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Effect of calcium, magnesium and sodium ions on *in vitro* nucleation of human gall bladder bile

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SUMMARY **The effect of increasing the calcium, magnesium and sodium concentration in gall bladder bile samples from 21 patients with gall stones and nine controls on the *in vitro* rate of formation of cholesterol microcrystals and numbers of cholesterol microcrystals formed was examined. Addition of these cations to raise the mean maximum concentration of calcium ions to 19·8 mmol/l, of magnesium ions to 20 mmol/l and sodium ions to 998 mmol/l did not trigger nucleation in control bile samples or samples from patients with gall stones. Increasing the mean concentration of calcium ions to 8·6 mmol/l and of sodium to 320 mmol/l increased the numbers of cholesterol monohydrate crystals/0·1mm^3 counted by light polarisation phase contrast microscopy at the time of nucleation in samples from patients with gall stones from a median of 2 (range 1–10) in control portions to 18 (range 2–128) for calcium ions and 10 (range 2–141) for sodium ions ($p<0·001$). Calcium and magnesium ions were more effective than sodium ions, and calcium ions could increase crystal numbers at concentrations found in samples from patients with gall stones, median 4·6 mmol/l (range 2·7–16·9 mmol/l). The concentrations of calcium and magnesium present in bile may therefore influence the rate of development of gall stones.**

The mechanism of formation of cholesterol microcrystals in bile is not yet understood but supersaturation of bile has been shown not to be the sole cause.[1] Human bile contains both unilamellar vesicles and mixed micelles.[2-5] Biliary cholesterol is associated with vesicles and micelles which are interconvertible.[3 4] Cholesterol microcrystals are thought to form when these supersaturated vesicles fuse to form multilamellar and microscopically visible liposomes.[5-7] From these the initial cholesterol monohydrate crystals may precipitate.[6 7]

The nucleation time of gall bladder bile from patients with gall stones has been shown to be more rapid than controls with similar cholesterol saturations.[1 8 9] Both pronucleation and antinucleation factors may be present in gall bladder bile.[9-11] Biliary cations may be nucleating factors as studies of model bile systems have shown that micellar growth occurs with increasing cation concentration and that vesicle fusion is enhanced by calcium ions.[12 13] In addition calcium bilirubinate, calcium phosphate and calcium carbonate have been found in cholesterol gall stones. If the fusion of supersaturated vesicles and micelles is caused by calcium ions and is due to interaction of the ions with vesicle surface charge then other cations may also promote vesicle aggregation. In this study the effect of the addition of calcium, magnesium, and sodium ions to gall bladder bile on the nucleation time and numbers of cholesterol microcrystals formed was examined.

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Methods

PATIENTS

Gall bladder bile was aspirated during elective cholecystectomy in 21 patients with cholesterol cholelithiasis. It was also collected from three patients during cholecystoduodenostomy which was undertaken in two patients for carcinoma of the head of the pancreas and in one for biliary obstruction as a result of chronic pancreatitis and pseudocyst formation. Gall bladder bile was also collected from four

patients with acalculous cholecystitis and two before chemotherapy for hepatic metastases all of whom also underwent elective cholecystectomy. The nine samples from patients undergoing cholecystoduodenostomy and cholecystectomy for acalculous cholecystitis or prior to chemotherapy were used as a control group. Seventeen of the samples collected during cholecystectomy for cholelithiasis were from women and four from men, mean age 44 (12) years. The nine control bile samples came from five women and four men, mean age 54 (23) years.

SAMPLE PREPARATION

Bile samples were collected in vacutainers (Becton Dickinson, Meglan Cedex, France). They were centrifuged at 100 000 *g* for two hours in a Beckman L5-65 preparative ultracentrifuge (Beckman, High Wycombe, Buckinghamshire) using a T65 rotor preheated to 37°C. The crystal free middle portion of the sample was then aspirated.[1] This was divided and 2 ml frozen at −20°C for measurement of Na^+, K^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, and Cu^{2+} concentrations. The remainder was examined microscopically to ensure the absence of microcrystals or other particulate material prior to nucleation and cation addition experiments. All glassware was heated to 37°C before use.

