and effective anti-obesity agents from plant materials. In the present study, we investigated the anti-obesity effects of *Bacillus subtilis* MORI-fermented soybean extracts (BTD-1) in high fat diet (HFD)-induced C57BL/6J obese mice.

MATERIALS AND METHODS

Chemicals used
All of the reagents used in this study were of analytical grade and were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Preparation of DNJ using *B. subtilis* MORI
Defatted (fat-free) soybean meal fermentation was performed according to Lim et al. (20) by isolating *B. subtilis* MORI (KCCM 10450P, the Korean Culture Collection of Microorganisms, Seoul, Korea) from the Korean traditional fermented soybean Cheonggukjang. Briefly, a single loop of seed culture was streaked on the surface of Difco[TM] yeast mold agar plates (BD, Franklin Lakes, NJ, USA) and incubated at 37°C for 24 h. A single colony from the plates was selected, and it was inoculated into Bacto[TM] tryptic soy broth (2.5 L) (BD) dispensed into four 6-L flasks with constant shaking (180 rpm) at 37°C for 17 h. Then, 10 L of the seed culture was taken and inoculated into 500 L of fermentation broth in a 2.5 metric ton fermenter containing 5% (w/v) defatted soybean meal and incubated at 37°C with constant shaking (80 rpm) for 5 days. After the 5th day, the fermented contents were centrifuged at 15,000 rpm using a high-speed centrifuge (Kansai Centrifugal Separator Mfg. Co., Ltd., Osaka, Japan). The supernatant was collected and concentrated to 60 L using a 500-L vacuum evaporator (Boksung Engineering, Incheon, Korea). The concentrated sample was mixed with corn starch as an excipient for drying using a spray dryer (Woojin, Anseong, Korea). The final products containing BTD-1 were stored for further analysis.

HPLC analysis of DNJ content in BTD-1
Extraction of the BTD-1 was performed using 1 g of BTD-1 in 100 mL deionized water at 60°C for 60 min in a water bath. Then, the contents were centrifuged at 10,000 g for 15 min using a Combi-514R apparatus (Hanil Science Industrial Co., Ltd., Incheon, Korea). The supernatant was diluted (1:10,000) with sterile distilled water at 25±2°C, and the diluted sample was used for subsequent derivatization. The DNJ content (mg/g) in BTD-1 was determined as described by Kim et al. (24) using 9-fluorenylmethyl chloroformate (FMOC-Cl; Fluka Chemie AG, St. Gallen, Switzerland) as a derivatizing agent. Ten μL of DNJ (Sigma-Aldrich Co.) was taken as a standard solution in a 1.5 mL microtube; 20 μL of 5 mM FMOC-Cl in CH_3CN was added with immediate mixing, and then the reaction was allowed to continue for 20 min at 20°C in a water bath (Jeiotech Co., Daejeon, Korea). The reaction was terminated by adding 0.1 M glycine (Sigma-Aldrich Co.) to stabilize the DNJ-FMOC, and then the mixture was filtered using a 0.2 μm syringe filter (Minisart RC4, Sartorius Stedim Biotech GmbH, Göttingen, Germany). High-performance liquid chromatography (HPLC) analysis was carried out using 10 μL aliquots from the filtrate using a LC-20A Prominence system (Shimadzu, Kyoto, Japan), consisting of a Capcell Pak C18 MG column (250×4.60 mm I.D., 5 μm) and a RF-10Axl fluorescence detector (excitation 254 nm and emission 322 nm, Shimadzu). The data analysis was performed using the LC solution data processing software. The analytes were eluted using the mobile phase of acetonitrile/0.1% aqueous acetic acid (1:1, v/v) at 1.0 mL/min for 40 min, and then the DNJ content was determined. The total DNJ content present in the BTD-1 was calculated using the standard DNJ sample by HPLC.

In vivo animal experiments
All experiments were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) of Chonbuk National University (CBU 2013-45, Jeonju, Korea). Sixty male mice (C57BL/6, 23~25 g) were procured from Koatech (Pyeongtaek, Korea). The mice were acclimatized for 2 weeks at ambient temperature (25±2°C) with alternative 12-h light/dark cycle. During that period, the mice were given standard food and water *ad libitum* for acclimatization. The normal diet (Rodent NIH-31 AUTO 18-4 diet, formulation no: 413110-12-30; Zeigler Bros., Inc., Gardners, PA, USA) and HFD (D12492, 60 kcal%, Research Diets Inc., New Brunswick, NJ, USA) were used to feed the respective groups. After 2 weeks, the animals were divided into 5 groups, each group containing 12 animals.

