Effects of Dietary Fats and Phytosterol on Serum Fatty Acid Composition and Lipoprotein Cholesterol in Rats

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Summary The effects of dietary fats and phytosterol on the fatty acid composition and lipoprotein cholesterol in serum were studied in female rats, with the following results. (1) The addition of 1% cholesterol to the 20% butter diet decreased the ratio of polyunsaturated fatty acid (PUFA) to saturated fatty acid (SFA) in serum. This phenomenon was negated when there was an intake of cod liver oil and wheat germ oil. (2) When cholesterol was added to the 20% butter diet, the serum total cholesterol increased 3.7-fold, due to an increase in the lower density lipoprotein (LDL+VLDL). (3) The addition of 5% phytosterol to the 10% butter-cholesterol diet reduced the total cholesterol level and increased the ratio of cholesterol in high density lipoprotein (HDL) to the cholesterol in LDL+VLDL. Although a 10% cod liver oil addition also reduced the total cholesterol level, the ratio of HDL/LDL+VLDL was similar to that of the 10% butter-cholesterol diet. (4) A direct relationship was found between the concentration of oleic acid (18:1) in serum and the total cholesterol level (r=0.947) and also the level of LDL+VLDL-cholesterol (r=0.935). These results show that cod liver oil, wheat germ oil, and phytosterol induce an increase in the PUFA/SFA ratio, promote hypocholesterolemia, and change lipoprotein concentration. However, there were indications that no relationship exists between the change in the total cholesterol level and the change in the ratio of HDL/LDL+VLDL, and that the increase of total cholesterol and LDL+VLDL-cholesterol was consistent with the increase of oleic acid in serum.

Key Words dietary fats, phytosterol, hypocholesterolemia, PUFA/SFA ratio, oleic acid and cholesterol, lipoprotein
Serum cholesterol seems to be a coronary risk factor in humans (1, 2) and its level is influenced by dietary factors (3, 4). Dietary cholesterol induced an increase in the serum cholesterol level, and the response to dietary cholesterol was greater in female rats than in males (5). Recent studies suggest that the cholesterol levels of high density lipoprotein (HDL) and low density lipoprotein (LDL) in serum play more important roles in human coronary artery disease than does the total cholesterol level (6).

Tallow raises the serum cholesterol level in humans while vegetable oils lower it (7, 8). The hypocholesterolemic effect of certain vegetable oils is due to their high polyunsaturated fatty acid (PUFA) and their low concentration of saturated fatty acid (SFA). An increase in the ratio of PUFA to SFA in diet is accompanied by reduction in serum cholesterol in humans (9,10). Another hypocholesterolemic factor to be considered in humans (3) and rats (11) is phytosterol. Corn oil has less PUFA than safflower oil but causes a lower level of human serum cholesterol, possibly because of its higher content of phytosterol (8). Some relationship between dietary PUFA and phytosterol may be a factor in the reduction of serum cholesterol.

In this study using female rats, we investigated the effects of dietary fats and phytosterol on fatty acid composition and lipoprotein cholesterol in serum. One part of the study examined the effects of butter as a source of SFA, cod liver oil as a source of PUFA which is low in phytosterol content, and wheat germ oil as a source for both PUFA and phytosterol. The other part of the study compared the effects of PUFA (cod liver oil) and phytosterol when they supplemented a butter-cholesterol diet.

**EXPERIMENTAL PROCEDURE**

*Animals.* Female Wistar rats (Clea Japan) weighing 180 to 213 g were housed individually in stainless steel wire-bottom cages and kept under standardized light (0800–2000) and temperature (22°C) conditions. All rats were fed an experimental diet *ad libitum* for 18 days and then fasted for 17–20 h prior to autopsy. After the rats were anesthetized with ethyl ether for about 1 min, blood was collected through the abdominal aorta with a syringe and then centrifuged to separate the serum.

