

Effect of phospholipids and bile acids on cholesterol nucleation time and vesicular/micellar cholesterol in gallbladder bile of patients with cholesterol stones

Dieter Jüngst,¹ Thomas Lang, Peter Huber, Volker Lange,* and Gustav Paumgartner

Departments of Medicine II and Surgery,* Klinikum Grosshadern, Ludwig-Maximilians-Universität of Munich, 8000 Munich 70, Germany

Abstract Supersaturation and rapid nucleation of cholesterol in bile are of key importance in the pathogenesis of cholesterol gallstones. While the effects of bile acids and phospholipids on cholesterol saturation of bile have been extensively studied, their influence on the cholesterol nucleation time has not been compared. We, therefore, investigated whether increases of bile acid or phospholipid concentrations in bile by in vitro supplementation affect the cholesterol nucleation time. Bile samples were obtained at surgery from patients with cholesterol gallstones. Prior to the nucleation assay the bile samples were divided into 0.5-ml aliquots and supplemented with 1.25, 2.5, 5.0, and 10.0 $\mu\text{mol/ml}$ of different phosphatidylcholines (PC-dimyristoyl, PC-dipalmitoyl, PC-distearoyl, and extracted biliary PCs) or with 5.0, 10.0, and 20.0 $\mu\text{mol/ml}$ of bile acids (glycine or taurine conjugates of cholic acid, deoxycholic acid, or chenodeoxycholic acid). The increase of phosphatidylcholine or bile acid concentration decreased the mean cholesterol saturation index to a similar extent (PC: 0.1–0.3; BA: 0.1–0.2). Supplementations of bile with increasing amounts of synthetic or biliary PCs caused a marked prolongation of the nucleation time in bile from 1.5 ± 0.2 up to ≥ 21 days or 2.5 ± 0.7 up to ≥ 21 days. Concurrently, biliary cholesterol was shifted from vesicles to mixed micelles and the cholesterol/phospholipid ratio of the remaining vesicles was progressively lowered. In contrast, the addition of bile acids to gallbladder bile did not affect the cholesterol nucleation time (2.2 ± 0.3 days), the percentage of vesicular cholesterol, or the cholesterol/phospholipid ratio of vesicles and micelles. One can speculate from these in vitro studies that increasing phospholipid concentrations in bile might be more effective for the prevention of cholesterol gallstones than increasing bile acid concentrations.—Jüngst, D., T. Lang, P. Huber, V. Lange, and G. Paumgartner. Effect of phospholipids and bile acids on cholesterol nucleation time and vesicular/micellar cholesterol in gallbladder bile of patients with cholesterol stones. *J. Lipid Res.* 1993. 34: 1457–1464.

Supplementary key words phosphatidylcholines • vesicles • micelles • intermicellar concentration

In gallbladder bile from cholesterol gallstone patients, cholesterol crystal formation is accelerated when compared to equally supersaturated bile from healthy controls

or patients with pigment stones (1–4). It is thought that nucleation of cholesterol monohydrate crystals from both model and native biles occurs mainly after fusion and aggregation of phospholipid-cholesterol vesicles to form birefringent liquid crystalline droplets (5). There seems to be a balance in bile between nucleation inhibitors and promoters and there is evidence that these may be proteins (6–8). Phospholipids represent quantitatively the second most important lipid fraction in bile and dissolve cholesterol in mixed bile acid-phospholipid micelles as well as in phospholipid vesicles (9–12). Phosphatidylcholines are the predominant biliary phospholipids and their major molecular species are the least hydrophobic found in liver cell membranes (13). Seventy to 80% of phosphatidylcholines in bile are *sn*-1 palmitoyl (16:0) with either *sn*-2 oleoyl (18:1) or linoleoyl (18:2) fatty acids (14, 15). Thus, particular molecular species of phosphatidylcholines are preferentially solubilized in bile salt micelles (16). Changes in the pattern of fatty acids of biliary phosphatidylcholines with an increase of arachidonic acid have been observed in bile from patients with cholecystolithiasis (17). In a recent study Nervi et al. (18) have indicated that an apparently selective reduction in biliary phospholipids by nutritional factors may be responsible for the high prevalence of cholesterol gallstones in certain populations. In many other previous studies, such a selective reduction in phospholipid concentration has not been implicated as a cause of cholesterol gallstone disease (19, 20). We were stimulated by the findings of Nervi et al. (18) to investigate whether an increase of biliary phospholipid

Abbreviations: CSI, cholesterol saturation index; CH, cholesterol; PC, phosphatidylcholine; PL, phospholipid; BA, bile acids; C, cholic acid; CDC, chenodeoxycholic acid; DC, deoxycholic acid; UDCA, ursodeoxycholic acid; TLC, thin-layer chromatography; IMC, intermicellar concentration.

