Effects of skim milk, skim milk yogurt, orotic acid, and uric acid on lipid metabolism in rats

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The effects of feeding two milk products (skim milk and skim milk yogurt) and two proposed hypocholesterolemic factors (orotic acid and uric acid) on serum cholesterol (HDL, LDL, total, HDL/Total and HDL/LDL), liver lipids (total liver lipids and liver cholesterol), and aortal cholesterol were studied. Ten groups, of nine rats each, were fed isocaloric Chow-based diets containing water, 45% skim milk (SM), 45% skim milk yogurt (SMY), and 0.0025% orotic acid (OA) or 0.001% uric acid (UA), without or with cholesterol. The SM diet (with cholesterol) resulted not only in lower total cholesterol ($P < 0.10$), LDL cholesterol ($P < 0.05$), aortal cholesterol ($P < 0.01$), and liver cholesterol ($P < 0.10$), but also in increased HDL ($P < 0.05$) and HDL/LDL ($P < 0.10$) cholesterol ratio. The SMY diet, on the other hand, resulted in lowered total serum cholesterol ($P < 0.05$) and aortal cholesterol ($P < 0.01$) and in higher LDL ($P < 0.05$) cholesterol. The hypocholesterolemic effects were more marked for SM than for SMY. Addition of OA and UA to diets increased serum cholesterol, LDL cholesterol, and total liver lipids; the OA diet also increased liver cholesterol. Neither OA nor UA alone was the factor responsible for the hypocholesterolemic effects seen with SM and SMY feeding.

Keywords: Skim milk; skim milk yogurt; orotic acid; uric acid; serum lipids; liver lipids; aortal lipid

Introduction

Total serum cholesterol has been identified as a major risk factor for coronary heart disease. Though many environmental and genetic variables influence serum cholesterol, a good correlation exists between total dietary fat intakes and mean serum cholesterol levels of different populations. Since dairy products make an appreciable contribution to saturated fat and cholesterol content of the diet, whole milk often has been implicated as a coronary health hazard. However, Mann and Spoerry,[1] reported a low serum cholesterol in East African Maasai men, even though their diets consisted chiefly of fermented whole milk (as much as 8 l/man/d). That observation has prompted a series of investigations on the hypocholesterolemic effects of milk. Various dairy products, such as whole milk, fermented milk, skim milk, yogurt, and buttermilk have been reported to lower serum cholesterol in man[2-6] and in experimental animals.[7-16] In contrast, little or no hypocholesterolemic effect of milk products was found in other human[17-20] and animal studies.[21-23]

Even those researchers who have confirmed the presence of a cholesterol-lowering effect are not certain about the responsible factors. Several milk constituents such as orotic acid,[24-29] uric acid,[30,31] calcium,[3,5,12,32] lactose,[33] milk fat globule membrane,[6] and products of bacterial fermentation[10,13] have been suggested.

This study was undertaken to determine the effects of two dairy products (skim milk and skim milk yogurt) and two proposed hypocholesterolemic factors (orotic acid and uric acid) on the cholesterol metabolism in normal and experimentally induced hypercholesterolemic rats. In an effort to ascertain the mechanism(s) responsible for the dairy product effect, serum

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cholesterol (HDL, LDL, total, HDL/Total, and HDL/LDL), liver lipids (total lipids and cholesterol), and aortal cholesterol were determined.

Materials and methods

Animals and diets

Ninety male weanling Sprague Dawley rats (Harlan Industries, Madison, WI) were housed individually in stainless steel metabolic cages in a room with controlled temperature (22°C) and 12-hour light-dark cycle. All the animals were fed a pelletized commercial nonpurified diet (Rodent Laboratory Chow #5001, Purina Mills Inc., St. Louis, MO) for a 7-day adjustment period after arrival. The rats then were divided into 10 groups of nine each, and fed the diets shown in *Table 1*. Water was added to diets A, B, G, H, I, and J, so that all diets would be similar in calorie density (*Table 2*). Cholesterol (0.5%) was added to diets B, D, F, H, and J to experimentally induce hypercholesterolemia in the rats. Food and water were fed ad libitum. Fresh diet was fed, and leftover feed was weighed and discarded on a daily basis. Animals were weighed weekly to ensure that all groups were gaining weight at the same rate.

In order to determine the length of time it took for the hypocholesterolemic effect (if any) to occur, at the end of 3, 5, and 7 weeks, three animals from each group were anesthetized in the fed state using sodium pentabarbital injected intraperitoneally. Blood samples were drawn from the abdominal aorta for determination of serum total, HDL, and LDL cholesterol. Livers were excised and weighted. Aorta pieces (approximately 2 cm) were cut between the diaphragm and bifurcation, freed from adhering connective tissue, and weighed. The serum, liver, and aorta samples were all stored at −20°C until further analysis.

