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Mucin and phospholipids determine viscosity of gallbladder bile in patients with gallstones

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Abstract

AIM An increased viscosity of gallbladder bile has been considered an important factor in the pathogenesis of gallstone disease. Besides lipids and proteins, mucin has been suggested to affect the viscosity of bile. To further clarify these issues we compared mucin, protein and the lipid componEnts of hepatic and gallbladder bile and its viscosity in patients with gallstones.

METHODS Viscosity of bile (mPa.s) was measured using rotation viscosimetry in regard to the non Newtonian property of bile at low shear rates.

RESULTS Biliary viscosity was markedly higher in gallbladder bile of patients with cholesterol (5.00 ± 0.60 mPa.s, mean ± SEM, *n* = 28) and mixed stones (3.50 ± 0.68 mPa.s; *n* = 8) compared to hepatic bile (0.92 ± 0.06 mPa.s, *n* = 6). A positive correlation between mucin and viscosity was found in gallbladder biles (*r* = 0.65; *P*<0.001) but not in hepatic biles. The addition of physiologic and supraphysiologic amounts of mucin to gallbladder bile resulted in a dose dependent non linear increase of its viscosity. A positive correlation was determined between phospholipid concentration and viscosity (*r* = 0.34, *P*<0.005) in gallbladder biles. However, no correlation was found between total protein or the other lipid concentrations and viscosity in both gallbladder and hepatic biles.

CONCLUSION The viscosity of gallbladder bile is markedly higher than that of hepatic bile in patients with gallstones. The concentration of mucin is the major determinant of biliary viscosity and may contribute by this mechanism to the role of mucin in the pathogenesis of gallstones.

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INTRODUCTION

Gallbladder mucin, a high molecular weight and densely glycosylated protein secreted by gallbladder epithelium, is the principal organic constituent of gallbladder mucus and biliary sludge[1-3]. It appears to play an important role in different stages of cholesterol or mixed gallstone formation[4]. Gallbladder mucin hypersecretion and accumulation in the gallbladder as a viscoelastic gel precedes the formation of gallstones in animals[5,6] and in humans[7,8]. Furthermore, the highly viscous gel on the luminal side of the gallbladder epithelium and the soluble mucin in bile are believed to enhance the residence time of lithogenic bile in the gallbladder, allowing the growth of cholesterol crystals and, thereby, serving as a nidus for gallstone formation[2,9]. In fact, an association between hexosamine concentrations in bile which derive mostly from soluble mucin and the viscosity of bile has been already described by Bouchier *et al*[10] many years ago. In a more recent study, capillary viscosimetry like Bouchier biliary viscosity at 37℃ was correlated closely with total protein concentration and hexosamine concentration in bile[11]. Few further studies dealing with bile viscosity are available, and in these studies capillary viscosimeters were also used[12-14]. According to these studies, bile behaves essentially as a Newtonian fluid at high shear rates. A Newtonian fluid has a constant viscosity, whereas a non-Newtonian fluid shows a disproportionate increase in viscosity at a low flow velocity. However, capillary viscosimeters lack standardization and are unable to measure the viscosity at low shear rates, at which bile shows a non-Newtonian behaviour. We used the Contraves LS-30 coaxial rotation viscosimeter to eliminate this problem. This

viscosimeter is very sensitive and has a wide measurement range, from 0.01 s^{-1} to more than 100 s^{-1} covering the range of both Newtonian and non-Newtonian flow.

Our study included bile samples from both hepatic and gallbladder biles from patients with mixed or cholesterol stones. The viscosity of bile was compared with the quantitatively most important constituents to investigate which component of bile affects its viscosity.

METHODS

Patients and collection of bile

Thirty-six patients, 25 women (mean age 40 ± 10.5 years) and 11 men (mean age 48.5 ± 11.4 years), who underwent laparoscopic surgery because of symptomatic gallstone disease and six patients with T-drainage after cholecystectomy were also included in the study. Gallstones were visualized by ultrasonography and reasonable gallbladder function was confirmed by determining that at least 30% of the fasting volume was emptied after the administration of a liquid test meal. Patients shown to have severely impaired gallbladder function or a loss of the gallbladder reservoir or cystic duct obstruction were excluded from the study. All patients gave informed consent after a detailed explanation of the procedure required for intraoperative bile collection. During laparoscopic surgery, the gallbladder was punctured with a 16-G needle and the bile was aspirated as completely as possible because of the known stratification of human gallbladder bile[15]. Stones were removed, washed with distilled water, dried, weighed and ground to a powder. The cholesterol content of the stones was measured chemically after extraction with organic solvent and expressed as percentage of dry weight[16]. T-tube hepatic bile was collected from 6 patients (3 women and 3 men) by gravity drainage within 24 hours after cholecystectomy.