*Diets.* The basal diet consisted of 20% casein, 4% Harper salt mixture (12), 1% Harper vitamin mixture (12), 2,000 IU retinyl palmitate, 1,000 IU cholecalciferol, 0.1% α-tocopherol, 0.5% choline chloride, and sucrose to 100%. All test diets were prepared by modifying the basal diet by reducing the amount of sucrose and stored at 6°C under nitrogen. For cholesterol-supplemented diets, 1% cholesterol (Sigma) and 0.25% cholic acid (Sigma) were added. Butter (Snow Brand Milk Products), cod liver oil (Wakasa), and wheat germ oil (Nisshin Seifun) were supplemented (2). The purity of the sterols was 95% and the composition of the sterol fraction was 45% β-sitosterol, 29% stigmasterol, 24% campesterol and 2% brassicasterol.

2 Oriental Yeast. 3 Wako. 4 The purity of the sterols was 95% and the composition of the sterol fraction was 45% β-sitosterol, 29% stigmasterol, 24% campesterol and 2% brassicasterol.
purchased from commercial sources and phytosterol was a gift from Riken Vitamin Co.4

Analysis of fatty acids by gas liquid chromatography. Saponification of serum lipids and preparation of fatty acid fraction were estimated by the Standard Method of the Japan Oil Chemist's Society for Analysis of Fats and Oils. Fatty acid methyl esters were prepared using BF3-methanol and analyzed by gas liquid chromatography as follows. A gas liquid chromatograph (Model 163, Hitachi Ltd.) equipped with flame ionization detector was used and fatty acid methyl esters were separated on a 3-m glass column (3 mm i.d.) packed with 5% SP-2300 coated on 60–80 mesh Uniport H.P. (13). The temperature was programed to increase from 180°C to 240°C at 4°C per min. The carrier gas (nitrogen) flow rate was 17.5 ml/min. Fatty acid methyl esters were identified by comparison of retention data with that of authentic standards and by the method of equivalent chain length. The contents of fatty acids were calculated on the recovery of the added internal standard of heptadecanoic acid (17:0). The ratios of PUFA/SFA in dietary fats using butter, cod liver oil, and wheat germ oil were 0.04, 1.08, and 4.09, respectively.

Determination of lipoprotein cholesterol by high performance liquid chromatography. Lipoproteins of four density classes were isolated from serum by high performance liquid chromatography (HPLC) (HLC 805, Toyo soda Mfg.) using aqueous gel permeation type columns (G5000PW + G3000SW × 2) (Toyo Soda Mfg.), and the quantitation of cholesterol in each lipoprotein fraction was performed by the enzymatic method as follows (14). For the separation of the lipoprotein fractions, 50 µl of whole serum was subjected to HPLC and the eluate were automatically mixed with the enzyme reagent (Cholesterol Reagent Set, Determiner TC555, Kyowa Hakko) in the reactor at a constant temperature. The purple color produced by cholesterol was determined by the absorbance at 550 nm after passage through the reactor. The concentration of cholesterol in each lipoprotein fraction was estimated from the peak area of the absorbance. The serum triglycerides were determined enzymatically (Triglycerides Reagent Set, Determiner TG, Kyowa Hakko).

RESULTS

Body and liver weights and food intake

The results are shown in Table 1. Cholesterol feeding in the 20% butter group resulted in a 1.5-fold increase in liver weight, although there were no significant differences in body weight and food consumption (group A versus group B, Table 1). With the cholesterol diets, rats that were fed 20% cod liver oil (C) had heavier livers than those fed 20% butter (B) or 20% wheat germ oil (D). The livers of rats fed the 10% butter diet supplemented with 10% cod liver oil also were significantly heavier than those of the other groups (F vs. E and G, Table 1). The supplement of 5% phytosterol to the 10% butter-cholesterol diet induced an approximately 20% decrease in the liver weights (E vs. G).
Table 1. Comparison of the effects of dietary fat and cholesterol on body and liver weights and food consumption.
Groups of four female rats each were fed the experimental diet for 18 days. The cholesterol diet group was fed 1% cholesterol with 0.25% cholic acid. Each value represents the mean ± SD for four female rats.

