Biliary lipid composition and gallstone formation in rabbits fed on soy protein, cholesterol, casein and modified casein

Tomris ÖZBEN

Department of Biochemistry, Faculty of Medicine, Akdeniz University, 07059 Topçular, Antalya, Turkey

In four experimental groups, rabbits were fed on diets containing soy beans, soy beans plus cholesterol (1 %, w/w), casein and modified casein for 8 weeks. Biliary lipid levels, lithogenic-index values and the rate of gallstone formation were determined. The highest mean relative concentrations (mol %) of cholesterol and phospholipid were found in the soy bean+cholesterol group, and the highest mean relative bile acid concentration was in the soy bean group. The lowest mean relative cholesterol and phospholipid values were found in the soy bean and modified casein groups respectively. The lowest mean relative bile acid level was in the soy bean+cholesterol group. The highest lithogenic index and rate of gallstone formation were in the soy bean+cholesterol group, and the lowest values were in the soy bean group. The modification of casein used was effective in decreasing the lithogenic effect of casein on gallstone formation.

INTRODUCTION

Clinical and epidemiological data reveal that diet plays a significant role in the development of gallstone diseases [1,2]. To date, most of the research emphasizing the effects of diet on gallstone formation has concentrated on dietary cholesterol, fat, carbohydrate and excessive energy intake [1,2]. Insufficient attention has been devoted to dietary proteins. Several studies have indicated that dietary vegetable proteins are hypocholesterolaemic, compared with animal proteins [1,3,4,5]. Since dietary protein can influence serum lipids, they may affect biliary cholesterol concentrations and cholelithiasis. Although there is clear evidence that dietary proteins have an effect on cholelithiasis, the mechanisms are not clearly defined. Kritchevsky [6] hypothesized that the hypocholesterolaemic effect of soy protein was based on the low lysine/arginine ratio in that protein. Other investigators considered that the atherogenicity of a protein depended on its lysine content [3]. Casein contains 40 % more lysine than soy protein does. So far, I failed to find any report in the literature about decreasing the lysine content of casein and addition of this form of casein to the diet of animals. In an attempt to decrease the lysine content of casein, I used the Maillard reaction [7,8]. This reaction, also known as the browning reaction, occurs between the glycosidic hydroxy group of sugars and free amino groups of amino acids and proteins. In my experiment, lactose reacts with the free amino groups of casein (e.g. the ϵ -amino group of lysine) and forms coloured products.

The purpose of this study was to examine the effect of dietary proteins (casein, modified casein and soy protein) on cholesterol gallstone formation in rabbits. The effect of dietary cholesterol on biliary lipids was also examined. In an effort to ascertain the possible mechanisms responsible for gallstone formation, the three primary bile constituents (bile acids, phospholipids and cholesterol) were quantitatively measured.

MATERIALS AND METHODS

Animals and diets

For the study, 40 random-bred male rabbits of the New Zealand White strain were obtained from the

Department of Laboratory Animal Science in our Medical Faculty. The rabbits were kept individually in cages with wire-mesh bases constructed of galvanized steel in a room with controlled lighting (14 h/day), constant temperature (16–20 °C) and relative humidity (55–65 %). On arrival, the animals, which were aged about 9 weeks, weighing 2203 ± 51 g, were maintained on commercial rabbit pellets for 2 weeks. After this initial equilibration period, they were assigned to one of the four experimental diets (Table 1) on the basis of their serum cholesterol concentrations measured 2 days before the end of this pre-experimental period, in such a way that the mean serum cholesterol levels of the groups were approximately similar. Only the protein components of the diets were different. The diets contained soy beans, soy beans + cholesterol (1 %, w/w), casein or modified casein. All diets were fed as pellets. Food was provided each day at 09:00 h on a restricted basis, the animals receiving 70 g/day. Most rabbits consumed all their food within 4 h. Water was provided *ad libitum*. The experimental period lasted 8 weeks. Food consumption and body weight for each animal were recorded four times per week.

Collection and preparation of samples for analysis

After having been fasted for 16 h, the rabbits were killed by cervical dislocation. The gall bladder was excised and examined for the presence of gallstones. The bile was collected and bile samples were stored at -20 °C for subsequent analysis.

Analytical techniques

Biliary cholesterol was extracted as described by Abell *et al.* [9] and was measured with a cholesterol oxidase kit (Dart reagent; Coulter Diagnostics, Hialeah, FL, U.S.A.) in a Dacos autoanalyser (Coulter Electronics). Biliary phospholipids were extracted and determined by the method of Bartlett [10]. After the hydrolysis and extraction of total bile acids in the bile [11,12], they were determined with the 3α -hydroxysteroid dehydrogenase kit (Merckotest; Merck, Darmstadt, Germany). The lithogenic index was calculated by using the formulae of Thomas & Hofmann [13] and data of Admirand & Small [14], Hegardt & Dam [15] and Holzbach [16].

