Effect of binding of ionised calcium on the *in vitro* nucleation of cholesterol and calcium bilirubinate in human gall bladder bile

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SUMMARY Biliary calcium may be a nucleating agent in cholesterol cholelithiasis. A study was designed to determine the effect of binding of ionised calcium on *in vitro* nucleation time. Ultracentrifuged and microscopically clear gall bladder bile from cholesterol gall stone patients was divided into two aliquots. One aliquot served as control and ionised calcium was bound in the second aliquot by addition of EDTA. Nucleation time was observed for the two groups. Addition of EDTA had no effect on lipid composition of the biles. EDTA bound all ionised calcium. Calcium bilirubinate precipitated from all controls on day 1 but was absent in all samples with EDTA. Addition of EDTA had no effect on cholesterol crystal nucleation time; nucleation time was rapid in both the controls and samples with EDTA. Ionised calcium is essential for calcium bilirubinate precipitation but is not responsible for the rapid nucleation time of bile from cholesterol gall stone patients.

Nucleation of cholesterol from bile is an important early step in cholesterol gall stone formation.¹ Gall bladder bile of patients with cholesterol gall stones has a rapid *in vitro* nucleation time compared with equally supersaturated bile from control patients.²⁻⁴ Bile appears to contain antinucleating⁵ and pronucleating substances.^{4 6-8} Calcium is a candidate pronucleating substance as calcium bilirubinate is at the centre of many cholesterol gall stones;⁹⁻¹² also calcium might contribute to vesicle aggregation¹³ and thus accelerate nucleation of cholesterol monohydrate crystals. On the other hand we have previously shown total and free calcium concentration to be similar in bile from cholesterol gall stone patients and controls.³ Unconjugated bilirubin concentrations are not higher in cholesterol gall stones biles³ and total bilirubin concentrations are higher in controls than in cholesterol stone patients.^{3 14} These studies, however, do not exclude the possibility that calcium ions or calcium bilirubinate are necessary for rapid *in vitro* nucleation of bile. The purpose of the present study was to determine the effect of binding of

calcium ions with EDTA on *in vitro* nucleation time. If calcium was an important pronucleator considerable lengthening of the nucleation time under these conditions would be expected.

Methods

PATIENTS

Gall bladder bile was collected at surgery from 10 patients undergoing elective cholecystectomy for cholesterol cholelithiasis.³ The patients were otherwise well and had normal liver function tests including SGOT, alkaline phosphatase, and total bilirubin. The age ($\pm$ SD) of the patients was 52.6 years $\pm$ 19.1 and weight 67.3 kg $\pm$ 10.4. There were nine women and one male in the patient group.

Each patient gave informed consent for bile collection. This study was approved by the Human Ethics Committee of the University of Toronto.

BILE PREPARATION

Fresh bile samples were divided into 6.0 ml aliquots. One aliquot served as control and the second aliquot was added to sterile vacutainers containing EDTA (K₃) in water (Becton Dickinson, Mississauga, Canada). The final concentration of EDTA in the biles was 3.5 mg/ml. This concentration is about

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three times that required to bind calcium in blood (approximately 1 mg/ml)¹⁵ and was chosen because gall bladder bile contains three to four times more calcium than blood.^{2 3 16} As expected, the pH of the bile samples fell to 5.0–6.0 after addition of EDTA; therefore the samples were buffered with 20–50 µl 1N NaOH to raise the pH to within 0.1 pH units of that of the parent control bile. Both the control and EDTA aliquots were then ultracentrifuged at 100 000 g for two hours to generate a bile specimen containing no crystals or other particulate matter by light microscopy.^{2 3}

NUCLEATION TIME TECHNIQUE

The control aliquots and those with added EDTA were observed daily for nucleation of cholesterol and calcium bilirubinate crystals using a modification³ of the technique of Holan *et al.*² The appearance of cholesterol crystals marks the nucleation time (in days) of a bile sample.

During bile preparation and nucleation time assay the samples were handled using sterile techniques and all containers and instruments were prewarmed to 37°C. The sterility of the controls and mixtures was assured by aerobic and anaerobic culture of the biles on the day of collection and upon nucleation. Only samples which were sterile were included in the studies.

