

## Young Persimmon Fruits Prevent the Rise in Plasma Lipids in a Diet-Induced Murine Obesity Model

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The effect of young and mature persimmon fruits on lipid metabolism was investigated in a diet-induced murine obesity model. A commercially purchased high fat diet (Quick Fat, CLEA Japan) was used as the basal diet. Dried and powdered young and mature fruits of two breeds of persimmon, Fuyu-kaki and Hachiya-kaki, were added to the basal diet at a concentration of 10%, respectively. Male C57BL/6 mice ( $n=4$ ) were divided into five groups and fed the basal diet or one of the persimmon-supplemented basal diets *ad libitum* for 14 weeks. Diets supplemented with both types of young fruit significantly reduced the rise in plasma lipids, including total cholesterol ( $p<0.005$ ), triglyceride ( $p<0.05$ ), and LDL cholesterol ( $p<0.05$ ), and the effect was almost equal between the two breeds. Real-time RT-PCR revealed that both of these young fruit-supplemented diets equally up-regulated expression of the cholesterol 7  $\alpha$ -hydroxylase (CYP7A1) gene in the liver by about three-fold ( $p<0.05$ ). CYP7A1 plays an important role in maintaining cholesterol homeostasis by regulating bile acid synthesis, suggesting that increased conversion of cholesterol to bile acids may have caused the cholesterol-lowering effect of the young fruits. The results indicate that young persimmon fruits are beneficial in the development of preventive and therapeutic agents against dyslipidemia.

**Key words** young persimmon fruits; dyslipidemia; cholesterol 7  $\alpha$ -hydroxylase (CYP7A1); bile acid synthesis

Dyslipidemia is generally characterized by increased fasting concentrations of total cholesterol, low-density lipoprotein (LDL) cholesterol, and triglyceride, in conjunction with decreased concentrations of high-density lipoprotein (HDL) cholesterol,<sup>1)</sup> and is the most important modifiable risk factor for coronary heart disease.<sup>2)</sup> Many reports have shown that dietary patterns characterized by a high intake of fruits and vegetables are associated with reduced risk of coronary heart disease.<sup>3–5)</sup>

Persimmon (*Diospyros kaki*) is an important fruit in Japan. Gorinstein *et al.* reported that whole mature fruit possesses hypolipidemic property in rats fed a cholesterol-supplemented diet.<sup>6)</sup> However, the effect of young fruit on lipid metabolism has not been investigated. In this study, we investigated the effect of young and mature persimmon fruits on lipid metabolism in a diet-induced murine obesity model, and found that the young fruits prevented the rise in plasma lipids and up-regulated the cholesterol 7  $\alpha$ -hydroxylase (CYP7A1) gene in the liver.

### MATERIALS AND METHODS

**Diets** Two breeds of persimmon, Fuyu-kaki and Hachiya-kaki, were used in this study. Fruits with green peel and 5–7 cm in diameter were used as young fruits. Whole fruits containing peel and seeds were dried by storage at a temperature below 42 °C using a decompression machine (BCD-2000U; Yahiro-sangyo, Minokamo, Gifu, Japan) and then powdered.

A commercially purchased high fat diet (Quick Fat, CLEA Japan, Tokyo, Japan) was used as the basal diet. Dried and powdered young and mature fruits of the two breeds of persimmon were added to the basal diet at a concentration of 10%, respectively.

**Animals** Six-week-old male mice (C57BL/6J strain) were purchased from CLEA Japan. They were randomly di-

vided into 5 groups of 4. Mice in each group were housed together and fed one of the above diets *ad libitum* for 14 weeks. Mice fed the basal diet were termed control mice, and those fed the diet supplemented with young Fuyu-kaki and Hachiya-kaki fruits, and mature Fuyu-kaki and Hachiya-kaki fruits as Y-Fuyu, Y-Hachiya, M-Fuyu, and M-Hachiya mice, respectively. After anesthetization with CO<sub>2</sub> gas with fasting for 24 h, mice were sacrificed and their organs were dissected for analysis. All experiments were performed in conformance of the International Guiding Principles for Biomedical Research Involving Animals.<sup>7)</sup>

**Blood Chemistry** Plasma glucose, total cholesterol, LDL cholesterol, triglyceride, and non-esterified fatty acid (NEFA) concentrations were analyzed using Glucose-CII, Cholesterol E, LDL-C, Triglyceride-E and NEFA-C kits (Wako Pure Chemical, Osaka, Japan), respectively.

