Stability of human gallbladder bile: Effect of freezing

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P Janowitz, R Mason, W Kratzer. Stability of human gallbladder bile: Effect of freezing. Can J Gastroenterol 2001;15(6):363-366. In the present study, the stability of the most essential biliary parameters of human gallbladder bile at -18°C was examined over several months. In 12 patients with gallstone disease (10 female, two male; 52.1 ± 13.3 years of age), bile was obtained through fine needle puncture of the gallbladder under local anesthetic. The concentrations of total lipids, cholesterol, phospholipids and bile acids, and the cholesterol saturation index and crystal appearance time were determined before and after freezing over a mean period of 4.38 ± 2.9 months. Gallbladder bile obtained by fine needle puncture has proved to be of excellent quality. The total lipid concentration was unchanged before (8.30 ± 4.16 g/dL) and after freezing (9.16 ± 4.54 g/dL, $P=0.6027$). The biliary cholesterol, phospholipids and bile acid concentrations, and cholesterol saturation index showed no statistically significant differences before and after freezing. A significant difference arises in the context of subdivision of the group to the nucleation time. Before freezing, most patients had a nucleation time between five and eight days, which shortened to between one and four days after thawing ($P=0.0100$). The authors conclude that, with the exception of the nucleation time, human gallbladder bile can be stored at -18°C for four months with stability of major lipid components.

Key Words: Bile acids; Cholesterol saturation index; Diagnosis; Gallstones; Nucleation time; Puncture

L'effet du gel sur la stabilité de la bile humaine, prélevée dans la vésicule

RÉSUMÉ : La présente étude avait pour objet la stabilité des principales caractéristiques de la bile humaine, prélevée dans la vésicule et conservée à une température de -18°C ; l'analyse s'est échelonnée sur plusieurs mois. Une ponction de la vésicule pratiquée sous anesthésie locale au moyen d'une aiguille fine a permis le prélèvement de bile chez 12 patients (10 femmes, 2 hommes; $52,1 \pm 13,3$ ans) souffrant de lithiase biliaire. On a procédé à différentes mesures avant et après le gel, et ce, sur une période moyenne de $4,38 \pm 2,9$ mois; il s'agit des taux de lipides totaux, de cholestérol, de phospholipides et d'acides biliaires ainsi que du degré de saturation du cholestérol et du temps d'apparition des cristaux. La bile prélevée s'est avérée d'excellente qualité. Le taux de lipides totaux est resté inchangé avant et après le gel ($8,30 \pm 4,16$ g/dl contre $9,16 \pm 4,54$ g/dl; $P=0,6027$). Les taux de cholestérol biliaire, de phospholipides et d'acides biliaires, de même que le degré de saturation du cholestérol, n'ont pas subi de différence significative sur le plan statistique. Le seul paramètre qui a montré une différence significative est le temps de nucléation. Avant le gel, ce temps oscillait entre cinq et huit jours chez la plupart des patients mais, après le dégel, il est passé de un à quatre jours ($P=0,0100$). Les auteurs en arrivent à la conclusion que la bile humaine prélevée dans la vésicule peut être conservée à une température de -18°C durant quatre mois tout en maintenant ses principales caractéristiques, sauf en ce qui concerne le temps de nucléation.

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The ongoing progress in the conservative therapy of gallstone disease has focused attention on the importance of adequate patient selection (1-6). Human gallbladder bile samples are often used in biliary research. The presence of cholesterol crystals and the determination of nucleation time (NT) in the gallbladder bile are the best methods of distinguishing between cholesterol and pigment gallstones, and correlate well with computed tomographic analysis (1,7,8). Furthermore, microscopic examination of bile is easier, quicker and cheaper than biochemical analysis of bile or computed tomography of the gallbladder (9-12). An immediate examination of the gallbladder bile at 37°C is considered a prerequisite for a microscopic examination. Conjugated bilirubin remains relatively stable at -70°C over several months but degrades rather quickly at -15°C (13). Other biliary parameters, such as bile acids, phospholipids and cholesterol, are stable at -30°C over a week (14). The aim of the present study was to examine the stability of the major lipid components at -18°C over several months.

