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Restoration of hepatocytes function following decompression therapy in extrahepatic biliary obstructed patients: Metabolite profiling of bile by NMR

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ABSTRACT

Prolonged biliary obstruction and infection cause retention of biliary constituents in liver followed by hepatocyte dysfunction. Decompression therapy is important for both management and prognostic reasons and restores hepatocyte function. Quantitative metabolite profiling of bile using NMR spectroscopy at the time of obstruction and serially following decompression therapy using percutaneous transhepatic biliary drainage (PTBD) from nineteen patients with extrahepatic malignant biliary obstruction are presented. Based on detailed history, clinical condition, total leucocyte counts (TLC) and microbiological cultures of bile, patients were classified in two groups depending upon absence or presence of cholangitis. Statistical analysis was performed for comparison within each group using Wilcoxan sign square rank test. TLC and liver function tests indicated a trend towards recovery following decompression by one week. While on day 0 biliary constituents were undetectable in both the groups of patients, they increased significantly following one week of drainage with better recovery in patients with cholangitis compared to without. Free amino acids' signals were detected in all specimens starting from day 1 after decompression. This indicates disruption of blood–bile barrier during cholestasis and slow restoration of tight junction of hepatocytes following decompression leading to the appearance of biliary constituents in bile. Decompression therapy tends to restore biliary constituents with a prompt recovery in patients with cholangitis further supports such therapy for clinical management and outcome. To our knowledge this is the first report on the detection of amino acids in bile taken from common bile duct though they have been reported in hepatic bile.

1. Introduction

Jaundice and cholangitis are common complications of majority of malignant extrahepatic biliary obstruction and are often considered as risk factors predicting advance disease and early mortality [1,2]. Surgery in jaundiced patients with a tumor is associated with a higher risk of postoperative complications compared with surgery in non jaundiced patients [3]. Moreover, most of the patients are not amenable for curative resection. Hence, prevention and palliation of debilitating symptoms is critically important for management of such patients. Therapeutic management of the patients with cholangitis who fail to respond to antibiotic treatment need urgent biliary decompression as definitive recommended treatment [4]. Percutaneous transhepatic biliary drainage (PTBD) and stenting, though a subject of discussion, yet it is believed to be a safe, effective and established technique of biliary decompression in modern routine practice despite the absence of convincing randomized data [5–7]. Adequate palliation is desirable as prolonged obstruction causes jaundice, pruritis and progressive hepatocellular dysfunction with alteration in host defence system due to retention of bile [8,9]. This follows altered permeability of blood–bile barrier allowing regurgitation of biliary constituents into blood circulation and endotoxemia [10–15]. Biliary drainage via PTBD alleviates icterus, resolves infection, restores most of the changes of the liver parenchyma and recovery of tight junction of hepatocytes and immune function [5–7,16,17]. It improves liver reserve function, nutritional and energy status [18–21] and reduces postoperative mortality [6]; patients with tumor could have more chances to receive chemotherapy/

Abbreviations: TBA, total bile acids; Chol, cholesterol; PDE, phosphodiester; PtdCho, phosphatidylcholine; Pi, inorganic phosphate; LysoPtdCho, lysophosphatidylcholine; GPC, glycerophosphocholine; DMSO-d_6, deuterated dimethyl sulphoxide; MDP, methylene diphosphonic acid sodium salt; PTBD, percutaneous transhepatic biliary drainage; TLC, total leucocyte counts; TSP, sodium salt of trimethylsilylpropionic acid-d_4; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; SA, serum albumin; PT, prothrombin time.

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radiotherapy/photodynamic therapy/radiofrequency ablation [22]. Drainage has been shown to improve prognosis and quality of life [23]. PTBD using aseptic technique in patients with malignant hilar obstruction allows relative hypertrophy of future liver remnant, thereby reducing the risk of postoperative liver failure [24].

However, the pathophysiological alterations from drainage in human obstructive jaundice are not clearly understood. The role of biliary decompression in restoration of hepatic tight junction might have clinical significance at molecular level. Little is known regarding its role in restorative function of hepatocytes reflecting alterations of metabolites in bile. Nuclear magnetic resonance (NMR) spectroscopy has obvious advantages over other analytical techniques in that it does not require any prior extraction/derivatization step, is not preselective for any particular metabolite and provides metabolic profile in a single experiment. We therefore, exploited application of NMR for metabolite profiling of bile [25]. In an earlier communication, it has been reported that reduced concentration indices of biliary constituents are indicator of jaundice and cholangitis [26]. However, the serial alterations in the biliary constituents in response to the decompression therapy in patients with malignant biliary obstruction and infection, has not yet been reported. Such studies have been reported herein. Specific aims addressed are: (a) indices of biliary constituents at presentation (day 0; at the time of PTBD); (b) metabolic profile of bile obtained serially following decompression; (c) serial changes in indices of biliary constituents following drainage; and (d) extent of restoration of biliary constituents indices in patients with/without cholangitis.

2. Materials and methods

2.1. Patients and the study protocol

Patients undergoing PTBD as a routine management for malignant extrahepatic biliary obstruction presenting jaundice in the Gastroenterology ward of one of the tertiary referral centers of northern India were the candidates for participation in this study. Evaluations included record of detailed history, physical examination for vital signs and elaborate admission laboratory analyses on each patient. Diagnosis of cause of obstruction was based on the basis of detailed history, initial clinical examination, liver function tests, ultrasonography and/or imaging modality (computed tomography/MRI). Patients diagnosed with malignant biliary obstruction with jaundice also met an extensive list of exclusion criteria including sensory findings, critical condition, hypotension, shock, altered mental state, cachectic or clinically debilitating symptoms. Serial bile specimens were collected aseptically from the catheter inserted in the common bile duct, on day 0 (at presentation while performing PTBD), thereafter, from external drains on day 1, midweek, end of 1 week and subsequently at the end of 2 weeks if the patient stayed in the hospital. Bile culture was performed with established techniques at each time points and various causative organisms were identified as described earlier [26]. The timing of the bile collection and handling it for microbiological culture were coordinated to optimize the results. In order to avoid any contamination with the external source, bile specimens were collected directly from the PTBD tube following drainage. All included patients were having documented evidence of jaundice (serum bilirubin level $\geq$1.0 mg/dL) as established by standard liver function tests [26–28]. Cholangitis was diagnosed clinically supported by laboratory and radiographic findings: clinical evidence of infection (fever $\geq$38.5 °C, abdominal pain or tenderness) with leucocytosis (in the absence of any other source of infection). The patients who had total leucocyte counts (TLC) > 11,100 cells/mm^3 and/or fever $\geq$38.5 °C with/without bile culture positivity were established as having cholangitis [26]. Patients with intrahepatic biliary obstruction, extrahepatic obstruction with benign cause, liver abscess, underlying cirrhosis, or patients in which bile specimens while sampling were mixed with blood while performing PTBD, patients to whom serial bile sampling could not be followed till the end of 1 week or patients who did not have cholangitis at presentation but developed it later because of any methodological flaws (complications related to stent placement principally sepsis) following PTBD were excluded from the study. For NMR analysis, about 1 mL of bile specimens collected aseptically, snap frozen in liquid nitrogen, kept in dark and stored in −80 °C, till ^{1}H and ^{31}P NMR experiments were performed.

The persons performing the NMR experiments were blinded to the clinical, microbiological and conventional laboratory results so as to have unbiased data analysis.

In the following study, nineteen patients with malignant biliary obstruction following drainage were included. Eight patients out of 19 were diagnosed as without cholangitis while eleven out of 19 diagnosed as having cholangitis. Bile specimens were collected in each patient on day 0, day 1, midweek and by end of 1 week. In two patients out of 19 they were additionally collected by the end of 2nd week also. Data for clinical, laboratory (liver function test and microbiological) and NMR were collected on these time points. A total of seventy-eight bile specimens were studied with thirty-two specimens in the group of patients without cholangitis and forty six bile specimens in the group of patients with cholangitis.

2.2. ^{1}H and ^{31}P NMR experiments

Deuterated dimethylsulphoxide 99.9% (DMSO-d_6), deuterium oxide 99.9% (D_2O), sodium salt of trimethylsilylpropionic acid-d_4 (TSP) and methylene diphosphonic acid sodium salt (MDP) were purchased from Sigma–Aldrich (Milwaukee, WI, USA).

