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Hypocholesterolemic effect of anhydrous milk fat ghee is mediated by increasing the secretion of biliary lipids

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The anhydrous milk fat ghee is one of the important sources of fat in the Indian diet. Our earlier studies showed that rats fed diets containing greater than 2.5 wt% of ghee had lower levels of serum cholesterol compared with rats fed diets containing groundnut oil. To evaluate the mechanism of the hypocholesterolemic effect of ghee, male Wistar rats were fed a diet containing 2.5 or 5.0 wt% ghee for a period of 8 weeks. The diets were made isocaloric with groundnut oil. Both native and ghee heated at 120°C containing oxidized lipids were included in the diet. The ghee in the diet did not affect the 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase activity in the liver microsomes, but it significantly increased biliary excretion of cholesterol, bile acids, uronic acid, and phospholipids. The rats fed ghee had lower levels of cholesterol esters in the serum as well as in the intestinal mucosa. Both native and oxidized ghee influenced cholesterol metabolism. These results indicate that supplementation of diets with ghee lipids would increase the excretion of bile constituents and lower serum cholesterol levels. (J. Nutr. Biochem. 11:69–75, 2000)

Keywords: ghee; saturated fatty acids; bile acids; cholesterol esters; HMG CoA reductase

Introduction

Ghee is the anhydrous milk fat prepared by heating butter at high temperatures. It is an important source of fat in the Indian diet. Ghee is consumed as such along with rice or other food preparations. It is also used for frying. Small quantities of ghee are also used as seasoning spices. Ghee lipids are not only rich in saturated fatty acids, but also contain cholesterol, which is oxidized when it is subjected to heating.[1,2] It is well established that saturated fats and cholesterol in the diet are risk factors for cardiovascular diseases.[3] Epidemiologic studies conducted in many Western nations have shown that the incidence of heart disease among the Indian immigrant population is higher than that among other ethnic populations.[4] Consumption of ghee along with a regular Indian dietary regimen has been attributed to increased the risk of cardiovascular disease in the Indian population.[5] However, this assumption has not been substantiated by any scientific data from either human or animal studies. In the Ayurvedic system of medicine, ghee is used extensively for therapeutic purposes.[6] Ghee has also been used for centuries in Indian diets without any reported adverse effects on health.

In our earlier studies we compared one group of animals fed a diet enriched with groundnut oil [rich in w6 polyunsaturated fatty acids (PUFA)] with another group fed ghee at levels ranging from 2.5 to 10%. We found lower concentrations of serum triglycerides, total cholesterol, and low density lipoprotein (LDL) cholesterol, which indicates hypolipidemic effect in the latter group of animals.[7] However, the mechanism for hypocholesterolemic activity of ghee is not yet clear. It has been postulated that cholesterol homeostasis in the body is maintained by the balance between the amounts of dietary cholesterol absorbed from the intestine, de novo synthesis in the liver, utilization by various tissues, excretion in the bile, and its reutilization by enterohepatic circulation. The synthesis of cholesterol is regulated by the activity of 3-hydroxy-3-methylglutaryl

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coenzyme A (HMG CoA) reductase. Cholesterol is utilized for membrane synthesis and in various metabolic products such as steroid hormones and bile acids by 7α-hydroxylase. The level of cholesterol in serum is also modulated by its clearance by hepatic receptors for LDL and by regulating esterification of cholesterol.[8–10]

Ghee contains cholesterol to an extent of 0.16% and upon heating generates oxysterols to an extent of 17.2% of total sterols present.[7] Oxysterols at high concentrations are known to depress HMG CoA reductase activity.[11] Oxysterols are also poor substrates for esterification reaction and interfere with lymphatic absorption of cholesterol and triglycerides.[12] Therefore, the present investigation was undertaken to study the effect of ghee on some of the important parameters involved in cholesterol metabolism to understand the mechanism by which anhydrous milk fat causes a reduction in cholesterol levels.