Microscopy of bile and crystal observation time

After the collection, bile samples were mixed thoroughly and one drop was immediately examined under polarized light microscopy for cholesterol crystals. Four mL of gallbladder bile was centrifuged in polycarbonate tubes with a 50 Ti rotor at 37℃ for 1 hour at 100 000 × *g* in a Beckman L5-65 ultracentrifuge (Beckman Instruments, Fullerton, CA) to precipitate biliary "sludge". The supernatant was microscopically free of cholesterol crystals. Aliquots were incubated at 37℃ and examined daily under polarized light microscopy for up to 21 days[17].The time taken for cholesterol microcrystals to appear was defined as the crystal observation time and measured in days.

Analysis of bile composition

For the analysis of bile composition, duplicate aliquots were stored at -30℃ prior to determination. Cholesterol was determined colorimetrically with the Liebermann-Burchard reaction after double extraction of 1 mL methanolic bile sample with petroleum ether[18]. Phospholipids were measured as total biliary phosphate after hydrolysis at 150℃ with sulfuric acid, using the colorimetric assay of Fiske-Subbarow, and total bile salts were determined by a modified 3-α-hydroxysteroid dehydrogenase method [19,20]. The saturation index of each sample was calculated in native bile by dividing the cholesterol concentration with the maximum cholesterol solubility according to Carey and was corrected for the total lipid content of each individual bile[21]. Total protein was analysed using the Lowry assay after purification of biliary proteins[22]. This method has been validated extensively by adding known amounts of different serum proteins (albumin or γ-globulin) to samples of gallbladder bile with unknown protein content. In contrast to the fluorimetric assay which underestimates the amount of added protein, protein recovery was determined quantitatively by the Lowry assay.

Biliary mucin concentration was determined according to an assay recently described[23]. The assay is a modification of the classical method of Pearson *et al*[24] and Harvey *et al*[25]. Briefly, fresh gallbladder bile was diluted 1:1 (v/v) with 0.1 mol/L Tris-HCl buffer (pH 7.5) containing 0.44mol/L potassium thiocyanate and 0.02% NaN-3. The formation of lipid aggregates in vesicular form was avoided by addition of sodium cholate to this buffer (final concentration 12.5 mmol/L). The mixture was shaken overnight and centrifuged at 12 000 × g for 10 min. One mL of the supernatant was fractioned on a Sepharose 2B column (40 cm × 1.0 cm, Pharmacia AB, Uppsala, Sweden) at a constant flow of 0.3 mL/min. As for elution buffer, we used 20 mmol/L Tris, 140 mmol/L NaCl, 3 mmol/L NaN-3, pH 8.0 buffer containing 25 mmol/L sodium cholate. Column fractions of 1mL were collected and analyzed for glycoprotein by periodic acid/Schiff (PAS) assay[26]. The excluded PAS-positive glycoprotein fractions were further pooled and dialyzed for 12 h against distilled water to remove bile acids. Mucin concentration was measured in this fraction by the periodic PAS-assay using purified human gallbladder mucin standard.

Determination of viscosity

The rheologic measurements were carried out on a calibrated Contraves Low Shear-30 rotation viscosimeter, using a coaxial-cylinder system with a gap width of 0.5 mm (Contraves AG, Zürich, Switzerland). The rotation viscosimeter allows accurate measurements of viscosity of both Newtonian and non-Newtonian fluids. Programming

of measurements and the processing of the measured data were done utilizing the Contraves Rheoscan 30. To obtain a rapid standardized measurement within the entire range of shear rates (0.1 to 118s^{-1}), a computer program was used to enable measurements in one sample within 5 min[27]. These were repeated twice after an interval of 60s each. Thus, the final result of biliary viscosity represented the mean value of a total of 30 determinations. The viscosity was measured within 3 hours after the collection of the bile sample at laparoscopic cholecystectomy. One mL of the bile sample was taken for any viscosity assay and all measurements were performed at 37℃. All samples were centrifuged for 2 min at 12 000 × *g*, to eliminate sediment that could interfere with the determinations.

To study the effect of mucin on the rheological properties of human bile, purified mucin from porcine stomach (0.24-2.24 g/L) was added to gallbladder bile and the viscosity measured as shown above.

