Composition Difference of Cholesterol-, Pigment- and Mixed-Type Gallstones, and Their Formation Mechanisms

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Abstract. Objective: The chemical compositions and formation mechanisms of different types of gallstones (cholesterol-, pigment-, and mixed-type) were studied. Methods: The components of gallstones were identified by Fourier transform infrared spectroscopy (FT-IR). The contents of cholesterol, bilirubin and bile acid in gallstones were determined by ultraviolet visible (UV-vis) spectrophotometry. Results: The majority of gallstones in Shenzhen area of China were cholesterol stones, followed by pigment stones. The incidence rate of female was significantly higher than that of male; the age of gallstone patients was mainly 30~50 years old. The pathogenesis of cholesterol gallstone is mainly the supersaturation of cholesterol in the bile. Pigment gallstone is closely related to the formation of calcium bilirubinate complex. Bile pigments can be deposited on cholesterol, then to form calcium bilirubinate, and finally form bile pigment-cholesterol mixed type gallstones. Conclusions: Cholesterol, bilirubin and bile acid play different roles in formation of different types of gallstones. Using UV-vis and FT-IR spectra can accurately detect the components of gallstones, and this analysis can provide inspiration for the effective prevention and treatment of gallstones.

Introduction

Gallstone disease is a common disease of biliary system, and its incidence is increasing [1, 2]. Literature [3] reported that the incidence of gallstones in male population was 7.9%, and in female was about 16.6%. However, the incidence rate of gallstones in African population was less than 5% [4]. At present, the pathogenesis of gallstones is not yet clear.

Gallstones can be divided into cholesterol gallstones, bile pigment stones, mixed stones and other types of stones (such as calcium carbonate, etc.). Cholesterol is present in all tissues of the body, and is also an important component of gallstones. About 70-80% of gallbladder stones are cholesterol stones. Cholesterol plays an important role in reducing the surface tension of fat. It is not easy for simple cholate to dissolve cholesterol; it needs 30 molecules of cholate to dissolve a molecule of cholesterol. Lecithin can greatly enhance the ability of cholate to dissolve cholesterol, so the lecithin plays a dominant role in the dissolution of cholesterol [5]. The formation of bile pigment stones is closely related to calcium bilirubinate.

The formation of gallstones is also closely related to the properties of bile acid and its salts. The side chain of the bile acid contains carboxyl groups, which can interact with calcium salts. The interaction between sodium cholate and hydroxyapatite was

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studied in vitro [6]. The results showed that sodium cholate could accelerate the formation of hydroxyl apatite. The reason is that the negatively-charged carboxyl C=O bond in sodium cholate can interact with Ca^{2+} ions, then speeding up the formation of HAP. Based on this, in this paper we study the main composition and content of 60 cases of gallstones, discusses the relationship between these components and the formation of different types of gallstones, and expected to provide inspiration for the effective prevention and treatment of gallstones in clinical medicine.

Experimental in Details

Reagents and Instruments

Cholesterol, bilirubin, and bile acid were purchased from Sigma Company.

FT-IR spectrometer (Nicolet-170 SX-type). UV-VIS-NIR UV-visible spectrophotometer (UV-3100 type, Shimadzu Corporation).

Collection and Characterization of Gallstones

60 cases of gallstones were obtained from surgery of Shenzhen People's Hospital, including 25 male and 35 female, aged between 24-71 years old, the average age is 43.9 years old. The stones were rinsed with water, and then disinfected with 95% alcohol, after washed twice with deionized water; they were dried at 45℃ in an oven and grinded into powder in a mortar. The component analysis by FT-IR indicated that 34 cases of gallstones were cholesterol type, 20 cases were pigment type, 4 cases were mixed type, and 2 cases were calcium carbonate type.

Determination of Cholesterol, Bilirubin and Bile Acid

All of them were determined with colorimetry, Cholesterol was determined at 555 nm using by iron chromogenic agent; bilirubin was measured in 533 nm by diazo reagent; cholic acid was directly measured at 387 nm using standard acid solution. All the experiments were repeated three times.

Statistical Analysis

The experimental data were analyzed using SPSS version 16.0 software and expressed as the mean ± standard deviation ($\bar{x}$ ±s). $p < 0.05$ was deemed to indicate a significant difference, $p < 0.01$ indicated an extremely significant difference, and $p > 0.05$ indicated no significant difference.

