A Freshwater Clam (Corbicula fluminea) Extract Improves Cholesterol Metabolism in Rats Fed on a High-Cholesterol Diet

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The effect of a freshwater clam (Corbicula fluminea) extract (FCE) on cholesterol metabolism in rats fed on a high-cholesterol diet was investigated. When rats were fed various amounts of FCE in addition to the high-cholesterol diet for 2 wk, the serum and hepatic cholesterol levels were gradually reduced in a dose-dependent manner, as compared with the control group. The excretion of neutral sterols and bile acids into the feces was increased by feeding FCE. Several phytosterols were detected in the feces of rats fed on the FCE-containing diet. In addition, substantial amounts of phytosterols were found in FCE. Cholesterol 7α-hydroxylase (CYP7A1) mRNA in the liver of the rats fed on the FCE-containing diets was higher than that of rats fed on the high-cholesterol diets without FCE. These results may suggest that enhanced cholesterol degradation and the excretion of neutral sterols and bile acids contributed to the hypocholesterolemic effect of FCE observed in the hypercholesterolemic rats fed on the high-cholesterol diet.

Key words: freshwater clam extract; cholesterol; bile acids; cholesterol 7α-hydroxylase

The freshwater clam, a popular edible shellfish in Asia, is said to be beneficial to the liver function. However, scientific evidence for such a benefit is limited. We have previously reported that a freshwater clam (Corbicula fluminea) extract (FCE) had desirable effects on the liver function of rats such as a protective effect against liver injury induced by D-galactosamine, an ameliorating effect on alcoholic fatty liver, and an accelerative effect on ethanol metabolism.1) Iritani et al. have reported the effect of the freshwater clam (Corbicula japonica) on the lipid metabolism in rats fed on a high-cholesterol diet.2,3) They used minced edible portions2) or triglycerides3) of the freshwater clam, and found that the freshwater clam reduced the serum and hepatic cholesterol and triglyceride levels in rats.2,3) In particular, they speculated that the hypocholesterolemic effect was due to inhibition of the absorption of cholesterol by several sterols (24-methylene cholesterol and β-sitosterol) in the freshwater clam.2) Several other previous studies have reported that shellfish other than the freshwater clam had hypocholesterolemic effects due to sterols;4–11) however, investigations on the effect of shellfish on bile acid metabolism have been limited.

We examined the hypocholesterolemic effect of FCE in rats fed on a high-cholesterol diet. As in previous studies that used the shellfish itself, we found in this study that FCE reduced the serum and hepatic cholesterol levels in rats. Moreover, we studied the expression of the genes involving cholesterol catabolism such as cholesterol 7α-hydroxylase (CYP7A1; EC1.14.13.7) in order to elucidate the degradation mechanism for absorbed cholesterol.

Materials and Methods

Preparation of the freshwater clam extract. Fifty kilograms of freshwater clams (Corbicula fluminea) were steamed until the shells opened, and the edible portions were removed. The edible portion (4.8 kg) was mashed and extracted with two-fold boiling water for 2 h, and extract filtered. The filtrate was spray-dried for use as FCE [57.0 g of protein, 21.3 g of carbohydrate, 8.1 g of moisture, 7.2 g of crude fat, and 6.4 g of ash were contained in 100 g of the powder]. The yield of FCE from the raw shellfish was approximately 1.5% (w/w).
Male Wistar rats (4 wk old, Japan SLC, Hamamatsu, Japan) weighing about 100 g were maintained at 23 °C with a 12 h light (8:00–20:00) and dark (20:00–8:00) cycle. To accustom the rats to the experimental conditions, they were initially fed on a commercial stock diet (5L37; Japan SLC) for 3 d, and then fed on a 20% casein diet for 4 d, before being divided into four groups of six animals each. The compositions of the test diets are shown in Table 1. The animals were fed on a high-cholesterol diet or on a high-cholesterol diet supplemented with various levels of FCE for 2 wk. The high-cholesterol diet contained 5 g of cholesterol and 2.5 g of sodium cholate per kg of diet. Various levels of FCE were added to the high-cholesterol diet at the expense of casein and sucrose. The protein level of all experimental diets was the same. The rats were individually housed in stainless steel cages and given free access to the experimental diets and water. Feces were collected over the final 3 d of the experimental period and used for determining fecal neutral sterols and bile acids.

The rats in all groups were anesthetized with diethyl ether and killed at 22:00, after 4-h of fasting, on the last day of the experimental period. Blood was collected by cardiac puncture for an analysis of the serum lipids. The liver was immediately removed for an analysis of the hepatic lipids and genes linked to cholesterol metabolism. The experimental procedures used in this study met the guidelines of the Animal Care and Use Committee of Oita University.

