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Effect of dietary ghee—the anhydrous milk fat, on blood and liver lipids in rats

Matam Vijaya Kumar, Kari Sambaiah, and Belur R. Lokesh

Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore, India

Dairy products are important sources of dietary fat in India. Anhydrous milk fat, viz., ghee, is consumed as such in the diet and also is used for frying the dishes. Ghee contains high levels of saturated fatty acids and cholesterol, which are considered risk factors for cardiovascular diseases. In the present study, ghee, at levels ranging from 0.25 to 10%, was included in a nutritionally balanced AIN-76 diet fed to Wistar rats for a period of 8 weeks. The serum lipid profiles of these animals showed a dose dependent decrease in total cholesterol, low density lipoproteins and very low density lipoproteins cholesterol, and triglyceride levels when ghee was present at levels greater than 2.5% in the diet. Liver cholesterol and triglycerides also were decreased in these animals. When ghee was included as a sole source of fat at a 10% level, polyunsaturated fatty acids in the serum and liver lipids were reduced significantly. Similar results were observed when ghee was subjected to a higher temperature (120°C) to generate cholesterol oxidation products and fed to the animals. Although cholesterol oxidation products were not accumulated in serum, significant amounts were accumulated in liver only when ghee was fed as a sole source of fat at a 10% level. This study revealed that the consumption of ghee up to a 10% level in the diet altered blood lipid profiles in such a manner as not to elevate the risk factors for cardiovascular diseases.

Keywords: ghee; saturated fatty acids; cholesterol oxidation products (COPS); blood lipids; liver lipids; essential fatty acids

Introduction

Laboratory studies with experimental animals and epidemiologic data on human populations have shown that dietary saturated fatty acids have atherogenic influence compared with unsaturated fatty acids.[1] Ghee, the anhydrous milk fat, is one of the important dietary fats consumed in India and other southeast Asian countries.[2,3] Ghee lipids contain not only saturated fatty acids to an extent of approximately 60% of total fatty acids but also contain approximately 0.15 to 0.30% cholesterol.[4] Cholesterol in food materials undergoes oxidation during processing, resulting in the formation of cholesterol oxidation products (COPS), which are reported to be atherogenic, mutagenic, cytotoxic, and angiotoxic.[5] Unlike other oxidized lipids, COPS are readily absorbed from the gut.[6] Some of the COPS are much more atherogenic than cholesterol itself.[7]

Recent epidemiologic studies have revealed that the incidence of coronary heart disease (CHD) is higher in immigrants of Indian origin in several countries compared with other ethnic populations settled in these countries. Although no definitive cause was attributed to this observation, it was suspected that dietary habits might play a role in rendering this group vulnerable to CHD.[8] The saturated fatty acids are known to enhance serum cholesterol levels. Indians, in general, consume saturated fats such as ghee, which also contain cholesterol. Normally, ghee is melted before it is served. It also is used for frying dishes. During these processes, the thermal and free radical reactions oxidize cholesterol, generating COPS.[5] Jacobson[8] suggested that these dietary COPS might be responsible for the higher incidence of CHD observed in Indian populations.

However, in the indigenous Ayurvedic system of medicine, ghee is used in the preparation of a number of formulations for treating allergy, skin, and respiratory diseases and is considered to induce many beneficial effects on human health.[9] In the United States, anhydrous milk fat without any non-fat solids was patented as hypoallergic butter.[10] Aneja and Murthy showed that milk fat is a source of conjugated linoleic acid[11] that has anticarcinogenic[12] and

Address correspondence to Dr. B.R. Lokesh, Department of Biochemistry and Nutrition, Central Food Technological Research Institute, Mysore-570 013, India
Received March 17, 1998; accepted September 29, 1998.

655 Avenue of the Americas, New York, NY 10010
0955-2863/99/$–see front matter
PII S0955-2863(98)00088-6

antiatherogenic properties.[13] Cope et al.[14] showed that butter and clarified butter oil protected hairless mice Skh-HR-1 from ultravoilet radiation-induced suppression of contact hypersensitivity. According to Ganguly,[15] milk fat is a good source of oleic acid, which can protect low density lipoproteins (LDL) from oxidation[16] and may prevent the initiation of atherosclerosis. Although milk fat is high in saturated fatty acids, it is argued that 59% of these total fatty acids do not have any hypercholesterolemic effect and contain antiatherogenic and anticarcinogenic compounds.[17–20] Milk fat is also a source of short chain fatty acids, which play an important role in the maintenance of mucosal integrity.[21] The short chain and medium chain fatty acids of milk fat are more readily absorbed, digested, and oxidized by carnitine independent pathway without hindering the formation of chylomicrons.[15,19]

In view of these controversial reports on both beneficial and deleterious effects of milk fat and their possible implications on health, the present investigation was undertaken to study the effects of anhydrous milk fat (ghee) on serum lipid profiles that have a bearing on cardiovascular function.

Materials and methods

Materials

Cholesterol, 5-α-cholestane, 7-β-hydroxycholesterol, cholesterol epoxide, cholestane triol, 7-ketocholesterol, 3-keto5-cholestene, 25-hydroxycholesterol, 20-α-hydroxycholesterol, dimethylformamide, trimethylchlorosilane, hexamethyldisilane, dipalmitoylphosphatidylcholine, triolein, thiobarbituric acid, vitamin E, and vitamin A were purchased from Sigma (St. Louis, MO USA). Heparin and manganese chloride were obtained from Sisco Research Laboratory (Bombay, India). Digitonin was purchased from BDH Chemicals (Bombay, India). All solvents used were of analytical grade and were distilled prior to use. Commercial ghee (brand name Nandini) prepared by the state-owned milk dairy in the State of Karnataka, India, was purchased from the local market. Preliminary studies also were conducted with ghee prepared from butter by the conventional method.[22] Ghee samples prepared in six different households were analyzed for their proximate composition.

