Egg Ovomucin Attenuates Hypercholesterolemia in Rats and Inhibits Cholesterol Absorption in Caco-2 Cells

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ABTRACT: This experiment was designed to evaluate the effect of casein or ovomucin (OV) on the micellar solubility of cholesterol and the taurocholate binding capacity *in vitro*. We also evaluated the effects of casein or OV on cholesterol metabolism in rats and Caco-2 cells. OV had a significantly greater bile acid-binding capacity than that of casein *in vitro*. Micellar cholesterol solubility *in vitro* was significantly lower in the presence of OV compared to casein. The cholesterol micelles containing OV significantly suppressed cholesterol uptake by Caco-2 cells compared to the cholesterol micelles containing casein. Consistent with these *in vitro* findings, OV-feeding significantly increased the fecal excretion of bile acids or cholesterol compared with casein-feeding. Serum total cholesterol was significantly lower in rats fed OV than in those fed casein. The concentrations of total lipids in liver were significantly lower in the OV-fed group compared with the casein group. These results suggest that the suppression of cholesterol absorption by direct interaction between cholesterol mixed micelles and OV in the jejunal epithelia is part of the mechanism underlying the hypocholesterolemic action of OV. OV may also inhibit the reabsorption of bile acids in the ileum, thus lowering the serum cholesterol level.

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Egg protein consists of well-balanced amino acids with high biological value. However, egg is a cholesterol-rich food whose use is always strictly or carefully advocated for the prevention of hypercholesterolemia and its related diseases. As the cholesterol exists exclusively in the egg yolk, egg white is cholesterol-free. Egg intake is thought to increase serum cholesterol concentrations in experimental animals (1) and humans (2).

Egg white contains a wide variety of proteins such as ovalbumin, ovomucin (OV), ovotransferrin, and lysozyme (3,4). Several reports have indicated that the quality and quantity of dietary protein affect the serum cholesterol level (1,5–9). Only a few reports have dealt with the effect of egg white protein (EWP) on the serum cholesterol level in rats (10) and humans (11). Moreover, the mechanism by which the hypocholesterolemic effect of EWP is exerted in rats is not well understood. Although OV is one of the EWP, there is no information about the hypocholesterolemic action of OV. In earlier papers (12,13) we used cultured Caco-2 cells and found that soy protein peptic hydrolysate (SPH) directly inhibited the absorption of micellar cholesterol. Our experimental system to evaluate cholesterol with Caco-2 cells is useful for clarifying the molecular mechanism underlying the mechanism for and effects of OV on cholesterol absorption from the small intestine. We postulate that OV-induced hypocholesterolemic action may have resulted in the inhibition of both cholesterol absorption in the intestinal epithelial cells and ileal reabsorption of bile acids. Thus, we used Caco-2 cells, rats, or *in vitro* assays to investigate the effects of the serum cholestrerol-lowering action of OV.

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Abbreviations: EWP, egg white protein; LTH, β-lactoglobulin tryptic hydrolysate; OV, ovomucin; SPH, soy protein peptic hydrolysate.

EXPERIMENTAL PROCEDURES

Preparation of OV. Fresh egg white was prepared from eggs (White Leghorn hens) according to the method of Xu *et al*. (14). OV from fresh egg white was prepared by the method of Kato *et al*. (15). Briefly, thick egg white separated from total egg white using a sieve was homogenized in a Waring blender for about 5 min and diluted with 3 vol of deionized water. The mixture was stirred for 1 h and then adjusted to pH 6.0 with 1 mol/L HCl. After the mixture stood overnight at 4°C, OV precipitated in this system and was then lyophilized.

Chemical analyses. Protein content was determined by the Kjeldahl method (16), with an N-to-protein conversion factor of 6.25. Lipids were extracted by using chloroform/methanol (2:1, vol/vol) and weighed. Sugar content was determined by the phenol-sulfonic acid method (17). Moisture was determined as the loss in weight after drying at 105°C for 24 h. Ash content was determined by the direct ignition method (550°C overnight). As shown in Table 1, amino acid composition was determined by the methods described previously (18). Tryptophan content was determined by the *p*-dimethyl- aminobenzaldehyde method (19,20). Casein was generously supplied by the Central Research Institute of Meiji Milk Products Co., Ltd. (Tokyo, Japan). The chemical composition of casein was as follows (g/kg): protein, 860; sugar 15; moisture, 110; lipid, 0; and ash, 15. The chemical composition of OV was as follows (g/kg): protein, 693; sugar 148; moisture, 120; lipid, 0; and ash, 39.