Biochemical analyses. Serum total cholesterol was determined with a commercial kit (T-CHO; KAINOS Laboratories, Tokyo, Japan). About 2.5 g of liver was homogenized, and the lipids were extracted with a chloroform:methanol mixture (2:1, v:v) as described by Folch et al. Total lipids in the liver were determined gravimetrically. The concentration of hepatic cholesterol in each lipid extract was determined by the methods just described. Agarose gel electrophoresis of the serum was carried out to separate the lipoproteins, after which lipoprotein-cholesterol was enzymatically stained. Fecal sterols were extracted by the method described by Delaney et al. Fecal neutral sterols were analyzed as the trimethyl silyl ester by using gas chromatography/mass spectrometry (GC 6890 equipped with 5973 MSD and a 30 m × 0.25 mm HP-5 ms capillary column; Agilent), with 5a-cholestone used as an internal standard. The injector and detector temperatures were set at 300 °C and 230 °C, respectively. The initial column temperature was 245 °C; it was held for 2 min and then increased to 300 °C at a rate of 2 °C/min. Fecal bile acids were enzymatically determined by the method of Sheltawy and Losowsky, with lithocholic acid used as a standard.

Total RNA was isolated according to the method described by Chomczynski and Sacchi, and 20 μg of total RNA was subjected to northern blot hybridization. The cDNA clones of rat CYP7A1, rat liver X receptor (LXR), rat small heterodimer partner (SHP), rat hepato-cyte nuclear factor 4 (HNF-4), rat apolipoprotein (apo) A-I, and mouse apo E were labeled with the Megaprime DNA labeling system (Amersham, Tokyo, Japan) and used for hybridization. Specific hybridization was quantified with an image analyzer (BAS 2000, Fuji Film, Tokyo, Japan). The apo E mRNA level was not affected by any treatment employed in this study (data not shown), so we used it as a normalization standard.

Statistics. The significance of differences among values was analyzed by a one-way analysis of variance (ANOVA) and then by Tukey’s multiple-range test. Differences were considered significant at *P* < 0.05.

Results

Table 2 shows the growth parameters of rats fed on the high-cholesterol diet or FCE-containing high-cholesterol diet. The food intake and relative liver weight did not differ among the groups. The final body weight and body weight gain of the rats fed on 150 g of FCE/kg or 300 g of FCE/kg of the high-cholesterol diet were significantly higher than of those fed on the high-cholesterol diet without FCE.

Figure 1 shows the levels of serum and hepatic cholesterol. Thirty grams of FCE per kilogram of the high-cholesterol diet did not affect the level of serum cholesterol, but the serum cholesterol of the rats fed with 150 g of FCE/kg or 300 g of FCE/kg of the high-cholesterol diet was significantly decreased, as compared with that of the rats fed on the high-cholesterol diet without FCE (Fig. 1A). Agarose gel electrophoresis revealed that VLDL cholesterol was substantially reduced by adding FCE to the high-cholesterol diet (data not shown). In all the groups of rats fed on the FCE-containing diets, the hepatic cholesterol level was significantly lower than that in the rats fed on the high-cholesterol diet without FCE. Hepatic cholesterol was not significantly different among the groups of rats fed on the high-cholesterol diet.
with FCE, although FCE tended to reduce it in a dose-dependent manner (Fig. 1B).

The fecal excretion of neutral sterols and bile acids was significantly higher by the rats fed on the FCE-containing diet in a dose-dependent manner, as compared with the rats fed on the high-cholesterol diet without FCE (Table 3).

Several phytosterols were found in the feces of rats fed FCE (Table 3), so we next analyzed the phytosterols pared with the rats fed on the high-cholesterol diet

### Table 2. Growth Parameters for Rats Fed on a Freshwater Clam Extract (FCE)-Supplemented High-Cholesterol Diet

<table>
<thead>
<tr>
<th></th>
<th>HC2</th>
<th>HCFCE2, g of FCE/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>150</td>
</tr>
<tr>
<td>Initial body weight (g)</td>
<td>109 ± 1.3</td>
<td>109 ± 1.2</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>182 ± 3.2a</td>
<td>188 ± 1.3b</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>73.5 ± 2.1a</td>
<td>79.3 ± 1.1a</td>
</tr>
<tr>
<td>Food intake (g for 14 d)</td>
<td>201 ± 5.2</td>
<td>199 ± 2.4</td>
</tr>
<tr>
<td>Relative liver weight</td>
<td>4.88 ± 0.09</td>
<td>5.14 ± 0.05</td>
</tr>
<tr>
<td>(g/100 g of body weight)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1Each value is the mean ± SEM for six rats in each dietary group. The statistical significance of differences among values was analyzed by ANOVA and then by Tukey’s multiple-range test. Values in a row with different letters indicate a statistically significant difference, P < 0.05.