Group I was the normal control, in which the animals were given a normal diet and water *ad libitum* throughout the experiments, while group II was the obesity con-

trol, in which animals were given a HFD. Group III served as the shame control treated with non-fermented soybean extracts [NFSE; 500 mg/kg body weight (BW)/d] along with a HFD. Group IV was administered a HFD along with BTD-1 (250 mg/kg BW/d) and group V was given HFD along with BTD-1 (500 mg/kg BW/d). All the groups were given water *ad libitum* throughout the experimental period. The experiments were performed for 12 weeks, during which time changes in body weight were monitored. At the end of the experiment, mice were anesthetized using chloral hydrate (400 mg/kg BW), sacrificed by decapitation, and samples were collected and stored for further investigation. Body weight was measured at 2 week intervals up to 12 weeks using an electronic balance (Precisa Instruments LLC, Clark, NJ, USA). The biochemical analysis was performed at end of the experimental period.

Biochemical analysis

Blood samples were collected from the tail vein nick for oral glucose tolerance test (OGTT) and retro-orbital plexus puncture glycated hemoglobin (HbA1c) and plasma glucagon. At the end of the 12th week, the mice were fasted for 16 h overnight, and 100 mg of glucose was administered orally using an oral gavage. Blood samples were collected at intervals of 0, 30, 60, 120, and 180 min after glucose administration. OGTT was measured using an Accu-Chek Compact® Meter (Roche Diagnostics, Indianapolis, IN, USA). Fasting insulin levels were measured with an ELISA kit (Shibayagi Co., Ltd., Shibukawa, Japan). Homeostasis model assessments for insulin resistance (HOMA-IR) values (25) were calculated using the following formula:

$$HOMA\text{-}IR = \frac{\text{fasting insulin } (\mu U/mL) \times \text{fasting glucose } (mmol/L)}{22.5}$$

HbA1c was measured using the In2it™ (I) Hemoglobin A1c test (Bio-Rad Laboratories Ltd., Deeside, UK). Plasma glucagon was measured using a glucagon assay kit (Glucagon Radioimmunoassay Kit, Linco Research, Inc., St. Charles, MO, USA).

G6Pase expression in liver tissue using Western blot analysis

Total protein content in the liver tissue was extracted using a cell lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, and 1% Triton X-100, pH 7.2) containing protease and phosphatase inhibitors, and the protein content was estimated using a bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL, USA). Briefly, an equal amount of extracted protein was loaded onto the 12% sodium dodecyl sul-

fate-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (Amersham Bioscience, Piscataway, NJ, USA). The membrane was blocked using 5% (w/v) skim milk in Tris-buffered saline (20 mM Tris and 0.9% NaCl, pH 7.4) and later incubated overnight with primary antibodies, glucose-6-phosphtase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA)] at 4°C. Later, the membrane was incubated with the respective secondary antibody (1:3,000, Cell Signaling Technology, Danvers, MA, USA). Following incubation, the membrane was detected using an enhanced chemiluminescence detection kit and images of the blots were captured using a gel imaging system (Alpha Innotech Fluorchem FC2, Alpha Innotech Corp., San Leandro, CA, USA). The membrane was re-probed for β-actin (C4: SC-47778, 1:2,000, Santa Cruz Biotechnology, Inc.) as a loading control, and the band intensity was quantified using the Image J software, version 1.48 (National Institutes of Health, Bethesda, MD, USA), then normalized to β-actin.

Hepatic metabolic activity of FAS

Hepatic metabolic activity of fatty acid synthase (FAS) was determined using the methods described by Nepokroeff et al. (26); activity was represented by oxidized nicotinamide adenine dinucleotide phosphate hydrate nmol/min/mg protein.

Lipid peroxidation and antioxidant defense systems

Lipid peroxidation in the liver sample was quantified based on the malondialdehyde (MDA) concentration, which was spectroscopically measured by calculating the absorbance of a red-colored end product formed with thiobarbituric acid (27). Superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) activities were determined using commercially available kits according to the manufacturers' instructions (Jiancheng Bioengineering, Co., Ltd., Nanjing, China).

Histology

The tissue preserved in 10% buffered formalin (pH 7.0) was washed with physiological saline and embedded into paraffin. Later, paraffin blocks were sectioned to a 5 μm thickness, mounted on a glass slide, and stained with hematoxylin and eosin (H&E). The sections were observed under a light microscope (Olympus, Tokyo, Japan) at 400× magnification.