Taurocholate-binding capacities. The binding capacity of cholestyramine, casein, or OV with taurocholate was measured by the method described previously (13). The mixtures containing 1.85 kBq of tauro [carbonyl-^{14}C]cholic acid (sodium salt) (1.89 Gbq/mmol; Amersham International, Buckinghamshire, United Kingdom), 0.1 mol/L sodium taurocholate in 5 mL of 0.1 mol/L Tris-HCl buffer (pH 7.4), and 1–500 mg binding substances [cholestyramine, casein: casein sodium (Wako Pure

TABLE 1
Amino Acid Compositions of Casein and Ovomucin (OV) (g/kg)

Amino acid	Casein	OV
Asp	89.5	102.8
Thr	56.2	83.9
Ser	53.8	92.8
Glu	201.6	97.7
Pro	75.6	90.8
Gly	19.8	67.1
Ala	38.8	51.3
Cys	2.4	39.5
Val	61.6	62.2
Met	32.4	21.7
Ile	53.1	45.4
Leu	94.3	64.2
Tyr	26.1	29.6
Phe	38.3	36.5
His	24.8	21.7
Lys	84.8	58.2
Arg	27.9	21.7
Trp	19.0	12.9

Chemical, Osaka, Japan), OV] were incubated at 37°C for 2 h, and the radioactivity in the supernatant (15,000 × *g* for 15 min) was measured by liquid scintillation counting.

Micellar solubility of cholesterol and taurocholate. Micellar solubility of cholesterol and taurocholate with proteins *in vitro* was measured by the method of Ikeda *et al*. (21) with some modifications. Micellar solutions (1 mL) containing 6.6 mmol/L sodium taurocholate, 0.5 mmol/L cholesterol, 1 mmol/L oleic acid, 0.5 mol/L monoolein, 0.6 mmol/L PC, 132 mmol/L NaCl; and 15 mmol/L sodium phosphate (pH 7.4), casein sodium or OV (5 mg/mL, respectively) were prepared by sonication. Then the mixture was incubated at 37°C for 24 h and ultracentrifuged at 100,000 × *g* for 60 min at 37°C. The supernatant was collected for the determination of cholesterol and taurocholate as described previously (13).

Cholesterol absorption in Caco-2 cells. Caco-2 cells were acquired from the American Type Culture Collection (Rockville, MD). The cells were maintained DMEM supplemented with 10% FBS, 4 mmol/L L-glutamine, 50 IU/mL of penicillin, and 50 mg/L streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The monolayers became confluent 3 to 4 d after seeding at between 7×10^5 and 1.2×10^6 cells per 100-mm diameter dish, and the cells were passed at a split ratio of 4 to 8 by trypsinizing with 0.25% trypsin and 0.8 mmol/L EDTA in PBS. Monolayers were grown in 24-well plastic dishes containing 1 mL of DMEM supplemented with FBS as described previously (13), fresh medium being added every 2 d. The experiments described usually used cultures 12–15 d after plating and were performed in medium-199/Earle's (GIBCO, Grand Island, NY) containing 1 mmol/L HEPES. Cell viability, as ascertained by trypan-blue exclusion, was unaffected by any of the experimental procedures. The number of passages of the cell line ranged from 70–85.

[^{14}C]-Labeled micellar cholesterol uptake in Caco-2 cells was measured by the method described previously (13). The final concentration of each [^{14}C]-labeled micellar solution (0.5 mL) was as follows: 3.7 kBq [4-^{14}C]cholesterol (2.1 Gbq/m mol; NEN, Boston, MA), 0.1 mmol/L cholesterol, 1 mmol/L oleic acid, 0.5 mmol/L monoolein, 6.6 mmol/L sodium taurocholate, 0.6 mmol/L PC, casein sodium, and OV (2.5 mg/0.5 mL, respectively). The micellar solution was mixed by ultrasonication.