Preparation and analysis of COPS in ghee

Ghee (25 g) was heated in a stainless steel mug in an electric oven at 120°C until its peroxide value reached approximately 25 mEq of oxygen (O_2)/kg fat.[23] The COPS from 0.5 g ghee were extracted after saponification for 18 hours at room temperature with 1 N methanolic KOH (10 mL). The unsaponifiable fraction was extracted with 10 mL hexane (three times and fractions pooled). The hexane layer was washed three times with 5 mL of 0.5 N methanolic KOH and 10 mL of water until it became free of alkali. The cholesterol and oxidized products were derivatized to trimethyl silylethers according to the Association of Official Analytical Chemists (AOAC) method.[24] They were separated and quantitated by capillary gas chromatography using DB-5 column (30 m × 0.32 mm; J&W Scientific, Folsom, CA USA). The gas chromatograph (GC) (Shimadzu model 14B) was fitted with flame ionization detector (FID) and connected to an online CR-4A chromatopac integrator. 5-α-Cholestane (10 μg) was used as an internal standard.

Analysis of vitamins E and A

Levels of vitamins E and A in ghee samples were quantitated by high performance liquid chromatography (HPLC) using cyanopropyl column as described by Rushing et al.[25] Conjugated linoleic acid was measured spectrophotometrically following AOAC procedure.[26]

Experimental animals

Male Wistar rats [OUTB-Wistar, IND-cft (2c)] 60.0 ±3.0 g were grouped by random distribution ($N = 8$ animals per group). They were placed in individual cages in an approved animal house facility and fed fresh diets daily. The animals had free access to food and water throughout the study.

Diet composition

The ingredients used in the basal diets were (g/100 g): casein 20; cellulose 5; sucrose 60; AIN 76 mineral mix 3.5; AIN 76 vitamin mix 1; methionine 0.3; choline chloride 0.2; and fat 10.[27] Ghee (unheated or heated) was incorporated into the diet at 0.25 to 10% levels replacing the equivalent amount of groundnut oil used to make the diet isocaloric. Food intake was monitored daily. After 8 weeks, rats were fasted overnight and sacrificed under ether anesthesia. Blood was drawn by cardiac puncture and serum was separated by centrifugation. Liver was removed, rinsed in ice-cold saline, blotted, weighed, and stored at −20°C until analyzed. Feces were collected during last 6 days of the experiment, dried, and stored for analysis.

Analysis of lipids

Lipids were extracted from serum and tissues by the method of Folch et al.[28] Serum lipid peroxides and liver thiobarbituric acid reactive substances (TBARS) were analyzed as described by Yagi[29] and Berg and Aust,[30] respectively.

Serum and liver cholesterol levels were quantitated by the method of Searcy and Bergquist.[31] High density lipoprotein (HDL) cholesterol was measured after precipitating apo B containing lipoproteins with heparin-$MnCl_2$ reagent.[32] Free cholesterol in serum and liver was estimated as per the above method after precipitating the free cholesterol with 0.5% digitonin.[33]

Phospholipids were analyzed by the method of Stewart[34] using dipalmitoylphosphatidylcholine as reference standard. Triglycerides were estimated by the method of Fletcher.[35] Total lipids were estimated by the gravimetric method.

The total fatty acids from serum and tissue lipids were analyzed as methyl esters[36] by capillary GC (Shimadzu 14B, fitted with FID) using fused silica capillary column 25 m × 0.25 mm (Parmabond FFAP-DF-0.25; Macherey-Nagel GmBH Co., Düren, West Germany). The operating conditions were column temperature 160°C, injector temperature 210°C, and detector temperature 250°C; column temperature was programmed to rise 6°C/min and final temperature was 240°C. Nitrogen gas (30 mL/min) was used as carrier. Individual fatty acids were identified by comparing retention times of authentic standards and were quantitated by an online chromatopac CR-6A integrator.

Lipids from feces were extracted by the method of Folch et al.[28] and quantitated by gravimetric method. COPS from serum and liver were analyzed as described earlier.

Statistical analysis

Results were analyzed by Student's *t*-test.[37]

Table 1 Proximate composition of homemade and commercial ghee samples

	Homemade	Commercial
Triglycerides (g/100 g)	97.8 ± 1.08	98.6 ± 0.07
Cholesterol (mg/100 g)	178.6 ± 7.2	161.7 ± 5.4
Phospholipids (mg/100 g)	28.6 ± 3.2	10.4 ± 1.8[a]
Vitamin E (μg/100 g)	305 ± 6.2	491 ± 21[a]
Vitamin A (μg/100 g)	447 ± 39	584 ± 18[a]
Conjugated linoleic acid (%)	0.95 ± 0.18	0.98 ± 0.06
Fatty acid composition (wt %)		
10:0	1.8	1.3
12:0	2.1	2.6
14:0	13.8	14.3
16:0	33.0	32.8
16:1	3.1	3.0
18:0	11.8	12.0
18:1	30.2	30.0
18:2	2.1	2.6

Values are mean ±SD of six samples.
[a]$P < 0.001$.

Results

Proximate composition of ghee: Homemade and commercial

Triglycerides are the major components of ghee (*Table 1*), which accounted for 98 to 99% of the total lipids. Ghee from commercial sources had marginally lesser amounts (9%) of cholesterol compared with those of homemade samples. Vitamins E and A were higher by 61% and 31%, respectively, in commercial ghee samples compared with homemade ghee samples. Both commercial and homemade ghee samples contained 0.95 to 0.98% conjugated linoleic acid. The commercial samples of ghee contained 64% fewer phospholipids than those that were homemade. The fatty acid composition of ghee lipids revealed high levels of palmitic and oleic acids, followed by myristic and stearic acids. Ghee had very little linoleic acid (2.1–2.6%). However, there was no significant difference in the overall fatty acid composition of homemade and commercial ghee samples. Because the overall composition of commercial and homemade ghee samples were comparable, further studies were carried out with commercial ghee samples only, which is used by the majority of households, particularly in urban areas.