CHEMICAL METHODS

Bile salts,¹⁷ cholesterol¹⁸ and phospholipid¹⁹ were measured in all samples and in some of the mixtures. Cholesterol saturation is expressed as the cholesterol saturation index (CSI).²⁰ In some controls and mixtures, total calcium was measured by atomic

absorption spectrophotometry (aa/ae spectrophotometer, Instrumentation Laboratory Inc, Lexington, Mass.). Ionised calcium was measured with an ion-selective electrode (ICA 1 ionised calcium analyser, Radiometer, Copenhagen). The pH of the samples was measured with a pH meter (Corning pH meter 125, Corning Science Products, Medfield, Ma.). The ionised calcium concentrations reported in this study are based on the pH of each particular sample – that is, not corrected to standard pH.

Gall stones were obtained from all the study patients. The stones were dried, crushed, weighed, extracted in hexane and cholesterol measured by GLC.¹⁸ All patients had cholesterol gall stones (>70% cholesterol by weight).

STATISTICAL ANALYSIS

Nucleation times of the gall bladder biles with and without added EDTA were compared by Wilcoxon's test.²¹

Results

EFFECTS OF EDTA ON LIPID COMPOSITION OF GALL BLADDER BILE

The lipid composition of the control gall bladder biles are shown in Table 1. The values are within the range of previous reports.^{2–5} Addition of EDTA had no discernible effects on lipid composition in four patients in whom lipid composition was measured both before and after addition of EDTA (Table 1).

EFFECTS OF EDTA ON IONISED CALCIUM CONCENTRATION OF GALL BLADDER BILE

In samples from the first four patients, total calcium

Table 1 Lipid composition,* total lipid concentration and cholesterol saturation index of gall bladder bile samples

Patient	Bile salts (mmol/dl)	Cholesterol (mmol/dl)	Phospholipid (mmol/dl)	Total lipid concentration (g/dl)	Cholesterol saturation index
1	9.62	1.47	3.33	7.87	1.42
2	6.53	0.69	1.83	4.89	1.26
3	20.56	3.87	7.65	17.52	1.42
4	5.44	0.68	1.73	4.28	1.39
4 with EDTA†	5.33	0.63	1.80	4.26	1.27
5	13.02	1.20	3.66	9.69	0.99
6	12.07	1.15	3.34	8.96	1.05
7	10.20	1.48	3.58	8.36	1.33
8	9.79	1.34	3.98	8.42	1.15
8 with EDTA	9.79	1.19	3.62	8.03	1.11
9	10.23	1.14	3.27	7.80	1.12
9 with EDTA	10.92	1.23	3.47	8.53	1.12
10	21.98	2.37	5.27	15.79	1.17
10 with EDTA	21.79	2.50	5.23	15.72	1.24

*To convert bile salt, cholesterol, and phospholipid concentration to mg/dl, multiply by 491, 387 and 775 respectively.

†Concentration of EDTA in mixtures=3.5 mg/ml.

concentrations were measured in the controls and ionised calcium concentrations in the controls and mixtures. The results are shown in Table 2. The values for total and ionised calcium concentrations in the control biles are within the range of previous reports.^{2 3 16} As shown, there was no detectable ionised calcium after mixing with EDTA. Ionised calcium was not measured in samples from the other six patients because it was assumed from the preceding results that the dose of EDTA was sufficient to reduce concentrations to very low levels.

EFFECT OF EDTA ON NUCLEATION OF CHOLESTEROL MONOHYDRATE CRYSTALS AND CALCIUM BILIRUBINATE CRYSTALS

Binding of calcium with EDTA had no effect on the *in vitro* nucleation time of cholesterol monohydrate.

Table 2 Effects of EDTA on ionised calcium concentration of gall bladder bile*

Patient	Control gall bladder bile		Gall bladder bile + EDTA	pH†
	Total calcium	Ionised calcium	Ionised calcium	
1	4.92	1.12	0	7.45
2	2.60	0.93	0	7.61
3	6.75	1.52	0	7.22
4	5.40	1.07	0	6.96

*All calcium concentrations are mmol/l. To convert to mg/dl multiply by 4.0.

†pH of bile samples after EDTA and NaOH added (Samples were buffered with IN NaOH to raise the pH to within 0.1 pH unit of the controls).