Bile was centrifuged at $6000 \times g \times 4\,^{\circ}C \times 10$ mins in order to remove any particulate material and cellular debris. Thereby, 500 μl of the clear supernatant was taken in a 5 mm NMR tube for performing NMR experiments on Bruker Avance 400 MHz spectrometer at 25 °C.

^{1}H and ^{31}P NMR experiments of neat bile specimens were performed using a capillary tube containing D_2O with known quantities of sodium salt of TSP and MDP separately, in each case. D_2O served as the "field-frequency-lock". One-dimensional ^{1}H NMR experiments using one pulse sequence were performed with water suppression by presaturation. Typical parameters were: spectral width: 8000 Hz; time domain data points: 32 K; flip angle of the radio frequency pulse: 45°; relaxation delay: 5 s to ensure maximum recovery of magnetization to equilibrium between the scans; number of scans: 128; line broadening function: 0.3 Hz. Exponential window function was used to Fourier transform the resulting data. Typical parameters of ^{31}P NMR spectra were: one pulse sequence with proton decoupling; flip angle of the radio frequency pulse: 90°; relaxation delay: 5 s; spectral width: 10,000 Hz; number of scans: 1000; time domain data points: 16 K; line broadening function: 3 Hz. Exponential window function was used to Fourier transform the resulting data. Two-dimensional (2D) NMR experiments, such as double quantum filtered correlated spectroscopy (DQF-COSY), total correlated spectroscopy (TOCSY) with parameters as reported earlier [29] were also performed on bile from one of the representative patient to unambiguously assign various metabolites.

^{1}H NMR spectra of native bile provides an overlapping resonance arising from C-18 methyl protons of cholesterol (Chol) and total bile acids (TBA). The signals for TBA and Chol can be separated by using a polar organic solvent (DMSO-d_6) [26,29] but the concentration of Chol is found to vary with the concentration of water [30] and hence it is better to employ the lyophilized samples. ^{1}H NMR experiments on all initially lyophilized bile specimens (50–500 μl)

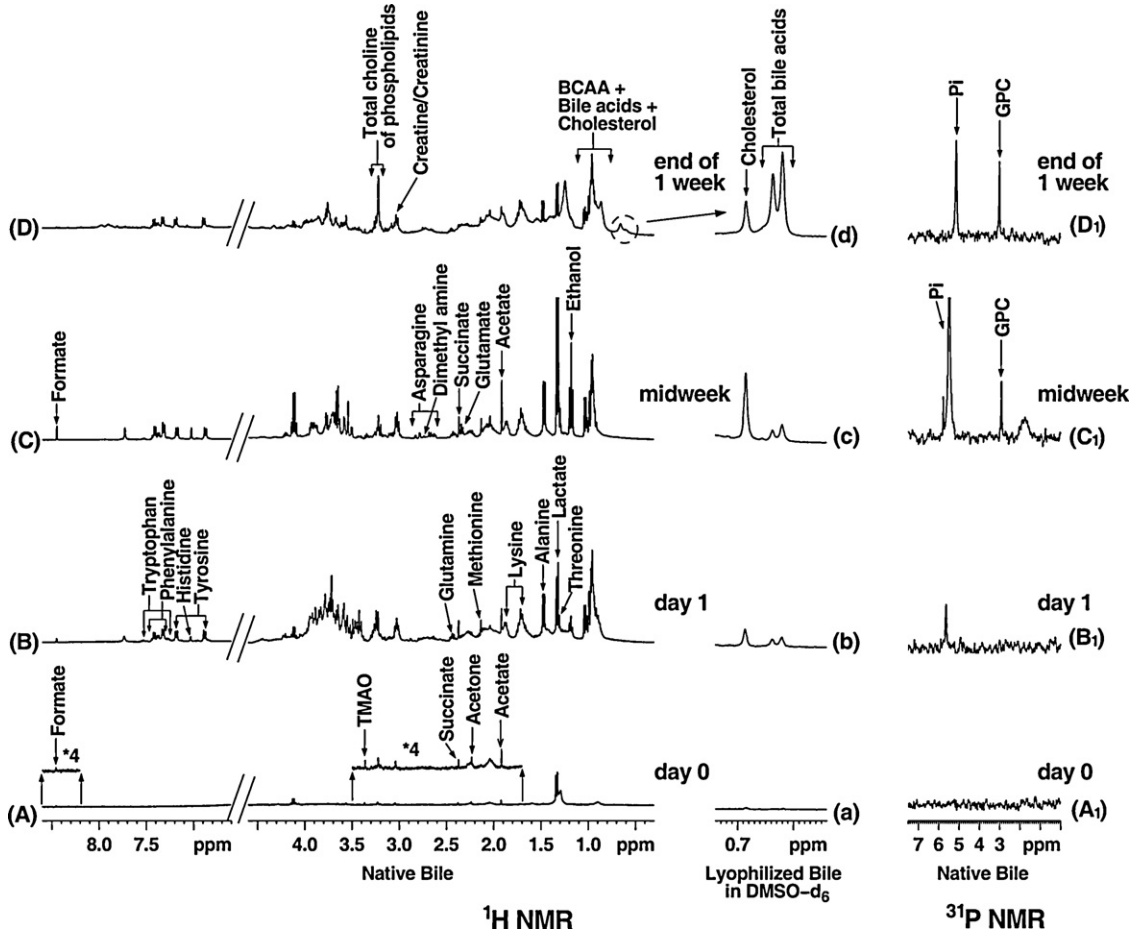

Fig. 1. ^{1}H NMR spectra of native bile specimens obtained serially on day 0 (A), day 1 (B), midweek (C) and end of 1 week (D) following decompression therapy in a representative patient with malignant biliary obstruction with jaundice and without cholangitis. Note the appearance of amino acids on day 1, which continues till the end of 1 week. BCAA: branched chain amino acid. Relevant portion of ^{1}H NMR spectra blow-up (lyophilized bile in DMSO-d_6) on respective time points shown by a, b, c, and d indicates restoration of biliary constituents: total bile acids (TBA), cholesterol (Chol), while ^{31}P NMR spectra of native bile shown as A_1, B_1, C_1 and D_1 at respective time points indicates restoration of phosphodiesters (PDE) and inorganic phosphate (Pi). All the spectra of bile specimens obtained serially are plotted on same vertical scale with respect to reference in each set.

were, therefore, performed by dissolving it in 500 μl of water free DMSO-d_6 (Fig. 1a).

^{1}H and ^{31}P NMR spectra of native bile as well as ^{1}H spectra of lyophilized bile (in order to obtain separate quantitative values of TBA and Chol) dissolved in DMSO-d_6 were recorded.

2.3. Statistical analysis

Observations for the clinical data are reported as median with range in parentheses ±standard error of mean (SEM) while NMR data are expressed as jackknife average (range) ± SEM; [jackknife interval]. Statistical differences in clinical parameters between different days were analyzed using the Wilcoxan sign square rank test for quantitative data without normal distribution. All *P*-values were two-tailed and a *P* value of less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSS statistical package Version 11.5 (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Clinical data

3.1.1. Demographic and etiological characteristics

Nineteen patients median age 54.5 years [range (36–76) years; male 7] with extrahepatic malignant biliary obstruction with/without infection were included in the study. The etiology of patients was as follows: carcinoma of the gall bladder (ten), cholangiocarcinoma (seven), malignant biliary stricture (two).

3.1.2. Data on microbiological culture of bile

3.1.2.1. (A) Day 0 bile. Bile specimens of eight patients out of the 19 were positive for various organisms. Five of these 8 had single bacteria while the remaining three had mixed bacterial population. Eight different types of bacteria were isolated in total: *E. coli* (three), *Enterococcus faecalis* (two), *Acinetobacter baumannii* (one), *Citrobacter freundii* (one), *Enterococcus faecium* (one), *Klebsiella pneumoniae* (one), *Enterobacter aerogenes* (one) and *Proteus mirabilis* (one).

Day 0 bile specimens were pale white (fifteen) and light yellowish (four) in colour.