Effect of heating on the composition of ghee

Ghee is normally melted before serving and also is used as a frying medium to prepare dishes. Accordingly, ghee samples were heated at 120°C for different intervals of time followed by estimating peroxide value and cholesterol levels (*Figure 1*). Peroxide value of the sample increased from an initial value of 2.73 to a value of 26.7 mEq O_2/kg by 50 hours (*Figure 1A*). However, heating of ghee samples for 50 hours decreased the cholesterol content to an overall loss of 66% (*Figure 1B*). Further analysis of heated samples indicated that there was no appreciable change in total triglyceride levels and the overall fatty acid composition of total lipids. However, the phospholipid content was decreased by 40%, and a marginal drop of 10% in vitamin E and vitamin A levels was observed in heated samples. There was no change in the conjugated linoleic acid content (*Table 2*). Thus, these studies indicated that there was a thermal degradation/oxidation of cholesterol and loss of phospholipids in ghee upon heating.

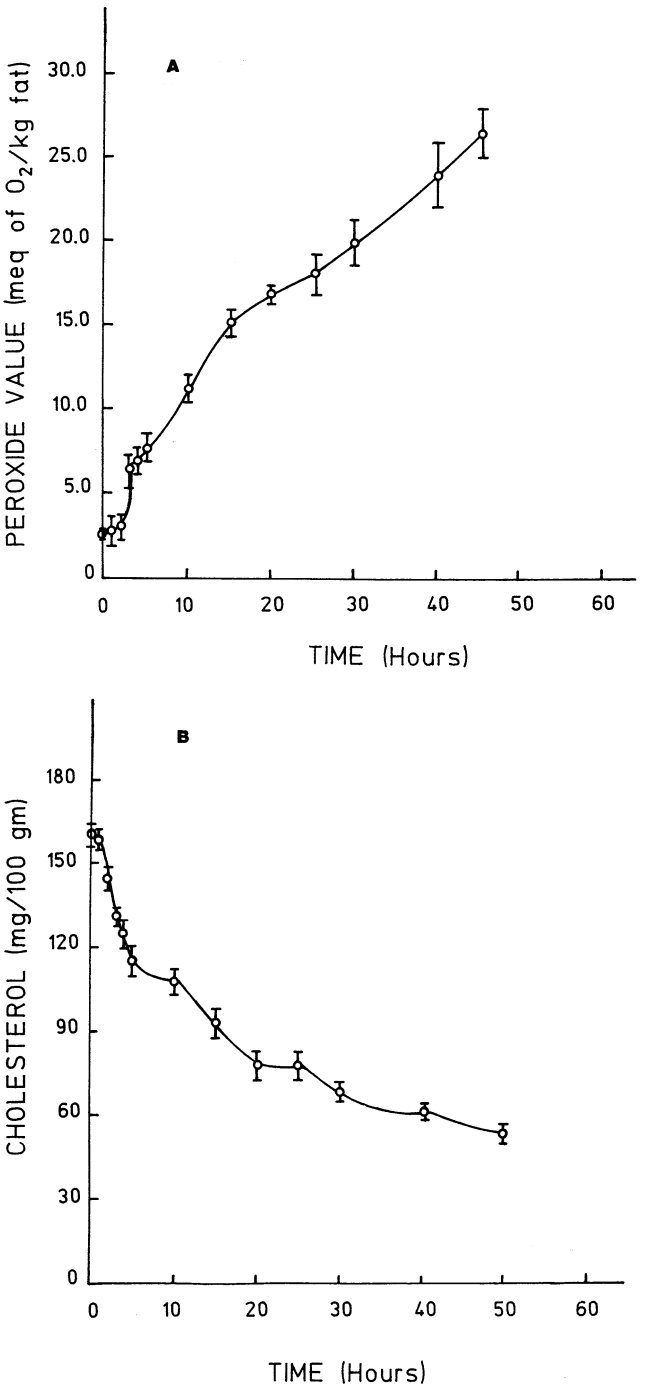

Figure 1 Effect of heating at 120°C at different intervals of time on peroxide value (*Figure 1A*) and cholesterol levels (*Figure 1B*) in ghee. Values are the mean ±SD determinations of three replicate ghee samples at each point.

Because there was no significant change in the overall fatty acid composition except for a drop in cholesterol levels

Table 2 Effect of heating on the composition of ghee

	Native	Heated*
Triglycerides (g/100 g)	98.1 ± 0.06	98.3 ± 0.08
Cholesterol (mg/100 g)	161.7 ± 3.2	54.4 ± 2.4[a]
Cholesterol oxidation products (% total sterols)	1.32 ± 0.56	17.6 ± 1.72[a]
Phospholipids (mg/100 g)	10.4 ± 0.71	6.0 ± 0.18[a]
Vitamin E (μg/100 g)	468 ± 21.3	423 ± 15.3
Vitamin A (μg/100 g)	561 ± 18.3	505 ± 14.2[b]
Peroxide value (mEq O_2/kg fat)	2.73 ± 0.18	26.7 ± 0.87[a]
Conjugated linoleic acid (%)	0.98 ± 0.02	0.97 ± 0.01
Fatty acid composition (wt %)		
10:0	1.3	1.3
12:0	2.6	2.5
14:0	14.3	12.5
16:0	37.8	34.2
16:1	3.0	2.8
18:0	12.0	11.2
18:1	30.0	28.0
18:2	2.6	1.4

Values are mean ±SD of three samples.
*Ghee was heated in a stainless steel mug at 120°C in an electric oven to reach the peroxide value of 25 mEq O_2/kg.
[a]$P < 0.001$.
[b]$P < 0.005$.