Materials and methods

Cholesterol, dipalmitoylphosphatidylcholine, cholic acid, deoxycholic acid, taurocholic acid, taurochenodeoxycholic acid, uronic acid, bovine serum albumin, HMG CoA, and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma Chemical Co. (St. Louis, MO USA). Digitonin was purchased from BDH (Mumbai, India) and Silica gel G from Sisco Research Laboratory (Mumbai, India). Carbazole and urethane were obtained from Aldrich (Milwaukee, WI USA). All other chemicals and solvents used in the experiment were of analytical grade and solvents were distilled before use. Commercial ghee (Nandini®) processed by a state owned milk dairy (Mysore) was purchased from the local market.

Animals and diets

Six week old male Wistar rats ($n = 7$) (OUTB–Wistar, IND-Cft[2c]) weighing 146.0 ± 5.12 g were maintained on AIN-76 diets[13] supplemented with 2.5% or 5% ghee (unheated or oxidized by heating, respectively) for a period of 8 weeks.[7] Food and water were given ad libitum. The unheated ghee contained 0.16% cholesterol, of which 1.0% of total sterols were oxysterols; the corresponding values in oxidized ghee were 0.051% and 17.2%, respectively.

Preparation of ghee with oxidized lipids

Ghee (1 kg) was heated in an electric oven in a stainless steel mug at 120°C for 50 hours to a peroxide value of 25.0 ± 1.0 mEq of oxygen/kg fat.[14]

Diets

The basal composition of the diet used in the study contained casein 20%, sucrose 60%, cellulose 5%, AIN-76 vitamin mixture 1%, AIN-76 mineral mixture 3.5%, choline chloride 0.2%, and methionine 0.3%,[13] and groundnut oil at 10 wt% was used in the control diet as source of fat. Experimental diets were prepared by adding unheated ghee (hereafter referred to as native ghee) or ghee heated at 120°C (referred to as oxidized ghee) at 2.5% or 5.0% levels in the diet and made isocaloric by making up to 10% with groundnut oil. Animals receiving diets containing 10.0% groundnut oil as dietary fat were the control group.

HMG CoA reductase assay

Rats were sacrificed between 9:00 and 10:00 PM by stunning. Liver was taken out, washed, and homogenized in 0.1 M triethanolamine buffer (pH 7.4) containing 0.02 M EDTA and 2 mM dithiothreitol. Liver microsomes were isolated as described by Shapiro and Rodwell[15] and HMG CoA reductase activity was estimated by following the formation of CoA as described by Hulcher and Oleson.[16]

Bile analysis

At the end of the 8-week experimental period, rats were anesthetized with urethane (1.2 g/kg body weight) by intraperitoneal injection. Laparatomy was performed and the common bile duct was cannulated for 3 hours with polyethylene tubing (PE-10; Thomas Scientific Co., Swedesboro, NJ USA) to collect the bile. After measuring the volume of the bile, it was kept frozen until used. All the analyses were completed within 2 weeks of collecting bile.

Bile solids (all solutes present in the bile) were determined by gravimetric method. Briefly, a known volume of bile was dried in a vacuum oven at 70°C in a preweighed planchet and weight of the bile solids after drying bile to a constant weight was noted.[17] Biliary lipids were extracted according to the method of Bligh and Dyer.[18] Chloroform layer was taken for estimation of biliary cholesterol and phospholipids by the methods of Zlatkis and Zak[19] and Stewart,[20] respectively. Uronic acid in methanolic layer was estimated by the method of Dische.[21] Total bile acids in the methanolic layer were estimated by using 3α-hydroxy steroid dehydrogenase as described by Turley and Dietschy.[22] Individual bile acids in the methanolic layer were separated by thin layer chromatography (TLC) using chloroform:methanol:acetic acid: water (65:24:15:9 v/v) and visualized by spraying 10% phosphomolybdic acid in ethanol. Individual bile acids were quantified by densitometry using a Camag TLC scanner (Model No. 111, Camag, Multenz, Switzerland) as described by Sambaiah et al.[17,23] Biliary fatty acids were analyzed by gas chromatography after methylation of fatty acids by BF_3- in methanol.[24]

Analysis of intestinal mucosal cell lipids

After fasting overnight, the rats were sacrificed under ether anesthesia and the small intestine was removed and washed thoroughly with ice-cold saline. The mucosal cells were scraped and lipids were extracted with chloroform:methanol (2:1, v/v) according to the method of Folch et al.[25] The extract was used for the analysis of cholesterol,[19] phospholipids,[20] and fatty acid composition of cholesterol ester fraction.[24] Cholesterol ester content was calculated by subtracting the free cholesterol (estimated by precipitating it with digitonin) from total cholesterol.

