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Anti-hyperlipidemic effect of soybean extract fermented by *Bacillus subtilis* MORI in *db*/*db* mice

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The purpose of this study was to investigate the anti-hyperlipidemic effect of soy bean extract solution fermented by *Bacillus subtilis* MORI (BTD-1E) in obese *db*/*db* mice. Eight-week-old male *db*/*db* mice were administered 33.3 mg/kg BTD-1E solution orally once a day for four weeks. The BTD-1E group showed significantly lower body weight compared with the db control group ($P<0.05$). The BTD-1E group showed significantly lower serum total cholesterol and LDL cholesterol levels compared with the db control group, respectively ($P<0.05$, $P<0.01$). The BTD-1E group showed significantly decreased liver weight relative to final body weight compared with the db control group ($P<0.01$). After four weeks of BTD-1E administration, lipid droplets in the liver were apparently decreased in the BTD-1E group compared to the db control group. In summary, our results suggest that BTD-1E has an anti-hyperlipidemic effect in the obese mouse model.

Key words: Fermented soybean extract, *Bacillus subtilis* MORI, 1-deoxynojirimycin, lipid-lowering effect, anti-obesity

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For the past decades, obesity has become a serious worldwide health problem largely due to high caloric intake and insufficient exercise [1]. Physiologically obesity is a disorder of energy balance and is fundamentally considered as a disorder of lipid metabolism [2]. It has been reported that obesity has a large effect on lipoprotein metabolism, leading to elevated levels of low-density lipoprotein cholesterol (LDL-C) and total cholesterol (T-CHOL) [3,4]. Many health risks such as heart disease, type 2 diabetes mellitus (T2DM), stroke, hypertension, osteoarthritis, fatty liver disease, and some forms of cancer are observed to be associate with excess weight [5,6]. Life-insurance data and epidemiological studies confirm that increasing degrees of overweight and obesity are important predictors of mortality [6,7]. According to a recent report from the World Health Organization (WHO), it was estimated that 45% of Korean men and 54% of women were overweight in 2005, and the percentages are expected to increase to 66% and 67%, respectively by 2015 [8]. Although diet control and lifestyle changes remain the first steps in obesity management, lifestyle modification (alone) as a treatment for obesity is widely regarded as ineffective [9]. Thus, the use of anti-obesity drugs is potentially important as a supplement to lifestyle modification [10].

At present, several medications are available for obesity treatment: Orlistat, a gastric and pancreatic lipase inhibitor [11]; Sibutramine, a centrally acting monoamine-reuptake inhibitor [12]; Rimonabant, the first endo-cannabinoid (CB_1)-receptor blocker [13]. Despite the

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anti-obesity effects, some critical adverse effects of these medications such as gastro-intestinal effects [14], increases in blood pressure and pulse rate [15], and increased incidence of mood-related disorder are reported [16].

1-deoxynojirimycin (DNJ) is known to be a strong intestinal α-glucosidase inhibitor (AGI) [17]. It has been reported that DNJ is a constituent part of mulberry leaves, dietary mulberry, and is also produced by several *Bacillus* and *Streptomyces* species isolated from soil [18,19]. DNJ potently inhibits α-glucosidase in the small intestine, binding to the active center of α-glucosidase [20]. It has been reported that DNJ reduces atherogenicity of LDL-C in patients with impaired glucose tolerance [21]. Cheik *et al.* have shown that soy product fermented by *Enterococcus faecium* and *Lactobacillus Jugurti* significantly decreases the lipogenesis rate and increases in lipolysis rate in tissue adipocytes and liver in rats with hypercholestrolemic diet [22]. The preventive effect of DNJ and AGI on T2DM has been extensively studied [23-24].

Recently, a paper has reported that a new species isolated from *Bacillus subtilis* MORI produces both AGI and DNJ in high quantities, and is present in traditionally fermented Korean soybean [25]. The DNJ content and AGI activity of *Bacillus subtilis* MORI-fermented soybean extract (BTD-1) were approximately 5-fold higher than those of mulberry leaves [26,27]. Our previous study showed the glucose-lowering effect of BTD-1 in *db/db* mice. However, the hypolipidemic effect of BTD-1 has not been completely studied.