Statistical analysis

Statistical analyses were performed using one way analysis of variance (ANOVA) followed by the Tukey's post hoc test and for the body weight changes and blood glucose test one way repeated measures ANOVA followed by Tukey's post hoc test was performed. Differences of *P*<
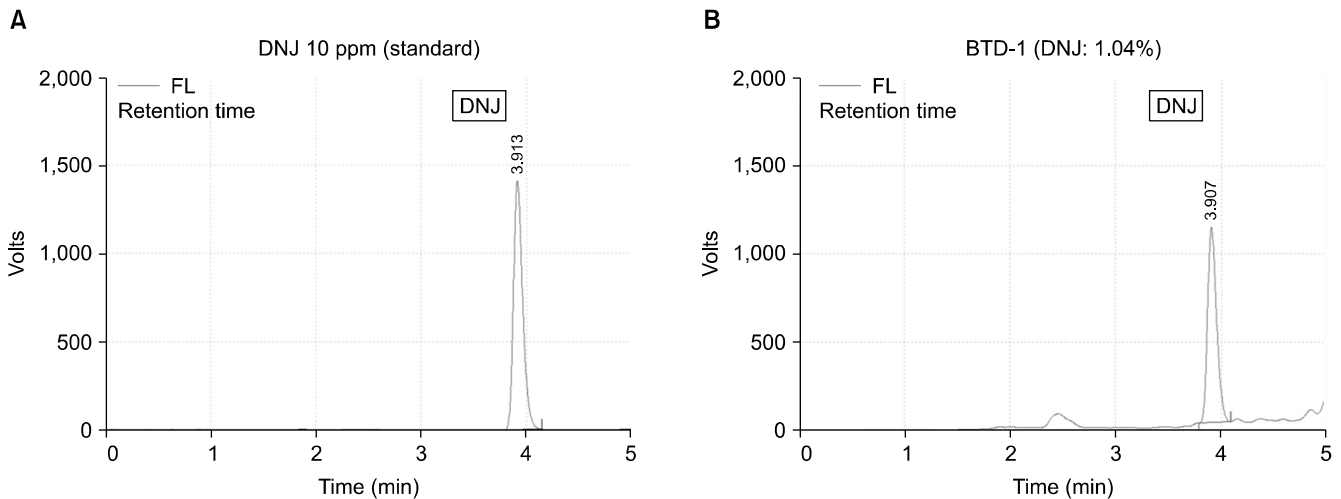
A

B

Fig. 1. High-performance liquid chromatography of the standard 1-deoxynojirimycin (DNJ) (A) and the DNJ presented in the *Bacillus subtilis* MORI-fermented soybean extracts (BTD-1) (B) with their respective retention times.

0.05 were considered to be statistically significant.

RESULTS

Quantification of DNJ content in BTD-1 from HPLC

The HPLC profile (Fig. 1) revealed that the DNJ content accounted for about 1.04% of the total BTD-1 content. Standard DNJ was successfully absorbed and eluted at a retention time of 3.913 min. The DNJ sample obtained from BTD-1 showed a retention time of 3.907 min. NFSE did not show a DNJ peak, confirming the absence of DNJ. Significant differences were observed between the DNJ content from the BTD-1 and NFSE (data not shown). This HPLC data clearly confirms that the fermented soybean extract has active DNJ.

Body weight and blood glucose parameters

The overall weight gain of the group III was lower than that of group II; this difference was significant ($P<0.01$) (Fig. 2). Group II showed increasing weight and body mass up to the 8th week and maintained a steady weight after that until the end of the experimental period, indicating HFD-induced obesity. Group IV and group V maintained a weight approximately equal to or lower than group I throughout the experimental period. When the experimental groups (IV and V) were compared, there was a significant weight difference, with group IV having a lower weight loss. Group V exhibited a significant weight reduction, indicating that BTD-1 had an effect on obesity. A significant weight increase was observed in group II throughout the experimental period compared to the control and the other groups ($P<0.01$). We performed OGTT at 12th week by administering oral glucose, and the blood glucose concentration was measured at 0, 30, 60, 120, and 180 min; the results are presented
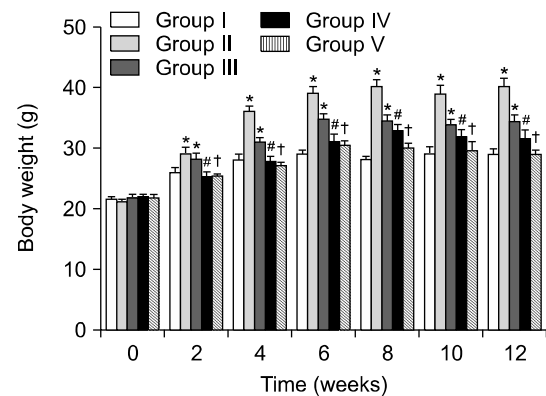

Fig. 2. Effect of *Bacillus subtilis* MORI-fermented soybean extracts (BTD-1) on the change in body weight of the mice throughout the 12 week period for all the groups (n=12). Group I, normal control; group II, obesity control [treated with high-fat diet (HFD)]; group III, treated with HFD+500 mg/kg body weight (BW)/d non-fermented soybean extracts (NFSE); group IV, treated with HFD+250 mg/kg BW/d BTD-1; group V, treated with HFD+ 500 mg/kg BW/d BTD-1. Statistical analysis was performed using one way repeated measures ANOVA followed by Tukey's post hoc test. Data are the mean±standard error (n=12). *$P<0.01$ compared with group I, #$P<0.05$ compared with group II, and †$P<0.01$ compared with group III.