Fatty acid analysis

Fatty acid composition of liver microsomal lipids and cholesterol ester fractions of intestinal mucosal cells were analyzed after saponification of lipids by methanolic potassium hydroxide and methylation of fatty acids by BF_3- methanol.[24] The fatty acid methyl esters were analyzed by gas chromatography (Shimadzu 14 B) fitted with fused Silica capillary column (25 m × 0.25 mm inner diameter, Supelco, Bellefonte, PA USA) and flame ionization detector. Individual fatty acids were identified by comparing with the retention times of authentic standards obtained from Nuchek Prep Inc. (Elysian, MN USA). Protein was estimated by the method of Lowry et al.[26]

Table 1 Effect of dietary ghee on cholesterol and HMG CoA reductase activity in rat liver microsomes

Addition of ghee to diet (%)	Cholesterol (μg/mg protein)	HMG CoA reductase (n moles CoA formed/min/mg protein)
0.0	32.5 ± 1.33	0.76 ± 0.05
Native		
2.5	32.4 ± 2.80	0.75 ± 0.04
5.0	33.1 ± 1.89	0.76 ± 0.10
Oxidized		
2.5	32.8 ± 1.28	0.74 ± 0.02
5.0	33.9 ± 1.89	0.72 ± 0.10

Values are mean ± SD; N = 7 rats/group.
HMG CoA–3-hydroxy-3-methylglutaryl enzyme A.

Statistical analysis

Results were statistically analyzed by one-way analysis of variance and Student's *t*-test.[27]

Results

The amount of food consumed (15.4 ± 1.8 g/rat/day) and gain in body weight (193.4 ± 10.3 g/rat) over 8 weeks were comparable in animals fed control diets and those fed different levels of ghee in the diet. The liver weights of animals in different groups were also comparable (3.07 ± 0.04 g/100 g body weight).

HMG CoA reductase activity

HMG CoA reductase is a rate-limiting enzyme in the biosynthesis of cholesterol, the activity of which is modulated by dietary lipids.[8,28] However, neither the cholesterol contents nor the HMG CoA reductase activities were affected when ghee was included as fat supplement in the diets (*Table 1*). Phospholipids (264 ± 5.7 μg/mg protein) also were not affected in liver microsomes after supplementing the diets with incremental amounts of ghee. The overall fatty acid composition of liver microsomes also was not affected significantly except for a decrease in 18:2 levels in animals fed ghee (*Table 2*).

Bile composition

Bile is one of the important routes through which cholesterol is excreted.[28,29] Hence, the effect of ghee in the diet on bile flow and its constituents was evaluated. Ghee in the diet marginally enhanced the volume of bile flow although it was not statistically significant. The cholesterol excretion in bile, however, significantly increased by 18 to 30% when increased amounts of ghee were included in the diet (*Table 3*). The bile solids also increased by 22 to 40% when ghee was added at 2.5% and 5% levels in the diet. Excretion of uronic acid increased by 26 to 47% in the bile under similar conditions. The excretion of total bile acids also increased by 30 to 86% when the level of ghee was increased from 2.5% to 5% in the diet. Most of these increases were reflected in the excretion of taurocholic acid and taurodeoxycholic acids. Although the excretion of taurocholic acid enhanced by 18 to 77%, taurodeoxycholic acid enhanced by 57 to 114%. Glycocholic acid excretion was also enhanced marginally by 15 to 30% in animals fed ghee. These observations indicated that consumption of ghee enhances the excretion of bile components and helps to lower serum cholesterol levels. It should also be mentioned here that although oxidized ghee contained 17-fold higher levels of oxysterols than were found in native ghee, its effect on the excretion of bile constituents was only marginally higher than that observed in animals given native ghee. The phospholipid excretion in bile was enhanced by 25 to 82% when ghee was included in the diet. Dietary ghee marginally elevated protein content in bile. The 18:2 and 20:4 levels in biliary phospholipids decreased when ghee was included in the diet (*Table 4*).

