Arch Pharm Res Vol 28, No 7, 859-865, 2005

Archives of Pharmacal Research
http://apr.psk.or.kr

Application of Microencapsulated Isoflavone into Milk

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(Received February 2, 2005)

This study was designed to develop a microencapsulated, water-soluble isoflavone for application into milk and to examine the hypocholesterolemic effect of such a milk product in a rat diet. The coating material was medium-chain triglyceride (MCT) and the core material was water-soluble isoflavone. The microencapsulation efficiency was 70.2% when the ratio (w/w) of coating material to core material was 15:1. The isoflavone release from the microcapsules was 8% after 3-day storage at 4°C. In *in vitro* study, 4.0-9.3% of water-soluble isoflavone in simulated gastric fluid was released in the pH range of 2 to 5 after 60 min incubation; however, in simulated intestinal fluid at pH 8, 87.6% of isoflavone was released from the capsules after 40 min incubation time. In sensory analysis, the scores of bitterness, astringency, and off-taste in the encapsulated isoflavone-added milk were slightly, but not significantly, different from those in uncapsulated, isoflavone-added milk. In blood analysis, total cholesterol was significantly decreased in the isoflavone-added group compared with that in the control after 6-week feeding. Therefore, this study confirmed the acceptability of MCT as a coating material in the microencapsulation of water-soluble isoflavone for application into milk, although a slight adverse effect was found in terms of sensory attributes. In addition, blood total cholesterol was lowered in rats which had been fed a cholesterol-reduced and microencapsulated, isoflavone-added milk for 6 weeks.

Key words: Microencapsulation, Water-soluble isoflavone, Medium-chain triglyceride, Milk

INTRODUCTION

The abundance of certain isoflavones in Asian diets and the lower rates of "Western" diseases such as coronary heart disease and certain cancers in Asian populations have suggested a protective role from these mostly soy-derived substances (Tikkanen and Aldercreutz, 2000). Several studies in animals and humans have shown that the consumption of soybean has beneficial effects in a variety of disorders including hypocholesterolemia as well as protection against cardiovascular disease and menopausal symptoms (Ali *et al.*, 2004; Lissin and Cooke, 2000). As well as soy protein (FDA, 1999), isoflavones containing in soybean having both weak estrogenic and antiestrogenic activity (Barns *et al.*, 2000) may also partly be responsible for the cholesterol lowering and cardioprotective effects. How soy protein and other components of soybean act to lower cholesterol and lipids is not known: it is possible that effect on endocrine system may be partly responsible since hormones do control lipid metabolism.

Isoflavones are phytoestrogens, which have potent biological activity (Aldercreutz, 1995) with similar chemical structure to mammalian estrogens and act as weak estrogens (Setchell and Cassidy, 1999). A recent study proposed that isoflavones may also be the factor responsible for the cholesterol-lowering property of some soy-foods (Anthony *et al.*, 1996; Fukui *et al.*, 2002). Soy protein lowers total cholesterol, low-density lipoprotein cholesterol, and triglycerides in humans, and inhibits atherosclerosis in animals (Arliss and Biermann, 2002). The acknowledged strong positive relation between cholesterol concentrations and coronary heart disease risk has increased consumer concern about excessive cholesterol intake (Grundy *et al.*, 1982; Gurr, 1992).

In addition, a recent study indicated that soy protein showed the beneficial effects on stimulation of bone formation, which is related to the bone mineral density especially in menopausal women (Fanti *et al.*, 1998). Therefore, the addition of isoflavone to milk is necessary for postmenopausal women, children and teenagers since milk is a great source of calcium. However, it is difficult to

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add isoflavone directly because it is bitter, with a beany off-flavor and brown color. A microencapsulation technique is therefore needed to overcome these problems.

Microencapsulation, which shows potential for the development of enzyme carriers in the food industry, could be a good vehicle for the addition of iron into milk (Berserieva *et al.*, 1990; Jackson and Lee, 1991). Currently, there is considerable interest in developing encapsulated flavors and enzymes. Among several factors to be considered, choice of coating material is the most important and this depends on the chemical and physical properties of the core material, and on the process used to form microcapsules. For the microencapsulation process, although several studies have used coating materials such as milk fat, agar, and gelatin for enzyme, flavor, and iron microencapsulation in food (Magee and Olson, 1981a; Magee and Olson, 1981b; Braun and Olson, 1986; Kwak *et al.*, 2001), no study has measured the efficiency of water-soluble, isoflavone microencapsulation, or the stability of the microcapsule itself.