3.1.2.2. (B) Day 1 bile. Bile specimens following drainage at day 1 of six patients out of the 19 were positive for various organisms. Five of these 6 had single bacteria while the remaining one had mixed bacterial population. Five different types of bacteria were isolated: *E. coli* were (two), *E. faecalis* (one), *A. baumannii* (one), *E. faecium* (two), *K. pneumoniae* (one).

Day 1 bile specimens were pale white (two), yellow (four) and yellowish-green (thirteen) in colour; the latter indicating the appearance of bilirubin in bile.

3.1.2.3. (C) Midweek bile. Bile specimens following drainage at midweek of four patients out of the 19 were positive for various organisms. Two of these 4 had single bacteria while the remaining two had mixed bacterial population. Five different types of bacteria were isolated in total: *E. coli* were (two), *E. faecalis* (one), *A. baumannii* (one), *E. faecium* (one) and *P. mirabilis* (one).

Midweek bile specimens were pale white (two), yellow (two), yellowish-green (two) and green (thirteen) in colour.

3.1.2.4. (D) Bile by the end of 1 week. Bile specimens following drainage of two patients out of the 19 were positive for various organisms. Both of these specimens had mixed bacterial population. Four different bacteria were isolated in total: *E. coli* were (two), *E. faecalis* (one), *E. faecium* (one) and *K. pneumoniae* (one).

Bile specimens by the end of 1 week were pale white (one), yellow (one), yellowish-green (two) and green (fifteen) in colour.

3.1.3. Biochemical, laboratory data and classification of patients

The biochemical, laboratory data indicating values of liver function tests in malignant biliary obstructed patients following drainage obtained serially is represented in Table 1. The results clearly indicate a significant progressive drop in bilirubin level, TLC and a trend of liver function tests returning towards normal. The study population ($n = 19$) was divided in two groups on the basis of presence and absence of cholangitis. Eight of the nineteen patients [median age 52.5 years, range (36–68) years; male 4] were identified without cholangitis, while eleven of nineteen patients [median age 56.5 years, range (45–76) years; male 3] were identified as having cholangitis. The clinical data of the two groups of the patients on different days following drainage is provided in Table 2.

3.1.3.1. Patients without cholangitis (n = 8). Eight patients were established as without cholangitis (TLC < 11,100 cells/mm^3) before inserting catheter via PTBD on day 0. None of the patients had fever and bile culture positivity. However, these patients had clinically proven extrahepatic malignant biliary obstruction and clinical symptoms of jaundice (bilirubin level >1.0 mg/dL).

3.1.3.2. Patients with cholangitis (n = 11). Eleven patients were established as having cholangitis before inserting catheter via PTBD on day 0. All the patients had leucocytosis (TLC > 11,000 cells/mm^3). Fever exceeded 38.5 °C in nine patients (81.8%). Out of nine, bile culture was positive for bacteria in eight patients (88.9%) and negative in one. Two had mild fever with negative bile culture but purulent bile. All eleven patients had clinical evidence of jaundice (bilirubin level >1.0 mg/dL) with proven malignant biliary obstruction.

3.2. ^{1}H and ^{31}P NMR data

3.2.1. Spectral assignments and quantitation and statistical analysis of bile specimens obtained serially

The signals of phosphodiesters (PDE) [glycerophosphocholine (GPC), lysophosphatidylcholine (LysoPtdCho), phosphatidylcholine (PtdCho)] and inorganic phosphate (Pi) were assigned in ^{31}P NMR spectra on neat bile based on chemical shift values reported in the literature [31] and subsequently, confirmed by the use of spiking with known compounds. Quantitative estimation of biliary constituents was performed using the integral area of each signal normalized to the intensity of known weight of an external reference as reported earlier [26]. The volume of the bile and number of protons (in the case of ^{1}H NMR) and phosphorus (in the case of ^{31}P NMR) were taken into consideration while performing quantitative estimation.

Representative ^{1}H and ^{31}P NMR spectra of bile specimens obtained serially from patients falling in each of the two groups are provided in Figs. 1(A–D and A_1–D_1) and 2(A–D and A_1–D_1), respectively, and quantitative values of biliary constituents are included in Tables 1 and 2. The sum of concentrations of GPC/LysoPtdCho/PtdCho is represented as PDE.

The unambiguous assignments of various resonances are given in Fig. 3. The resonances of succinate and pyruvate were confirmed by experimental procedure reported earlier [32]. The metabolites with overlapping resonances were not considered for quantitation.

3.2.1.1. Day 0 bile. From the ^{1}H NMR spectra (Fig. 1A), it is seen that concentration of various metabolites in neat index bile are low with lactate, acetate and formate detected in almost all bile specimens. Signals from creatine/creatinine were present in nine, ethanol in three, acetone in six, acetoacetate in four, succinate in six, dimethylamine (DMA) in one, trimethylamine-oxide (TMAO) in one and choline in two and glycerophosphocholine in two. Prominent signals arising from added contrast agent (Fig. 2A) (Urovideo 75%, Bracco SPA, Miano, Italy and Imaging Products Pvt. Ltd., Mumbai, India) were also present in a few (five). However, there were no contributing resonances from the contrast agent to the ^{31}P NMR spectra of native bile (because of absence of phosphorus atom) and relevant portion of ^{1}H NMR spectra (0.60–0.70 ppm) of bile in DMSO-d_6, it was more straightforward to analyze these spectra for quantitative estimation of biliary constituents. The non-interference of contrast agent for analysis of biliary constituents has also been reported earlier [33].

The biliary constituents were undetectable in most of the day 0 bile specimens, however, they were present in a few e.g. TBA (three), Chol (five) and phospholipid GPC (one), Pi (three), as shown in Figs. 1(a and A_1) and 2(a and A_1).

3.2.1.2. Day 1 bile. ^{1}H NMR spectra of day 1, bile obtained after 24 hrs of PTBD procedure appeared complex with overlapping signals arising from most of the free amino acids, biliary constituents and other metabolites in almost all bile specimens. However, no signals from the contrast agent were observed (indicating it possibly elute by one day) Figs. 1B and 2B. Most of the free amino acids were present in almost all bile specimens. Apart from these, other metabolites were present in a few patients: acetate (seventeen), formate (fourteen), lactate (seventeen), creatine/creatinine (eighteen), ethanol (two), DMA (seven), acetone (five), acetoacetate (six), succinate (eight) and pyruvate (six). Furthermore, ^{1}H and ^{31}P NMR spectra indicate that biliary constituents started appearing in almost all bile specimens in significant concentration as given in Figs. 1(b, B_1) and 2(b, B_1). However, biliary constituents were undectable in a few bile specimens: Chol (six), PDE (seven), and Pi (four). The PDE resonances present in bile specimens were identified as PtdCho (three), LysoPtdCho (two), and GPC (seven).

3.2.1.3. Midweek bile. ^{1}H NMR spectra indicate that various metabolites were present, by and large similar to day 1 bile.

Furthermore, biliary constituents as indicated in Fig. 1(c and C_1) were significantly different from day 1 bile (Fig. 1(B)) with the presence of TBA in all, and absence of Chol in one, PDE in five and Pi in three patients. The number of patients in whom the PDE resonances present were: PtdCho (two), LysoPtdCho (one), and GPC (six). The signals of PtdCho, LysoPtdCho or GPC were present either alone or in combination.

3.2.1.4. Bile by the end of 1 week. By and large similar trend was observed as in midweek bile. The median concentration indices of biliary constituents were higher compared to midweek bile.

^{1}H and ^{31}P NMR of bile indicate that by now biliary constituents appeared in significantly high concentration indices with respect to day 0 with presence of TBA and cholesterol in all and absence of PDE in four and Pi in one patient. The number of patients in whom the PDE resonances present were: PtdCho (two), LysoPtdCho (six), GPC

Table 1
Comparison of clinical, laboratory and NMR parameters (concentration of chief biliary constituents) in patients with extra-hepatic malignant biliary obstruction and infection ($n = 19$) following percutaneous transhepatic biliary decompression therapy (PTBD) on days 0, 1, midweek, and end of 1 week. Data are expressed in median and (range) ± standard error of mean (SEM); ND = not detected; TLC = total leucocyte counts; ALP = alkaline phosphatase; ALT = alanine transaminase; AST = aspartate transaminase; TBA = total bile acids; Chol = cholesterol; PDE = phosphodiester; Pi = inorganic phosphate.