Effect of ghee on cholesterol and cholesterol ester levels in serum and intestinal mucosa

The endogenous levels of cholesterol esters in serum give an indication of the atherogenic potentials of dietary lipids.[30] The cholesterol ester level in the plasma is an indication of the combined activity of lecithin:cholesterol acyltransferase (LCAT) and acyl CoA:cholesterol acyl transferase (ACAT) activities, whereas that of intestinal mucosa gives an indication of ACAT activity.[31]

Table 2 Effect of dietary ghee on fatty acid composition of rat liver microsomes

Fatty acid (%)	Control	Addition of ghee in the diet (%)			
		Native		Oxidized	
		2.5	5.0	2.5	5.0
14:0	0.50 ± 0.16	0.69 ± 0.15	0.70 ± 0.16	0.60 ± 0.07	0.61 ± 0.18
16:0	23.70 ± 2.5	24.10 ± 1.04	25.60 ± 1.76	25.40 ± 0.64	26.10 ± 0.8
16:1	0.98 ± 0.27	0.69 ± 0.18	0.560 ± 0.26	0.70 ± 0.08	0.87 ± 0.18
18:0	22.60 ± 2.7	23.90 ± 1.12	26.10 ± 2.5	23.00 ± 0.98	25.00 ± 1.54
18:1	13.50 ± 1.89	12.80 ± 0.81	12.80 ± 1.76	13.40 ± 1.16	14.00 ± 0.69
18:2	12.80^{a} ± 0.76	11.10^{b} ± 0.52	09.90^{b} ± 1.2	11.00^{b} ± 0.73	9.30^{bc} ± 0.67
20:4	26.10 ± 1.5	26.80 ± 1.7	24.10 ± 0.89	25.40 ± 1.5	23.80 ± 1.34

Values are mean ± SD; N = 7 rats/group.
Values with different superscripts in a row are significantly different from one another at $P < 0.05$.

Table 3 Influence of dietary ghee on bile secretion and bile composition in rats

Parameters	Control	Addition of ghee to the diet (%)			
		Native		Oxidized	
		2.5	5.0	2.5	5.0
Bile flow (ml/h)	0.518 ± 0.082	0.528 ± 0.060	0.530 ± 0.054	0.558 ± 0.071	0.589 ± 0.094
Cholesterol (μ moles/h)	0.102^{a} ± 0.026	0.120^{b} ± 0.016	0.125^{b} ± 0.026	0.130^{bc} ± 0.026	0.133^{bc} ± 0.020
Bile solids (g %)	2.86^{a} ± 0.44	3.48^{b} ± 0.24	4.02^{bc} ± 0.36	3.49^{bd} ± 0.29	3.89^{bcd} ± 0.23
Uronic acids (μ moles/h)	3.26^{a} ± 0.67	4.11^{a} ± 0.64	4.37^{b} ± 0.76	4.37^{a} ± 0.76	4.79^{b} ± 0.61
Total bile acids (μ moles/h)	5.87^{a} ± 0.98	7.61^{b} ± 0.61	10.10^{c} ± 1.10	8.44^{d} ± 0.78	10.90^{c} ± 1.40
Taurocholic acid (μ moles/h)	2.79^{a} ± 0.41	3.24^{b} ± 0.21	4.43^{c} ± 0.14	3.52^{d} ± 0.44	4.95^{c} ± 0.28
Taurodeoxycholic acid (μ moles/h)	1.27^{a} ± 0.34	2.00^{b} ± 0.18	2.60^{c} ± 0.12	2.24^{d} ± 0.06	2.72^{c} ± 0.45
Glycocholic acid (μ moles/h)	0.534^{a} ± 0.20	0.612^{b} ± 0.26	0.683^{bc} ± 0.31	0.580^{abd} ± 0.14	0.695^{bce} ± 0.09
Phospholipids (μ moles/h)	0.741^{a} ± 0.169	0.920^{a} ± 0.258	1.270^{ba} ± 0.209	0.995^{ba} ± 0.243	1.348^{bcd} ± 0.337
Protein (mg/h)	3.02^{a} ± 0.41	3.30^{b} ± 0.38	3.11^{ac} ± 0.46	3.21^{abc} ± 0.45	3.70^{bcde} ± 0.56

Values are mean ± SD; N = 7 rats/group.
Values with different superscript in a row are significantly different from one another at $P < 0.05$.

