Effects of casein, soy, and whey proteins and amino acid supplementation on cholesterol metabolism in rats

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Cholesterol metabolism was studied in male rats fed high-fat, cholesterol-enriched diets containing casein, soy, or whey protein. Liver weight, total lipids, and cholesterol were higher for rats fed casein and whey than for those fed soy. Serum total, HDL, and LDL cholesterol were not significantly altered by the protein fed. VLDL cholesterol was lower for rats fed soy than for those fed casein, but there were no significant differences between whey-fed rats and the other two groups. During week 1, fecal output was highest for the soy-fed group, but during week 7, it was highest for the whey-fed group. During week 1, fecal cholesterol, coprostanol, and total steroids for the soy-fed group were higher than for the other two groups. During week 7, the soy-fed group was still excreting the most coprostanol, but the least cholesterol. Addition of amino acids had little impact on liver total lipids or cholesterol, except they decreased with addition of methionine to soy and whey diets. Serum total and LDL cholesterol were not altered by amino acid supplementation. Addition of cystine to casein decreased VLDL cholesterol. Addition of methionine to soy and whey increased HDL cholesterol. Little effect of amino acid supplementation was found on fecal steroids.

Keywords: dietary protein; serum lipids; liver lipids; fecal lipids; amino acid supplementation

Introduction

The role of dietary protein in lipid metabolism has become the focus of much attention during the last decade. Much of the evidence has been produced in experimental animals such as rabbits,[1,2] rats,[3-10] pigs,[11] chickens,[12,13] guinea pigs,[14] and monkeys.[15] In general, those studies have indicated that feeding animal protein, as opposed to vegetable protein, induced hypercholesterolemia. In many of those studies, casein was used as the animal protein and soy as the vegetable protein. However, the effect of dietary protein on serum cholesterol level may not always be attributed to its animal or vegetable origin, since in one study[16] whey protein was found to be as hypocholesterolemic as soy protein.

Kritchevsky[17] suggested that the ratio of arginine to lysine in a protein might play an important role in determining the cholesterolemic effect of protein. This was confirmed by Park and Liepa,[18] who showed that the addition of arginine to casein reduced serum cholesterol level, whereas the addition of lysine to soy protein increased serum cholesterol level. However, such modifications were not found by other workers.[19]

Addition of sulfur-containing amino acids was found to influence serum cholesterol level, but again the results of studies were inconclusive.[20-23] Comparison of the metabolic effect of methionine and cystine supplementation in rats revealed that methionine stimulated lipogenesis, whereas cystine stimulated gluconeogenesis.[24]

The purpose of this study was to try to confirm the hypocholesterolemic effects of whey protein and to investigate whether modification of ratios of arginine to lysine and methionine to cystine in casein, soy, and whey protein influences cholesterol metabolism.

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Table 1 Composition of diets (wt%)

	Casein			Soy			Whey		
Ingredient[a]	1	2	3	4	5	6	7	8	9
Protein (23%)									
Casein	26.12	26.12	26.12	—	—	—	—	—	—
Soy Isolate	—	—	—	26.42	26.42	26.42	—	—	—
Whey Concentrate[b]	—	—	—	—	—	—	30.99	30.99	30.99
Amino acid									
Lysine	—	—	—	—	1.94	—	—	1.11	—
Arginine	—	1.27	—	—	—	—	—	—	—
Methionine[c]	—	—	—	—	—	4.25	—	—	1.87
Cystine	—	—	1.31	—	—	—	—	—	—
Corn oil	14.40	14.40	14.40	14.40	14.40	14.40	14.40	14.40	14.40
Cholesterol	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20	1.20
Alphacel	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Mineral mix[d]	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50
Vitamin mix[e]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Choline	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Cornstarch	48.56	47.29	47.3	48.27	46.33	44.02	43.71	42.64	41.84

[a] ICN Nutritional Biochemicals, Cleveland, OH, unless otherwise noted.
[b] Donated by Zealand Milk Products Inc., Petaluma, CA.
[c] NCR Teklad Diets, Madison, WI.
[d] AIN mineral mixture 76.
[e] AIN vitamin mixture 76; 0.0001% menadione and 0.001% ethoxyquin were added to each diet.

