Introduction

Hypercholesterolemia has been well known as a proven risk factor for cardiovascular disease (CVD), which is the number one cause of death globally. It is also one of many factors causing non-alcoholic fatty liver disease (NAFLD) (1), one of the most common chronic liver disorders in the Western world (2, 3). NAFLD has a broad spectrum of symptoms ranging from simple steatosis to cirrhosis (4, 5), even in patients with a normal body-mass index (6). This raises concerns that patients with NAFLD could also be at a greater risk of CVD (7, 8). One of the primary goals of clinical management to reduce the risk for CVD is achieving therapeutic target levels of all lipid parameters (9).

During the progression of hypercholesterolemia, the expression of genes and proteins related to lipid metabolism, including retinoid-binding protein (RBP) (10), and fatty acid–binding proteins (FABPs) changes (11, 12). Cutaneous fatty acid–binding protein (C-FABP) and heart fatty acid–binding protein (H-FABP) belong to FABPs which are members of the superfamily of lipid-binding proteins (13). RBP is synthesized in the liver and delivers retinol from liver stores to peripheral tissues, and elevated serum levels of RBP4 have been linked to NAFLD in a cohort of subjects with and without diabetes (14, 15). Fat intake induces changes in the expression of several genes related to fat and energy metabolism, but the progression to obesity is still not well understood. Monitoring mRNA levels of RBP, C-FABP, and H-FABP may afford a simple and convenient method of detecting hypercholesterolemia.

Citrus flavonoids are particularly promising, with a large body of research in humans and animals showing...
Materials and Methods

Rats and feeding method

Sixty 8-week-age male Wistar rats (purchased from Shimizu Laboratory Supplies Co., Ltd., Kyoto) were acclimated in an air-conditioned room at 25°C with 55% humidity and given standard chow for 2 days. The rats were divided into four groups: control (C, n = 15) group, hesperidin (H, n = 15) group, high-cholesterol diet (HC, n = 15) group, and high-cholesterol diet with hesperidin (HHC, n = 15) group. The rats in each group were numbered from 1 to 15. The body weights of rats 1 – 5, 6 – 10, and 11 – 15 in the 4 groups averaged 283.2 ± 7.1, 281.4 ± 10.4, and 285.3 ± 11.6 g, respectively. The C group was fed a standard rat diet (CE-2; Japan Clea, Inc., Tokyo). A high-cholesterol diet (21, 22) was supplied for the HC group; it was made by adding 2% cholesterol and 0.5% cholic acid to the standard diet. The hesperidin diet and high-cholesterol diet with hesperidin were made by adding 0.08% hesperidin (Wako Pure Chemical Industries, Osaka) to the standard diet and high-cholesterol diet, respectively. The amount of feed for each rat was regulated to 25 g/day and water was supplied ad libitum. Body weights, systolic and diastolic blood pressure, and heart rate were measured when the experiment started and on the day of sacrifice. Blood pressure and heart rate were measured by a noninvasive computerized tail-cuff method (BP-98A; Softron, Tokyo).

Sample collection

On days 28, 56, and 84, 5 rats of each group in order of their number were sacrificed by collecting blood from the heart under pentobarbital anesthesia following a 12-h fast. Liver tissue and adipose tissue around the left kidney were removed, and then portions of the liver samples were stored in a 10% formalin solution for HE (hematoxylin and eosin) and oil red O staining (23). The remaining samples were immediately transferred into EP tubes containing 500 μl of RNAlater (Ambion, Inc., Austin, TX, USA), quickly frozen in liquid nitrogen, and stored at −80°C. Serum ALT (alanine aminotransferase), ALP (alkaline phosphatase), γ-GTP (γ-glutamyl transpeptidase), cholinesterase, total bilirubin, creatinine, total cholesterol, HDL (high-density lipoprotein) cholesterol, and TG levels were analyzed using an auto analyzer (7180 Clinical Analyzer; Hitachi High-Technologies, Tokyo) at an accredited clinical laboratory (SRL, Tokyo).

RBP4 enzyme-linked immunosorbent assay (ELISA)

Serum samples were applied for an ELISA of RBP4 (Adipogen Inc., Incheon, Korea) according to the manufacturer’s instructions.

