Crystallogeny fundamentals of the cholesterol gallstone*

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Abstract The nucleation mechanism and crystal growth process of the cholesterol gallstone are studied and a systematic theory expounded by crystallogeny is proposed. Normal feed and stone-forming feed were used to raise guinea pigs in the control and stone-causing groups respectively. The state and transformation of liquid crystal vesicles, the appearance of crystal nuclei, and the formation of microcrystal grains were observed under a polarizing microscope during the experimental period. It was found that the liquid crystal vesicles in the bile of the control group were small, scattered, and always existed as single forms, and no shaped gallstone crystals were formed. While in the stone-causing group, liquid crystal vesicles grew to larger ones, and then aggregated to form large liquid crystal cells. Solid crystal growth along the edge of these liquid crystal cells formed microcrystal grains. These demonstrated that bile liquid crystal vesicles form the basic nuclei of cholesterol gallstone. Heterogeneous nucleation is the common process in the formation of crystal nuclei and crystal growth.

Keywords: **cholesterol gallstone, liquid crystal vesicles, heterogeneous nucleation.**

Cholelithiasis is a frequently-occurring disease, and the incidence of this disease in adult is 15%—20% in western countries. In China, the incidence has reached 7%—10% and is still rising[1—3]. Therefore the research on the pathogenesis of cholelithiasis has attracted increasing attention. In Caucasians, about 90% of gallbladder stones are cholesterol-stone calculi, which is higher than that in Chinese population[1—3]. While with the changes of food structure, the proportion of cholesterol-stone calculi in Chinese is increasing year after year[1—3]. When studying the mechanism of gallstone formation, several hypotheses have emerged[4—6], which involve the absorption and excretion of cholesterol and the chemical environment of bile.

From the viewpoint of crystallogeny, a key stage in the initial formation of cholesterol stone calculi is the formation of cholesterol nuclei from supersaturated bile. Since the 1990s, extensive studies on model bile experiments have been conducted. Some of the experiments showed that the liquid crystal vesicles are the basic nucleus and play an important role in gallstone formation. And some others inferred that the liquid crystal vesicles are the predecessor of gallstone. However, these results should be confirmed by animal experiments or even human experiments.

In this study we investigated the nucleation mechanism and the crystal growth process in guinea pigs. We observed the process of gallstone formation by a polarizing microscope and tried to explain our findings by crystallogeny theories.

1 Materials and methods

1.1 Materials

Forty-six guinea pigs, half male and half female, each weighing about 300 g, were randomly divided into control ($n=23$) and stone-causing groups ($n=23$). The guinea pigs were provided by the animal branch of the Scientific Research Department in Kunming Medical College, with qualified animal testing certificate issued by Yunnan Province.

1.2 Methods

Animals care: Normal feed was provided by the animal branch of the Scientific Research Department of Kunming Medical College (a qualified unit for the equipments of animal's experiments) to the control group, while the stone-causing group was given stone-forming feed. The two groups were raised separately in flocks with free access to water containing 1% vitamin C.

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The components of the normal feed were as follows: bran 28%, maize 20%, horsebean haulm bran 30%, bean cake 10%, leaven 4%, powdered whey 3%, fish meal 3%, bone meal 1%, salt 0.5%, V_{AD3} 0.2%, lysine 0.1%, methionine 0.1%, mineral accession 0.1%; and the components of the stone-forming feed were as follows: starch 25%, bran 25%, glucose 12%, lard 1.5%, cholesterol 1.5%, normal feed 35%. The mechanism of gallstone formation caused by stone-forming feed is mainly due to the too much intake of cholesterol and grease. The components were mixed evenly and made into granules with a feed machine.

Sample preparation and observation: The guinea pigs were sacrified at three time points: 10 days after feeding (with 5 guinea pigs in each group), 25 days after feeding (also with 5 guinea pigs in each group), 60 days after feeding (with 13 guinea pigs in each group). Their gallbladder was taken out and the bile was collected by a standard surgery procedure.

Using a micro-sample advancer with precision of 0.2 μL (W-104 model of Shanghai Medical Instruments Factory, China), 6.0 μL of the bile samples was dropped onto a slide and an advanced polarizing microscope (BHSP model of Olympus, Japan) was used to observe the state of the liquid crystal nuclei and the growth process of gallstone crystals.