Therefore, the objectives of this study were to examine the optimum conditions of water-soluble isoflavone microencapsulation, to measure the stability of the microcapsule retaining the isoflavone in milk during storage and *in vitro* study, and to confirm it's effect in lowering blood cholesterol in rats.

MATERIALS AND METHODS

Materials

Medium-chain triglyceride (MCT) as a coating material was purchased from Il-Shin Emulsifier Co., LTD. (Seoul, Korea), water-soluble isoflavone (30% purity) as a core material from Amorepacific Co., LTD. (Seoul, Korea), genistin (4', 5, 7-trihydroxyisoflavone-7-glucoside) from Sigma Chemical (St. Louis, MO, U.S.A.) and daidzin (4'-hydroxyisoflavone-7-glucoside) from Fujico Co., LTD. (Tokyo, Japan). All ingredients were of food grade.

Milk treatment

For cholesterol removal, milk was added with 1.0% β-cyclodextrin, and the mixture was stirred with 800 rpm blending for 10 min at 4°C, and centrifuged at 166×g for 10 min. The cholesterol removal rate in milk was 98.3%.

Microencapsulation

Microcapsules of water-soluble isoflavone were made by MCT, which was selected as a coating material in a previous study (Kwak *et al.*, 2001). The ratios of coating material to core material were 5:1, 10:1, 15:1, and 20:1(w/w) to maximize the content of water-soluble isoflavone and the stability of the microcapsules. The materials were mixed at 1,200 rpm for 1 min with a stirrer. An airless paint sprayer (W-300 Wagner Spray Tech. Co., Markdorf, Germany) nebulized a coating material-water soluble isoflavone emulsion at 45°C into a cylinder containing a solution (0.05% Tween-60) at 5°C (Kwak *et al.*, 2001). The diameter of the nozzle orifice was 0.4 mm. The chilled fluid was centrifuged at 166×g for 10 min to separate microcapsules. Microcapsules were formed as lipid solidification on the chilled fluid. To remove residual isoflavone adhering to the outside walls of the microcapsules, the microcapsules were washed with 0.05% Tween-60 solution and centrifuged again.

HPLC analysis

Quantification and identification of isoflavone were performed using a m-Bondapak C_{18} column (5 mm, 25 m× 4. 6 mm, i.d.: Waters, U.S.A.) and two-solvent system at a flow rate of 1 mL/min. The column was equilibrated with 20% methanol. After the injection of 20 μL of sample, the ratio of solvent B (60% methanol) to solvent A (20% methanol) was increased by a linear gradient to 100% of solvent B in 50 min and to 100% of solvent A in 60 min. Analyses were performed using a Shimazu solvent delivery system equipped with a UV detector (Shimazu, Tokyo, Japan) to monitor absorbance at 254 nm.

The stock solution of isoflavone was prepared by dissolving pure standards in 70% methanol. Small portions of the solution were injected into the HPLC column and each chemical composition of isoflavone was determined from the peak area in the HPLC chromatogram.

Stability of microcapsule during storage

To measure the stability of the water-soluble, isoflavone microcapsules, 10 mL of distilled water was added into the same amount of microcapsule solution, and stored at 4, 20, and 30°C for 12 days. The samples were centrifuged and the collected supernatant was analyzed for determination of the water-soluble isoflavone content released from the microcapsules. All measurements were done in triplicate.

In vitro study

To determine the stability in the human stomach and intestine, the following two simulated gastrointestinal solutions were prepared: 1) gastric fluid prepared in a sample solution containing pepsin (pH 1.2) and simulated into 4 different fluids with pH 2, 3, 4, and 5 using 2 N HCl and NaOH, and 2) intestinal fluid prepared in 0.1 M PBS buffer (100 mL, pH 7.4) containing 20 mg pancreatin, 5 mg lipase, 10 mM cholic acid, and 10 mM deoxycholic acid, and simulated into 3 different intestinal solutions as pH 6, 7, and 8 (Freund *et al.*, 2000; Kwak *et al.*, 2002).

The microcapsules of water-soluble isoflavone in distilled water were incubated with 100 rpm shaking at 37°C for 10

min in the gastric fluid, and at 37°C for 60 min with samples collected at 20-min intervals in the intestinal fluid. The collected samples were centrifuged at 166×g and the supernatant was measured for water-soluble isoflavone content released from the microcapsules. All treatments were performed in triplicate.