Ghee at 2.5% and 5% levels in the diet significantly decreased the total cholesterol levels in the serum (*Table 5*). Serum cholesterol contents in rats fed native ghee at the 2.5% level decreased by 10% whereas in animals fed heated ghee, the decrease was 13%. At the 5% level, the native ghee decreased serum cholesterol levels by 16% whereas the heated ghee lowered it by 25% compared with that in animals fed groundnut oil. All the decreases observed in total cholesterol levels reflected on the cholesterol ester fraction (*Table 5*). Free cholesterol levels, however, remained unaffected as a result of inclusion of ghee in the diet.

Similarly, native ghee at 2.5% and 5% levels in the diet decreased total cholesterol levels moderately by 7% and 13% in the intestinal mucosal cells (*Table 5*). The inclusion of oxidized ghee at 2.5% and 5% levels in the diet decreased total cholesterol levels by 11% and 14% compared with those in animals fed groundnut oil. Inclusion of oxidized ghee in the diet, however, enhanced free cholesterol significantly, but decreased cholesterol ester fractions in mucosal cells, indicating that esterification process of cholesterol in the intestine was inhibited by ghee lipids, particularly those found in heated samples.

The decrease in the cholesterol ester formation in the serum and intestinal mucosa could have been caused by (1) a decrease in the availability of cholesterol as an acceptor, (2) a decrease in the availability of fatty acids such as 16:0, 18:2, or 20:4, which normally are utilized for esterification of cholesterol, or (3) the presence of oxysterols, which are poor substrates for the esterification reaction because of the presence of the hindering hydroxy, keto, and epoxy groups. The mean cholesterol level in the serum was 62.1 mg/dL in control animals, whereas it was 52.2 mg/dL (combined mean from *Table 5*) in ghee fed animals. Similarly, the mean cholesterol content in intestinal mucosal cells was 79.8 μg/mg protein in control animals, whereas it was 69.5 μg/mg protein (combined mean from *Table 5*) in ghee fed animals. Hence, total cholesterol was available in adequate amounts both in serum and in mucosal cells for esterification process. Similarly, sufficient amounts of fatty acids (16:0 and 18:2) that are normally utilized for cholesterol esterification (with the exception of arachidonic acid) were available in the diet (*Table 6*). There was no deficiency of major fatty acids found in cholesterol ester fractions of serum and intestinal mucosal cells when control and ghee fed animals were compared. These observations indicate that there was no limitation on the availability of substrates for cholesterol esterification process in all the groups of animals. It should, however, be pointed out that the levels of cholesterol ester fractions observed in animals fed oxidized ghee were lower than those found in animals fed native ghee. Whether the higher levels of oxysterols found in oxidized ghee have any role in influencing the activity of enzyme that esterifies cholesterol is not clear. In the present study, however, no appreciable elevation in the levels of oxysterols in liver was observed when ghee was fed at 2.5% and 5.0% levels.[7]

Table 4 Essential fatty acid composition of biliary phospholipids of rats fed groundnut oil and ghee

Fatty acid (%)	Control	Addition of ghee to the diet (%)			
		Native		Oxidized	
		2.5	5.0	2.5	5.0
18:2	19.70^{a} ± 0.90	17.70^{b} ± 1.10	12.90^{bc} ± 2.40	17.00^{bd} ± 2.40	13.40^{bce} ± 2.30
20:4	22.70^{a} ± 2.40	19.20^{b} ± 1.50	17.10^{bc} ± 2.10	20.00^{bd} ± 1.80	18.60^{bde} ± 1.70

Values are mean ± SD; N = 7 rats/group.
Values with different superscript in a row are significantly different from one another at $P < 0.05$.