After 14 d, the cells were rinsed two times with 1 mL of PBS. A [^{14}C]-labeled micellar solution (0.5 mL) containing casein sodium or OV was then added to the dishes, which were incubated at 37°C for 20 min in a CO_2 incubator. After this incubation, the cells were rinsed two times with 1 mL of PBS. The cells were finally lysed in 0.1% SDS solution, after which 7.5 mL of Aquasol-2 (NEN) was added, and the radioactivity in the cellular debris was counted to determine the amount of cholesterol absorbed into the cells. The cellular protein was determined by a commercially available kit (Bio-Rad Protein Assay; Bio-Rad, Tokyo, Japan). The amount of cholesterol absorbed into the cells was expressed as pmol/mg protein.

Effects of OV or casein on lipid metabolism in rats (animals and diets). Male rats of the Wistar strain (Japan SLC, Hamamatsu, Japan) were used in the present animal studies. Room temperature was maintained at 22 ± 2°C with a 12-h cycle of light (0800–2000) and dark. The approval of the Gifu University Animal Care and Use Committee was given for our animal experiments. All the rats were housed individually in metal cages and were allowed free access to food and water. After acclimation to a commercial stock diet (CE-2; Japan CLEA, Tokyo, Japan) for 3 d, 5-wk-old rats weighing 115–130 g were divided into two groups of six rats each on the basis of body weight. The composition of the basal diet recommended by the American Institute of Nutrition (22) is shown in Table 2. OV was added to the diet at a nitrogen level equivalent to that of a casein diet at the expense of carbohydrates. Each group had free access to one of the respective test diets (Table 2) containing casein or OV as the protein source for 10 d. After 24 h without food, the rats were anesthetized with

TABLE 2
Composition of Experimental Diets (g/kg)

	Diet group	
Ingredient	Casein	OV[a]
Casein	232.56	232.56
OV	—	72.15
Lard	50	50
Corn oil	10	10
Mineral mixture[b]	35	35
Vitamin mixture[c]	10	10
Choline chloride	2	2
Sucrose	200.98	176.93
Starch	401.96	353.86
Cellulose	50	50
Cholesterol	5	5
Sodium cholate	2.5	2.5

[a]See Table 1 for abbreviation.
[b]AIN-76 mineral mixture (21).
[c]AIN-76 vitamin mixture (21).

diethyl ether and killed. Blood was collected by cardiac puncture, and the liver was removed. Fecal collections (d 7–9) were completed prior to the 24-h food restriction and blood sampling. Feces were used for determining fecal steroids.

Rat lipid analyses. Various lipid concentrations were determined using commercially available kits as follows: serum and liver cholesterol with Monotest cholesterol (Boehringer Mannheim Yamanouchi, Tokyo, Japan); HDL-cholesterol with HDL-cholestase (Nissui, Tokyo, Japan); serum and liver TG with Triglycolor III (Boehringer Mannheim Yamanouchi); and serum phospholipid with Phospholipid C-Test Wako (Wako Pure Chemical, Osaka, Japan). Liver lipids were extracted by the method of Folch *et al.* (23), and total lipids were determined gravimetrically as described previously (24). Fecal acidic steroids were measured according to the method of Bruusgaard *et al.* (25) and Malchow-Moller *et al.* (26), whereas fecal neutral steroids were assayed with trimethylsilyl ether by using 1.5% OV-17 with a GC-14A instrument (Shimadzu, Kyoto, Japan) and 5 α-cholestane as the internal standard (27).

Statistical analysis. Results are expressed as mean ± SEM. The statistical significance of differences was evaluated by Student's *t*-test (28).

RESULTS

Taurocholate binding capacities. From 200 to 500 mg, the bile acid-binding capacity of OV was significantly greater than that of casein (Fig. 1).

Micellar solubility of cholesterol and taurocholate. The micellar solubility of cholesterol in the presence of OV was significantly lower than with casein. Micellar solubility of taurocholate was unchanged (Fig. 2).

Cholesterol absorption in Caco-2 cells. The cholesterol micelles containing OV induced a significant suppression of cholesterol absorption in Caco-2 cells compared to the cholesterol micelles containing casein (Fig. 3).