CATION ADDITION EXPERIMENTS

The concentrations of sodium, calcium and magnesium were augmented in samples of bile from controls and patients with gall stones. Stock 5 mol/l NaCl was used to prepare working solutions of 5 mol/l, 2·5 mol/l, 1·25 mol/l, and 0·625 mol/l NaCl. The pH was adjusted to 7·4 with 0·1 mol/l NaOH. One hundred microlitre portions of these solutions at 37°C were added to 500 µl portions of each centrifuged bile fraction. To a control portion 100 µl distilled water was added. Additionally 50 µl of 160 mmol/l $CaCl_2$ and $MgCl_2$ solutions were added to 500 µl portions of bile. A 50 µl addition with distilled water as control was also made. In a second experiment to investigate the effect of different concentrations of calcium on nucleation 50 µl portions of 160 mmol/l, 80 mmol/l, 40 mmol/l, 20 mmol/l, and 10 mmol/l $CaCl_2$ and distilled water were added at 37°C to 450 µl portions of the centrifuged bile, also at 37°C.

In these experiments the bile portions were incubated at 37°C in a waterbath in the dark under a nitrogen atmosphere. Each portion was examined daily for the appearance of crystals. On termination of these experiments all portions were frozen at −20°C before measurement of the final concentrations of sodium and calcium ions. All chemicals were Analar grade.

NUCLEATION

The portions of bile were incubated in glass vials (Magnus Scientific Instrumentation Ltd, Aylesbury, Buckinghamshire) under a nitrogen atmosphere sealed with a screwtop containing a rubber septum. One drop of incubate was aspirated daily by aseptic technique and examined on a glass slide at a magnification of ×400 using light polarisation phase contrast microscopy (Leitz, Wetzlar, Germany). When cholesterol microcrystals were first observed they were counted in triplicate using an improved Newbauer microscope counting chamber (Hawksley, Lancing, Sussex). If no crystals were observed by 21 days the incubation was terminated. Portions of bile used in these experiments were cultured anaerobically and aerobically, only sterile samples were used.

CATION CONCENTRATIONS

The concentrations of sodium and potassium in the 100 000 *g* bile fractions were measured with an Instrumentation Laboratory 343 flame photometer (Instrumentation Laboratory, Warrington, Cheshire). Concentrations of Ca^{2+}, Mg^{2+}, Zn^{2+}, and Cu^{2+} were measured using an Instrumentation Laboratory Video II atomic absorption spectrophotometer.

STATISTICAL ANALYSIS

The sign rank Wilcoxon's pairs test and the Mann Whitney test were used to compare crystal numbers in the incubates.

Results

Not all of the samples were used in each experiment because of variation in sample size. Figure 1 shows examples of cholesterol monohydrate crystals and calcium bilirubinate crystals photographed during light polarisation phase contrast microscopy.

CATION CONCENTRATIONS

The range of concentrations of each cation in bile from patients with gall stones and controls is shown in Figure 2. The highest magnesium and calcium concentrations in samples from patients with gall stones were double those of the controls. Using the Mann Whitney test there was no statistical difference in the concentrations of the biliary cations between controls and patients with gall stones.