Table 5 Influence of dietary ghee on cholesterol levels in serum and intestinal mucosa

Parameters analyzed	Control	Addition of ghee to the diet (%)			
		Native		Oxidized	
		2.5	5.0	2.5	5.0
I. Serum					
Total cholesterol (mg/dL)	$62.1^{a} \pm 2.8$	$56.2^{b} \pm 3.3$	$52.2^{c} \pm 2.1$	$54.1^{bd} \pm 2.5$	$46.3^{e} \pm 1.9$
Free cholesterol (mg/dL)	12.9 ± 0.81	11.3 ± 2.5	10.8 ± 0.98	12.8 ± 1.3	12.6 ± 1.25
Cholesterol ester (mg/dL)	$49.2^{a} \pm 3.10$	$44.9^{b} \pm 2.3$	$41.4^{bc} \pm 2.50$	$41.3^{bc} \pm 2.10$	$33.7^{bcd} \pm 2.12$
II. Intestinal mucosa					
Total cholesterol (μg/mg protein)	$79.8^{a} \pm 3.8$	$74.3^{b} \pm 4.1$	$69.5^{c} \pm 4.2$	$71.3^{d} \pm 2.5$	$68.3^{e} \pm 3.1$
Free cholesterol (μg/mg protein)	$32.8^{a} \pm 2.1$	$34.3^{b} \pm 2.4$	$33.8^{ab} \pm 1.9$	$38.3^{bcd} \pm 1.84$	$41.3^{bcd} \pm 1.45$
Cholesterol esters (μg/mg protein)	$47.8^{a} \pm 2.4$	$40.0^{b} \pm 1.8$	$35.7^{c} \pm 1.56$	$33.0^{d} \pm 2.6$	$27.0^{e} \pm 1.71$

Values are mean ± SD; N = 7 rats/group.
Values with different superscript in a row are significantly different from one another at $P < 0.05$.

Discussion

The goal of the present study was to explore the mechanism of action of ghee on the hypocholesterolemic effect in experimental animals. One of the important factors that regulates cholesterol level in the body is its intake from the diet and efficacy of its absorption. It has been reported that rats absorb 50 to 80% of dietary cholesterol. Humans absorb 45 to 56% of dietary cholesterol.[32–34] The absorption rate depends on the presence of bile salts. However, there is some controversy regarding the influence of accompanying dietary lipids on the absorption of cholesterol. It has been reported that dietary unsaturated fatty acids reduce the intestinal absorption of cholesterol in monkeys.[35] However, Berr et al.,[36] did not find any differences in the fractional absorption of cholesterol in hamsters when the intake of cholesterol was low. In the present study, rats consumed 0.6 mg or 1.2 mg cholesterol per day from native ghee or 0.19 mg or 0.38 mg cholesterol per day from oxidized ghee when these two types of ghee were included in the diet at levels of 2.5% and 5%, respectively. This was much less than the intake of dietary cholesterol by hamsters in a study where nature of dietary lipid did not influence absorption of cholesterol.[36] Hence, it is unlikely that ghee lipids would interfere with the absorption of dietary cholesterol and lower serum cholesterol levels.

Liver is the primary site for the biosynthesis of cholesterol in which HMG CoA reductase plays an important role as the rate-limiting regulatory enzyme.[37] This enzyme is down-regulated by cholesterol level in the diet.[29,38] The oxysterols and PUFA are also known to inhibit HMG CoA reductase.[11,39] Even though heated ghee contained 27 mg cholesterol oxides/100 g (17.1% of total sterols as oxides),

Table 6 Fatty acids of dietary lipids and cholesterol ester fractions in serum and intestinal mucosa