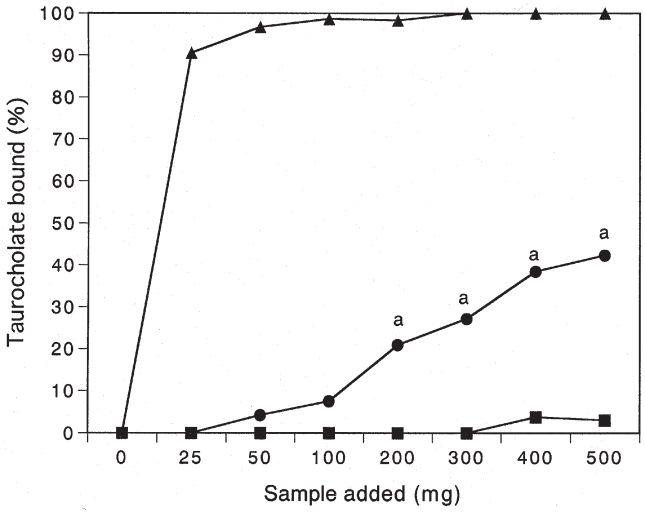

FIG. 1. Binding of cholestyramine (▲), casein (■), or ovomucin (●) with taurocholate. Individual values represent means of assays performed in duplicate. Error bars (SEM) are too small to show. Statistical significance compared to casein by Student's *t*-test (a, $P < 0.05$).

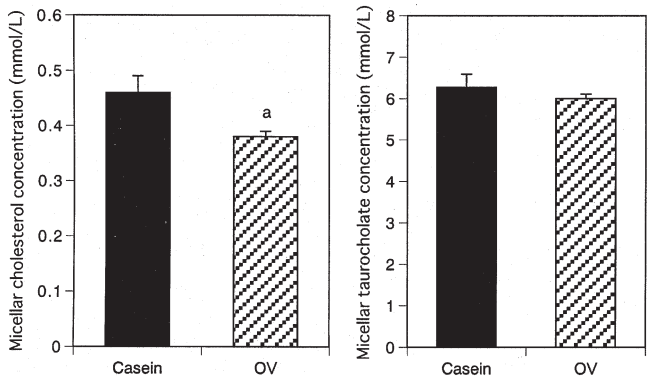

FIG. 2. Effect of casein or ovomucin (OV) on micellar solubility of cholesterol and taurocholate. Each value is expressed as mean ± SEM of three determinations. Statistical significance compared to casein by Student's *t*-test (a, $P < 0.05$).

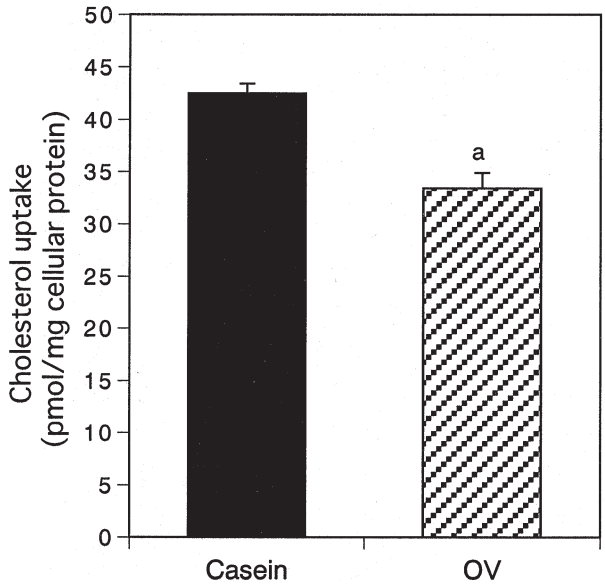

FIG. 3. Effect of casein or ovomucin (OV) on cholesterol absorption in Caco-2 cells. Each value is expressed as mean ± SEM of five determinations. Statistical significance compared to casein by Student's *t*-test (a, $P < 0.001$).

Effects of OV or casein on lipid metabolism in rats. Food intake, growth rates, and the relative liver weight were unaffected by dietary OV (Table 3). Serum total cholesterol levels in the OV groups were significantly lower than in the casein group. The HDL-cholesterol in the OV group tended to increase compared with the casein group. The proportion in the OV group of HDL-cholesterol to serum total cholesterol [(b)/(a)], which is known as the atherogenic index, was significantly higher than in the casein group. The liver total lipids level was significantly lower in the OV group than in the casein group. Fecal dry weight was unchanged by OV feeding. The fecal excretion of bile acids and cholesterol was significantly increased by OV feeding compared with casein feeding.

