Anti-Obesity Effect of Fish Oil and Fish Oil-Fenofibrate Combination in Female KK Mice

Takeshi Arai, Hyoun-ju Kim, Hiroshige Chiba, and Akiyo Matsumoto

Faculty of Pharmaceutical Sciences, Josai University, Saitama, Japan

Aim: The aim of our study is to elucidate the effects of EPA- or DHA-rich fish oil, and of the latter plus fenofibrate, on lipid metabolism in female KK mice.

Methods: Female KK mice were fed purified experimental diets containing lard/safflower oil (4:6, Lard/SO), EPA-rich fish oil (EPA), DHA-rich fish oil (DHA), or DHA-rich fish oil plus 0.2% (w/w) fenofibrate (DHA + FF) for 8 weeks. At the end of the experiments, we measured levels of plasma lipids, hepatic triglycerides, and cholesterol, as well as the hepatic mRNA expression of lipogenic and lipidolytic genes.

Results: The final body weight of EPA- and DHA-fed groups was significantly lower than that of the Lard/SO-fed group, and that of the DHA + FF-fed group was the lowest. All three fish oil treatments significantly reduced plasma insulin levels. Hepatic lipid levels significantly decreased in all three of these groups compared with the Lard/SO-fed group. Plasma adiponectin increased in both the EPA- and DHA-fed groups, but the increase was suppressed in the DHA + FF-fed group. Hepatocytes of Lard/SO-fed mice were filled with numerous fat droplets, but fat accumulation was inhibited in both EPA- and DHA-fed mice and was significantly prevented by fenofibrate treatment. SREBP-1c mRNA levels were decreased by about half in EPA- and DHA-fed mice compared with Lard/SO-fed mice. FAS, Insig-1, HMG-CoA reductase, and LDL-receptor mRNA levels also markedly decreased in both EPA- and DHA-fed mice, but there was no additional decrease in DHA + FF fed mice. Fenofibrate treatment significantly induced mRNA expression of AOX and UCP-2, but not of PPARα.

Conclusion: These data suggest that fish oil inhibited body weight gain and exhibited an anti-obesity effect through the inhibition of lipid synthesis in female KK mice. Furthermore, fenofibrate treatment markedly inhibited body weight gain by the induction of fatty acid oxidation. Plasma adiponectin levels did not increase in mice fed DHA-rich fish oil with fenofibrate, although white adipose tissue (WAT) weight significantly decreased. We considered that adiponectin sensitivity increased more in mice fed DHA-rich fish oil with fenofibrate than in mice fed DHA-rich fish oil alone.


Key words: Hepatic lipid, Adiponectin, Fat accumulation

Introduction

Consumption of fish reduces the risk of coronary heart disease. The beneficial effect of fish can be attributed to n-3 polyunsaturated fatty acids, such as eicosapentaenoic acid (EPA, 20:5) or docosahexaenoic acid (DHA, 22:6) in fish oil. Fish oil consumption decreases lipogenesis and increases fatty acid oxidation in liver, and improves plasma and hepatic lipid levels in hyperlipidemia patients. The synthesis of fatty acids and cholesterol in the liver are mainly regulated by sterol regulatory element-binding proteins (SREBPs), a transcription factor of genes related to lipogenesis. It was reported that fish oil inhibited the maturation of SREBP-1 in vivo and reduced the transcription of genes coding for lipogenic enzymes, such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), stearoyl-CoA desaturase-1 (SCD-1), 3-hydroxy-
3-methylglutaryl coenzyme A (HMG-CoA) reductase, HMG-CoA synthase, and LDL-receptor. Meanwhile, peroxisome proliferator-activated receptor α (PPARα) is activated by fish oil consumption. PPARα is a ligand-activated transcription factor that regulates the expression of genes involved in triglyceride hydrolysis and fatty acid oxidation, such as acyl-CoA oxidase (AOX), lipoprotein lipase (LPL), medium-chain acyl-CoA dehydrogenase (MCAD), acyl-CoA synthetase (ACS), and uncoupling protein-2 (UCP-2).

