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An Extended Analysis of Gall Stones: Nagpur, India

KEYWORDS: Gallstones, Total bilirubin, Fatty acids,Triglyceride

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ABSTRACT *Introduction: The impaired metabolism of cholesterol, bile acids & bilirubin, characterized by gallstone formation. The prevalence of cholelithasis varies and has been reported as 2% to 29% in different region of India and increased in the recent years. It is essential to know chemical composition of gal stone for better understanding of etiophatology which include quantization of total cholesterol bilirubin, fatty acid, bile acid, soluble proteins, oxalate and chloride, calcium, iron, phosphate (inorganic). The composition of these ingredients varied in different type of stone. Present study was oriented to find out the chemical composition of various stone received after cholycystectomy/and other clinical procedures. Objective: To study the chemical analysis of gallstones in relation to the type of diet. Material & Method: Department of Surgery at NPK Salve Institute of Medical Sciences, Nagpur, from September 2010 to October 2012 on 50 patients fulfilling the inclusion criteria of the study. Biochemical analysis of gallstones was recorded. Results: Overall chemical composition of gallstones has not changed in spite of life style changes. Cholesterol stones had significantly higher concentration of total cholesterol, inorganic phosphorus, magnesium and phospholipids, Pigment stones had significantly higher concentration of bile acids and potassium while mixed stones had significantly higher concentration of bile acids, soluble proteins, calcium and magnesium.*

Introduction

Gallstones are major causes of morbidity and mortality throughout the world[1]. At least 10% adult have gallstone[2]. The estimated prevalence of gallstone disease in India has been reported as 2% to 29%[3, 4].

In India, this disease is seven times more common in the North (stone belt) than in South India[5]. Gallstones vary in their composition, a majority being cholesterol stone (80%) and the remaining being mixed, combined or pigmented stone[6]. There are many researches on etiology, clinical presentation, management specifically evaluating the modalities of treatment but chemical analysis though age old investigation was not given much importance in spite that they could give an insight into pathogenesis and presentation.

The present study describes an extensive quantitative analysis of gallstones, change in its chemical composition from Nagpur which includes metabolites like fatty acids, triglycerides, phospholipids, bile acids, soluble protein and cations and anions like sodium, potassium, inorganic phosphorus, calcium, Magnesium, Chloride, Iron, oxalate and Copper

Material & Method

This was a hospital based, “cohort” study. Gallstones from 50 the patients were selected by random method (Computer generated), from the patients were collected after cholycestectomy at NPK Salve Institute of Medical Sciences, Nagpur, (Department of Surgery) from September 2010 to October 2012. The stones were divided into 3 groups depending upon their color: pale yellow and whitish stones as cholesterol calculi, black and blackish brown as pigment calculi and brownish yellow or greenish with laminated features as mixed calculi. A total of 15 biochemical parameters were analyzed. The other relevant information about number of calculi and diet pattern were obtained from hospital record.

Random selection of 50 patients from ongoing study at the Institute. The patients excluded from the study who did not gave consented to join study, with primary CBD stones.i.e. no calculus in gallbladder or asymptomatic gallstones. Ethical clearance was obtained from Institutional ethical committee of the hospital. Considering the cost of analysis a representative sample of stones removed during surgical management were selected and chemically analyzed.

Study Factors

A total of 15 biochemical parameters were studied in the 3 type of stones, the concentration of cholesterol, bile acids, soluble proteins, potassium, calcium, inorganic phosphorus, magnesium and phospholipids was measured.

Biochemical analysis of gallstones

The stones were powdered in a pestle and mortar and dissolved in different solvents depending upon the type of chemical constituent to be analyzed. To determine total cholesterol and total bilirubin, 30mg stone powder was dissolved in 3 ml chloroform in a test tube. The tube was kept in boiling water bath for 2 min. The stone solution thus obtained was used for determination of total cholesterol and total bilirubin. To determine calcium, oxalate, inorganic phosphate, magnesium, chloride, soluble protein, triglycerides, iron, copper, sodium and potassium, 30 mg stone powder was dissolved in 3 ml IN HCl in graduated 10 ml tube and its final volume was made up to 10 ml with distilled water. The tube was kept in boiling water bath for 1 hr. To analyze phospholipids, stone powder (20 mg) was dissolved in 15 ml $CHCl_3$+CH_3OH in 2:1 ratio, containing 1N HCl. To measure bile acids and fatty acids, the stones were dissolved in chloroform-methanol (2:1) mixture and ethyl alcohol-solvent ether in (3:1 mixture) respectively.