DISCUSSION

In this study we found, for the first time, that hen egg OV is a hypocholesterolemic protein. OV clearly demonstrated serum

TABLE 3
Effects of Dietary Casein and OV on Body and Liver Weights, Food Intake, Serum and Liver Lipids, and Fecal Steroid Excretion in Rats[a]

	Diet group	
	Casein	OV
Body weight gain (g/10 d)	25.9 ± 1.1	22.4 ± 2.5
Liver weight (g/100 g body wt)	3.81 ± 0.06	3.61 ± 0.07
Food intake, d 6 (g/d)	14.2 ± 0.7	13.6 ± 0.6
Serum (mmol/L)		
Total cholesterol (a)	2.83 ± 0.21	1.96 ± 0.07[b]
HDL cholesterol (b)	0.53 ± 0.04	0.66 ± 0.06
LDL + VLDL cholesterol	2.30 ± 0.22	1.30 ± 0.08[b]
Atherogenic index: (b)/(a) (mol/mol)	0.19 ± 0.02	0.34 ± 0.03[b]
TG	0.38 ± 0.04	0.37 ± 0.03
Phospholipids	1.13 ± 0.08	1.04 ± 0.05
Liver		
Total lipids (mg/g liver)	142.8 ± 4.41	124.0 ± 2.47[b]
Cholesterol (μmol/g liver)	70.8 ± 2.4	66.9 ± 3.2
TG (μmol/g liver)	26.8 ± 2.9	24.8 ± 2.1
Phospholipid (μmol/g liver)	118.8 ± 3.2	98.7 ±1.6
Feces		
Dry weight (g/3 d)	2.42 ± 0.06	2.58 ± 0.10
Cholesterol (μmol/3 d)	252.5 ± 6.7	281.3 ± 9.5[c]
Bile acids (μmol/3 d)	126.5 ± 5.8	151.9 ± 7.2[c]

[a]Mean ± SEM of six rats. For abbreviation see Table 1.
[b]Significantly different from casein group at $P < 0.01$.
[c]Significantly different from casein group at $P < 0.05$.

cholesterol-lowering effects compared with casein. The major differences in amino acid compositions between casein and OV are in the level of glycine and cystine (Table 1). The relationship between the serum cholesterol-lowering activity of dietary protein and the amino acid contents of protein has been reported previously (29–31). Sugiyama *et al*. (31) reported a significant negative correlation between blood cholesterol levels and the level of cystine in intact dietary proteins. Thus, as OV contains higher levels of cystine than casein, the differences in amino acids content may relate to the differences in serum cholesterol level in the present study.

The amount of OV in egg white is 3.5% (w/w) of the total EWP. Reportedly, OV, which is macromolecular and a highly glycosylated glycoprotein, has two subunits, one a protein-rich α-subunit (M.W. 220 kDa) and one a carbohydrate-rich β-subunit (M.W. 400 kDa) (15,32). Recent studies suggest that OV exhibits both antiviral (18,33) and antitumor activities (4). All the sialic acid present in OV was described as *N*-acetylneuraminic acid (34). Previous studies (4,18,33) discussed the relationships between the *N*-acetylneuraminic acid of OV and its physiological activity. Our preliminary observations suggested that cholesterol absorption in Caco-2 cells or micellar solubility of cholesterol is unaffected by the *N*-acetylneuraminic acid *in vitro*. Whether the hypocholesterolemic action induced by OV *in vivo* is related to *N*-acetylneuraminic acid is currently being studied.

Taking dietary utilization of OV into account, OV is appropriate to use as a supplement in the diet containing an adequate protein rather than a main protein source in the diet. It is not easy to prepare a large amount of OV, which contains only 3.5% (w/w) of the EWP. Thus, we simply added OV to the casein diet (20% casein), increasing the protein content of the diet (Table 2). We also found that the serum cholesterol level was significantly decreased in rats fed the diet containing 15% casein supplemented with 5% OV compared to that of 20% casein diet (Nagaoka, S., and Watanabe, K., unpublished results).