in Fig. 3. After 12 weeks, the base-line (0-min, before glucose administration) revealed an elevated blood glucose level in groups II and III compared to the other groups. Group II showed a significant increase ($P<0.01$) in blood glucose levels compared to group I and other experimental groups (III~V). Blood glucose levels in groups IV and V were statistically similar to group I. After administration of glucose, the blood glucose levels were monitored at different time intervals. Fig. 3 clearly shows that blood glucose levels were significantly elevated at 30 min in all groups ($P<0.01$) and were found to decrease after 30 min except in group II, which reached a maximum of 500 mg/dL at 60 min and then decreased gradually. A significant decrease in blood glucose levels was observed
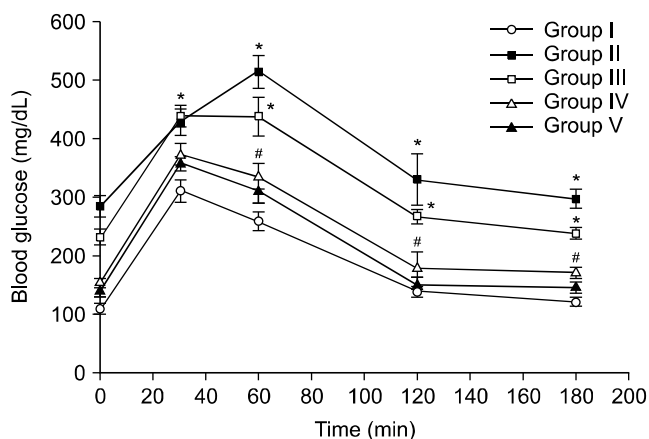
Fig. 3. Effect of *Bacillus subtilis* MORI-fermented soybean extracts (BTD-1) on oral glucose tolerance test. Group I, normal control; group II, obesity control [treated with high-fat diet (HFD)]; group III, treated with HFD+500 mg/kg body weight (BW)/d non-fermented soybean extracts (NFSE); group IV, treated with HFD+250 mg/kg BW/d BTD-1; group V, treated with HFD+500 mg/kg BW/d BTD-1. Statistical analysis was performed using one way repeated measures ANOVA followed by Tukey's post hoc test. Data are the mean±standard error (n=12). *$P<0.01$ compared with group I and #$P<0.05$ compared with group II.
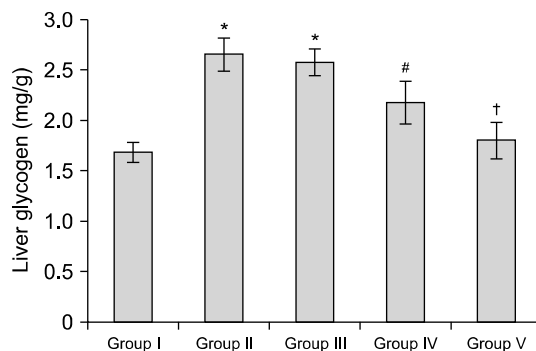

Fig. 4. Effect of *Bacillus subtilis* MORI-fermented soybean extracts (BTD-1) on the glycogen levels in the liver tissue of the C57BL/6J mice. Group I, normal control; group II, obesity control [treated with high-fat diet (HFD)]; group III, treated with HFD+500 mg/kg body weight (BW)/d non-fermented soybean extracts (NFSE); group IV, treated with HFD+250 mg/kg BW/d BTD-1; group V, treated with HFD+500 mg/kg BW/d BTD-1. Statistical analysis was performed using one way ANOVA followed by Tukey's post hoc test. Data are the means mean± standard error (n=12). *$P<0.01$ compared with group I, #$P<0.05$ compared with group II, and †$P<0.01$ compared with group III.

in group IV and group V ($P<0.01$), which was similar to that displayed by the normal control group. At the end of 180 min, the blood glucose levels in group I, IV, and V had recovered nearly to the 0-min level, while the other groups (II and III) were still significantly elevated from base-line, taking longer to recover due to HFD intake and obesity.

Hepatic glycogen metabolism
Hepatic glycogen levels were studied after 12 weeks, and the results are presented in Fig. 4. The graph clearly shows that glycogen levels of group II were significantly the most higher ($P<0.01$), while group V showed a dramatic decrease in hepatic glycogen content compared to the other test groups (II, III, and IV) indicating the effects of BTD-1 on glycogen metabolism.

Liver SOD, GSH-Px, CAT, and MDA levels
Changes in antioxidant defense system levels in the liver are shown in Table 1. SOD plays an important role in cellular defense against oxidative stress, thereby converting the superoxide anion to H_2O_2. The SOD enzyme activity of group II was found to be significantly reduced compared to the control group after 12 weeks of HFD administration, whereas a significantly higher ($P<0.01$) SOD level was observed in group V compared to the other groups (I~IV). The same pattern of enzymatic expression was seen for GSH-Px and CAT. GSH-Px and CAT activities were significantly reduced in group II, while levels were found to be significantly increased in group III, and a significant increase in GSH-Px and CAT was observed in group V after 12 weeks ($P<0.01$). The data

clearly indicate the impact of BTD-1 on the antioxidant defense system in the liver. MDA levels were significantly increased ($P<0.01$) in group II and group III when compared to group I. Between group II and III, there was a significant difference in MDA levels suggesting that NFSE itself did not reduce MDA levels. On the other hand, group V showed a significant reduction in MDA levels. A significantly lower MDA level was observed in group V compared to group IV.