Results

Composition Detection of Different Types of Gallstones

The compositions of 60 cases of gallstones were detected using FT-IR spectrum and the results were shown in Table 1. It can be seen that 34 cases were cholesterol type, 20 cases were pigment type, 4 cases were mixed type, and 2 cases were calcium carbonate type. The prevalence rate of female patients with gallstones (58.3%) was about 1.4 times higher than that of men (41.7%).

Figure 1 shows the representative FT-IR spectra of all types of gallstones. For cholesterol gallstone (Figures 1A-D), the peak at v = 2934 and 2870 cm^{-1} belong to v_{as} (CH_2, CH_3) and v_s (CH_2, CH_3) of cholesterol, respectively, 1469 and 1377 cm^{-1} belong to δ_{as} (CH_2, CH_3) and δ_s (CH_2, CH_3) of methylene, 1050 cm^{-1} belongs to C-O bond stretching vibration v (C-O) of cholesterol. The weak absorption peak at wave number

ν = 1637 cm^{-1} was the absorption of amide I of proteins. All these results showed that their main composition was cholesterol.

Table 1. Composition analyses of 60 cases of gallstones and sex distribution of these gallstone patients

Type of gallstone	Number	Detection rate (%)	Male (%)	Female sex (%)
Cholesterol	34	56.7	14 (41.2%)	20(58.8%)
Pigment	20	33.3	207 (35%)	13 (65%)
Mixed type	4	6.7	42 (50.0%)	2 (50.0%)
Calcium carbonate	2	3.3	22 (100.0%)	0 (0%)
Total	60	100	6025 (41.7%)	35 (58.3%)

In the FT-IR spectra of pigment stones (Figures 1E-G), the peak at ν = 3406 cm^{-1} was assigned to pyrrole ring N-H stretching vibration in calcium bilirubinate; 1669 and 1627 cm^{-1} were assigned to lactam ν(C=O, C-N, C=C); 1570 cm^{-1} was v_{as} (COO-); 1440 cm^{-1} was δ (CH_2) of the pyrrole of bilirubin; the absorption peak at 1250 cm^{-1} belonged to carbon nitrogen bond (C-N) stretching vibration of pyrrole ring. That is, the main component of these samples was calcium bilirubinate.

In the FT-IR spectra of calcium bilirubinate - cholesterol mixed stones (Figure 1H), the peaks at ν = 3406, 1669, 1627, 1570, 1440, 1250, 702 cm^{-1} were attributed to the characteristic absorption of bilirubin, and the peaks at ν = 1050 cm^{-1} was the characteristics absorption of cholesterol. It indicated the samples contained cholesterol and calcium bilirubinate simultaneously.

Figure 1I is a representative FT-IR spectrum of calcium carbonate calculus. the peaks at ν = 3422, 2928, 1487, 875, 720 cm^{-1} were assigned as the characteristics absorption of calcium carbonate.