2HC, high-cholesterol diet group; HCFCE, freshwater clam extract (FCE)-supplemented high-cholesterol diet group

### Table 3. Excretion of Sterols into the Feces of Rats Fed on a Freshwater Clam Extract (FCE)-Supplemented High-Cholesterol Diet

<table>
<thead>
<tr>
<th></th>
<th>HC2</th>
<th>HCFCE2, g of FCE/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>150</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>8.0 ± 1.2c</td>
<td>10.6 ± 1.9d</td>
</tr>
<tr>
<td>Coprostanol</td>
<td>2.1 ± 0.7c</td>
<td>5.7 ± 2.4ab</td>
</tr>
<tr>
<td>Coprostanone</td>
<td>0.0c</td>
<td>0.2 ± 0.1bc</td>
</tr>
<tr>
<td>Brassicasterol</td>
<td>0.1 ± 0.0d</td>
<td>0.3 ± 0.0a</td>
</tr>
<tr>
<td>Campesterol</td>
<td>0.0c</td>
<td>0.1 ± 0.0a</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>0.0c</td>
<td>0.1 ± 0.0a</td>
</tr>
<tr>
<td>β-Sitosterol</td>
<td>0.2 ± 0.0d</td>
<td>0.2 ± 0.0a</td>
</tr>
<tr>
<td>Total neutral sterols</td>
<td>10.0 ± 0.9a</td>
<td>16.5 ± 1.0a</td>
</tr>
<tr>
<td>Total phytosterols</td>
<td>0.3 ± 0.1a</td>
<td>0.7 ± 0.1a</td>
</tr>
<tr>
<td>Total bile acids</td>
<td>241 ± 28c</td>
<td>333 ± 29c</td>
</tr>
</tbody>
</table>

1Each value is the mean ± SEM for six rats in each dietary group. The statistical significance of differences among values was analyzed by ANOVA and then by Tukey’s multiple-range test. Values in a row with different letters indicate a statistically significant difference, P < 0.05.

2HC, high-cholesterol diet group; HCFCE, freshwater clam extract (FCE)-supplemented high-cholesterol diet group

3Total neutral sterols, cholesterol + coprostanol + coprostanone

4Total phytosterol, brassicasterol + campesterol + stigmasterol + β-sitosterol

Fig. 1. Effect of the Freshwater Clam Extract (FCE) on (A) Serum and (B) Hepatic Cholesterol in Rats Fed on a High-Cholesterol Diet. Each value is the mean ± SEM for six rats in each dietary group. The statistical significance of differences among values was analyzed by ANOVA and then by Tukey’s multiple-range test. In each graph, different letters indicate a statistically significant difference, P < 0.05. HC, high-cholesterol diet group; HCFCE, freshwater clam extract (FCE)-supplemented high-cholesterol diet group.
terol, stigmasterol, and β-sitosterol were detected in FCE (Table 4).

The mRNA level of hepatic CYP7A1, the limiting enzyme on the metabolic pathway from cholesterol to bile acid, tended to be higher in the rats fed with 30 g of FCE/kg or 150 g of FCE/kg of the high-cholesterol diet. The hepatic CYP7A1 mRNA level in the 300 g of FCE/kg of the high-cholesterol diet group was significantly higher than that of the group fed on the high-cholesterol diet without FCE (Fig. 2A). The hepatic level of SHP mRNA, a negative regulator of CYP7A1, was not altered (Fig. 2B). As positive regulators, the HNF-4 mRNA level in the liver was increased by FCE in a dose-dependent manner (Fig. 2C), while the mRNA level of LXR was unchanged (Fig. 2D).