**Morphological Analysis** The dissected liver samples were fixed with 4% paraformaldehyde in 0.1 M sodium-phosphate buffer, pH 7.4 (PB), overnight at 4 °C, followed by immersion in phosphate-buffered saline (PBS) containing 20% sucrose for 24 h at 4 °C. The fixed liver samples were embedded in TISSU MOUNT (Shiraimatsukiki, Osaka, Japan) and quickly frozen with liquid nitrogen. Frozen sections (6  $\mu$ m thick) were prepared using a cryostat, followed by fixation with 4% paraformaldehyde in PB for 20 min at room temperature. After rinsing the fixative in PBS, hematoxylin and eosin (HE) staining was carried out for microscopic observation with an NIKON E600 microscope (Nikon, Tokyo, Japan).

**Quantitative Real-Time PCR** RNA extraction, cDNA synthesis and SYBR Green-based real-time PCR were conducted as previously described by Matsumoto *et al.*<sup>8)</sup> Briefly, total RNA samples were isolated from liver tissues using a QuickPrep Total RNA extraction kit (GE Healthcare Bioscience, Piscataway, NJ, U.S.A.). For preparation of cDNA, 2  $\mu$ g of each total RNA sample was reverse-transcribed using

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Table 1. Primers Used for Real-Time RT-PCR

Gene	Forward (5'→3')	Reverse (5'→3')
EF-1 $\alpha$	CTCAGGTGATTATCCTGAACCATC	AACAGTTCTGAGACCGTTCTTCCA
SREBP-1	TGCTCCTGTGTGATCTACTTCTTG	TGTAGGAATACCCTCCTCATAGCA
PPAR- $\alpha$	AATGGGCACTTCTAAGACTACCTG	GTGCAGATTAGTTTCAGGGATT
HMGCR	AAGGTGGTGAGAGAGGTGTAAAG	AATACAGTTTGAACCTCCACATT
CYP7A1	GGATCAAGAGCAACTAAACAACCT	GCTATGATGTCATCTTTCGAATG

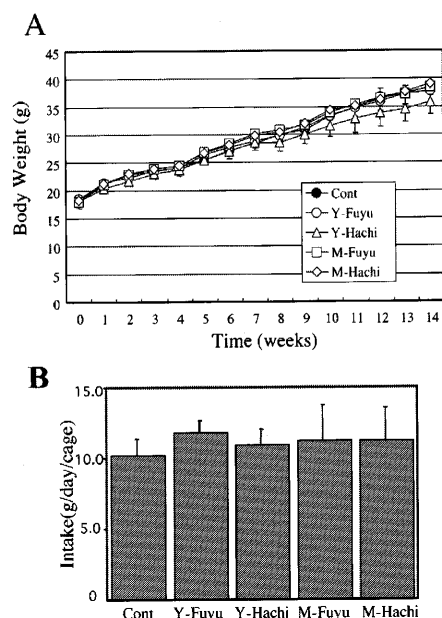


Fig. 1. Body Weight Development (A) and Average Food Intake (B) during the 14-Week Study Period

Results represent the mean  $\pm$  S.D.

Super Script III Rnase H<sup>-</sup>-reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) and an oligo(dT) primer (Invitrogen). Prepared cDNA samples were then purified using a PCR Purification kit (QIAGEN, Cambridge, MA, U.S.A.).

