Biochemical studies on patients with Gallstones

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Abstract: Chemical composition of gall stones is essential for aetiopathogensis of gallstone disease. We have reported quantitative chemical analysis of total cholesterol bilirubin, calcium, total cholesterol, total bilirubin, triglycerides, phospholipids, bile acids and soluble proteins in 90 gallstones from Gastroenterology Center, Mansoura, of biliary calculi (35 cholesterol, 30 mixed and 25 pigment) retrieved from surgical operation of 90 patients. Total cholesterol as the major component and total bilirubin, phospholipids, triglycerides, bile acids, soluble protein, calcium as minor components were found in all types of calculi. The cholesterol stones had higher content of total cholesterol and phospholipids compared to mixed and pigment stones. The mixed stones had triglycerides than to cholesterol and pigment stones. The pigment stones were richer in total bilirubin; bile acids, calcium, and soluble protein compared to cholesterol and mixed stones. [El-Shahat A. Toson and Marihan A.Helal **Biochemical studies on patients with Gallstones**. Nature and Science 2011;9(12):88-93]. (ISSN: 1545-0740). http://www.sciencepub.net

Key words Gallstone, Bile acid, phospholipids, Triglyceride, bilirubin.

Introduction

Gallstones are concretions that form in the biliary tract, usually in the gallbladder. Their development is insidious, and they may remain asymptomatic for decades. Migration of gallstones may lead to occlusion of the biliary and pancreatic ducts, causing pain (biliary colic) and producing acute complication **Heuman DM et al.,(1996),** such as acute cholecystitis, ascending cholangitis, or acute pancreatitis **Center SA (2009).**Chronic gallstone disease may lead to fibrosis and loss of function of the gallbladder and predisposes to gallbladder cancer.

Gallstone formation occurs because certain substances in bile are present in concentrations that approach the limits of their solubility. When bile is concentrated in the gallbladder, it can become supersaturated with these substances, which then precipitate from solution as microscopic crystals. The crystals are trapped in gallbladder mucus, producing gallbladder sludge. Over time, the crystals grow, aggregate, and fuse to form macroscopic stones. Occlusion of the ducts by sludge and stones produces the complications of gallstone disease. The 2 main substances involved in gallstone formation are cholesterol and calcium bilirubinate.

One of the causes of posthepatic hyperbilirubinemia is cholestasis where there is failure of bile to reach the small intestine. Cholesterol is virtually insoluble in water and is maintained in an aqueous environment in vesicles combined with phospholipids and bile salts. In normal conditions, the vesicles maintain the concentration of cholesterol in bile near its saturation point. Cholesterol monohydrate crystals form when the ratio of cholesterol, phospholipids and bile salts exceeds the normal range and results in the formation of gallstones in a process termed cholelithiasis. Eighty per cent of gallstones are composed largely of cholesterol; the remaining 20% consist of calcium and bilirubin.

They vary in size from that of a grain of sand to the diameter of a golf ball.

Researchers believe that gallstones may be caused by a combination of factors, including inherited body chemistry, body weight, gallbladder motility (movement), and perhaps diet. The absence of such risk factors does not, however, preclude the formation of gallstones.

No clear relationship has been proven between diet and gallstone formation; however, low-fiber, high-cholesterol diets and diets high in starchy foods have been suggested as contributing to gallstone formation. Other nutritional factors that may increase risk of gallstones include rapid weight loss, constipation, eating fewer meals per day, eating less fish, and low intakes of the nutrients folate, magnesium, calcium, and vitamin C**. Ortega RM et al.,(1997)** On the other hand, wine and whole-grain bread may decrease the risk of gallstones**. Misciagna et al., (1996).**

Pigment gallstones are most commonly seen in the developing world. Risk factors for pigment stones include hemolytic anemia's (such as sickle-cell disease and hereditary spherocytosis), cirrhosis, and biliary tract infections. **Trotman et al., (1980).** So that, the aim of present study is describe an extensive quantitative analysis of gallstones to show possible mechanisms of gallstone formation.

Material and Methods

Material

This study was conducted on 90 patients with gallstones (18 male and 72 female; aged 23– 60 years), were prospectively recruited from the Gastroenterology

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The stones were divided into 3 groups depending upon their colour: 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.