No.	Variables	(A) Day 0	(B) Day 1	(C) Midweek	(D) End of 1 week
Clinical and laboratory parameters					
1	Bilirubin total (mg/dL)	12.4 (3–24.9) ± 1.6•	10.3 (0.5–22.2) ± 2.0*	8.9 (0.4–18.5) ± 1.5	7.4 (0.4–15.7) ± 1.3■
4	TLC (cells/mm^3)	12,606 (4900–21,900) ± 1151••	9942 (4800–15,200) ± 842	9815 (5500–16,000) ± 692	9343 (6000–15,600) ± 730■■
5	ALP (U/L)	636 (229–2184) ± 114.6	563 (222–1002) ± 101.4	339 (146–867) ± 63.6	291 (101–1208) ± 68.2■■■
6	ALT (U/L)	87 (28–179) ± 9•	71 (20–120) ± 5.3	60 (33–139) ± 6.3	44 (14–194) ± 9.9■■
7	AST (U/L)	91 (35–176) ± 9.4	77 (10–232) ± 10.7	65 (31–226) ± 11.3	52 (27–197) ± 11.3
8	Serum Albumin (g/dL)	2.8 (2.1–4.1) ± 0.1	2.8 (2.0–3.6) ± 0.1	2.8 (1.4–3.6) ± 0.1	2.9 (2.0–3.7) ± 0.1
9	Prothrombin time (s)	15.6 (9.5–30.9) ± 1.1	14.8 (11.4–19.9) ± 0.6	14.3 (11.8–19.6) ± 0.4	14.1 (11.6–18.9) ± 0.5
NMR parameters					
1	Total BA (mM/L)	0.1 (ND–2.0) ± 0.1•••	0.7 (0.1–3.4) ± 0.2**	2.0 (0.1–10.1) ± 0.8¶	7.8 (0.2–69.6) ± 4.3■■■
2	Cholesterol (mM/L)	0.1 (ND–0.5)••	0.1 (ND–0.4)***	0.3 (ND–1.0) ± 0.1	1.2 (0.1–6.6) ± 0.5■■■
3	PDE (mM/L)	ND (ND–0.1)•••	0.1 (ND–0.7)	0.1 (ND–0.7) ± 0.1	0.3 (ND–1.08) ± 0.1■■■
4	Pi (mM/L)	ND (ND–0.2)•••	0.3 (ND–0.9)	0.3 (ND–1.3) ± 0.1	0.6 (ND–2.8) ± 0.2■■■

A vs. B •••$P < 0.001$, ••$P < 0.01$, •$P < 0.05$; B vs. C ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$; C vs. D ¶$P < 0.05$; A vs. D ■■■$P < 0.001$, ■■$P < 0.01$, ■$P < 0.05$.

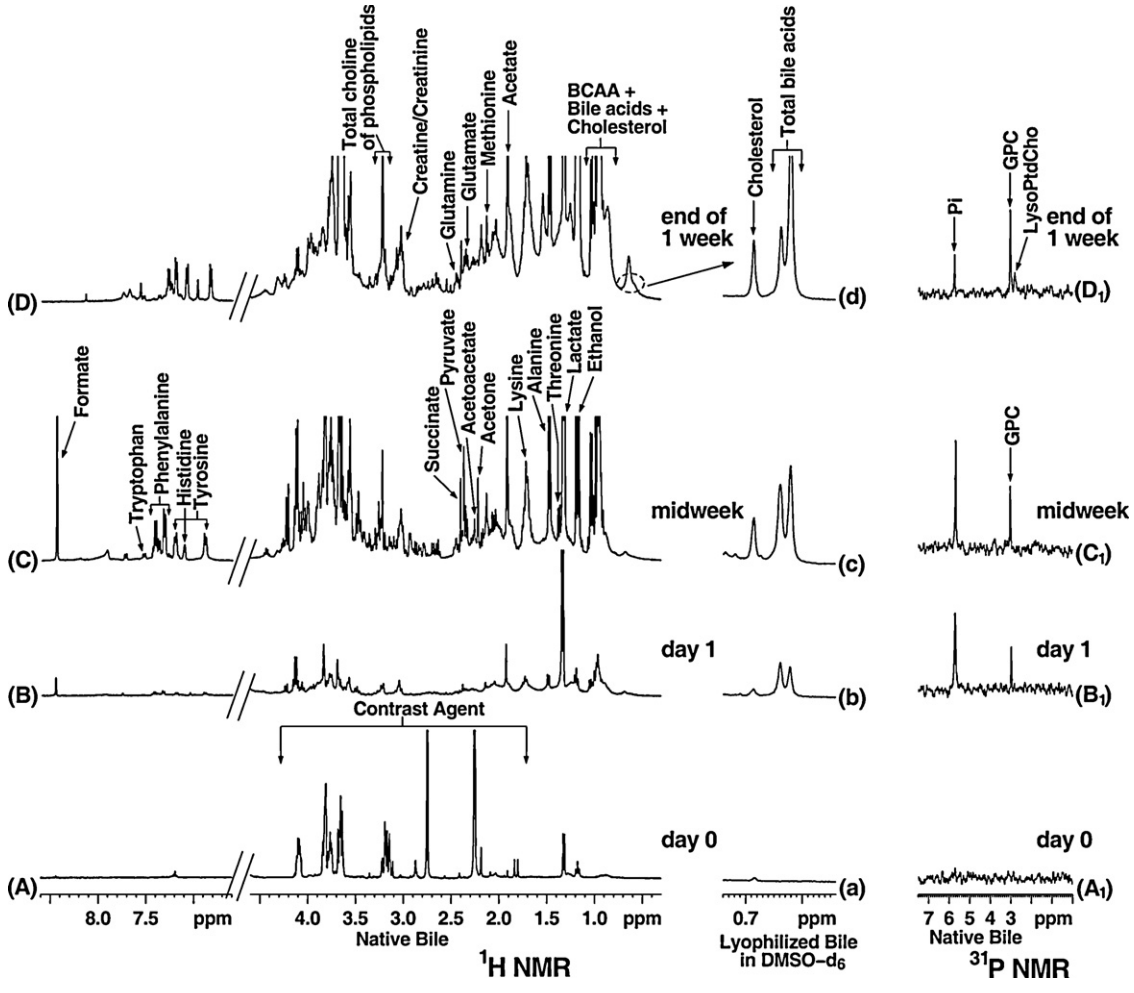

Fig. 2. ^{1}H NMR spectra of native bile specimens obtained serially on day 0 (A), day 1 (B), midweek (C) and end of 1 week (D) following decompression therapy in a representative patient with malignant biliary obstruction with jaundice and with cholangitis. Note the appearance of amino acids on day 1, which continues till the end of 1 week. BCAA: branched chain amino acid. Relevant portion of ^{1}H NMR spectra blow-up (lyophilized bile in DMSO-d_6) on respective time points shown by a, b, c, and d indicates restoration of biliary constituents: total bile acids (TBA), cholesterol (Chol), while ^{31}P NMR spectra of native bile shown as A_1, B_1, C_1 and D_1 indicates restoration of phosphodiesters (PDE) and inorganic phosphate (Pi). All the spectra are plotted on same vertical scale with respect to reference in each set.

Table 2
Comparison of clinical, laboratory and NMR parameters (concentration indices of chief biliary constituents in bile) between two groups of patients with malignant biliary obstruction presenting jaundice without cholangitis ($n = 8$) and patients with malignant biliary obstruction presenting jaundice with cholangitis ($n = 11$) following percutaneous transhepatic biliary decompression therapy (PTBD) on days 0, 1, midweek, end of 1 week. Clinical data are expressed in median and (range) ± standard error of mean (SEM); NMR data are expressed as jackknife average (range) ± SEM [jackknife intervals]. ND = not detected; TLC = total leucocyte counts; ALP = alkaline phosphatase; ALT = alanine transaminase; AST = aspartate transaminase; TBA = total bile acids; Chol = cholesterol; PDE = phosphodiester; Pi = inorganic phosphate.

