

Dissolution of Cholesterol Gallstones in Mice by the Oral Administration of a Fatty Acid Bile Acid Conjugate

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Gallstones, mostly cholesterol stones, affect some 15% of the population. Oral bile salts dissolve human cholesterol gallstones, but with low efficacy, and surgery remains the main therapeutic option. Fatty acid bile acid conjugates (FABACs) were shown to prevent formation of cholesterol gallstones in experimental animals. The aim of this study was to test whether these compounds could dissolve preexisting cholesterol gallstones via oral administration. Inbred, gallstone-susceptible C57J/L mice were given a lithogenic diet for 2 months, and the presence of gallstones was ascertained. The mice were then switched to a regular diet while part of them were given in addition C20-FABAC, by gavage, at a dose of 0.5 or 3 mg per animal per day. All mice tested had cholesterol gallstones after 2 months on the lithogenic diet. In study I, after 2 months on the regular diet, 3 of 4 (75%) of the controls had gallstones, whereas none of the 6 FABAC-fed animals (3 mg/d) had stones ($P = .033$). In study II, evaluating 2 FABAC doses, after 2 months on the regular diet, 8 of 8 (100%) of the controls had gallstones, which were found in 2 of 7 (28%) and 1 of 8 (12%) of the mice supplemented with 0.5 mg/d ($P = .007$) or 3 mg/d ($P = .001$) FABAC, respectively. On a molar basis, the dose of 0.5 mg FABAC is equivalent to 14 mg/kg/d of a bile acid. In conclusion, FABACs given orally can dissolve preexisting cholesterol gallstones in mice. This was accomplished with a dose of FABAC equivalent to the dose of bile acids used in human gallstone dissolution. (HEPATOLOGY 2002;35:597-600.)

Gallstones are found in about 15% of the population in most industrialized countries.¹ The great majority are cholesterol gallstones. Oral bile salt therapy can dissolve such gallstones, but was largely abandoned because of low efficacy. Surgery is currently the only practical option for patients with symptomatic and/or complicated gallstone disease. Fatty acid bile acid conjugates (FABACs) are a new family of synthetic molecules designed to solubilize biliary cholesterol. They prolong the nucleation time of model and human bile and markedly reduce the final crystal mass.² They dissolve preexisting cholesterol crystals *in vitro* and *in vivo*, and prevent the formation of cholesterol crystals in bile. Finally, they were shown to prevent the formation of cholesterol gallstones in inbred mice.³

In clinical practice, potential gallstone dissolution rather than prevention is the main problem. The present study was therefore designed to test whether the oral administration of FABACs is able to dissolve preexisting formed cholesterol gallstones. Arachidyl

amido cholanoic acid (Aramchol), an amide conjugate of arachidic and cholic acid, is one of the most effective FABACs³ and was used in this investigation. Inbred gallstone susceptible C57J/L mice rapidly and reliably develop cholesterol gallstones when fed a lithogenic diet⁴ and were used in the present study.

Materials and Methods

Animals and Diets. Inbred gallstone-susceptible C57J/L male mice, 4 to 5 weeks old, weighing approximately 20 g were used in all experiments. The lithogenic diet⁴ consisted of butter fat 15%, cholesterol 1%, cholic acid 0.5%, and corn oil 2% wt/wt, which were added to their regular diet (Koffolk; Petach Tikva, Israel). The mice were kept on a constant 12-hour day/night cycle, at room temperature (22°C). All examinations were performed after a 20-hour fast. Animals were operated on after ketamine anesthesia and sacrificed by overdose. The study was approved by the institutional committee for animal experiments.

Study Protocol. In study I, 17 inbred mice were given the lithogenic diet for 2 months. A group of the mice were then sacrificed and their gallstone status ascertained. The remaining mice were then reverted to a regular chow diet. Part served as controls, and the others were given in addition Aramchol, suspended in 0.5 mL saline, at a dose of 3 mg per animal per day by gavage. The controls were given an equal volume of saline only. After 1 month, some control and test animals were sacrificed and their gallstone status ascertained. After 2 months on the regular diet, all animals were sacrificed. The gallbladder was ligated, excised, and inspected. Bile was aspirated with a thin needle, and the gallbladder was then opened and again inspected for the presence of gallstones and sludge by using a stereoscopic light microscope and polarized light.

Abbreviations: FABAC, fatty acid bile acid conjugate; Aramchol, arachidyl amido cholanoic acid (C20-FABAC).