Materials and methods

The nine test diets included: (1) casein, (2) casein + arginine, (3) casein + cystine, (4) soy, (5) soy + lysine, (6) soy + methionine, (7) whey, (8) whey + lysine, and (9) whey + methionine. The diets were formulated to contain 23% protein and the energy distribution was 30% from fat, 20% from protein, and 50% from carbohydrate. *Table 1* lists the complete composition of the diets. The three sources of protein were: casein, high nitrogen (ICN Nutritional Biochemicals, Cleveland, OH); soy protein isolate (ICN); and whey protein concentrate, low lactose (New Zealand Milk Products, Inc., Petaluma, CA).

Amino acids were added to some of the diets. L-lysine hydrochloride (ICN) was added to soy (diet 5) and whey (diet 8) proteins to simulate the ratio of lysine to arginine in casein (diet 1). L-arginine hydrochloride (ICN) was added to casein (diet 2) to simulate the ratio of lysine to arginine in soy protein (diet 4). L-methionine (NRC Teklad Diets, Madison, WI) was added to soy (diet 6) and whey (diet 9) proteins to simulate the ratio of methionine to cystine in casein. L-cystine (ICN) was added to casein (diet 3) to simulate the ratio of methionine to cystine in soy (diet 4) protein. The addition of amino acids was done at the expense of carbohydrate. Amino acid ratios and amounts of amino acids added to the three proteins are shown in *Table 2*.

Male weanling rats (Harlan Sprague Dawley, Madison, WI) were housed in individual stainless steel cages with raised mesh floors in a temperature- and light-controlled environment. Diet and water were offered ad libitum. Body weight and feed intake were recorded on a weekly basis. Feces were collected daily, weighed, and stored at −20° C during the first and seventh weeks. A total of 72 rats (received in two shipments, two weeks apart) were fed the test diets for 48 days. Upon arrival of each shipment, rats were weighed and fed chow for five days. Then they were divided randomly into two groups of 18 each. The first group was assigned immediately to the test diets, while the second group continued on chow for three additional days and then was assigned to the test diets.

At the end of the experimental period, fasting blood samples were collected from the abdominal aorta after anesthetizing the rats with Barb-Euthol injected intraperitoneally. Serum was obtained by centrifugation and kept at refrigeration temperature for the separation of lipoproteins. After three days, the remaining serum was stored at −20° C. After euthanation of the rats, livers were removed and stored at −20° C. Epididymal fat pads were removed, blotted dry, and weighed.

The separation of lipoprotein fractions was done by a density gradient ultracentrifugation technique as described by Terpstra et al.[25] The lipoprotein bands were made visible by prestaining of serum with Sudan Black B. The samples were centrifuged at 20° C for 24 hours at 35,000 rpm in a Beckman L3-50 ultracentrifuge using a SW40 rotor and cellulose nitrate tubes.

Table 2 Amino acid ratios and added amino acids

	Amino acid ratios		Added amino acids (wt%)			
Protein	Lys:Arg	Meth:Cys	Lys	Arg	Meth	Cys
Casein	1.95	8.25	—	5.53	—	5.69
Soy	0.84	0.54	8.43	—	18.50	—
Whey	0.83	0.89	4.66	—	8.13	—

Lipoprotein fractions were removed by aspiration with a syringe and the volume of the fractions was measured. Fractions were then stored individually in capped tubes at −20° C for future analysis.