The present study was thus designed to investigate the lipid-lowering effect of water extract of BTD-1 (BTD-1E) in *db/db* mice.

Materials and Methods

Animals

Twelve male *db/db* mice (five weeks old, Central Lab. Animal, Inc., Seoul, Korea) were used after a three weeks acclimatization period. The mice were divided into two groups of six. The db control group was administered with 0.9% saline only, whereas the BTD-1E group was administered with 33.3 mg/kg of BTD-1E solution orally once a day for four weeks. The animals were maintained in the experimental animal center at Hallym University, Korea. The facility was maintained at 22±2°C, in 55±10% relative humidity, and an artificial 12 h light and 12 h dark cycle was maintained. A normal rodent pellet diet (Jeiljedang, Seoul, Korea) and water were supplied *ad libitum*. This animal study was conducted in accordance with the guidelines and the approval of the Institutional Animal Care and Use Committee of Hallym University (Hallym-2010-74).

Preparation of the BTD-1E

BTD-1E was supplied by Biotopia Co. (Chuncheon, Korea). The 5% (w/v) defatted soybean meal was fermented for five days at 37°C by *Bacillus subtilis* MORI (KCCM-10450P), which had been isolated from *chungkookjang*, a Korean traditional food. BTD-1 powder was made into a spray by drying the culture supernatant and the excipient so that they were 1:1 (v/v). Twenty grams of BTD-1 powder was well mixed with 100 mL distilled water. The mixture was extracted for 1 hr at 60°C in a waterbath. Then the reactant was centrifuged at 10,000 g for 10 min. The supernatant of BTD-1E was used in this animal study. The DNJ component of BTD-1 powder is 0.944±0.04% (w/w). The α-glucosidase inhibitor unit of BTD-1 was 593,200 GIU/g and the 50% inhibitory concentration (IC_{50}) was 1.7 μg/mL. The dosage of the active ingredients in BTD-1E was considered with the differences between human (60 kg adult) and mouse metabolic in mind. The dosage of oral administration of BTD-1E was 2,000 GIU/kg (mouse body weight) of GIU and the DNJ concentration was 0.37 mg/kg.

Measurement of body weight and diet intake

Throughout the four week experimentation period, body weight and food intake were measured weekly.

Serum lipids analyses

Blood was collected from the orbital plexus after an overnight fast (15 h), weekly. We measured T-CHOL, LDL-C, HDL cholesterol (HDL-C), and triglyceride (TG). For these measurements we used a biochemical analyzer (Kornelab20XT, Thermo, Espoo, Finland) with commercial reagents from Thermo Fisher Scientific (Espoo, Finland).

Organ weight

Organ weights, including that of the liver, kidney, testis, spleen, and epiderminal fats (EPDF) were carefully separated and weighed. The relative organ weight (%) was calculated based on final body weight.

Histopathological analysis by Oil Red O staining in liver

At the end of the experiment, livers were collected from mice under anesthesia with diethyl ether after 15 h of fasting. The livers were fixed in 4% paraformaldehyde for 24 h. The fixed livers were then transferred to a 20% sucrose solution for 48 h. Then the livers were treated with OCT compound for 24 h. The tissues were processed routinely for frozen-sectioned 10 μm in thickness and stained with Oil Red O solution (O-0625, SIGMA-ALDRICH, St. Louis, USA). For nucleus staining, the tissues were stained with hematoxylin and eosin according to general procedures. Morphology of the livers was examined under an inverted microscope (ZEISS, Oberkochen, Germany) and the images were captured using the Axiovision Rel. 4.7 program.

Statistical analysis

Data are presented as the mean±SDM. To determine statistical significance, data were analyzed using student's *t* test. A value of $P<0.05$ was considered to be statistically significant.

Results

Change of body weight and food consumption

Body weight and food consumption were measured once a week during the experimental period. BTD-1E treatment significantly decreased mean body weight in the mice throughout the experimental period compared with that of db control group ($P<0.05$) (Figure 1A). Average food consumption was slightly higher in the BTD-1E group compared with the db control group (Figure 1B). The difference in the food consumption of the mice treated with BTD-1E was not significant when compared with that of the db control group.