Fenofibrate, one of the fibrate classes of hypolipidemic drugs, is known as an agonist for PPARα; it lowers plasma levels of triglyceride and LDL-cholesterol, and raises plasma HDL-cholesterol concentrations.

Previous studies have demonstrated the respective effects of EPA and DHA on metabolic control. Botolin et al. incubated rat hepatocytes using EPA or DHA and observed that DHA suppressed nuclear SREBP-1 production strongly and more than EPA. Conversely, EPA consistently induced PPARα compared with DHA in vitro. In vivo, Froyland et al. suggested that EPA possesses a potent triglyceride-lowering property and stimulates greater oxidation of fatty acids than DHA. In addition, the combination of EPA and DHA provided a stronger hypolipidemic effect than each alone. In our previous study, consumption of different fish oils, specifically EPA- or DHA-rich fish oil, did not produce different effects on lipogenesis in C57BL/6J mice. In this study, we decided to use KK mice that develop non-insulin-dependent diabetes mellitus with moderate obesity, insulin resistance and hyperlipidemia.

The aims of this study were to determine differences in the effects of administering two fish oils, EPA-rich and DHA-rich, as well as the latter plus fenofibrate, on lipid metabolism in female KK mice, and to elucidate the mechanism underlying lipid metabolism.

### Materials and Methods

#### Animals

Female KK mice were obtained from Tokyo Laboratory Animals Science Co. (Tokyo, Japan) at 5 weeks of age and fed a standard rodent diet (CE2; Clea Japan, Tokyo, Japan) for 1 week to stabilize the metabolic conditions. Mice were exposed to a 12-h light/12-h dark cycle and maintained at a constant temperature of 22 ± 2°C and humidity of 55 ± 10%. Mice were divided into four groups (n = 5 in each group). The animal experiments were approved by the Institutional Animal Care and Use Committee at Josai University Life Science Center.

#### Diets

The three diets used a different fat source, lard/safflower oil (4:6, Lard/SO), EPA-rich fish oil (EPA), or DHA-rich fish oil (DHA), and another used DHA-rich fish oil supplemented with 0.2% (w/w) fenofibrate (DHA + FF). The diet details are shown in Table 1. Mice were fed each diet for 8 weeks. The purified experimental diets contained, on an energy basis, 54% carbohydrate, 25% fat and 21% protein. Lard (Oriental Yeast, Tokyo, Japan) contained 44% oleic acid (18:1n-9), 24% palmitic acid (16:0) and 14% stearic acid (18:0) as the main fatty acids; safflower oil (Benihana Foods, Tokyo, Japan) contained 78% oleic acid and 14% linoleic acid (18:2n-6); EPA-rich fish oil contained 20% EPA (20:5n-3) and 13% DHA (22:6n-3); and DHA-rich fish oil contained 7% EPA and 24% DHA. Fenofibrate at 0.2% in this study corresponds to 200 mg/kg/day and is 1.5 times the dosage of fenofibrate used in a previous study with C57BL/6 mice. It was found that the final body weight decreased 10% compared to the initial weight in female KK-Ay mice fed the 10% fat diet with 0.2% fenofibrate (data not shown). In our present study, we considered that 0.2% dosage of fenofibrate is an effective amount to change the lipid metabolism in female KK mice. Fish oil was provided by NOF Corporation (Tokyo, Japan). Fenofibrate was purchased from Sigma-Aldrich (St Louis, MO).

#### CT Scan Analysis

The body fat composition of mice was examined radiographically using X-ray CT for experimental ani-
animals with a mouse mode (La Theta LCT100; ALOKA, Tokyo, Japan). At the end of the experiments, animals were anesthetized at about 10:00 a.m. after 3 h fasting by intraperitoneal injection of pentobarbital sodium (Nembutal; Dainippon Sumitomo Pharma, Osaka, Japan). We performed CT scanning at 2 mm intervals from the diaphragm to the bottom of the abdominal cavity. The fat amount was divided into visceral and subcutaneous fat and evaluated quantitatively using La Theta software.