¹To whom correspondence should be addressed.

concentrations by in vitro supplementation would prolong the abnormally rapid nucleation of cholesterol in the gallbladder bile of patients with cholesterol gallstones. Three synthetic molecular species of saturated phosphatidylcholines with different patterns of fatty acids and phosphatidylcholines extracted from human gallbladder bile of patients with cholesterol or pigment stones were used for the supplementation experiments. In control experiments we investigated the effect of an increase of bile acid concentrations on the cholesterol nucleation time by adding taurine or glycine conjugates of cholic, deoxycholic, and chenodeoxycholic acid to bile.

MATERIALS AND METHODS

Patients and collection of biles

Fifty-two patients, 37 women and 15 men who underwent elective surgery because of symptomatic gallstone disease, were selected for the study. Gallbladder bile was aspirated with an 18-gauge needle by puncture of the gallbladder during surgery within 3 min after ligation of the cystic duct. Particular care was taken to collect gallbladder bile completely, because of the possibility of stratification (21) and to avoid disruption of the mucosa by clamping of the gallbladder wall prior to this procedure. The stones were removed with the gallbladder, washed with distilled water, dried, and weighed. The cholesterol content was measured chemically after extraction with organic solvents and expressed as percentage of the dry weight (22).

Microscopy of bile and nucleation time

After collection, bile samples were mixed thoroughly and one drop was immediately examined by polarized light microscopy for cholesterol crystals. Cholesterol nucleation time was determined as described recently (23) with a slight modification of the original method of Holan et al. (1).

Analysis of biliary lipids

For the analysis of bile lipids, duplicate aliquots were stored at -70°C prior to determination. Cholesterol was determined colorimetrically by the Liebermann-Burchard reaction after double extraction of 1 ml methanolic bile sample with petroleum ether (24). Phospholipids were measured as total biliary phosphate after hydrolysis at 150°C with sulfuric acid, using the colorimetric assay of Fiske-SubbaRow (25). Inorganic phosphate contributes only minor amounts to total biliary phosphate; therefore, total phosphate adequately reflects lipid-bound phosphate. Total bile salts were determined by a modified 3α -hydroxysteroid dehydrogenase method (26). The saturation index of each sample was calculated by dividing the cholesterol concentration by the maxi-

mum cholesterol solubility according to Carey (27) and corrected for the total lipid content of each individual bile.

Separation of vesicles and micelles

Micelles were separated from vesicles by gel filtration as described by Sömjen and Gilat (11). Glass columns (30×1.2 cm) were filled with Sephacryl-S-300 superfine gel containing 0.04% sodium azide in distilled water (Pharmacia AB, Uppsala, Sweden). The void volume of the column was determined with 2% Dextran Blue 2000 (Pharmacia). After ultracentrifugation ($100,000 g$ for 1 h) of the bile, 0.5 ml of the isotropic phase was applied to the column and eluted in 1.2-ml fractions using Tris-HCl buffer (pH 8.0) containing 10 mM sodium cholate to prevent disruption of micellar lipids. Thirty-five fractions were collected and analyzed for cholesterol and phospholipid content. One hour after supplementation of bile with the respective amounts of PC or BA aliquots, 0.5 ml was processed as described above, without further ultracentrifugation prior to the column chromatography.

