Effects of dietary fat energy restriction and fish oil feeding on hepatic metabolic abnormalities and insulin resistance in KK mice with high-fat diet-induced obesity

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Abstract

We investigated the effects of dietary fat energy restriction and fish oil intake on glucose and lipid metabolism in female KK mice with high-fat (HF) diet-induced obesity. Mice were fed a LSO50 diet consisting of 50 energy% (en%) lard/safflower oil as the fat source for 12 weeks. Then, the mice were fed various fat energy restriction (25 en% fat) diets—LSO, FO2.5, FO12.5, or FO25—containing 0, 2.5, 12.5, or 25 en% fish oil (FO), respectively, for 9 weeks. Conversion from a HF diet to each fat energy restriction diet significantly decreased final body weights and visceral and subcutaneous fat mass in all fat energy restriction groups, regardless of fish oil contents. Hepatic triglyceride and cholesterol levels markedly decreased in the FO12.5 and FO25 groups, but not in the LSO group. Although plasma insulin levels did not differ among groups, the blood glucose areas under the curve in the oral glucose tolerance test were significantly lower in the FO12.5 and FO25 groups. Real-time polymerase chain reaction analysis showed fatty acid synthase mRNA levels significantly decreased in the FO25 group, and stearoyl-CoA desaturase 1 mRNA levels markedly decreased in the FO12.5 and FO25 groups. These results demonstrate that body weight gains were suppressed by dietary fat energy restriction even in KK mice with HF diet-induced obesity. We also suggested that the combination of fat energy restriction and fish oil feeding decreased fat droplets and ameliorated hepatic hypertrophy and insulin resistance with suppression of de novo lipogenesis in these mice.

1. Introduction

Excess dietary energy and fat intake increases de novo lipid synthesis and causes obesity, which leads to hyperlipidemia, diabetes, and hypertension, often resulting in coronary heart disease or stroke [1-3]. Adipose tissue mass increase and adipose cell hypertrophy in obesity cause abnormal secretion of adipocyte-derived hormones such as adiponectin, leptin, tumor necrosis factor α (TNFα), and resistin [4-5]. In several studies, subjects with obesity and diabetes showed decreased plasma adiponectin levels and increased leptin and TNFα levels [6-8]. Adiponectin stimulates AMP-activated protein kinase (AMPK) activation, inhibits gluconeogenesis, and increases glucose uptake and fatty acid oxidation, thus enhancing insulin sensitivity [9-11]. We previously demonstrated that fish oil (FO) feeding increased plasma adiponectin levels and decreased plasma insulin and leptin levels, leading to ameliorated insulin sensitivity in female C57BL/6J and KK mice [12, 13]. FO contains n-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and its ingestion reduces plasma and hepatic lipid levels [14, 15]. FO-induced lipid-lowering actions have been shown to cause lipogenesis inhibition and fatty acid oxidation stimulation in the liver [16-18]. The synthesis of fatty acids and cholesterol is mainly regulated by sterol regulatory element-binding proteins (SREBPs), which are transcription factors of genes related to lipogenesis [19]. FO feeding decreases SREBP1c mRNA expression and/or mature protein, and results in the inhibition of SREBP-1 target genes such as those that encode acetyl-CoA carboxylase, fatty acid synthase (FAS), and stearoyl-CoA desaturase 1 (SCD-1) [18, 20]. On the other hand, fatty acid oxidation is stimulated by FO intake and caused by activation of peroxisome proliferator-activated receptor (PPAR)α, a nuclear receptor that regulates the target genes for acyl-CoA oxidase (AOX) and uncoupling protein 2 (UCP-2) [20, 21]. In previous studies, we suggested the changes in these mRNA expressions by FO differed in female C57BL/6J or KK mice [12, 13].

In 20–25 energy% (en%) FO feeding, hepatic SREBP-1 mRNA levels did not change in C57BL/6J mice but decreased in KK mice. Insulin-induced gene 1 (Insig-1) and FAS mRNA levels significantly decreased in both FO-fed C57BL/6J and KK mice. However, body weight gains and fat accumulations were markedly decreased with FO feeding in KK mice but not in C57BL/6J mice. These data imply that the effects of FO on lipid metabolism vary considerably with physical conditions such as the degrees of obesity. Therefore, we evaluated the potential beneficial effects of FO feeding with dietary fat energy restriction on glucose and lipid metabolism in female KK mice with high-fat (HF) diet-induced obesity.

2. Materials and Methods

2.1. Mice and diets

Female KK mice were obtained from Tokyo Laboratory Animals Science Co. (Tokyo, Japan) at 5 weeks of age and fed a standard pelleted diet (CE2; Clea, Tokyo, Japan) for 1 week to acclimate. Mice were exposed to a 12-h light–dark cycle and maintained at a constant temperature of $22 \pm 2°C$ and humidity of $55 \pm 10\%$. To induce obesity, the mice were fed a HF diet consisting of 50 en% lard/safflower oil (LSO50) as the fat source for 12 weeks. Then, the mice with HF diet-induced obesity were divided into 5 groups (n = 5 in each group). For another 9 weeks, 1 group was maintained with LSO50 feeding, and the other 4 groups were fed various 25 en% fat diets—LSO, FO2.5, FO12.5, or FO25—containing 0, 2.5, 12.5, or 25 en% FO (fish oil), respectively. The composition of experimental diets is summarized in Table 1. Lard/safflower oil, which was a mixture with the six to four ratio of lard and safflower oil, was used as a base oil of all diets. Lard (Oriental Yeast, Tokyo, Japan) contained 44% oleic acid (18:1, n-9), 24% palmitic acid (16:0), and 14% stearic acid (18:0) as the main fatty acids; safflower oil (Benibana Foods, Tokyo, Japan) contained 78% oleic acid and 14% linoleic acid (18:2, n-6); and fish oil (NOF Co., Tokyo, Japan) contained about 7% EPA (20:5, n-3) and 24% DHA (22:6, n-3). All diets were made into a soft pellet with water and stored at $-30°C$ until each meal is supplied at 10:00 AM daily freshly. The mice were allowed free access to water and feed. During the 9 weeks, body weight was measured weekly and food intake was recorded daily. Food intake energy was represented by the daily mean intake calculated from total intake energy in the feeding period for 9 weeks. The animal experiments were approved by the Institutional Animal Care and Use Committee of Josai University.

2.2. CT scan analysis

At the end of the experiments, the mice were fasted for 3 hours and anesthetized by intraperitoneal injection of pentobarbital sodium (Nembutal; Dainippon Sumitomo Pharma, Osaka, Japan). The body composition of the abdomens of mice was radiographically examined using computed tomography (CT) scan for experimental animals in the mouse mode (La Theta LCT100; ALOKA, Tokyo, Japan). Contiguous 2-mm slice images between L2 and L4 were used for quantitative assessment using LaTheta software (version 2.10). Details of the CT-scanning procedures have been described elsewhere [13].

2.3. Collection of blood and tissue samples

After CT scanning, blood samples were drawn from the inferior vena cava and treated with EDTA 2Na. The liver, white adipose tissue (WAT) around the uterus, and brown adipose tissue (BAT) from the interscapular region were removed, immediately weighed, froze in liquid nitrogen, and then stored at −80°C. The liver tissue was homogenized with Trizol (Invitrogen, Carlsbad, CA), and RNA was prepared by the method described by Chirgwin et al. [22].