| Exp. Group | Diet | Fat | Cholesterol | Body weight (g) | Liver weight (g/100 g Body weight) | Food intake (g/18 days) |
|------------|------|-----|-------------|----------------|-----------------------------------|------------------------|
|            |      |     |             | Initial       | Final                |                        |
| I          | A    | 20% Butter | –           | 199 ± 11      | 254 ± 20              | 3.1 ± 0.1<sub>abc</sub> | 297 ± 34<sup>f</sup> |
| I          | B    | 20% Butter | +           | 199 ± 10      | 242 ± 10              | 4.6 ± 0.2<sub>ad</sub> | 260 ± 24 |
| I          | C    | 20% Cod liver oil | +        | 199 ± 10      | 230 ± 15<sup>f</sup> | 5.4 ± 0.1<sub>bde</sub> | 224 ± 31<sup>fg</sup> |
| I          | D    | 20% Wheat germ oil | +      | 199 ± 10      | 260 ± 15<sup>f</sup> | 4.3 ± 0.3<sub>ce</sub> | 295 ± 39<sup>g</sup> |
| II         | E    | 10% Butter | +           | 199 ± 10      | 252 ± 8               | 4.4 ± 0.3<sub>ac</sub> | 294 ± 17 |
| II         | F    | 10% Butter + 10% cod liver oil | +     | 199 ± 8       | 248 ± 18              | 5.3 ± 0.4<sub>ab</sub> | 263 ± 27 |
| II         | G    | 10% Butter + 5% phytosterol | +    | 199 ± 6       | 240 ± 8               | 3.5 ± 0.3<sub>bc</sub> | 304 ± 15 |

Values in the same column of each experiment with the same superscript letter are significantly different (t-test, <sup>a,b,c,d,e</sup> p < 0.001, <sup>f,g</sup> p < 0.05 in experiment I. <sup>a,b</sup> p < 0.01, <sup>c</sup> p < 0.05 in experiment II.).
Effects of dietary cholesterol, fats, and phytosterol on serum triglycerides and fatty acid composition

The effects of butter, cod liver oil, wheat germ oil, and phytosterol on the levels of triglycerides and the ratio of polyunsaturated fatty acid (PUFA) to saturated fatty acid (SFA) in serum were compared (Table 2). The triglyceride content was not significantly changed by the addition of cholesterol to the 20% butter diet (group A versus group B, Table 2). Whereas no significant changes were found in the amounts of both PUFA and SFA, dietary cholesterol induced a decrease in PUFA content and an increase in SFA content, and resulted in a decrease in the PUFA/SFA ratio from 1.90 to 1.15. When cod liver oil or wheat germ oil was substituted for butter in the 20% fat-cholesterol diets, the PUFA/SFA ratio was observed to be higher than the ratio in the 20% butter-cholesterol diet but was about the same or higher than the ratio of the butter diet without cholesterol (Table 2).

Supplementation of 10% cod liver oil and 5% phytosterol to the 10% butter-cholesterol diet resulted in a decrease in triglycerides (E vs. F and G, Table 2), although the addition of 5% phytosterol did not reduce the serum triglycerides significantly. Observed was the tendency for both an increase in the PUFA content with the addition of phytosterol to the cholesterol diet and a decrease in SFA content with the addition of cod liver oil. Despite the considerable individual variation in the concentration of PUFA and SFA, the PUFA/SFA ratios in groups F and G increased about 1.7-fold compared with those in group E. These results suggest that no direct correlation exists between the change in triglycerides levels and the change in fatty acid composition in serum, and demonstrate that cod liver oil, wheat germ oil, and phytosterol all negated a decrease in the ratio of PUFA to SFA in serum.

Effects of dietary cholesterol and fats on cholesterol concentration in serum lipoproteins

The effects of dietary cholesterol on the cholesterol levels and the cholestrol pattern in the lipoprotein fractions separated by HPLC are shown in Table 3. Cholesterol feeding to the 20% butter group increased the total serum cholesterol and this rise was due to the increase in the cholesterol in the LDL+VLDL fraction (group A versus group B). The distribution of LDL+VLDL-cholesterol increased, and that of HDL-cholesterol decreased with cholesterol in the 20% butter diet (A vs. B). Therefore, the ratio of HDL-cholesterol to LDL+VLDL-cholesterol was reduced in the cholesterol feeding.

