Effects of Soy Milk and Bifidobacterium Fermented Soy Milk on Lipid Metabolism in Aged Ovariectomized Rats

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The effects of soy milk and fermented soy milk on lipid metabolism were studied in aged ovariectomized rats. Twenty 8- to 10-month old Wistar rats were randomly assigned to four treatment groups: sham-operated + control diet (sham-C); ovariectomized (OVX) + control diet (OVX-C); OVX + soy milk diet (OVX-SM); and OVX + fermented soy milk diet (OVX-FSM). The rats were fed on these diets for 6 weeks. Ovariectomy induced an increase in the plasma cholesterol level by 40%. The plasma total cholesterol level of the OVX-FSM rats was decreased by 20% compared to that of the OVX-C rats. The plasma total cholesterol level of the OVX-SM group was not significantly different from that of the OVX-C and sham-C rats. The plasma triglyceride level of the OVX-FSM rats was lower than that of the sham-C rats. The liver cholesterol content in OVX-SM and OVX-FSM rats was lower than that of the OVX-C rats. The liver triglyceride contents of the sham-C, OVX-SM, and OVX-FSM groups were lower than that of the OVX-C group. Fecal steroid excretion did not differ among the groups. Ovariectomy decreased the uterus weight. The OVX-SM and OVX-FSM groups had the same uterus weights as those of the OVX-C group.

Thus, the diet including fermented soy milk prevented the cholesterol elevation induced in rats by ovarian hormone deficiency.

Key words: soy milk; cholesterol; Bifidobacterium; ovariectomy; isoflavones

The potential roles of dietary soy in the prevention and treatment of chronic diseases, notably heart disease and cancer, have long been known. Soy protein, isoflavones, phospholipid, and phytate have been investigated as components responsible for the anti-atherogenic effect of soy, although the mechanism was not completely established. Many types of soy food are consumed throughout the world. A new type of soy food is being developed to reduce the soy’s bean-like flavor, for incorporation into human foods. Soy milk is an aqueous extract of whole soybeans. However, many people find the taste of soy milk undesirable. Soy milk contains soy protein and isoflavones, which are thought to have an anti-atherogenic effect. The fermentation of soy results in compositional changes in isoflavones, phytate, and saponins. Bifidobacterium breve YIT 4065, which is used as a commercial fermented milk starter, is suitable for the fermentation of soy milk. Bifidobacterium produces lactate and acetate, which change the physicochemical character of soy protein. Bifidobacterium breve YIT 4065 causes the release of aglycones from isoflavone glucosides by β-glucosidase. Soy milk fermented by Bifidobacterium breve YIT 4065 increased the HDL-cholesterol level and decreased the VLDL+LDL-cholesterol level, and consequently decreased the atherogenic index value in growing male hamsters fed a high-fat diet that was cholesterol-free or cholesterol enriched.

Isoflavones have weak estrogenic activities, and their functions as both estrogen agonists and antagonists in vitro have been reported. It has also been reported that genistein is absorbed more rapidly than its glucoside. Postmenopausal women have a high risk of coronary heart disease, which has been found to be at least partially attributable to an unfavorable lipid metabolism. Postmenopausal women who take estrogen generally have lower rates of cardiovascular disease than women of a similar age who do not take estrogen.

The purpose of this study was to evaluate the effects of soy milk and fermented soy milk on the lipid metabolism in aged ovariectomized estrogen-deficient rats.

Materials and Methods

Diet. Crude soy milk (crude protein 4.83%, crude fat 2.68%, brix 11.9) from Shikokukakouki Co. (Tokushima, Japan) was used as the starting material for fermented soy milk. Bifidobacterium breve YIT4065 was obtained from the collection of the Culture Collection Research Laboratory of the Yakult Central Institute (Tokyo, Japan). A seed culture prepared anaerobically in the soy milk was freshly added to the soy milk at 1% (vol/vol) and fermented statically for 30 h at 37°C. The titratable acidity, pH and viable cell counts of the fermented soy milk were 0.970%, 4.66 and 1.2 × 10⁹ cfu/ml, respectively. The original unfermented soy milk and the fermented soy milk were both freeze-dried and milled until the products could be passed through a 0.84-mm sieve (#20 mesh). The crude protein and crude fat levels in the freeze-dried soy milk were 44.8% and 24.9%, respectively. The composition of the control diet was casein (25%), corn oil (10%), lard (7%), cellulose (5%), vitamin mixture (1%), mineral mixture (4%), choline bitartrate (0.2%), α-corn starch (32.1%), su-