No.	Variables	Without cholangitis ($n = 8$)				With cholangitis ($n = 11$)			
		(A) Day 0	(B) Day 1	(C) Midweek	(D) End of 1 week	(A′) Day 0	(B′) Day 1	(C′) Midweek	(D′) End of 1 week
Clinical and laboratory parameters									
1	Bilirubin total (mg/dL)	9.1 (3.0–24.9) ± 2.8	5.8 (0.5–21.6) ± 2.7*	6.0 (0.4–18.5) ± 3.1	4.0 (0.4–15.7) ± 2.5	12.4 (8.8–23.0) ± 1.8•	9.1 (7.1–22.2) ± 2.9	7.6 (5.0–14.9) ± 1.7¶	7.0 (3.5–12.3) ± 1.4■
2	TLC (cells/mm^3)	10,750 (4900–11,000) ± 805	10,200 (4800–10,700) ± 838	10,400 (5500–11,000) ± 1080	8200 (6600–10,100) ± 571	15,400 (11,100–21,900) ± 1577•	9100 (7500–15,200) ± 1646	9600 (7500–16,000) ± 935	9400 (6000–15,600) ± 1050■
3	ALP (U/L)	666 (297–2184) ± 255.3	489 (222–1002) ± 182.4	267 (245–867) ± 119.2	234.5 (101–1208) ± 136.9■	612.5(229–1066) ± 77.2	563 (385–625) ± 71.9	364 (146–607) ± 69.8	341 (104–853) ± 69.2■
4	ALT (U/L)	83.5 (28–130) ± 11.8	68.5 (36–84) ± 6.1	54.5 (33–139) ± 12.3	42.5 (29–92) ± 7.1■	99(41–179) ± 13.2	71 (20–120) ± 8.2	62 (33–109) ± 6.7	49 (14–194) ± 16.0
5	AST (U/L)	88.5 (35–113) ± 8.7	71.5 (21–107) ± 9.1	61 (37–226) ± 22	53 (31–123) ± 11.9	97 (37–176) ± 14.9	79 (10–232) ± 17.2	69 (31–173) ± 12.3	51 (27–197) ± 17.8
6	Serum albumin (g/dL)	2.7 (2.1–3.0) ± 0.1	2.7 (2.0–3.6) ± 0.2	2.8 (2.2–3.3) ± 0.1	2.9 (2.2–3.7) ± 0.2	2.8 (2.4–4.1) ± 0.2	2.8 (2.1–3.4) ± 0.1	2.9 (1.4–3.6) ± 0.2	2.9 (2.0–3.7) ± 0.2
7	Prothrombin time (s)	15.5 (11.4–18.9) ± 1.0	14.6 (11.4–19.1) ± 1.0	14.1 (12.0–16.3) ± 0.5	14.1 (11.6–18.5) ± 0.9	15.6 (9.5–30.9) ± 1.8	15.5 (12.9–19.9) ± 0.7	14.3 (11.8–19.6) ± 0.6	14.2 (11.9–18.9) ± 0.7
NMR parameters									
1	TBA (mM/L)	0.3 (ND–0.5) ± 0.1 [0.2–0.3]	0.6 (0.1–3.4) ± 0.4 [0.6–0.7]	1.9 (0.1–10.1) ± 1.3 [1.7–2.0]	3.8 (0.2–15.9) ± 2.1 [3.6–4.0]	ND (ND–0.1) [ND–ND]	0.7 (0.1–1.7) ± 0.2 [0.7–0.7]	2.2 (0.2–9.2) ± 1.1 [2.0–2.3]	10.6 (0.2–69.6) ± 7.4 [9.6–11.5]
2	Chol (mM/L)	0.1 (ND–0.5) ± 0.1 0.1 [0.1–0.1]	0.1 (ND–0.37) ± 0.1 [0.1–0.1]	0.3 (ND–1.0) ± 0.1 [0.3–0.3]	0.8 (0.1–2.6) ± 0.4 [0.7–0.8]	ND (ND–ND) [ND–ND]	0.1 (ND–0.3) ± 0.1 [0.1–0.1]	0.3 (0.1–0.6) ± 0.1 [0.3–0.3]	1.4 (0.1–6.6) ± 0.8 [1.3–1.5]
3	PDE (mM/L)	ND (ND–ND) [ND–ND]	0.1 (ND–0.2) ± 0.1 [0.1–0.1]	0.1 (ND–0.3) ± 0.1 [0.1–0.1]	0.2 (ND–0.7) ± 0.1 [0.2–0.2]	ND (ND–0.1) [ND–ND]	0.2 (ND–0.7) ± 0.1 [0.2–0.2]	0.2 (ND–0.7) ± 0.1 [0.2–0.2]	0.4 (ND–1.1) ± 0.1 [0.4–0.4]
4	Pi (mM/L)	ND (ND–ND) [ND–ND]	0.4 (ND–0.9) ± 0.1 [0.4–0.4]	0.4 (0.1–1.3) ± 0.2 [0.4–0.5]	1.0 (0.3–2.8) ± 0.4 [0.9–1.0]	ND (ND–0.2) [ND–ND]	0.2 (ND–0.4) ± 0.1 [0.2–0.2]	0.2 (ND–0.6) ± 0.1 [0.2–0.2]	0.2 (ND–0.4) ± 0.1 [0.2–0.2]

A vs. B and A′ vs. B′, •$P < 0.05$; B vs. C and B′ vs. C′ *$P < 0.05$; C vs. D and C′ vs. D′ ¶$P < 0.05$; A vs. D and A′ vs. D′ ■$P < 0.05$ (Wilcoxan signed square test).

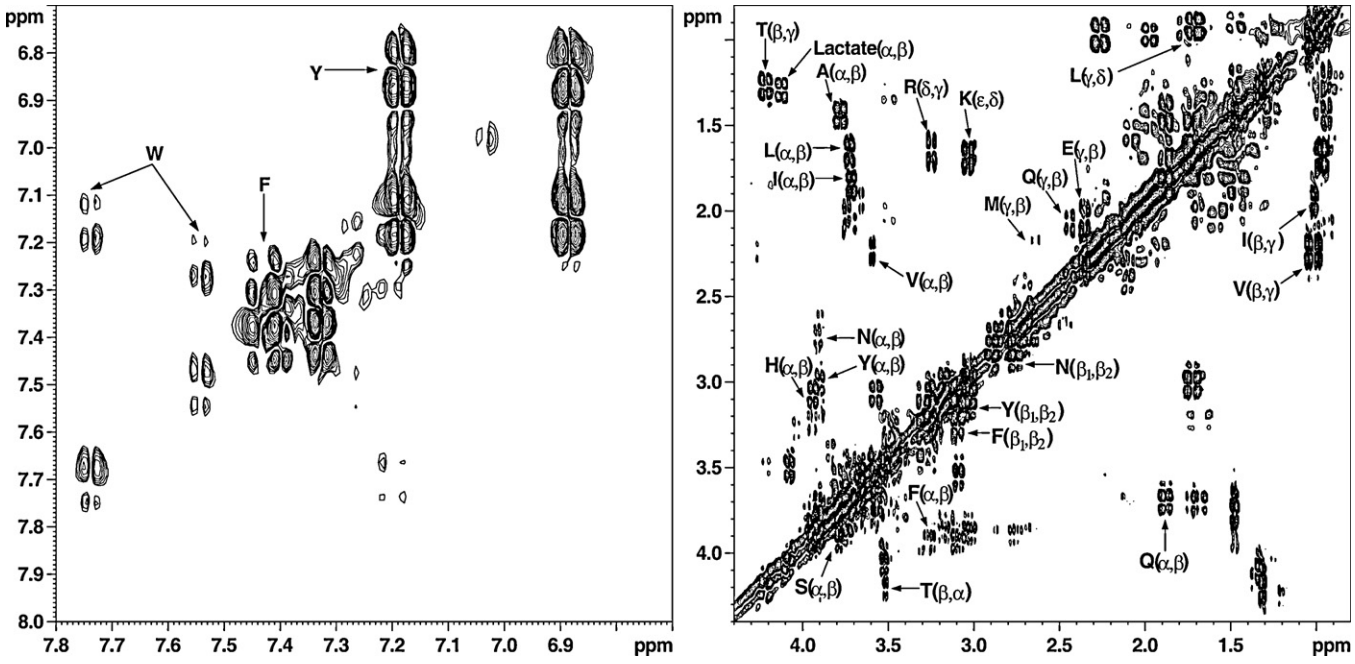

Fig. 3. 2D DQF-COSY spectrum of a typical bile specimen obtained on day 1 from a patient. All the cross peaks between protons, within each amino acid, are marked using single letter codes. The abbreviations are alanine (A), valine (V), leucine (L), isoleucine (I), aspargine (N), glutamate (E), glutamine (Q), serine (S), threonine (T), Methionine (M), lysine (K), arginine (R), histidine (H), tryptophan (W), phenylalanine (F) and tyrosine (Y).