NUCLEATION

All portions of one control bile sample nucleated on day 10. The addition of calcium, magnesium, or sodium did not alter the nucleation time of any control samples. The median nucleation time of bile samples from patients with gall stones was one day

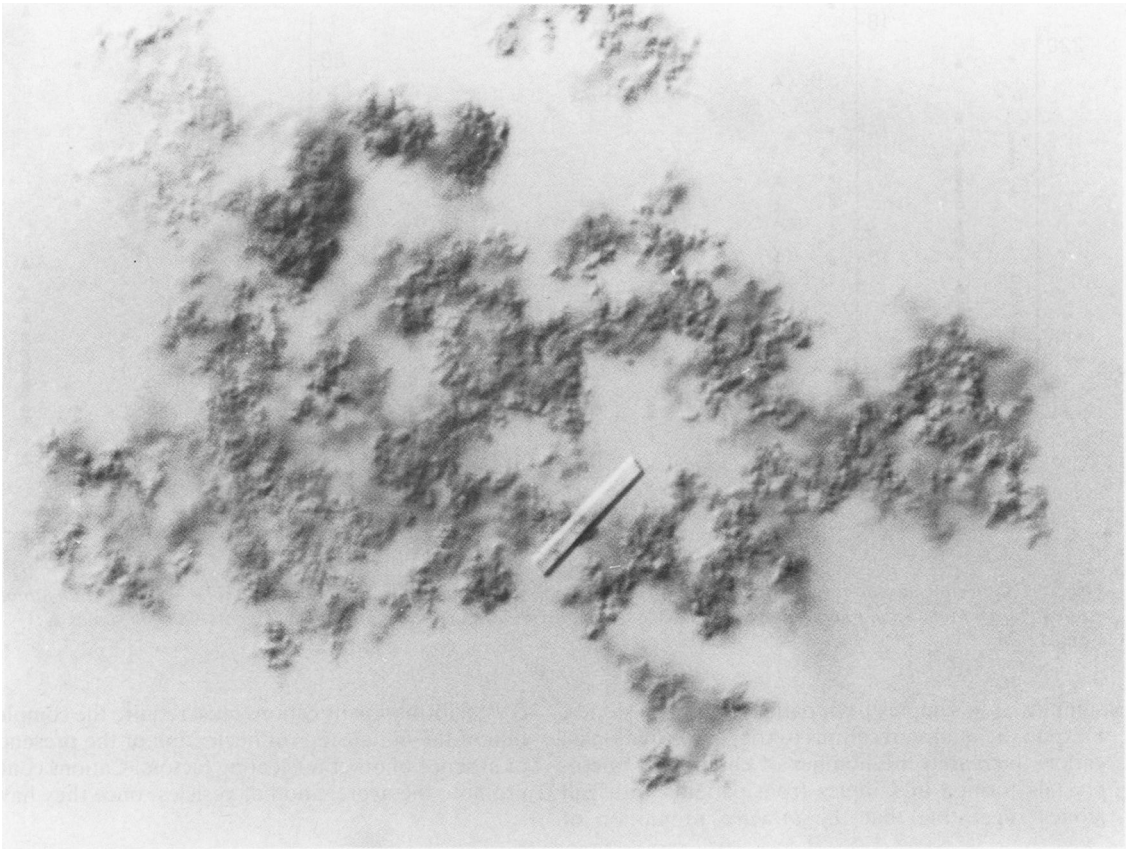

Fig. 1 *Example cholesterol monohydrate and calcium bilirubinate crystals seen under light polarisation phase contrast microscopy.*

(range one to four days). The addition of calcium, magnesium, or sodium ions to portions of these bile samples did not alter the nucleation time of these cation augmented portions when compared with control portions. The numbers of cholesterol microcrystals present at nucleation in portions with 100 µl 5 mol/l NaCl or 50 µl 160 mmol/l $CaCl_2$ or $MgCl_2$ added were significantly greater than in control portions ($p<0{\cdot}001$) (Table 1). The median numbers of microcrystals formed in sodium, 15 (range 3–160) and calcium, 20 (range 8–254) incubates were not statistically different nor were the numbers of crystals in the sodium and magnesium, 14 range (6–120), incubates.

CHOLESTEROL MICROCRYSTALS

When increasing quantities of sodium ions were added to 14 bile samples (Table 2) the numbers of cholesterol microcrystals were significantly greater in all portions than in the control portion ($p<0{\cdot}001$). The numbers of crystals tended to increase with increasing sodium concentration. The increase in the crystal numbers was statistically significant ($p<0{\cdot}01$) at each increment in the sodium concentration.