**Collection of Blood and Tissue Samples**

After CT scan, blood samples were obtained from the inferior vena cava and treated with EDTA 2Na. Liver, white adipose tissue (WAT) and brown adipose tissue (BAT) were removed immediately, weighed, and homogenized with Trizol (Invitrogen, Carlsbad, CA), and RNA was prepared by the method described by Chirgwin et al. A part of the liver of each mouse was frozen for measurement of triglyceride and cholesterol. Plasma triglyceride, total cholesterol and HDL-cholesterol concentrations were measured by enzymatic assays, each Etest Wako (Wako Pure Chemical Industries, Osaka, Japan). Plasma insulin and leptin were quantified with enzyme-linked immunosorbent assays (ELISA) using the Insulin ELISA kit and Leptin/mouse ELISA kit, respectively (Morinaga Institute of Biological Science, Tokyo, Japan). Plasma Adiponectin was quantified using the mouse/rat adiponectin ELISA kit (Otsuka Pharmaceutical, Tokyo, Japan).

**Liver Histopathology**

A piece of liver tissue was excised from the median lobe of a mouse. Five mouse samples for each group were collected individually. 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.

**Real-Time Quantitative One-Step RT-PCR**

Messenger RNA expressions of genes related with lipogenesis and fatty acid oxidation were quantified by real-time PCR, using a Sequence Detector, from the cycle number (Ct) for threshold signal detection. PCR was performed using the ABI Prism 7000 Sequence Detection Systems (Applied Biosystems, CA), using a QuantiTect SYBR Green Real-time PCR kit (QIAGEN, Hilden, Germany). mRNA was amplified in QuantiTect SYBR Green RT-PCR Master Mix containing PCR primers. The thermal cycling conditions were as follows: reverse transcription at 50°C for 30 min, initial activation at 95°C for 15 min plus 40 cycles at 94°C for 15 s, 55°C for 30 s and at 72°C for 1 min. The PCR primers used were as follows: SREBP-1c, 5’ primer, 5’-GGAGCCATGATTGCA-CATT-3’ and 3’ primer, 5’-GGCCCGGGAAGTCA-CTGT-3’; SREBP-2, 5’ primer, 5’-GGGTTCGAGG-ACCATTGA-3’ and 3’ primer, 5’-ACAAAGTGTGC-CTGAAAAACATCA-3’; FAS, 5’ primer, 5’-TCACCACGTGAGCTCTGCAGAGA-AGCGAG-3’ and 3’ primer, 5’-TGTCATTGGCCT-CCTCAAAGGGGCGTCCA-3’; LDL-receptor, 5’ primer, 5’-AGGCTGTGGGGCTCCATAGG-3’ and 3’ primer, 5’-TGCGGTCCAGGGTCATCT-3’; HMG-CoA reductase, 5’ primer, 5’-CTTGTTGGAATTGCT-TGTGATTG-3’ and 3’ primer, 5’-AGCCGAACGAC-CACATGAT-3’; AOX, 5’ primer, 5’-TCAACAGCCCCAATCTGACCTCATT-3’ and 3’ primer, 5’-TCA-GGTACCATATCCTCCTCCT-3’, UCP-2, 5’ primer, 5’-GGTCCTCTGTCTCCTGTACTGC-3’ and 3’ primer, 5’-GGGTTCGAGGAGTCATTGA-3’; PPARα, 5’ primer, 5’-GGTGGCTGCTATAATTTGCTGT-3’ and 3’ primer, 5’-GAGGTGTCACTCTGATGTT-3’; AdipoR2, 5’ primer, 5’-ACCCACAACCTTGGCTCATTAC-3’ and 3’ primer, 5’-CCATAAGCAGTTAGCCAGCTAT-3’.

A housekeeping transcript, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5’ primer, 5’-TGTGTCCGTCGTGGATCTGA-3’ and 3’ primer, 5’-CCTGCTTCACCACCTTTGCTCA-3’, was used as an endogenous control gene.

**Statistical Analysis**

Data from multiple groups were compared by one-way analysis of variance. Each group was compared with the others by Fisher’s protected least significant difference test (SYSTAT 11; Systat Software, Chicago). Values are the mean ± S.D. Statistical significance is defined as p < 0.05.