2 Results

2.1 Liquid crystal vesicles in gallbladder bile of guinea pigs in the control group

Under the perpendicular polarizing microscope, the bile liquid crystal vesicles of guinea pigs in the control group were small, scattered and occurred as single vesicles. With feeding days prolonged, bile liquid crystal vesicles increased and became larger, and occasionally were densely distributed in some regions. However, the bile liquid crystal vesicles did not merge into large liquid crystal bodies, as shown in Fig. 1.

2.2 Bile liquid crystal inclusions in gallbladder of guinea pigs in the stone-causing group

Under the perpendicular polarizing microscope, the bile liquid crystal vesicles of guinea pigs in the stone-causing group increased and became larger. With the feeding days prolonged, these liquid crystal vesicles gathered gradually and merged into large polymeric bodies (Fig. 2). Particularly, the interior of these inclusions still remained in a liquid crystal state, surrounded by well grown solid crystal substances. This indicated that crystals grew outward from a core formed by these liquid crystal inclusions, forming some micro-gallstone crystal grains, as shown in Fig. 3.

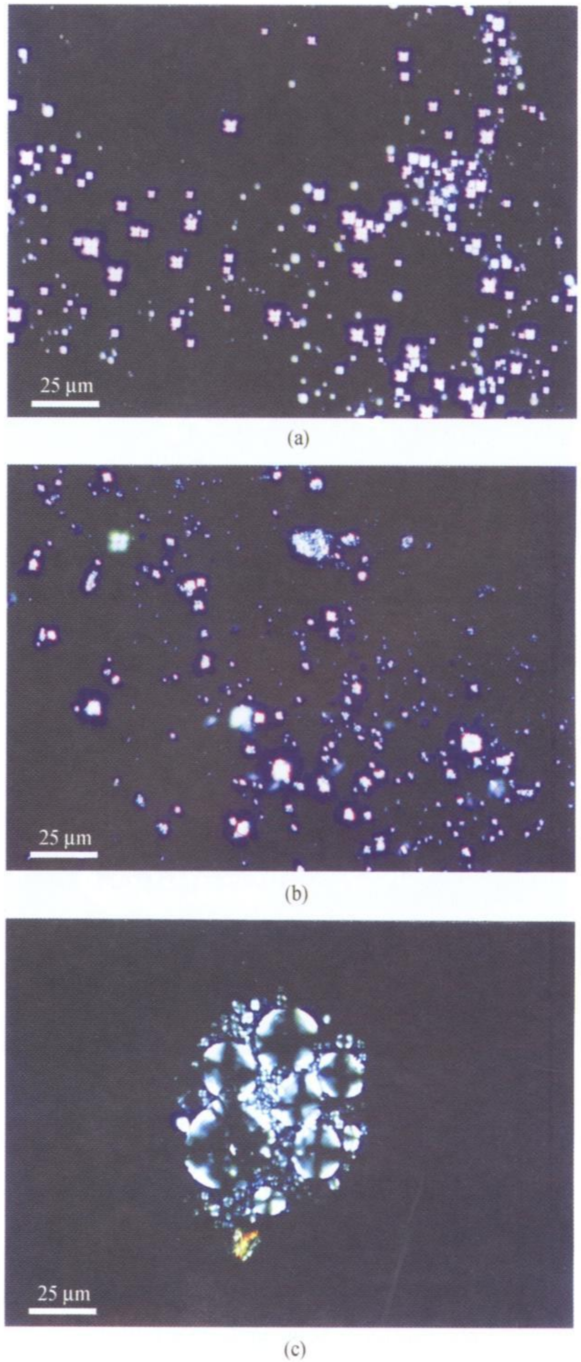
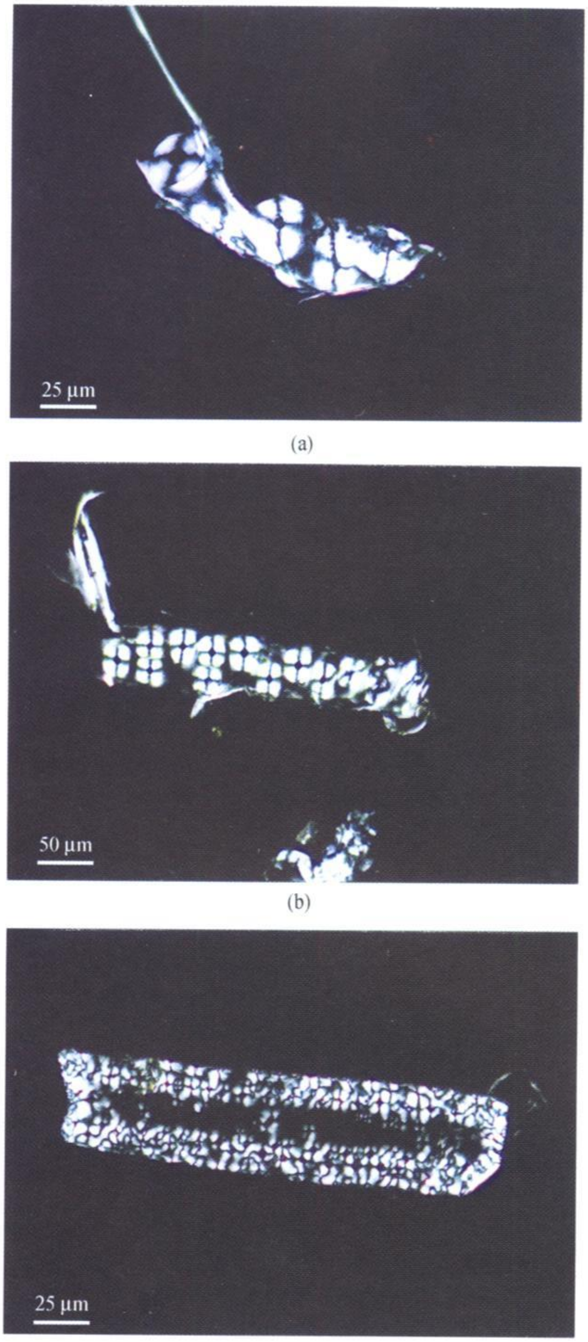