A portion of the liver tissue of each mouse was used for analyzing triglyceride (TG) and total cholesterol (TC) contents. Hepatic lipids were extracted from approximately 100 mg of liver tissue for each mouse in accordance with the method of Folch et al. [23]. For the measurements of triglyceride and total cholesterol in the liver, Wako Triglyceride E-Test and Cholesterol E-Test kits (Wako Pure Chemical Industries, Osaka, Japan) were used, respectively. Plasma TG, TC, and HDL cholesterol (HDL-C) levels were measured using the same E-Test Wako kits. Plasma insulin and leptin levels were quantified by enzyme-linked immunosorbent assays (ELISA) using the Insulin ELISA kit and the Leptin/mouse ELISA kit, respectively (Morinaga Institute of Biological Science, Tokyo, Japan). Plasma adiponectin

levels were quantified using the mouse/rat adiponectin ELISA kit (Otsuka Pharmaceutical, Tokyo, Japan).

2.4. Liver histopathology

A piece of liver tissue was excised from the median lobe of the liver. Samples from five mice were collected for each group. The liver tissues were immersed in 10% formaldehyde and embedded in paraffin, cut into sections, stained with hematoxylin eosin (H&E), and examined under a microscope.

2.5. Measurement of mRNA in the liver

mRNA levels were quantified by real-time reverse transcription polymerase chain reaction (RT-PCR) using a sequence detector by the cycle number (Ct) for threshold signal detection. PCR was performed using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) using a QuantiTect SYBR Green Real-time PCR kit (QIAGEN, Hilden, Germany) according to the manufacture's instruction. The thermal cycling conditions were as follows: reverse transcription at 50°C for 30 min, PCR initial activation at 95°C for 15 min plus 40 cycles of denaturation at 94°C for 15 s, annealing at the optimum temperature of each primer for 30 s, and extension at 72°C for 1 min. The PCR primer sequences were showed in Table 2.

2.6. Oral glucose tolerance test (OGTT)

At 25 weeks of age, OGTTs were performed by gavage administration of 1g glucose/kg body weight after 4 hours fasting. Blood samples were collected from the tail vein before glucose administration (time 0) and at 30, 60, 90, and 120 min afterward. Glucose levels were determined by a blood glucose monitoring system (One Touch, Lifescan, Milpitas, CA).

2.7. Statistical analysis

Data from multiple groups were compared by one-way or two-way analysis of variance (ANOVA). Each group was compared with the others using Fisher's protected least significant difference test (SYSTAT 11; Systat Software, Chicago, USA). Values were reported as the mean ± S.D. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Fat energy restriction decreased body weight and adipose tissue mass in female KK mice with HF diet-induced obesity

Body weights in the LSO50 group continued to increase until the end of the experiment. In the LSO and all FO groups that switched to fat energy restriction (25 en% fat) diets, body weights were decreased, although these changes were similar between the LSO and all FO groups. And, there were significant differences in time effect and in interaction between diet and time ($P < 0.05$, two-way ANOVA) (Fig. 1A). Although liver weights did not differ between the LSO50 and LSO groups, they significantly decreased in the FO12.5 and FO25 groups and also showed a decreasing tendency ($P = 0.066$) in the FO2.5 group compared with the LSO group. There was no difference in parametrial WAT weights between the LSO50 and LSO groups or between the LSO and all FO groups. Interscapular BAT weights decreased significantly in the LSO group compared with the LSO50 group (Table 3). Meanwhile, on CT scan analysis, visceral and subcutaneous fat mass significantly decreased in the LSO group compared with the LSO50 group (Fig. 2). There were no differences in interscapular BAT weights and visceral and subcutaneous fat mass between the LSO and all FO groups.

3.2. FO feeding decreased blood glucose levels in female KK mice with HF diet-induced obesity

To investigated the effects of FO and fat energy restriction in acquired-cause induced obesity, we used KK mice with high-fat diet induced obesity, insulin resistance and hyperlipidemia. Body weights and insulin levels of KK mice used in this study were higher than other KK or KK-Ay mice [13, 24, 25].

Although there was no difference in the blood glucose levels between the LSO50 and LSO groups, the levels significantly decreased in the FO25 group compared with the LSO group. Plasma insulin levels did not differ among groups. However, plasma adiponectin levels significantly decreased in the FO2.5 group compared with the LSO group. Plasma leptin levels significantly decreased in the FO2.5 and FO12.5 groups compared with the LSO group (Table 3). To examine the effects of FO on glucose clearance in obese KK mice, the oral glucose tolerance test (OGTT) was performed. In the blood glucose levels, there were significant differences in effects of diet, time and interaction between diet and time ($P < 0.05$, two-way ANOVA) (Fig. 1B). The blood glucose area under the curve (AUC) values did not vary in the LSO group compared with the LSO50 group. However, blood glucose levels at the 30, 60, 90 and 120 min. were low in the FO12.5 and FO25 group compared with the LSO group (Fig. 1B) and the glucose AUCs significantly decreased by 88% and 72% in the FO12.5 and FO25 groups, respectively, compared with the LSO group (Fig. 1C).

3.3. FO feeding decreased hepatic triglyceride and total cholesterol levels

To elucidate the effects of FO and fat energy restriction in obese female KK mice, we measured plasma and hepatic lipid levels, and the results are shown in Table 4.

The plasma TG levels significantly increased in the FO2.5 group, but there was no difference in the FO12.5 and FO25 groups compared with the LSO group. On the other hand,

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plasma TC and non HDL-C levels decreased in the FO2.5, FO12.5, and FO25 groups compared with the LSO group. Plasma HDL-C levels showed comparable levels in all groups. Hepatic TG contents in the FO12.5 and FO25 groups significantly decreased to about 50% and 30% of that in the LSO group, respectively. Hepatic TC contents showed a significant decrease in the FO2.5, FO12.5, and FO25 groups compared with the LSO group (35%, FO2.5 diet; 18%, FO12.5 diet; 12%, FO25 diet, respectively). These hepatic lipid contents reductions were confirmed in the microscopic images of liver tissue (Fig. 2). In the LSO50 group, the liver tissues showed grossly developed hepatic cell hypertrophy, with all of the hepatic cells filled with numerous fat droplets. Although the liver tissues in the LSO and FO2.5 groups did not show a change in the deposition of fat droplets, the FO12.5 and FO25 groups showed markedly decreased fat droplets, suggesting the effects of FO on inhibition of hepatic lipids accumulation.

3.4. 25 en% FO feeding decreased mRNA levels of fatty acid synthesis-regulating genes

The hepatic mRNA levels of lipid metabolism-regulating genes are shown in Table 5. The LSO50 group showed significantly decreased SREBP-1c, Insig-1, and FAS mRNA levels but no change in SCD-1 or Insig-2a mRNA levels compared with the LSO group. There were no significant differences in SREBP-1c mRNA levels among the 25 en% fat diets groups; however, the FO25 group showed a decreasing tendency ($P = 0.061$) compared with the LSO group. In addition, the FO25 group showed significantly decreased FAS, SCD-1, Insig-1 and Insig-2a mRNA levels, whereas the FO12.5 group only showed significantly decreased SCD-1 mRNA levels, compared with the LSO group.