**Results**

**Phenotypic Comparison**

After one-week consumption of the experimental diets, the body weight gain of DHA + FF-fed mice was about half that of mice fed the other diets, and body weight was significantly lower than that of Lard/SO-fed mice (Fig. 1). The final body weight of both fish oil-fed groups was significantly lower than that of the Lard/SO-fed group, and that of the DHA + FF-fed group was lowest of all (Table 2). Between the two fish oils, there was no difference in final body weight after 8 weeks.
Liver weight significantly decreased in both the EPA- and DHA-fed groups compared with the Lard/SO-fed group, but not in the DHA + FF-fed group (Table 2). WAT weight significantly decreased in the EPA-, DHA-, and DHA + FF-fed groups compared with the Lard/SO-fed group. The reduction in WAT weight was greater in the EPA-fed group than in the DHA-fed group, and was greatest of all in the DHA + FF-fed group. BAT weight decreased in the DHA + FF group, but did not change in the EPA- and DHA-fed groups. CT analysis showed that visceral and subcutaneous fatty deposits were significantly decreased in both fish oil-fed groups compared with the Lard/SO-fed group, but not subcutaneous fat in the EPA-fed group, and the DHA + FF-fed group was lower than both fish oil groups (Fig. 2A, B, C).

Effects of Fish Oils and Fenofibrate on Blood Glucose Level and Plasma Insulin, Leptin, and Adiponectin

Blood glucose levels did not change among groups regardless of fat type or fenofibrate treatment. Plasma insulin levels were significantly lower in EPA-, DHA- and DHA + FF-fed groups compared to the Lard/SO-fed group (Table 3). Plasma leptin levels were practically unchanged in both the fish oil groups, but decreased significantly in the DHA + FF-fed group compared with the Lard/SO group. In contrast, plasma adiponectin levels significantly increased in both the fish oil groups relative to the Lard/SO diet group, but this increase was suppressed by fenofibrate treatment.

Effects of Fish Oils and Fenofibrate on Plasma and Hepatic Lipid Levels

Plasma triglyceride levels did not change in the EPA- and DHA-fed groups compared with the Lard/SO-fed group, but the DHA + FF-fed group significantly reduced them (Table 3). Plasma total cholesterol significantly decreased in the EPA- and DHA-fed groups, but not in the DHA + FF-fed group. Moreover, DHA + FF-fed mice showed significantly increased plasma HDL-cholesterol. Liver triglyceride levels remarkably decreased in both EPA- and DHA-fed mice by about 90% and 70% compared with Lard/SO-fed mice, and the levels were lowest in DHA + FF-fed mice (Fig. 3B). There was no difference in these levels between the two fish oil groups. Liver total cholesterol levels also remarkably decreased in both EPA- and DHA-fed mice by about 90% in both cases compared with Lard/SO-fed mice, and the decrease in these levels in DHA + FF-fed mice was similar to those in both the fish oil groups (Fig. 3C); however, there was no difference in liver cholesterol levels between the two fish oil groups.

![Fig. 1. Body weight curves for female KK mice fed lard/safflower oil, EPA-rich fish oil, and DHA-rich fish oil with or without 0.2% fenofibrate for 8 weeks.](image)

Values represent the mean ± S.D. (n = 5). Statistical significance is indicated for the *-EPA-rich fish oil group, **-DHA-rich fish oil group, --DHA-rich fish oil with fenofibrate group compared to the Lard/SO group. ANOVA by Fisher’s protected least significant difference (PLSD) test, p < 0.05.