Assays

Determination of serum cholesterol. Total, HDL, and LDL cholesterols were measured using the LDL-Direct-Plus Cholesterol Ratio System (Isolab, Akron, OH). Total cholesterol was determined by adding serum directly to the cholesterol reagent (enzyme re-

Table 1 Diet ingredients (g/100 g)[a]

	Diet groups									
	A	B	C	D	E	F	G	H	I	J
Chow[b]	70.0	69.5	55.0	54.5	55.0	54.5	69.9975	69.4975	69.999	69.499
Water	30.0	30.0					30.0	30.0	30.0	30.0
SM			45.0	45.0						
SMY					45.0	45.0				
OA							0.0025	0.0025		
UA									0.001	0.001
Chol		0.5		0.5		0.5		0.5		0.5

[a] SM, skim milk; SMY, skim milk yogurt; OA, orotic acid; UA, uric acid; chol, cholesterol.
[b] Rodent Laboratory Chow 5001 (Purina Mills Inc., St. Louis, MO): ground extruded corn, soybean meal, dried beet pulp, fish meal, ground oats, brewer's dried yeast, dehydrated alfalfa meal, cane molasses, wheat germ meal, dried whey, meat and bone meal, animal fat preserved with BHA, dicalcium phosphate salt, wheat middlings, calcium carbonate, vitamin B_{12} supplement, DL-methionine, CA pantothenate, choline chloride, folic acid, riboflavin supplement, thiamin, niacin supplement, pyridoxine hydrochloride, ferrous sulfate, vitamin A supplement, D-activated animal sterol, vitamin E supplement, Ca iodate, cobalt carbonate, copper sulfate, zinc sulfate, zinc oxide.

Table 2 Nutrient composition (g/100 g) and calorie density (kcal/100 g) of the experimental diets

	Diets					
	Chow + Water		Chow + SM[a]		Chow + SMY[a]	
Nutrients	70%	30%	55%	45%	55%	45%
Protein	16.4	—	12.9	4.4	12.9	4.5
Fat	3.2	—	2.3	—	2.3	—
Carbohydrate	34.3	—	27.0	6.7	27.0	6.4
Vitamins	0.7	—	0.6	—	0.6	—
Ash	5.1	—	4.0	0.9	4.0	0.9
Fiber	4.1	—	3.2	—	3.2	—
Moisture	7.0	30.0	5.5	32.4	5.5	32.4
Calorie density	232		227		226	

[a] SM, skim milk; SMY, skim milk yogurt.

agent and activator). For HDL and LDL cholesterol, serum was added to an affinity column, and eluted sequentially with alpha reagent for HDL cholesterol and with Beta reagent for LDL cholesterol. The fractions collected were added to cholesterol reagent, mixed, and incubated at 37°C for 5 minutes. Absorbance of the pink chromophore was read at 505 nm.

Determination of total liver lipid and cholesterol. Liver lipids were determined by a modification of the Folch gravimetric method.[34,35] An aliquot of the organic layer was analyzed for total cholesterol by the ferric chloride-sulfuric acid method.[36]

Determination of aortal cholesterol. The aortal sample was soaked in 5 ml of choloroform-methanol (2:1). After 48 hours, the aorta sample was removed, and the extraction solvent was evaporated.[37] The lipid then was resolved in 0.5 ml of isopropanol. Aliquots then were analyzed for total cholesterol by the ferric chloride-sulfuric acid method.[36]

Statistical Analysis. A two-factor fixed-effects statistical model was employed with three rats randomly assigned to each of the 30 diet × period combinations. The ANOVA was run on a SAS GLM procedure,[38] and attention was paid to differences at 0.01, 0.05, and 0.10 levels of significance.

Results

Feed intake, feed efficiency, and weight gains

There were no significant differences among diet groups in feed intake (24 to 27 g) or feed efficiency (0.19 to 0.25). Weight gains of the animals were not significantly different between diets for weeks 1 to 3 and 6 to 7; however, during weeks 4 to 5, a significant decrease in weight gain was observed for groups receiving the OA and the UA diets.