Sensory analysis

A panel of eight reviewers experienced in judging dairy products evaluated the milk samples throughout the study. The microcapsules with MCT were added into the milk and stored for 1, 3, 6, 9, and 12 days at 4°C. The following three sensory characteristics were tested: the intensity of off-flavor (beany flavor), bitterness, and color were scored on a seven-point scale (1=very slight, 2=slight, 3=slight-moderate, 4=moderate, 5=moderate-strong, 6=strong, and 7=very strong).

Animals and diets

Male Sprague-Dawley rats obtained from the Jung-Ang Lab. Animal, Inc. (Seoul, Korea) weighing 60 to 75 g were placed individually in stainless-steel wire cages in a windowless room and were subjected to a 12/12 h light/dark cycle. The rats were acclimatized for 1 week and fed a commercial rat chow during this period. All diets were formulated as recommended by the American Institute of Nutrition (Tables I and II).

All animals were fed ad libitum a 40% beef tallow modified rodent diet with 5% cholesterol and 0.5% cholic acid for 7 weeks, then fed a fat-free AIN-76A diet containing encapsulated isoflavone for 6 weeks. Animals were given free access to tap water *via* a stainless steel delivery system.

Table I. *Composition of 40% beef tallow modified AIN-76A purified rodent diet with 5% cholesterol and 0.5% cholic acid*

Ingredient	g/kg
Casein, high nitrogen	200
Corn starch	150
Beef tallow	400
Sucrose	95
Cholesterol	50
Cellulose	50
Mineral mix[1]	35
Vitamin mix[2]	10
Cholic acid	5
DL methionine	3
Choline bitartrate	2

[1]AIN-76 Mineral mix (g/kg) : $CaHPO_4$ 500, NaCl 74, K citrate monohydrate 220, K_2SO_4 52, MgO, Mn carbohydrate 3.5, Fe citrate 6.0, Zn carbonate 1.6, Cu carbonate 0.3, KIO_3 0.01, $Na_2SeO_4 \cdot PH_2O$0.01, $CrK(SO_4) \cdot P12H_2O$0.55, Sucrose 118.

[2]AIN-76 Vitamin mix (g/kg) : thiamin·PHCl 0.6, riboflavin 0.6, pydoxine·PHCl 0.7, nicotinic acid 3, D-calcium pantothenate 1.6, folic acid 0.2, D-biotin 0.02, cyanocobalamin 0.001, retinyl palmitate 0.8, DL-α-tocopheryl acetate 20, cholecalciferol 0.00025, menaquinone 0.005.

Table II. Composition of fat-free AIN-76A

Ingredient (%)	g/kg
Casein	200
DL methionine	3
Corn starch	150
Sucrose	550
Cellulose	50
Salt mix[1]	35
Vitamin mix[2]	10
Choline bitartrate	2

[1]AIN-76 Mineral mix (g/kg): $CaHPO_4$ 500, NaCl 74, K citrate monohydrate 220, K_2SO_4 52, MgO, Mn carbohydrate 3.5, Fe citrate 6.0, Zn carbonate 1.6, Cu carbonate 0.3, KIO_3 0.01, Na_2SeO_4 H_2O 0.01, $CrK(SO_4) \cdot P12H_2O$ 0.55, Sucrose 118.

[2]AIN-76 Vitamin Mix (g/kg): thiamin·PHCl 0.6, riboflavin 0.6, pydoxine·PHCl 0.7, nicotinic acid 3, D-calcium pantothenate 1.6, folic acid 0.2, D-biotin 0.02, cyanocobalamin 0.001, retinyl palmitate 0.8, DL-α-tocopheryl acetate 20, cholecalciferol 0.00025, menaquinone 0.005.

The animals were assigned randomly to the following two groups: 1) control, fed a fat-free diet (Table II) containing 1 mL/day of commercial milk, and 2) isoflavone, encapsulated isoflavone-added group, fed a normal diet with 1 mL/day cholesterol-reduced milk containing 10 mg of isoflavone.

For blood analysis, the animals were fasted for 12 h after which a 1.5 mL blood sample was withdrawn from the tail, centrifuged at 3,000 rpm for 10 min, and stored at -20°C until analysis.

Statistical analysis

Data from each experiment were analyzed by analysis of variance (ANOVA) using a SAS program (1985) and differences among the treatments were determined by Duncan's multiple test at $p<0.05$, unless otherwise stated.