Methods

The stones were washed by distilled water, dried and 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, 30 mg stone powder was dissolved in 1 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, soluble protein and triglycerides, 30 mg stone powder was dissolved in 1.5 ml IN HCl in 5 ml measuring flask and its final volume was made up to 5 ml with distilled water. The tube was kept in boiling water bath for 1hr. To analyze phospholipids, stone powder (20 mg) was dissolved in 7 ml $CHCl_3$+CH_3OH in 2:1 ratio, containing 1N HCl. To measure bile acids the stones were dissolved in chloroform-methanol (2:1) mixture.

Total cholesterol by enzymatic colorimetric kit method of spinreact according to (**Natio HK cholesterol *et al*.,1984;Burtis Aet *al*.,1999 andTietz NW *et al*,1995**), triglycerides by enzymatic colorimetric kit method of spinreact according to (**Buccolo G *et al*, 1973;Burtis Aet *al*.,1999 and Tietz *et al*,1995**)total bilirubin by modified van den Bergh and Muller method kit of spectrum according to **Bailisteri WF and Shaw LM (1987) and Malloy HT, Evelyn KA,(1973),** soluble protein by colorimetric method of **Lowry *et al***, calcium by colorimetric method kit of **Gindler M and King J D (1972),** phospholipids by enzymatic colorimetric method according to **Tietz N W and Saunders W B (1999)**. and bile acids by enzymatic colorimetric method according to **Tietz N W and Saunders W B (1999)**. The dissolved stone solutions were stored at 2-8°C, when not in use.

Results

The total cholesterol

The total cholesterol was significantly higher in cholesterol stones, compared to pigment stones ($p<0.0001$) and in mixed stones as compared to pigment stones. However, there was a significant difference between total cholesterol content of cholesterol stones and mixed stones ($p<0.001$).

The triglyceride

The triglyceride content was highest in mixed stones and lowest in pigment stones. The triglyceride content was significantly higher ($p<0.0001$) in cholesterol stones and mixed stones as compared to pigment stones However, the difference was insignificant between cholesterol stones and mixed stones ($p>0.05$).

The phospholipids

The phospholipids content was highest in cholesterol stones and lowest in mixed stones. It was significantly higher in cholesterol stones compared to mixed stones and pigment stones ($p < 0.0001$). However, the difference was insignificant between pigment stones and mixed stones ($p>0.05$).

The bile acid

The bile acid content in pigment stones was comparatively higher than that in cholesterol stones and mixed stones and the difference was highly significant ($p < 0.0001$).

The total bilirubin

The total bilirubin concentration was highest in pigment stones and lowest in mixed stones. It was significantly higher in pigment stones compared to mixed stones and cholesterol stones ($p<0.0001$) and insignificantly higher in cholesterol stones as compared to mixed stones ($p>0.05$).

The soluble protein

The soluble protein content was highest in pigment stones and lowest in mixed stones. The protein content was significantly higher in pigment stones ($p<0.0001$) as compared to mixed stones and cholesterol stones and in cholesterol stones as compared to mixed stones .

The calcium

The mean calcium content was highest in pigment stones and lowest in cholesterol stones. It was significantly higher in pigment stones and mixed stones as compared to cholesterol stones ($p<0.0001$). However, there was an insignificant difference between calcium content of pigment stones as compared to mixed stones ($p>0.05$).

Table 1: Means of total cholesterol, triglycerides, bile acids and phospholipids mean levels in stones of patient with gallstone.

Parameters / Groups	Total cholesterol mg/gm	Bile acids µmol/L.	phospholipids mg/gm	Triglycerides mg/gm
Cholesterol stones				
Mean ± S D	283.7 ± 62.6	109.4 ± 35.3	145.8 ± 27.7	37.4 ± 12.1
Range (n =35)	(200 - 400)	(80 -170)	(100 – 186.2)	(24 - 60.6)
Pigment stones				
Mean ± S D	130.6 ± 11.6	218.7 ± 32.8	31.7 ± 8	11.25 ± 4.7
Range (n =25)	(118 – 160)	(164.8 -274)	(24 – 50)	(6 – 20.6)
P	< 0.0001 ii	< 0.0001 ii	< 0.0001 ii	< 0.0001 ii
P2	< 0.0001 **	< 0.0001 **	>0.05 Ns	< 0.0001 **
Mixed stones				
Mean ± S D	220 ± 73.3	44.4 ± 20.6	26 ± 4	42.8 ± 13.7
Range (n =30)	(120- 350)	(20.8 - 80)	(20 - 31)	(34 - 82.7)
P	< 0.001i	< 0.0001 ii	< 0.0001 ii	>0.05 Ns

ii= highly significant with those having cholesterol gallstones. i= significant with those having cholesterol gallstones **=highly significant between mixed and pigment gallstones and Ns= non significant with those having cholesterol gallstones.