Table 1. Compositions of the experimental diets

Diets: I, soy bean; II, soy bean + cholesterol; III, casein; IV, modified casein. The constant components consisted of (g): $\text{CaHPO}_4 \cdot \text{H}_2\text{O}$, 2.9; NaCl , 0.8 (in diets containing soy isolate, 0.6); KHCO_3 , 1.8; MgCO_3 , 0.3; MgO , 0.2; vitamin premix, 1.2; mineral premix, 1.0. The vitamin premix contained (in mg, except as noted): thiamin, 6; riboflavin, 2.25; niacin, 15.2; calcium pantothenate, 5.6; choline chloride, 200; inositol, 100; folic acid, 0.85; biotin, 0.06; pyridoxine/HCl, 2.25; *p*-aminobenzoic acid, 50; vitamin B-12, 1.5 μg ; DL- α -tocopheryl acetate, 5; menadione, 0.4; vitamin A, 1500 i.u.; cholecalciferol, 300 i.u.; vitamin C, 40. The mineral premix contained (in mg): sodium citrate, $2\text{H}_2\text{O}$, 153.5; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 90; MnO_2 , 14; $\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 10; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 20; KBr , 2; $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$, 0.85; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 10; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.5; KI , 0.5; As_2O_3 , 0.02; NaF , 0.85; $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 0.5.

Ingredients (g/100 g of diet)	Amount for diet (%)			
	I	II	III	IV
Soy bean protein*	20.8	20.8	—	—
Casein protein†	—	—	21	—
Modified casein protein‡	—	—	—	21
Cholesterol	—	1	—	—
Methionine	0.2	0.2	—	—
Corn starch	17	17	17	17
Sucrose	21	21	21	21
Soy bean oil	10	9	10	10
Sawdust	18	18	17.8	17.8
Molasses	5	5	5	5
Constant components	8	8	8.2	8.2
Total	100	100	100	100

* Obtained from soy protein isolate, consisting of 86.0 % protein, 3.8 % carbohydrate, 0.8 % fat, 0.1 % fibre, 3.8 % ash and 5.5 % moisture.

† Obtained from casein isolate, consisting of 49 % protein, 32 % carbohydrate (lactose), 1.0 % fat, 0.1 % fibre, 8 % ash and 9.9 % moisture.

‡ Prepared by dry heating of casein isolate at 190 °C for 3 min [7,8].

Statistical analysis

The data were analysed by a non-parametrical Mann-Whitney U test.

RESULTS

In four experimental groups, rabbits were fed on diets containing soy bean, soy bean + cholesterol (1 %, w/w), casein and modified casein, for 8 weeks. Biliary lipid levels, lithogenic-index values and the rate of gallstone formation were determined (Table 2 and 3).

The mean absolute and relative concentrations of biliary cholesterol were significantly different in all groups (Table 3), and were highest in the soy bean + cholesterol group. The lowest values were found in the soy bean group (Table 2).

The mean absolute and relative concentrations of phospholipids present in bile were highest in the soy bean + cholesterol group. The lowest values were found in the bile of rabbits fed on modified casein (Table 2). The mean absolute concentration of biliary phospholipids was significantly higher in the soy bean + cholesterol group than that of animals fed on soy beans or modified casein (Tables 2 and 3). Significant differences were observed between the mean relative concentrations of biliary phospholipids in the soy bean + cholesterol and modified casein groups (Table 3).

The highest mean absolute concentration of bile acids was found in the soy bean + cholesterol group, and the lowest value was in the modified-casein group (Table 2). The mean absolute concentration of bile acids in the bile of animals fed on modified casein was significantly different from those of the soy bean + cholesterol and soy bean groups (Table 3). No significant differences were observed between the absolute concentrations of bile acids in the other groups (Table 3). The highest and the lowest mean relative concentrations of bile acids were found in the soy bean and soy bean + cholesterol groups respectively (Table 2). The relative concentrations of bile acids in bile of animals fed on soy beans, casein or modified casein did not differ significantly (Table 3). Significant differences were observed between the relative

Table 2. Influence of dietary protein and cholesterol on gallstone formation, bile lipids and lithogenicity in rabbits

Results are expressed as means $\pm$ S.D. for ten animals in each experimental group. Values in parentheses are relative concentrations (mol %).

Protein source	No. of rabbits with gallstones	Absolute concn. (mm)			Lithogenic index	
		Cholesterol	Phospholipid	Bile acid	A.S.*	H.D.H.†
Soy bean	1	3.0 $\pm$ 0.4 (1.5 $\pm$ 0.4)*	15.3 $\pm$ 3.4 (8.1 $\pm$ 2.5)	175.0 $\pm$ 22.9 (90.4 $\pm$ 2.3)	0.20 $\pm$ 0.06	0.41 $\pm$ 0.13
Soy bean + cholesterol	6	9.9 $\pm$ 1.2 (4.7 $\pm$ 0.8)	21.2 $\pm$ 4.8 (9.9 $\pm$ 2.1)	184.0 $\pm$ 25.6 (85.5 $\pm$ 2.6)	0.58 $\pm$ 0.09	1.16 $\pm$ 0.17
Casein	4	6.2 $\pm$ 0.9 (3.3 $\pm$ 0.2)	16.7 $\pm$ 5.3 (8.6 $\pm$ 1.5)	167.0 $\pm$ 25.7 (88.1 $\pm$ 1.3)	0.42 $\pm$ 0.05	0.86 $\pm$ 0.10
Modified casein	2	4.4 $\pm$ 1.2 (2.6 $\pm$ 0.5)	12.6 $\pm$ 1.2 (7.6 $\pm$ 0.3)	148.0 $\pm$ 10.9 (89.8 $\pm$ 0.6)	0.35 $\pm$ 0.06	0.72 $\pm$ 0.14

* Using equation derived from data of Admirand & Small [14].