Statistical analysis

Values of each group of parametric data are expressed as means ± SEM. Non-parametric data (crystal observation time) are expressed as median and range. Group comparison was performed for parametric data using unpaired Student's *t* test and for non-parametric data using the Mann-Whitney U test. Spearman's correlation coefficient were calculated between variables and expressed as significant at the $P<0.05$ level.

RESULTS

Individual and total lipids, CSI, crystal observation time, protein, mucin and viscosity in gallbladder bile of patients with cholesterol and mixed stones and in hepatic bile are shown in Table 1. As expected, protein, mucin and lipid concentrations and viscosities were markedly higher in gallbladder bile compared to hepatic bile. A positive correlation between mucin and viscosity was found in gallbladder biles (Figure 1) but not in hepatic biles ($r = 0.01$;n.s.). Figure 2 shows the effect of adding physiological and supraphysiological amounts of mucin to gallbladder bile in relation to the viscosity. There was a dose dependent non linear increase of its viscosity by adding external mucin to native gallbladder bile at three different shear rates including the prestatic region (shear rate 0.1s^{-1}) in this experiment.

Furthermore, a positive correlation was determined between phospholipid concentration and viscosity ($r = 0.34$, $P<0.05$) in gallbladder biles (Figure 3). However, no significant correlation was found between total protein (Figure 4) or the other lipid concentrations and viscosity in both gallbladder (Table 2) and hepatic biles.

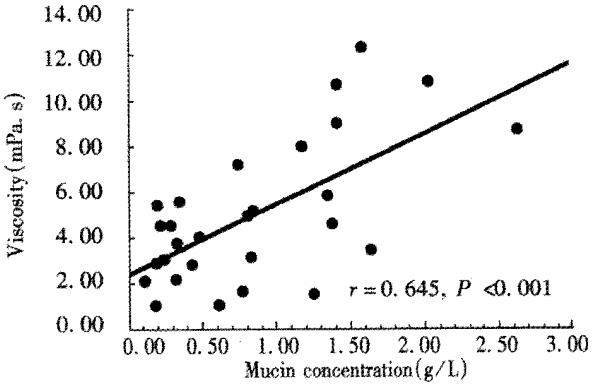

Figure 1 Correlation of viscosity and mucin concentration in 36 gallbladder biles from patients with gallstones.

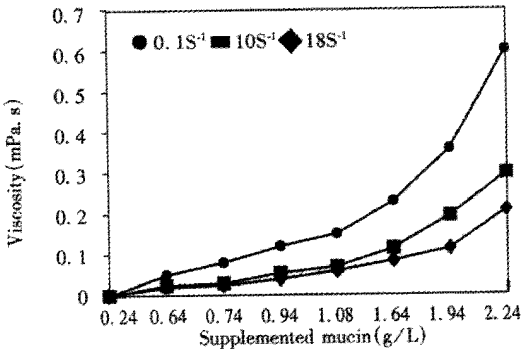

Figure 2 Increase of viscosity at 3 different shear rates including the prestatic region after supplementation of gallbladder bile with purified porcine mucin.

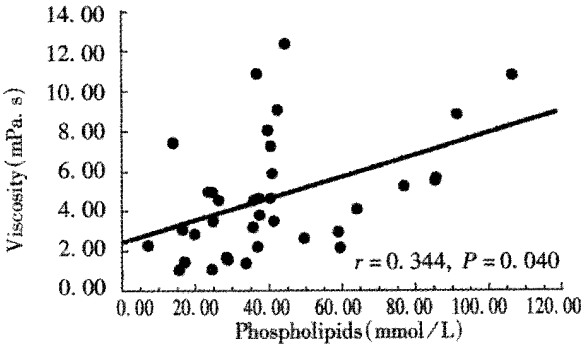

Figure 3 Correlation of viscosity and phospholipid concentration in 36 gallbladder biles from patients with gallstones.

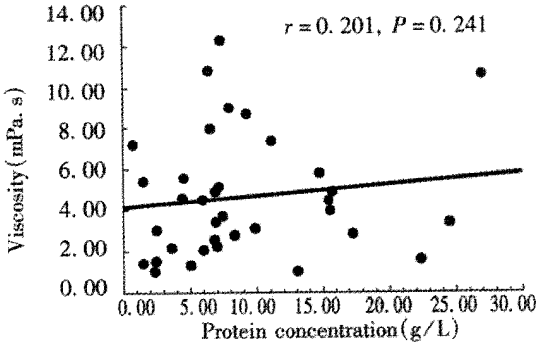

Figure 4 Correlation of viscosity and total protein concentration in 36 gallbladder biles from patients with gallstones.