Serum HDL, LDL, Total, HDL/Total, and HDL/LDL cholesterol levels

Since high positive correlations were found between the lipid values obtained after weeks 3, 5, and 7 (*Table 3*), an overall value was calculated by averaging values of all nine rats over the three time periods. The overall mean HDL, LDL, and total cholesterol values are shown in *Table 4*, and the significant differences for various diet comparisons are shown in *Table 5*. Comparing diets without cholesterol addition (A, C, E, G, I) to diets with cholesterol addition (B, D, F, H, J), showed that addition of 0.5% cholesterol produced hypercholesterolemia after 3 weeks of feeding. The overall means showed significant increases in the total and LDL cholesterol levels with addition of 0.5% cholesterol to Chow, SM, SMY, and OA diets ($P < 0.01$). The HDL/Total cholesterol ratio was lowered by the Chow ($P < 0.01$) and UA ($P < 0.10$) diets; whereas the HDL/LDL cholesterol ratio was lowered by the Chow, SM, and SMY diets ($P < 0.01$), with 0.5% added cholesterol.

Table 3 Spearman's rank-order linear correlation for serum HDL, LDL, and total serum cholesterol

Periods	HDL	LDL	Total
Week 3 with week 5	+0.167	+0.672	+0.224
Week 3 with week 7	+0.210	+0.627	+0.297
Week 5 with week 7	+0.148	+0.552	+0.879
Week 3 with overall[a]	+0.382	+0.818	+0.455
Week 5 with overall[a]	+0.445	+0.855	+0.867
Week 7 with overall[a]	+0.818	+0.782	+0.940

[a] Partially spurious being part with whole, but indicates general agreement.

Comparing experimental diets without cholesterol addition (C, E, G, I) to the Chow diet without cholesterol addition (A), it showed that, overall, the SM and SMY diets lowered total serum cholesterol and HDL cholesterol ($P < 0.05$). OA and UA diets, on the other hand, increased total and LDL cholesterol ($P < 0.01$) when compared to the Chow diet. The overall HDL/Total cholesterol ratio was not significantly different when the SM, SMY, OA, and UA groups were compared to the Chow group, but the overall, HDL/LDL ratio was lowered by the SMY ($P < 0.05$), OA, and UA diets ($P < 0.01$).

Comparing diets with cholesterol addition D, F, H, J) to the Chow diet with cholesterol addition (B), overall the SM and SMY diet groups had lower total serum cholesterol values ($P < 0.10$, $P < 0.05$). For SM, this lowered total cholesterol was reflected in lowered LDL cholesterol ($P < 0.05$) and increased HDL cholesterol ($P < 0.05$); whereas for SMY increased serum LDL cholesterol was found ($P < 0.05$). The SM diet also produced a higher ($P < 0.10$) HDL/LDL cholesterol ratio, whereas the SMY diet lowered ($P < 0.10$) the HDL/LDL cholesterol ratio. The OA diet increased the Total, LDL, and HDL cholesterol levels ($P < 0.01$, $P < 0.10$, $P < 0.01$ respectively), but had no significant effect on the HDL/Total and HDL/LDL cholesterol ratios.

Total liver lipid, liver cholesterol, and aortal cholesterol

Addition of 0.5% cholesterol to diets increased liver lipid and liver cholesterol in Chow, SM, SMY, OA, and UA groups ($P < 0.01$) (*Tables 3* and *4*). The aortal cholesterol was also higher with the Chow, SM, SMY, OA, and UA diets ($P < 0.01$) when 0.5% cholesterol was added. The differences for aortal cholesterol were apparent from the third week onward, whereas the liver lipid and liver cholesterol changes took longer to show up, suggesting that hepatic alterations take time to develop.

When SM, SMY, OA, and UA diets without cholesterol (C, E, G, I) were compared to the Chow diet without cholesterol (A), no significant differences were seen in the total liver lipid and liver cholesterol

Table 4 Effects of experimental diets, without or with cholesterol addition, on various biochemical parameters[a,b]

Diets	Serum cholesterol (mg/dl) HDL	LDL	Total	HDL/Total	HDL/LDL	Liver (mg/g) Lipid	Chol[c]	Aorta (mg/g) Chol[c]
				Without cholesterol				
Chow	46.07	22.07	69.83	0.66	2.20	32.50	0.85	2.74
SM	41.64	21.03	66.75	0.62	2.06	33.66	0.72	1.97
SMY	42.23	23.74	65.42	0.65	1.82	32.84	0.81	1.82
OA	46.18	34.37	79.33	0.58	1.38	32.58	0.81	2.27
UA	48.18	33.36	79.97	0.60	1.49	34.04	0.87	2.31
				With cholesterol				
Chow	39.08	35.88	78.71	0.50	1.13	35.56	1.49	3.63
SM	43.98	30.05	74.81	0.57	1.46	35.09	1.07	2.98
SMY	39.30	41.81	74.66	0.53	0.97	37.37	1.51	2.50
OA	44.29	40.49	86.24	0.52	1.28	38.63	2.43	2.87
UA	42.01	34.94	80.89	0.52	1.24	37.79	1.48	2.95

[a] Values are means of nine animals/group. SEM for HDL, LDL, total, HDL/Total, HDL/LDL, liver lipid, liver cholesterol, and aortal cholesterol: 1.35, 1.71, 1.36, 0.035, 0.020, 0.77, 0.16, and 0.12, respectively.
[b] For significant differences between various diet comparisons, see *Table 5*.
[c] Chol: cholesterol.