RESULTS AND DISCUSSION

Microencapsulation

The optimum ratio of MCT to water-soluble isoflavone was examined as shown in Table III. Microencapsulation efficiency was the highest at 70.2% for a coating to core material ratio of 15:1, and was significantly lower at ratios of 5:1 and 10:1. The decrease in efficiency with increasing coating material over a 15% ratio may have resulted from the left over MCT forming an upper layer of dispersion fluid after centrifugation (Kwak *et al.*, 2001).

Table III. Efficiency of microencapsulation with different ratios of MCT and water-soluble isoflavone[1]

Ratio (w/w)		Efficiency (%)
MCT[2]	Water-soluble isoflavone	
5	1	57.3[c]
10	1	63.1[b]
15	1	70.2[a]
20	1	68.6[ab]

[1]Means of triplicated experiments. Means in a column by the same letter are not significantly different (R<0.05)
[2]Medium-chain triglyceride

Stability of microcapsules during storage

The microcapsules were examined for their ability to retain water-soluble isoflavone at different temperatures during storage as shown in Fig. 1. In all samples, the percentage water-soluble isoflavone release increased with the length of storage period. At all temperatures, the release of water-soluble isoflavone from the microcapsules increased significantly from 0- to 3-day storage and increased slightly thereafter up to 12-day storage. At 3-day, 8, 9, and 11% of water-soluble isoflavones were released at 4, 20, and 30°C, respectively, which was not significantly different. At 12-day storage, 11.5, 12.9, and 17% of water-soluble isoflavones were released at 4, 20, and 30°C, respectively.

In vitro study

Metabolites of isoflavones appeared in the blood within 30 min of ingestion, indicating the rapid absorption in the small intestine. Therefore, an experiment should be performed to determine how stable the microcapsules are in the stomach and how effectively they are released in the intestine, which is the primary site of water-soluble isoflavone absorption and regulation.

The release of water-soluble isoflavone from microcapsules showed a similar trend at every pH of 2, 3, 4, and 5 (Fig. 2), when incubated in simulated gastric fluid. When incubated at pH 2, the release of water-soluble isoflavone was initially less than 4%, but increased up to 6% at 60 min. Incubation at pHs of 3, 4, and 5 at 37°C showed an initial release of water-soluble isoflavone in the range of 4.6-5.5%, but increased up to 8, 9, and 9.3% at pHs of 3, 4, and 5 at 60 min incubation, respectively. The present study indicated that, as expected, only a small amount of isoflavone was released from the microcapsules in the simulated gastric environment.

To determine how effectively water-soluble isoflavone is released in the intestine, a simulated intestinal fluid was prepared with the presence of pancreatin and bile salts, and microcapsules were incubated at 37°C for 60 min (Fig. 3). The release of water-soluble isoflavone increased dramatically with both an increase of pH and an increase of incubation time. About 19% of the entrapped, water-soluble isoflavone was released at pH 6 after 60 min incubation. When incubated at pH 7, there was a dramatic increase from 5.8 to 40.2% between 0 and 20 min incubation and this continued increasing to 71% at 60 min. When incubated at pHs of 7 and 8, 61.2, and 87.6% of water-soluble isoflavones were released from the microcapsules at 40 min incubation, respectively.

The present data were in agreement with other reported results (Kwak *et al.*, 2003; Seok *et al.*, 2003) of over 80% of isoflavone being released from microcapsules made by

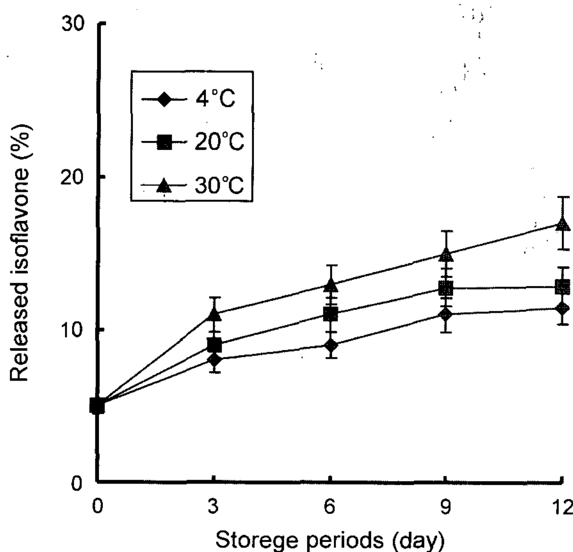

Fig. 1. Effect of temperature on isoflavone release from microcapsules stored at 4°C for 12 days in milk