There were no differences in AOX and UCP-1 mRNA levels between the LSO50 and LSO groups. However, mRNA levels of AOX, a target gene of PPARα related to fatty acid oxidation, significantly increased in the FO12.5 group but not in the other FO groups.

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AdipoR2 mRNA levels significantly decreased in all FO groups compared with the LSO group. There were no differences in AdipoR2 mRNA levels between the LSO50 and LSO groups.

4. Discussion

This study showed that a change from a HF diet (50 en% fat) to fat energy restriction (25 en% fat) diets decreased the body weight and visceral and subcutaneous fat mass in female KK mice with HF diet-induced obesity. In addition, a 25 en% fish oil containing diet (FO25) significantly decreased hepatic mRNA levels of fatty acid synthesis-regulating genes compared with the LSO diet. Furthermore, mice fed the FO12.5 and FO25 diets showed smaller fat droplets in the liver tissues than those fed the LSO diet. These tissue specimen results were reflected in the reduced hepatic TG and TC contents. In OGTT, the FO12.5 and FO25 diets decreased plasma glucose AUC compared with the LSO diet.

As a result of the LSO50 diet, body weight of female KK mice increased by about 2.5 fold for 12 weeks leading to obesity (23.6 ± 1.0 g in 6-week-old mice at the starting point, 55.9 ± 4.3 g in 18-week-old mice at the end point). A change to the 25 en% fat diets, such as LSO and all FO diets, for another 9 weeks resulted in significant reductions in final body weight. Also, visceral and subcutaneous fat mass and interscapular BAT weight decreased in the LSO and each FO diet-fed mice. These decreases in final body weight and adipose tissue weight probably were due to the restricted fat energy ratio of 25 en% (Fig. 1A). Meanwhile, all FO diets did not particularly affect body weight reduction in the female KK mice with HF diet-induced obesity. In previous studies, we showed that body weights were markedly decreased in C57BL/6J mice fed 20 en% FO diet and regular KK mice, are free from the prior induction by high-fat diet, fed 25 en% FO diet [13, 26]. These results

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suggested that a 25 en% FO diet in the female KK mice with HF diet-induced obesity did not enough to modify body weight and WAT weight.

Kim et al. [27] reported that body weight and visceral fat mass significantly increased with 36 en% fat diet for 12 weeks in C57BL/6J mice, but the expressions of lipogenesis-regulating genes, such as ACS 2 (acetyl-CoA synthetase 2), FAS and Insig-1, were decreased in 36 en% fat diet-fed mice compared to 17 en% fat diet-fed mice. These results are consistent with our observation that SREBP-1c, Insig-1 and FAS mRNA levels in the liver significantly decreased in the LSO50 diet-fed obese mice compared with the LSO diet-fed mice. These results revealed that fatty acid synthesis is suppressed to maintain the lipid metabolism homeostasis under excess body fat accumulation conditions. In rodent studies, diets containing 25 en% FO or 60 en% FO decreased SREBP-1c mRNA, compared with their respective controls, while 40 en% FO decreased mature SREBP-1 protein but not its mRNA [18, 28-30]. These data suggested that FO is probably associated with modification in the maturation process of the SREBP-1 precursor. Our previous studies with C57BL/6J mice showed that 20 en% FO did not decrease SREBP-1c mRNA levels, but markedly reduced mRNA levels of SREBP-targeting genes such as FAS and SCD-1. Also, FO intake decreased the mRNA levels of Insig-1, which is regulated coordinately with SREBP-1 mature protein levels [26, 31]. In agreement with previous studies, the FO25 group significantly decreased FAS, SCD-1, Insig-1 and Insig-2a mRNA levels compared with those of the LSO group, whereas no difference was observed in SREBP-1c mRNA level.

mRNA levels of fatty acid oxidation-regulating genes, such as AOX and UCP-2, did not change between the LSO50 and LSO groups. AOX and UCP-2 mRNA levels in the liver increased as an adaptive response to the excess fat droplet accumulation in Wistar rats and C57BL/6J mice [32, 33]. Also, AOX and UCP-2 mRNA levels were markedly increased following 30–60 en% FO feeding for 1 week or 60 en% FO feeding for 5 months in

C57BL/6J mice [20, 21]. In the present study, UCP-2 mRNA levels did not show large difference in any of the group, and AOX mRNA levels significantly increased in the FO12.5 group but not in the other FO groups. These results indicate that body weight loss by fat intake restriction in the KK mice with HF diet-induced obesity appears to have occurred independently from the reduction of lipogenesis and the stimulation of fatty acid oxidation. Also, our findings demonstrate that 2.5–25 en% FO intake is not sufficient to induce fatty acid oxidation in KK mice with HF diet-induced obesity.

SCD-1 mainly converts palmitic acid to oleic acid, a monounsaturated fatty acid, and SCD activation induces triglyceride synthesis [34]. SCD-1 deficiency prevented adiposity and hepatic steatosis on diet-induced obesity, and increased insulin sensitivity [35, 36]. In our present study, SCD-1 mRNA levels were significantly decreased in the FO12.5 and FO25 groups, compared with the LSO group. Also, hepatic TG and TC contents significantly decreased in the FO12.5 and FO25 groups. These results suggest that 12.5–25 en% FO intake effectively decreased SCD-1 mRNA levels, resulting in the inhibition of fatty acid synthesis and the amelioration of hepatic lipid accumulation even in the HF diet-induced obesity KK mice. These changes with SCD-1 mRNA reduction might have improved the OGTT levels even without decline of plasma insulin level. Further studies are necessary to determine a link between plasma insulin level, insulin sensitivity and diet-induced obesity.

Muurling et al. [37] showed that a 17 en% fat diet ameliorated insulin resistance and decreased plasma TG levels in C57BL/6J mice, although a 45 en% fat diet worsened insulin resistance. In our previous study, the elevations of plasma insulin level in the regular KK mice were sufficiently inhibited with the 25 en% FO diet, indicating that body weight loss may ameliorate insulin resistance [13]. However, in this study with the HF diet-induced obesity KK mice, the 25 en% FO did not affect plasma insulin level. These data indicate that fat energy restriction and/or 25 en% FO were not sufficient to alter the elevated plasma

insulin levels caused by excessive body weight gain in KK mice with HF diet-induced obesity.