Supplementation experiments

Synthetic crystalline L- α -phosphatidylcholinedimyristoyl (P-6392), L- α -phosphatidylcholinedipalmitoyl (P-1652), and L- α -phosphatidylcholinedistearoyl (P-6517) were obtained from Sigma (St. Louis, MO). Biliary phospholipids were extracted from gallbladder bile (chloroform-methanol 2:1, v/v) and fractionated by TLC on 20×20 cm glass plates coated with silica gel (Merck). After one-dimensional TLC in the solvent system (chloroform-methanol-25% aq. ammonia, 60:20:5) the separated PCs were made visible with iodine vapor, recovered, and assayed for phosphorus content. Prior to the nucleation assay, bile samples were divided into 0.5-ml aliquots and supplemented with 1.25, 2.5, 5.0, and 10.0 $\mu\text{mol/ml}$ of the different types of PCs. For supplementation the PCs were dissolved in methanol (50 $\mu\text{mol/ml}$) and the respective amounts were pipetted into the test tubes, dried with a stream of N_2 , and redissolved in the bile aliquots. Complete dissolution was achieved after 1 h as documented by the determination of phospholipid concentration in each sample. After 1 h, samples of the bile were taken for nucleation studies and chromatographic separation.

Sodium salts of taurocholic acid (T-4009), taurochenodeoxycholic acid (T-6260), taurodeoxycholic acid (T-0875), glycocholic acid (G-2878), glycochenodeoxycholic acid (G-0759), and glycodeoxycholic acid (G-6132) were obtained from Sigma (St. Louis, MO). These bile acids were dissolved in methanol (100 $\mu\text{mol/ml}$) and 2.5, 5.0, and 10.0 μmol were pipetted into the test tubes. After drying with a stream of N_2 , bile acids were redissolved in 0.5-ml bile aliquots. Complete dissolution was assessed after 1 h by measurement of bile acid concentration in each sample. After 1 h, samples of the bile were taken for nucleation studies and chromatographic separation.

Statistical analysis

Data are expressed as means \pm SEM. Means between groups were compared using Student's *t* test for parametric data and Wilcoxon's rank test and Mann-Whitney test for non-parametric data (nucleation time). A *P* value < 0.05 was considered statistically significant.

RESULTS

Stone analysis and biliary microscopy

The cholesterol content of the stones ranged from 53 to 96% (mean $69 \pm 2.2\%$). Biliary microscopy showed typical cholesterol crystals in all native biles as well as in the sediment after ultracentrifugation.

Lipid analysis, cholesterol saturation and nucleation time

Mean values \pm SEM for lipids, CSI, and cholesterol nucleation time in the bile samples used for the different supplementation experiments are given in Table 1.

Supplementation experiments

After addition of the PCs, the calculated CSI declined from 1.6 ± 0.3 to 1.5 ± 0.2 with the minimum amount of PC supplementation ($1.25 \mu\text{mol/ml}$ bile) and reached the lowest value of 1.3 ± 0.2 when the maximum amount of PCs ($10.0 \mu\text{mol/ml}$ bile) was added. A marked prolongation of the cholesterol nucleation time was achieved in all bile samples after the supplementation with all types of PCs (Fig. 1). PC-dipalmitoyl and PC-distearoyl were more effective than PC-dimyristoyl (Fig. 1). Even when only low amounts of PC-dipalmitoyl or PC-distearoyl ($1.25 \mu\text{mol/ml}$ bile) were added, a considerable prolongation of the cholesterol nucleation times from 1.5 ± 0.2 days to 6.5 ± 2.1 days and 7.5 ± 2.4 days, respectively, was seen. After supplementation with higher amounts of both PCs, no cholesterol crystals were observed during the observation period of 21 days, despite the fact that the

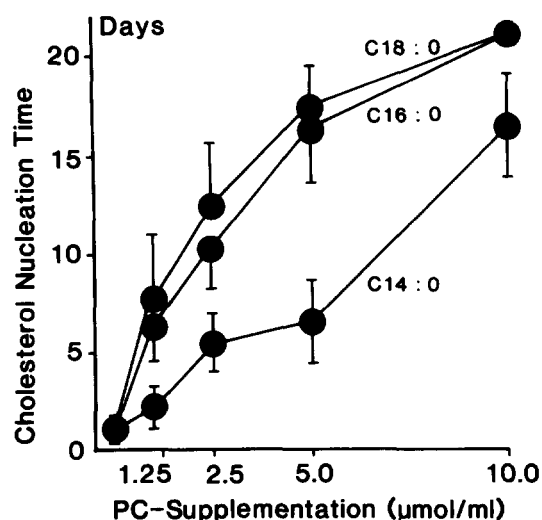


Fig. 1. Effect of bile supplementation with L- α -PC-dimyristoyl (C 14:0), L- α -PC-dipalmitoyl (C 16:0), and L- α -PC-distearoyl (C 18:0) on cholesterol nucleation time in gallbladder biles of 15 patients with cholesterol gallstones.

biles were still supersaturated ($\text{CSI} > 1.0$) in the majority of the samples.

