CHAPTER - 2

Cholesterol, Phospholipid and Bile Salt Composition of Gallbladder Bile of Controls and Patients with Gallstone Belonging to the Indian Ganges Delta.

INTRODUCTION

Cholesterol is the major constituent of gallstone. It is considered that in bile, cholesterol remains in the form of mixed micelles with bile salt and phospholipid as well as in phospholipid vesicles\(^1,2\); the vesicles undergo aggregation to form cholesterol microcrystals, a prerequisite of gallstone. Therefore, a major consideration is that the biles of patients with cholesterol gallstone are supersaturated in cholesterol\(^3-9\). Under subtle environmental changes, such biles may release cholesterol and the subjects are susceptible to the gallstone disease. So far most of the studies on bile composition on patients with cholesterol gallstones have been reported from well nourished populations of the economically developed western countries. The subjects of this study in whom the gallbladder bile composition has been studied belong to the Indian Ganges delta, where the incidence of gallbladder disease is known to be high\(^10\). The population under study has a marginal nutritional status, and subsists on low calorie, infrequent meals, the main staple being rice. The composition of bile
has been analysed with reference to the maximum micellar solubility of cholesterol (described by Admirand and Small\textsuperscript{3} and Holzbach et al.\textsuperscript{4}) lithogenic index\textsuperscript{11} and the capacity of bile to solubilise added cholesterol\textsuperscript{3}.

**EXPERIMENTAL**

**Materials:**

Bile samples from the gallbladder were obtained at surgery by needle aspiration in 64 subjects. Of these, 27 were controls (Male:Female = 10:17) who were undergoing upper gastrointestinal surgery and had no evidence of hepatic and gallbladder disease. The remaining 34 (Male:Female = 19:15) had cholesterol gallstones. Due care was taken to avoid contamination of gallbladder bile with hepatic bile, by clamping the cystic duct prior to aspiration and emptying the gallbladder completely. The bile samples were either frozen at -20°C or extracted immediately. All the samples were well shaken and were not subjected to centrifugation prior to analysis. The presence of free bile acids was checked by thin layer chromatography and cholesterol microcrystals by light microscopy.

**Analysis of Bile Salts:**

1 ml of bile was taken in a stoppered bottle and to it was added 1 ml of saturated barium hydroxide and 20 ml of
extraction mixture (12 ml of ethanol and 8 ml of A. R. Grade diethylether). This was kept at 60°C and shaken for 10 minutes, after which it was centrifuged. The supernatant was separated and the residue extracted two more times in a similar manner. The pooled extract was adjusted to pH 8 to 9 by the passage of CO₂. This was centrifuged and the supernatant dried in a 60°C water bath. The dried samples were dissolved in 1:1 ethanol:water mixture and shaken with 4 ml N-heptane in a separating funnel to remove neutral lipids. Adequate aliquots of the ethanolic layer were chromatographed on 0.25 mm silica gel G thin layer plates along with known standards of bile acids. The developing solvent was a mixture of iso-amyl acetate, pro-pionic acid, n-propanol and water (4:3:2:1 v/v) as described by Hofmann¹²,¹³. The plates were then dried, sprayed with 30% (w/v) sulphuric acid, and placed in a hot air oven at 90°C to 100°C to make the bands faintly visible in daylight. The bands containing the bile acids were carefully and completely scraped into 5 ml of 65% (W/W) sulphuric acid, incubated in a water bath for one hour at 60°C, and then centrifuged. Optical density of the supernatant was read spectrophotometrically at 385 nm and the bile salt concentrations were calculated according to the method of Ganshirt et al.¹⁴. The recovery of known amounts of taurocholate, taurochenodeoxycholate and taurodeoxycholate, glycocholate, glycochenodeoxycholate and glycodeoxycholate ranged from 81-85%.
**Estimation of total cholesterol:**

Total cholesterol was estimated according to Abell et al.\(^{15}\) modified for bile. 0.5 ml of bile was taken in a stoppered centrifuge tube incubated at 37°C for 55 mins, vigorously shaken with 10 ml of n-hexane for 1 min, centrifuged for 5 min. at 1000 r.p.m. the supernatant dried and colour developed with Liebermann Burchard reagent and measured colorimetrically at 620 nm.