The OGTT at the 7th week from the transition to the experimental diets showed that the FO12.5 and FO25 diets significantly decreased the glucose AUC compared with the LSO diet. Adiponectin secreted from adipocytes stimulates fatty acid oxidation in the liver and induces AMPK activation in muscle, which decreases plasma and hepatic lipid levels, inhibits gluconeogenesis, and improves peripheral uptake of glucose [9, 38]. An elevation of insulin secretion or a decline of insulin sensitivity shows an inverse correlation with plasma adiponectin level or hepatic AdipoR2 mRNA expression [39-42]. Also, an increase of the body mass index and/or body fat suppresses adiponectin secretion [7, 43]. On the other hand, in the insulin-resistant rat, plasma adiponectin increases following treatment with FO [44]. Our previous study also showed that plasma adiponectin levels were significantly increased in 25 en% FO diet-fed KK mice [13]. In our present study with the HF diet-induced obesity KK mice, fat energy restriction resulted in decreased body weight and adipose tissue weight, however plasma adiponectin levels were not increased. Even in 2.5–25 en% FO diets similar results showed, which, caused an inhibition of hepatic AdipoR2 mRNA. Huang et al. [24] reported that exercise training suppressed hepatic AdipoR2 mRNA levels and then enhanced insulin sensitivity; however, plasma adiponectin levels were not changed. These variations might be due to differences between the congenital obesity and the congenital plus diet-induced obesity. We may need to elucidate the details of this obesity models. And, further investigations are required to clarify the relationship among plasma adiponectin, hepatic AdipoR2 mRNA levels and insulin resistance on glucose and lipid metabolism in the various physical conditions.

In conclusion, we have showed that dietary fat restriction is effective in suppressing body weight gain even in the HF diet-induced obesity KK mice, and the combination of dietary fat

energy restriction and fish oil feeding produced a suppression of de novo lipogenesis, resulting in a decrease of hepatic fat droplet and an amelioration of hepatic hypertrophy. Also, 12.5–25 en% FO diets might be effective to improve marked glucose tolerence caused by HF diet -induced obesity. These results may provide data and information useful to understanding the difference of obesity treatments between the congenital obesity and the congenital plus diet-induced obesity.

Acknowledgments

References

[1] Wilson PW, D'Agostino RB, Sullivan L, Parise H, Kannel WB. Overweight and obesity as determinants of cardiovascular risk: the Framingham experience. Arch Intern Med. 2002;162:1867-72.

[2] Gotto AM Jr. Triglyceride as a risk factor for coronary artery disease. Am J Cardiol. 1998;82:22Q-25Q.

[3] Meshkani R, Adeli K. Hepatic insulin resistance, metabolic syndrome and cardiovascular disease. Clin Biochem. 2009;42:1331-46.

[4] Greenberg AS, Obin MS. Obesity and the role of adipose tissue in inflammation and metabolism. Am J Clin Nutr. 2006;83:S461-5.

[5] Kang JH, Goto T, Han IS, Kawada T, Kim YM. Dietary capsaicin reduces obesity-induced insulin resistance and hepatic steatosis in obese mice fed a high-fat diet. Obesity. 2010;18:780-7.

[6] Havel PJ, Kasim-Karakas S, Mueller W, Johnson PR, Gingerich RL, Stern JS. Relationship of plasma leptin to plasma insulin and adiposity in normal weight and overweight women: effects of dietary fat content and sustained weight loss. J Clin Endocrinol Metab. 1996;81:4406-13.

[7] Weyer C, Funahashi T, Tanaka S, Hotta K, Matsuzawa Y, Pratley RE, et al. Hypoadiponectinemia in obesity and type 2 diabetes: close association with insulin resistance and hyperinsulinemia. J Clin Endocrinol Metab. 2001;86:1930-5.

[8] Suzuki R, Watanabe S, Hirai Y, Akiyama K, Nishide T, Matsushima Y, et al. Abdominal wall fat index, estimated by ultrasonography, for assessment of the ratio of visceral fat to subcutaneous fat in the abdomen. Am J Med. 1993;95:309-14.

[9] Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, et al. Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid

oxidation in muscle and causes weight loss in mice. Proc Natl Acad Sci U S A. 2001;98:2005-10.

[10] Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, et al. The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. Nat Med. 2001;7:941-6.

[11] Yamauchi T, Kamon J, Minokoshi Y, Ito Y, Waki H, Uchida S, et al. Adiponectin stimulates glucose utilization and fatty-acid oxidation by activating AMP-activated protein kinase. Nat Med. 2002;8:1288-95.

[12] Arai T, Kim HJ, Chiba H, Matsumoto A. Interaction of fenofibrate and fish oil in relation to lipid metabolism in mice. J Atheroscler Thromb. 2009;16:283-91.

[13] Arai T, Kim HJ, Chiba H, Matsumoto A. Anti-obesity effect of fish oil and fish oil-fenofibrate combination in female KK mice. J Atheroscler Thromb. 2009;16:674-83.

[14] Iso H, Kobayashi M, Ishihara J, Sasaki S, Okada K, Kita Y, et al.; JPHC Study Group. Intake of fish and n3 fatty acids and risk of coronary heart disease among Japanese. The Japan Public Health Center-Based (JPHC) Study Cohort I. Circulation. 2006;113:195-202.

[15] Nestel PJ. Effects of N-3 fatty acids on lipid metabolism. Annu Rev Nutr. 1990;10:149-67.

[16] Halminski MA, Marsh JB, Harrison EH. Differential effects of fish oil, safflower oil and palm oil on fatty acid oxidation and glycerolipid synthesis in rat liver. J Nutr. 1991;121: 1554-61.

[17] Ide T, Kobayashi H, Ashakumary L, Rouyer IA, Takahashi Y, Aoyama T, et al. Comparative effects of perilla and fish oils on the activity and gene expression of fatty acid oxidation enzymes in rat liver. Biochim Biophys Acta. 2000;1485:23-35.

[18] Kim HJ, Takahashi M, Ezaki O. Fish oil feeding decreases mature sterol regulatory

element-binding protein 1 (SREBP-1) by down-regulation of SREBP-1c mRNA in mouse liver. A possible mechanism for down-regulation of lipogenic enzyme mRNAs. J Biol Chem. 1999;274:25892-8.

[19] Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest. 2002;109:1125-31.

[20] Nakatani T, Kim HJ, Kaburagi Y, Yasuda K, Ezaki O. A low fish oil inhibits SREBP-1 proteolytic cascade, while a high-fish-oil feeding decreases SREBP-1 mRNA in mice liver. Relationship to anti-obesity. J Lipid Res. 2003;44:369-79.

[21] Tsuboyama-Kasaoka N, Takahashi M, Kim HJ, Ezaki O. Up-regulation of liver uncoupling protein-2 mRNA by either fish oil feeding or fibrate administration in mice. Biochem Biophys Res Commun. 1999;257:879-85.

[22] Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry. 1979;18:5294-9.

[23] Folch J, Lees M, Sloane Stanley G. H. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem. 1957;226:497-509.

[24] Huang H, Iida KT, Sone H, Yokoo T, Yamada N, Ajisaka R. The effect of exercise training on adiponectin receptor expression in KKAy obese/diabetic mice. J Endocrinol. 2006;189:643-53.

[25] Yamada K, Hosokawa M, Yamada M, Watanabe R, Fujimoto S, Fujiwara H, et al. Dietary corosolic acid ameliorates obesity and hepatic steatosis in KK-Ay mice. Biol Pharm Bull. 2008;31:651-5.

[26] Hirako S, Kim HJ, Arai T, Chiba H, Matsumoto A. Effect of concomitantly used fish oil and cholesterol on lipid metabolism. J Nutr Biochem. 2010;21:573-9.

[27] Kim S, Sohn I, Ahn JI, Lee KH, Lee YS, Lee YS. Hepatic gene expression profiles in a long-term high-fat diet-induced obesity mouse model. Gene. 2004;340:99-109.