Table 5 Significant differences for various diet comparisons[a,b]

Diet difference[c]	Serum cholesterol HDL	LDL	Total	H/T[d]	H/L[d]	Liver Lipid	Chol[d]	Aorta Chol[d]
				Diets without vs with cholesterol				
A–B	+**	−**	−**	+**	+**	−**	−**	−**
C–D		−**	−**		+**			−**
E–F		−**	−**		+**	−**	−**	−**
G–H		−*	−**			−**	−**	−**
I–J	+**			+⁺		−**	−**	−**
				Diets without cholesterol				
A–C	+*		+*					+**
A–E	+*		+*		+*			+**
A–G		−**	−**		+**			+**
A–I		−**	−**		+**			+**
C–E								
				Diets with cholesterol				
B–D	−*	+*	+⁺		−⁺		+⁺	+**
B–F		−*	+*		+⁺	−⁺		+**
B–H	−**	−⁺	−**			−**	−**	+**
B–J						−*		+**
D–F	+*	−**			+**	−*	−⁺	+**

[a] **: $P < 0.01$; *: $P < 0.05$; +: $P < 0.10$.
[b] + sign indicates first diet value to be greater than second diet value; − sign indicates first diet value to be less than second diet value.
[c] A: chow; B: chow + cholesterol; C: skim milk; D: skim milk + cholesterol; E: skim milk yogurt; F: skim milk yogurt + cholesterol; G: orotic acid; H: orotic acid + cholesterol; I: uric acid; J: uric acid + cholesterol.
[d] Chol: cholesterol; H/T: HDL/Total; H/L: HDL/LDL.

content. However, aortal cholesterol was lowered by the SM, SMY, OA, and UA diets ($P < 0.01$).

Comparing the SM, SMY, OA, and UA diets with cholesterol (D, F, H, J) to the Chow diet with cholesterol (B), total liver lipid was greater for the SMY ($P < 0.10$), OA, and UA diets ($P < 0.01$). The liver cholesterol was lower in the SM group ($P < 0.10$), but was higher in the OA group ($P < 0.01$). Aortal cholesterol was lower in the SM, SMY, OA, and UA groups ($P < 0.01$).

Discussion

The results indicate both SM and SMY to have a hypocholesterolemic effect. Other researchers[4,9,14] have reported hypocholesterolemia when dairy products were providing 24 to 25% of the caloric intake, whereas in this study the SM and SMY diets were found to exert a hypocholesterolemic effect when the supplementation was reduced to 20% of caloric intake (making consumption more feasible and realistic).

The growth and feed efficiencies of animals in our study fed the SM and SMY diets were not different from those of rats fed the Chow diet. This is in agreement with the reports by other researchers[13,22] who also found dairy products to offer no nutritional advantage for weight gains. However, some workers[10,39] have reported dairy products to give greater weight gains and feed efficiency ratios when compared with controls.

In our study, the hypocholesterolemic effects of SM and SMY were evident after 5 weeks of feeding. After 3 weeks, there were no significant differences when SM and SMY diets were compared to the Chow diet, indicating that this length of time was not sufficient to see any effect in serum cholesterol. However, some workers[3] have reported a cholesterol-lowering effect after 1 week of feeding, whereas others[14] have reported 90 days as the length of time taken to observe hypocholesterolemia.

Addition of 0.5% cholesterol to the Chow, SM, SMY, and OA groups produced hypercholesterolemia after 3 weeks of feeding. This hypercholesterolemia was found to persist even after 7 weeks of feeding, showing that there was no adaptation or negative feedback mechanism taking effect.