Table 2: Means of total biliuribin, soluble proteins and Ca mean levels in stones of patient with gallstone

Parameters / Groups	Total biliurbin mg/gm	Soluble protein mg/gm	calcium mg/gm
Cholesterol stones			
Mean ± S D	4.1 ± 0.6	11.4 ± 0.76	4 ± 1
Range (n =35)	(3.3 - 5.3)	(10.3 - 12.2)	(2.3 -5.6)
Pigment stones			
Mean ± S D	8.6 ± 0.68	32.2 ± 5.4	10 ± 0.83
Range (n =25)	(7.4 -9.5)	(20.2 - 37.7)	(8.3 – 11)
P	< 0.0001 ii	< 0.0001 ii	< 0.0001 ii
P2	< 0.0001 **	< 0.0001 **	>0.05Ns
Mixed stones			
Mean ± S D	3.9 ± 0.66	8.8 ± 1.5	9.7 ± 0.81
Range (n =30)	(3 - 5.4)	(6 – 10.9)	(8.6 – 9.7)
P	>0.05Ns	< 0.0001 ii	< 0.0001ii

ii= highly significant with those having cholesterol gallstones. **=highly significant between mixed and pigment gallstones and Ns= non significant with those having cholesterol gallstones.

In the human gallbladder, three types of gallstones exist, depending on the major constituents: pure cholesterol, pure pigment, and mixed (small amounts of calcium and bilirubin salts). Pigment stones appear in two major forms: black and brown. Whereas black primarily of calcium bilirubinate, brown pigment stones are associated with infections of the biliary tract .**Sherlock S and Dooley J (2002).**

The biliary calculi collected from 90 gallstone patients were divided into 3 groups based on their colour: cholesterol calculi, mixed calculi and pigment calculi. Out of the 90 stones collected, 30 were mixed calculi, 25 were pigment calculi and 35 were cholesterol calculi.

The cholesterol, bilirubin, bile acids, triglycerides, phospholipids, soluble protein and calcium were found in all 90 gallstones patient. The results of quantitative analysis of the constituents of gallstones are expressed as mg/gm dry wt.

The total cholesterol was significantly higher in cholesterol stones, compared to pigment stones and in mixed stones ($p<0.0001$) as compared to pigment stones. and cholesterol stones and mixed stones ($p<0.001$). Gallstones are believed to form, when the concentration of cholesterol exceeded that which can be held in mixed micellar solution with bile acids and phospholipids.

Supersaturation of cholesterol is believed to be due to Precipitation of excess cholesterol in bile as solid crystalsis **(Hofmann AF et al., 1993; LaMont JT and Carey MC 1992).** The high level of cholesterol in cholesterol calculi has been related to high carbohydrate diet **Cuevas A (2004).**

The triglyceride content was highest in mixed stones and lowest in pigment stones. The triglyceride content was significantly higher ($p<0.0001$) in cholesterol stones and mixed stones as compared to pigment stones However, the difference was insignificant between cholesterol stones and mixed stones ($p>0.05$).

The triglycerides get accumulated along with cholesterol salts to form gallstones. The higher content of triglyceride in mixed stone or cholesterol stone compared to pigment stone might be due to more deposition of calcium salts of cholesterol and esters of fatty acids in mixed and cholesterol stone as compared to pigment stone, where calcium bilirubinate is the major salt **Chandran, P.*et al.,(*2007).**

The phospholipids content was highest in cholesterol stones and lowest in mixed stones. It was significantly higher in cholesterol stones compared to mixed stones and pigment stones ($p < 0.0001$). However, the difference was insignificant between pigment stones and mixed stones ($p>0.05$). There was a positive correlation between phospholipids and viscosity in gallbladder bile. Thus, increases in bile viscosity may lower the emptying of the gallbladder, thereby allowing more time for cholesterol crystal growth. Mucin has been shown to hydrophobically bind cholesterol in vesicles, promoting nucleation of solid cholesterol monohydrate crystals **Smith BF (1987);**

Afdhal NH (1995)

The bile acid content in pigment stones was comparatively higher than that in cholesterol stones and mixed stones and the difference was highly significant ($p < 0.0001$). That mean the higher bile acid the lower formation of cholesterol gallstones due to Vlahcevic and co-workers found that patients with cholesterol gallstones had a significantly decreased pool size of bile salts **lahcevic Z R (1970).**

The total bilirubin concentration was highest in pigment stones and lowest in mixed stones. It was significantly higher in pigment stones compared to mixed stones and cholesterol stones ($p<0.0001$) and insignificantly higher in cholesterol stones as compared to mixed stones ($p>0.05$).