Serum lipid profiles

The contents of various types of lipid in serum including T-CHOL, LDL-C, HDL-C, and TG were analyzed in the *db/db* mice treated with the BTD-1E solution. At 3 and 4 weeks, the BTD-1E group showed significantly lower T-CHOL levels: 74.6 and 64.8% of that of the db control group, respectively ($P<0.05$) (Figure 2A). At 2, 3, and 4 weeks, the BTD-1E group showed significantly lower LDL-C levels: 63.4, 39.4, and 64.8% of the db control group, respectively ($P<0.05$, $P<0.01$, $P<0.01$) (Figure 2B). The HDL-C level was slightly lower in the BTD-1E group compared with the db control group (Figure 2C). TG level was slightly higher in the BTD-1E group compared with the db control group (Figure 2D). The differences in the HDL-C and TG levels of the mice treated with BTD-1E were not significant when compared with the db control group.

Relative organ weights

Changes of liver, spleen, testis, kidney, and EPDF

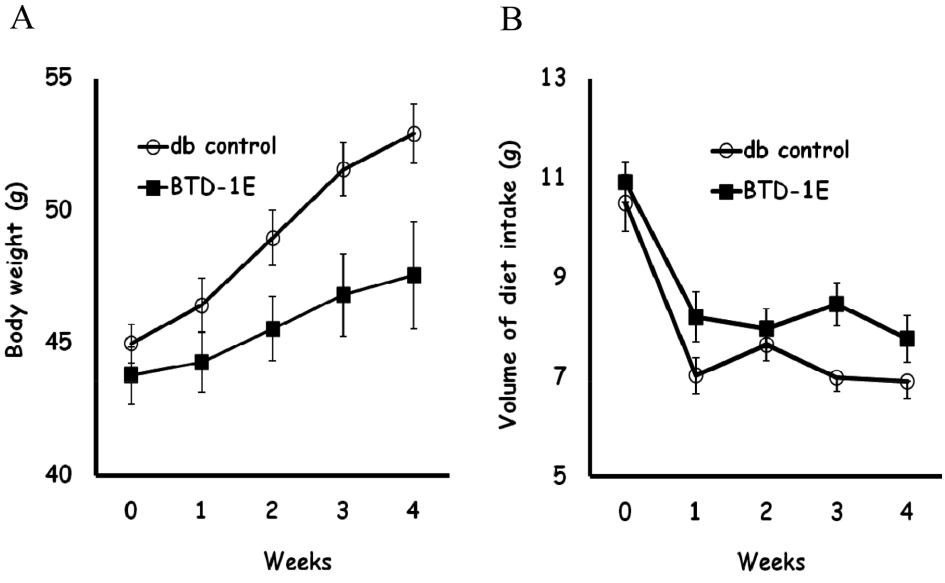

Figure 1. Effects of BTD-1E on body weight and diet intake. (A) body weight, and (B) dietary consumption after administration of BTD-1E in *db /db* mice. Data represent the means±SD (n=6). *$P<0.05$ as compared to the db control group.