[28] Xu J, Nakamura MT, Cho HP, Clarke SD. Sterol regulatory element binding protein-1 expression is suppressed by dietary polyunsaturated fatty acids. A mechanism for the coordinate suppression of lipogenic genes by polyunsaturated fats. J Biol Chem. 1999;274:23577-83.

[29] Mater MK, Thelen AP, Pan DA, Jump DB. Sterol response element-binding protein 1c (SREBP1c) is involved in the polyunsaturated fatty acid suppression of hepatic S14 gene transcription. J Biol Chem. 1999;274:32725-32

[30] Yahagi N, Shimano H, Hasty AH, Amemiya-Kudo M, Okazaki H, Tamura Y, et al. A crucial role of sterol regulatory element-binding protein-1 in the regulation of lipogenic gene expression by polyunsaturated fatty acids. J Biol Chem. 1999;274:35840-4.

[31] Yang T, Espenshade PJ, Wright ME, Yabe D, Gong Y, Aebersold R, et al. Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. Cell. 2002;110:489-500

[32] Chavin KD, Yang S, Lin HZ, Chatham J, Chacko VP, Hoek JB, et al. Obesity induces expression of uncoupling protein-2 in hepatocytes and promotes liver ATP depletion. J Biol Chem. 1999;274:5692-700.

[33] Hsu SC, Huang CJ. Changes in liver PPARalpha mRNA expression in response to two levels of high-safflower-oil diets correlate with changes in adiposity and serum leptin in rats and mice. J Nutr Biochem. 2007;18:86-96.

[34] Flowers MT, Ntambi JM. Stearoyl-CoA desaturase and its relation to high-carbohydrate diets and obesity. Biochim Biophys Acta. 2009;1791:85-91.

[35] Miyazaki M, Flowers MT, Sampath H, Chu K, Otzelberger C, Liu X, Ntambi JM. Hepatic stearoyl-CoA desaturase-1 deficiency protects mice from carbohydrate-induced adiposity and hepatic steatosis. Cell Metab 2007;6:484-96.

[36] Rahman SM, Dobrzyn A, Dobrzyn P, Lee SH, Miyazaki M, Ntambi JM. Stearoyl-CoA

desaturase 1 deficiency elevates insulin-signaling components and down-regulates protein-tyrosine phosphatase 1B in muscle. Proc Natl Acad Sci U S A. 2003;100:11110-15.

[37] Muurling M, Jong MC, Mensink RP, Hornstra G, Dahlmans VE, Pijl H, et al. A low-fat diet has a higher potential than energy restriction to improve high-fat diet-induced insulin resistance in mice. Metabolism. 2002;51:695-701.

[38] Bueno AA, Oyama LM, de Oliveira C, Pisani LP, Ribeiro EB, Silveira VL, et al. Effects of different fatty acids and dietary lipids on adiponectin gene expression in 3T3-L1 cells and C57BL/6J mice adipose tissue. Pflugers Arch. 2008;455:701-9.

[39] Tsuchida A, Yamauchi T, Ito Y, Hada Y, Maki T, Takekawa S, et al. Insulin/Foxo1 pathway regulates expression levels of adiponectin receptors and adiponectin sensitivity. J Biol Chem. 2004;279:30817-22.

[40] Pajvani UB, Du X, Combs TP, Berg AH, Rajala MW, Schulthess T, et al. Structure-function studies of the adipocyte-secreted hormone Acrp30/adiponectin. Implications fpr metabolic regulation and bioactivity. J Biol Chem. 2003;278: 9073-85.

[41] Möhlig M, Wegewitz U, Osterhoff M, Isken F, Ristow M, Pfeiffer AF, et al. Insulin decreases human adiponectin plasma levels. Horm Metab Res. 2002;34: 655-8.

[42] Fasshauer M, Klein J, Neumann S, Eszlinger M, Paschke R. Hormonal regulation of adiponectin gene expression in 3T3-L1 adipocytes. Biochem Biophys Res Commun. 2002;290:1084-9.

[43] Hosoda K, Masuzaki H, Ogawa Y, Miyawaki T, Hiraoka J, Hanaoka I, et al. Development of radioimmunoassay for human leptin. Biochem Biophys Res Commun. 1996;221:234-9.

[44] Rossi AS, Lombardo YB, Lacorte JM, Chicco AG, Rouault C, Slama G, et al. Dietary fish oil positively regulates plasma leptin and adiponectin levels in sucrose-fed,

insulin-resistant rats. Am J Physiol Regul Integr Comp Physiol. 2005;289:R486-94.

Legends for figures

Fig. 1

Growth curves and glucose level changes during the oral glucose tolerance tests (OGTTs) in obese KK mice treated with dietary fat energy restriction and/or fish oil.

Growth curves (A), the time course of blood glucose levels (B), and glucose area under the curve(C) during OGTT. Growth curves were plotted until 8 weeks. OGTT was performed at the 7th week of treatment with the experimental diets. After 4 h of fasting, OGTT was performed by administration of a 10% glucose solution (1g/kg). Blood samples were obtained immediately before glucose administration and 30, 60, 90, and 120 min after glucose loading. Values represent the mean ± S.D. (n = 5). Two-way ANOVA P values are shown in parentheses with D, diet effect; T, time effect; D×T, interaction between diet and time. (A) * means $P < 0.05$ versus the LSO50 group. (C) Groups with different letters are significantly different. ANOVA with Fisher's protected least significant difference (PLSD) test, $P < 0.05$.

Fig. 2

CT-based body fat composition and the alteration of hepatic condition in obese KK mice treated with dietary fat energy restriction and/or fish oil for 9 weeks.