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Fermented Soy Milk and Lipid Metabolism

Plasma lipids. Plasma total cholesterol, high density lipoprotein (HDL) cholesterol, triglyceride, and non-esterified fatty acid were measured enzymatically with commercial kits (Determiner TC555, Kyowa Medics, Tokyo; HDL cholesterol test Wako, Wako Junyaku, Osaka, Japan; and Triglyceride G test Wako, and NEFA C-test Wako, Wako Junyaku, respectively). Low density lipoprotein cholesterol plus very low density lipoprotein (LDL + VLDL) cholesterol was calculated as the difference between total cholesterol and HDL cholesterol. The atherogenic index (AI) was calculated as the VLDL + LDL-cholesterol: HDL cholesterol ratio. Beta-hydroxybutyric acid was measured by the method of Kientsch-Engel and Siess.14

Liver lipid. Liver lipid was extracted by the method of Folch et al.15 The liver total cholesterol and triglyceride concentrations were measured as described above. The liver free cholesterol was measured enzymatically with a commercial kit (Free cholesterol E-test, Wako Junyaku).

Fecal neutral steroid excretion. Feces were lyophilized, and 30-70 mg of homogenized feces, with 5α-cholestan as an internal standard was saponified and extracted by the method of Grundy et al.16 Neutral steroids were measured by a previously described method.17

Fecal bile acid excretion. First, 40-60 mg of lyophilized feces with 200 μl of 1 mm 5β-pregn-3α, 17α, 20α-triol was extracted with 5 ml of ethanol at 80°C. The ethanol was evaporated under nitrogen gas, and the residue was dissolved in methanol and then passed through a 0.45-μm filter (C3 LH; Millipore Japan, Tokyo). The bile acids were measured by a previously described method.18

Statistical analysis. The results are expressed as means and SD. Means were compared with Statistica software (StatSoft, Inc., Oklahoma, USA), by variance analysis and subsequent Tukey's HSD comparisons after logarithmic transformation to stabilize the variance, if the variance was significantly different (Bartlett test).19 For nonparametric data, i.e., for body weight at 4 wk and 5 wk, the Kruskal-Wallis test and subsequent nonparametric Tukey's test were done.

Results

Chemical compositions of soy milk and fermented soy milk

Lactic, formic, and acetic acid in the freeze-dried soy milk amounted to 2.1, 1.6, and 18.2 μmol/g, respectively. Lactic, formic, and acetic acid in the freeze-dried fermented soy milk amounted to 339.1, 27.3, and 358.3 μmol/g, respectively. The isoflavone compositions of the soy milk and fermented soy milk are shown in Table 1. The soy milk and fermented soy milk contained the same amount of total isoflavones. The soy milk had greater levels of glucosides (98.5%), while, in contrast, great levels of aglycons were found in the fermented soy milk. The fermented soy milk contained 17- and 70-fold

crose (15%), and CaCO3 (1.25%). The composition of the soy milk (SM) diet and fermented soy milk (FSM) diet was casein (11.55%), corn oil (2.53%), lard (7%), cellulose (5%), vitamin mixture (1%), mineral mixture (4%), choline bitartrate (0.2%), α-corn starch (32.05%), sucrose (6.063 and 6.061%, respectively), CaCO3 (1.097 and 1.099%, respectively), and soy milk or fermented soy milk powder (30%), respectively. The mineral and vitamin mixture was based on the AIN-76 formulation except that CaHPO4 was substituted for sucrose.

Animals. Twenty female Wistar rats (8-mo-old, CLEA Japan, Tokyo, Japan) were housed individually in wire-bottomed cages. They were kept in a room with controlled lighting (lights on 08:00-20:00 hours), temperature (24 ± 1), and humidity (60 ± 5%). The animals were given free access to a stock diet (MF; Oriental Yeast, Tokyo) for 12 d. After the menopause of all rats had been confirmed by the observation of epithelial cells in the vagina for six days, fifteen rats were ovariectomized and five had a sham operation.12 After a seven-day recovery from the operation, the absence of menstruation in the ovariectomized rats and the regular menstruation of the sham-operated rats were confirmed for six days.