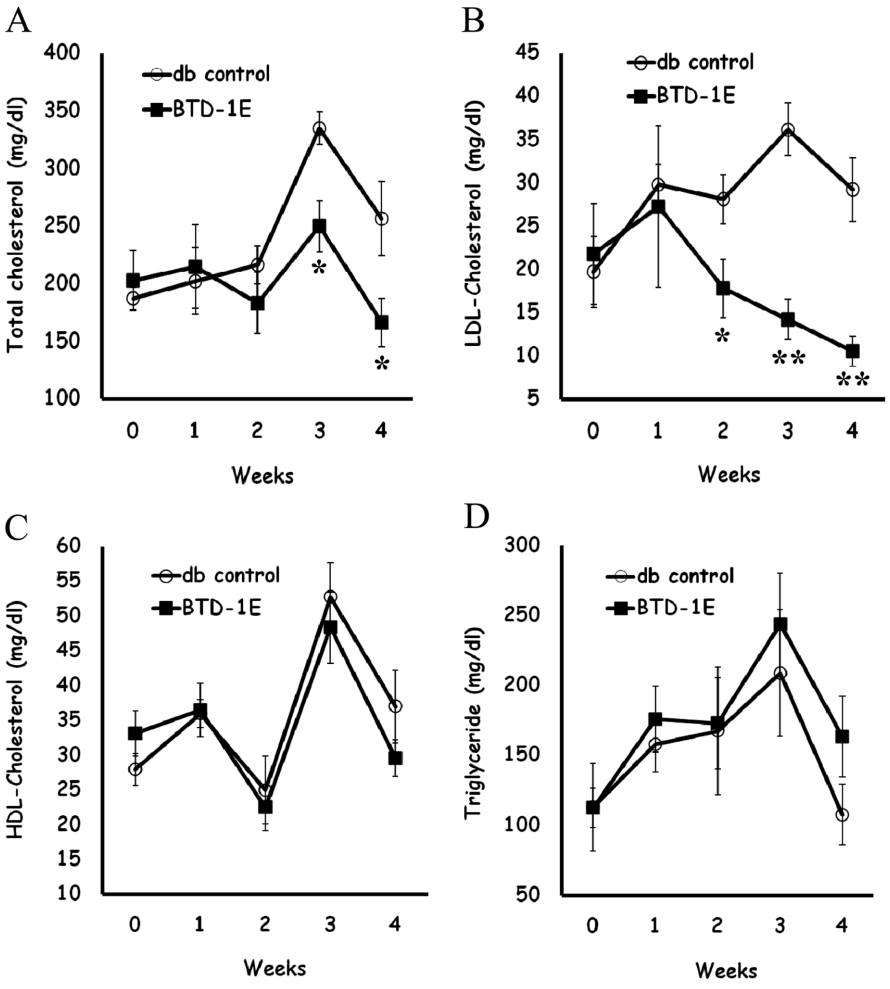

Figure 2. Effects of BTD-1E on serum lipid profiles. (A) total cholesterol, (B) LDL cholesterol, (C) HDL-cholesterol, and (D) triglyceride levels. Data represent the means±SD (n=6). *P<0.05 and *P<0.01 as compared to the db control group.

weights were measured in relation to final body weight (Figure 3). The relative final body weights and liver weights of the BTD-1E group were only 88.1 and 79.7% of those of the db control group, respectively (P<0.05, P<0.01) (Figure 3A and 3B). The relative spleen, testis, and kidney weights of the BTD-1E group were 124, 105.7, and 128.2% of those of the db control group, respectively (P<0.01) (Figure 3C-3E). There were no significant differences in EPDF weight among experimental groups (Figure 3F). However, these results show the relative values to body weight, all actual weights of organs in BTD-1E group were lower than those of the *db* control group.

Lipid accumulation in liver

There were no lipid droplets in the livers of the C57BL/6J mice detected by Oil Red O stains (Figure 4A). Large fat droplets were present extensively in the livers of the db control mice (Figure 4B). The lipid droplets were remarkably less in the BTD-1E administered mice compared with the db control mice (Figure 4C).

Discussion

We evaluated the inhibitory effect on lipid metabolism of feeding BTD-1E through the analysis of serum lipid contents and lipid accumulation in the liver in *db/db* mice for four weeks. The major findings of our studies are that BTD-1E decreases body weight due to its effects on lipid metabolism, as a dietary therapy for obesity. Overweight and obesity are usually defined using BMI [28]. Thus, decreases in body weight and body fat are important and are the primary targets in obesity therapy [29,30]. The BTD-1E group showed significantly lower body weight compared with the db control group. This