**Estimation of Phospholipid:**

The phospholipids were extracted from the bile samples following the method of Bligh and Dyer\(^{16}\). 0.5 ml of the bile was mixed with 5 ml of methanol-chloroform mixture (2:1, v/v) in a separating funnel to form a monophasic solution. The solution was well shaken; to it was added 1.3 ml each of chloroform and water and the phases were allowed to separate. The lower organic layer was withdrawn and the solvent removed in a rotary evaporator at 30-35°C. The small amount of water left was removed by repeated addition and evaporation of small amounts of benzene. The residue was redissolved in a suitable volume of chloroform and the total phosphorus was measured in an aliquot according to Ames and Dubin\(^{17}\) and expressed as distearyl lecithin. (32 mg P is equivalent to 790 mg of distearyl lecithin).
Cholesterol solubility:

Cholesterol (J. T. Baker, U.S.A.) recrystallised thrice from hot methanol (m.p 147.0°C) and checked by melting point determination, was added to bile in sufficient quantity and the mixture was equilibrated for 72 hours at 37°C in a shaking water bath. After equilibration, the bile was centrifuged at 16,000 r.p.m. for 30 minutes and was then filtered through a millipore, 0.22 micron filter (Millipore Corpn. Bedford. Mass). The filtrate was separated and analysed for cholesterol by Abell et al's\textsuperscript{15} method.

RESULTS

Bile composition and phase diagrams:

Table I shows the mean values (\pm SE) of cholesterol, bile salts and phospholipids of gallbladder bile in subjects with cholesterol-gallstone and in controls. The relative proportions of cholesterol, bile acids and lecithin are same in the two group of subjects. However, the concentration of bile salts is lower and that of cholesterol higher in controls than those reported from the West\textsuperscript{18}. In gallstone patients, concentrations of the major constituents are lower than those of the controls, suggesting a lower concentrating capacity of the gallbladder in the former.
Table 1

Mean Values of the major components of Gallbladder Bile in controls and in patients with gallstone.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 27)</th>
<th>Patients (n = 34)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/100 ml</td>
<td>mM/L</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Mean</td>
<td>SE*</td>
</tr>
<tr>
<td></td>
<td>769.0</td>
<td>72.7</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>1920.0</td>
<td>205.5</td>
</tr>
<tr>
<td>Total Bile Acid</td>
<td>4813.0</td>
<td>613.0</td>
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*SE*: Standard error of the mean
In figure 1 and 1a, results from both stone patients and controls have been plotted on triangular coordinates. In these diagrams, AB and CD represent the lines of maximum cholesterol solubility derived from the model systems of Admirand and Small$^3$ and Holzbach et al.$^4$, respectively. The points are mostly above the line AB and almost absolutely above the line CD. The scatter of the points is not different between the control and the patients. Thus 32% of the patients with gallstone have values below both the lines while as many as 61% of the controls are above the lines of maximum micellar solubility. One interesting feature, hitherto unreported, is that there is a trend towards a shift of the points to the right side of the phase diagram. This is in contrast to the findings of Admirand and Small$^3$, where most of the points were to the left of the triangle touching the base line of cholesterol at a point as high as 15%. Our data are at variance, being 10% less in bile salts, although maximal cholesterol content was as high as 15%.

In figure 2 the arithmetic mean of the three constituents of bile from controls as well as patients with gallstone have been plotted on the triangular co-ordinate along with their standard deviations. The area covered has been shown by joining the limits of these deviations. Mean values of the same constituents as reported by Heller & Bouchier$^{18}$ and Small and Rapo$^9$, have also been plotted on the same graph for comparison. The values obtained by us for controls as well as from
Figure 1: Major constituents of gallbladder bile in controls and patients with gallstone are plotted on triangular co-ordinates. Closed circles: Patients. Open circles: Controls.