Fig. 1. Bile liquid crystal vesicles in gallbladder bile of guinea pigs in the control group. (a) Small, scattered crystal vesicles (after 10 days feeding); (b) larger crystal vesicles (after 25 days feeding); (c) densely distributed crystal vesicles (after 60 days feeding).



(c)

Fig. 2. Bile liquid crystal vesicles gathered and merged into large polymeric bodies in stone-causing group. After feeding for 10 days (a), 25 days (b), 60 days (c).

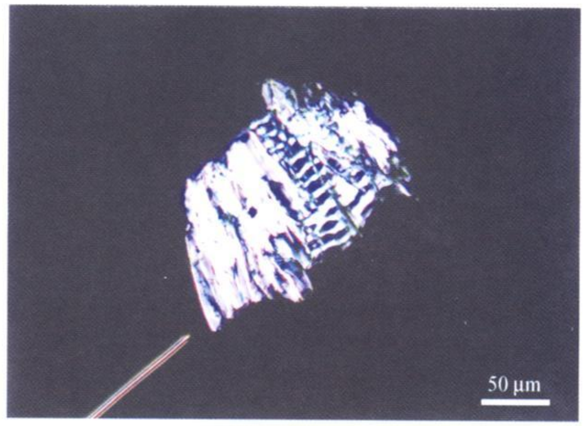


Fig. 3. Crystal growth from the cores of bile liquid crystal inclusions in stone-causing group.

3 Discussion

Bile is a kind of polynary solution system composed of cholesterol, cholic acid, bile bilirubin, phospholipid, inorganic salt, etc. Cholesterol is a kind of lipid that cannot dissolve in water, but it has a certain solubility in a cholate-phospholipid water solution system[3,4]. According to the micelles and vesicles theory, micelles are a polymer of cholesterol, cholate and phospholipid[4—7], and within a suitable proportional range, cholesterol stays in a dissolved state. However, as the proportion changes, cholesterol separates out from the bile to form a crystal gallstone[8—12]. Vesicles exist in a monolayer or multilayer structure lipid body composed of cholesterol and phospholipid[13—15]. Phospholipid is a biological macromolecule and has smectic liquid crystal character. It has a liposoluble hydrophobic radical which easily combines with cholesterol to form a cholesterol-phospholipid amphiphilic molecule and takes on a liquid crystal state in water solution. These amphiphilic molecules will form a globular or ellipsoid grain automatically under the action of molecular forces, and this process will occur automatically even in low saturation bile. With the increase of amphiphilic molecule concentration, they can gather, merge, and further form a smectic liquid crystal structure.