**Discussion**

Iritani et al. have shown in previous studies that feeding freshwater clams significantly reduced the cholesterol level in rats fed on a high-cholesterol diet. We have demonstrated here that the extract of freshwater clam (FCE) significantly reduced the serum and hepatic cholesterol levels (Fig. 1). Iritani et al. have indicated that freshwater clams contained several sterols that competitively interfered with cholesterol absorption. Therefore, they attributed the hypocholesterolemic effect of freshwater clams to competitive interference with cholesterol absorption. However, it is unclear whether plant sterols in FCE were present in a sufficient amount to decrease the cholesterol level in the serum and liver. The contribution of plant sterols to the

| Table 4. Phytosterols in the Freshwater Clam Extract (FCE) |
|-----------------|-----------------|
| Phytosterol     | FCE (μmol/g)    |
| Brassicasterol  | 1.80            |
| Campesterol     | 3.31            |
| Stigmasterol    | 1.42            |
| β-Sitosterol    | 0.33            |

Fig. 2. Effect of the Freshwater Clam Extract (FCE) on the mRNA Levels of CYP7A1 (A), SHP (B), HNF-4 (C), and LXR (D) in Rats Fed on a High-Cholesterol Diet. Each value is the mean ± SEM for six rats in each dietary group. The statistical significance of differences among values was analyzed by ANOVA and then by Tukey’s multiple-range test. In each graph, different letters indicate statistically significant differences, \( P < 0.05 \). HC, high-cholesterol diet group; HCFCE, freshwater clam extract (FCE)-supplemented high-cholesterol diet group.
reduction of serum and liver cholesterol should be quantitatively determined. Sugano et al. have conducted a study on the dosage level of plant sterols having an effect on cholesterol metabolism in rats fed on a high-cholesterol diet.\(^{16}\) Based on their study, the amount of 300 g of FCE/kg of diet used in this study might have provided enough of such phytosterols as brassicasterol, \(\beta\)-sitosterol, campesterol, and stigmasterol (Table 4) to reduce liver cholesterol but not to reduce serum cholesterol.\(^{19}\) However, phytosterol in 30 g or 150 g of FCE/kg of diet was not sufficient to reduce serum and hepatic cholesterol in this study. This suggests that the hypocholesterolemic effect due to phytosterol in FCE may not be very potent. However, the fecal excretion of neutral sterols was dramatically increased by feeding the FCE-containing diets (Table 3). These results suggest that constituents other than phytosterols in FCE contributed to the accelerative effect on neutral sterol excretion in the rats fed with a high-cholesterol diet.

The fecal excretion of bile acids in this study was elevated by the FCE-containing diet (Table 3). It is not known how phytosterols influence the excretion of bile acids into feces.\(^{10-21}\) We have assumed that FCE would have other effects related to bile acid metabolism that would contribute to its hypocholesterolemic action. Tanaka et al.\(^{5}\) have reported that dietary oyster accelerated the fecal excretion of neutral sterols and bile acids. They speculated that the hypocholesterolemic effect of oyster may have been due to a disturbance of bile salt micelles in the intestines.\(^{5}\) We found that the level of CYP7A1 mRNA, the rate-limiting enzyme for bile acid synthesis, was significantly increased by supplementing FCE to the high-cholesterol diet (Fig. 2A). This might have been responsible for the reduction in the serum level of cholesterol. Many transcriptional factors have been reported to play an important role in regulating CYP7A1 transcription.\(^{22}\) CYP7A1 gene expression is negatively regulated by bile acids through SHP, whereas HNF-4 and LXR positively regulate gene expression.\(^{22}\) The induction of the gene occurred without any change in the SHP mRNA level (Fig. 2B). Additionally, we determined in this study the mRNA levels of HNF-4 and LXR to elucidate how CYP7A1 was up-regulated in rats fed on the FCE-containing diet. However, the gene expression of LXR, which binds to a DR4 (direct repeat 4) and stimulates rat CYP7A1 gene transcription,\(^{23}\) was not altered by feeding FCE (Fig. 2D). HNF-4 gene expression was up-regulated by supplementing FCE to rats fed on the high-cholesterol diet (Fig. 2C). HNF-4 binds to a DR1 sequence in BARE-II and stimulates the CYP7A1 promoter activity.\(^{24,25}\) The up-regulation of CYP7A1 might have been due to the induction of the HNF-4 gene by feeding FCE. HNF-4 also regulates apo A-I, a major apolipoprotein in the HDL particle.\(^{26}\) In this study, however, the expression level of apo A-I mRNA was not altered by feeding FCE (data not shown), despite the finding that the HNF-4 gene was up-regulated. This leads us to speculate that other mechanisms involved in the induction of CYP7A1 gene expression might be important. We are now investigating other possibilities.

In conclusion, the hypocholesterolemic effect of FCE may have been due not only to competitive interference with cholesterol absorption by several plant sterols, but also to enhancement of the cholesterol excretion and degradation by constituents other than plant sterols. In particular, cholesterol degradation to bile acids was through the induction of CYP7A1. We are at present investigating the effect of FCE on cholesterol metabolism in other hypercholesterolemic models, e.g., the endogenous hypercholesterolemia induced by xenobiotics.

References