Similarly, supplementation with increasing amounts of extracted biliary phosphatidylcholines from patients with cholesterol or pigment stones progressively prolonged the nucleation time from 2.5 ± 0.7 to > 21 days (Fig. 2). Concurrently, the percentage of total biliary cholesterol in vesicles decreased from $20.2 \pm 3.2\%$ to $5.1 \pm 2.6\%$ and the cholesterol/phospholipid ratio of the remaining vesicles was progressively lowered from 1.3 ± 0.1 to 0.4 ± 0.1 (Fig. 3).

The addition of single taurine or glycine conjugates of chenodeoxycholic acid, deoxycholic acid, or cholic acid lowered the CSI from 1.7 ± 0.2 to 1.6 ± 0.2 , 1.6 ± 0.2 , and 1.5 ± 0.2 when the respective amounts of 5.0, 10.0, and $20.0 \mu\text{mol/ml}$ bile were added. However, supplementation of bile with these different bile acids did not affect

TABLE 1. Concentrations of bile acids, phospholipids, cholesterol, and total lipids, cholesterol saturation index, and cholesterol nucleation time of gallbladder biles for phospholipid and bile acid supplementation experiments

Variable	Supplementation with PC-dimyristoyl, PC-dipalmitoyl, or PC-distearoyl (n = 15)	Supplementation with extracted biliary PCs (n=12)	Supplementation with single glycine or taurine conjugates of CDC, DC, or C (n = 15)	Supplementation with mixtures of glycine: taurine (3:1) conjugates of CDC (38%), DC (18%), or C (44%) (n = 10)
Bile acids (mmol/l)	72.0 ± 8.0	71.1 ± 10.1	65.2 ± 8.5	84.8 ± 14.5
Phospholipids (mmol/l)	26.0 ± 2.7	18.7 ± 2.2	23.0 ± 2.8	33.9 ± 5.4
Cholesterol (mmol/l)	10.0 ± 0.9	9.6 ± 1.2	9.1 ± 1.2	10.9 ± 2.3
CSI	1.6 ± 0.2	1.7 ± 0.2	1.7 ± 0.2	1.2 ± 0.1
Total lipids (g/dl)	5.9 ± 0.6	5.3 ± 0.6	5.3 ± 0.6	7.2 ± 1.1
Nucleation time (days)	1.5 ± 0.2	2.5 ± 0.7	2.2 ± 0.3	2.0 ± 0.5

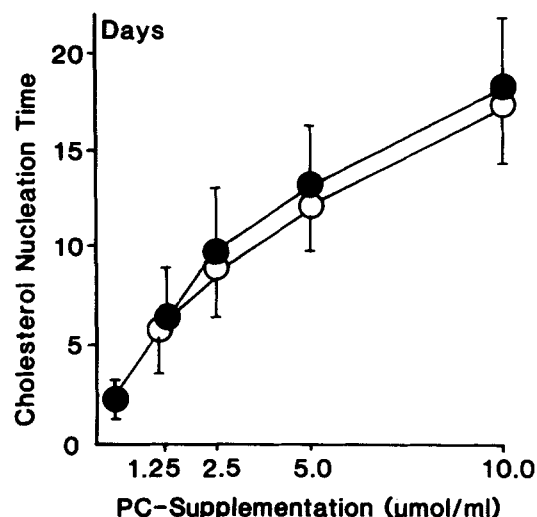


Fig. 2. Effect of bile supplementation with extracted phosphatidylcholines from gallbladder bile of patients with cholesterol (○) or pigment (●) stones on cholesterol nucleation time in gallbladder biles of 12 patients with cholesterol gallstones.

the cholesterol nucleation time (2.2 ± 0.3 days) significantly (**Fig. 4**). In additional experiments the effect of bile acids on vesicular/micellar cholesterol in gallbladder bile of patients with cholesterol stones was investigated.