From the viewpoint of crystallogeny, in the process of phase transition or crystal growth in a solution system, the occurring and growing up of new nucleus is called nucleation. It can be divided into spontaneous and heterogeneous nucleation[16,17]. Spontaneous nucleation is the process whereby the same probability of nucleation exists everywhere in the parent phase, but spontaneous nucleation must overcome a very high surface potential, as well as needing a very high degree of supersaturation[17], which is not possible in the gallbladder. Heterogeneous nucleation is a process in which a certain type of non-uniformity exists in the parent phase solution system. For instance, liquid crystal vesicles, cholesterol separation, bilirubin separation, inflammation secretion of gallbladder, relict parasite, calcium salt deposits, etc. This non-uniformity initiates the role of nucleation to form crystal growth. Among which, the liquid crystal vesicles composed of cholesterol-phospholipid amphiphilic molecules form the basic nucleus. It plays a very important and even key role in gallstone formation. In the polynary bile system, different temperatures and concentrations of amphiphilic molecules will cause several basic mesomorphic liquid crystals to form. At different concentrations of amphiphilic molecule, globular S_1 and S_2 phases, ellipsoidal M_1 and M_2 phases, and smectic G phase can be formed, they have been all observed in our previous experiments[18, 19].

Because of the presence of these amphiphilic molecules, and under the action of molecular forces, cholesterol-phospholipid amphiphilic molecules can collect into globular or ellipsoidal grains automatically. This process will occur spontaneously even at lower concentrations of bile. Thus, as long as the cholesterol-phospholipid amphiphilic molecules exist, and however high or low their concentration, liquid crystal vesicles would form. This is why we find liquid crystal vesicles existing in the bile of the control and stone-causing groups. The difference is, with the increase in concentration of cholesterol in the bile of the stone-causing group, the density of cholesterol-phospholipid amphiphilic molecules increases. Therefore bile liquid crystal vesicles gather gradually and then merge into a large single polymeric body. This single polymeric body can be as large as several hundreds microns[18, 19].

The formation of bile liquid crystal vesicles results in the state that cholesterol concentration is far higher in this region than in surrounding bile. Thus, these bile liquid crystal vesicles and their polymeric bodies intersperse in the bile to form the crystal nuclei. So that, growth of multinucleate crystals based on these nuclei is possible. Of course, because of the complexity and changeability of the bile environment, crystal nuclei cannot grow to form integrate crystal but only minicrystal cells. This is why we can find a great deal of minicrystal cells or aggregate minicrystal cells in gallbladder stones under the electron microscope or polarizing microscope[19, 20]. Under suitable conditions, these micro-crystals constantly sediment, grow, dissolute and ablate, leading to the formation of various shapes of gallstone crystals[19, 20].

It is necessary to emphasize that the growth and dissolution of crystal is a dynamic process occurring simultaneously, so it is uncertain that a shaped stone can form from a nucleus existing in the gallbladder. With a small nucleus size, a higher degree of supersaturation or super-cooling is needed for crystal growth. Otherwise, a nucleus smaller than critical size may be dissolved earlier or later. In this experiment, either in the control or in the stone-causing group, liquid crystal vesicles could always be observed in the gallbladder bile of guinea pigs. However, the difference is that in the gallbladder bile of the control group, the bile liquid crystal vesicles always existed in single forms, and the crystal growth extending from these single liquid crystal vesicles never appeared. This is because the size of these single liquid crystal vesicles is not big enough, in other words, it is smaller than the critical size for crystal growth. But in the bile of the stone-causing group, except single liquid crystal vesicles, we observed that liquid crystal vesicles tended to aggregate and merged in strings, forming liquid crystal inclusions, which are large enough to reach the critical size needed for crystal growth. The crystals grow rapidly extending from the edge of these liquid crystal inclusions, thus, a great number of micro-crystal grains formed. If these micro-crystal grains cannot be drained in time, further growth of crystals and the formation of shaped stone crystals cannot be avoided. So, gathering and merging of bile liquid crystal vesicles is the key to crystal growth.

In conclusion, the bile liquid crystal vesicles form the nuclei of the cholesterol gallstone. Heterogeneous nucleation is the common process in the formation of crystal nuclei and crystal growth.

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