Table 4 Fatty acid composition of dietary lipids

Fatty acid (%)	Addition to the diet: ghee (%)					
	0*	0.25	1.0	2.5	5.0	10.0
10:0	ND	ND	ND	ND	0.5	0.9
12:0	ND	ND	0.3	0.7	0.9	3.1
14:0	0.5	0.8	1.2	2.9	4.5	14.7
16:0	13.5	14.2	16.6	19.8	23.9	32.7
16:1	ND	ND	0.5	0.8	2.9	4.3
18:0	3.3	3.5	4.4	5.8	8.8	12.5
18:1	44.6	44.0	42.1	41.9	37.1	28.5
18:2 (n−6)	36.5	36.0	33.5	26.7	19.9	2.1
20:0	1.9	1.7	1.4	1.0	0.8	ND
Saturated fatty acid (S)	17.3	20.2	22.5	29.2	38.6	63.9
PUFA (P)	36.5	36.0	33.5	26.7	19.9	2.1
P/S ratio	2.11	1.78	1.49	0.91	0.52	0.033

Note: There was no change in the fatty acid composition of dietary lipids when heated ghee (PV 26 mEq O_2/kg fat) was substituted for native ghee (PV < 1 mEq O_2/kg fat).
*Control: fat is exclusively from groundnut oil.
Values are mean ±SD of three samples.
ND–not detected. PUFA–polyunsaturated fatty acids.
P/S–ratio of polyunsaturated fatty acids to saturated fatty acids in the diet.

of heated samples, the oxidation products of cholesterol were analyzed by GC. The COPS showed an increase from an initial value of 2.14 mg/100 g to 28.5 mg/100 g ghee after heating. Even though unheated ghee samples contained small amounts of COPS amounting to 1.32% of total sterols, in heated ghee it increased to 17.6% of total sterols. There were significant increases in the levels of 7-ketocholesterol, 3-keto5-cholestene, 20-α-hydroxycholesterol, and 7-β-hydroxycholesterol after heating the ghee samples under specified conditions (*Table 3*). Fresh ghee that was not subjected to any further heating will henceforth be referred to as native ghee, whereas that was heated at 120°C for obtaining peroxide value of approximately 26 mEq O_2/kg will be referred to as heated ghee.

Effect of feeding native and heated ghee on growth parameters

Ghee was incorporated in the diet at different levels replacing equivalent amount of groundnut oil from the control diets. The fat level was kept constant at 10% in all the groups. Groundnut oil was chosen for the control diets because it is the most commonly used edible oil in southern India. The fatty acid composition of dietary lipids is shown in *Table 4*. As the amount of ghee in the diet increased, there was a corresponding decrease in the P/S ratio of dietary lipids. Except in the group of animals that received ghee as the sole source of fat, all groups of animals received adequate amounts of the essential fatty acid (EFA) linoleic acid in the diet.

The amount of diet consumed in different groups were comparable (15.2 ±2 g/rat/day, mean ±SD, $N = 8$ rats/group). The overall gain in body weight (259 ±9.1 g), food efficiency ratio (30.6 ±1; calculated as the percent ratio of weight gained by the animal to the food consumed), and percent fat absorption (96.6 ±1.1) were also comparable in all groups of animals. There were no significant changes in the weights of liver, heart, and other organs among different groups of animals. The hematologic parameters and histologic observations of liver and aorta were normal in different groups of animals (data not shown). Similar results were observed when either native ghee or heated ghee was used in the diet. These studies have indicated that ghee at different levels in the diet has no adverse effect on normal growth and physiology of animals.

Table 3 Cholesterol oxidation products in ghee

	Native (mg/100 g)	Heated (mg/100 g)
7-β-hydroxy cholesterol	0.092 ± 0.021	1.8 ± 0.23
3-keto5-cholestene	0.11 ± 0.012	6.08 ± 0.56
Epoxycholesterol	0.72 ± 0.027	3.05 ± 0.05
20-α-hydroxycholesterol	0.66 ± 0.008	8.43 ± 0.13
25-hydroxycholesterol	0.32 ± 0.003	0.69 ± 0.005
Cholesterol triol	ND	0.4 ± 0.08
7-ketocholesterol	0.24 ± 0.022	8.05 ± 0.34
Total COPS (% total sterols)	1.32	17.6

Values are mean ±SD three samples.
ND–not detected. COPS–cholesterol oxidation products.

Effect of feeding native and heated ghee on serum lipid profiles

When ghee was included in the diet at levels of greater than 2.5%, a significant drop in the total cholesterol levels in the serum was observed (*Table 5*). Thus, at 2.5%, 5%, and 10% levels in the diet, native ghee decreased serum cholesterol levels by 12%, 16%, and 30%, respectively, and the corresponding values for animals fed heated ghee were 16%, 21%, and 32%, respectively, as compared with those