(fourteen). The signals of PtdCho, LysoPtdCho or GPC were present either alone or in combination.

3.2.2. Patients without and with cholangitis

The restoration of biliary constituents serially with time (day 0, day 1, midweek and end of 1 week) in relation to recovery of jaundice and infection (as reflected by declining concentrations of bilirubin and TLC) following decompression in two groups of the patients with/without cholangitis, are presented in Fig. 4(A and B) and Table 2. The restoration of bile acids and cholesterol is more pronounced in general compared to PDE in both the groups. However, in patients with cholangitis compared to without, restoration of TBA, Chol and PDE is better by one week.

Metabolite profiling of bile using NMR provides an opportunity to qualitatively and quantitatively assess alterations in indices of biliary constituents during the course of human malignant biliary obstruction and following decompression.

The observation of undetectable to significantly lower indices of biliary constituents in index bile (day 0) in all cases (Tables 1 and 2) can be explained as follows: bile is synthesized in liver and concentration of biliary constituents are maintained via a complex network of signals that regulate synthesis, expression and function of specific transporters located at the canalicular side of hepatocyte that determine their export from biliary sinusoids to canaliculus [28,34]. Bile is normally secreted at a pressure of about 15–25 cm H_2O. Extrahepatic malignancy renders persistent mechanical obstruction to bile ducts and impedence to normal flow of bile and as such causes an increase in the back pressure on the liver. Pressure rise to about 35 cm H_2O results in suppression of bile flow and therefore jaundice [28]. Experimental studies in cholestatic models have indicated that infection of the bile above the obstruction leads to cholangitis and further suppresses bile flow via down regulation of expression of transporters in canalicular side [35].

Our studies further report significant restoration of biliary constituents following drainage. This is in conformity with earlier report indicating upregulation of expression levels of the multidrug resistance-associated proteins of the canalicular bilirubin conjugate export pump (MRP2) and the canalicular bile salt export pump (BSEP) in the liver following decompression [13]. The recovery in biliary constituents following decompression was more pronounced in patients with cholangitis compared to those without cholangitis. This indicates decompression therapy may have important clinical implication at molecular level in patients with cholangitis, in not only resolving infection but also restoring biliary constituents. In clinical practice, biliary decompression remains the cornerstone of treatment to patients with acute cholangitis who fail to respond to antibiotic treatment [36–39]. Our results on bile culture clearly demonstrate resolution of bacterial infection in most of the patients with cholangitis following drainage by one week. Relief of jaundice and cholangitis with drainage has also been reported earlier in animal and human studies [17,36–39].

The presence of at least one of the PDE constituents, viz GPC, LysoPtdCho and PtdCho in bile following drainage indicates that decompression therapy clears accumulated bile in liver which are in various stages of synthesis in hepatocytes [8,28]. PtdCho has been reported to be chief PDE resonance in bile [30]. However, the present study revealed that GPC was present in most of bile specimens following drainage while PtdCho was present only in few. Mann et al. evaluated hepatic metabolism by employing localised ^{31}P MRS (1.5 T) in patients with malignant obstruction with jaundice and reported reduction in the content of PDE in liver following one week of drainage. However, in order to explain these changes the need of in vitro studies of bile at higher field strength over the course of biliary obstruction and infection following decompression was desired [21]. The present studies (at higher field) demonstrating a consequent increase in PDE resonances in the bile following drainage justify their finding of reduced PDE resonances in liver following drainage indicating clearance of accumulated bile. The changes observed in concentration of small metabolites (acetate, DMA) with time are attributed to the possible bacterial growth [40,41].

The results on clinical data in our study clearly show a trend of liver function tests, bilirubin levels and parameters of cholangitis returning towards normalization (reached an optimal value) after one week following decompression which are in accordance

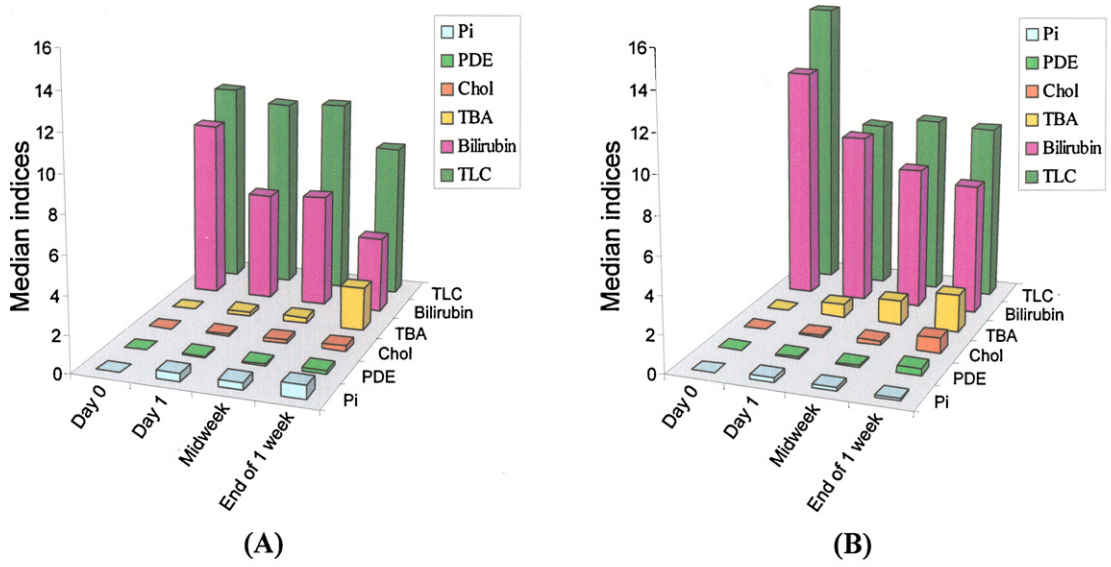

Fig. 4. Bar diagrams indicating median indices of total bile acids (TBA), cholesterol (Chol), phosphodiesters (PDE) and inorganic phosphate (Pi) (mM/L) in two groups of patients: (A) without cholangitis and (B) with cholangitis at day 0, day 1, midweek and end of 1 week. The restoration of chief biliary constituents (mM/L) appearing following decompression is clearly shown (bilirubin is represented in mg/dL and TLC as 1 unit = 1000 cells/mm^3).

to various animal and human studies [18–21,39]. The greenish colour of bile in majority of the patients following one week of drainage further indicates alleviation in the severity of jaundice. Plasma albumin levels indicate synthetic function of hepatocytes that, however, does not increase significantly and possibly might take longer time to restore.

The presence of amino acids in bile was observed in the present study from day 1 following drainage. To our knowledge this is the first report of the observation of amino acids in bile obtained from common bile duct, though they have earlier been reported in hepatic bile [42]. In bile mixed with blood (hemobilia), the occurrence of amino acids has been reported [43,44]. In our study, the endogenous origin of amino acids in bile is established from the fact that right from day 0 amino acids were not observed and the question of mixing of blood in bile in the subsequent days does not arise. The origin of amino acids in the bile could be attributed to bacterial metabolism [45] and/or (ii) disruption of 'blood–bile (canaliculosinusoidal) barrier' function resulting into increased permeability between blood and bile. The results reported herein show the presence of amino acids even in cases without cholangitis and this rule out the first possibility. Therefore, the second cause appears to be the most likely explanation. It is further confirmed from the fact that tight junctions between hepatocytes are the only structures responsible for the barrier between blood and the lumen of bile canaliculus [10] and extrahepatic biliary obstruction causes discontinuities in the junctional meshwork providing a direct pathway resulting in leakage of amino acids in the bile [11]. Furthermore, in the two patients who were in the hospital beyond one week (Supplementary Fig. S1), the presence of amino acids was observed in the bile at the end of 2 weeks but in considerably reduced indices (Supplementary Fig. S1D). The studies that PTBD restores the function of tight junctions have been reported [16]. It appears that such restoration of junctional gap might take longer time of decompression.