The effects of butter, cod liver oil, or wheat germ oil on the concentration and percentage of cholesterol in each of the lipoproteins were compared (B, C, and D, Table 3). The total cholesterol level decreased and the concentration of lipoprotein cholesterol was much lower in all lipoprotein fractions in the 20% cod liver oil- and 20% wheat germ oil-cholesterol diets compared with the 20% butter-cholesterol diet. The ratios of HDL-cholesterol to LDL+VLDL-cholesterol in the cod liver oil-
Table 2. Comparison of the effects of dietary fat and cholesterol on serum triglycerides and fatty acid composition.

| Exp. Group | Diet | Fat | Cholesterol | Triglycerides (mg/100 ml) | Fatty acid (mg/100 ml) | Ratio of PUFA/SFA |
|------------|------|-----|-------------|--------------------------|------------------------|------------------|
| A          | 20% Butter | -   | +           | 100.8 ± 35.0             | 108.8 ± 53.5          | 95.4 ± 17.9      |
| B          | 20% Butter | +   | +           | 105.7 ± 11.9             | 105.7 ± 11.9          | 105.7 ± 11.9     |
| C          | 20% Cod liver oil | +   | +           | 119 ± 25                 | 119 ± 25              | 119 ± 25         |
| D          | 20% Wheat germ oil | +   | +           | 125 ± 41                 | 125 ± 41              | 125 ± 41         |
| E          | 10% Butter | +   | +           | 168.0 ± 25.1             | 168.0 ± 25.1          | 168.0 ± 25.1     |
| F          | 10% Butter + 10% cod liver oil | +   | +           | 248 ± 73                 | 248 ± 73              | 248 ± 73         |
| G          | 10% Butter + 5% phytosterol | +   | +           | 255 ± 60                 | 255 ± 60              | 255 ± 60         |

Data shown are mean ± SD and values in the same column of the each experiment with the same superscript letter are significantly different (p<0.001, b,c p<0.01, d,e p<0.05 in experiment I. a p<0.001, b,c p<0.05 in experiment II.).

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Table 3. Comparison of the effects of dietary fat and cholesterol on cholesterol in serum lipoproteins. Groups of four rats each were fed the experimental diet for 18 days as described in Table 1. Cholesterol distribution (%) is shown in parenthesis.

| Exp. Group | Diet | Cholesterol (mg/100 ml) | Ratio of HDL/ LDL + VLDL |
|------------|------|-------------------------|--------------------------|
|            | Fat  | Cholesterol Total       | VHDL     | HDL      | LDL + VLDL |                     |
| I          | A    | 20% Butter –            | 81.9 ± 8.3<sup>f</sup> | 4.12 ± 1.2<sup>f</sup> | 61.1 ± 2.4<sup>abc</sup> | 16.7 ± 6.7<sup>fg</sup> | 4.19 ± 1.81<sup>fg</sup> |
|            |      | (100)                   | (5.0)    | (74.6)   | (20.4)     |                       |
|            | B    | 20% Butter +            | 306.2 ± 125.2<sup>f</sup> | 6.03 ± 4.08 | 37.4 ± 5.1<sup>ad</sup> | 262.7 ± 118.8<sup>fh</sup> | 0.16 ± 0.05<sup>eh</sup> |
|            |      | (100)                   | (2.0)    | (12.2)   | (85.8)     |                       |
|            | C    | 20% Cod liver oil +     | 80.6 ± 13.5<sup>se</sup> | 1.45 ± 0.94<sup>f</sup> | 17.5 ± 8.1<sup>bf</sup> | 61.7 ± 21.2<sup>sh</sup> | 0.35 ± 0.25<sup>f</sup> |
|            |      | (100)                   | (1.8)    | (21.7)   | (76.5)     |                       |
|            | D    | 20% Wheat germ oil +    | 63.4 ± 24.7<sup>h</sup> | 1.65 ± 0.41<sup>e</sup> | 17.9 ± 4.5<sup>cd</sup> | 43.8 ± 21.3<sup>l</sup> | 0.46 ± 0.15<sup>sh</sup> |
|            |      | (100)                   | (2.6)    | (28.3)   | (69.1)     |                       |
| II         | E    | 10% Butter +            | 387.7 ± 22.2<sup>ab</sup> | 10.47 ± 9.18 | 88.6 ± 27.6<sup>d</sup> | 288.8 ± 53.5<sup>ab</sup> | 0.33 ± 0.17<sup>d</sup> |
|            |      | (100)                   | (2.7)    | (22.8)   | (74.5)     |                       |
|            | F    | 10% Butter + 10% cod liver oil | 145.4 ± 30.5<sup>a</sup> | 4.65 ± 1.41 | 30.7 ± 4.6<sup>ad</sup> | 110.0 ± 32.6<sup>ce</sup> | 0.30 ± 0.11<sup>se</sup> |
|            |      | (100)                   | (3.2)    | (21.1)   | (75.7)     |                       |
|            | G    | 10% Butter + 5% phytosterol | 122.5 ± 11.9<sup>b</sup> | 4.50 ± 1.71 | 88.8 ± 5.9<sup>a</sup> | 29.1 ± 10.9<sup>bce</sup> | 3.47 ± 1.63<sup>de</sup> |
|            |      | (100)                   | (3.7)    | (72.5)   | (23.8)     |                       |