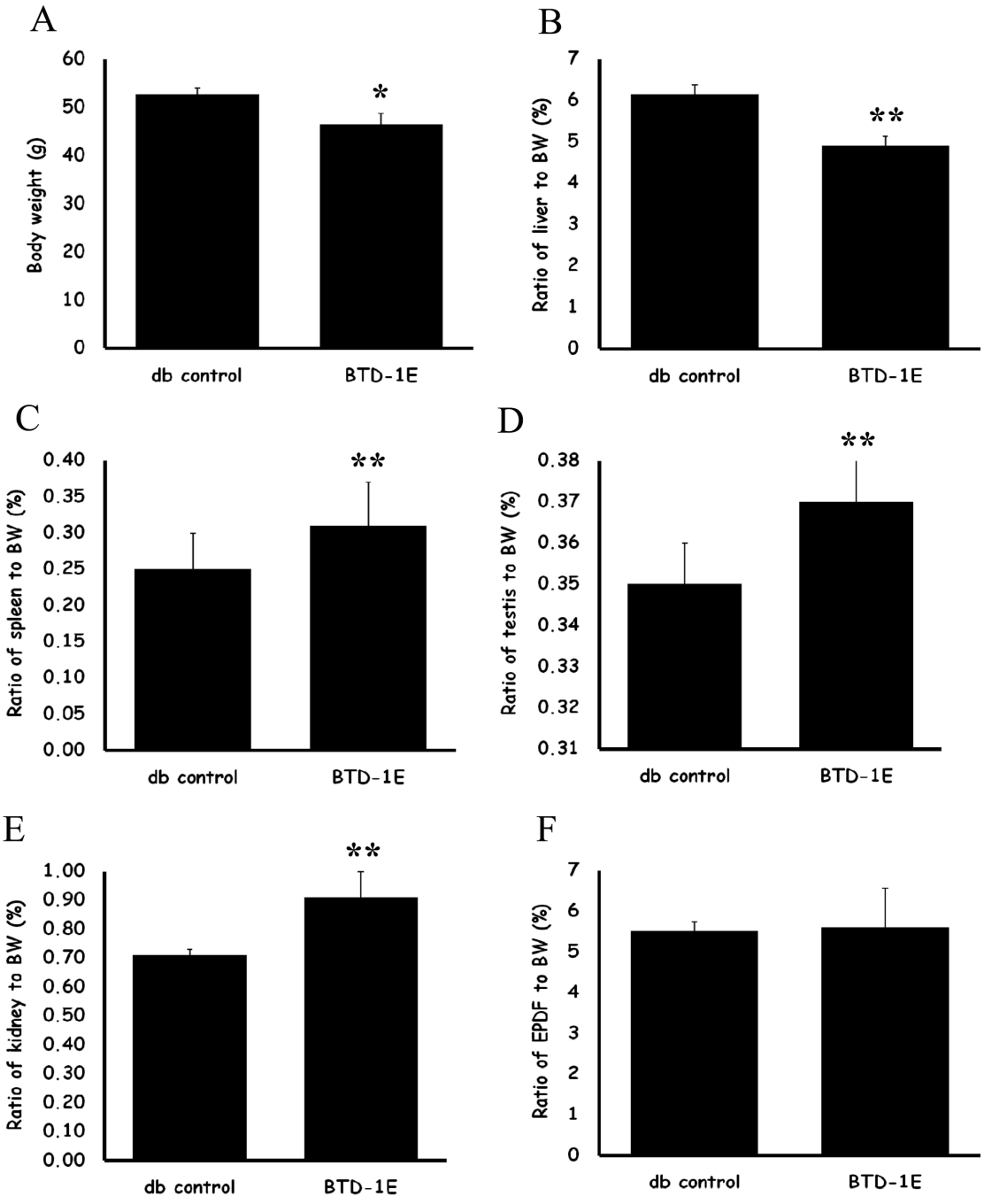

Figure 3. Effects of BTD-1E on relative organ weight. (A) final body weight, (B) relative liver weight: (liver weight/final body weight) X 100%, (C) relative spleen weight, (D) relative testis weight, (E) relative kidney weight, and (F) relative epidermal fat weight. Data represent the means±SD (n=6). **P*<0.05 and **P*<0.01 as compared to the db control group.

anti-hyperlipedemic effect was not associated with the inhibition of diet consumption because the BTD-1E group showed a slightly higher level of diet consumption compared with the db control group.