Blood glucose, insulin, and glucose metabolic enzymes
Fasting blood glucose and insulin levels were measured at the end of the 12th week, and these data are presented in Table 2. There was a significant increase in the fasting blood glucose levels in group II compared to group I and the other treated groups ($P<0.01$). Group V showed a significant decrease in blood glucose levels compared to the other positive control and experimental groups. Group III exhibited a significantly lower fasting blood glucose levels compared to group II, while the same value was significantly higher than in groups IV and V ($P<0.01$). Fasting insulin levels were found to be significantly elevated ($P<0.01$) in group II compared to group I and experimental groups. Group V showed a significant difference in the insulin reduction levels. The HOMA-IR index was calculated on the basis of final blood glucose and insulin concentrations. Group II had a significantly higher HOMA-IR index than the control and experimental groups, whereas group V had a dramatically lower HOMA-IR index compared to groups III and IV. We also measured blood-HbA1c to determine whether BTD-1 improves blood hyperglycemia. From Table 2, it is clear that treatment with a high dose of BTD-1 lowered blood-HbA1c levels as compared to the normal con-

Table 1. Antioxidant enzyme activity and lipid peroxidation levels in the liver of C57BL/6J mice after oral administration of *Bacillus subtilis* MORI-fermented soybean extracts (BTD-1) for 12 weeks

Group[1]	Antioxidant defense system[2]			Lipid peroxidation[3]
	SOD (U/mg tissue)	GSH-Px (μM/mg tissue)	CAT (U/mg tissue)	MDA (μM/mg tissue)
I	3.84±0.46	33.42±2.58	19.48±1.74	14.86±1.87
II	1.75±0.32*	15.76±3.15*	10.68±2.32*	58.74±7.43*
III	1.73±0.36[NS]	14.84±3.42[NS]	11.03±2.79[NS]	61.54±8.07[NS]
IV	2.42±0.42[#]	22.69±4.36[#]	14.87±3.41[#]	42.69±9.43[#]
V	2.98±0.35[†]	25.86±3.84[†]	17.69±2.97[†]	29.47±7.02[†]

Results are expressed as the mean±SD in each group (n=12 mice). Statistical analysis was performed using one way ANOVA followed by Tukey's post hoc test.
*Significantly different ($P<0.01$) from normal control.
[#]Significantly different ($P<0.05$) from HFD.
[†]Significantly different ($P<0.01$) from HFD.
[NS]Not significantly different ($P>0.05$) from HFD.
[1]I, normal control; II, obesity control [treated with high-fat diet (HFD)]; III, treated with HFD+500 mg/kg body weight (BW)/d non-fermented soybean extracts (NFSE); IV, treated with HFD+250 mg/kg BW/d BTD-1; V, treated with HFD+500 mg/kg BW/d BTD-1.
[2]SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase.
[3]MDA, malondialdehyde.

Table 2. Blood glucose, insulin, and glucose metabolic enzymes in C57BL/6J mice after oral administration of *Bacillus subtilis* MORI-fermented soybean extracts (BTD-1) for 12 weeks

Group[1]	Fasting blood glucose (mg/dL)	Fasting insulin (pg/mL)	HbA1c (%)	HOMA-IR[2] index
I	79.6±5.9	1,054.9±137.6	5.01±0.46	4.2±0.8
II	125.4±6.7*	1,984.7±206.4*	8.25±0.57*	7.3±1.3*
III	119.8±7.4[NS]	1,875.9±198.7[NS]	8.13±0.84[NS]	7.1±1.2[NS]
IV	107.2±9.6[#]	1,473.6±372.7[#]	7.02±0.76[#]	5.1±1.4[#]
V	94.7±7.8[†]	1,309.4±228.3[†]	6.17±0.69[†]	4.8±0.9[†]

Results are presented as the mean±SD in each group (n=12 mice). Statistical analysis was performed using one way ANOVA followed by Tukey's post hoc test.
*Significantly different ($P<0.01$) from normal control.
[#]Significantly different ($P<0.05$) from HFD.
[†]Significantly different ($P<0.01$) from HFD.
[NS]Not significantly different ($P>0.05$) from HFD.
[1]I, normal control; II, obesity control [treated with high-fat diet (HFD)]; III, treated with HFD+500 mg/kg body weight (BW)/d non-fermented soybean extracts (NFSE); IV, treated with HFD+250 mg/kg BW/d BTD-1; V, treated with HFD+500 mg/kg BW/d BTD-1.
[2]HOMA-IR, Homeostasis model assessments for insulin resistance.

trol and the other treated groups. G6Pase and PEPCK expression was analyzed and the results are presented in Fig. 5. Gluconeogenesis is controlled by the activities of 2 vital enzymes, G6Pase and PEPCK (28). The rate of PEPCK and G6Pase expression was significantly higher in group II compared to group I and other experimental groups, including group III. PEPCK and G6Pase expression was significantly lower in group V compared to all the other groups except group I, which indicates that the expression of glucose metabolic enzymes were directly regulated by BTD-1.