Table 5 Effect of native and heated ghee on serum lipid profiles

Parameters analyzed	Addition to the diet: Ghee (%)									
		Native				Heated				
	0	0.25	1.0	2.5	5.0	10.0	0.25	1.0	2.5	5.0	10.0
Total cholesterol (mg %)	65.2 ± 4.3	64.4 ± 5.5	66.7 ± 5.7	57.1[b] ± 4.1	54.8[b] ± 6.4	45.2[a] ± 4.6	63.8 ± 7.8	62.3 ± 4.2	54.7[a] ± 3.6	51.2[a] ± 4.6	44.3[a] ± 4.5
Free cholesterol (mg %)	19.2 ± 1.3	20.1 ± 2.3	18.1 ± 2.4	15.8[a] ± 0.93	16.6[a] ± 2.3	12.7[a] ± 1.6	20.8 ± 1.9	20.2 ± 2.0	20.6 ± 1.5	21.6 ± 1.6	17.9 ± 2.3
Cholesterol esters (mg %)	45.7 ± 5.6	44.3 ± 1.8	48.1 ± 1.9	41.3 ± 4.1	38.5 ± 5.7	32.8[a] ± 4.9	43.0 ± 2.1	42.3 ± 2.1	34.8[b] ± 5.7	30.2[a] ± 3.4	29.3[a] ± 4.7
HDL cholesterol (mg %)	35.8 ± 3.1	37.3 ± 6.7	33.7 ± 7.4	26.9[a] ± 3.4	24.9[a] ± 3.2	22.0[a] ± 3.2	38.9 ± 3.4	37.1 ± 3.9	30.6 ± 4.1	32.6 ± 3.8	28.7[a] ± 4.6
VLDL+LDL cholesterol (mg %)	29.3 ± 6.2	27.1 ± 6.3	27.9 ± 6.0	30.2 ± 2.6	29.7 ± 3.9	23.5 ± 4.9	29.5 ± 4.5	25.6 ± 4.9	24.6 ± 3.6	19.8[b] ± 2.8	19.0[a] ± 2.7
Triglycerides (mg %)	182 ± 17.9	162 ± 32.9	167 ± 23.1	150[b] ± 15.8	156[c] ± 12.8	135[a] ± 9.8	166 ± 25.8	155 ± 32.7	143[a] ± 8.7	147[a] ± 13.5	143[a] ± 16.2
Phospholipids (mg %)	73.4 ± 5.5	73.3 ± 9.5	73.2 ± 7.3	67.1 ± 3.1	66.2 ± 2.0	69.6 ± 4.3	75.0 ± 7.2	74.1 ± 6.6	73.1 ± 4.6	78.4 ± 8.3	66.6 ± 5.8
Lipid peroxides (nmoles/mL)	8.61 ± 1.07	7.1 ± 1.3	6.8 ± 0.1	7.2 ± 1.2	8.2 ± 1.6	10.2 ± 1.3	7.8 ± 1.0	7.4 ± 1.1	8.6 ± 1.9	12.0 ± 2.9	17.6[a] ± 8.0

Values are mean ±SD of eight rats.
[a]$P < 0.001$.
[b]$P < 0.005$.
[c]$P < 0.01$.
HDL–high density lipoprotein. VLDL–very low density lipoprotein. LDL–low density lipoprotein.

observed in control animals. No significant changes in serum cholesterol levels were observed when ghee was fed at 0.25% and 1% levels in the diet. However, there were significant differences in cholesterol levels in lipoprotein fractions in the animals fed native and heated ghee. Although native ghee in the diet decreased both free and cholesterol ester levels, in animals fed heated ghee, a significant drop was observed only in cholesterol ester fraction of the serum (*Table 5*). Although HDL cholesterol was not affected in animals fed up to 5% heated ghee, in those fed native ghee at 5% level in the diet, an approximately 30% drop in HDL cholesterol was observed. On the other hand, cholesterol associated with apoB fractions [very low density lipoprotein (VLDL) + LDL] was dropped by 32% in animals fed heated ghee at 5% level, but in animals fed native ghee at 5% level, there was no such decrease in cholesterol associated with apoB fraction. Serum triglycerides showed a drop of approximately 20 to 25% in animals fed native and heated ghee at levels higher than 2.5% in the diet. Serum phospholipids did not show any significant changes in animals fed native and heated ghee. Although heated ghee contained a 10-fold higher peroxide value, the lipid peroxides in serum did not change significantly except in animals fed 10% heated ghee. Similarly, although heated ghee contained levels of COPS that were 14-fold higher than native ghee, there was no increase in the COPS level in the serum when heated ghee was fed to animals. The following COPS were identified in control as well as in ghee fed animals: In control animals, 7-β-hydroxycholesterol and 7-α-hydroxycholesterol were detected at levels of 0.9 ±0.1 mg/dL and 0.28±0.04 mg/dL (mean ±SD, $N = 8$); for those fed ghee, the corresponding values were 0.99 ±0.13 mg/dL and 0.30 ±0.02 mg/dL, respectively (combined mean ±SD, $N = 8$ rats in each group). No other COPS were detected in the serum of control or ghee fed animals.

The fatty acid profiles of serum lipids in control animals and in animals fed ghee were analyzed (*Table 6*). Very significant changes were observed when ghee was used as the sole source of dietary fat. In this group of animals, oleic acid levels increased by 36 to 40% compared with those found in control animals. However, linoleic acid and arachidonic acid levels decreased significantly in serum lipids when ghee was used as the sole source of fat. Although linoleic acid levels decreased by 78%, the arachidonic acid levels decreased by 65% in the animals fed ghee at a 10% level. In addition, eicosatrienoic acid also was observed in these animals to an extent of 1.8 to 2.8% of total fatty acids. However, eicosatrienoic acid was not observed in other experimental groups. In addition, the decrease in the EFA levels in animals fed ghee at 2.5% and 5.0% levels was marginal compared with those fed 10% ghee in the diet. These studies indicated that ghee as a sole source of fat in the diet could deplete EFA levels and might induce EFS deficiency. However, even under these conditions, no external symptoms of EFA deficiency were observed. The ratio of triene to tetraene was less than 0.4, indicating that complete EFA deficiency was not induced in the animals in spite of lowering of EFAs.