PATIENTS AND METHODS

In 12 patients with gallstone disease (10 female, two male; 52.1±13.3 years of age; five cholesterol and seven mixed pigment gallstones), bile was obtained through fine needle puncture of the gallbladder under local anesthetic. In one female patient, the gallbladder puncture was performed twice.

Bile aspiration was performed for research purposes; therefore, appropriate informed consent was obtained from each patient. Patients with a history of chronic or acute cholecystitis, or sonographically or computed tomographically confirmed cholecystitis were excluded from the study.

The fine needle puncture of the gallbladder was performed between 08:00 and 09:00 after a 12 h fasting period. The method has been described elsewhere (1,3,15). The transducer was equipped with a commercially available lateral puncture attachment to provide a biopsy guide for all punctures. To avoid bile leakage, the gallbladder bile was completely aspirated in each patient. Aerobic and anaerobic cultures were performed immediately after aspiration. The positive samples contaminated by bacteria have been excluded from the present study. The bile samples were ultracentrifuged at 100,000 g (37°C) for 2 h. The isotropic (cholesterol- and liquid crystal-free) interphase was incubated at 37°C afterwards. The NT was determined by applying the method of Holan et al (8), defined as the earliest time required for detection of cholesterol monohydrate crystals in aliquots of the incubated isotropic phase, expressed in days. One drop of incubated aliquots was examined daily on a glass slide at a magnification of 100× to 400× under crossed polars (Leitz, Germany). Total biliary bile acids were determined by enzymatic testing (Sterognost-3- α , Nycomed N-0401 Oslo 4, Norway). Bile acid analysis was carried out using high performance liquid chromatography with dexamethasone as the internal standard (16). The biliary cholesterol and phospholipid concentrations were determined using an enzymatic colour

TABLE 1

Biliary parameters before and after freezing at -18°C over a mean period of 4.38±2.9 months (n=13)

	Before	After	P
Nucleation time (days)	7.9±7.5	5.4±7.9	0.4236
Cholesterol saturation index	1.36±0.33	1.37±0.31	0.8904
Total lipid concentration (g/dL)	8.3±4.1	9.1±4.5	0.6027
Biliary bile acids (mmol/L)	107.1±56.0	114.3±61.0	0.7567
Biliary bile acids (mol%)	68.6±3.8	67.0±5.5	0.4171
Phospholipids (mmol/L)	36.4±14.5	37.9±18.9	0.8195
Phospholipids (mol%)	21.5±1.8	22.7±3.3	0.2808
Biliary cholesterol (mmol/L)	13.9±6.5	16.0±7.8	0.4768
Biliary cholesterol (mol%)	9.8±2.7	10.2±2.8	0.7257

test (CHOD-PAP method, Boehringer Mannheim, Germany). The total lipid concentration of bile (TLCO) is the sum of the cholesterol, bile acid and phospholipid concentrations, and is expressed in g/dL (conversion factors: cholesterol 386 g/mol, bile acids 495 g/mol, phospholipids 735 g/mol). The cholesterol saturation index, as defined by Admirand and Small (17), was calculated using a microcomputer program described by Kuroki et al (18). The rest of each gallbladder bile sample was stored at -18°C over a mean period of 4.38±2.9 months (range 0.3 to 13 months). After thawing at room temperature over 12 h, the bile samples were ultracentrifuged at 100,000 g (37°C) for 2 h; the isotropic interphase was then incubated at 37°C, and the renewed determination of the biliary bile parameters was carried out according to the methods described above.

All data are presented as mean ± SD. Statistical significance was calculated using the Student's *t* test for dependent random samples.

RESULTS

The gallbladder puncture was successful in all 12 patients (100%). Complications due to the fine needle puncture, such as bleeding, bile leak or inflammation, were not observed. The TLCO was unchanged before (8.30±4.16 g/dL; range 1.38 to 15.3 g/dL) and after freezing (9.16±4.54 g/dL; range 1.41 to 16.4 g/dL) (P=0.6027). There was no significant correlation between lipid concentration and the length of the storage period ($r=-0.0704$, $P=0.8194$). Other biliary components also showed no statistically significant differences before and after freezing (Tables 1 and 2).