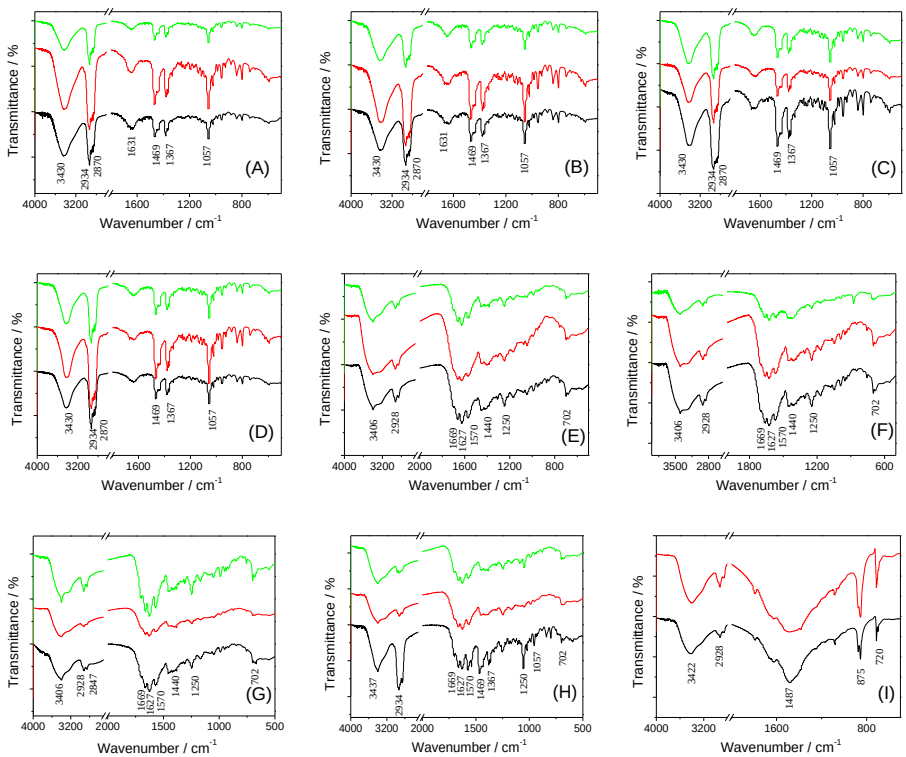

Figure 1. Representative FT-IR spectra of different types of gallstones. (A-D) cholesterol type; (E-G): calcium bilirubinate type; (H): mixed type; (I): calcium carbonate type.

Content of Cholesterol, Bilirubin and Bile Acid in Different Types of Gallstones

The absorption peaks of cholesterol, bilirubin and bile acid were at 555, 533 and 387 nm, respectively, and their standard curves were shown in Figure 2. It can be seen that the three standard curves were linear, and the correlation coefficients of these data were stronger, and their regression equations were significant. Figure 3 showed the absorption peaks for different concentrations of bile acid solution.

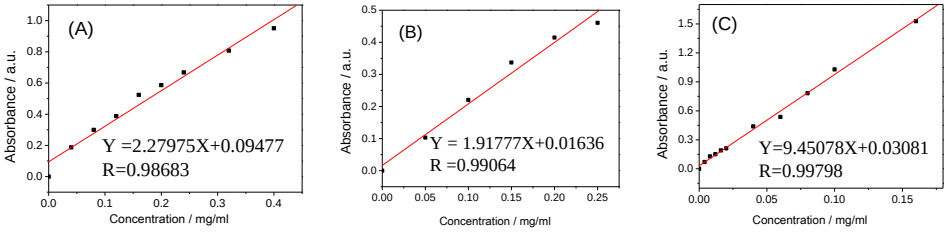

Figure 2. Standard curves for detection of cholesterol (A), bilirubin (B) and bile acid (C).

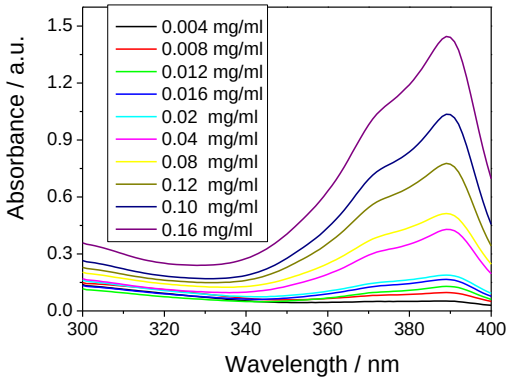

Figure 3. Absorption curves of different concentrations of bile acid.

Table 2 lists the content of cholesterol, bilirubin, and bile acid in 60 cases of different types of gallstones. It can be seen that content of cholesterol levels in cholesterol gallstone is significantly higher than that in pigment stones (252.2 ± 17.0 vs 73.2 ± 11.9 mg/L, P < 0.01). The content of bilirubin in pigment stones is significantly higher than that in cholesterol stones (316.6 ± 15.5 vs 24.3 ± 7.9 mg/L, P < 0.01). The content of bile acid in cholesterol gallstone is significantly less than that in pigment stones (17.1 ± 2.2 vs 22.4 ± 3.2 mg/L, P < 0.05). The content of various components in mixed type stones is similar to that of pigment stones; and the contents of both bilirubin and cholesterol are very little in calcium carbonate stones.

Table 2. Content of cholesterol, bilirubin, bile acid in 60 cases of different types of gallstones.

Gallstone type	n	Cholesterol (mg/L)	Bilirubin (mg/L)	Bile acid (mg/L)
Cholesterol	34	252.2 ± 17.0	24.3 ± 7.9	17.1 ± 2.2
Pigment	20	73.2 ± 11.9	316.6 ± 15.5	22.4 ± 3.2
Mixed	4	73.4 ± 32.5	249.3 ± 65.9	11.9 ± 3.4
Calcium carbonate	2	10.0 ± 0.8	55.4 ± 8.6	13.8 ± 0.2
P		$p_{1,2} < 0.01$; $p_{1,3} < 0.01$;	$p_{1,2} < 0.01$; $p_{1,3} < 0.01$	$p_{1,2} < 0.05$, $p_{2,3} < 0.05$

Discussion

Age and Sex Distribution of 60 Cases of Gallstones Patients

In this paper, the chemical compositions of gallstones in Shenzhen area were analyzed. It was found that cholesterol gallstones accounted for the majority, followed by type of pigment gallstones.