The finding of bacterial proteins in stones, therefore, confirms these observations.

The ability of bacterial β-glucuronidase enzymes to hydrolyse the bilirubin glucuronide complex thus releasing a poorly soluble bilirubin has been proposed to explain the formation of pigment stones(**Aydogdu I et al.,2001**; **Buch S et al., 2010)** The same mechanism may also be contributing to the formation of mixed cholesterol stones as most of these have pigmented areas in which bilirubin mostly unconjugated would disturb the fragile equilibrium of a cholesterol supersaturated bile and coprecipitate with cholesterol, proteins and bacteria which are found in these pigmented areas **(Diehl AK et al.,1995; Aydogdu I et al., 2001)**

The soluble protein content was highest in pigment stones and lowest in mixed stones. The protein content was significantly higher in pigment stones ($p<0.0001$) as compared to mixed stones and cholesterol stones and in cholesterol stones as compared to mixed stones.

Protein was highest in pigment stones due to gallstones grow in the layer of glycoproteins, and calcium bilirubinate is bounded with glycoproteins, with structure or configuration of both the salt and the proteins altered during binding **Soloway R D et al.,(1987).**These findings lend support to the theory that proteins and glycoproteins form a net-work serving as traps upon which calcium bilirubinate polymers and other undissolved salts first bind and stone nidation and growth take place **(Wu E et al., 1989 and Azemoto R 1996)**. **Buch S et al., (2010)** 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 pigment stones as well as cholesterol stones.

The mean calcium content was highest in pigment stones and lowest in cholesterol stones. It was significantly higher in pigment stones and mixed stones as compared to cholesterol stones ($p<0.0001$). However, there was an insignificant difference between calcium content of pigment stones as compared to mixed stones ($p>0.05$).

As the critical component of pigment gallstone, calcium bilirubinate is a calcium coordination compound with a very complex structure **Wagner A J et al., (1987)** The pyrrole NH, lactam NH, and carboxyl group of bilirubin bind to calcium ions to form a nonstoichiometric bilirubinate complex **Yang Z L et al., (1991).**

Conclusion

Gallstones may be prevented by increasing consumption of both soluble and insoluble fiber which reduces the absorption of deoxycholic acid by producing a favorable shift in the triad of factors that control cholesterol's solubility in bile. People who may be at risk for developing gallstones may want to try modifying their diet to decrease their risk.

References

Afdhal, N.H., Niu, N., Nunes, D.P., Bansil, R., Cao, XX., Gantz, D., Small D.M., and Offner G.D. (1995): Mucin vesicle interactions in model bile: evidence for vesicle aggregation and fusion before cholesterol crystal formation. Hepatology; 22:856-865.

Aydogdu, I., Sari, R., Ulu, R., and Sevinc, A. (2001): The frequency of gallbladder stones in patients with pernicious anemia. J Surg Res; 101:120-3

Azemoto, R., Tsuchiya, Y., Ai, T., Murayama, H., Nakagawa, Y., Saisho, H., et al. (1996): Does gallstone formation after open cardiac surgery result only from latent hemolysis by replaced valves? Am J Gastroenterol; 91:2185-9.

Bailisteri, W.F., and Shaw, L.M., (1987): Fundamentals of clinical chemistry 3[ed]ed. Philadelphia: WB Saunders; 729-761.

Buccolo, G. *et al*, (1973): Quantitative determination of serum triglycerides by use of enzymes. Clin Chem; 19(5):476-482.

Burtis Aet *al., (*1999): Tietz textbook of clinical chemistry, 3[ed]

Buch, S., Schafmayer, C., Volzke, H., Seeger, M., Miquel, J.F., Sookoian S.C., et al. (2010): Loci from a genome-wide analysis of bilirubin levels are associated with gallstone risk and composition. Gastroenterology; 139:1942-51.

Center, S.A. (2009): Diseases of the gallbladder and biliary tree. Vet Clin North Am Small Anim Pract; 39(3):543-98.

Chandran, P., Kuchhal, N. K., Garg, P., and Pundir, C.S., (2007): An extended chemical analysis of gallstone, Indian Journal of Clinical Biochemistry; 22 (2) 145-150

Cuevas, A., Miquel, J.F., and Reyes, M.S. (2004): Diet as a risk factor for cholesterol gall stone disease. J Am Coll Nutr; 23:187-96.