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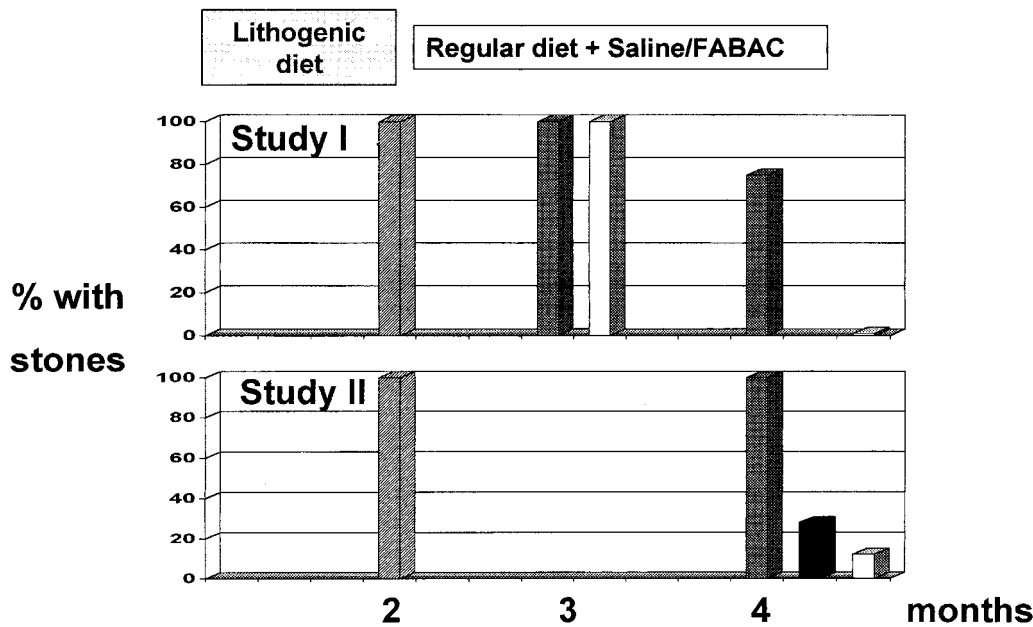


Fig. 1. Gallstone dissolution by FABAC in mice. Percent of mice with gallstones over time (▨) after 2 months on a lithogenic diet; or after reverting to a regular diet with (□) FABAC 3 mg/d; (■) FABAC 0.5 mg/d; **Upper panel** (study I): At 4 months none of the FABAC-treated animals had gallstones, compared with 75% in controls. **Lower panel** (study II): At 4 months 28% and 12% of the FABAC-treated animals (0.5 mg/d and 3 mg/d, respectively) had gallstones, compared with 100% in controls.

Stones were collected for chemical analysis. Blood was aspirated from the aorta. Liver and gallbladder were placed in formalin for histologic examination, and part of the liver was rapidly frozen for further tests.

In study II, which examined 2 different doses of Aramchol, the lithogenic diet was given as described above. After 2 months on the lithogenic diet all mice were reverted to a regular diet. Some served as controls and 2 test groups were given, in addition, 3 mg or 0.5 mg of Aramchol per animal per day by gavage. The interim examination after 1 month on the regular diet was omitted in study II. After 2 months all animals were sacrificed and tested as described above.

Analytical Methods. Biliary lipids were extracted by chloroform/methanol (2:1, vol/vol). Bile salts⁵ and cholesterol⁶ were quantitated enzymatically, while phospholipids were determined by the method of Bartlett.⁷ Cholesterol content of the gallstones was determined enzymatically (as described above in biles) after drying, pulverization of the stones, and dissolution in chloroform/methanol.

Statistical Analysis. In study I, the data of treated animals were compared with those of control animals using Fisher's exact test. $P < .05$ was considered statistically significant. In study II, according to the Bonferroni adjustment used to control for the experiment-wise error, only P values smaller than .017 were considered significant.

Results

Study I. After 2 months on the lithogenic diet all mice examined (4 of 4 = 100%) had abundant cholesterol gallstones in their gallbladders. After 1 month on the regular diet 1 control and 2 Aramchol-treated animals were sacrificed. All 3 had gallstones. After another month all animals were sacrificed. Three of 4 (75%) of the controls (diet only) had gallstones. None of the 6 mice given Aramchol had gallstones ($P = .033$) (Fig. 1). Biliary sludge was found in 1 of 4 (25%) of the control animals and 3 of 6 (50%) of the Aramchol-treated animals.

Study II. After 2 months on the lithogenic diet, all 13 mice then sacrificed had gallstones. The remaining 23 mice were switched to the regular diet. Eight served as controls, 7 received 0.5 mg per animal per day of Aramchol, and 8 received 3 mg per animal per day of the compound. All animals were sacrificed after 2 months on the regular diet. All 8 controls had multiple cholesterol gallstones in their gallbladders. Two of 7 mice (28%) given 0.5 mg/d of Aramchol had a single small gallstone in their gallbladders ($P = .007$ vs. controls). One of 8 (12%) mice given 3 mg/d of Aramchol had cholesterol gallstones ($P = .001$ vs. controls). The difference between the 2 Aramchol-treated groups was not significant ($P = .57$). Comparison of the 3 groups simultaneously revealed significant differences ($P = .001$) (Fig. 1). Biliary sludge only was found in 4 of 8 mice given Aramchol 3 mg/d.

The mean weight of the mice at the end of the experiments was 31.6 g in the controls, 30.2 g in the Aramchol 0.5 mg/d group, and 33.1 g in the Aramchol 3 mg/d group. There was no evidence of toxicity in the Aramchol-fed animals. Fatty liver was noted in many of the animals, as expected after a high-fat diet. Histologic examination of the livers did not show any pathologic findings attributable to Aramchol.