Table 3 Effects of EDTA on nucleation time of cholesterol crystals from gall bladder bile

Patient	Nucleation time (d)	
	Control bile	Bile + EDTA
1	2	3
2	3	2
3	1	1
4	1	1
5	1	1
6	1	2
7	3	2
8	2	1
9	2	2
10	1	1

p=not significant (Wilcoxon's test).

Biles with added EDTA nucleated cholesterol monohydrate crystals on the same day as control biles, or one day earlier or one day later than control biles (Table 3). There was no difference in appearance of the crystals which precipitated from biles containing EDTA (Fig. 1).

We have previously reported that calcium bilirubinate crystals appear in bile samples on day one of the *in vitro* nucleation time test.³ This occurs in almost all biles whether from patients with cholesterol gall stones or from controls without biliary tract disease. Calcium bilirubinate precipitates were present in all control aliquots on day one in this study. None of the EDTA containing aliquots precipitated calcium bilirubinate. The observation for calcium bilirubinate crystals was continued for two days after the nucleation of cholesterol crystals.

Discussion

Calcium is present in bile in ionised form or bound to bile salts or proteins.^{22 23} Calcium salts are major constituents of pigment stones and their importance in pigment stone formation is well documented.²⁴ Calcium may also be important in cholesterol gall stone formation. Calcium salts are found in the centre of cholesterol gall stones⁹⁻¹² and it has been proposed that the nidus for cholesterol nucleation is made of calcium salts.²³ Recently vesicles have been shown to be a second cholesterol carrier in human bile²⁵ and calcium ions can act as a vesicle aggregating agent¹³ and might accelerate nucleation of cholesterol from bile by this mechanism.

Patients with cholesterol gall stones are readily distinguished from controls without gall stones and from patients with pigment gall stones by the *in vitro* nucleation time test.²⁻⁴ EDTA can reduce ionised calcium to undetectable levels in human bile but this has no effect on the *in vitro* nucleation time. Thus this study cannot support a role for calcium ions in the nucleation of cholesterol from bile.

Other studies from this laboratory have also looked for and failed to find a connection between biliary calcium and the rapid *in vitro* nucleation time of gall bladder bile from patients with stones. Gollish *et al*³ found that calcium bilirubinate crystals precipitate equally rapidly in abnormal and normal bile and that there was no correlation between the time for calcium bilirubinate precipitation and cholesterol crystal nucleation time. The levels of total and ionised calcium are not higher in the gall bladder bile of cholesterol stone patients compared with normal subjects.^{2 3 26} Unconjugated bilirubin, the anion which complexes with ionised calcium to form calcium bilirubinate, is present in similar concentrations in cholesterol gall stone and normal

Fig. 1 Photomicrographs of human gall bladder bile with (top and bottom right panels) and without (top and bottom left panels) addition of EDTA. Note typical polarising cholesterol monohydrate crystals in all samples but lack of calcium bilirubinate precipitates (granular background precipitates) in bile samples with added EDTA. Binding of ionised calcium with EDTA did not affect cholesterol crystal nucleation time. Magnification $\times 250$ with polarised light.

gall bladder bile.³ Only in pigment gall stone bile are concentrations of unconjugated bilirubin abnormally high.²⁴

As opposed to cholesterol, the precipitation of calcium bilirubinate was very sensitive to chelation of ionised calcium. To our knowledge this is the first demonstration that precipitation of these typical crystals can be prevented in human gall bladder bile by EDTA. This finding provides encouragement to those seeking to prevent or treat pigment stones by reducing calcium levels in bile.

Finally, it is now known that cholesterol is transported in human bile both by mixed micelles and unilamellar vesicles.²⁵ The latter are not removed by the short period of ultracentrifugation used in these studies and are too small to be seen by light microscopy. The specimens used in this study were free of crystals or other particulate matter by

light microscopy. This had been previously referred to as the isotropic phase in the *in vitro* nucleation time technique.²⁻⁵ Although isotropic by light microscopy, it is not truly isotropic and we have avoided the term. There is some evidence that nucleation occurs from the vesicular phase.²⁷ This study indicates that Ca^{++} is not necessary for this to occur.

In summary, ionised calcium does not appear responsible for the rapid *in vitro* nucleation time of bile from cholesterol gall stone patients. We have reaffirmed the importance of ionised calcium in the precipitation of calcium bilirubinate from bile.

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