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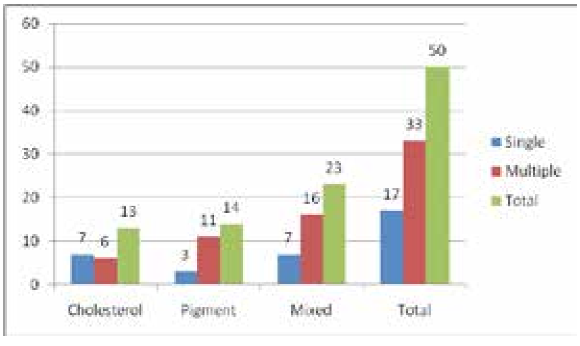
Analysis of Total cholesterol was done by enzymic colorimetric method of Bayer Diagnostic India Ltd[7], analysis of total bilirubin was done by colorimetric method of Accurex Biomedical Pvt. Ltd[8], analysis of triglycerides was done by enzymic colorimetric method of Bayer Diagnostics India Ltd[9], analysis of soluble protein was done by colorimetric method of Lowry et al[10], analysis of calcium was done by OCPC kit method of Miles India Ltd[11], analysis of phospholipid & inorganic phosphate was done by colorimetric method of Fiske and Subba Row[12], analysis of magnesium was done by colorimetric method of Neill and Neely[13], analysis of chloride was done by colorimetric method of Bayer Diagnostics India Ltd.[14], analysis of iron & copper was done by Atomic Absorption Spectrophotometer (Make Hitachi, Japan), analysis of sodium &potassium was done by Flame photometer (Systronics 'Mediflame' 127, Hyderabad), analysis of fatty acids was done by colorimetric method of Stern and Shapiro[15] and analysis of bile acids was done by colorimetric method of Carey[16] were estimated. The dissolved stone solutions were stored at 2-8°C, when not in use.

Statistical Analysis
Categorical variable were analysed using Fisher's exact test and Chi-square test. Comparison of values of biochemical analysis were done using Kruskel wall is One way ANOVA. The data was presented in tabular form using tables, pie & bar diagrams for descriptive statistics.

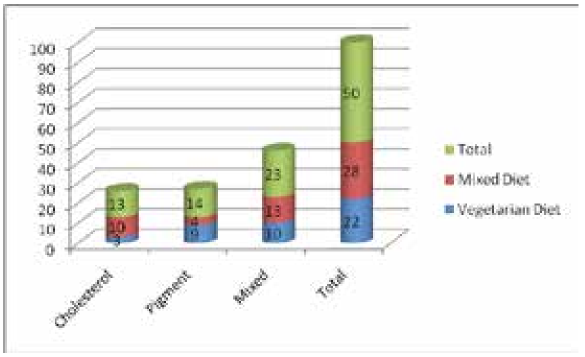
Result and discussion:
Number and Type of stone
A correlation between number of stones and chemical type of stone was carried out. Out of 17 patients of single stone, 7 had cholesterol stones, 7 mixed stones while 3 had pigment stones. Of 33 patients of multiple stones, 16 had mixed stones, 11 had pigment stones and 6 had cholesterol stones. The total percentage of Cholestrol, mixed and pigmented stones was 26%, 28% and 46% respectively. While a study done in Haryana by P. Chandran et al showed 26%, 38% and 36%, respectively[17]. In Haryana region study by Pundir CS et al showed the prevalence respectively 14.2%, 68.6% and 17.2% [18].

It could be inferred from the results that 69.5 % multiple stones are mixed stones, 78.5 % multiple stones are pigment stones while 53.8 % cholesterol stones are single.(Bar diagram. 1).



Bar diagram:- 1 Showing Number and Type of stone

Diet and Type of stone
Cholesterol stones are almost 3 times more common in patients consuming mixed diet (35.7%) than patients consuming vegetarian diet (13.6%).Pigment stones are more common in patients consuming vegetarian diet (40.9%) than patients consuming mixed diet (17.8%). Mixed stones are similar in both groups. (Bar diagram 2.)



