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Biochemical Study of Gallstones Compositions in Iraqi patients

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Abstract

The aim of this study is to determine the organic and inorganic components of bile and gallstones in Iraqi patients. Forty seven patients were included in this study with mean age (53$\pm$7) years and BMI (30.82$\pm$4.18) Kg/m^2. Bile was classified according to its corresponding stones into: Bile of Mixed stones and Bile of pigment stones.

IR spectra were studied for both types of stones and their bile in addition to biochemical analysis for organic and inorganic components. The organic components include: (cholesterol, bilirubin, bile salts, and phospholipids), while inorganic components include salts of: (calcium, phosphorus, iron, cupper and magnesium). The results reveal to there was significant low levels ($p<0.005$) of bile salts and phospholipids in bile of patients with mixed stones in comparison to their levels in bile of pigment stones ($p<0.001$). There is significant increase in the salts levels of calcium, phosphorus, iron, cupper, magnesium, and bilirubin, in bile of pigment stones as compared to their levels in mixed stones bile.

It was concluded that most types of stones that are formed in Iraqi patients are mixed stones when cholesterol is the main component and pigment stones are more less incidence to occur when bilirubin salts form their main component and they are mostly tend to occur in hemolytic diseases such as: sickle cell anemia, thalassaemia and so on .

Key words: Gallstones, causes and risk factors, chemical composition of gallstones.

Introduction:

Gallstones are solid crystals. Small, hard pellets that are formed in the gallbladder which is a pear shaped organ that stores bile [1].Gallstones are one of the common diseases of the gallbladder, they occur at any age even children, but mostly at age(35-65)years and in both males and females. Females are affected more than males in a ratio 4:1 .Gallstones are formed due to super saturation of bile, which is viscous, greenish – yellow colour fluid contains water, bile salts, phospholipids, bile pigments and electrolytes, it is synthesized and secreted by liver and stored in gallbladder [2]. Bile contains no digestive enzymes but it considered itself as a digestive secretion. When saturation of bile with one or more of its components for a reason or another, precipitation of the crystals starts and acts as a nidus for stones formation [3]. There are many causes for gallstones to be formed which include [4]: Hypomotility of gallbladder, high cholesterol diet, diseases that are causing haemolysis of erythrocytes like: thalassaemia, sickle cell anaemia and so on, and other miscellaneous causes (like age, gender, multiparity due to hormonal changes during pregnancy, rapid weight loss as in heavy diet, obesity, medications like: oral contraceptive pills, Genetic factor may play a role). Gallstones are

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classified according to their constituents into[5]:

- Mixed stones which are the commonest type and formed from 50% cholesterol and 20% bile pigments and other salts and compounds, they are small in size, white to faint grey colour and multifaceted.
- Pigment stones: composed mostly of bilirubin in more than 60% and less than 10% cholesterol, they are small in size, soft friable, they are furtherly divided into:Brown stones which contains calcium bilirubinate and formed due to bacterial infection and black stones which contains calcium carbonate, calcium phosphate, cupper bilirubinate and formed due to haemolysis of blood.

Aim of the work:

The aim was studying the chemical compositions of both bile and gallstones that are formed in the gallbladder of Iraqi patients, which included organic and inorganic components. In addition to fined the correlations among these compositions which lead to stone formation.

Materials and methods:

Chemicals :

Sulphuric acid, Nitric acid, Hydrochloric acid, Glacial acetic acid, Trichloro acetic acid, Sulphanilic acid, Ascorbic acid, Sodium nitrite, Ammonium molybdate, Cholesterol, Calcium carbonate, Chloroform, Ethanol, Methanol, Petroleum ether(40-60)C^0 , Diethyl ether, Methyl red, Ferric chloride.6H2O, Sodium acetate, Sodium hydroxide, Ethyl acetate and Sodium arsenite , all of the chemicals are from BDH and Fluka company.

Samples collection:

Forty seven patients were included in this study, nine males and thirty-eight females with mean age(53$\pm$7) years and BMI (30.82$\pm$4.18) all of them were diagnosed with gallstones disease and operative surgery was decided as a treatment, stones with their bile were collected after the cholecystectomy.

Preparation of samples:

After obtaining of stones they were washed with distilled water many times and then they were left to dry at the room temperature for few days then they were pulverized and the powder of stones kept in dissicator for complete drying then the powder was extracted by organic solvents (Methanol, chloroform, ethanol and petroleum ether)[6], and biochemical tests were done.