Ten gallbladder biles were supplemented with a mixture (3:1) of glycine/taurine conjugates of cholic (44%), chenodeoxycholic (38%), and deoxycholic (18%) acid. As expected from the previous experiments, the addition of this bile acid mixture (5.0, 10.0, and 20.0 $\mu\text{mol/ml}$ bile) did not affect the cholesterol nucleation time (2.0 ± 0.5 days). Furthermore, the percentage of total biliary cholesterol in vesicles ($25.5 \pm 4.1\%$) or the cholesterol/phospholipid ratio in vesicles (0.7 ± 0.1) did not change (**Fig. 5**).

DISCUSSION

Phosphatidylcholine is the predominant phospholipid in bile and has a major role in the solubilization and transport of biliary cholesterol. Although the bile acids are generally recognized as the principal solubilizing agents in bile, their capacity to render cholesterol soluble in bile is severely reduced in the absence of phospholipids (28). The supplementation experiments of this study using pure synthetic crystalline disaturated phosphatidylcholines showed that the addition of increasing doses to human biles progressively prolonged the nucleation time. Slight differences in the antinucleatory effect of these phosphatidylcholines might be related to the different length of the fatty acid chains. It has to be noted that the completely saturated phosphatidylcholines used for sup-

plementation in the study do not occur physiologically in bile. Therefore, additional studies in gallbladder biles from patients with cholesterol gallstones using extracted phosphatidylcholines from human gallbladder bile were performed. These supplementation experiments showed a comparable increase of the cholesterol nucleation time as in the studies using synthetic disaturated phosphatidylcholines. Moreover, phosphatidylcholines extracted from bile from patients with cholesterol stones seemed to be as effective as phosphatidylcholines extracted from bile from patients with pigment stones. However, gallbladder bile phosphatidylcholines were recovered after iodine visualization of the chromatography plates. Iodination occurs across double bonds and may lead to a relative decrease in polyunsaturated fatty acids with a proportional increase in more saturated fatty acids (29). Subjects with cholesterol gallstones were reported to have alterations in the molecular species of PC in bile, although the results of various studies are conflicting (15, 17, 30–32). Therefore, our findings do not exclude that a different pattern of the molecular species of PC in bile is responsible for the

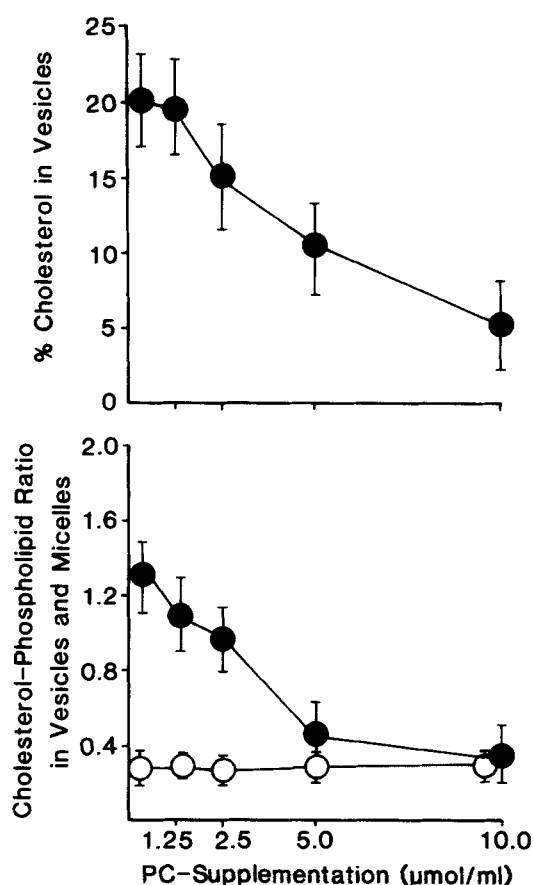


Fig. 3. Effect of bile supplementation with extracted phosphatidylcholines from gallbladder bile on the percentage of total biliary cholesterol in vesicles and cholesterol/phospholipid ratio in vesicles (●) and micelles (○) in gallbladder biles of 12 patients with cholesterol gallstones.