Real-time PCR

Total RNA from the liver and adipose tissue around the left kidney was extracted using TRizol reagent according to the manufacturer’s instructions (Promega, Carlsbad, CA, USA). A semiquantitative real-time polymerase chain reaction (RT–PCR) was performed using Line-Gene (Toyobo, Tokyo) and SYBR Green I (Roche, Basel, Switzerland). The detection was executed at the extension reaction stage in each cycle. The RT–PCR was performed as follows: denaturation for 30 s at 90°C, followed by a 20-min incubation at 61°C, then 95°C for 10 min, 50 cycles at 99°C (15 s), 55°C (45 s), and 72°C (30 s), melting at 95°C for 2 min, 65°C for 1 min, and 95°C for 30 s. The primer sets for RBP, H-FABP, C-FABP, and β-actin mRNA were all synthesized by Hokkaido System Science (Sapporo). The sequence of each primer is listed in Table 1. Using the 2−ΔΔCT method, mRNA expression was semi-quantitatively measured as a relative amount of each target RNA to a known house keeping gene (β-actin) expression level (24, 25).

Extraction of sterols from analytical samples for gas chromatography

Fecal samples collected during the last 3 days using metabolic cages (Sugiyama-gen, Tokyo) were used for extraction of sterols. Fecal samples were dried by lyophilization, and the sterol fraction was extracted from 100 mg of sample using dichloromethane. The sterol fraction was analyzed by gas chromatography.

Table 1.  The sequence of each PCR primer

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
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<tbody>
<tr>
<td>RBP</td>
<td>Forward 5′-gacaaggetctttctctgg-3′ Reverse 5′-gactcgtcccttggctgtag-3′</td>
</tr>
<tr>
<td>H-FABP</td>
<td>Forward 5′-ctagcatgagggaagcaagg-3′ Reverse 5′-tgcttcatccagacaagtgg-3′</td>
</tr>
<tr>
<td>C-FABP</td>
<td>Forward 5′-ggctccgtcttgcttgctgg-3′ Reverse 5′-gggctggctcttaggaagat-3′</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward 5′-ggctccgtcttgcttgctgg-3′ Reverse 5′-gggctggctcttaggaagat-3′</td>
</tr>
</tbody>
</table>

chocolate- and triglyceride (TG)-lowering effects of citrus fruits and juices, as well as purified flavonoids (16, 17). Hesperidin, a citrus bioflavonoid, has biological and pharmacological properties including anti-inflammatory, anti-carcinogenic, antioxidative, vascular protective, and lipid-lowering activities (18–20). In this experiment, we examined the effects of hesperidin on the development of hypercholesterolemia and fatty liver induced by a high-cholesterol diet in rats and investigated mRNA expressions of lipid metabolism–related proteins (RBP, H-FABP, and C-FABP) and the synthesis and absorption indices of cholesterol by gas chromatography.
determining the fecal cholesterol concentration by gas chromatography. A 50-mg sample of pulverized feces with 300 μg of 19-hydroxycholesterol as an internal standard (26) was hydrolyzed with 1 ml of 1 M ethanolic sodium hydroxide for 30 min at 80°C. After the addition of 0.5 ml distilled water to the hydrolysate, nonsaponifiable materials (sterols) were extracted with n-hexane, and the solvent was evaporated. Meanwhile, 300 μl of serum was also processed by the same method, except that 19-hydroxycholesterol, 1 M ethanolic sodium hydroxide, and distilled water were applied at 100 μl and 300 μl, respectively.

Gas chromatography

The gas chromatograph (Model G5000; Hitachi, Tokyo) was equipped with a HiCap CBP-1 capillary column (25 m × 0.25 mm I.D.; Shimadzu, Kyoto) and a solventless injector (Shimadzu). The temperature of the capillary column was programmed as follows: 260°C for 0 min, an increase from 260°C to 280°C at a rate of 2°C/min, and then at 280°C for 30 min. Helium was used as the carrier gas. Capillary gas chromatograms and analytical data for the sterols were obtained using a computerized data system (D-2500 Chromatopac, Hitachi) connected with the gas chromatograph.