<table>
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<tr>
<th></th>
<th>Lard/SO</th>
<th>EPA</th>
<th>DHA</th>
<th>DHA + FF</th>
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</thead>
<tbody>
<tr>
<td>Feeding energy (kcal/mouse/day)</td>
<td>17.40 ± 1.42</td>
<td>15.34 ± 1.27</td>
<td>16.50 ± 0.93</td>
<td>13.90 ± 1.60</td>
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<td>Initial body weight (g)</td>
<td>21.7 ± 1.0</td>
<td>21.8 ± 1.0</td>
<td>21.6 ± 1.1</td>
<td>21.7 ± 1.1</td>
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<td>Final body weight (g)</td>
<td>46.91 ± 3.23 (a)</td>
<td>40.04 ± 3.56 (b)</td>
<td>41.59 ± 2.99 (b)</td>
<td>30.34 ± 2.39 (c)</td>
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<tr>
<td>Liver weight (g)</td>
<td>3.27 ± 0.50 (a)</td>
<td>1.36 ± 0.14 (b)</td>
<td>1.70 ± 0.29 (b)</td>
<td>3.24 ± 0.13 (a)</td>
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<tr>
<td>WAT weight (g)</td>
<td>4.20 ± 0.31 (a)</td>
<td>3.02 ± 0.16 (c)</td>
<td>3.64 ± 0.33 (b)</td>
<td>1.34 ± 0.42 (d)</td>
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<tr>
<td>BAT weight (mg)</td>
<td>207.3 ± 53.4 (a)</td>
<td>243.2 ± 60.3 (a)</td>
<td>256.1 ± 24.6 (a)</td>
<td>78.0 ± 21.8 (b)</td>
</tr>
</tbody>
</table>

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.
Fat Droplet Accumulation in Liver

The enlarged cytoplasm in the Lard/SO-fed group was filled with numerous fat droplets (Fig. 3A); however, fat droplet accumulation was inhibited in both the EPA- and DHA-fed groups, and was significantly prevented by fenofibrate treatment.

Messenger RNA Expression of Genes Involving Lipid Metabolism in Liver

SREBP-1c mRNA levels significantly decreased in EPA- and DHA-fed mice by 69% and 29% compared with Lard/SO-fed mice (Fig. 4A), while FAS mRNA levels decreased in both the fish oil groups by 97% and 94% (Fig. 4B). mRNA levels of Insig-1, which is up-regulated by mature SREBP-1, decreased in EPA-fed mice and in DHA-fed mice by 78% and...
61%, respectively, compared with Lard/SO-fed mice (Fig. 4C). The addition of fenofibrate to the DHA diet did not alter mRNA expression of these lipid synthesis genes.

SREBP-2 mRNA expression significantly decreased only in EPA-fed mice (Fig. 5A). mRNA levels of HMG-CoA reductase, a rate-limiting enzyme of cholesterol synthesis, significantly decreased in both the fish oil groups, mice by 79% and 53%, compared with Lard/SO-fed mice, and fenofibrate treatment did not influence the levels (Fig. 5B). LDL receptor mRNA levels decreased in EPA- and DHA-fed mice by 67% and 48% compared with Lard/SO-fed mice, and were not influenced by fenofibrate treatment (Fig. 5C).

AOX mRNA levels were unaffected by either oil. On the other hand, DHA-FF-fed mice exhibited 4.0-fold higher levels than Lard/SO-fed mice (Fig. 6A). UCP-2 mRNA levels did not increase with either fish oil, but the levels in DHA-FF-fed mice were 4.8-fold those of Lard/SO-fed mice (Fig. 6B); however, PPARα levels were unaffected by fat type or fenofibrate treatment (Fig. 6C). Adiponectin receptor 2 (AdipoR2) mRNA levels significantly decreased in both the fish oil groups as well as in the DHA-FF-fed group compared with the Lard/SO-fed group (Fig. 6D).

Discussion
In the present study, we examined the effects of fish oils and fenofibrate on lipid metabolism in female KK mice, a model that develops moderate obesity, insulin resistance and hyperlipidemia, and clearly showed the following: 1) consumption of fish oil inhibited body weight gain and exhibited an anti-obesity effect through the inhibition of lipid synthesis in female KK mice. Furthermore, fenofibrate treatment markedly inhibited body weight gain by inducing fatty acid oxidation; 2) fish oil consumption markedly improved hyperinsulinemic status; 3) plasma adiponectin levels increased in mice fed either EPA- or
DHA-rich fish oil; however, in mice fed DHA-rich fish oil with fenofibrate, the increment declined, with a marked decrease in WAT weight.