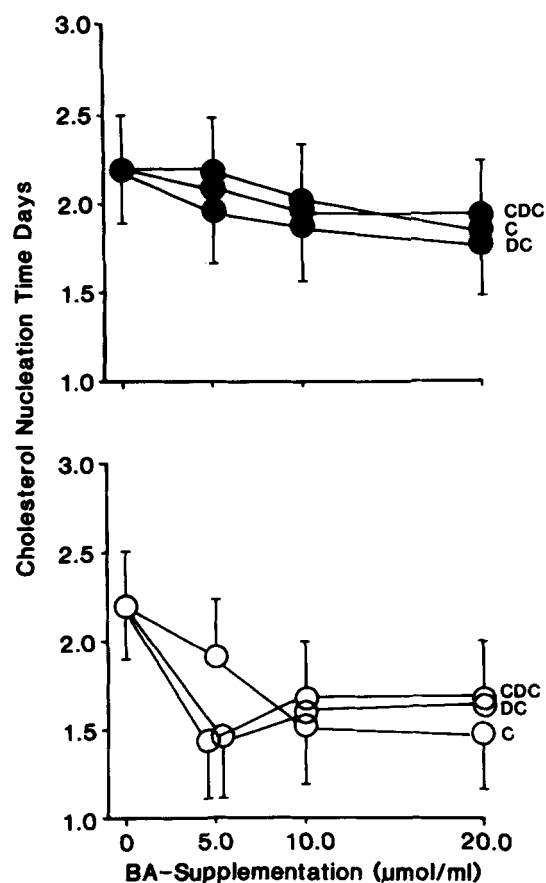


Fig. 4. Effect of bile supplementation with single glycine (O) or taurine (●) conjugates of cholic, chenodeoxycholic, and deoxycholic acid on cholesterol nucleation time in gallbladder bile of 15 patients with cholesterol gallstones.

more rapid nucleation of cholesterol in gallbladder bile of patients with cholesterol stones compared to biles from patients with pigment stones.

Several studies suggest that cholesterol crystals in bile derive mainly from cholesterol-rich phospholipid vesicles (5, 33) and a close association of crystals and these vesicles has been reported (34). The addition of phosphatidylcholines to bile might stabilize these vesicles and enhance their capacity to transport cholesterol. In our study even small amounts (1.25 μmol/ml) of added PCs prolonged cholesterol nucleation significantly. A decrease of the CH/PL ratio in isolated vesicles was observed which might be responsible for the increased cholesterol nucleation time. Furthermore, our experiments demonstrate a shift of biliary cholesterol from the less stable vesicular phase to the more stable mixed micellar phase after the addition of increasing doses of phospholipids extracted from gallbladder bile. Thus, the observed prolongation and normalization of the cholesterol nucleation time after adding these phosphatidylcholines might be caused by both a phase transition of biliary cholesterol and a decreased CH/PL ratio in the remaining vesicles.

Kibe et al. (35) have studied extensively factors affecting cholesterol monohydrate crystal nucleation time in model systems of supersaturated bile. Increasing the degree of cholesterol supersaturation as an independent variable shortened the nucleation time in concentrated but not in dilute systems. A decrease in bile BA/PL molar ratio within the physiologically relevant range from 4.0 to 2.0 was accompanied by a prolonged nucleation time and favored spontaneous vesicle formation.

Phospholipid supplementation in our study decreased the BA/PL molar ratio from 2.8 to 2.0 within the physiologically relevant range, prolonged nucleation time but did not favor vesicle formation. In contrast to the study of Kibe et al. (35), a significant decrease of vesicular cholesterol was observed particularly in biles supplemented with higher doses of PC (5–10 μmol/ml). The reason for this discrepancy is unclear but demonstrates that observations in model biles may not be relevant for human biles.