† Using equation derived from data of Hegardt & Dam [15] and Holzbach [16].

Table 3. Significance of differences in the absolute and relative concentrations of bile lipids and lithogenic-index values in rabbits fed on four different diets

The Mann-Whitney U test was used. Values in parentheses are significances of differences in the relative concentrations. A.S. and H.D.H. are explained in Table 2.

Protein source	Absolute concentration (mmol/l)			Lithogenic index	
	Cholesterol	Phospholipid	Bile acid	A.S.	H.D.H.
Soy bean versus soy bean + cholesterol	$P < 0.01$ ($P < 0.01$)	$P < 0.05$ ($P > 0.05$)	$P > 0.05$ ($P < 0.01$)	$P < 0.01$	$P < 0.01$
Soy bean versus casein	$P < 0.05$ ($P < 0.01$)	$P > 0.05$ ($P > 0.05$)	$P > 0.05$ ($P > 0.05$)	$P < 0.01$	$P < 0.01$
Soy bean versus modified casein	$P < 0.05$ ($P < 0.01$)	$P > 0.05$ ($P > 0.05$)	$P < 0.05$ ($P > 0.05$)	$P < 0.01$	$P < 0.01$
Soy bean + cholesterol versus casein	$P < 0.05$ ($P < 0.01$)	$P > 0.05$ ($P > 0.05$)	$P > 0.05$ ($P < 0.05$)	$P < 0.01$	$P < 0.01$
Soy bean + cholesterol versus modified casein	$P < 0.01$ ($P < 0.01$)	$P < 0.05$ ($P < 0.05$)	$P < 0.05$ ($P < 0.01$)	$P < 0.01$	$P < 0.01$
Casein versus modified casein	$P < 0.05$ ($P < 0.05$)	$P > 0.05$ ($P > 0.05$)	$P > 0.05$ ($P < 0.05$)	$P < 0.05$	$P > 0.05$

concentrations of bile acids in the other groups (Table 3).

The lithogenic-index values calculated from the formula of Thomas & Hofmann [13] and data of Hegardt & Dam [15] and of Holzbach [16] were twice as high as with those of Admirand & Small [14] (Table 2). The highest lithogenic index and rate of gallstone formation were in the soy bean + cholesterol group and the lowest values were in the soy bean group. The lithogenic index of the rabbits fed on casein was higher than that of those fed on modified casein. The incidence of gallstone formation in the animals fed on modified casein was half of that in those fed on casein (Table 2).

DISCUSSION

It is clear from the present study that the type of dietary protein affects biliary lipid composition in rabbits. Casein, compared with soy protein, caused increased levels of biliary cholesterol and, as a result, an increase in the lithogenic index of saturation. Likewise, dietary cholesterol was found to cause a greater increase in biliary cholesterol and in the lithogenic index. No significant differences in biliary phospholipids and bile acids were observed between the animals fed on casein or soy protein. Similar results have been reported by other workers [1,2,6,17,18].

The hypercholesterolaemic effect of dietary casein versus soy protein in rabbits has been well established [3–6,19]. However, the mechanisms responsible for these effects are not yet clear. Beynen *et al.* [2] proposed that the effects of dietary protein on biliary lipid composition are mediated through the concentration of serum cholesterol. Dam *et al.* [20] found that a number of dietary modifications affected gallstone formation in hamsters, and concluded that the lithogenicity of the standard diet was due to increased cholesterol synthesis. Ginsberg *et al.* [21] found that hepatic 3-hydroxy-3-methylglutaryl-CoA reductase activity in hamsters fed on the lithogenic diet was more than 10 times greater than that observed in controls. Kritchevsky & Klurfeld [17] showed the inhibitory influence of soy protein on gallstone formation when included in a lithogenic diet. Our results obtained

from diets containing modified casein showed lower biliary cholesterol concentration and lithogenic index than those on casein diet. We conclude that the lysine content of casein is, at least partly, responsible for its hypercholesterolaemic and lithogenic effects, and the Maillard reaction is effective in decreasing the lysine content of casein.

The lithogenic-index values, calculated by the equation derived from data of Hegardt & Dam [15] and Holzbach [16], were greater than those calculated by using the equation derived from data of Admirand & Small [14]. These results are in accordance with the suggestion by Holzbach *et al.* [16] that the micellar zone for cholesterol solubility was significantly lower than that previously reported by Admirand & Small [14].

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