Table 6 Effect of ghee on serum fatty acid composition

Fatty acid (%)	Addition to the diet: ghee (%)						
		Native			Heated		
	0	2.5	5.0	10.0	2.5	5.0	10.0
12:0	0.27 ± 0.15	0.22 ± 0.10	0.12 ± 0.02	0.3 ± 0.1	0.17 ± 0.03	0.18 ± 0.01	0.40 ± 0.04
14:0	0.76 ± 0.48	0.64 ± 0.10	0.72 ± 0.10	0.88 ± 0.30	0.7 ± 0.1	0.73 ± 0.10	1.08 ± 0.14
16:0	21.7 ± 1.7	21.7 ± 1.3	23.0 ± 0.7	23.9 ± 3.0	21.6 ± 1.4	22.6 ± 1.8	25.00 ± 1.60
16:1	3.8 ± 0.9	3.7 ± 0.4	4.8 ± 0.6	6.8[a] ± 0.9	4.0 ± 0.4	5.0 ± 0.9	6.3[a] ± 0.9
18:0	5.7 ± 0.3	6.4 ± 0.7	6.1 ± 0.57	6.1 ± 0.8	5.7 ± 0.4	5.1 ± 0.9	6.0 ± 0.8
18:1	27.7 ± 1.3	29.2 ± 1.0	32.2[a] ± 1.5	37.6[a] ± 2.3	28.5 ± 1.4	30.5 ± 2.5	38.8[a] ± 3.1
18:2 (n-6)	19.8 ± 1.3	17.3 ± 1.3	14.6[a] ± 1.5	4.35[a] ± 0.9	16.5 ± 1.0	13.7[a] ± 1.0	4.2[a] ± 0.96
20:3 (n-9)	ND	ND	ND	1.8 ± 0.8	ND	ND	2.87 ± 0.7
20:4 (n-6)	19.8 ± 2.0	19.1 ± 1.1	16.8 ± 1.9	7.4[a] ± 1.4	18.2 ± 1.4	17.0 ± 2.0	6.6[a] ± 1.9

Values are mean ±SD of eight rats.
[a]$P < 0.001$.
ND–not detected.

Effect of feeding native and heated ghee on liver lipid profiles

Liver is an important organ for the metabolism of cholesterol and is also sensitive to changes in the dietary lipids. Hence, the lipid profiles in liver were analyzed after feeding the animals different levels of ghee in the diet. Similar to that observed in serum, the total cholesterol levels in liver was decreased by 14 to 16% when ghee was included in the diet at levels greater than 2.5% (*Table 7*). However, unlike in serum, there was no further decrease in cholesterol levels of liver when ghee was increased to 10% in the diet. The free cholesterol levels in liver marginally increased when higher levels of ghee were included in the diet, but a significant drop in liver cholesterol ester levels was observed when ghee was fed to the animals. Thus, at 2.5%, 5%, and 10% levels, native ghee decreased liver cholesterol ester levels by 21%, 25%, and 32%, respectively, whereas in those in animals fed heated ghee, the corresponding values were 34%, 34%, and 43%, respectively. Similarly, triglyceride levels decreased by 14%, 20%, and 27% in animals receiving 2.5%, 5%, and 10% of native ghee, respectively, whereas the corresponding decreases in animals fed heated ghee were 16%, 24%, and 29%, respectively. There were no significant changes in the liver phospholipid levels in all the experimental groups. The ascorbate/Fe^{2+} induced lipid peroxidation in liver homogenate was lower in animals fed increasing amounts of ghee than in the control animals (*Table 7*). However, small amounts of COPS were identified in the livers of animals fed ghee, particularly those fed at 10% levels in the diet (*Table 8*). Major increases that were not observed in control animals were observed in cholesterol epoxides, 20-α-hydroxycholesterol, and cholestane triol. There were no significant differences in COPS present in the liver of animals fed native and heated ghee.

Pure ghee is deficient in EFAs (*Table 1*). This in turn may influence the levels of polyunsaturated fatty acid (PUFA) composition of tissue lipids and thereby affect the biochemical functions. Lowering of lipid peroxidation in liver gives one such indication (*Table 7*). Hence, the fatty acid changes were monitored in the liver (*Table 9*). Significant changes were observed in PUFA levels of livers compared with those found in control animals. Linoleic acid levels decreased by 12%, 25%, and 63% when ghee was at 2.5%, 5%, and 10% levels, respectively. Similarly, arachidonic acid levels decreased by 0, 19%, and 42% in liver

Table 7 Effect of ghee on liver lipids

Parameters analyzed	Addition to the diet: ghee (%)						
		Native			Heated		
	0	2.5	5.0	10.0	2.5	5.0	10.0
Total cholesterol (mg/g)	6.0 ± 0.67	5.2[a] ± 0.63	5.08[a] ± 0.5	5.03[a] ± 0.42	5.14[a] ± 0.5	5.01[a] ± 0.44	5.1[a] ± 0.82
Free cholesterol (mg/g)	1.98 ± 0.37	2.04 ± 0.45	2.08 ± 0.31	2.31[a] ± 0.50	1.97 ± 0.39	2.35[a] ± 0.44	2.86[a] ± 0.40
Cholesterol ester (mg/g)	4.02 ± 0.64	3.16[b] ± 0.59	3.00[b] ± 0.47	2.72[a] ± 0.19	2.67[a] ± 0.28	2.66[a] ± 0.39	2.31[a] ± 0.50
Triglycerides (mg/g)	6.85 ± 1.4	5.90 ± 1.2	5.50[b] ± 1.7	4.98[a] ± 1.7	5.75 ± 0.5	5.20[b] ± 1.7	4.87[a] ± 0.96
Phospholipids (mg/g)	21.1 ± 2.7	20.1 ± 1.1	20.3 ± 4.4	21.2 ± 2.3	17.8 ± 2.1	18.1 ± 2.3	19.4 ± 1.9
Lipid peroxides (nmoles/mg protein)	1.81 ± 0.23	1.25 ± 0.37	1.16[a] ± 0.13	0.94[a] ± 0.19	1.51 ± 0.35	1.40[b] ± 0.21	1.00[a] ± 0.15

Values are mean ±SD of eight rats.
[a]$P < 0.001$.
[b]$P < 0.005$.