Quantitative real-time RT-PCR was performed on a BioFlux LineGene (TOYOBO, Osaka, Japan) using a SYBR Green Real-time PCR Master Mix (TOYOBO) according to the manufacturer's instructions. A housekeeping transcript, elongation factor-1 $\alpha$  (EF-1 $\alpha$ ), was used as an internal control because of its stable expression *in vivo*.<sup>9)</sup> The primers used for each gene are summarized in Table 1. The PCR products were evaluated by inspection of their melting curves (data not shown).

**Statistics** One-way ANOVA and Fisher's PLSD test were performed with Stat view software (SAS Institute, Cary, NC, U.S.A.).

## RESULTS AND DISCUSSION

**Development of Body Weight** Figure 1A shows the body weight development of mice during the 14-week study period. Although that of Y-Hachiya mice was slightly lower than those of the other groups, no significant difference was observed among groups. Figure 1B shows the daily average food intake of each group throughout the experimental period. There was no significant difference in food intake among groups. These results suggest that supplementation of the basal diet with young and mature persimmon fruits does

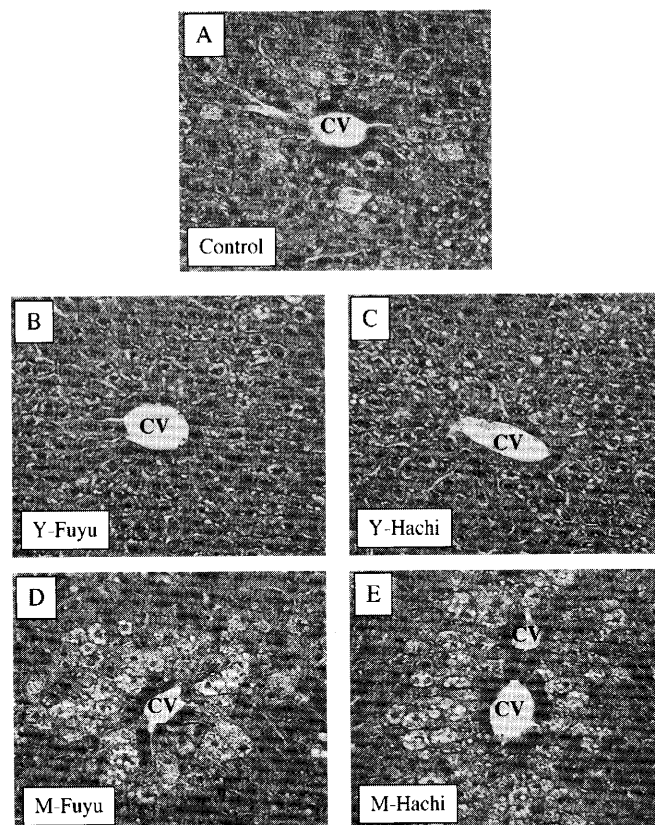


Fig. 2. Hematoxylin and Eosin Staining of Liver Tissues of Control (A), Y-Fuyu (B), Y-Hachiya (C), M-Fuyu (D), and M-Hachiya Mice (E)

CV: central vein. Magnification,  $\times 200$ .

not affect body weight development or food intake at the concentration examined.

**Morphology of the Liver** To examine the histopathology of the liver, we performed HE staining (Fig. 2). Many enlarged hepatocytes rich in unstained cytoplasm around the central vein were observed in control mice (Fig. 2A), suggesting that fatty liver was induced by intake of the high fat diet. The same abnormality was observed in M-Fuyu and M-Hachiya mice (Figs. 2D, E); however, unstained cytoplasmic regions were diminished and enlarged hepatocytes were scarcely observed in Y-Fuyu and Y-Hachiya mice (Figs. 2B, C). These results indicate that both types of young fruit, but not the mature fruits, prevent hepatocyte steatosis induced by intake of a high fat diet.

**Prevention of the Rise in Plasma Lipids by the Young Fruits** To investigate the effect of the young and mature fruits on blood disorders, we examined the concentrations of plasma glucose, total cholesterol, LDL cholesterol, triglyceride, and NEFA, a rise of which leads to glucose tolerance and lipid disorders (Fig. 3). No significant differences were observed in the glucose concentration among groups (Fig.