Expression of FAS in the liver
In addition, in study for expression of FAS in the liver, the activity was found to be reduced in group V (4.35± 1.12 nmol/min/mg protein) compared to group II (9.76 ±1.34 nmol/min/mg protein). The differences were observed between the HFD group and the treated groups IV (6.26±1.87 nmol/min/mg protein) and V. No signifi-

cant differences were observed between groups II and III (10.01±1.75 nmol/min/mg protein). But compared to the control (3.45±0.78 nmol/min/mg protein), significant differences were observed between group II and V treated with BTD-1. But, importantly, more repeat studies were demanded for reproducibility.

BTD-1 reduces accumulation of lipids in the liver and kidney
Representative microscopic images of both the liver and kidney are presented in Fig. 6. Group I had normal cell architecture, whereas group II showed significant morphological differences. Compared to the control group, group II exhibited significant vascular degeneration of hepatocytes, normal hepatic lobule distribution, and infiltration of inflammatory cells, which were observed in H&E-stained liver and kidney cell sections. Groups IV and V showed reduced hepatic droplets compared to the control group. Hepatic lipid accumulation was signifi-
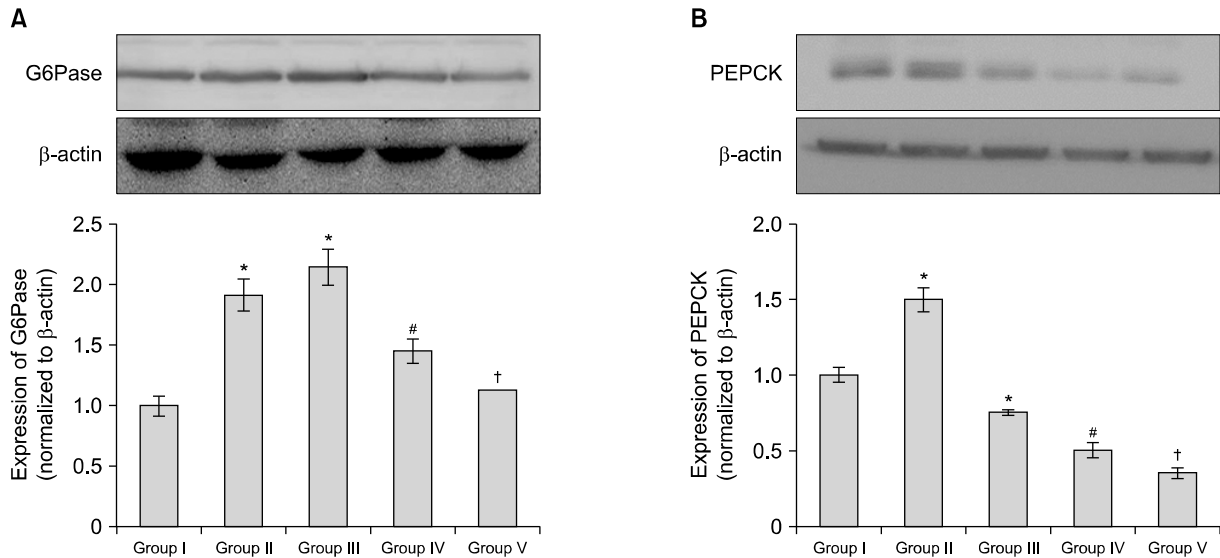
Fig. 5. Western blot analysis of the effect of *Bacillus subtilis* MORI-fermented soybean extracts (BTD-1) on (A) glucose-6-phospha-tase (G6Pase) and (B) phosphoenolpyruvate carboxykinase (PEPCK) expressions in the liver tissue using western blot analysis then normalized to β-actin. Group I, normal control; group II, obesity control [treated with high-fat diet (HFD)]; group III, treated with HFD+500 mg/kg body weight (BW)/d non-fermented soybean extracts (NFSE); group IV, treated with HFD+250 mg/kg BW/d BTD-1; group V, treated with HFD+500 mg/kg BW/d BTD-1. *$P<0.01$ compared with group I, #$P<0.05$ compared with group II, and †$P<0.01$ compared with group II.

cantly higher in group II, and was dramatically reduced in group V. Group V was shown to inhibit the accumulation of lipids in hepatocytes and kidney cells.