The ovariectomized rats were separated into three groups of five animals each, with similar mean body weights of 333-339 g. The ovariectomized rats were assigned to control (OVX-C), soy milk (OVX-SM), and fermented soy milk (OVX-FSM) groups. The sham-operated rats were fed the control diet (sham-C). Before the test diet was started, all rats were fed the control diet for 1 wk. Then, they, given free access to the test diet and deionized water for 6 wk. Body weight was recorded once a week, and food consumption was recorded every 2 or 3 d. After 5 weeks of the experimental diets, feces were collected for 3 d. The apparent digestibility of dry matter was estimated from the weight of dried food intake and dried fecal excretion. The animals were maintained in accordance with the guidelines of the Ethical Committee for Animal Experiments of the Yakult Institute.

At the end of the 6-wk test diet period, the rats were anaesthetized by intraperitoneal injection of pentobarbital sodium (Nembutal; Abbott Laboratories, North Chicago, IL, USA), 25 mg/kg body weight. Food was removed 4 h before killing. Blood was collected from the aorta ventralis in tubes containing EDTA and then separated by centrifugation at 2000 × g for 15 min at 4°C. The livers were perfused in situ with saline (9 g NaCl/1), removed, weighed, and then kept at −20°C until the liver lipid analysis. The uteri were removed and weighed.

Analytical methods

Diet. Genistein and daizein in the SM and FSM diets were analyzed with an HPLC system (LC Module 1, Waters, Tokyo) with a YMC-Pack C4 column (YMC Co., Kyoto, Japan) and a UV detector (260 nm).7 Organic acid was measured by a previously reported method.13

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higher amounts of daidzein and genistin compared to the soy milk, respectively.

**Growth and digestibility**

No significant differences were detected in the animals' final body weight, the apparent digestibility of dry matter, or the food intake (Table 2). The feed efficiency was lower in the OVX-FSM group than in the sham-C and OVX-C groups. The body weights of the sham-C rats were lower than those of the OVX-C rats at 4 and 5 wk.

**Plasma lipids**

The plasma total cholesterol level in the OVX-C rats was increased by 40% compared to that in the sham-C rats (Table 3), but the level of the OVX-FSM group did not increase. The OVX-FSM rats showed plasma total cholesterol levels that were decreased by 20% compared to the levels of the in OVX-C rats. The OVX-SM values were not significantly different from those of the sham-C or OVX-C groups.

Rats fed SM and FSM had lower plasma triglyceride levels compared to the rats fed the control diet. A significant difference was detected between the sham-C and OVX-FSM rats in the plasma triglyceride level.

The OVX-C rats had higher HDL-cholesterol levels than those of the sham-C rats. The HDL-cholesterol levels of the OVX-SM and OVX-FSM rats were between those of the sham-C and OVX-C rats. The VLDL + LDL-cholesterol levels of the OVX-FSM rats was lower than that in OVX-C rats. The atherogenic index did not differ among groups.

**Liver lipid**

The liver total cholesterol contents of the OVX-SM and OVX-FSM rats were lower than that in the OVX-C rats and were not significantly different from the sham-C values (Table 4). The liver free cholesterol content in the OVX-FSM rats was lower than that in the OVX-C rats. The liver triglyceride contents of the sham-C, OVX-SM and OVX-FSM rats were lower than that of the OVX-C rats. The liver triglyceride concentration of the OVX-SM group was lower than that in the OVX-C group.

**Fecal steroid excretion**

The fecal neutral steroid and bile acid excretions did

| Table 1: Isoflavone Concentrations in Soy Milk and Fermented Soy Milk* |
|-----------------------------|-----------------------------|
| Soy milk | Fermented soy milk |
| Daidzin | 777.8 | 521.9 |
| Genistin | 887.6 | 184.5 |
| Daidzein | 14.5 | 248.1 |
| Genistein | 11.1 | 771.2 |
| Total | 1691.0 | 1725.7 |

Values are expressed in μg/g.

* Values present as the amounts of aglycones.

| Table 2: Body Weight, Food Intake, and Apparent Digestibility of Dry Matter in Ovariectomized (OVX) Rats Fed the Control, Soy Milk, or Fermented Soy Milk Diet, and in Sham-operated (Sham) Rats Fed the Control Diet |
|-----------------------------|-----------------------------|-----------------------------|
| Sham | OVX | ANOVA |
| Initial body weight (g) | control | control | soy milk | fermented soy milk | P < |
| 301±48 | 338±26 | 339±21 | 333±8 | NS |
| Final body weight (g) | 326±53 | 374±31 | 356±13 | 331±15 | NS |
| Food intake (g/day) | 12.8±1.1 | 13.6±1.0 | 13.1±0.4 | 12.5±0.8 | NS |
| Feed efficiency | 0.045±0.016 a | 0.060±0.040 a | 0.030±0.016 ab | 0.006±0.020 b | 0.01 |
| Dry matter digestibility (%) | 94.4±0.6 | 94.5±1.1 | 93.1±2 | 93.1±2.3 | NS |

Values are mean and SD for five rats.