Table 8 Effect of ghee on cholesterol oxides in liver

Cholesterol oxides (μg/g)	Addition to the diet: ghee (%)						
		Native			Heated		
	0	2.5	5.0	10.0	2.5	5.0	10.0
7β-hydroxy cholesterol	27.2 ± 6.2	30.1 ± 0.9	29.5 ± 1.0	34.9 ± 2.9	28.3 ± 0.4	32.0 ± 1.8	39.0 ± 4.6
7-α-hydroxy cholesterol	20.8 ± 3.0	25.0 ± 3.0	28.6 ± 4.0	30.1 ± 4.2	26.3 ± 4.8	29.2 ± 3.7	31.7 ± 3.6
7-keto cholesterol	ND	0.20 ± 0.03	0.30 ± 0.02	0.45 ± 0.02	0.25 ± 0.06	0.35 ± 0.04	0.50 ± 0.02
Cholesterol epoxides	ND	ND	ND	7.00 ± 0.12	ND	ND	9.80 ± 0.32
20-α-hydroxy cholesterol	ND	ND	ND	9.00 ± 2.9	ND	ND	11.00 ± 0.3
Cholestane triol	ND	ND	ND	8.00 ± 0.3	ND	ND	10.0 ± 0.32
3-keto5-cholestene	ND	ND	ND	0.15 ± 0.04	ND	ND	0.2 ± 0.01

Values are mean ±SD of eight rats.
ND–not detected.

when ghee was included in the diet at 2.5%, 5%, and 10% levels, respectively. It also was observed that eicosatrienoic acid was detected in the tissue only when ghee was given as a sole source of fat at 10%. However, the ratio of triene to tetraene was less than 0.4 and no overt symptoms of EFA deficiency were observed in any of these animals. Similar changes were seen in heart and adipose tissue (data not shown).

Discussion

The results of the present study indicate that ghee underwent oxidative changes when heated to higher temperatures, as is practiced in Indian households. Heating did not significantly change the triglyceride levels, the major constituent of ghee, and the overall fatty acid composition. However, heated ghee showed a 66% drop in total cholesterol level and a significant increase in COPS. However, COPS accounted for only 17.6% of total sterols in heated ghee. Similarly, Chen et al.[38] observed that when lard was heated for 72 hours at 180°C, its cholesterol content reduced by 70% but without concomitant formation of COPS in equivalent amounts. Similar results also were reported by Kumar and Singhal[39] and Jacobson,[8] who found that heating of ghee led to the development of COPS to the extent of 9 to 12% of total sterols. These studies indicate that in addition to being converted to COPS, cholesterol in food materials has undergone thermal degradation and part of it may be converted into volatile compounds. Among the various COPS found in heated ghee, 20-α-hydroxycholesterol, 7-ketocholesterol, and 3-keto5-cholestene accounted for approximately 80% of oxidized products formed. Even though native ghee contained small amounts of COPS, it accounted for only 1.3% of total sterols present. These results are in accordance with those reported by Kumar and Singhal[39] and Jacobson.[8] The COPS are considered to be highly atherogenic in nature.[40,41]

However, the ghee lipids containing COPS incorporated in the diet at levels ranging from 0.25 to 10% did not show any deleterious effects on experimental animals in terms of growth, Food Efficiency Ratio (FER), fat intake, fat absorption, and organ weights. No lipid deposition was observed either in the arteries or in the livers of animals fed a diet containing ghee. Interestingly, serum cholesterol levels decreased in a dose dependent manner when ghee was incorporated at levels ranging from 2.5 to 10% in the diet. Although cholesterol ester fraction decreased in animals fed both native and heated ghee, the decrease in free cholesterol level was observed only in animals fed native ghee. Although significant changes were observed in HDL choles-

Table 9 Effect of ghee on liver lipid fatty acid composition

Fatty acid (%)	Addition to the diet: ghee (%)						
		Native			Heated		
	0	2.5	5.0	10.0	2.5	5.0	10.0
14:0	0.38 ± 0.3	0.36 ± 0.06	0.48 ± 0.16	0.48 ± 0.0	0.41 ± 0.2	0.50 ± 0.09	0.58 ± 0.17
16:0	17.2 ± 1.6	16.6 ± 0.9	18.5 ± 1.3	19.8 ± 1.6	17.5 ± 0.5	18.9 ± 2.2	19.7 ± 1.1
16:1	4.8 ± 1.2	3.8 ± 0.9	5.5 ± 1.2	6.6 ± 0.6	4.3 ± 0.6	5.7 ± 1.4	6.0 ± 0.8
18:0	8.9 ± 1.2	10.0 ± 0.7	10.0 ± 0.6	10.5 ± 0.7	9.9 ± 1.1	8.3 ± 0.6	12.5[a] ± 1.5
18:1	28.2 ± 1.5	27.1 ± 2.1	29.0 ± 1.3	34.7[a] ± 1.5	27.3 ± 2.4	29.3 ± 1.9	33.5 ± 2.1
18:2 (n-6)	19.0 ± 1.4	16.8 ± 2.0	14.3[a] ± 0.57	7.0[a] ± 0.9	16.7 ± 1.7	12.7[a] ± 1.2	6.5[a] ± 0.9
20:3 (n-9)	ND	ND	ND	2.1 ± 0.32	ND	ND	3.4 ± 0.48
20:4 (n-6)	20.5 ± 2.1	21.0 ± 2.8	16.6[b] ± 1.9	11.8[a] ± 0.3	20.0 ± 1.5	15.3[b] ± 1.9	11.7[a] ± 1.6

Values are mean ±SD of eight rats.
[a]$P < 0.001$.
[b]$P < 0.005$.
ND–not detected.