When calcium ions were added in increasing concentration (Table 3) the numbers of cholesterol microcrystals present at nucleation was greater than the control when the mean final calcium concentration was 8·6 mmol/l or greater ($p<0{\cdot}001$). The numbers of cholesterol microcrystals in the 9·9 and 19·6 mmol/l portions were also significantly greater than in the 8·6 mmol/l portions ($p<0{\cdot}001$).

Discussion

Although calcium ions have been shown to act as vesicle aggregating agents in model bile systems, and in doing so accelerate nucleation[12] in the experiments reported here the addition of calcium, magnesium and sodium did not accelerate nucleation, in control

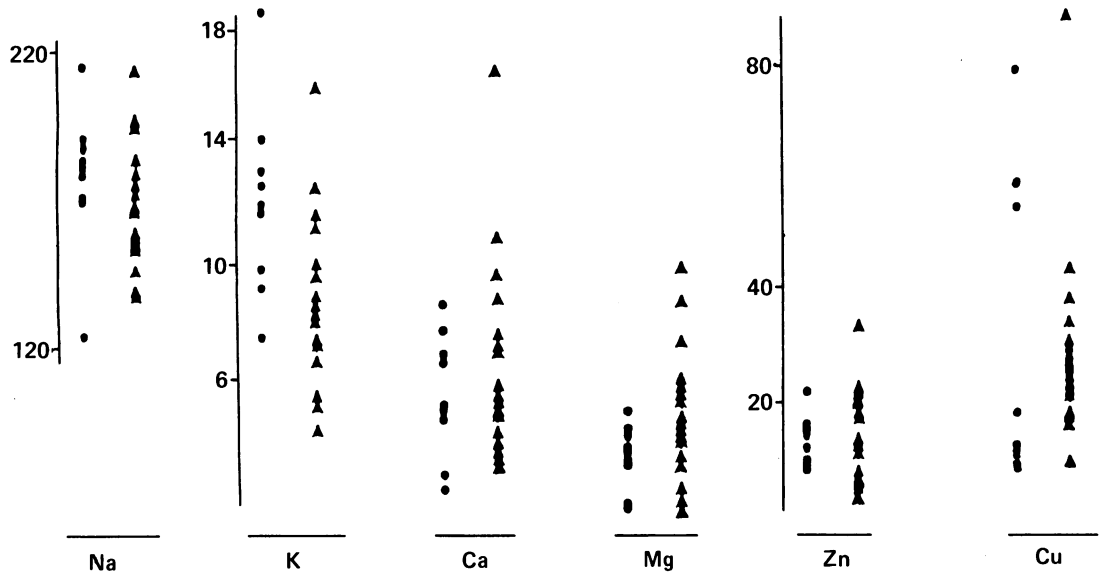

Fig. 2 *Cation concentrations in samples from patients with gall stones and controls. Sodium mmol/l – Na; potassium mmol/l – K; calcium mmol/l – Ca; magnesium mmol/l – Mg; zinc μmol/l – Zn; copper μmol/l – Cu. Patients with gall stones ▲; Controls ●.*

samples or in samples from patients with gall stones. Despite the failure of cations to trigger nucleation all cations increased the number of cholesterol microcrystals formed in samples from patients with gall stones suggesting that the *in vitro* promotion of crystal formation by cations must require the completion of the initial steps of nucleation or the presence or absence of other nucleating factors. Cations could promote the aggregation of vesicles, once they have

Table 1 *Number of cholesterol crystals/0·1 mm³ in bile samples from patients with gall stones, on the day of nucleation when incubated with 5 mol/l sodium, 160 mmol/l calcium, or 160 mmol/l magnesium*

		Number of crystals/0·1mm²		
Sample	*Control*	*Sodium*	*Calcium*	*Magnesium*
1	1	3	22	14
2	2	8	21	18
3	3	3	15	13
4	2	10	8	6
5	3	20	10	7
6	1	3	15	10
7	1	20	16	8
8	5	160	135	14
9	8	93	119	120
10	2	10	166	48
11	10	111	254	120
12	8	34	19	16
Median number of crystals	2·5	15*	20*	14*
Range	1–10	3–160	8–254	6–120

*Significantly different from control by sign rank Wilcoxon's test ($p<0·001$).