AB: Line of maximum solubility of cholesterol according to Admirand and Small.

CD: Line of maximum solubility of cholesterol according to Holzbach et al.
Figure 2. △, Mean compositions of hepatic bile from patients with gallstone, according to Small and Rapo\textsuperscript{9}.

▲, Mean compositions of gallbladder bile from patients with gallstone according to Small and Rapo\textsuperscript{9}.

○, Mean compositions of gallbladder bile from controls according to Heller and Bouchier\textsuperscript{18}.

●, Mean compositions of gallbladder bile from patients with gallstone according to Heller and Bouchier\textsuperscript{18}.

□, Mean compositions of gallbladder bile from controls, the present study.

■, Mean compositions of gallbladder bile from patients with gallstone, the present study.
patients with gallstone are almost at the same point. The standard deviations cover a much wider area indicating a wider scatter than those reported earlier. Mean values of our controls as well as patients are between the mean values reported by Small and Rapo by Small and Rapo for hepatic and gallbladder biles.

It can also be seen that the mean values for gallbladder bile from patients as well as controls described by Heller et al. and patients with gallstone described by Small and Rapo are below the line of maximum cholesterol solubility defined by Admirand and Small but above the line of Holzbach et al.

**Trihydroxy/Dihydroxy ratio and Glycine/Taurine ratio:**

The proportion of Trihydroxy bile salts both in controls as well as in patients is higher than those reported previously. However, there is no difference in the trihydroxy/dihydroxy ratio between the control (2.1:1) and the patients (2.0:1).

The glycine:taurine ratio in controls is 2.30:1, whereas in patients 2.38:1.

**Relationship of lithogenic index to bile salt composition:**

Lithogenic index has been devised to give a numerical expression to micellar and nonmicellar characteristics of bile with regard to cholesterol solubility. An index greater than
than one in any patient indicates that the bile is supersaturated with respect to cholesterol. The mean lithogenic index derived from the equation of Holzbach et al.\textsuperscript{11} is comparable in the two group of subjects (control 2.59, stone 2.10). Figure 3 shows the relationship of lithogenic index to the ratio of dihydroxy:trihydroxy bile salts (D:T ratio). The proportion of dihydroxy bile salts increases with an increase in the lithogenic index with a poor correlation coefficient of 0.4. Such a relationship is not clearly observed with G:T ratio (results not shown).

Cholesterol solubility and its relation to lithogenic index, D:T ratio and G:T ratio.

The results presented in Fig.1 support undersaturation and supersaturation of cholesterol both in control as well as in patients with gallstones. It is to be noted that all the controls are supersaturated with respect to cholesterol i.e. addition of extra cholesterol has yielded significant negative solubility in these subjects. Of the 18 observations made, 7 have positive solubility, 5 negative solubility while the rest do not solubilise any extra cholesterol. Cholesterol solubility does not show any relationship with lithogenic index, G:T ratio as well as D:T ratio (results not shown).
Relation of lithogenic index (L.I) to dihydroxy/trihydroxy ratio of bile acids (D/T ratio) in gallbladder bile of controls (O) and in patients with gallstone (●). The least square line (not drawn) follows the relation, \( y = 1.864 + 1.246 x \), the correlation coefficient being 0.4.
Analysis of gallstones:

Gallstones from 41 patients are analysed for cholesterol\textsuperscript{15} bile acids\textsuperscript{12-14}, phospholipid\textsuperscript{16}, calcium\textsuperscript{22} and iron\textsuperscript{23} by standard methods. The mean cholesterol content is 65.8 ± 24.1 mg per g of stone. Significant amounts of bile salts are present in 12\% of the stones analysed. In ten patients phospholipid is also present; it is in traces in three while in the rest, it varies from 37 mg to 260 mg per g of stone. Mean calcium content of the stones is 11.8 ± 2.3 mg per g of stone. Calcium is not measurable in 7. Mean iron content is 101 ± 10.9 mg per g of stone. In 7 of the stones iron is not present in measurable quantity.