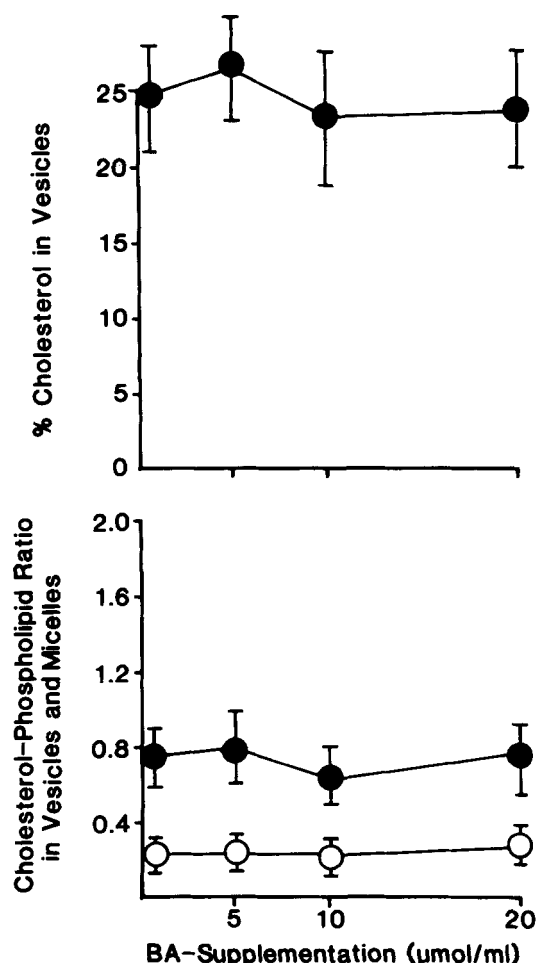


Fig. 5. Effect of bile supplementation with a mixture of glycine/taurine (3:1) conjugates of cholic (44%), chenodeoxycholic (38%), and deoxycholic (18%) acid on the percentage of total biliary cholesterol in vesicles and cholesterol/phospholipid ratio in vesicles (●) and micelles (O) in gallbladder biles of 10 patients with cholesterol gallstones.

The effect of phospholipids and their molecular species on cholesterol solubility and nucleation in human and model bile has been studied recently by Halpern et al. (36). In agreement with our results they found that the addition of egg phosphatidylcholine to human bile prolongs the nucleation time, shifts cholesterol from the vesicular phase to the non-vesicular phase, and reduces the CH/PL ratio of the remaining vesicles. Donovan, Timofeyeva, and Carey (37) have monitored the formation of liquid crystals and cholesterol monohydrate crystals in supersaturated model bile (10% total lipid) at comparable cholesterol saturation indices for various egg yolk PC/BA ratios (0, 0.25, 0.67). For fixed PC/BA ratios and CSI, cholesterol nucleation times increased at high PC/BA ratios. Therefore, several studies in human or model bile demonstrate a beneficial effect of increasing PC concentrations on biliary lithogenicity.

Furthermore, Cohen and Carey (38) have shown that acyl chain unsaturation modulates distribution of lecithin molecular species between mixed micelles and vesicles in model bile. Vesicles were enriched with lecithins containing the most saturated *sn*-1 or *sn*-2 chains by as much as 2.4-fold whereas mixed micelles were enriched with the more unsaturated lecithins. They suggest that modifications of biliary lecithins species may profoundly influence the physical chemistry of native bile as well as gallstone formation and prevention. Similar results have been reported more recently by Booker et al. (39) for human gallbladder bile. As in our study, bile was fractionated by Sephacryl S-300 SF gel filtration chromatography, and a sodium cholate concentration of 10 mM was chosen for all chromatographic separation of lipid particles. Gel filtration is capable of providing separation of discrete populations of micelles and vesicles, but the procedure may not ensure the integrity of each (40). If the correct IMC of BA were present in the eluant buffer and assuming there is no interaction between the column matrix and BA, then micelles and vesicles of native bile could be separated without alterations in the size or the composition of each phase. Donovan, Timofeyeva, and Carey (41) have studied the influence of total lipid concentration, bile salt:lecithin ratio, and cholesterol content on inter-mixed micellar/vesicular (non-lecithin-associated) BA concentrations in model bile. They concluded that by using an eluant containing 10 mM sodium cholate, separation of vesicles and micelles from model bile with total lipid concentrations similar to gallbladder bile may overestimate the proportion of vesicular lipids. Therefore, it appears that, in absolute terms, an accurate assessment of cholesterol transport in terms of a micelle:vesicle ratio for a given system is difficult. However, if experimental conditions are kept constant for the same study, micelle:vesicle ratios are reasonably accurate in relative terms (42). This seems to be particularly relevant for our study where changes of the micelle:vesicle ratios are determined before

and after PC or BA supplementation in different aliquots from one original gallbladder bile.