Within the same column, means with the same letter are not significantly different by Tukey's test.

| Table 3: Plasma Lipid Concentrations in Ovariectomized (OVX) Rats Fed the Control, Soy Milk, or Fermented Soy Milk Diet, and in Sham-operated (Sham) Rats Fed the Control Diet |
|-----------------------------|-----------------------------|-----------------------------|
| Sham | OVX | ANOVA |
| Total cholesterol (mg/dl) | control | control | soy milk | fermented soy milk | P < |
| 78.3±6.8 b | 108.9±13.1 a | 92.8±12.4 ab | 87.2±12.8 b | 0.01 |
| Triglyceride (mg/dl) | 149.6±48 a | 139.3±84.4 ab | 99.0±19.9 ab | 70.0±17.2 b | 0.05 |
| HDL-cholesterol (mg/dl) | 35.0±3.6 b | 56.2±14.2 a | 50.1±6.3 ab | 46.9±6.2 ab | 0.01 |
| VLDL+LDL-cholesterol (mg/dl) | 43.3±5.9 ab | 52.7±6.2 a | 42.7±6.2 ab | 40.3±7.5 b | 0.05 |
| Atherogenic index* | 1.24±0.20 | 1.00±0.30 | 0.80±0.04 | 0.90±0.10 | NS |
| NEFA (mEq/l) | 0.294±0.054 | 0.324±0.066 | 0.254±0.055 | 0.263±0.050 | NS |
| β-HBA (mg/dl) | 49.0±3.7 b | 53.3±5.7 ab | 55.8±5.4 ab | 57.7±3.7 a | 0.06 |

Values are mean and SD for five rats.

Within the same column, means with the same letter are not significantly different by Tukey's test.

* Means were compared after logarithmic transformation of the data.

* Atherogenic index = VLDL+LDL-cholesterol/HDL-cholesterol
Table 4. Liver Lipid Contents in Ovariectomized (OVX) Rats Fed the Control, Soy Milk, or Fermented Soy Milk Diet, and in Sham-operated (Sham) Rats Fed the Control Diet.

<table>
<thead>
<tr>
<th></th>
<th>Sham control</th>
<th>OVX soy milk</th>
<th>Fermented soy milk</th>
<th>ANOVA</th>
<th>V&lt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol (mg/g)</td>
<td>7.2±0.1</td>
<td>7.6±1.1</td>
<td>6.7±0.5</td>
<td>7.0±0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Free cholesterol (mg/g)</td>
<td>6.6±0.1</td>
<td>6.5±0.6</td>
<td>6.6±1.2</td>
<td>6.3±0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Esterified cholesterol (mg/g)</td>
<td>0.5±0.2</td>
<td>1.1±0.8</td>
<td>0.7±0.5</td>
<td>0.7±0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Triglyceride (mg/g)</td>
<td>63.2±10.8 b</td>
<td>119.5±29.3 a</td>
<td>76.2±18.5 b</td>
<td>82.7±19.8 ab</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Total cholesterol (mg/liver):  
- Sham control: 20.0±2.3 ab  
- OVX soy milk: 22.6±2.3 a  
- Fermented soy milk: 18.7±2.2 b  
- ANOVA: 17.7±1.3 b, 0.05

Values are mean and SD for five rats. 
Within the same column, means with the same letter are not significantly different by Tukey's test.

not differ significantly among the groups. The levels of fecal neutral steroid excretion in the sham-C, OVX-C, OVX-SM, and OVX-FSM groups were 14.0±4.2, 11.6±2.7, 10.2±2.0, and 10.4±2.1 mg/3d, respectively. The levels of fecal bile acid excretion in the sham-C, OVX-C, OVX-SM, and OVX-FSM groups were 4.1±0.7, 6.8±6.8, 6.0±3.0, and 5.1±2.4 μmol/3d.

Reproductive tissue weights

The uterus weights in the sham-C, OVX-C, OVX-SM, and OVX-FSM groups were 0.456±0.067, 0.124±0.024, 0.135±0.018, and 0.139±0.018 g, respectively. The uterus weight in the OVX-C group was 27% of that in the sham-C group. The uterus weights in the OVX-SM and OVX-FSM groups were the same as that in the OVX-C group.