All gallstones analyzed contained greater than 65% of cholesterol per dry weight of stone. The biliary lipid composition of the mice at the end of both studies is shown in Table 1. There were no significant or consistent changes.

Discussion

This study has shown that preexisting formed cholesterol gallstones can be dissolved by the oral administration of a FABAC. The dosage of Aramchol of 0.5 mg per animal per day (=25 mg/kg/d) is equivalent on a molar basis to approximately 14 mg/kg/d of ursodeoxycholic acid. Hence, gallstone dissolution was accomplished with a FABAC dosage that is similar to the dosages of bile acids (cheno- or ursodeoxycholic) used in human gallstone dissolution.⁸⁻¹⁰

Table 1. Biliary Lipids at the End of the Study

	Cholesterol (mmol/L)	Phospholipid (mmol/L)	Bile Salt (mmol/L)	Total Lipids (g/dL)	CSI
Study I					
Controls LD + RD	2.3 ± 1	6.4 ± 5.9	76.7 ± 46	4.7 ± 3	1.16 ± 0.5
FABAC 3 mg	4.2 ± 1.2	9.6 ± 4.9	90.7 ± 19.3	5.8 ± 1.1	1.26 ± 0.74
Study II					
Controls LD + RD	1.4 ± 0.3	6.6 ± 1.3	48.7 ± 13.6	3.2 ± 0.8	0.67 ± 0.1
FABAC 0.5mg	1 ± 0.3	6.1 ± 2.1	51.5 ± 11.7	3.3 ± 0.7	0.52 ± 0.2
3mg	0.9 ± 0.2	4.2 ± 1.7	42.2 ± 10.4	2.6 ± 0.6	0.65 ± 0.2

NOTE. Values are means ± SD.

Abbreviations: CSI, cholesterol saturation index; RD, regular diet; LD, lithogenic diet.

Beynen¹¹ has shown in C57BL/U mice, that diet-induced gallstones persisted for more than 107 days after return to a regular diet. Gallstones continued to be present in 66% to 100% of animals sequentially examined during this time period. Our findings in C57J/L mice confirm this. After 2 months on a regular diet, gallstones were found in 75% of controls in study I and 100% of controls in study II. This stone persistence in the controls allowed us to show the dissolution potency of the FABACs.

These inbred mice, when fed the lithogenic diet, have an extremely rapid and potent lithogenic drive, much stronger than in known human "models." In our experience, close to 100% of these mice developed cholesterol crystals within 1 to 2 weeks and cholesterol gallstones within 3 to 4 weeks.^{2,3} These gallstones keep increasing in size and never disappear if the lithogenic diet is continued. In comparison, pregnant women develop biliary sludge (containing crystals) in some 30% of cases and gallstones in approximately 3% to 4%¹² but only after 9 months of pregnancy. After 3 to 6 months of rapid weight loss about 30% of humans develop gallstones.¹³ Because of these major differences, we chose to test the gallstone-dissolving capacity of FABACs in these inbred mice while on a regular diet.

It is noteworthy that gallstone dissolution was accomplished without any consistent effect on the biliary lipid composition. There were fluctuations in the concentrations of several biliary lipids in both studies. The changes were, however, devoid of statistical significance and did not show any consistent trend. This confirms our previous observations that FABACs do not significantly modify biliary lipids.^{14,15} Their stone-dissolving capacity must therefore be related to their direct effect on cholesterol solubilization. However, additional effects on nonlipid components in bile cannot be excluded. FABAC concentrations in gallbladder bile could not be measured in the present study because of the small volumes of bile, which were all used for lipid analyses. In previous studies the levels, a few hours after gavage, were in the range of 0.4 to 0.7 mmol/L and some 30 hours later were about 0.1 to 0.2 mmol/L.³

Biliary sludge was found in the gallbladder of 25% to 50% of the mice at the end of the trial period. Sludge is a known finding, related to gallstones. It may precede and accompany gallstone formation.^{12,16} It may apparently also be found during and after gallstone dissolution, suggesting a reversal of the stone formation process.

All or most of the gallstones in this study were dissolved within 2 months. In human gallstone dissolution, using ursodeoxycholic

acid, the process usually lasts many months and even years.⁹ The differences in species, diet, lithogenic drive, size of gallstones, etc., do not permit us even to guess how long such a dissolution might take in humans. To be clinically useful, medical dissolution using FABACs would have to be considerably more rapid than that obtained with cheno- and ursodeoxycholic acid. Prevention of subsequent recurrence would be another prerequisite, which was not adequately fulfilled when using bile salts. FABACs were proven to prevent gallstone formation in mice.³ This implies a potential for the eventual dissolution and prevention of gallstone recurrence using a single medical agent. So far, no toxicity was noted in the experimental animals tested.¹ Our subsequent, much more extensive experience confirms this apparent lack of toxicity.^{15,17} A trial of dissolution in humans will, however, have to await formal toxicity studies and the appropriate permits.

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