Female KK mice show substantial body weight gains from an early stage. In this study, fish oil-fed mice showed moderate body weight gain, and DHA-rich fish oil with fenofibrate significantly suppressed weight gain. The change in SREBP-1c expression altered the transcription of SREBP-1 target genes, such as FAS and SCD-1, which regulate fatty acid synthesis. Our data indicated that fish oil feeding significantly decreased SREBP-1c and FAS mRNA in female KK mice as well as in female C57BL/6J mice. Moreover, mRNA levels of Insig-1, which is induced by SREBP-1 mature protein, were inhibited significantly in both the fish oil groups. This suggests that fish oil induces the suppression of SREBP-1 transcription and protein activity, and consequently inhibits fatty acid synthesis.

Fenofibrate is known as an agonist of PPARα, which acts as a ligand-activated transcriptional factor. AOX and UCP-2, genes coding for fatty acid oxidation and thermogenesis, are induced by PPARα activation. Several studies have reported that fenofibrate did not induce PPARα mRNA and protein expression, although it did induce PPARα target genes expression. Our results indicated that fenofibrate treatment with fish oil markedly stimulates lipid catabolism by stimulating mainly PPARα target genes, such as AOX and UCP-2, more than by inducing the mRNA expression of PPARα itself. Therefore, the combination of DHA-rich fish oil and fenofibrate may stimulate fatty acid oxidation, and thus prevent increased body weight and WAT weight, while also improving insulin sensitivity.

Both fish oils markedly lowered plasma insulin levels, although blood glucose levels did not differ among the four groups. These results indicate that fish oil consumption can improve the hyperinsulinemic status in female KK mice. Krebs et al. reported that weight loss improves insulin sensitivity. Our data suggest that fish oil feeding resulted in a decrease of WAT and body weight and improved insulin sensitivity in female KK mice.
Rossi et al. reported that shifting the source of fat to fish oil for 2 months increased plasma levels of both leptin and adiponectin in rats\textsuperscript{25}. Haluzik et al. reported that fenofibrate treatment did not increase serum adiponectin in mice fed a carbohydrate diet\textsuperscript{26}. This is also consistent with our previous observation using C57BL/6J mice that plasma adiponectin increased significantly in fish oil groups, but not in the fish oil with fenofibrate group. There are two types of adiponectin receptors, AdipoR1 and AdipoR2. AdipoR1 is ubiquitously expressed with predominant expression in muscle, whereas AdipoR2 is mainly expressed in the liver\textsuperscript{27}. Tsuchida et al. reported that PPAR\(\alpha\) activation by treatment with its agonist, Wy-14,643, up-regulated the expressions of AdipoR1 and AdipoR2 in WAT, but did not increase serum adiponectin concentrations\textsuperscript{28}. In another study, AdipoR2 mRNA level in the liver was decreased by exercise training. This study also shows that exercise training improves insulin resistance but does not change plasma adiponectin concentration\textsuperscript{29}. In the present study, AdipoR2 mRNA in the liver significantly decreased in both the fish oil groups as well as in the fenofibrate-added group; however, the DHA-rich fish oil with fenofibrate group did not increase plasma adiponectin levels compared with the Lard/SO group. These data suggest that the decline of plasma adiponectin by fenofibrate treatment is due to decrease adiponectin formation by a significant decrease in WAT. This suggests that plasma leptin levels also decreased by WAT decrease.

In humans, treatment with fibrates is consistently associated with an increase in plasma HDL-cholesterol\textsuperscript{10}. Our previous study also showed that fenofibrate treatment markedly increased plasma HDL-cholesterol in C57BL/6J mice\textsuperscript{17}. The data presented here indicate that the increase of plasma total cholesterol in DHA+FF fed mice is due to the increase of plasma HDL-cholesterol by the effect of fenofibrate.

Previous studies have reported that EPA and DHA exert their lipid-lowering effects in different ways\textsuperscript{13-16}. In our study using female KK mice, the liver triglyceride level in the EPA group was one-third that in the DHA group. Frøyland et al. suggested that
EPA possesses a potent triglyceride-lowering property more than DHA\(^1\). These data support our finding that EPA-rich fish oil possesses stronger hypolipidemic ability than DHA-rich fish oil in female KK mice; however, the difference was not significant, and both EPA-rich and DHA-rich fish oil showed effective hypolipidemic ability in this study.

To clarify the differences between EPA and DHA in fish oil, further examinations of the effects of these fatty acids on lipid metabolism in obesity are required.

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