Statistical analyses

The data are expressed as the mean ± standard error (S.E.M.). For the continuous variables, differences in responses among groups were compared using Mann-Whitney’s U test for non-parametric variables by SPSS 11.0J (SPSS Japan, Inc., Tokyo). P < 0.05 was considered statistically significant.

Results

Histological examination of liver tissues

As Fig. 1 shows, the fatty degeneration (steatosis) of liver was observed in the HC and HHC groups, but not in the C and H groups. These changes comprising tiny and large vacuoles and pleomorphic nuclei were more prominent in HC than in HHC (Fig. 1A). Oil red O staining revealed that the vacuoles contained lipids, with lipid droplets accumulation observed in all high-cholesterol diet supplemented groups (Fig. 1B). Overall, fatty liver changes were more prominent in the HC group than the HHC group (×400).

Body, liver, and adipose tissue weights

At the beginning of the experiment, there were no significant differences in the baseline of body weights. As Table 2 shows, a high-cholesterol diet increased body weights in the HC and HHC groups compared with the C and H groups at 8 and 12 weeks (P < 0.05). Liver weights were significantly lower in the HHC group than the HC group after 8 and 12 weeks (P < 0.05), but higher in the HHC and HC groups than the C and H groups during the 12 weeks.

Blood chemistry and cholesterol concentrations

No significant changes in systolic or diastolic blood pressure and heart rates were observed during the experiment. Serum ALT, ALP, γ-GTP, cholinesterase, total bilirubin, and creatinine concentrations were normal in all groups (not shown). After 4 and 12 weeks, the concentration of blood sugar in HC (122.0 ± 6.1 and 119.67 ± 2.85 mg/dl, respectively) and HHC (121.7 ± 2.4 and 114.3 ± 5.9 mg/dl, respectively) groups were significantly elevated compared with those in the C (91.4 ± 4.7 and 98.7 ± 3.0 mg/dl, respectively) and H (104.4 ± 4.6 and 110.4 ± 3.3 mg/dl, respectively) groups (P < 0.05). The concentration of blood sugar in the HHC group tended to be lower than that in the HC group.

The high-cholesterol supplemented groups showed significant differences with the C and H groups in TC (higher), LDL cholesterol (higher), and HDL cholesterol (lower) concentrations (Fig. 2, P < 0.05). At 12 weeks, the total cholesterol concentration differed significantly between the HC and HHC groups (P < 0.05). TG levels tended to be lower in the HHC group than the HC groups.

RBP4 in serum

RBP4 concentrations were increased in the HC group compared to the other groups (including the HHC group) after 8 and 12 weeks (Fig. 3, P < 0.05).

mRNA expression in liver and adipose tissue

Figure 4 demonstrates the changes of mRNA expression in liver and adipose tissue around the left kidney of rats for 12 weeks. Data for the HC and HHC groups is shown taking the level of mRNA in the C group as 1 at each time point. Values for the H group, similar to those of the control group, are not shown.

RBP mRNA expression in liver in the HC group was significantly increased compared to that in the C and H groups after 4 weeks (P < 0.05) and then gradually decreased to be significantly lower than levels in the C, H, and HHC groups until 12 weeks (P < 0.05). Compared with the other three groups, the mRNA expression of...
CFABP in liver in the HC group remained high during the entire experiment ($P < 0.05$). HFABP mRNA expression in the HC group was significantly up-regulated after 12 weeks ($P < 0.05$), whereas it was down-regulated at 8 weeks ($P < 0.05$).

Consistent with RBP mRNA expression in liver, a down-regulation of expression in the HC group also appeared in adipose tissue around the left kidney after 8 and 12 weeks (vs. the other groups $P < 0.05$). CFABP mRNA expression in the HC group gradually increased in adipose tissue. Similar changes in HFABP mRNA expression were observed in adipose tissue of the HC group: down-regulation at 4 weeks (vs. the other groups, $P < 0.05$) and 8 weeks, then marked up-regulation at 12 weeks.
weeks (vs. the other groups, $P < 0.05$). For these mRNA expression levels of lipid metabolism–related proteins, the changes in the HC group were attenuated in the HHC group.