DISCUSSION

This study on the composition of bile in patients with cholesterol gallstone and controls is the only one of its kind from a geographic area which is completely different in its ethnic composition, environment and dietary habits than most of the studies hitherto reported. The population from whom the study subjects have been selected, experiences a very high incidence of gallstone disease\textsuperscript{10} and most of the patients are young, thin and have borderline nutritional status. Furthermore, they subsist on infrequent meals, low in calories, fat and protein, containing predominantly carbohydrates. It is
therefore not surprising that the bile composition with respect to its three major lipid constituents expressed either as mM/L or as relative proportions are different from the ones reported in the western subjects\textsuperscript{3-9}. These differences comprise a relative increase of cholesterol, and a decrease in both bile acids and phospholipids among the controls. In patients with gallstone, the concentrations of these three components are much lower reflecting a diminished concentrating capacity of the gallbladder. This is in spite of our efforts to exclude patients from the study with evidence of nonfunctioning gallbladder on cholecystography. When proportions of these three constituents are plotted on a triangular co-ordinate, no distinction can be made between patients with cholesterol stone and controls (Fig. 1). The mean values of both the controls as well as subjects with cholesterol stones fall almost at the same location in the phase diagram (Fig. 2). However, the standard deviations of the mean have a much wider range than the other studies reported. This may be a reflection of a larger number of samples studied rather than a difference between this population and those of the western countries. The mean values, both in controls as well as in patients, have also fallen above the previously described lines of cholesterol saturation, there is also a shift of the mean to the right. This is a reflection of a proportionate increase in phospholipid and decrease in bile salts. Although with regard to the position of the points in the phase diagram no real
distinction can be made between the control and the subjects with gallstone; the latter had cholesterol micro-crystals whereas the controls had none.

The results of analysis of biles in this study suggest that cholesterol supersaturation of bile may no longer be regarded as the only factor in the mechanism of gallstone formation. It is then important to search for additional factors which can either promote or prevent precipitation of cholesterol from gallbladder bile. One such factor which has been suggested\textsuperscript{6,18-20} is an increase of dihydroxy/trihydroxy ratio of bile acids in patients with gallstone disease. Although we have been unable to show such an increase in the patients compared with the controls, there is however a trend towards a positive relation between lithogenic index and the concentration of dihydroxy bile acids (Fig.3). However, both control as well as the patients have a much higher proportion of trihydroxy bile acids than that in the western subjects. We cannot satisfactorily explain this in the absence of data on the bile acid kinetics in our population with special reference to its faecal losses\textsuperscript{24}. It is possible that our population, having a substantially different dietary pattern with particular reference to its fibre content, looses bile acids in the faeces at a higher rate and the increased proportion of trihydroxy bile acids may only be a reflection of this situation.

Composition of natural bile is complex. In addition to
the three major lipid constituents, bile contains polyvalent cations and protein polyions. The discrepancy between the findings based on the in vitro models (so elegantly and accurately done) and in vivo bile analysis from control and the patients with cholesterol stone, may be due to the fact that the role of these ionic constituents has not been taken into account in the model systems. If supersaturation of bile with respect to cholesterol is the only important factor in lithogenesis, then the stones so formed should have cholesterol as its only constituent, because phase rule would allow only the precipitation of the constituent which rises above its maximum solubility level. The fact that the majority of the stones contained polyvalent cations such as Ca$^+$, Fe$^{+++}$ and some of the stones also contained significant amounts of phospholipid and bile salts suggests that a satisfactory model to explain lithogenesis has to take into account the role of such ionic constituents present in the natural bile. The contribution of the micro-constituents in the mechanism of gallstone formation has been also the subject of our study and will be reported in a subsequent communication.
REFERENCES