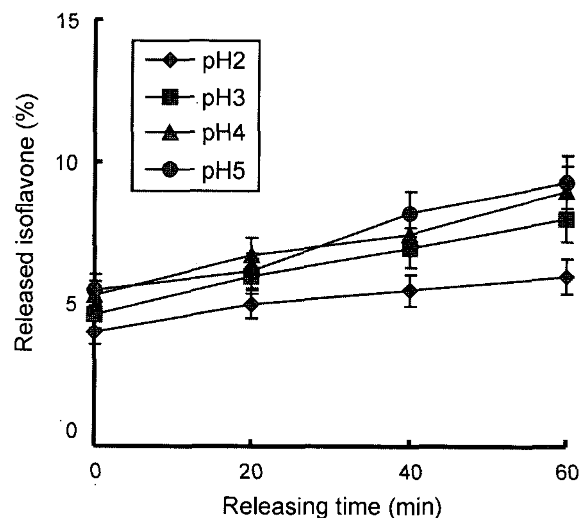

Fig. 2. Effect of pH on isoflavone release from microcapsules incubated under simulated-gastric condition *in vitro.* Simulated-gastric fluid contained pepsin and was adjusted to different pH values with HCl and NaOH and incubated at 37°C for 60 min.

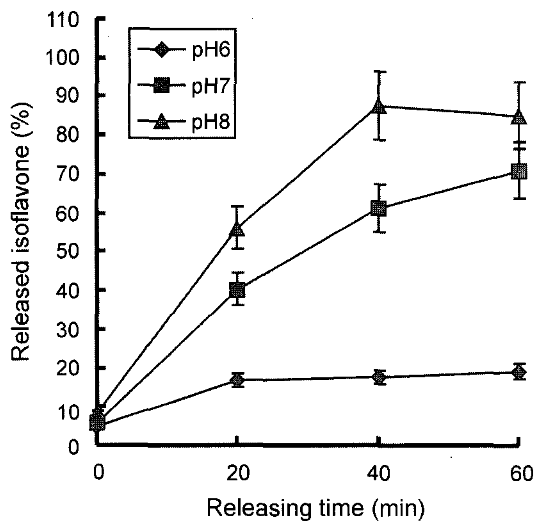

Fig. 3. Effect of pH on isoflavone release from microcapsules incubated under simulated-intestinal fluid *in vitro*. Simulated-intestinal fluid contained enzymes such as lipase (5 mg) and pancreatin (20 mg) and was incubated at 37°C for 60 min.

polyglycerol monostearate in simulated gastric environment at pHs of 7 and 8. In addition, a similar study (Freund *et al.*, 2000) investigated the stability of spherulites (liposomes) in gastric or intestinal fluid in different incubation pHs. Less than 3% of the spherulites were broken in acidic fluid (pHs 3, 4, 5, and 6) at 37°C after 2 h incubation. Their results were in agreement with our *in vitro* results, which demonstrated a microcapsule release rate of less than 15% in an acidic condition.

It is generally accepted that for an effective uptake of nutrition from microcapsules, the following three problems need to be resolved: the capsules have to contain as much nutrition as possible, resist the gastric and intestinal fluids, and be captured by the enterocytes before being released into the general circulation. Our results suggested that microcapsules are convenient tools for water-soluble isoflavone addition due to the increased water-soluble isoflavone absorption which results from the favored uptake and effective release in the intestine. In conclusion, the present study results have supported the application of microencapsulation of water-soluble isoflavone into milk.

Sensory analysis

The sensory characteristics in milk containing uncapsulated or encapsulated, water-soluble isoflavone were examined when stored for 12 days at 4°C (Table IV). There was a slight difference in color and off-flavor between the treatments. For off-flavor, uncapsulated, water-soluble, isoflavone-added milk showed a significant difference compared with those of control and encapsulated groups at 0-day. At 3-day storage, both uncapsulated and encapsulated water-soluble isoflavone-added milk showed a significantly different bitterness, astringency, and off-taste.

Table IV. Sensory scores of microencapsulated, isoflavone-fortified milk during storage at 4°C for 12 days[1]