Fatty acids	Control GNO 10%	Diet with native ghee		Diet with oxidized ghee	
		2.5%	5.0%	2.5%	5.0%
Ghee					
10:0	ND	ND	0.5	ND	0.5
12:0	ND	0.7	0.9	0.67	1.1
14:0	0.5	2.9	4.5	3.1	4.9
16:0	13.5	19.7	24.2	20.4	24.4
18:1	44.6	41.4	38.1	40.8	38.5
18:2	36.5	26.7	19.9	26.4	19.3
Cholesterol ester fractions					
Serum					
14:0	1.61 ± 0.27	1.7 ± 0.42	2.5 ± 0.78	1.89 ± 0.65	3.2 ± 0.91
16:0	14.2 ± 0.9	12.8 ± 1.2	13.3 ± 1.1	13.2 ± 1.08	14.1 ± 0.56
18:1	$10.7^{a} \pm 0.42$	$12.2^{b} \pm 1.6$	$14.6^{bc} \pm 1.89$	$10.4^{acd} \pm 1.3$	$13.9^{bce} \pm 1.8$
18:2	$12.7^{a} \pm 0.53$	$12.0^{a} \pm 1.64$	$9.5^{b} \pm 0.71$	$11.8^{bc} \pm 1.5$	$8.4^{bcd} \pm 1.1$
20:4	50.8 ± 0.33	49.6 ± 1.89	43.7 ± 1.51	49.1 ± 2.1	47.8 ± 2.1
Intestinal mucosa					
14:0	1.98 ± 0.06	1.95 ± 0.76	2.81 ± 0.65	1.56 ± 0.41	3.0 ± 0.71
16:0	$20.1^{a} \pm 2.9$	$22.3^{b} \pm 4.1$	$23.3^{bc} \pm 0.89$	$23.4^{bc} \pm 3.1$	$26.0^{bcde} \pm 1.81$
18:1	$43.8^{a} \pm 2.9$	$41.2^{b} \pm 2.1$	$39.0^{c} \pm 4.4$	$37.6^{d} \pm 2.9$	$33.0^{e} \pm 3.89$
18:2	10.2 ± 2.31	10.0 ± 1.83	9.89 ± 2.1	10.8 ± 2.1	11.1 ± 0.92
20:4	8.4 ± 0.91	8.9 ± 1.91	9.9 ± 0.74	9.0 ± 0.87	10.3 ± 1.53

Values are mean ± SD; N = 7 rats/group.
Values with different superscript in a row are significantly different from one another at $P < 0.05$.
ND–not detected.

it did not affect HMG CoA reductase activity compared with that found in animals fed groundnut oil and those fed native ghee, which contained less than 1% of cholesterol oxides. Even though ghee containing diets (at 2.5% and 5% levels) had 27% and 45% less linoleic acid compared with that of groundnut oil diets, it did not drastically alter essential fatty acid levels in liver microsomes, indicating that changes in dietary PUFA and cholesterol oxides did not influence HMG CoA reductase activity in liver microsomes.

Bile is an important vehicle for the excretion of cholesterol and its metabolites.[40,41] Even though the volume of the bile secreted did not increase significantly upon feeding ghee containing diets, it did enhance the excretion of cholesterol, phospholipids, bile solids, uronic acid, total bile acids, taurocholic acid, and taurodeoxycholic acids. Melchior et al.[42] found that saturated fats such as butter fat and coconut oil enhanced bile acid pools in squirrel monkeys. Similarly, Cheema et al.[43] showed that saturated fats such as beef tallow significantly increased bile acid, cholesterol, and phospholipid output in mice compared with these levels in animals fed unsaturated lipids. Oshima et al.[44] also observed an increased secretion of phospholipids and cholesterol in the bile of hamsters fed saturated fats such as butter fat. These studies indicated that saturated fats may enhance bile flow and increase the excretion of bile components. The results of the present study are in agreement with these findings. It is also interesting to note that squirrel monkeys fed butterfat and cholesterol developed less cholelithiasis than those fed cholesterol with PUFA containing oils such as safflower oil.[42] In contrast to these studies, Ramesha et al.[28] reported an increase of bile acid secretion when rats were fed diet containing a PUFA. Similarly Berr et al.[36] also reported an increase in the hepatic excretion of cholesterol when hamsters were fed a diet enriched in n-6 PUFA compared with those fed a diet containing saturated fat. Interestingly, they also observed that biliary excretion decreased in animals fed a n-3 PUFA enriched diet. In addition, the basal outputs of bile and bile salt levels were comparable in animals fed saturated fat and those on a n-6 PUFA diet. However, the rate of synthesis for cholic acid in animals fed saturated fat or n-3 PUFA containing diet was comparable. Further, the dietary fatty acids also differentially changed the fatty acid composition of biliary phospholipids and the secretory ratio of cholesterol, phospholipids, and bile acids in bile, indicating a complex interactive influence of dietary lipids on the excretion of cholesterol and biliary constituents, which were differentially affected by dietary lipids.