Body weight is usually related with lipid metabolism. Thus we analyzed the contents of serum lipid after BTD-1E treatment. In the present study, BTD-1E resulted in remarkably lower serum T-CHOL and LDL-C levels compared with the db control group. These results show that BTD-1E can regulate lipogenesis and lipolysis to maintain serum T-CHOL and LDL-C levels in the blood of the mice. It was reported that *Monascus*-fermented soybean extract reduced T-CHOL and LDL-C levels in rats fed a high cholesterol diet [31]. In several studies, a

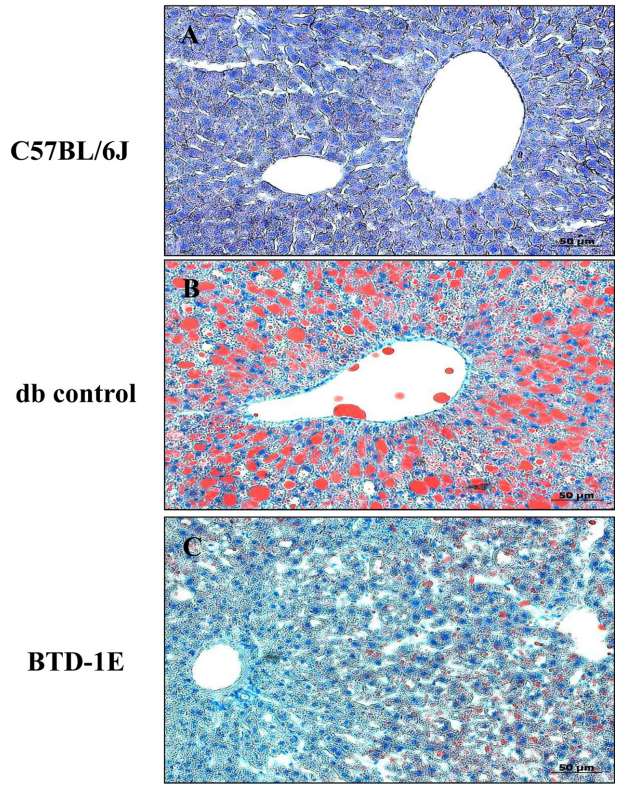

Figure 4. Effect of BTD-1E on fat accumulation in the liver. (A) C57BL/6J, (B) db control, and (C) BTD-1E treated groups. Many fat droplets were diffusely present in the liver of the db control mice. In contrast, the fat droplets were remarkably decreased in the BTD-1E administered mice. Oil red O (red) and hematoxylin-eosin stain (blue).

small increase of HDL-C is observed after feeding soybean or isoflavones [32], whereas in others no change or a small decrease in HDL-C is observed [33]. Yousef *et al.* reported HDL-C increased in a dose dependent manner [33]. This study was performed only for four weeks. A long term study is required to elucidate the mechanisms underlying the improvement effect on HDL-C levels by BTD-1E. TG levels usually change according to diet consumption. Since BTD-1E group showed slightly higher diet consumption compared with the db control group, thus TG levels of BTD-1E group slightly higher than db control group.

Lipid accumulation in adipocytes is determined by a balance of lipogenesis and lipolysis [34]. In this study, the liver weight of the BTD-1E group in relation to final body weight was significantly lower when compared with that of the db control group. In the liver, lipid droplets were obviously less common in the BTD-1E administered mice compared with the db control mice. Hepatomegaly and increases in the hepatic lipid accumulation are commonly encountered in obese and diabetic animals with hyperinsulinemia [34]. A long term study is required to explain clearly the mechanisms underlying the effect on organ weights, specially EPDF, by BTD-1E.

In conclusion, our results suggest that BTD-1E has the beneficial effect of preventing obesity in *db/db* mice. BTD-1E administration resulted in lower body weight, serum lipid levels, liver weight, and the deposition of neutral lipid in the liver without appetite modulation in *db /db* mice. BTD-1E thus might be used for prevention and/or therapy for overweight, obesity, and further cardiovascular diseases related with hyperlipidemia.

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