Storage period (day)	Treatment	Sensory description				
		Color	Off-flavor	Bitterness	Astringency	Off-taste[2]
0	Control[3]	5.0^{ab}	5.0^{efg}	5.0^{f}	5.0^{f}	5.0^{f}
	Uncapsulated	5.1^{ab}	5.5^{defg}	6.2^{abcd}	6.5^{bcd}	6.4^{bcd}
	Encapsulated	5.1^{ab}	4.8^{fg}	5.6^{cdef}	5.7^{def}	6.0^{de}
3	Control	4.9^{b}	4.9^{efg}	5.4^{def}	5.2^{ef}	5.2^{ef}
	Uncapsulated	5.1^{ab}	5.9^{cde}	6.8^{ab}	6.9^{abc}	6.9^{bc}
	Encapsulated	5.0^{ab}	4.9^{efg}	6.1^{bcde}	6.0^{cde}	6.1^{cd}
6	Control	5.0^{ab}	4.7^{g}	5.1^{f}	5.0^{f}	5.2^{ef}
	Uncapsulated	5.3^{ab}	5.5^{defg}	6.1^{bcde}	6.5^{bcd}	6.5^{cd}
	Encapsulated	5.3^{ab}	5.4^{defg}	5.8^{cdef}	6.0^{cde}	6.3^{cd}
9	Control	5.2^{ab}	5.4^{defg}	5.2^{ef}	5.4^{ef}	5.8^{def}
	Uncapsulated	5.4^{ab}	7.0^{ab}	6.2^{abcd}	6.8^{abc}	8.0^{a}
	Encapsulated	5.2^{ab}	5.8^{defg}	6.3^{abc}	6.6^{bcd}	7.5^{ab}
12	Control	5.3^{ab}	6.2^{bcd}	5.9^{bcdef}	5.5^{ef}	6.4^{cd}
	Uncapsulated	5.6^{a}	7.7^{a}	5.9^{bcdef}	7.6^{a}	8.3^{a}
	Encapsulated	5.3^{ab}	6.6^{bc}	7.1^{a}	7.1^{ab}	7.7^{ab}

[1]Means of 10 replicates. Means in a column by the same letter are not significantly different (R<0.01)
The scale of each score: 1=very slight, 2=slight, 3=slight-moderate, 4=moderate, 5=moderate-strong, 6=strong and 7=very strong
[2]Off-taste except for bitter and astringency
[3]Control : Market milk at 4°C for 12day storage

Table V. Effects of experimental diet on food intake and body weight gain

Treatment	Food intake (g/day)	Body weight gain (g/6week)
Control[1]	19.21	94.73
Isoflavone[2]	23.85	112.58

[1] Milk with no treatment
[2] Isoflavone-added, cholesterol-reduced milk (10 mg/day)

The scores in the encapsulated, isoflavone-added milk were a little lower than those in the uncapsulated, isoflavone-added milk after 12-day storage. As for all the sensory characteristics, no remarkable increase was found after the 12-day storage.

Animal study

After 7 weeks feeding with the diet of 40% beef tallow and 5% cholesterol, the average food intake was 19.21 and 23.85 g/day in the control group consuming 1 mL/day of commercial whole milk and in the isoflavone group consuming 10 mg/day isoflavone in 1 mL milk, respectively. The body weight gain was not significantly different between the control and isoflavone-added group, at 94.73 and 112.58 g, respectively, after the 6-week period (Table V).

During the 6-week feeding period of the experimental diet, the total blood cholesterol was significantly higher in the control group at 209.0 mg/dL than in the isoflavone-added group at 174.1 mg/dL. However, blood triacylglyceride was not significantly different between the control and isoflavone-added group, at 35.8 and 37.0 mg/dL, respectively (Table VI). In addition, no difference was found between the two groups in blood high density lipoprotein (HDL)-cholesterol, at 43.6 and 41.6 mg/dL in the control and isoflavone-added group, respectively.

The present data indicated that 1 mL/day cholesterol-reduced milk containing 10 mg isoflavone lowered the total blood cholesterol significantly, but did not affect the blood triglyceride and HDL-cholesterol levels. These results suggest that the addition of isoflavone into cholesterol-reduced milk in the form of microcapsules has the potential to promote a hypocholesterolemic effect. In addition, there is further potential for the development of other dairy products exhibiting a lowering effect on total blood cholesterol.

Table VI. Effect of experimental diets on the change of blood triacylglycerol, total cholesterol and high-density lipoprotein-cholesterol in rats fed for 6 weeks

Treatment	Total CH		TG		HDL CH	
	Initial	Final	Initial (mg/dL)	Final	Initial	Final
Control[1]	112.5	209.0	30.0	35.8	33.0	43.6
Isoflavone[2]	124.6	174.1	38.3	37.0	31.6	41.6

[1] Milk with no treatment
[2] Isoflavone-added, cholesterol-reduced milk (10 mg/day)

ACKNOWLEDGEMENT

This research was supported by a grant from the Korea Research Foundation, Republic of Korea (KRF-2003-041-F00038).

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