Data shown are mean ± SD and values in the same column of each experiment with the same superscript letter are significantly different (<sup>a,b,c,d</sup>p < 0.001, <sup>*</sup>p < 0.01, <sup>f,s,h,i</sup>p < 0.05 in experiment I, <sup>a,b</sup>p < 0.001, <sup>*</sup>p < 0.01, <sup>f,e</sup>p < 0.05 in experiment II.).
and wheat germ oil-cholesterol diets (C and D) were higher than those in the butter-cholesterol diets (B). As shown in Table 3, the pattern of cholesterol distribution in the lipoprotein fractions of the 20% cod liver oil- and 20% wheat germ oil-cholesterol diets (C and D) was similar to the pattern in the 20% butter-cholesterol diet (B), rather than being similar to the pattern of 20% butter-cholesterol free diet (A).

Effects of polyunsaturated fat and phytosterol supplementation on the cholesterol concentration in serum lipoproteins

The effects of polyunsaturated fat (cod liver oil) and phytosterol supplementation on cholesterol levels and lipoprotein patterns were studied (Table 3). The serum total cholesterol concentration was reduced by the addition of 10% cod liver oil to the 10% butter-cholesterol diet (group E versus group F). This decrease was primarily due to the reduction in the LDL+VLDL fraction and also due to a concomitant decrease in the HDL-cholesterol concentration. The pattern of the cholesterol distribution in group F was like that in group E, and the ratio of HDL-cholesterol to LDL+VLDL-cholesterol was also almost the same as the ratio in the 10% butter-cholesterol diet. These results showed that addition of cod liver oil did not induce a change in the cholesterol distribution in each lipoprotein. As for the supplementation of 5% phytosterol, in particular, this reduction was caused by the decrease in the LDL+VLDL fraction to about one-tenth of the level of the 10% butter-cholesterol diet (E vs. G). When compared with those of the other two diets, groups E and F, the addition of phytosterol to the 10% butter-cholesterol diet (G) caused a marked difference in the cholesterol distribution in the lipoproteins. The cholesterol concentration in the HDL fraction was 72.5% in the phytosterol group (G) and much greater than the 22.8% and 21.1% of groups E and F, respectively. The ratio of HDL-cholesterol to LDL+VLDL-cholesterol was more than 10 times greater than the ratios of the diets without phytosterol. The pattern of cholesterol distribution in the phytosterol-added diet was almost the same as that of the butter diet without cholesterol (A and G, Table 3). These findings indicated that phytosterol supplementation caused a change in the distribution of cholesterol in lipoprotein fractions.

Correlation between the cholesterol level and the oleic acid content in serum

Dietary cholesterol, phytosterol, and polyunsaturated fat induced changes in the composition of the fatty acid in serum as shown in Table 2. When comparing the cholesterol level to the change of each fatty acid content, the concentration of oleic acid (18:1) in serum showed a correlation to the total cholesterol in serum in all the rats from groups A to G (r=0.947, Fig. 1a). The scatter diagrams of the results from individual animals also indicated a correlation between the LDL+VLDL-cholesterol and the oleic acid concentration (r=0.935, Fig. 1b). These results demonstrated that the change in the levels of total cholesterol and LDL+VLDL-cholesterol were accompanied by the change in the concentration of oleic acid in serum.
Fig. 1. Comparison of the levels of total and LDL+VLDL-cholesterol and oleic acid content in serum. Whole serum were subjected to high performance liquid chromatography (HPLC) and to gas liquid chromatography (GLC) for determination of cholesterol and oleic acid (18:1), respectively. For 18 days, the rats were fed 20% butter diet (A, ◊), 20% butter-cholesterol diet (B, ●), 20% cod liver oil-cholesterol diet (C, △), 20% wheat germ oil-cholesterol diet (D, ▼), 20% butter-cholesterol diet (E, ■), 10% butter-cholesterol diet with 10% cod liver oil (F, ▲), or 10% butter-cholesterol diet with 5% phytosterol (G, △) as described in Tables 1 and 2. (r=0.947 and r=0.935 in Fig. 1a and 1b, respectively.)