DISCUSSION

In this study, the anti-obesity effect of BTD-1 was investigated using a diet-induced obese mouse model and a normal control reference. In the present investigation, 60% kcal HFD was fed to mice to induce obesity, which was confirmed by pathophysiological symptoms. At the end of the 12-week experimental period, HFD-induced mice were obese when compared to the normal control and the HFD-fed groups treated with BTD-1. From this study, it is clear that BTD-1 has a direct effect on body weight, glucose, and lipid metabolism, which play an important role in preventing obesity. DNJ from mulberry and soybean has been reported to be beneficial in suppressing high glucose levels (20,29), increasing plasma adiponectin, activated the β-oxidation system and suppressed lipid accumulation in the liver (13). HbA1c levels were significantly reduced in BTD-1-treated groups, suggesting that BTD-1 has specific antiglycation properties that contribute to the reduction of HbA1c levels. HbA1c levels have been used to predict an individual's risk of diabetes. Persistent hyperglycemia leads to non-enzymatic glycosylation of proteins (30). The increased levels of glycosylated hemoglobin observed in HFD-fed mice were significantly reduced in the HFD-fed groups treated with BTD-1. This is due to enhanced glucose homeostasis, which is further supported by the OGTT da-

ta. Deviations in lipid metabolism slowly decrease the strength of antioxidative defense mechanisms (31). Group III was not significantly different from group II in body weight, glucose metabolism or lipid metabolism results, whereas BTD-1-treated groups had significantly lower body weight and mass ($P<0.01$). Fasting glucose and insulin levels were found to be significantly lower in the BTD-treated groups compared to group II. This result clearly confirms that BTD-1 increases insulin sensitivity, which plays a major role in reducing blood glucose levels. The enzymatic antioxidant mechanism plays a major role in the elimination of free radicals. The present study confirms that BTD-1 treatment significantly increased SOD activity and CAT along with enhancing GSH-Px activity, confirming that BTD-1 scavenges oxygen free radicals. Lim et al. (20) proposed a possible mechanism of BTD-1 suggesting that its hypoglycemic action may be due to the protection of pancreatic β-cells from oxidative stress. BTD-1 consumption elevated SOD, CAT, and GSH-Px levels, and reduced MDA levels by inducing lipid and sugar metabolism, which was achieved through the enhancement of antioxidant activities. The results were further in agreement with the histopathological H&E result that clearly shows the reduction of lipid accumulation in liver and kidney sections in the BTD-1 treated groups in a dose-dependent manner. Obvious fat accumulation in the kidney and liver was observed after feeding mice a HFD for 12 weeks. The liver plays an essential role in lipid homeostasis via regulation of lipogenesis and oxidative stress (32). The effects of DNJ on lipid metabolism were examined; the activation of the fatty acid β-oxidation system in the liver can reduce lipid
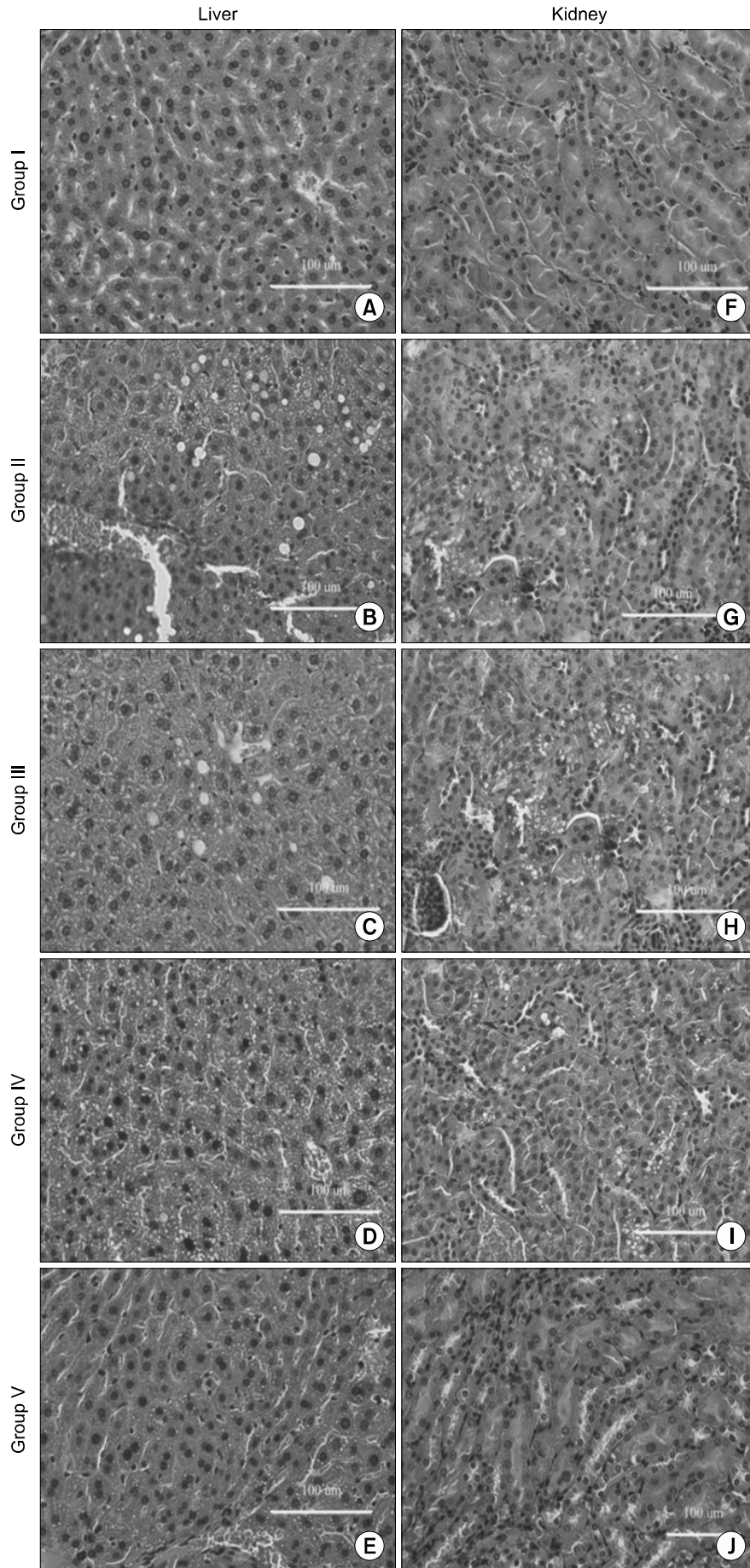
Liver Kidney