Serum total and lipoprotein cholesterol were estimated enzymatically using a Sigma Diagnostic Kit.[26] Liver lipids were extracted by the Folch et al.[27] procedure using a chloroform:methanol (2:1) solvent. Liver cholesterol was determined by the ferric chloride-sulfuric acid procedure.[28] Liver total lipid was determined by weighing the lipid on a Gram-Atic Balance (Fisher Scientific Co.) after evaporating the solvent.

Fecal steroids were analyzed for the feces collected during days 3–7 and days 41–47, which will be referred to as week 1 and week 7, respectively. Fecal neutral steroids were extracted by the method described by Beynen et al.[29] Thin layer chromatography was utilized to separate cholesterol and coprostanol, which were then measured by densitometry.

The standard SAS GLM program was used to perform the analyses of variance and the covariance analyses of weight gains adjusting for feed intake and fecal total steroids adjusting for cholesterol intake. This program also produced the least-squares means and the pooled estimates of standard deviations within diet and measurement. Fisher's LSD analysis was done on differences among means for all parameters. Cholesterol intake, serum cholesterol, liver cholesterol, and fecal steroids were subjected to correlation coefficient analysis.[30]

Results

Mean feed intakes, weight gains, and epididymal fat pad weights are shown in *Table 3*. Feed intake was similar for rats fed casein or soy protein, but it was lower ($P < 0.04$) for rats fed whey protein as compared to soy protein. Weight gain (both unadjusted and adjusted for feed intake) were not significantly different for rats fed casein or whey proteins, but were higher for those fed casein ($P < 0.002$) and whey ($P < 0.04$) than for rats fed soy protein. Epididymal fat pad weight was not significantly different among the three protein groups.

Supplementation of casein with arginine or cystine exerted no significant effects on feed intake, weight gain, or epididymal fat pad weight. Similarly, the addition of lysine to either soy or whey proteins had no significant effects on those three parameters. However, the addition of methionine to soy and whey proteins drastically reduced ($P < 0.0001$) feed intake and unadjusted weight gain. When weight gain was adjusted for feed intake, there was less difference ($P < 0.05$) between the feed intake of the soy − and soy + methionine-fed groups and no significant difference between the whey − and whey + methionine-fed groups. Addition of methionine to the diets resulted in lower epididymal fat pad weights with both the soy ($P < 0.001$) and whey ($P < 0.003$) proteins.

Table 4 presents mean liver weight, total lipids, and cholesterol. Liver weights were greater in rats fed casein ($P < 0.0001$) and whey protein ($P < 0.03$) than in those fed soy protein. Total liver lipids were not significantly different among the three protein groups, but liver cholesterol was lower in rats fed soy protein than in rats fed casein ($P < 0.05$) and whey protein ($P < 0.003$).

The addition of arginine and cystine to casein or lysine to soy and whey proteins did not significantly modify liver weight, total lipids, or liver cholesterol, with one exception. Liver weight was lower ($P < 0.05$) when lysine was added to the whey. The addition of

Table 3 Least squares mean feed intake (g), weight gain (g), weight gain adjusted for feed intake (g), and epididymal fat pad (EFP) weight (g)

Diet group	Feed intake	Wt gain	Adj. wt gain	EFP wt
Casein	751.8^{ab}	260.8^{ab}	242.0^{ab}	3.21^{ab}
Casein + arg	752.1^{ab}	266.9^{a}	248.0^{a}	3.56^{b}
Casein + cys	731.0^{abc}	253.4^{abc}	239.5^{ab}	3.25^{ab}
Soy	755.3^{a}	227.1^{c}	207.5^{c}	3.35^{ab}
Soy + lys	750.5^{ab}	237.0^{abc}	218.5^{bc}	3.46^{ab}
Soy + met	398.9^{d}	81.9^{d}	146.7^{d}	1.35^{c}
Whey	701.8^{bc}	256.9^{ab}	250.0^{a}	3.58^{bd}
Whey + lys	681.1^{c}	235.0^{bc}	233.0^{ac}	3.40^{ab}
Whey + met	550.2^{e}	198.1^{e}	227.1^{ac}	2.88^{a}
SD	52.24	28.48	25.86	0.62

Note. Values in the same column not sharing any common superscript are significantly different at $P \leq 0.05$. In a few instances, the numbers of rats per diet per measurement were less than eight as a result of two rat deaths and several unavoidable missing measurements.