DISCUSSION

An increase in dietary cholesterol resulted in higher serum total cholesterol levels in humans (15). We observed that dietary cholesterol induced an increment in the serum total cholesterol concentration, but had no significant effect on the serum triglycerides when cholesterol was added to the 20% butter diet in female rats (group A versus group B, Tables 2 and 3). These findings were consistent with the results reported by O’Brien et al. using female rats (5). When wheat germ oil or cod liver oil replaced butter as the fat source, the increase of serum total cholesterol by cholesterol feeding was not significant (A vs. C and D, Table 3). These facts indicated that the values of cholesterol in serum were not directly reflected in the amounts in diets.

The cholesterol-reducing effect of cod liver oil in this study appears to be due to the higher content of PUFA in cod liver oil than in butter, and this was consistent with observations with other polyunsaturated fat diets in swine (16) and in humans (12). The mechanism by which PUFA alters the serum cholesterol levels is
unknown, but the decrease of the SFA level in serum by the addition of cod liver oil to the 10% butter-cholesterol diet (E vs. F, Table 2) may explain that the lower concentration of cholesterol is caused by an inhibition of the synthesis of fatty acid by PUFA (18). In addition, the supplementation of polyunsaturated fat (cod liver oil) did not induce any differences in the lipoprotein distributions from those in the saturated fat (butter) diet, in spite of a decrease of total serum cholesterol (E vs. F, Table 3). This fact may indicate that dietary PUFA induces a change in the cholesterol concentration not only in the lower density lipoproteins but in all lipoprotein fractions.

The hypocholesterolemic effect of phytosterol is generally assumed to be caused by its interference with cholesterol absorption (19, 20) and by its effect on cholesterol metabolism within the body (21). In our study, supplementation of phytosterol to the butter-cholesterol diet resulted in an increase in the PUFA level in serum (E vs. G, Table 2) and did not induce fatty liver, although a higher level of serum triglycerides was found compared with the polyunsaturated fat-supplemented group (F vs. G, Table 2). In addition, a marked decrease of LDL+VLDL-cholesterol was observed in phytosterol-fed rats (E vs. G, Table 3). Given these results, the phytosterol seems to alter the PUFA level in serum and the amount of fatty acid in the liver, and then appears to induce a change in the cholesterol concentration specifically of the lower density lipoproteins, unlike dietary PUFA which affects all lipoprotein fractions.

The high level in the total cholesterol concentration and the low level in the PUFA/SFA ratio in serum in the 10% butter group with cholesterol feeding was mitigated by a supplementation either with polyunsaturated fat or phytosterol (E vs. F and G, Tables 2 and 3). On the other hand, the pattern of lipoprotein cholesterol distribution in the phytosterol-added group (G) was very different from that in group F which had been supplemented with polyunsaturated fat (Table 3). Group G had a much higher cholesterol percentage in the HDL fraction and a smaller percentage in the LDL+VLDL fraction than did group F. Therefore, phytosterol supplementation caused more than a 10-fold enhancement of the ratio of HDL-cholesterol to LDL+VLDL-cholesterol compared with the ratio in the 10% butter-cholesterol diet without phytosterol (E vs. G, Table 3). In contrast, the cholesterol ratio of HDL-cholesterol to LDL+VLDL-cholesterol in group F supplemented with polyunsaturated fat was almost the same as the ratio in the 10% butter-cholesterol diet (E vs. F, Table 3). These observations suggest that there is no correlation between the changes in the amount of total cholesterol and the changes in cholesterol distribution in lipoproteins. Thus, different mechanisms must be involved in the control of lipoprotein metabolism and hypocholesterolemia.

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