Diehl, A.K., Schwesinger, W.H., Holleman, Jr. DR., Chapman, JB., and Kuron, WE.(1995): Clinical correlates of gallstone composition: distinguishing pigment from cholesterol stones. Am J Gastroenterol; 90:967—72.

Gindler, M. and King, J.D. (1972): Am. J. Clin. Path; 58,376.

Heuman, D.M., Moore, E.L. and Vlahcevic, Z.R. (1996): Pathogenesis and dissolution of gallstones. In: Zakim D, Boyer TD, eds. Hepatology: A Textbook of Liver Disease. 3[rd] ed. Philadelphia, Pa: WB Saunders; 1996:376-417.

Hofmann, A.F., Amelsberg, A., and Vansonnenberg, E. (1993): Pathogenesis and treatment of gallstones. *N Engl J Med*; 328**:** 1854–55.

lahcevic, Z. R., Bell, C. C. Buhac, Jr., I., Farrar, J. T. and Swell, L. (1970): Diminished bile acid pool size in patients with gallstones. Gastroenterology; 59: 165-173.

LaMont, J.T., and Carey, M.C. (1992): Cholesterol gallstone formation. Pathobiology and pathomechanics. *Prog Liver Dis*; 10**:** 165–91.

Lowry, OH., Rosenbrough, N.J., Farr A.L., and Randall, R.J. (1951): Protein measurement with the folin phenol reagent. J Biol Chem; 193: 265-75.

Malloy, H.T., Evelyn, K.A., (1973): The determination of bilirubin with the photoelectric colorimetric method J Biol Chem; 119:481-490.

Misciagna, Giovanni, Leoci, Claudio, Guerra, Vito, Chiloiro, Marisa,Elba, Silvana, Petruzzi, José, Mossa, Ascanio, Noviello, Maria R. et al. (1996): Epidemiology of cholelithiasis in southern Italy. Part II. European Journal of Gastroenterology & Hepatology; 8: 585–93.

Natio, H.K., cholesterol., Kaplan, A., *et al*., (1984): Clin Chem the C.V. Mosby Co. St Louis Toronto. Princeton; 1194-11206 and 437.

Ortega, R.M., Fernández-Azuela, M., Encinas-Sotillos, A., Andrés, P.and López-Sobaler, A.M. (1997): Differences in diet and food habits between patients with gallstones and controls. Journal of the American College of Nutrition; 16 (1): 88–95.

Sherlock, S., and Dooley, J. (2002): Diseases of the liver and biliary system. Oxford: Blackwell Science.

Smith, B.F. (1987): Human gallbladder mucin binds biliary lipids and promotes cholesterol crystal nucleation in model bile. J Lipid Res; 28:1088-1097.

Soloway, R. D., Wu, J. G., Yang, Z. L. et al., (1994): Why does Ca++ vary from 3 to 12% in non-stoichiometric calcium bilirubinates? Gastroenterology; 106(4): 359.

Tietz, N. W., (1999): Textbook of clinical chemistry, 3[ed]ed. Philadelphia: WB Saunders; 268-273.

Tietz, N.W., *et al, (*1995): Clinical guide to laboratory tests, 3[ed]

Trotman, Bruce, W., Bernstein, Seldon,E., Bove, Kevin E., Wirt and Gary D. (1980): Studies on the Pathogenesis of Pigment Gallstones in Hemolytic Anemia. Journal of Clinical Investigation; 65 (6): 1301–8.

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Wagner, A. J., Wu, J. G., Soloway, R. D. et al., (1987): Does the structure of calcium bilirubinate provide special properties contributing to its precipitation in bile? A comparison with other metal bilirubinates by Fourier transform infrared spectroscopy (FT-IR), Hepatology; 7(5): 1138—1139.

Weight, L.M., Byrne M.J., and Jacobs, P. (1991): Hemolytic effects of exercise. Clin Sci (Lond); 81:147—52.

Wu, E., Wu, J. G., Xu, G. X. et al., (1989): Gallstone analysis by ESR spectroscopy, Chinese Science Bulletin; 34(23):1996—2000.

Yang, Z. L., Soloway, R. D., Wu, J. G. et al., (1991): Calcium binds with both the carboxyl and pyrrole groups of bilirubinate invitro formation of non-stoichoimetric salt, Gastroenterology; 100(5): A813.