The last row represents pooled estimate of the standard deviation (SD) within diet and measurement. Because of the unequal numbers of observations among diets, pooled SEMS cannot be given for each measurement.

Table 4 Least-squares mean liver weight (g), total lipids (g/g), and cholesterol (g/g)

	Liver		
Diet group	Wt	Total lipids	Cholesterol
Casein	16.67^{a}	0.20^{ab}	0.064^{a}
Casein + arg	15.77^{a}	0.20^{ab}	0.062^{ab}
Casein + cys	15.35^{a}	0.20^{ab}	0.062^{ac}
Soy	12.67^{bc}	0.18^{bc}	0.051^{bcd}
Soy + lys	11.51^{bc}	0.16^{cd}	0.044^{de}
Soy + met	6.18^{d}	0.07^{e}	0.010^{f}
Whey	14.85^{a}	0.20^{ab}	0.071^{a}
Whey + lys	12.85^{b}	0.20^{ab}	0.066^{a}
Whey + met	10.85^{c}	0.13^{d}	0.031^{e}
SD	1.95	0.03	0.013

Note. Values in the same column not sharing any common superscript are significantly different at $P \leq 0.05$. In a few instances, the numbers of rats per diet per measurement were less than eight as a result of two rat deaths and several unavoidable missing measurements.

The last row represents pooled estimate of the standard deviation (SD) within diet and measurement. Because of the unequal numbers of observations among diets, pooled SEMS cannot be given for each measurement.

Table 5 Least-squares mean serum total and lipoprotein cholesterol (mmol/L)

Diet group	Serum cholesterol			
	Total	VLDL	LDL	HDL
Casein	3.16	1.42^{a}	0.17	0.52^{a}
Casein + arg	3.05	1.10^{ab}	0.50	0.44^{a}
Casein + cys	2.94	0.96^{bc}	0.28	0.50^{a}
Soy	2.66	0.96^{bc}	0.38	0.41^{a}
Soy + lys	2.61	1.05^{abc}	0.06	0.47^{a}
Soy + met	3.17	0.55^{c}	0.37	0.87^{b}
Whey	2.83	1.01^{abc}	0.28	0.47^{a}
Whey + lys	2.63	0.84^{bc}	0.23	0.41^{a}
Whey + met	2.85	0.67^{bc}	0.25	1.06^{b}
SD	0.49	0.44	0.32	0.22

Note. Values in the same column not sharing any common superscript are significantly different at $P \leq 0.05$. In a few instances, the numbers of rats per diet per measurement were less than eight as a result of two rat deaths and several unavoidable missing measurements.

The last row represents pooled estimate of the standard deviation (SD) within diet and measurement. Because of the unequal numbers of observations among diets, pooled SEMS cannot be given for each measurement.

methionine to soy and to whey reduced ($P < 0.0001$) all three parameters.

Mean serum total and lipoprotein cholesterol levels are shown in *Table 5*. Serum total cholesterol of the casein-fed rats was higher than that of either the soy- or whey-fed groups, but the differences were not significant. Only two lipoprotein classes, namely VLDL and HDL, were observable in serum of all rats in each group. The LDL band was observed in serum of all rats in the methionine-supplemented groups, but in serum of only some rats in the other groups. Cholesterol concentration in the VLDL band was higher ($P < 0.04$) in the casein-fed rats than in the soy-fed group. VLDL cholesterol in the whey-fed rats was only slightly higher than that in the soy-fed rats, but it was not significantly lower than that of the casein-fed group. LDL and HDL cholesterol concentrations were not significantly different among the three protein groups.