Bar diagram – 2. Showing Diet and Type of stone

Biochemical Analysis
A total of 15 biochemical parameters were studied in the 3 types of stones. Amongst the three types, cholesterol, pigment and mixed stones. The concentration of cholesterol, bile acids, total bilirubin, fatty acid, triglyceride, soluble proteins, potassium, calcium, inorganic phosphorus, magnesium, iron, chloride, sodium, copper, potassium and phospholipids had statistically significant difference (Table.2).

Chemical / Type of Stone	Cholestrol	Pigment	Mixed	P-Value
Cholesterol	605.79±42.73	480.77±23.65	483.63±36.36	0.02 Ch vs Pg 0.05 Ch vs Mx 0.05 Pg vs Mx
Bile acid	6.44±1.72	16.34±1.65	20.47±2.24	0.08 Ch vs Pg 0.03 Ch vs Mx 0.02 Pg vs Mx
Total bilirubin	2.13±0.65	1.43±0.34	5.87±0.37	0.05 Ch vs Pg 0.06 Ch vs Mx 0.05 Pg vs Mx
Trigyceride	68.03±4.28	33.7±5.06	72.06±3.65	0.03 Ch vs Pg 0.06 Ch vs Mx 0.05 Pg vs Mx
Fatty acids	23.36±1.38	12.37±1.29	13.04±1.35	0.05 Ch vs Pg 0.05 Ch vs Mx 0.07 Pg vs Mx
Soluble Protein	22.37±4.36	96.74±11.27	10.86±1.84	0.05 Ch vs Pg 0.05 Ch vs Mx 0.05 Pg vs Mx
Phospholipid	9.10±1.05	6.36±0.57	5.16±0.46	0.01 Ch vs Pg 0.03 Ch vs Mx 0.04 Pg vs Mx
Sodium	1.02±0.16	3.80±0.42	1.18±0.32	0.04 Ch vs Pg 0.001Ch vs Mx 0.001Pg vs Mx
Potassium	0.226±0.02	0.863±0.08	0.268±0.02	0.001Ch vs Pg 0.001Ch vs Mx 0.05 Pg vs Mx
Calcium	12.32±1.86	27.65±3.50	25.48±2.35	0.05Ch vs Pg 0.002Ch vs Mx 0.05Pg vs Mx
Inorganic Phosphorus	21.46±2.47	8.45±1.32	15.71±1.16	0.001Ch vs Pg 0.05 Ch vs Mx 0.001Pg vs Mx
Magnesium	8.56±1.05	12.84±2.08	7.82±0.86	0.05 Ch vs Pg 0.07 Ch vs Mx 0.05 Pg vs Mx
Chloride	20.04±1.62	35.29±2.05	22.66±1.48	0.09 Ch vs Pg 0.001Ch vs Mx 0.001Pg vs Mx
Iron	0.66±0.79	0.72±0.07	1.27±1.02	0.05 Ch vs Pg 0.05 Ch vs Mx 0.10 Pg vs Mx
Copper	0.79±0.18	0.36±0.04	0.33±0.02	0.05Ch vs Pg 0.05Ch vs Mx 0.08Pg vs Mx

Table: 2. Showing Results of Biochemical Analysis

Cholesterol: The cholesterol level was significantly higher in cholesterol stone (605.79±42.73) than the pigmented (480.77±23.65) and (483.63±36.36) in mixed stone ($p < 0.001$). A study conducted in Rohtak India by P. Chandran et al showed the cholesterol level in pigmented stone was (489.85±28.68) while there is no significant difference between cholesterol and mixed stone type[17]. This is also confirmed by a study carried out by Pundir C.S. et al in Haryana[18].

Bile acids: In our study bile acid concentration in choles-

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terol, pigmented and mixed type of stones was as follow 6.44, 16.34 and 20.47. It was significantly high in mixed type of calculi while lower in cholesterol calculi. A study conducted by P. Chandran in Haryana showed the bile acid was higher in pigmented than the cholesterol and mixed type of stone[17]. This may be different diet pattern of two different regions.