It can be seen from Table 1 that the incidence rate of female patients with gallstones in Shenzhen area was significantly higher than that of male (Male: Female = 7:5), which was consistent with the research result of literature [7].

Age is one of the most important factors to lead gallstone disease. It is reported that children under 16 years old have almost no gallstone disease, and the incidence rate increases with the increase of age [8]. Figure 4 shows the age and sex distribution of gallstone patients. We can see that the patients was distributed between the ages of 24 to 71 years old, and the male patients of 40-49 years old were the largest, accounting for 32%, followed by patients of 30-39 years old, accounting for 24%. The distribution of female patients in 30-39 years old was the largest, accounting for 31.4%. Our results were consistent with the literature [7]. The prevalence of gallstones in young people in this study is relative high; it was mainly related to the lifestyle of young people in Shenzhen area. The young people in this area have a busy life and less exercise, they like high calorie, high fat, and high sugar diet. All these factors are the cause of the high incidence of gallstones for the younger.

Generally speaking, the probability of a woman to suffer from gallstones in a life is 2-3 times higher than male. Estrogen can influence liver lipid metabolism, increase cholesterol saturation in bile, thus promote the formation of gallstones.

Role of Cholesterol, Bilirubin and Bile Acid in Gallstone Formation

1) Role of cholesterol.

It can be seen from Table 2 that the cholesterol content in cholesterol stones was significantly higher than that in pigment stones (252.2 ± 17.0 vs 73.2 ± 11.9 mg/L, $P < 0.01$), and also higher than that in mixed type and calcium carbonate type gallstones, it suggests that cholesterol plays an very important role in formation of cholesterol gallstones.

About 70-80% gallbladder stones are cholesterol type. Hypersecretion cholesterol in liver is the main pathological mechanism of cholesterol gallstone formation. Excess cholesterol secretion may be accompanied by an abnormal secretion of cholates and phospholipids [9]. Excess cholesterol can be precipitated as a solid crystal, which is the essential condition for formation of cholesterol stones.

There is a small amount of calcium bilirubinate complex in cholesterol stones. A small amount of bilirubin can lead to formation of cholesterol crystals.

2) Role of bilirubin.

In pigment stones, the bilirubin level was significantly higher than that in cholesterol stones (316.6 ± 15.5 vs 24.3 ± 7.9 mg/L, $P < 0.01$), it indicates that bilirubin plays a crucial role in formation of pigment stones. The bacteria in infected bile easily produce a large number of β-glucose acid glycosides, which can hydrolyze bilirubinate into non-conjugated bilirubin (UCB); UCB can react with calcium ions to form calcium bilirubinate precipitation. When the activity of β-glucose increases, the concentration of UCB will increase, it leads an increase of the incidence of pigment gallstones [10].

The main composition of mixed type gallstones is calcium bilirubinate. Most of the mixed type gallstones have cholesterol core structure [11], and the outer layer is mainly composed of calcium bilirubinate and calcium carbonate. After cholesterol core is formed, calcium bilirubinate can deposited on the core, and then a cholesterol-cholesterol mixed type gallstones was formed.

3) Role bile acid.

A small amount of bile acid was detected in all types of gallstones. Cholate is an important biological surfactant in bile; it can make cholesterol be dissolved in bile easily. The low concentrations of bile acid in gallstones of cholesterol type and mixed type suggest the concentration of cholate is low, which creates favorable condition for the precipitation of cholesterol crystals. Pigment gallstone is mainly calcium bilirubinate complex, and less affected by cholate.

Conclusions

FT-IR was used to analyze the compositions of 60 cases of gallstones in Shenzhen area of China. The majority of gallstones were cholesterol stones, followed by pigment stones. The incidence rate of female was significantly higher than that of male; the age of the patients was mainly between 30 and 50 years old. Cholesterol, bilirubin and bile acid play different roles in the formation of different type's gallstones. Analysis of each composition in gallstones is the key to reveal the formation mechanism and to provide the effective prevention and treatment of gallstones.

Acknowledgments

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