The addition of amino acids to the three proteins had no significant effects on serum total or LDL cholesterol. However, addition of cystine to casein resulted in a reduction ($P < 0.05$) of VLDL cholesterol and addition of methionine resulted in increases in HDL cholesterol when it was added to soy ($P < 0.001$) and to whey ($P < 0.0001$) proteins.

During the first week of the study, average daily fecal output was higher ($P < 0.04$) for the soy-diet group than for the casein- and whey-diet groups (*Table 6*). However, during the seventh week, average daily fecal output had increased for the whey-diet group so that it was higher ($P < 0.05$) than that of the soy-diet group. More cholesterol was excreted by the soy-fed rats than by the casein-fed ($P < 0.01$) or whey-fed rats ($P < 0.02$) during the first week. During the seventh week, more cholesterol was excreted by the casein-fed rats ($P < 0.02$) and the whey-fed rats ($P < 0.003$) than by the soy-fed rats. Only one significant effect of amino acid supplementation on fecal output, cholesterol, and coprostanol was observed (i.e., with methionine supplementation). Values for all those parameters were significantly lower when methionine was added to soy. Fecal output and cholesterol, but neither coprostanol nor total neutral steroids (cholesterol + coprostanol), were significantly lower when whey was supplemented with methionine.

Discussion

Effect of protein

Several previous studies[31,32] suggested that dietary protein may influence cholesterol metabolism. In general, low-fat diets with or without cholesterol were used in those studies. Sautier et al.[16] provided evidence that whey protein exerted a hypocholesterolemic effect similar to that of plant proteins (soy and

Table 6 Least-squares mean fecal weight (g/d), cholesterol (g/d), coprostanol (g/d), and total steroids (g/d)

Diet	Wet weight		Cholesterol		Coprostanol		Total steroids*	
	wk 1	wk 7	wk 1	wk 7	wk 1	wk 7	wk 1	wk 7
Casein	1.07^{a}	1.53^{abc}	0.050^{ab}	0.072^{abe}	0.013^{ab}	0.021^{ab}	0.063^{a}	0.093^{ab}
Casein + arg	1.12^{a}	1.52^{abc}	0.052^{ab}	0.078^{ae}	0.015^{a}	0.021^{ab}	0.067^{a}	0.100^{a}
Casein + cys	1.05^{a}	1.35^{b}	0.048^{be}	0.066^{abc}	0.011^{abc}	0.019^{ac}	0.060^{ab}	0.085^{ab}
Soy	1.37^{b}	1.39^{ab}	0.064^{c}	0.057^{c}	0.027^{d}	0.027^{b}	0.091^{c}	0.084^{ab}
Soy + lys	1.37^{bc}	1.53^{abc}	0.064^{c}	0.062^{bc}	0.028^{d}	0.029^{b}	0.092^{c}	0.090^{ab}
Soy + met	0.53^{d}	0.89^{d}	0.021^{d}	0.033^{d}	0.009^{bc}	0.016^{a}	0.030^{d}	0.049^{c}
Whey	1.04^{a}	1.63^{c}	0.051^{ab}	0.081^{e}	0.008^{c}	0.016^{ac}	0.059^{ab}	0.097^{a}
Whey + lys	1.18^{ac}	1.56^{abc}	0.059^{ac}	0.076^{e}	0.008^{c}	0.014^{a}	0.068^{a}	0.090^{ab}
Whey + met	0.82^{e}	1.33^{ab}	0.039^{e}	0.056^{c}	0.010^{bc}	0.022^{bc}	0.049^{b}	0.078^{ab}
SD	0.20	0.24	0.011	0.012	0.004	0.008	0.011	0.017

Note. Values in the same column not sharing any common superscript are significantly different at $P \leq 0.05$. In a few instances, the numbers of rats per diet per measurement were less than eight as a result of two rat deaths and several unavoidable missing measurements.