Table 2 *Number of cholesterol microcrystals/0·1 mm³ in bile samples from patients with gall stones on the day of nucleation when incubated at 37°C with increasing concentrations of sodium ions. The number of crystals present are listed below the mean concentration of sodium in each portion*

Mean sodium concentrations (mmol/l)		*Number of crystals/0·1mm²*			
	Control	*320*	*420*	*640*	*998*
Sample		*	*	*	*
1	1	2	9	1	3
2	2	6	6	12	8
3	3	10	6	1	3
4	2	6	4	4	10
5	3	5	8	11	20
6	1	10	12	6	3
7	1	3	4	11	20
8	5	141	200	226	160
9	8	70	91	63	93
10	2	6	7	7	10
11	10	18	23	53	111
12	8	24	26	25	34
13	2	8	36	245	321
14	1	4	10	24	43

Crystal number in column marked * are significantly greater ($p<0·001$) than control by sign rank Wilcoxon's test.

Table 3 *Number of cholesterol microcrystals/0·1 mm³ in samples from patients with gall stones on the day of nucleation when incubated at 37°C with increasing concentrations of calcium ions. The number of crystals present are listed below the mean concentration of calcium in each portion*

Mean calcium concentration (mmol/l)	*Number of crystals/0·1 mm³*					
	Control	*6·2*	*7·0*	*8·6*	*9·9*	*19·4*
Sample				*	*	*
1	2	2	7	24	54	37
2	5	4	3	11	55	108
3	7	5	9	7	17	19
4	2	3	9	128	260	324
5	1	1	1	2	4	12
6	2	4	10	60	120	200

Crystal numbers in column marked * are significantly greater ($p<0·01$) than control by sign rank Wilcoxon's test.

formed, by a reduction of their net negative surface charge thereby reducing intervesicle repulsive forces.[14] This may promote the formation of multilamellar liposomes from which crystallisation may occur.

Calcium and magnesium ions increased cholesterol microcrystal numbers at lower concentrations than sodium ions. If the nucleating effect of cations is caused by a reduction of vesicular surface charge then this difference in efficiency is predicted by colloid and emulsion theory.[15] The greater efficiency of calcium and magnesium indicate that they could contribute to the formation of large numbers of cholesterol microcrystals at the pathophysiological concentrations found in samples from patients with gall stones. The other divalent and trivalent cations (Zn^{2+}, Cu^{2+}, Fe^{3+}) are present in micromolar concentration and are unlikely to contribute significantly to cholesterol crystal formation.[16]

Although the middle portions of centrifuged bile samples used were free of particulate material by light microscopy vesicles have been shown to be present in these preparations of both control and abnormal bile samples.[5]

Gall bladder water absorption increases during the early stages of cholelithiasis. The high concentrations of cations found in some samples from patients with gall stones may be due to this rather than increased secretion of cations.[17,18] If this is the case then high concentrations of calcium and magnesium may not be unusual during the early stages of cholelithiasis. Similarly gall bladder absorption of water is reduced during the later stages of cholelithiasis and the low concentrations of cations found in some samples may represent longstanding cholelithiasis.

In conclusion, the concentrations of the divalent cations, calcium and magnesium, present in bile samples from patients with cholesterol cholelithiasis could promote the formation of large numbers of cholesterol monohydrate microcrystals. This could increase the rate of development of gall stones or retard dissolution of gall stones during treatment with bile salts.

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