1-Biochemical tests of stones :

- Measuring the levels of total cholesterol by colorimetric method (Liberman burchard reaction), the principle of this method is[7] :

$$\text{Cholesterol} \xrightarrow[\text{Conc. } H_2So_4]{FeCl_3} \text{Cholesta polyene (carbocation)} \; \{\text{red colour}\}$$

- Weight percentage for bile salts in stones[8] :

Extraction of stone powder was done by organic solvents, then bile salts were obtained in the ethanol layer, then evaporation of solvent and weight percentage calculated as follow:

$$\text{Bile salts w/w\%} = \frac{\text{wt. of bile salts in ethanol phase}}{\text{Wt. of stone powder}} \times 100$$

- Measuring of total bilirubin by colorimetric method(Van Den Bergh reaction)[9]:

Bilirubin +Diazotized sulphanilic acid → Azo bilirubin {coloured compound}

- Measuring the levels of inorganic phosphorus by Kit method (BioMerieux Kit)[10]:

$$\text{mg /dl of P} = \frac{A_t}{A_S} \times 5 \text{ (mg/dL)}$$

At : Absorbance of test As : Absorbance of standard

- Measuring of concentrations of some elements in the stones (Ca, Fe, Cu, and Mg) by atomic absorption spectroscope[11].
- IR spectra for both types of stones were recorded by compounding the powder of stones with KBr disc[12].

2- Biochemical tests of bile:

- Measuring of level of phospholipids by colorimetric method after extraction of phospholipids and calculated as follow [7]:

$$\text{Phospholipids(mg/dL)} = \frac{At - AB}{As} \times 250 \text{ (mg/dL)}$$

At: Absorbance of test As: Absorbance of standard A_B: Absorbance of blank

- Measuring of total cholesterol, weight percentage of bile salts, total bilirubin, concentrations of (Ca, Fe, Mg , Cu and phosphorus) by using the same methods that were used in stones.

Statistical analysis:

The data was analyzed on computer statistical programme SPSS version 10. The mean ±SD was also computed for the comparison of results. The comparison of mean between two groups was tested by student's t test. Results were considered statistically significant if P value is less than 0.05.

Results and Discussion:

- Results of IR spectra of gallstones: for both types of stones were studied and compared to pure IR spectra of cholesterol, calcium bilirubinate, calcium carbonate to determine the compounds in stones. For cholesterol it showed broad stretching absorption band at (3380 cm^{-1}) which refers to **OH** group in cholesterol ring and a broad spectrum band in (1470-1455)cm^{-1} refers to the **C—H** bonds in cholesterol rings. Presnce of absorptive band in (2910 cm^{-1}) due to symmetric and asymmetric vibration which refers to **CH_2—CH_3** groups that bound to cholesterol ring.
  The broad bands in (1630cm^{-1}), (1670 cm^{-1}), and (1575cm^{-1}) refer to **–C═C–, C═O, C—N** which refers to bilirubin salts, same results were seen for bile of both types of stones in addition to spectrum band in (785cm^{-1})for calcium carbonate and also in (1435cm^{-1}) and in(1485cm^{-1})[13]. Table (1) refers to w/w % for cholesterol in both types of bile and stones.

Table (1): % for cholesterol in both types of stones and bile

w/w% for cholesterol			
	Number of samples	Mean ±SD	P value
Mixed stones	35	45.1 ± 14.86	P<0.001
Pigment stones	12	2.4 ± 1.74	
Bile of mixed stones	35	8.27 ± 3.96	P<0.001
Bile of pigment stones	12	3.99 ± 1.3	

Levels of cholesterol in both types of stones and their bile expressed in w/w% the levels in mixed stones is significantly higher in those of pigment type which is about double with p <0.001, this high levels considered the main reason for precipitation in bile and stone formation due to supersaturation of bile with cholesterol[14]. Table (2) shows significant high levels of bile salts in pigment stones and their corresponding bile in comparison to those of mixed stones with p<0.001for stones and p<0.005 for bile :

Table-2: % of bile salts in both types of stone and their bile

w/w% for bile salts			
	Number of samples	Mean ±SD	P value
Mixed stones	35	1.008 ± 0.5	P<0.001
Pigment stones	12	3.09 ± 0.97	
Bile of mixed stones	35	31.51 ± 10.29	P<0.001
Bile of pigment stones	12	34.25 ± 2.86	

The results shows levels of bile salts which are significantly higher in the bile of pigment stones with p <0.001, this can be explained by the increase in bile salts leads to decrease in the levels of cholesterol in bile because they form with the phospholipids the micelle molecules which are responsible for emulsifying and digestion of cholesterol and as long as pigment stones have low levels of cholesterol so they have high contents of bile salts which cause their precipitation and forming a nidus for stone formation[15]. Table (3) shows significant high levels of phospholipids in bile of mixed stones in comparison to that of pigment stones with p<0.001:

Table (3): % of phospholipids in both types of stones' bile

w/w% for phospholipids			
	Number of samples	Mean ±SD	P value
Bile of mixed stones	35	17.79 ± 6.48	P<0.001
Bile of pigment stones	12	14.34 ± 5.32	

Results in table (3) shows the levels of phospholipids in the bile of both types of stones its significantly higher in mixed type than in pigment stones' bile with p <0.001 this is because the levels of cholesterol in this type of stones is higher than pigment stones and the levels of bile salts are low which are important factor for digestion and emulsifying of lipids in bile and that leads to supersaturation of bile with lipids[16]. Table (4) shows the levels of total bilirubin which are significant in pigment stones and their corresponding bile when compared to those of mixed stones and their bile with p <0.001 for stones and p <0.005 for bile :

Table (4): % of total bilirubin in both stones type and their bile

w/w% for total bilirubin			
	Number of samples	Mean ±SD	P value
Mixed stones	35	17.6 ± 13.91	P<0.001
Pigment stones	12	65.85 ± 10.64	
Bile of mixed stones	35	4.67 ± 3.28	P<0.005
Bile of pigment stones	12	8.29 ± 2.69	

In Table (4) the levels of bilirubin are significant in pigment stones because the main source of bilirubin is haemolysis of erythrocytes, myoglobin and some types of bacteria and as long as this type of stones formed due to haemolytic diseases or due to infection , this is because the presence of unconjugated bilirubin which is insoluble and supersaturation of bile with it and precipitation ending with stone formation, in bacterial infection, the bacteria has the ability to coverts conjugated bilirubin to unconjugated one by the effect of B-glucuronidase enzyme, the unconjugated bilirubin will combined with other compounds and precipitate to form the stones[17,18]. Table (5) shows levels of calcium salts in bile and stones of both types:

Table (5): levels of calcium salts in bile and stones of both types

Levels of calcium salts in mmol/L			
	Number of samples	Mean ± SD	P value
Mixed stones	35	3.58 ± 1.55	P<0.001
Pigment stones	12	11.6 ±74.9	
Bile of mixed stones	35	1.1 ± 0.41	P<0.005
Bile of pigment stones	12	2.36 ± 0.68	

It is significantly higher in pigment stones and bile than those in mixed stones and their bile because the ability of calcium ion to bind with bilirubinate ion which is high in pigment type to form insoluble calcium bilirubinate[19]. Table (6) shows the levels of phosphorus, its significantly high in pigment stones and their bile than in mixed type :

Table (6): % of phosphorus

Levels of phosphorus in mmol/L in bile and w/w% in stones			
	Number of samples	Mean ±SD	P value
Mixed stones	35	2.01 ± 0.62	P<0.001
Pigment stones	12	4.62 ± 1.35	
Bile of mixed stones	35	0.3 ± 0.11	P<0.005
Bile of pigment stones	12	0.5 ± 0.22	

It is higher in pigment stones also because the increase in phosphorus is related to the increase in calcium and the disturbance for any reason causing disturbance in ionic constant and causing calcium phosphate salts to be precipitated in high levels in the bile and stone formation[20]. Table -7- shows levels of magnesium salts in stones and bile, it has high levels in stones and bile of pigment type than in mixed type with p <0.005, p<0.01 respectively :

Table (7): % of levels of magnesium salts

Levels of magnesium salts in mmol/L in bile and w/w% in stones			
	Number of samples	Mean ±SD	P value
Mixed stones	35	0.15 ± 0.07	P<0.005
Pigment stones	12	0.61 ± 0.25	
Bile of mixed stones	35	0.042 ± 0.15	P<0.01
Bile of pigment stones	12	0.108 ± 0.025	

Its also higher in pigment stones because magnesium plays as a co-factor for active transport for calcium through the cell membrane and as the calcium is found in high concentration in pigment type so the magnesium, so the extra levels of magnesium bind to bilirubin to form magnesium bilirubinate which are in low concentration in comparison to that of calcium salts but inspite of that they precipitate as a component of stones [21]. Table (8) shows the levels of iron salts in stones and bile its significant high levels had been seen in pigment type than in mixed type:

Table (8): % levels of iron salts in stones and bile

Levels of iron salts in mmol/L in bile and w/w% in stones			
	Number of samples	Mean ±SD	P value
Mixed stones	35	0.072 ± 0.037	P<0.001
Pigment stones	12	0.15 ± 0.078	
Bile of mixed stones	35	0.07 ± 0.01	P<0.005
Bile of pigment stones	12	0.12 ± 0.063	