Without cholesterol addition, SM (based on the overall analysis of data) was found to significantly lower total cholesterol, HDL cholesterol, and aortal cholesterol. The SMY diet without cholesterol lowered total cholesterol, HDL cholesterol, HDL/LDL cholesterol ratio, and aortal cholesterol. Since the rat chow in our study contained only a negligible amount of cholesterol, the groups that had no cholesterol added to the diets, had no exogenous source of cholesterol. Therefore, the plasma cholesterol of animals in these groups would be expected to be mainly biosynthetic in origin. The major regulator of cholesterol biosynthesis is the conversion of 3-hydroxy-3-methylglutaryl coenzyme-A to mevalonic acid, which is catalyzed by 3-hydroxy-3-methyl-glutaric acid CoA (HMG CoA) reductase. NcNamara et al[40] have shown that rat milk contains a thermostable protein, which depresses the activity of HMG CoA reductase in livers of rats. Boguslawski and Wrobel[27] also found that additions of cow's milk to rat liver homogenate completely inhibited sterol synthesis from acetate to mevalonate. Chawla and Kansal[14] showed that supplementation of diet with cow's milk caused about 50% inhibition of hepatic steroid genesis. Thus, diminution of cholesterol genesis is undoubtedly one of the mechanisms resulting in the hypocholesterolemia observed for the SM and SMY diets without cholesterol addition in our study.

With cholesterol addition, the SM diet resulted in lower total cholesterol, LDL cholesterol, aortal cholesterol, and liver cholesterol; and increased HDL cholesterol, and HDL/LDL cholesterol ratio. The SMY diet, in the presence of cholesterol, resulted in lower total cholesterol, and aortal cholesterol; and in increased LDL cholesterol as compared to the Chow diet. Thus, the SM and SMY diets exerted hypocholesterolemia to a greater extent when provided to hyperlipemic rats (i.e., when SM and SMY were added to diets in the presence of dietary cholesterol the hypocholesterolemic effects of SM and SMY were more marked). This indicates that along with inhibition of endogenous cholesterol biosynthesis, the hypocholesterolemic effect also could be due to an increased excretion of fecal neutral sterols and bile acids.

In our study, not only were the cholesterol-lowering effects more pronounced in the hyperlipemic rats, but also the effects of SM, generally, were greater than those of SMY. SM diets (with cholesterol) not only lowered total, LDL, aortal, and liver cholesterol, but also increased HDL cholesterol and HDL/LDL cholesterol ratio. The SMY diet on the other hand, resulted in lower total cholesterol and aortal cholesterol, and higher LDL cholesterol. The increase in LDL seen in the rats fed the SMY diet versus the lowering of LDL cholesterol in rats fed the SM diet, led to our conclusion that the SM diet was more effective. Thus, our data suggest that the cholesterol-lowering effect is due to the milk component and not due to any chemical modification of milk during the fermentation process of yogurt formation, as reported by other researchers.[4,10,13,14]

Even though OA and UA were added to the diets in amounts similar to those found in SMY,[41] the effects produced by these two organic acids when added alone to the diets were different from those of SM and SMY. OA addition at a level of 0.0025% (with cholesterol) results in increased HDL, LDL, total cholesterol, liver lipids, and liver cholesterol; and lowered aortal cholesterol. Okonkwo and Kinsella[24] reported steatosis and some hepatic hypertrophy in rats fed 0.15% OA in the diets, which increased with progressive increase of OA. Though hepatic hypertrophy and fatty livers were not found in our study, we did see significant increases in liver lipids and liver cholesterol in the OA fed groups. The UA diet without cholesterol resulted in increased LDL and total cholesterol, and decreased aortal cholesterol. These results indicate that neither OA nor UA alone is the factor responsible for producing the hypocholesterolemic effect. However, this does not exclude the possibility of both OA and UA acting in a synergistic way with other milk components to produce the effect.

Thus, we conclude that both SM and SMY produce a hypocholesterolemic effect with the effect being more pronounced under hyperlipemic conditions. This suggests that both SM and SMY tend to have a therapeutic rather than a prophylactic effect. The effect is more marked for SM than for SMY, suggesting that it is due to some component of milk and is not produced and/or enhanced by chemical modification during the fermentation process of yogurt formation. The observed hypocholesterolemic effect is thought to be caused by: (a) depressed cholesterol biosynthesis and (b) increased degradation and excretion of cholesterol

and its metabolites. Neither OA nor UA alone is the factor responsible for this hypocholesterolemic effect, which suggests that it may be caused by the total combination of ingredients in milk or some other single component.

The authors would like to finally add a note on the relevance of these data to humans. This study was carried out in the rat, an animal notoriously resistant to atherosclerosis, and whose lipoproteins are characterized by very low LDL and other atherogenic lipoproteins. Furthermore, the effect of addition of 0.5% cholesterol to the diet of rats is miniscule as compared to other animals. This study confirms those effects and also the effects of skim milk and yogurt on reducing the hypercholesterolemia. Certainly, the major effect in the diets without cholesterol is on the HDL fraction, but it is more complex in the animals fed cholesterol.

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