**Table 2.** Body, liver, and adipose tissue weights

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>H</th>
<th>HC</th>
<th>HHC</th>
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<tr>
<td><strong>Body weights (g)</strong></td>
<td></td>
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<tr>
<td>4 weeks</td>
<td>399.6 ± 6.8</td>
<td>404.8 ± 4.4</td>
<td>431.9 ± 9.4</td>
<td>420.6 ± 9.5</td>
</tr>
<tr>
<td>8 weeks</td>
<td>499.4 ± 5.5</td>
<td>484.5 ± 11.2</td>
<td>512.8 ± 2.6$^b$</td>
<td>505.9 ± 4.4</td>
</tr>
<tr>
<td>12 weeks</td>
<td>506.2 ± 9.0</td>
<td>498.2 ± 9.1</td>
<td>544.5 ± 4.9$^b$</td>
<td>542.0 ± 9.0$^b$</td>
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<tr>
<td><strong>Liver weights (g)</strong></td>
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<tr>
<td>4 weeks</td>
<td>13.58 ± 0.52</td>
<td>12.65 ± 0.54</td>
<td>18.27 ± 0.61$^b$</td>
<td>17.15 ± 0.37$^b$</td>
</tr>
<tr>
<td>8 weeks</td>
<td>13.71 ± 0.48</td>
<td>13.92 ± 0.58</td>
<td>24.80 ± 1.60$^b$</td>
<td>20.95 ± 0.22$^{bc}$</td>
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<tr>
<td>12 weeks</td>
<td>14.58 ± 0.43</td>
<td>14.25 ± 0.41</td>
<td>26.68 ± 0.56$^b$</td>
<td>23.03 ± 0.52$^{bc}$</td>
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<tr>
<td><strong>Adipose tissue weights (g)</strong></td>
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<tr>
<td>4 weeks</td>
<td>2.70 ± 0.19</td>
<td>2.58 ± 0.33</td>
<td>3.74 ± 0.09$^b$</td>
<td>3.64 ± 0.47</td>
</tr>
<tr>
<td>8 weeks</td>
<td>3.99 ± 0.25</td>
<td>3.77 ± 0.62</td>
<td>4.72 ± 0.31</td>
<td>4.71 ± 0.35</td>
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<tr>
<td>12 weeks</td>
<td>4.19 ± 0.51</td>
<td>3.95 ± 0.42</td>
<td>6.18 ± 0.41$^b$</td>
<td>4.92 ± 0.26$^c$</td>
</tr>
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</table>

C: control, standard diet group (n = 5); H: standard diet with hesperidin group (n = 5); HC: high-cholesterol diet group (n = 5); HHC: high-cholesterol diet with hesperidin group (n = 5). a: $P < 0.05$ vs. C group; b: $P < 0.05$ vs. H group; c: $P < 0.05$ vs. HC group. Data are expressed as the mean ± S.E.M.

**Fig. 2.** Effect of hesperidin on the changes in lipid profiles. Total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) concentrations in serum are shown. C: control, standard diet group (n = 5); H: standard diet with hesperidin group (n = 5); HC: high-cholesterol diet group (n = 5); HHC: high-cholesterol diet with hesperidin group (n = 5). Dotted, hatched, and shaded columns represent 4, 8, and 12 weeks, respectively. a: $P < 0.05$ vs. C group; b: $P < 0.05$ vs. H group; c: $P < 0.05$ vs. HC group; *$P = 0.078$ vs. HC group.

Cholesterol, lathosterol, campesterol, and $\beta$-sitosterol concentrations determined by gas chromatography

Phytosterols are naturally occurring substances found in plants and structurally similar to cholesterol (27). Campesterol and $\beta$-sitosterol indicate cholesterol intestinal absorption (28), whereas the serum lathosterol concentration is an indicator of whole-body cholesterol synthesis (29). We used gas chromatography to measure these three substances in the serum of rats. The results are shown in Table 3. Serum lathosterol concentrations decreased in the HHC group (vs. HC group, $P < 0.05$ at 12 weeks). Concentrations of serum campesterol and $\beta$-sitosterol were lower in the HHC group than the HC group, but the difference in $\beta$-sitosterol concentrations was not significant at 8 and 12 weeks.
Analysis of rat feces showed that the HC and HHC groups contained more cholesterol than the C and H groups. Furthermore, feces of the HHC group contained much more cholesterol than the HC group at 8 and 12 weeks (Table 4, \( P < 0.05 \)).