CT images (A), visceral fat (B), subcutaneous fat (C), liver megascopic condition (D, upper) and histopathology (D, lower). In CT images, purple and yellow areas represent visceral and subcutaneous fat, respectively. Liver tissues from each group embedded in paraffin, stained with hematoxylin and eosin (H&E), and examined under a microscope at 400-fold magnification (D, lower). Values represent the mean ± S.D. (n = 5). Groups with different letters are significantly different. ANOVA with Fisher's protected least significant difference (PLSD) test, $P < 0.05$.
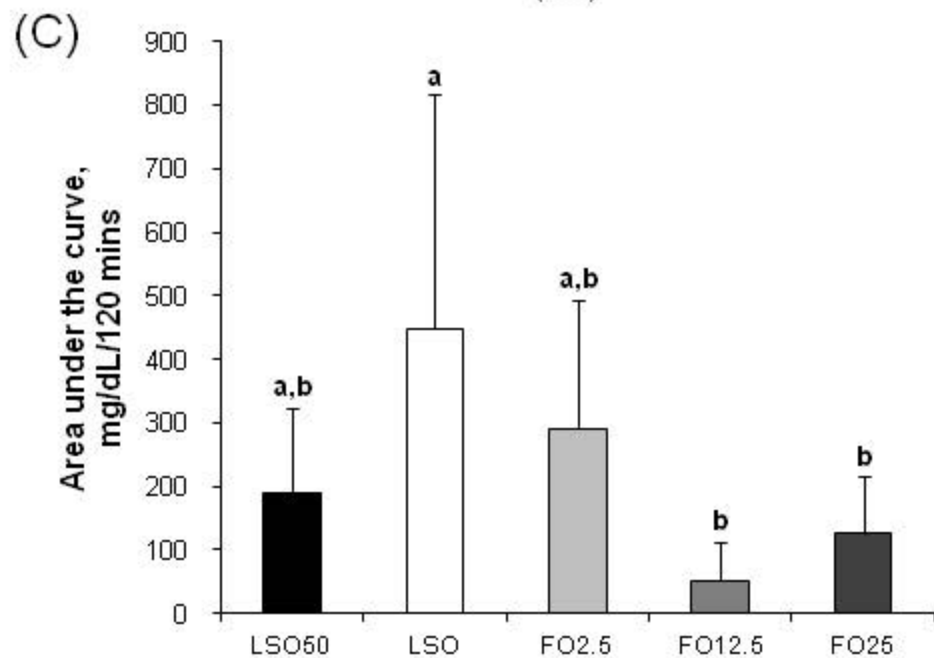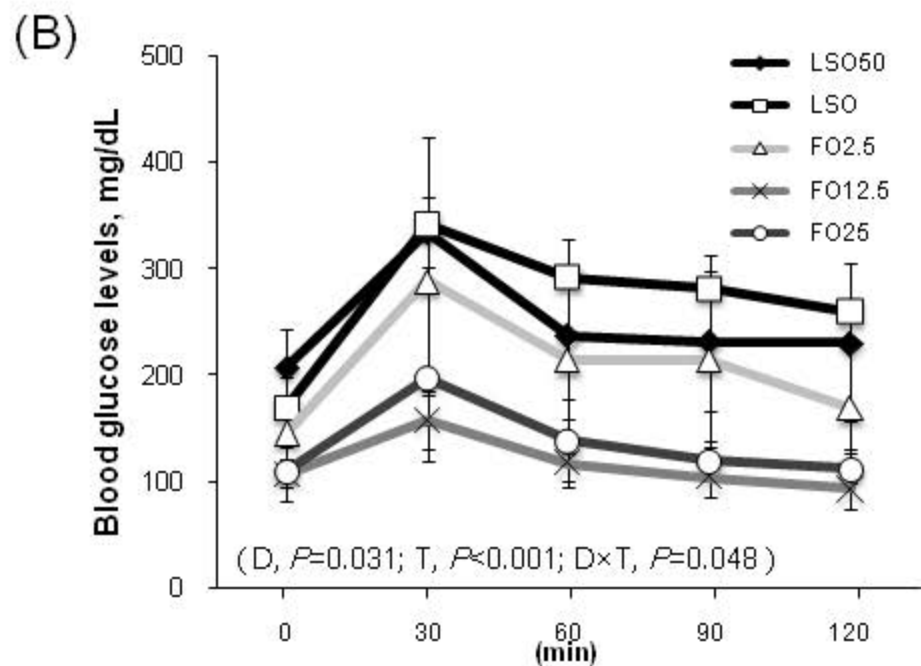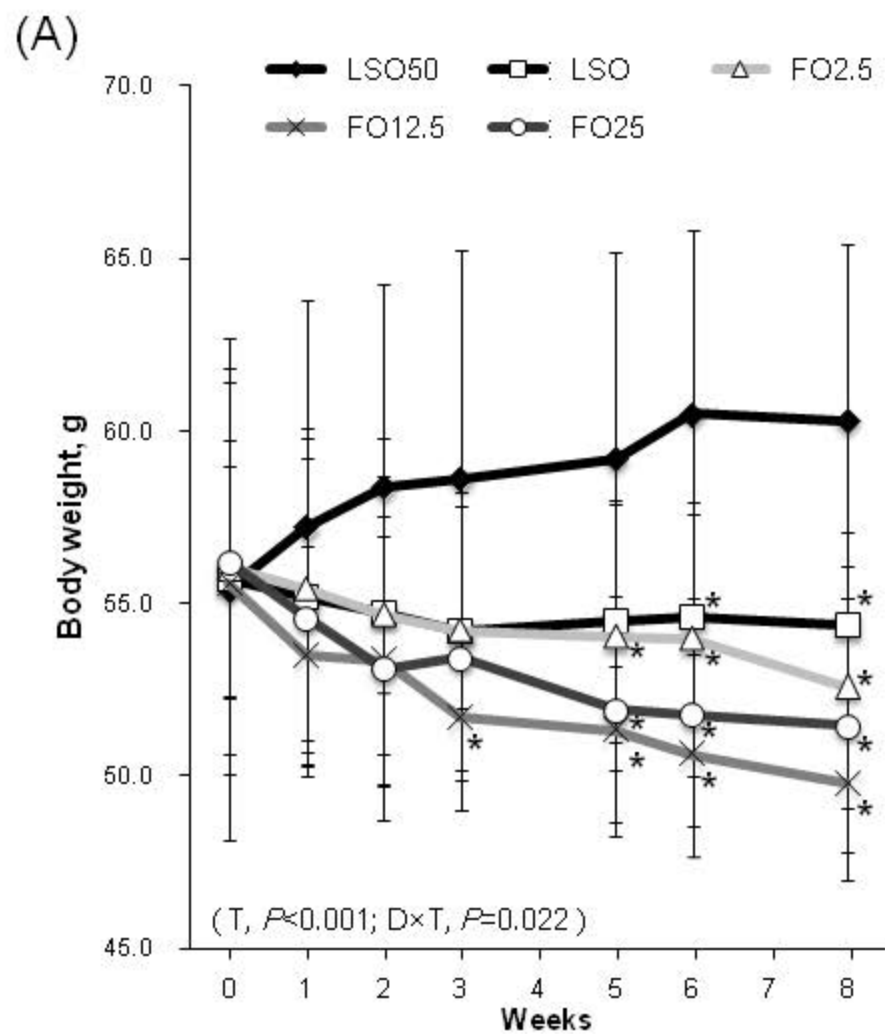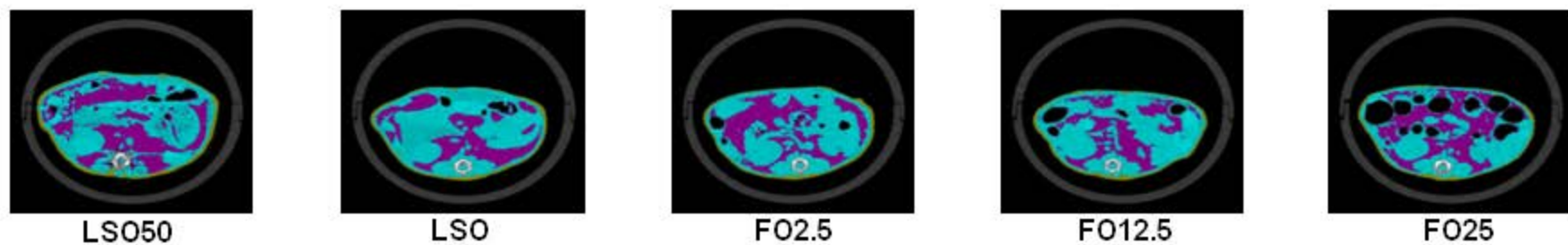
22

Fig. 1

Fig.2

(A)