Total bilirubin: The total bilirubin concentration was highest in mixed calculi and lowest in pigmented calculi. It was significantly higher in mixed calculi compared to cholesterol and pigmented calculi ($p<0.05$) and insignificantly higher in pigmented calculi than than the cholesterol calculi ($p>0.05$). This study opposes the previous study done by Kumar D et al[19].

Triglyceride: The triglyceride content was significantly ($p<0.05$) higher in cholesterol (68.03±4.28) and mixed calculi (72.06±3.65) as compared to pigmented calculi (33.7±5.06). However the difference was insignificant between cholesterol and mixed calculi ($p>0.05$). This result is similar to study by Chandran et al.[17] and Mohammed Helmy et al.[20]. The higher content of triglycerides in mixed or cholesterol stones compared to pigmented might be due to higher deposition of calcium salts of cholesterol and esters of fatty acids in mixed and cholesterol stone when compared to pigmented stones in which calcium bilirubinate is the major salt[21].

Fatty acid: The mean fatty acid content was highest in cholesterol (23.36±1.38mg/gm) and lowest in pigmented (12.57±1.29mg.gm) calculi. The difference of fatty acid content was significant ($p<0.05$) between cholesterol vs pigmented and cholesterol vs mixed calculi but insignificant between pigmented and mixed calculi ($p>0.05$). But Chandran et al.[17] and Mohammed Helmy et al.[20]. found highest in cholesterol and lowest in mixed type of calculi. The highest content of fatty acids in cholesterol calculi might be due to interaction between excessive cholesterol and fatty acids.

Soluble proteins: The soluble protein content was highest in pigmented (96.74±11.27mg/gm) calculi and lowest in mixed calculi (10.86±1.84mg/gm). The protein content was significantly higher in pigmented calculi ($p<0.05$) as compared to mixed calculi and cholesterol calculi and cholesterol calculi as compared to mixed calculi ($p<0.05$). This study revealed the study of P.Chandran et al.[17]. Binette et al.[22] suggested that the proteins to be the candidates either to facilitate or hinder the formation of stones. Maki[23] proposed the ability of bacterial β-glucuronidase enzymes (protein) to hydrolyse the bilirubin glucuronide complex thus releasing a poorly soluble bilirubin to explain the formation of PS as well as CS.

Phospholipids: The phospholipids content was highest in cholesterol stones and lowest in mixed stones. There was a significant difference between phospholipid content of cholesterol calculi and mixed calculi ($p< 0.03$), mixed calculi and pigment calculi ($p < 0.04$) and between cholesterol calculi and pigment calculi ($p < 0.03$). Similar result was found by P. Chandran et al.[17]. This might be due to accumulation of phospholipids alongwith cholesterol during cholesterol stone formation. The accumulation of phospholipids could either be due to their enhanced biosynthesis or decreased utilization.

Sodium: The mean sodium content was highest in pigment calculi (3.80±0.42mg/gm) and lowest in cholesterol calculi (1.02±0.16mg/gm). The difference in sodium content was highly significant ($p<0.04$) in pigment calculi as compared to cholesterol calculi and mixed calculi but insignificant ($p>0.001$) between cholesterol calculi and mixed calculi. This study revealed the study of P. Chandran et al.[17]. Sodium forms salt with bile acid which gets accumulated during cholelithiasis. The more the content of bile acid, the more content of sodium as was observed in pigment calculi compared to other calculi.

Potassium: The mean potassium content was highest in pigment calculi and lowest in cholesterol calculi. The potassium content was significantly higher ($p<0.001$) in pigment calculi as compared to mixed and cholesterol calculi. The difference of potassium content of cholesterol calculi and mixed calculi ($p>0.05$) was however insignificant. A certain ratio of sodium and potassium is maintained in bile of gall bladder. Higher the sodium content, higher the potassium content. This might be the reason for higher potassium content in pigment calculi compared to other calculi. This was same as in study of P. Chandran et al.[17].