terol levels in animals fed native ghee, the changes were more pronounced in VLDL + LDL fractions in heated ghee fed animals. Serum triglycerides decreased in animals fed both native and heated ghee. Phospholipids remained unchanged in animals fed native and heated ghee. Lipid peroxides increased only marginally in animals fed heated ghee as a sole source of fat but no increase of COPS in serum were detected in the present study, indicating differential influence of ghee lipids on serum lipid profiles. It is interesting to note that although Asian immigrants are found to be susceptible to CVDs, they have lower serum cholesterol levels and higher HDL cholesterol levels compared with British and other ethnic populations.[42] Similarly, lower serum cholesterol levels have been observed in East African Maasai men who consume large quantities of dairy products.[43] These results also were confirmed in experimental animals fed milk and yogurt.[44] Milk is known to contain factors that inhibit cholesterol biosynthesis.[45] Our unpublished studies also indicated that feeding ghee to rats increased formation of bile acid, which is a metabolite of cholesterol. Navder et al.[44] suggested that the hypocholesterolemic effect of dairy products may be mediated through inhibition of cholesterol biosynthesis, enhancing the fecal excretion of sterols and bile acids. However, the factors responsible for these effects are yet to be identified. Recently, Aneja and Murthy[11] and Pariza[12] showed that conjugated linoleic acid at 2% levels in the diet decreased serum LDL cholesterol and atherogenesis in rabbit model. Ghee contains approximately 1% conjugated linoleic acid.

Osada et al.[46] reported that oxysterols inhibited HMG-CoA reductase and cholesterol absorption in rats. Oxysterols also inhibited absorption of triglycerides.[47] Smith[5] has reported that epoxycholesterol of dietary origin is converted to cholestane triol, which is a potent inhibitor of HMG-CoA reductase. Although heated ghee contained approximately 17.6% of oxysterols, its effect in lowering serum cholesterol was similar to that of native ghee, which contained only 1.32% oxysterols. Therefore, the levels of oxysterols alone cannot explain the hypocholesterolemic effect of ghee lipids. The liver lipids also were altered in a fashion similar to that observed in serum after giving ghee in the diet at levels of 2.5 to 10%. Total cholesterol, cholesterol ester, and triglycerides decreased in ghee fed animals. However, the levels of free cholesterol were enhanced significantly in rats fed a 10% level of ghee. This indicates that ghee as a sole source of dietary fat may inhibit the activity of esterifying enzyme such as acyl CoA-cholesterol acetyltransferase (ACAT) activity and prevent the conversion of cholesterol to its ester form. As in the case of serum, phospholipids remained unchanged but lipid peroxidation marginally reduced after feeding ghee. Liver also accumulated various cholesterol oxides when ghee was given as a sole source of fat.

In addition to influencing serum cholesterol and triglyceride levels, ghee also altered serum and tissue fatty acid composition. The most significant changes were observed in PUFA levels when ghee was used as the sole source of fat at a 10% level in the diet. At this level of feeding, both linoleic acid and arachidonic acid levels decreased drastically in serum and liver lipids. A small amount of eicosatrienoic acid was observed in these tissues. However, the ratio of triene to tetraene fatty acids did not indicate EFA deficiency in animals. It is also interesting to note that in the presence of linoleic acid, ghee did not drastically affect either the PUFA levels or induce the formation of eicosatrienoic acid (Mead acid), but still its influence in lowering serum lipids remained. Fat in the Indian diet contributes only 15% of total calories. Approximately 60 to 70% of this is contributed by invisible fat such as those derived from cereals, pulses, vegetables, spices, and condiments.[3,48] These are very rich in linoleic acid.[49] In fact, the average total contribution of linoleic acid in Indian diets are of the order of 4.8 energy percent and that from linolenic acid is 0.28 energy percent, which is much higher than the minimum requirement of these EFAs.[3,48] Hence the presence of ghee in Indian diet is unlikely to influence EFA status of individuals. It is also interesting to note that oleic acid levels increased in serum when diets containing ghee were fed to animals. Oleic acid enriched LDL were known to resist oxidation.[16] This, in turn, may prevent plaque formation. Populations living in the Mediterranean region that consume oleic acid rich olive oil have a lower incidence of CVD.[1]

The amount of ghee used in the present study ranged from 0.25 to 10% of total dietary fat. Based on the food consumption by rats, it was calculated that, on average, rats consumed ghee to an extent of 0.125 g/kg to 5g/kg body weight, which is equivalent to the consumption of 7.5 to 300 g/day/adult. The amount of ghee consumed in the Indian diet shows a wide variation depending on the region and socioeconomic status. Ghee is consumed not only as such but also is used in the preparation of many dishes. In the Ayurvedic system of medicine, ghee is given in graded amounts to patients to reach a level of 350 to 500 g/day in the treatment of skin diseases and allergy. Our unpublished results show that serum cholesterol levels were decreased by 8 to 15% in these patients after ghee was consumed as a part of the therapy. Similar results of 15 to 20% decrease in serum cholesterol levels also were observed in studies on Indian desert gerbils (unpublished data).

Conclusion

The present studies indicate that consumption of ghee in the diet even at very high concentrations do not increase serum lipids, which are considered to be a risk factor for heart disease. In fact, several epidemiologic studies have suggested that Asian immigrants have higher risk for heart disease, probably due to increased incidence of noninsulin dependent diabetes and other factors.[50–52] A strong suggestion was also made to link the consumption of anhydrous milk fat (viz., ghee and its associated COPS) with increased risk of heart diseases.[8] The present findings with experimental animals do not give any support for linking ghee consumption to hypercholesterolemia and hyperlipidemia, which are considered to be risk factors for heart diseases. Interestingly, consuming increased levels of ghee reduced serum cholesterol and triglyceride levels. However, use of excess intake of ghee as a means for lowering serum cholesterol is not recommended, but the study indicates that there is no reason for apprehension for consuming ghee in the diet, which is an age-old practice that is relished in Indian culinary.

Acknowledgments

The authors thank the head of the Department of Biochemistry and Nutrition and the director of CFTRI, Mysore, for encouragement, and Mr. V.A. Daniel for editing the manuscript. Mr. M. Vijay Kumar acknowledges the grant of the Junior Research Fellowship by UGC and Senior Research Fellowship by CSIR, New Delhi, India.

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