The presence of cholangitis over and above jaundice renders additional stress on hepatocytes [46,47]. The degree of clinical severity of cholangitis represents a balance between the type and virulence of the bacterial species, degree of obstruction and purulent transformation of bile on one hand and host resistance, nutritional state and cardiopulmonary status on the other. The presence of organic acids (lactate, succinate, pyruvate etc.), short chain fatty acids (formate, acetate etc.), DMA could arise as a result of bacterial metabolism or anaerobic conditions [29,48] which confirms our results. An intense signal of lactate also indicates low respiration and high glycolytic activity of rapidly dividing tumor cells [49] and has been utilized for detection of hepatobiliary malignancies [50]. However, such studies have ignored the presence of cholangitis. The studies reported herein, showing presence of intense signal of lactate in patients with cholangitis indicate its origin due to bacterial metabolism (Fig. 2(B)) [25,26].

In some cases, random changes of biliary constituents in serial studies were observed (Supplementary Fig. S2C and E). However, in such patients cholangitis was not indicated at presentation (in index bile) and septic complications were observed after a few days. Interestingly, infection (Supplementary Fig. S2C, c and C_1 and E, e and E_1) indicates suppression of biliary constituents which is in conformity with our earlier report [26]. Such complications, though uncommon [51], yet often debated and attributed to the methodological flaws [6,7,52], hence such patients have been excluded from the present study. Realizing the importance of decompression therapy, various advancements are taking place and a closed drainage system has been evaluated to eliminate contamination with environmental pathogens [53].

4. Concluding remarks

The studies demonstrate the reappearance of the biliary constituents following drainage within one week. The restoration of biliary constituents following one day of drainage was more pronounced in patients with cholangitis indicating important implication of decompression therapy in such patients.

Conflict of interest

No conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.04.010.