One can speculate from these *in vitro* studies that increasing phosphatidylcholine concentration in bile might be a promising approach in preventing cholesterol gallstone disease. However, we must consider that our *in vitro* experiments might be quite unphysiological. As we know from Gallinger et al. (43) and Pattinson and Willis (44) ultracentrifugation of bile samples prior to the nucleation assay to remove cholesterol monohydrate crystals does reduce considerably the amount of mucin in the remaining isotropic phase. Mucin is considered as a potent nucleation factor (45) and its partial reduction in the bile samples by ultracentrifugation might affect the results of our study. Furthermore, phospholipids do not enter bile from a thin film applied to a surface but are most likely secreted in vesicular form (46, 47). Micellar cholesterol is solubilized in the phospholipid bilayer of the mixed micelles and if phospholipid is secreted in vesicles, then it must be transferred to micelles. It seems entirely possible that provision of a thin film of phospholipids might permit transfer of these lipids mostly or exclusively to micelles and thus produce very beneficial micelles in respect to cholesterol transport. However, such a selective enrichment of micelles with phospholipids has not been observed in our study. The CH/PL ratio in mixed micelles remained rather constant (0.26–0.31) in our PC-supplementation experiments with a nonsignificant trend to increase as a result of dissolving CH/PL vesicles with higher CH/PL ratio. In fact, our results demonstrate that PCs provided as a thin film on the surface of the test tube are incorporated in the vesicles, which results in a marked decrease from 1.3 to 0.4 of their CH/PL ratio.

An antinucleating effect such as that demonstrated with phosphatidylcholine could not be observed after the addition of different molecular species of bile salts. These were added as physiologically occurring sodium salts of glycine or taurine conjugates of cholic acid, deoxycholic acid, or chenodeoxycholic acid. At a decrease of saturation of bile comparable to that in the phospholipid experiments, no effect on the cholesterol nucleation time was observed.

To investigate why addition of bile acids did not increase nucleation time, a mixture of physiologically occurring sodium salts of glycine:taurine (3:1) conjugates of cholic acid, deoxycholic acid, and chenodeoxycholic acid was added to the bile samples and the distribution and composition of vesicles and mixed micelles in bile were studied. No dissolution of phospholipid-cholesterol vesicles was observed when up to 20 $\mu\text{mol/ml}$ bile acids was added and the cholesterol-phospholipid ratio in vesicles and micelles did not change significantly in these experiments. These results could explain why cholesterol nucleation time in human bile was not affected by supplementation of different bile acids in amounts from 5–20 $\mu\text{mol/ml}$ bile.

Donovan et al. (37) have studied the effects of bile salt hydrophobicity on cholesterol nucleation in model bile. For fixed PC/BA concentration, cholesterol nucleation time increased with the hydrophilicity of the bile salts: tauroursodeoxycholic acid > taurocholic acid and taurodeoxycholic acid. The authors concluded that increasing bile salt hydrophobicity promotes cholesterol crystal formation and hydrophilic bile salts retard nucleation of cholesterol.

In our study, supplementation of bile with glycine or taurine conjugates of chenodeoxycholic acid, deoxycholic acid, or cholic acid had no significant effect on formation of cholesterol crystals. The effect of the hydrophilic bile acid ursodeoxycholic acid was not investigated in our study because of its physiologically low amounts in human bile. However, it has been demonstrated recently in several independent studies that oral administration of ursodeoxycholic acid prolongs nucleation time in human gallbladder bile (48–51). In all these studies a higher PL/CH ratio in gallbladder bile was found in UDCA-treated patients compared to untreated controls. This may be a further mechanism besides the observed absolute increase of UDCA in bile that could be responsible for the retarded nucleation of cholesterol. Similarly, oral administration of chenodeoxycholic acid to patients with gallstones prolongs cholesterol nucleation time and increases PL/CH ratio in bile (50–52).

We conclude that the addition of different phosphatidylcholines including extracted mixtures from human gallbladder bile but not physiologically occurring bile acids to bile in amounts that comparably decrease the CSI prolong the cholesterol nucleation time. This is caused by both a phase transition of cholesterol from vesicles to mixed micelles and a decreased cholesterol phospholipid ratio of the remaining vesicles. One can speculate from these in vitro studies that increasing phospholipid concentrations in bile might be more effective for the prevention of cholesterol gallstones than increasing bile acid concentrations. 

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