The last row represents pooled estimate of the standard deviation (SD) within diet and measurement. Because of the unequal numbers of observations among diets, pooled SEMS cannot be given for each measurement.

* Total steroids = cholsterol + coprostanol.

sunflower). Their diets contained no cholesterol and 1% corn oil.

In the present study, 1.2% cholesterol and 14.4% corn oil were included in the diet. No significant differences in serum cholesterol level were found among the casein-, soy-, and whey-protein groups, probably because of the high level of dietary fat. Nagata et al.[9] observed that in a low fat diet, soy protein exerted a marked effect on serum cholesterol level, but the effect became unclear with higher dietary fat (5%). Tanaka et al.[33] showed that rats fed soy protein were hypocholesterolemic even when dietary fat was increased to 5%.

VLDL cholesterol decreased significantly with feeding soy protein and slightly with feeding whey protein as compared to feeding casein. In other studies, HDL cholesterol decreased in rats fed soy, whey, and sunflower proteins[16] and cottonseed protein.[18]

A decrease in liver cholesterol upon feeding a soy protein diet also was reported in other studies.[4,5,19] However, Sautier et al[16] found that a whey protein diet produced a lower amount of liver cholesterol than casein, soy, or sunflower protein diets, whereas in our study, there was no significant difference between the whey and casein diets. Such a discrepancy in the two studies could be related to the incorporation of cholesterol in our diets.

Our findings in regard to fecal steroids for week 7 does not agree with previous reports. Tanaka et al.[33] observed an increase in coprostanol and total neutral steroids in rats fed soy protein compared to those fed casein. Similar observations were made in another study,[16] in which feces of rats fed soy protein contained more neutral steroids than those of rats fed casein, whey, or sunflower proteins. The high level of dietary fat and cholesterol and longer period of fecal collection in our study could be responsible for the differences in observations.

Effect of amino acids

Modification of serum cholesterol level by addition of certain amino acids was reported in some studies,[8,17-19] but not in others.[19,20] In our study, no significant differences were found in serum cholesterol level upon the addition of amino acids. However, we observed a slight increase in serum cholesterol with methionine supplementation of soy protein and a slight decrease with cystine supplementation of casein. These small modifications in serum cholesterol level reflected the significant changes in lipoprotein cholesterol with addition of methionine and cystine. Arginine and lysine supplementation did not have an effect on serum or lipoprotein cholesterol.

In order to simulate the ratio of methionine to cystine of casein, methionine supplementation (4% and 2%, respectively) of both soy and whey protein was high. Feed intake was drastically reduced, especially in the soy-methionine group. Consequently, most of the parameters measured also were lower than those for the other groups, except serum total cholesterol, which was not significantly different. The increase in HDL cholesterol in the methionine-supplemented groups may be explained by increased transport of cholesterol to steroid synthesizing organs.[34,35] This is possible since, in those groups, feed intake was not adequate to maintain the requirement of those organs for cholesterol synthesis. Excess methionine has been reported to increase fat deposition in the liver. However, in our study no such observation was obtained. Excess methionine produced amino acid imbalance and with the inadequate feed intake, amino acids probably were used for energy rather than for fatty acid synthesis.

The only plausible explanation for our results is that at this level of corn oil, cholesterol was redistributed between plasma and other tissues, such as the liver. Grundy and Ahrens[36] reported that the hypocholesterolemic effect of PUFA in human subjects was best explained by the mechanism of redistribution of cholesterol between plasma and tissues.

Conclusions

In summary, although type of dietary protein (casein, soy and whey) in high-fat, cholesterol-containing diets did not have a significant effect on serum total cholesterol or fecal neutral steroids, other cholesterol parameters were affected. As compared to casein protein alone, soy protein decreased both VLDL and liver cholesterol. Supplementation with sulfur-containing amino acids had more effect on cholesterol parameters than did supplementation with arginine and lysine. Methionine supplementation increased HDL cholesterol, whereas cystine decreased VLDL cholesterol.

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