Discussion

The purpose of this study was to find whether fermented soy milk is effective in preventing the increase in the blood cholesterol increase due to ovariectomy (OVX). OVX induced an increase in the plasma cholesterol level of rats. The fermented soy milk had a favorable effect on the plasma cholesterol level without an increase in steroid excretion. The soy milk was also effective in preventing the increase in the blood cholesterol, but not significantly.

A reduction in cholesterol is observed with soy protein in experimental animals and human subjects. Metabolic changes that have been observed following soy protein feeding in a variety of animal models. 3-Hydroxy-3-methylglutaryl CoA reductase activity was increased when soy was fed to rats. The consumption of soy protein is associated with an increase in the removal of LDL and very low density lipoproteins.

Fermented soy milk contains elevated levels of the aglycones formed through enzymatic hydrolysis during fermentation. Similar tendencies have been noted for soybean paste (miso) and other fermented soy foods. Anthony et al. reported that rhesus monkey fed soy protein with isoflavones had lower plasma VLDL + LDL-cholesterol and lower atherogenic index values than those fed soy protein without isoflavones. King et al. reported that the plasma genistein concentra-

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at 2 h after dosing in genistein-treated rats was two-fold higher than that in soy-extract-treated rats, although there were no significant differences at 8 h or later. These data showed that genistein (aglycone form) was absorbed more rapidly than its glucoside. These findings may explain why the fermented soy milk induced a greater decrease in plasma cholesterol than soy milk in this study. Isoflavones have weak estrogenic activity. Fremenopausal women have a much lower incidence of coronary heart disease than men of similar age, but after menopause the difference between men and women decreases. Estrogen reduces the risk of cardiovascular disease. Sex hormone deficiency is associated with an increased coronary heart disease risk in women. Stevenson et al. reported that the change from the premenopausal to the postmenopausal status caused increases in total cholesterol, LDL-cholesterol, and triglyceride, with a reduction in HDL-C cholesterol. The serum total cholesterol and LDL-cholesterol levels were observed to increase as a consequence of menopause in a 7-year longitudinal study of Japanese women. Estrogen treatment decreased LDL-cholesterol and increased HDL-cholesterol in menopausal women.

Estrogen injection into OVX rats reduced plasma cholesterol to a lower level than that of sham-operated rats. In this study, however, the fermented soy milk reduced the plasma lipids in OVX rats to the same level as those of the sham group. The increase in the plasma HDL level caused by OVX in this study did not agree with that in postmenopausal women. This discrepancy may be due to the difference in species or differences between ovariectomy and postmenopause.

Phospholipids, phytate, and plant sterols can reduce blood cholesterol. Bifidobacteria cells can remove cholesterol through both assimilation and coprecipitation with deconjugated bile salts, as found in an in vitro study. Bifidobacterium breve YIT 4065 produces lactate and acetate, which can modify the physicochemical properties of soy protein. The viscosity of the fermented soy milk we used was very high (1573 mPas), but that of soy milk, which is the starting material of fermented soy milk, was only 13.4 mPas. In this study, ex vivo viscosity was not estimated. The viscosity of a meal was found...
to be significantly reverse correlated with the blood cholesterol concentration.\(^{27}\)

Fecal steroid excretion was not affected by the experimental diets in this study, which might indicate that the mechanism of the cholesterol lowering effect was not associated with cholesterol absorption or bile acid reabsorption in this study.

Estrogen-replacement therapy is effective for the prevention of osteoporosis in postmenopausal women. However, estrogens also substantially increase the incidences of endometrial cancer\(^{28}\) and breast cancer.\(^{29}\) Soy milk and fermented soy milk did not affect the uterus weight in the rats examined here. This finding agrees with the results in soy protein-fed peripubertal rhesus monkeys.\(^{30}\) However, a large amount of genisteen increased the uterus weight in young ovariectomized rats because of its estrogenic properties.\(^{30}\)

In this study, the sham-C rats had lower the body weights than those of the O VX-C rats at 4 and 5 wks. This finding is in agreement with a previous report.\(^{13}\) The body weights of the OVX-SM and OVX-FSM rats were between those of the OVX-C and sham-C rats. However, no significant differences were detected in food intake or dry matter digestibility. The feed efficiency was lower in the OVX-FSM group than in the sham-C and OVX-C groups. OVX and soy diet lead to modifications of metabolic efficiency. This may be associated with cholesterol metabolism. In conclusion, fermented soy milk had a cholesterol lowering effect, and it is likely that this effect was greater than that of soy milk.

**Acknowledgment**

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**References**