It has been postulated that the degree of serum cholesterol-lowering activity depends on the degree of fecal steroid excretion (acidic steroids + neutral steroids) (35). The present study demonstrated a higher fecal excretion of cholesterol (11.4% change) and acidic steroids by OV feeding (Table 3), indicating that the effect is due at least in part to an enhancement of fecal steroid excretion. Smith (36) reported that human gallbladder mucin binds to cholesterol in model bile. Thus, we speculate that an increased fecal excretion of cholesterol may be induced by the binding of cholesterol to OV in the intestine. There have been many studies on the hypocholesterolemic effects of proteins except for OV, most of which emphasized the hypothesis that a peptide with binding capacity to bile acid could inhibit the reabsorption of bile acid in the ileum and decrease the blood cholesterol level (37). These possibilities may be applicable to the case of OV on the basis of the evidence of fecal bile acid excretion (Table 3) and taurocholate-binding capacity (Fig. 1) in the present study.

Cholesterol is rendered soluble in bile salt-mixed micelles and then absorbed (38). The present study indicated that the micellar solubility of cholesterol in the presence of OV was significantly lower than with casein. Very interestingly, we found for the first time that the presence of SPH (13) or

β-lactoglobulin tryptic hydrolysate (LTH) (39) significantly suppressed micellar solubility of cholesterol *in vitro*. Sitosterol (21), sesamine (40), or catechin (41) also lowered the micellar solubility of cholesterol in conjunction with the serum cholesterol-lowering effects in rats. These findings, including those with OV, suggest that the suppression of micellar solubility of cholesterol induces the inhibition of cholesterol absorption in the jejunum, and this may be closely related to the lowering action of serum cholesterol. As shown in the cases of OV, LTH (39), or SPH (13), other dietary proteins or peptides may also affect such solubility.

In recent studies, monolayers of Caco-2 cell cultures were used as a model system to examine the process of lipid metabolism (42–44). For example, Field *et al*. (42) reported that Caco-2 cells, like the small intestine, had the ability to absorb micellar cholesterol and to express marker enzymes such as alkaline phosphatase as small intestinal epithelial cells. The experiments described usually used cultures 12–15 d after plating. This is optimal to determine the cholesterol uptake of the Caco-2 cell culture concomitant with the optimal expression of marker enzymes as small intestinal epithelial cells. In our previous paper (12), we found by using the Caco-2 cultured cell strain that the cholesterol micelles containing SPH (12) or LTH (39) significantly suppressed cholesterol absorption by Caco-2 cells *in vitro*. There is no information about the effects of OV on cholesterol absorption. We therefore used Caco-2 cells or rats to investigate the mechanisms of the serum cholesterol-lowering action of OV. Our experimental system to evaluate cholesterol absorption with Caco-2 cells is very useful for clarifying the molecular mechanism underlying the inhibitory effect of OV on cholesterol absorption from the small intestine, which was previously unknown. There have so far been a few experimental studies to evaluate some effects of proteins or peptides on cholesterol absorption by using cultured intestinal cells (12,13,39).

In this study, we found that OV lowers serum cholesterol levels in rats and inhibits cholesterol absorption in Caco-2 cells. The present results suggest that the suppression of cholesterol absorption by direct interaction between cholesterol-mixed micelles and OV in the jejunal epithelia is part of the mechanism of hypocholesterolemic action induced by OV. Whether OV micelles act directly to lower cholesterol absorption in rat jejunum epithelium *in vivo* is currently being studied.

There have been many studies on the hypocholesterolemic effects of proteins, most of which emphasized the hypothesis that a peptide with high bile acid-binding capacity could inhibit the reabsorption of bile acid in the ileum and decrease the blood cholesterol level (ileal effects). These possibilities may be applicable to the case of OV on the basis of the evidence of fecal bile acid excretion and bile acid-binding capacity in this study. Fecal excretion of bile acids was significantly increased by OV feeding compared with casein feeding, and the bile acid-binding capacity of OV was significantly greater than that of casein. However, our earlier studies (13,39), together with this study, suggest that the reduction of micellar solubility of cholesterol, which may cause the suppression of cholesterol absorption by direct interaction between cholesterol-mixed micelles and OV in the jejunal epithelia, is part of the mechanism of hypocholesterolemic action induced by OV (jejunal effects). Thus, the hypocholesterolemic action of OV may involve both jejunal and ileal effects.

This study clearly indicates the hypocholesterolemic action of OV compared to casein in the animal model. Present findings concerning the hypocholesterolemic action of OV may enhance both industrial utilization of egg or egg proteins and their value for health enhancement.

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