LSO50 LSO FO2.5 FO12.5 FO25

(B)

(C)

(D)

LSO50 LSO FO2.5 FO12.5 FO25
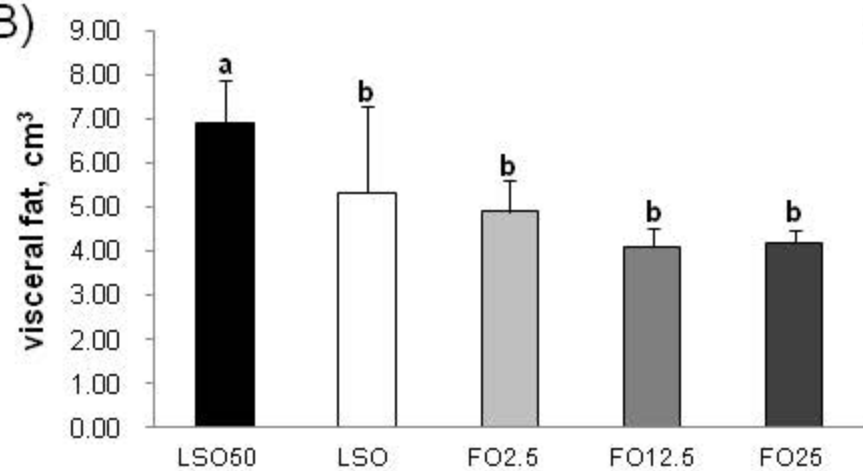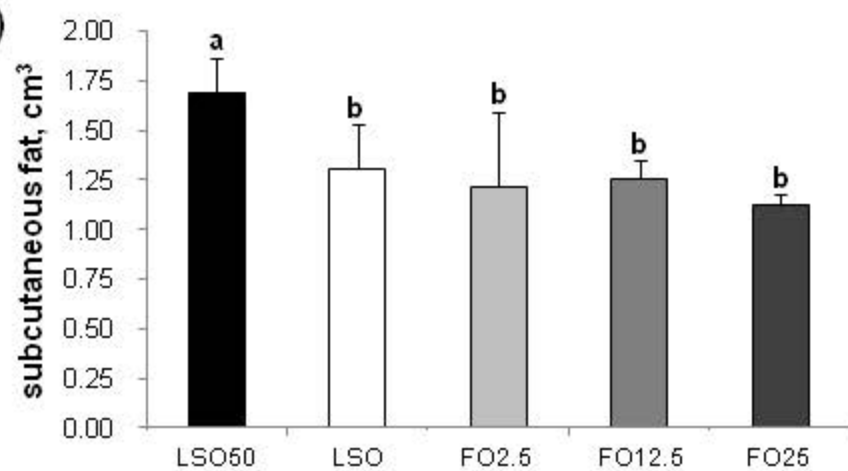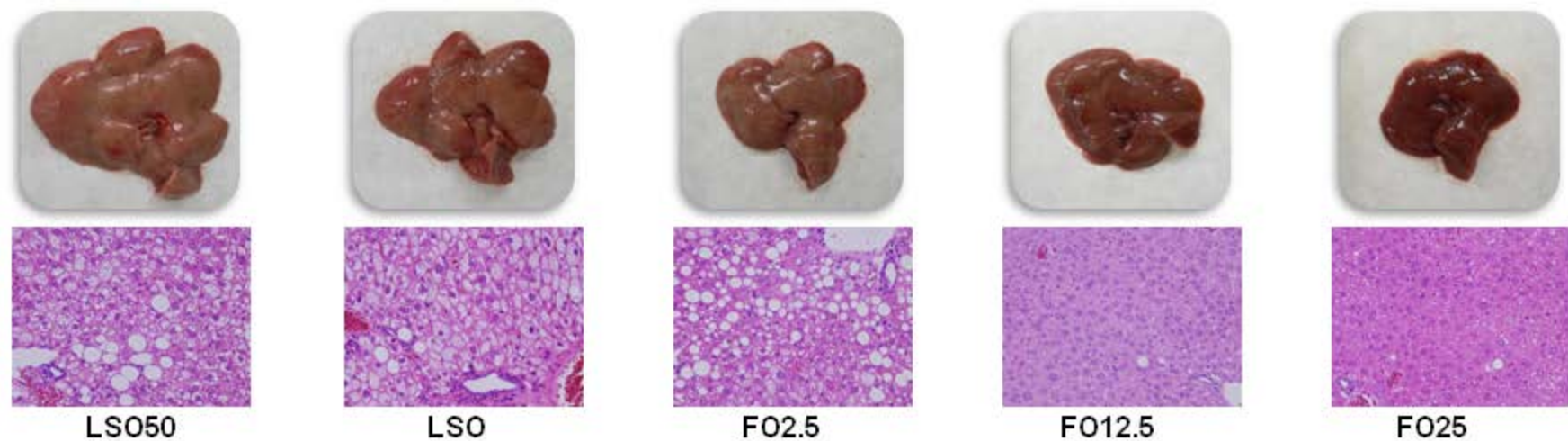
Table 1. Composition of the experimental diets

Ingredient	LSO50	LSO	FO2.5	FO12.5	FO25
Lard	10.00	4.20	3.78	2.10	
Safflower oil	15.00	6.30	5.67	3.15	
Fish oil			1.05	5.25	10.50
Casein	25.00	21.00	21.00	21.00	21.00
Sucrose	12.50	10.50	10.50	10.50	10.50
β-starch	24.93	47.45	47.45	47.45	47.45
Vitamin mix	1.56	1.31	1.31	1.31	1.31
Mineral mix	4.38	3.68	3.68	3.68	3.68
Cellulose powder	6.25	5.25	5.25	5.25	5.25
L-cystine	0.38	0.32	0.32	0.32	0.32
t-Butylhydroquinone	0.0050	0.0021	0.0021	0.0021	0.0021
Total (g)	100.0	100.0	100.0	100.0	100.0
Total energy(kcal/100g)	464.8	386.7	386.7	386.3	386.0
Fat energy(kcal/100g)	232.3	97.6	97.5	97.1	96.7
Fat energy(%)	50.0	25.2	25.2	25.1	25.1

Abbreviations: LSO, lard /safflower oil; FO, fish oil.

Table 2. Primer sequences for real-time PCR amplification

Genes	Sense (5'→3')	Antisense (3'→5')
SREBP-1c	GGAGCCATGGATTGCACATT	GGCCCGGGAAGTCACTGT
Insig-1	TCACAGTGACTGAGCTTCAGCA	TCATCTTCATCACACCCAGGAC
Insig-2a	CCCTCAATGAATGTACTGAAGGATT	TGTGAAGTGAAGCAGACCAATGT
FAS	TCACCACTGTGGGCTCTGCAGAGAAGCGAG	TGTCATTGGCCTCCTCAAAAAGGGCGTCCA
SCD-1	CCGGAGACCCCTTAGATCGA	TAGCCTGTAAAAGATTTCTGCAAACC
PPARα	GTGGCTGCTATAATTTGCTGTG	GAAGGTGTCATCTGGATGGTT
AOX	TCAACAGCCCAACTGTGACTTCCATTA	TCAGGTAGCCATTATCCATCTCTTCA
UCP-2	GTTCCTCTGTCTCGTCTTGC	GGCCTTGAAACCAACCA
AdipoR2	ACCCACAACCTTGCTTCATCTAC	CCATAAGCATTAGCCAGCCTATC
GAPDH	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGAT

Table 3. Phenotypic comparison in obese female KK mice treated with dietary fat energy restriction and/or fish oil for 9 weeks.