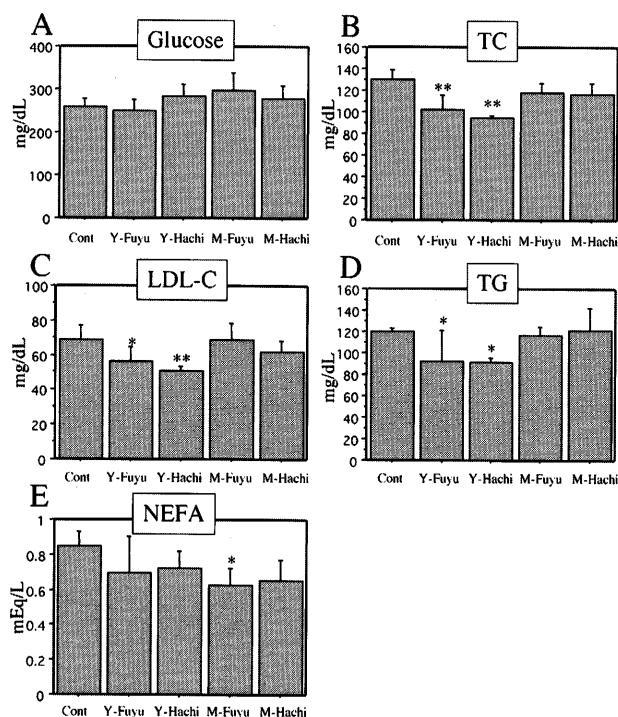


Fig. 3. Plasma Biochemical Values of Glucose (A), Total Cholesterol (B), Low-Density Lipoprotein Cholesterol (C), Triglyceride (D), and Non-esterified Fatty Acid (E) in the Mice

Data represent the mean  $\pm$  S.D. ( $n=4$ ). \* $p<0.05$ , \*\* $p<0.005$  compared with control mice.

3A); however, interesting differences in the lipid concentration were observed. Both types of young fruit equally lowered total cholesterol (Fig. 3B, Y-Fuyu and Y-Hachiya:  $p<0.005$ ), LDL cholesterol (Fig. 3C, Y-Fuyu:  $p<0.05$ , Y-Hachiya:  $p<0.005$ ), and triglyceride (Fig. 3D, Y-Fuyu and Y-Hachiya:  $p<0.05$ ) with statistical significance. Although there was no statistically significant difference between results, except for M-Fuyu mice ( $p<0.05$ ), young and mature fruits tended to lower NEFA (Fig. 3E).

Free fatty acids (FFAs), absorbed from the diet or released by adipocytes, are a major energy source for most tissues in fasting animal. Most FFAs taken up by the liver are rapidly incorporated into complex lipids, including triglycerides, phospholipids, glycolipids, and cholesterol esters; however, excessive accumulation of these lipids in the liver causes fatty liver, with triglyceride accumulating most commonly.<sup>10)</sup> The results of blood chemistry showed that the young fruits lowered plasma triglyceride and NEFA, which might have resulted in the prevention of hepatocyte steatosis.

**Gene Expression in the Liver** The liver plays a central role in lipid metabolism, including synthesis, storage, and degradation.<sup>10)</sup> We therefore examined expression changes in lipid metabolism-related genes, sterol regulatory element binding protein-1 (SREBP-1), peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ), cholesterol 7 $\alpha$ -hydroxylase (CYP7A1), and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), in the liver tissues using SYBR Green-based real-time PCR (Fig. 4). SREBP-1 regulates the expression of several lipogenic enzymes, and PPAR $\alpha$  controls fatty acid oxidative metabolism in the liver.<sup>11)</sup> CYP7A1 is the initial and rate-limiting enzyme in bile acid biosynthesis,<sup>12)</sup> and HMGCR is the rate-limiting enzyme in cholesterol biosyn-