**Discussion**

Hypercholesterolemia had been established as one of major risk factors contributing to CVD, and a recent report strongly suggested that NAFLD is also associated with an increased risk of CVD (30, 31). LDL cholesterol was reported to be linked to atheromas; on the other hand, plasma levels of HDL cholesterol are a negative risk factor for CVD (32). The antiatherogenic function of HDL is mediated at least in part by the promotion of

![Diagram](image-url)

**Fig. 3.** Retinol-binding protein 4 (RBP4) concentration in serum. RBP4 concentrations in control, standard diet group (C, \( n = 5 \)), standard diet with hesperidin group (H, \( n = 5 \)), high-cholesterol diet group (HC, \( n = 5 \)), and high-cholesterol diet with hesperidin group (HHC, \( n = 5 \)) for 4, 8, and 12 weeks. Dotted, hatched, and shaded columns represent 4, 8, and 12 weeks, respectively. a: \( P < 0.05 \) vs. C group; b: \( P < 0.05 \) vs. H group; c: \( P < 0.05 \) vs. HC group.

![Diagram](image-url)

**Fig. 4.** Changes in mRNA expression with time in liver and adipose tissue. Levels of RBP, C-FABP, and H-FABP against \( \beta \)-actin mRNA expression are shown taking the level of mRNA expression in the control (C) group as 1 at each time point. The left-side graphs show the expression in liver. The right-side graphs show the expression in adipose tissue around the left kidney. Triangles represent values in the HC group and squares, those in the HHC group. HC: high-cholesterol diet group (\( n = 5 \)); HHC: high-cholesterol diet with hesperidin group (\( n = 5 \)). a: \( P < 0.05 \) vs. C group; b: \( P < 0.05 \) vs. H group; c: \( P < 0.05 \) vs. HC group. 4W: 4 weeks, 8W: 8 weeks, 12W: 12 weeks.
Hesperidin Ameliorates Dyslipidemia

Current approaches to raise plasma HDL cholesterol levels have limitations; for example, niacin can modestly increase HDL cholesterol levels but it is easy to induce tolerability, and fibrates have relatively small effects on HDL cholesterol in most patients. As well, LDL cholesterol reduction therapies also remain a substantial risk according to clinical evidence. Statins, hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, are well-established drugs for the treatment of hypercholesterolemia, though they sometimes cause adverse reactions such as rhabdomyolysis and hepatitis. Chinese herbal medicines would be an efficient and relatively safe way to achieve the balance between LDL and HDL cholesterol concentrations through daily diet in combination with drugs. In the present study, we observed a decrease in all lipid parameters (total cholesterol, TG, and LDL cholesterol concentrations) and an increase of HDL cholesterol concentrations in the serum of rats fed high-cholesterol diet with hesperidin. Hesperidin effectively alleviated the steatosis of fatty liver caused by a high-cholesterol diet, as shown by histopathological examinations. According to these results, to some extent, hesperidin might play an important role in reducing the risk of CVD.

RBP mRNA is mainly expressed in liver, kidney, and adipose tissue. RBP mRNA expression in white adipose tissue was up-regulated in obese rats fed a high-fat diet. In our experiment, RBP mRNA expression in the liver of rats fed a high-cholesterol diet was up-regulated and then down-regulated, while in adipose tissues, it was down-regulated. In the case of a high-cholesterol diet, the high concentration of RBP protein circulating in the serum may reduce the mRNA expression; this speculation was proved by subsequent RBP4 measurements.

RBP4 is a protein secreted by hepatocytes and adipose tissue, and it takes part in the control of metabolic and proliferative cell functions, including steatogenesis. Stefan et al. found a direct relation between hepatic fat content and blood levels of RBP4 in healthy subjects. Thus, the measurement of serum or plasma RBP4 levels is a useful means for understanding metabolic disorders. Rosuvastatin significantly decreased the serum level of RBP4 in type 2 diabetes patients with hyperlipidemia, in addition to exerting a strong LDL-cholesterol lowering effect, and resulted in significant regression of atherosclerosis. In our experiment, hesperidin showed weak, but similar effects to statins, suggesting its specificity in alleviating hypercholesterolemia to be partly induced through a lowering of the RBP4 concentration. The observed lower RBP4 concentration in hesperidin-supplemented groups might