Groups	LSO50	LSO	FO2.5	FO12.5	FO25
Food intake[*] (kcal/mouse/day)	17.92	15.87	16.44	15.42	14.74
Final body weight (g)	58.61 ± 5.31^{a}	53.15 ± 3.54^{b}	51.98 ± 3.68^{b}	49.07 ± 3.18^{b}	50.45 ± 3.36^{b}
Body weight gain (g)	3.22 ± 2.74^{a}	-2.57 ± 5.08^{b}	-4.06 ± 2.17^{b}	-7.13 ± 3.89^{b}	-5.77 ± 2.72^{b}
Liver weight (g)	$3.20 \pm 0.81^{a,b}$	3.85 ± 1.41^{a}	$2.80 \pm 0.87^{a,c}$	$2.14 \pm 0.44^{b,c}$	1.99 ± 0.16^{c}
WAT weight (g)	3.30 ± 0.79^{a}	$2.63 \pm 1.48^{a,b}$	$2.70 \pm 0.34^{a,b}$	2.24 ± 0.38^{b}	$2.48 \pm 0.22^{a,b}$
BAT weight (mg)	462.6 ± 92.6^{a}	365.7 ± 41.2^{b}	278.6 ± 21.3^{c}	$320.6 \pm 31.1^{b,c}$	352.5 ± 58.3^{b}
Blood glucose (mg/dL)	$150 \pm 56^{a,b}$	180 ± 60^{a}	$156 \pm 38^{a,b}$	$148 \pm 25^{a,b}$	117 ± 14^{b}
Plasma insulin (μg/L)	38 ± 25	53 ± 66	29 ± 33	83 ± 64	52 ± 40
Plasma leptin (μg/L)	179 ± 25^{a}	$142 \pm 45^{a,b}$	94 ± 27^{c}	77 ± 25^{c}	$105 \pm 39^{b,c}$
Plasma adiponectin (mg/L)	16.3 ± 4.1^{a}	$13.5 \pm 2.5^{a,b}$	9.5 ± 1.8^{c}	$11.4 \pm 2.4^{b,c}$	$12.1 \pm 2.3^{b,c}$

Abbreviations: LSO, lard/safflower oil; FO, fish oil; WAT, white adipose tissue; BAT, brown adipose tissue. Values represent the mean ± S.D. (n = 4-5). Groups with different letters are significantly different. ANOVA with Fisher's protected least significant difference (PLSD) test, P < 0.05. *means daily food intake energy calculated from total intake energy in the feeding period for 9 weeks.

Table 4. Plasma and hepatic lipid levels in obese female KK mice treated with dietary fat energy restriction and/or fish oil for 9 weeks.

Groups	LSO50	LSO	FO2.5	FO12.5	FO25
Plasma triglyceride (mmol/L)	1.46 ± 0.14^b	1.67 ± 0.14^b	2.50 ± 0.67^a	1.67 ± 0.23^b	1.51 ± 0.42^b
Plasma free fatty acid (mmol/L)	1.32 ± 0.48	1.32 ± 0.45	1.83 ± 0.74	1.54 ± 0.17	1.13 ± 0.22
Plasma total cholesterol (mmol/L)	3.70 ± 0.60^a	3.76 ± 0.65^a	2.85 ± 0.67^b	$2.98\pm0.57^{a,b}$	2.38 ± 0.49^b
Plasma HDL-cholesterol (mmol/L)	1.92 ± 0.48	1.35 ± 0.78	1.50 ± 0.86	1.81 ± 0.75	1.48 ± 0.70
Plasma non HDL-cholesterol (mmol/L)	$1.81\pm0.80^{a,b}$	2.46 ± 0.88^a	1.40 ± 0.62^b	1.24 ± 0.57^b	0.98 ± 0.47^b
Liver triglyceride (μmol/g liver)	104.83 ± 12.70^a	121.57 ± 2.94^a	112.45 ± 10.81^a	75.27 ± 21.23^b	33.81 ± 11.66^c
Liver total cholesterol (μmol/g liver)	$25.91\pm19.20^{a,b}$	44.14 ± 23.69^a	$16.70\pm1.51^{b,c}$	$8.52\pm1.63^{b,c}$	5.57 ± 0.67^c

Abbreviations: LSO, lard/safflower oil; FO, fish oil; HDL, high density lipoprotein. Values represent the mean ± S.D. (n = 4-5). Groups with different letters are significantly different. ANOVA with Fisher's protected least significant difference (PLSD) test, $P < 0.05$.

Table 5. Hepatic mRNA levels of lipid metabolism regulating genes in obese female KK mice treated with dietary fat energy restriction and/or fish oil for 9 weeks.

Groups	LSO50	LSO	FO2.5	FO12.5	FO25
SREBP-1c	0.53±0.08[c]	1.00±0.24[a,b]	1.18±0.38[a]	1.28±0.44[a]	0.63±0.19[b,c]
Insig-1	0.58±0.18[b]	1.00±0.12[a]	0.93±0.31[a]	0.82±0.30[a,b]	0.54±0.09[b]
Insig-2a	0.91±0.26[a]	1.00±0.40[a]	0.51±0.21[b]	0.61±0.40[a,b]	0.14±0.04[c]
FAS	0.52±0.11[b,c]	1.00±0.26[a]	0.71±0.36[a,b]	0.68±0.49[a,b]	0.16±0.04[c]
SCD-1	0.79±0.25[a]	1.00±0.19[a]	0.75±0.28[a]	0.28±0.26[b]	0.05±0.05[b]
PPARα	1.10±0.22[b,c]	1.00±0.10[c]	1.37±0.26[a,b]	1.53±0.27[a]	1.48±0.16[a]
AOX	1.20±0.33[b]	1.00±0.24[b]	1.16±0.15[b]	1.65±0.32[a]	1.26±0.23[b]
UCP-2	0.98±0.40	1.00±0.40	1.10±0.67	0.71±0.15	0.73±0.17
AdipoR2	0.87±0.07[a,b]	1.00±0.17[a]	0.81±0.16[b]	0.77±0.10[b]	0.60±0.10[c]

Abbreviations: LSO, lard/safflower oil; FO, fish oil; SREBP-1c, sterol regulatory element binding protein-1c; Insig-1, insulin-induced gene-1; Insig-2a, insulin-induced gene-2a; FAS, fatty acid synthase; SCD-1, stearoyl-CoA desaturase; PPARα, peroxisome proliferator-activated receptor α; AOX, acyl-CoA oxidase; UCP-2, uncoupling protein-2; AdipoR2, adiponectin receptor 2. mRNA levels were determined by real-time quantitative RT-PCR. Results are expressed as the ratio of the obtained value to that of the LSO group. Values represent the mean ± S.D. (n = 4-5). Groups with different letters are significantly different. ANOVA with Fisher's protected least significant difference (PLSD) test, $P < 0.05$.