References

[1] W.G. Hawkins, R.P. DeMatteo, W.R. Jarnagin, L. Ben-Porat, L.H. Blumgart, Y. Fong, Jaundice predicts advanced disease and early mortality in patients with gallbladder cancer, Ann. Surg. Oncol. 11 (2004) 310–315.
[2] J.H. Boey, L.W. Way, Acute cholangitis, Ann. Surg. 191 (1980) 264–270.
[3] C.P. Armstrong, J.M. Dixon, T.V. Taylor, G.C. Davies, Surgical experience of deeply jaundiced patients with bile duct obstruction, Br. J. Surg. 71 (1984) 234–238.
[4] E.A. Aly, C.D. Johnson, Preoperative biliary drainage before resection in obstructive jaundice, Dig. Surg. 18 (2001) 84–89.
[5] O.M. van Delden, J.S. Lameris, Percutaneous drainage and stenting for palliation of malignant bile duct obstruction, Eur. Radiol. 18 (2008) 448–456.
[6] N.A. van der Gaag, J.J. Kloek, S.M. de Castro, O.R. Busch, T.M. van Gulik, D.J. Gouma, Preoperative biliary drainage in patients with obstructive jaundice: history and current status, J. Gastrointest. Surg. 13 (2009) 814–820.
[7] A. Coss, M.F. Byrne, Preoperative biliary drainage in malignant obstruction: indications, technique and the debate over risk, Curr. Gastroenterol. Rep. 11 (2009) 145–149.
[8] V.J Desmet, Cholestasis: extrahepatic obstruction and secondary biliary cirrhosis, in: R.N.M. Mc Sween, P.P. Antony, P.J. Scheurer (Eds.), Pathology of the Liver, second ed., Churchill-Livingstone, Edinburgh, 1987, pp. 364–423.
[9] C.E. Scott-Conner, J.B. Grogen, The pathophysiology of biliary obstruction and its effect on phagocytic and immune function, J. Surg. Res. 57 (1994) 316–336.
[10] T. Kojima, T. Yamamoto, M. Murata, H. Chiba, Y. Kokai, N. Sawada, Regulation of the blood–biliary barrier: interaction between gap and tight junctions in hepatocytes, Med. Electron Microsc. 36 (2003) 157–164.
[11] H. Robenek, J. Herwig, H. Themann, The morphologic characteristics of intercellular junctions between normal human liver cells and cells from patients with extrahepatic cholestasis, Am. J. Pathol. 100 (1980) 93–114.
[12] S.A. Azer, S.A. Coverdale, K. Byth, G.C. Farrell, N.H. Stacey, Sequential changes in serum levels of individual bile acids in patients with chronic cholestatic liver disease, J. Gastroenterol. Hepatol. 11 (1996) 208–215.
[13] J. Shoda, M. Kano, K. Oda, J. Kamiya, Y. Nimura, H. Suzuki, Y. Sugiyama, H. Miyazaki, T. Todoroki, S. Stengelin, W. Kramer, Y. Matsuzaki, N. Tanaka, The expression levels of plasma membrane transporters in the cholestatic liver of patients undergoing biliary drainage and their association with the impairment of biliary secretory function, Am. J. Gastroenterol. 96 (2001) 3368–3378.
[14] G. Ohshio, T. Manabe, T. Tobe, H. Yoshioka, Y. Hamashima, Circulating immune complex, endotoxin and biliary infection in patients with biliary obstruction, Am. J. Surg. 155 (1988) 343–347.
[15] T.M Karsten, T.M. van Gulik, L. Spanjaard, A. Bosma, M.A. van der Bergh Weerman, K.P. Dingemans, J. Dankert, D.J. Gouma, Bacterial translocation from the biliary tract to blood and lymph in rats with obstructive jaundice, J. Sur. Res. 74 (1998) 125–130.
[16] G. Ohshio, T. Manabe, K. Tamura, H. Kudo, H. Yoshioka, T. Tobe, Effects of percutaneous transhepatic biliary drainage on blood–bile permeability and selective IgA transport in patients with biliary obstructions, Ann. Surg. 211 (1990) 428–432.
[17] S. Katz, R. Yang, M.J. Rodefeld, W.J. Folkening, J.L. Grosfeld, Impaired hepatic bacterial clearance is reversed by surgical relief of obstructive jaundice, J. Pediatric. Surg. 26 (1991) 401–406.
[18] R.N. Younes, N.A. Vydelingum, P. Derooij, F. Scognamiglio, L. Andrade, M.C. Posner, M.F. Brennan, Metabolic alterations in obstructive jaundice: effect of duration of jaundice and bile-duct decompression, HPB Surg. 5 (1991) 35–48.
[19] I.A. Fraser, P. Shaffer, S.V. Tuttle, M.A. Lessler, E.C. Ellison, L.C. Carey, Hepatic recovery after biliary decompression of experimental obstructive jaundice, Am. J. Surg. 158 (1989) 423–427.
[20] G.R. Xu, C.J. Kirk, A.W. Goode, Changes in biliary lipid concentrations in bile duct obstruction: an experimental study, J. R. Soc. Med. 79 (1986) 522–527.
[21] D.V. Mann, W.W. Lam, N.M. Hjelm, N.M. So, D.K. Yeung, C. Metreweli, W.Y. Lau, Biliary drainage for obstructive jaundice enhances hepatic energy status in humans: a 31-phosphorus magnetic resonance spectroscopy study, Gut 50 (2002) 118–122.
[22] S. Suzuki, K. Inaba, Y. Yokoi, K. Ohata, S. Ota, M. Azuma, T. Tanaka, H. Konno, S. Baba, T. Hirano, S. Nakamura, Photodynamic therapy for malignant biliary obstruction: a case series, Endoscopy 36 (2004) 83–87.
[23] N.S. Abraham, J.S. Barkun, A.N. Barkun, Palliation of malignant biliary obstruction: a prospective trial examining impact on quality of life, Gastrointest. Endosc. 56 (2002) 835–841.
[24] J. Belghiti, S. Ogata, Preoperative optimization of the liver for resection in patients with hilar cholangiocarcinoma, HPB (Oxford) 7 (2005) 252–253.
[25] L. Bala, C.L. Khetrapal, In-vitro NMR spectroscopic studies of body fluids in human gastro-enterological diseases, in: C.L. Khetrapal, A. Kumar, K.V. Ramanathan (Eds.), Future Directions in NMR, Springer Verlag, Germany, 2009, pp. 33–49.
[26] L. Bala, P. Tripathi, G. Bhatt, K. Das, R. Roy, G. Choudhuri, C.L. Khetrapal, ^{1}H and ^{31}P NMR studies indicate reduced bile constituents in patients with biliary obstruction and infection, NMR Biomed. 22 (2009) 220–228.
[27] B.R. Thapa, A. Walia, Liver function tests and their interpretation, Indian J. Pediatr. 74 (2007) 663–671.
[28] M.H. Sleisenger, J.S. Fordtran, Gastrointestinal Disease, W.B. Saunders Company, Philadelphia, 1993, pp. 1106–1118.
[29] L. Bala, U.C. Ghoshal, U. Ghoshal, P. Tripathi, A. Misra, G.A. Gowda, C.L. Khetrapal, Malabsorption syndrome with and without small intestinal bacterial overgrowth: a study on upper-gut aspirate using ^{1}H NMR spectroscopy, Magn. Reson. Med. 56 (2006) 738–744.
[30] G.A. Nagana Gowda, B.S. Somashekar, O.B. Ijare, A. Sharma, V.K. Kapoor, C.L. Khetrapal, One step analysis of major bile components in human bile using ^{1}H NMR spectroscopy, Lipids 41 (2006) 577–589.
[31] J. Schiller, K. Arnold, Application of high resolution ^{31}P NMR spectroscopy to the characterization of the phospholipid composition of tissues and body fluids – a methodological review, Med. Sci. Monit. 8 (2002) MT205–222.
[32] L. Bala, A. Sharma, R.K. Yellapa, R. Roy, G. Choudhuri, C.L. Khetrapal, ^{1}H NMR spectroscopy of ascitic fluid: discrimination between malignant and benign ascites and comparison of the results with conventional methods, NMR Biomed. 21 (2008) 606–614.
[33] S.A. Khan, I.J. Cox, A.V. Thillainayagam, D.S. Bansi, H.C. Thomas, S.D. Taylor-Robinson, Proton and phosphorus-31 nuclear magnetic resonance spectroscopy of human bile in hepatopancreaticobiliary cancer, Eur. J. Gastroenterol. Hepatol. 17 (2005) 733–738.
[34] A. Geier, M. Wagner, C.G. Dietrich, M. Trauner, Principles of hepatic organic anion transporter regulation during cholestasis, inflammation and liver regeneration, Biochim. Biophys. Acta 1773 (2007) 283–308.
[35] M Trauner, M. Arrese, C.J. Soroka, M. Ananthanarayanan, T.A. Koeppel, S.F. Schlosser, F.J. Suchy, D. Keppler, J.L. Boyer, The rat canalicular conjugate export pump (Mrp2) is down-regulated in intrahepatic and obstructive cholestasis, Gastroenterology 113 (1997) 255–264.
[36] M. Pessa, I. Hawkins, S.B. Vogel, The treatment of acute cholangitis: percutaneous transhepatic biliary drainage before definitive therapy, Ann. Surg. 205 (1987) 389–392.
[37] W.A. Qureshi, Approach to a patient who has suspected acute bacterial cholangitis, Gastroenterol. Clin. North Am. 35 (2006) 409–423.
[38] P.A. Lipsett, H.A. Pitt, Acute cholangitis, Front. Biosci. 8s (2003) 1229–1239.
[39] S.S. Baijal, R.K. Dhiman, S. Gupta, B.C. Sharma, S. Roy, D.K. Agarwal, G. Choudhuri, V.A. Saraswat, S.R. Naik, Percutaneous transhepatic biliary drainage in management of obstructive jaundice, Trop. Gastroenterol. 18 (1997) 167–171.
[40] O. Teahan, S. Gamble, E. Holmes, J. Waxman, J.K. Nicholson, C. Bevan, H.C. Keun, Impact of analytical bias in metabonomic studies of human blood serum and plasma, Anal. Chem. 78 (2006) 4307–4318.
[41] Q.N. Van, H.J. Issaq, Q. Jiang, Q. Li, G.M. Muschik, T.J. Waybright, H. Lou, M. Dean, J. Uitto, T.D. Veenstra, Comparison of 1D and 2D NMR spectroscopy for metabolic profiling, J. Proteome Res. 7 (2008) 630–639.
[42] I.F. Duarte, C. Legido-Quigley, D.A. Parker, J.R. Swann, M. Spraul, U. Braumann, A.M. Gil, E. Holmes, J.K. Nicholson, G.M. Murphy, H. Vilca-Melendez, N. Heaton, J.C. Lindon, Identification of metabolites in human hepatic bile using 800 MHz ^{1}H NMR spectroscopy, HPLC–NMR/MS and UPLC-MS, Mol. Biosyst. 5 (2009) 180–190.
[43] Y.J. Lou, C.X. Chen, J.Y. Jiang, Y.M. Li, Hepatobiliary and pancreatic: hemobilia, J. Gastroenterol. Hepatol. 21 (2006) 474.
[44] O.B. Ijare, T. Bezabeh, N. Albiin, U. Arnelo, A. Bergquist, B. Lindberg, I.C. Smith, Absence of glycochenodeoxycholic acid (GCDCA) in human bile is an indication of cholestasis: a ^{1}H MRS study, NMR Biomed. 22 (2009) 471–479.
[45] M. Garg, M.K. Misra, S. Chawla, K.N. Prasad, R. Roy, R.K. Gupta, Broad identification of bacterial type from pus by ^{1}H MR spectroscopy, Eur. J. Clin. Invest. 33 (2003) 518–524.
[46] R. Kumar, B.C. Sharma, J. Singh, S.K. Sarin, Endoscopic biliary drainage for severe acute cholangitis in biliary obstruction as a result of malignant and benign diseases, J. Gastroenterol. Hepatol. 19 (2004) 994–997.
[47] J.F. Gigot, T. Leese, T. Dereme, J. Coutinho, D. Castaing, H. Bismuth, Acute cholangitis. Multivariate analysis of risk factors, Ann. Surg. 209 (1989) 435–438.
[48] L.M. Prescott, J.P. Harley, D.A. Klein, Microbiology, W.C. Brown, Dubuque, 1993, pp. 155–158.
[49] H. Haug, C. Schramm, Metabolic pattern of free amino acids, glucose, lactic acid, pyruvic acid, fatty acids, BUN, uric acid, etc. in serum of 21 healthy persons and 24 patients with malignant tumors, Clin. Chem. 21 (1975) 1025.
[50] T Nishijima, M. Nishina, K. Fujiwara, Measurement of lactate levels in serum and bile using proton nuclear magnetic resonance in patients with hepatobiliary diseases: its utility in detection of malignancies, Jpn. J. Clin. Oncol. 27 (1997) 13–17.
[51] V. Vidal, C.S. Ho, P. Petit, Early cholangitis complicating percutaneous biliary drainage, J. Radiol. 84 (2004) 1707–1709.

[52] N.A. van der Gaag, S.M. de castro, E.A. Rauws, M.J. Bruno, C.H. van Eijck, E.J. Kuipers, J.J. Gerritsen, J.P. Rutten, J.W. Greve, E.J. Hesselink, J.H. Klinkenbijl, I.H. Rinkes, D. Boerma, B.A. Bonsing, C.J. van Laarhoven, F.J. Kubben, E. van der Harst, M.N. Sosef, K. Bosscha, I.H. de Hingh, L. Th de Wit, O.M. van Delden, O.R. Busch, T.M. van Gulik, P.M. Bossuyt, D.J. Gouma, Preoperative biliary drainage for periampullary tumors causing obstructive jaundice; drainage vs. (direct) operation (DROP-trial), BMC Surg. 7 (2007) 3–12.

[53] J.I. Blenkharn, G.A. Mcpherson, L.H. Blumgart, Septic complications of percutaneous transhepatic biliary drainage. Evaluation of a new closed drainage system, Am. J. Surg. 147 (1984) 318–321.