| Table 3. Lathosterol, campesterol, and β-sitosterol concentrations in serum determined by gas chromatography |
|---------------------------------|---|---|---|---|
|                               | C  | H   | HC  | HHC |
| Lathosterol (µg/µl)           |    |     |     |     |
| 4 weeks                       | 0.64 ± 0.02 | 0.52 ± 0.08 | 0.63 ± 0.18 | 0.75 ± 0.42 |
| 8 weeks                       | 0.22 ± 0.15 | 0.19 ± 0.05 | 1.47 ± 0.42<sup>b</sup> | 0.57 ± 0.08<sup>b</sup> |
| 12 weeks                      | 0.64 ± 0.04 | 0.59 ± 0.13 | 2.41 ± 0.07<sup>b</sup> | 1.72 ± 0.22<sup>c</sup> |
| Campesterol (µg/µl)           |    |     |     |     |
| 4 weeks                       | 0.89 ± 0.04 | 0.52 ± 0.04<sup>a</sup> | 1.43 ± 0.41<sup>b</sup> | 0.74 ± 0.03<sup>a</sup> |
| 8 weeks                       | 2.14 ± 0.78 | 1.31 ± 0.38 | 11.52 ± 2.92<sup>b</sup> | 2.66 ± 0.77<sup>c</sup> |
| 12 weeks                      | 0.80 ± 0.17 | 0.67 ± 0.09 | 8.69 ± 1.80<sup>b</sup> | 3.46 ± 0.57<sup>b</sup> |
| β-Sitosterol (µg/µl)          |    |     |     |     |
| 4 weeks                       | 0.71 ± 0.06 | 0.52 ± 0.05<sup>a</sup> | 1.18 ± 0.25<sup>b</sup> | 0.13 ± 0.03<sup>abc</sup> |
| 8 weeks                       | 0.66 ± 0.17 | 0.61 ± 0.08 | 0.81 ± 0.61 | 0.54 ± 0.17 |
| 12 weeks                      | 0.91 ± 0.14 | 0.95 ± 0.11 | 1.06 ± 0.16 | 0.50 ± 0.24 |

C: control, standard diet group (n = 5); H: standard diet with hesperidin group (n = 5); HC: high-cholesterol diet group (n = 5); HHC: high-cholesterol diet with hesperidin group (n = 5). a: P < 0.05 vs. C group, b: P < 0.05 vs. H group, c: P < 0.05 vs. HC group. Data are expressed as the mean ± S.E.M.

| Table 4. Cholesterol concentrations in feces determined by gas chromatography |
|---------------------------------|---|---|---|---|
|                               | C  | H   | HC  | HHC |
|                               |    |     |     |     |
| 4 weeks                       | 1.93 ± 0.31 | 0.57 ± 0.09<sup>a</sup> | 25.33 ± 2.02<sup>ab</sup> | 26.56 ± 3.41<sup>ab</sup> |
| 8 weeks                       | 0.90 ± 0.07 | 0.77 ± 0.10 | 16.95 ± 1.32<sup>ab</sup> | 30.95 ± 4.76<sup>abc</sup> |
| 12 weeks                      | 0.86 ± 0.07 | 0.61 ± 0.09<sup>a</sup> | 26.07 ± 1.23<sup>ab</sup> | 37.81 ± 1.80<sup>abc</sup> |

C: control, standard diet group (n = 5); H: standard diet with hesperidin group (n = 5); HC: high-cholesterol diet group (n = 5); HHC: high-cholesterol diet with hesperidin group (n = 5). a: P < 0.05 vs. C; b: P < 0.05 vs. H group; c: P < 0.05 vs. HC group. Data are expressed as the mean ± S.E.M. in mg.
explain up-regulation of RBP mRNA compared to the high-cholesterol diet groups (at 12 weeks in liver and at 8 and 12 weeks in adipose tissue).