Table 1 Cholesterol, phospholipids, bile acids, total lipids, CSI, protein, mucin, viscosity (mean ± SEM) and crystal observation time (median and range) in gallbladder bile of patients with gallstones and in hepatic bile

	Cholesterol stones (n = 28)	Mixed stones (n = 8)	Hepatic bile (n = 6)
Cholesterol (mmol / L)	21.1 ± 2.2	12.8 ± 1.7	1.9 ± 1.1
Phospholipids (mmol / L)	46.0 ± 4.7	26.6 ± 4.7	3.4 ± 1.3
Bile acids (mmol / L)	141.2 ± 15.1	65.9 ± 7.7	13.7 ± 7.4
Total lipids (g / dL)	11.3 ± 1.1	5.7 ± 0.7	1.0 ± 0.5
CSI	1.43 ± 0.09	1.79 ± 0.34	2.61 ± 1.68
Total protein (g / L)	10.4 ± 1.8	6.2 ± 0.9	2.8 ± 1.6
Mucin (g / L)	0.85 ± 0.12	0.39 ± 0.12	0.08 ± 0.04
Viscosity (mPa·s)	5.00 ± 0.60	3.50 ± 0.68	0.92 ± 0.06
Crystal observation time (days)	2.0 (1-13)	2.5 (1-14)	>21 (>21)

Table 2 Correlation coefficients between viscosity and mucin, protein, lipids and CSI in gallbladder bile of 36 patients with cholesterol or mixed gallstones

Viscosity (mPa·s)	Correlation coefficient (r)	Significance level
Mucin (g / L)	0.645	P<0.001
Total protein (g / L)	0.201	P = 0.24
Total lipids (g / dL)	0.238	P = 0.16
Cholesterol (mmol / L)	0.223	P = 0.19
Phospholipids (mmol / L)	0.344	P = 0.04
Bile acids (mmol / L)	0.161	P = 0.34
CSI	-0.141	P = 0.41

DISCUSSION

The aim of this study is to elucidate the relationship between viscosity and the main constituents of gallbladder and hepatic bile of patients with gallstones.

A major finding of our study is that mucin concentration is positively correlated to the viscosity of gallbladder bile. Similar results were obtained by Shoda *et al*[11], using a capillary viscosimeter, who determined a positive correlation between biliary viscosity and hexosamine concentration of gallbladder bile. In contrast to this report, we did not find a significant correlation between viscosity and the total protein concentration in gallbladder bile of patients with gallstones. These discrepancies might be explained by the different methods of protein determination in both studies. As stated above, the fluorescamine method used in the study of Shoda *et al*. might underestimate the total amount of protein in bile. The use of capillary viscosimeter instead of rotation viscosimetry in the study of Shoda is unlikely to explain the different findings, since bile behaves over a wide range of shear rates like a Newtonian fluid.

A further finding of our study was the positive correlation between phospholipids and viscosity in gallbladder bile. This correlation might be affected by the degree of concentration of gallbladder bile, although the other lipid components bile acids and cholesterol were not positively correlated to the viscosity. Furthermore, higher ratios of phospholipid to bile acids would favor the development of higher molecular weight micelles or vesicles which could favor a higher viscosity of bile. Although further studies have measured bile viscosity in patients with biliary drainage[28,29], the number of studies concerning gallbladder bile is very limited. This is astonishing, since an increased viscosity may play an important role in the formation of cholesterol crystals in gallbladder bile. According to Poiseuille's law bile flux through the cystic duct is inversely correlated to bile viscosity. 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[30-32] and may also contribute thereby to an increase of the viscosity of gallbladder bile.

We investigated further the relation between viscosity and mucin concentration in human bile by adding purified porcine stomach mucin (range between 0.24 and 2.24 g/L) to native gallbladder bile. A non-linear increase of viscosity with the addition of mucin at 3 different shear rates including the prestatic region was observed. Particularly, after reaching a mucin concentration above 2 g/L, a greater increase in viscosity was found. Cholesterol crystal growth was promoted by already physiological concentrations of bovine gallbladder mucin and appeared to be maximal at 4 g/L [33]. Very high mucin concentrations (up to 20 g/L) have been found in mucin gels and sludge[34] and it has been proposed that crystal growth occurs within the mucin gel that is freqently seen before gallstone formation[2]. The concomitant increase in viscosity may contribute further to the retention of crystals in the gel phase of biliary mucin.

In summary, the most relevant finding of this study suggests that the concentration of mucin is the major determinant of biliary viscosity. Thus, an increased secretion of mucin by the gallbladder epithelium might contribute to a vicious cycle by inhibiting the emptying of the gallbladder and by this mechanism favoring the formation of gallstones.

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