Cholesterol esters are important constituents of serum lipoproteins and are implicated in the process of atherogenesis.[45] Cholesterol ester formation is mediated by two important groups of enzymes, LCAT and ACAT.[31,46] The LCAT enzyme plays an important role in the formation of cholesterol esters in serum whereas ACAT plays a similar role in intestinal mucosa.[31] Even though ghee in the diet significantly reduced the total serum cholesterol levels, it was mostly reflected in the cholesterol ester fraction. This might have been caused by limiting the availability of the substrates or by inhibiting the enzyme activities responsible for esterification of cholesterol. The mean level of free cholesterol in serum of control animals was 12.9 $\pm$ 0.81 mg/dL whereas that in animals fed ghee was 11.88 $\pm$ 0.98 mg/dL (combined mean $\pm$ SD of all animals on ghee diet). Similarly, the amount of free cholesterol in the intestinal mucosal cells of control animals was 32.8 $\pm$ 2.1 μg/mg protein whereas that in animals fed ghee diets was 36.93 $\pm$ 3.54 μg/mg protein (combined mean $\pm$ SD from all animals on ghee diets). Although 79.2% of the total cholesterol available was esterified in serum of control animals, 77.1% of total cholesterol available in ghee fed animals was esterified. These studies indicated that the esterification process per se in serum was not severely hampered in ghee fed animals and that further ghee lipids might not be exerting direct influence on LCAT activity in serum. This is in contrast to the results of Baudet and Jacotot,[47] who observed lower LCAT activity in human volunteers consuming milk fats compared with that found in subjects given ω6 PUFA rich oils such as sunflower oil or peanut oil. However, it should be pointed out that, unlike in the present study, the milk fat used in the studies of Baudet and Jacotot was not supplemented with essential fatty acids. In the case of intestinal mucosa, 59.9% of total cholesterol was esterified in control animals whereas 47.7% of total cholesterol available was esterified in ghee fed animals. Lowering of the cholesterol ester fraction in the intestinal mucosa in ghee fed animals indicated that ghee lipids might have inhibited ACAT activity. The intestinal ACAT activity is regulated by many factors.[45] The effect of dietary lipids on ACAT activity is equivocal. A diet containing 10% corn oil did not change ACAT activity in rats. However, a diet containing 20% sunflower oil enhanced the enzyme activity. Unsaturated fat in diet also increased ACAT activity in rabbits and rats.[45,48] Because the diets containing ghee had higher levels of saturated fatty acids than those in groundnut oil diets, it is likely that the ACAT activity was affected by ghee, resulting in lower esterification reactions. It might be possible that intestinal mucosal cells would come in direct contact with cholesterol oxides present in ghee, which is a poor substrate for esterification process would affect ACAT activity.

In conclusion, the present study indicated that the ghee in the diet of rats exhibited hypocholesterolemic effects by enhancing the secretion of biliary constituents. It also reduced the levels of cholesterol ester fractions in serum as well as in intestine. However, the activity of key biosynthetic enzyme HMG CoA reductase was not affected by ghee. However, caution should be exercised when extrapolating these mechanistic data to humans because cholesterol metabolism in the rat differs significantly from that in humans. However, a recent study from our laboratory indicated a similar hypocholesterolemic effect of ghee in humans who were given incremental doses of ghee for a period of 7 days.[49] Whether a similar mechanism as has been observed in rats operates for hypocholesterolemic effect of ghee has yet to be determined.

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