Fig. 6. Effects of *Bacillus subtilis* MORI-fermented soybean extracts (BTD-1) on lipid accumulation of liver and kidney cells of C57BL/6J mice using hematoxylin and eosin staining. Group I, normal control; group II, obesity control [treated with high-fat diet (HFD)]; group III, treated with HFD+500 mg/kg body weight (BW)/d non-fermented soybean extracts (NFSE); group IV, treated with HFD+250 mg/kg BW/d BTD-1; group V, treated with HFD+ 500 mg/kg BW /d BTD-1.

accumulation (33). Intake of a HFD itself contributes to the development of obesity, hyperinsulinemia, hypertriglyceridemia, and insulin resistance in humans and rodents by altering triglycerides (TG) and cholesterol levels in the plasma and tissues (34). Both groups treated with BTD-1 exhibited reduced metabolic enzyme activity. The activity of the lipogenic enzyme FAS was reduced in the group V, while it was significantly higher in group II. This clearly indicates that BTD-1 inhibits the accumulation of TG and cholesterol in the liver. In the previous study, the treatment of BTD-1 was significantly increased phosphorylation of acetyl-CoA carboxylase (ACC) protein expression in 3T3-L1 preadipocytes (35). Therefore, mode of action on anti-obesity of BTD-1 was postulated that related to suppress ACC and FAS expression which are involved in adipogenesis and lipid metabolism. Several studies have reported that increasing endogenous glucose production correlates closely with elevated levels of 2 important gluconeogenic enzymes, PEPCK and G6Pase (36,37). These statements confirm that the upregulation of these enzymes will lead to the development of diabetes (36,37). In gluconeogenesis, glucose is synthesized by these enzymes (G6Pase and PEPCK) from noncarbohydrate precursors. G6Pase catalyzes dephosphorylation of glucose-6-phosphate to get free glucose to other organs influenced by diabetes, starvation, or prolonged fasting (38). Enhanced activities and expression of G6Pase and PEPCK in the liver have been observed in many diabetic models and are believed to contribute to the increased hepatic glucose levels seen in several diseases (39). Similarly, in the present investigation, DNJ present in BTD-1 improved glucose homeostasis by inhibiting gluconeogenic enzymes. PEPCK and G6Pase were mainly controlled by gene transcription in the liver tissue of the BTD-1-treated groups; protein expression in the BTD-1-treated groups revealed a significant reduction in both of these enzymes. The PEPCK and G6Pase expression levels as shown in Fig. 5, clearly confirm that BTD-1 had a significant role in controlling blood glucose levels in group V mice. In conclusion, BTD-1 effectively reduced body mass and prevented obesity by downregulating the key enzymes involved in glucose metabolism (PEPCK and G6Pase), lipid metabolism (FAS, HMG-CoA, and ACAT), and by upregulating those involved in the antioxidant defense system (SOD, GSH-Px, and CAT), playing a key role in the control of blood glucose and lipid levels in the treated groups. BTD-1 can be used as a potential functional food ingredient. The present study suggests that BTD-1 could be effectively used as an anti-hyperglycemic and antihyperlipidemic agent in the control of obesity and its related secondary complications, mainly diabetes.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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