Levels of iron salts can be noticed which formed in very low concentration (trace) in both types but still higher in pigment stones because it comes from haemolysis of erythrocytes whether physiological or pathological. Some of the iron can pass to the bile and unite with its salts to precipitate and be a part of a stone [22]. Table (9) shows levels of cupper salts which are significant in pigment type than in mixed type:

Table (9): % of levels of cupper salts

Levels of cupper salts in mmol/L in bile and w/w% in stones			
	Number of samples	Mean ±SD	P value
Mixed stones	35	0.065 ± 0.04	P<0.001
Pigment stones	12	0.128 ± 0.095	
Bile of mixed stones	35	0.032 ± 0.007	P<0.005
Bile of pigment stones	12	0.076 ± 0.019	

The results shows the levels of copper salts which are higher in pigment stones also this can be explained as that for iron salts formation, copper binds to bilirubinate ion to form copper bilirubinate salts [23]. This subject was chosen because it becomes the commonest gallbladder diseased not in Iraq only but all over the world. The main mechanism for stone formation is supersaturation of bile with its contents that leads to their precipitation and stone formation, in this study analysis of some organic and inorganic components of stones and their corresponding bile. Most attention was focused on presence of organic compounds as cholesterol, bile salts, phospholipids and bilirubin which play a major role in stone formation while the inorganic compounds play a co-precipitator role, the inorganic compounds are: calcium phosphate, magnesium phosphate, calcium oxalate and other inorganic salts. The study showed that mixed stones composed mostly from cholesterol while pigment stones composed of bilirubin and calcium salts.

Conclusion:

From the results that had been obtained it concluded that mixed stones are the commonest type in Iraqi patients due to the dietary habit of our people .The main mechanism of stone formation is supersaturation of bile with its contents which can be precipitated forming the nidus for stone formation. Calcium is an important factor for stone formation in both types because it forms crystals (nidus) for stone and accelerate the precipitation around that nidus.

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دراسة كيميائية حياتية لمكونات حصى المرارة لدى العراقيين

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الخلاصة

يهدف البحث إلى دراسة المكونات العضوية واللاعضوية في العصارة الصفراوية (bile) وحصى المرارة لمرضى حصى المرارة في العراق(Gallstones). جمعت العينات لسبعة وأربعون مريضاً يتراوح معدل أعمارهم (53±7) سنة و معدل كتلة الجسم BMI (30.82±4.18) كغم /م ^{2}. صنفت العصارة الصفراوية إلى نوعين طبقاً إلى مكونات الحصى العائدة لها: العصارة الصفراوية لمرضى حصى المرارة الخليطة(Mixed stone bile) والعصارة الصفراوية لمرضى حصى الصبغة الصفراوية (Pigment stone bile) .

درست أطياف الأشعة تحت الحمراء (IR) لكلا النوعين والتحاليل الكيميائية لبعض مكونات الحصى والعصارة الصفراوية العائدة لها ،تضمنت المكونات العضوية : (الكوليسترول و البيليروبين والأملاح الصفراوية والدهون المفسفرة). أما المكونات اللاعضوية فتضمنت أملاح كل من: (الكالسيوم و الفسفور والحديد والنحاس والمغنيسيوم). أشارت النتائج إلى وجود انخفاض ملحوظ في مستوى الأملاح الصفراوية والدهون المفسفرة في العصارة الصفراوية لمرضى الحصى الخليطة عند مقارنتها مع العصارة الصفراوية لمرضى حصى الصبغة الصفراوية بقيمة $p<0.005$ و $p<0.001$ على التوالي ، كما وأشارت النتائج إلى وجود زيادة معنوية ($P<0.005$) في نسبة أملاح كل من الكالسيوم والفسفوروالحديد والنحاس والمغنيسيوم في العصارة الصفراوية لمرضى حصى الصبغة الصفراوية مقارنة مع نسبتيهما في العصارة الصفراوية للحصى الخليطة.

استنتج من خلال هذا البحث أن غالبية حصى المرارة المتكونة لدى العراقيين هي من نوع الحصى الخليطة Mixed stones و النسبة الأعلى في مكوناتها هي الكوليسترول أما أملاح البيليروبين وأملاح العناصر الأخرى تمثل النسبة الأقل ، بينما حصى الصبغة الصفراوية تمثل النسبة الأقل حدوثًا لدى العراقيين وتكون أملاح البيليروبين هي النسبة الأعلى في مكوناتها إضافة إلى أملاح بعض العناصر الأخرى وهذا النوع من الحصى كثير الحدوث لدى المرضى المصابين بإمراض تكسر الدم كفقر الدم المنجلي و فقر دم البحر المتوسط وغيرها.