The NT was not significantly different in the complete group before freezing with 7.9±7.5 days and after freezing with 5.5±7.9 days (P=0.4236); however, individual fluctuations have been observed (Figure 1). A significant difference arises in the context of subdivision of the group for the NT (P=0.0100, χ^2 test). Before freezing, most patients had an NT of between five and eight days. After thawing, this peak lay between one and four days.

TABLE 2
Percentage of biliary bile acids before and after freezing
(n=13)

Biliary bile acids (mol%)	Before	After	P
Cholic acid	29.6±6.8	30.7±6.3	0.6581
Glycocholic acid	18.9±5.7	20.4±3.4	0.4274
Taurocholic acid	10.8±6.0	10.3±4.3	0.8005
Chenodeoxycholic acid	36.0±10.3	35.9±10.3	0.9760
Glycochenodeoxycholic acid	26.9±8.5	26.8±8.5	0.9673
Taurochenodeoxycholic acid	8.6±3.7	9.1±3.9	0.7555
Deoxycholic acid	28.8±9.6	28.8±9.5	0.9903
Glycodeoxycholic acid	22.7±8.6	22.7±8.4	0.9891
Taurodeoxycholic acid	6.1±3.4	6.1±3.4	0.9504
Lithocholic acid	2.6±1.6	2.6±1.7	0.9631
Glycolithocholic acid	1.5±1.2	1.4±0.8	0.6641
Taurolithocholic acid	1.0±0.7	1.2±1.0	0.5173
Ursodeoxycholic acid	3.0±2.8	1.9±1.7	0.2353
Glyoursodeoxycholic acid	1.1±1.2	1.0±1.0	0.8011
Tauroursodeoxycholic acid	1.8±2.9	0.8±1.7	0.2860

DISCUSSION

In the present study, the stability of human gallbladder bile remained unaltered at -18°C over a mean period of 4.38 ± 2.9 months. Cholesterol, phospholipids, bile acids, total lipid concentration and the cholesterol saturation index showed no trends or significant changes. Other studies had already shown that biliary components are stable at -30°C over one week (14). In a study of porcine gallbladder bile, the pH and concentrations of total and ionized calcium, phospholipid, cholesterol and bile salts were stable over 17 days at -17°C (19). The total bilirubin concentration was also stable at -15°C (19).

The NT was the only parameter that showed clear individual fluctuations. Before freezing, most patients had an NT between five and eight days; after freezing this peak was between one and four days. In the initial stage of cholesterol gallstone formation, the nucleation of cholesterol monohydrate crystals occurs within the mucous gel (20). The results of other studies suggest that qualitative differences in individual proteins of gallbladder bile are responsible for nucleation-promoting activity in vitro (20-24). On the other hand, the purified apolipoproteins A-1 and A-2 are able to prolong the NT (25-27). Apolipoprotein A-1 stabilizes the nonmicellar fraction by forming an apolipoprotein A-1 and lipid complexed particle, resulting in prolonged cholesterol crystal nucleation (27). The close association of vesicles and crystals in human gallbladder bile supports the contention that vesicles are important in the initial nucleation of cholesterol monohydrate crystals (28). Either the glycoprotein activity becomes strengthened after freezing or the activity of the nucleation inhibitor factors is reduced. In the present study, the glyco-

Figure 1) Distribution of the nucleation time in days before and after freezing (n=13)

proteins and the apolipoproteins were not measured; therefore, it is not possible to comment on this. The prerequisite for a longer storage of the gallbladder bile is presumably an extraction of the samples without bacterial contamination. In another study, the gallbladder bile was contaminated in 13.3% of patients with gallstones without history and without evidence of acute or chronic cholecystitis (29).

CONCLUSION

With the exception of the NT, human gallbladder bile can be stored at -18°C for four months with stability of major lipid components.

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