FABP family members are in charge of regulating fatty acid uptake and intracellular transport (14). H-FABP regulates metabolic homeostasis by modulating intracellular lipid transport in cardiac muscles (45). FABP mRNA expression has been reported to increase in abdominal white adipose tissue in rats fed a high-fat diet for 30 days (12). However, in cardiac muscle, H-FABP mRNA expression was down-regulated in rats fed a high-fat diet for 65 days (46). This phenomenon of H-FABP mRNA expression in livers of rats fed a high-cholesterol diet for 28 days was also demonstrated in our study. Compared with control rats, H-FABP mRNA of high-cholesterol diet rats was markedly up-regulated in liver (at 8 and 12 weeks) and adipose tissue (at 12 weeks). This result is partly consistent with our previous study (47).

C-FABP, also known as psoriasis-associated FABP, keratinocyte FABP, or epidermal FABP, is a member of the intracellular lipid-binding protein multigene family (48). Here, in livers of high-cholesterol diet rats, it maintained high expression levels for 12 weeks; and in adipose tissue, first down-regulation (at 4 and 8 weeks), then up-regulation (at 12 weeks) was displayed. This is in accordance with not only our previous study, but also another study with obese rats (49). In conclusion, compared with HC groups, hesperidin supplementation (HHC group) attenuated the marked changes in mRNA expression at 12 weeks in adipose tissue. 

Plasma cholesterol homeostasis relies mainly on two physiologic mechanisms: intestinal cholesterol absorption and liver cholesterol synthesis, which are cross-regulated to maintain plasma and serum cholesterol levels (50). Liver cholesterol synthesis has been found to be related to plasma or serum levels of lathosterol (29, 51), a precursor sterol in the cholesterol synthetic chain, whereas cholesterol intestinal absorption is proportional to plasma levels of phytosterols (mainly campesterol and sitosterol), which are plant-derived sterols that cannot be synthesized but only absorbed by the intestine (50).

The relationship between biomarkers of cholesterol metabolism and cholesterol concentration is still controversial. Patients with non-alcoholic fatty liver disease exists higher cholesterol synthesis and low absorption (52), but modest association of high cholesterol absorption and low cholesterol synthesis with an increased severity of coronary artery disease is also reported (53). In an experiment, which takes plasma non-cholesterol sterols as a diagnostic tool, Noto et al. also found increases in both cholesterol synthesis and absorption in pediatric hypercholesterolemia as we found in the high-cholesterol diet group (54).

Concentrations of lathosterol, campesterol, and β-sitosterol were lower in the HHC group than the HC group, consistent with the simultaneous results of the fecal tests. As Table 4 shows, data of fecal gas chromatography in HC groups showed irregular changes with time, but the relationship among the groups kept the same tendency in each experimental period. From the results of gas chromatography, we presume that hesperidin could efficiently decrease cholesterol concentrations by inhibiting both absorption and synthesis and might work by regulating enzymes or mRNA related with cholesterol synthesis or absorption.

Hepatic HMG-CoA reductase is a key enzyme in cholesterol synthesis, and acyl CoA cholesterol acyltransferase (ACAT) is another key cholesterol-regulating enzyme involved in the esterification and absorption of cholesterol, secretion of hepatic LDL-cholesterol, and accumulation of cholesterol in the arterial wall (55). Our hypothesis is supported by studies showing that hesperidin could inhibit both hepatic HMG-CoA reductase and ACAT activities, while increasing fecal cholesterol and LDL receptor mRNA levels in animals and cells (56 – 59). Furthermore, it also suppressed the mRNA expression of steroyl-CoA desaturase, a key enzyme in lipid synthesis in rat primary hepatocytes (60).

Hesperidin, as a flavanone glycoside found abundantly in citrus fruits, is easy to be absorbed in the daily diet for humans. Rizza et al. reported oral hesperidin administration (500 mg once daily for 3 weeks) favorably altered lipid profiles in subjects with the metabolic syndrome (61); in individuals with hypercholesterolemia, consumption of orange juice or hesperidin for 4 – 6 weeks increases HDL and lowers TGs, respectively (62, 63). A more recent study found no effect of hesperidin (800 mg/day for 4 weeks) on serum cholesterol or TG levels (64). Consequently, differences among results of these various studies are not easily explained. Further studies should be designed to specifically examine the effects of hesperidin on lipid metabolism and the pharmacokinetic process of hesperidin in humans.

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