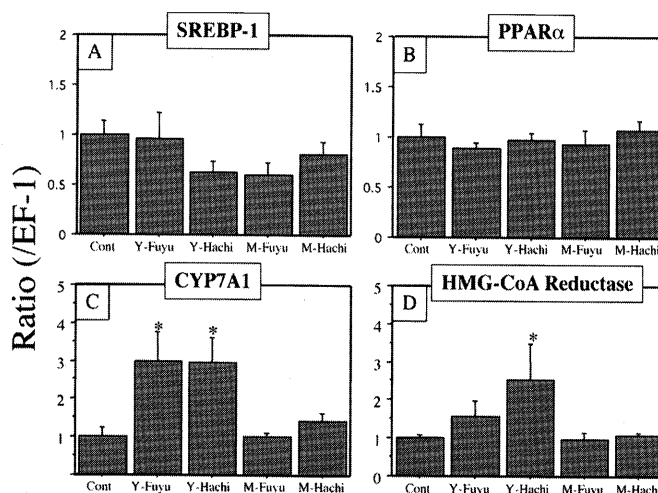


Fig. 4. Gene Expression of SREBP-1 (A), PPAR- $\alpha$  (B), CYP7A1 (C), and HMGCR (D) in the Liver Determined by Real-Time RT-PCR

Elongation factor-1 $\alpha$  (EF-1 $\alpha$ ) was used as a control to standardize the efficiency of each reaction. Results represent the mean  $\pm$  S.E. ( $n=4$ ). \* $p<0.05$  compared with control mice.

thesis.<sup>13)</sup> Although some scattering was observed in the data, there was no significant difference in SREBP-1 and PPAR $\alpha$  expression between groups (Figs. 4A, B). The CYP7A1 gene was strongly up-regulated by about three-fold in Y-Fuyu and Y-Hachiya mice with statistical significance ( $p<0.05$ ), but not in M-Fuyu and M-Hachiya.

Conversion of cholesterol to bile acids plays an important role in cholesterol homeostasis, and the initial and rate-limiting enzyme CYP7A1 regulates bile acid synthesis.<sup>14)</sup> Up-regulation of the CYP7A1 gene by the young fruits therefore indicates that increased conversion of cholesterol to bile acids occurred, which might be one mechanism causing the cholesterol-lowering effect. Miyake *et al.* reported that over-expression of CYP7A1 in the liver of transgenic mice blocked the formation of diet-induced atherosclerosis and gallstone,<sup>15)</sup> suggesting that CYP7A1 might be an excellent therapeutic target against lipid disorders. On the other hand, the HMGCR gene was also up-regulated in these two groups (Y-Hachiya:  $p<0.05$ ). Considering the lowered, not increased, plasma lipids in the mice fed young fruits, it is possible that cholesterol deficiency by excessive conversion of cholesterol to bile acids might have caused up-regulation of the HMGCR gene. These results indicate that intake of young fruits is effective in preventing dyslipidemia from a genetic point of view; however, further studies on protein levels and enzymatic activities are required to further explore the effect of young fruits; such studies are currently under way in our laboratory.

The results obtained in this study suggested that neither types of mature fruit improved lipid metabolism, although Gorinstein *et al.* reported that mature persimmon-supplemented diet (7.0% w/w) significantly lowered plasma lipids, including total cholesterol, LDL cholesterol, and triglyceride, in rats fed diets containing cholesterol.<sup>6)</sup> Mature persimmon fruits might therefore be effective in improving lipid metabolism in particular cases. On the other hand, the young fruits of both breeds of persimmon exerted an equal effect on the plasma chemistry, histopathology of the liver, and gene expression in the liver, suggesting that these beneficial effects

might be common to all young persimmon fruits.

In summary, the present study showed that two breeds of young persimmon equally exert beneficial effects, such as preventing hepatocyte steatosis and lowering plasma cholesterol. Although the functional constituents are not identified, young persimmon fruits might be beneficial in the development of preventative and therapeutic agents against dyslipidemia.

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