Calcium: The mean calcium content was highest in pigment calculi (27.65±3.50mg/gm) and lowest in cholesterol calculi (12.32±1.86mg/gm). It was significantly higher in pigment calculi and mixed calculi as compared to cholesterol calculi ($p<0.05$). The calcium content in various gallstones was in the following order. Pigmented calculi > Mixed calculi > Cholesterol calculi. It is in agreement with our earlier report by Pundir CS et al.[18] and reports from Singapore[24] and Chandigarh[25]. Earlier calcium carbonate was identified as the most frequently occurring compound in pigment stones[26]. This could be attributed to the suggestion that the copper and iron may act as chelating agents for calcium bilirubinate. The central aggregates of calcium salts constitute hard foreign bodies which may lead to ulceration of gallbladder mucosa and microscopic haemorrhage. The iron released by this process may be another source of its deposition in gallstones. Injury to gallbladder mucosa also provides an opportunity for release of epithelium -glucorunidase, an additional enzyme contributing towards precipitation of calcium bilirubinate[25]. It has been postulated that calcium precipitation in bile is a critical event in cholelithiasis[27].

Inorganic phosphorus: The inorganic phosphate content was highest in cholesterol calculi (21.46.±2.47) and lowest in pigment (calculi 8.45±1.32). There significant difference of inorganic phosphorous content between mixed calculi and pigment calculi ($p>0.001$). This is in agreement with report from Haryana P.Chandran et al.[17], but differed from our earlier report Pundir CS et al.[18] where mixed calculi had highest inorganic phosphate content, however, there was no significant difference between mixed and cholesterol calculi. Inorganic phosphate might be playing an important role in the formation of cholesterol gallstones by forming salt with calcium.

Magnesium: The magnesium composition was highest in pigmented calculi (12.84±2.08mg/gm) and lowest in mixed calculi (7.82±0.86mg/gm). There was significant difference of magnesium between cholesterol calculi and pigmented calculi ($p<0.05$) and between pigmented and mixed calculi but there was no significant difference between cholesterol and mixed calculi.

Chloride: The mean chloride content was highest in pigmented calculi (35.29±2.05mg/gm) and lowest in cholesterol calculi (20.04±1.62 mg/gm), which might be due

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to their easy deposition with calcium bilirubinate. The chloride content was significantly higher ($p<0.001$) in pigmented calculi as compared to cholesterol and mixed calculi but insignificant between cholesterol calculi and mixed calculi ($p>0.05$). This result was similar as P. Chandran et al.[17]. Chloride ions are always present in biological fluids in human being including bile, which might get deposited in the form of sodium chloride salt along with major salts of the gallstones.

Iron: The mean iron content was highest in mixed calculi (1.27±1.02mg/gm) and lowest in cholesterol calculi (0.66±0.79 mg/gm). The iron content was significantaly higher in mixed calculi as compared to cholesterol calculi ($p<0.001$) and pigment calculi ($p<0.05$). However, there was an insignificant difference ($p>0.05$) between iron content of cholesterol calculi and pigment calculi. These observations are in agreement with our earlier report Pundir CS et al.[18], report from Chandigarh Verma GR et al.[25], and P Chandran et al.[17]. Been et al. [28] reported the presence of small amount of iron in a thick black shell around the central dark inclusion of gallstones. The high iron content in mixed gallstones might be promoting aggregation of calcium bilirubinate particles which are the major constituents especially due to the polyelectrolytic nature of iron. Verma et al.[25] suggested that central aggregates of calcium salts may lead to ulceration of gall bladder mucosa and microscopic haemorrhage. The iron released by this process might be another source of its deposition in gallstones.

Copper: The copper content was highest in cholesterol calculi and lowest in pigment calculi. The content of copper in cholesterol calculi was significantly higher as compared to mixed calculi ($p<0.05$) and pigment calculi ($p<0.05$). However, the difference in copper content of mixed calculi and pigment calculi ($p>0.05$) was insignificant. The study favors the P. Chandran et al.[17]. The role of copper in pathogenesis of calcium bilirubinate in gallstones is suggested Been JM et al.[28].

Conclusion

Our study that amongst the three types, cholesterol, pigment and mixed stones: the concentration of cholesterol, bile acids, soluble proteins, potassium, calcium, inorganic phosphorus, magnesium and phospholipids had statistically significant difference.

Cholesterol stones had significantly higher concentration of total cholesterol, fatty acid, phospholipids, inorganic phosphorus and copper, Pigment stones had significantly higher concentration soluble protein, calcium, sodium, potassium, magnesium and chloride while mixed stones had significantly higher concentration of bile acids, total bilirubin, triglyceride and iron.

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