Abstract

Background and Aims: Plausible reasons for the failure of liver graft in liver transplantation are explored. \(^1\)H-NMR spectroscopy of serum is employed for assessment of liver graft function. Differences in concentrations of specific metabolites between patients with successful and unsuccessful liver grafts following transplantation were used as possible markers to assess the graft quality. Methods: Blood samples from the patients undergoing liver transplantation were obtained preoperatively, immediately after transplant followed by every 24 hrs of post-transplantation until patients were discharged or expired. \(^1\)H-NMR spectroscopic studies of serum were performed at each time point and concentrations of various metabolites measured. Conventional biological tests were also performed at each time point. Results: Elevation of concentrations of the nine metabolites (lactate, alanine, lysine, glutamine, methionine, asparagine, tyrosine, histidine and phenylalanine) in non-survivors using NMR was attributed to the graft dysfunction. The information on the graft dysfunction using conventional biological tests was obtained much later. However, elevation in aminotransferases and bilirubin levels was indicated after about one week and 3 days respectively in non-survivors. Hepatic failure causes alteration in the concentrations of amino acids due to impairment of amino acid metabolism and urea cycle. \(^1\)H-NMR spectroscopy provides the information of all the metabolites in a single step without involving any chemical pretreatment implying better accuracy since each step involved can introduce its own experimental error. Conclusion: Distinct metabolic profile in non-survivors compared to survivors following transplantation promises potential of \(^1\)H-NMR studies in the assessment of liver graft function.

Key words

\(^1\)H NMR – liver transplantation – serum metabolic profile – amino acids.

Introduction

The liver performs multiple functions, the important ones are synthetic, metabolic, secretory and excretory. The liver fails to perform such functions irreversibly in various diseases e.g. chronic to end stage liver diseases. Liver transplantation has been established as a viable treatment under such conditions [1]. A significant cause of mortality and morbidity in liver transplantation is failure of donor graft function. The widening of indications for liver transplantation, shortage of liver grafts and the incidence of primary dysfunction of liver grafts at an unacceptable rate have renewed interest in the identification of new biomarkers for predicting graft viability, monitoring its functioning and metabolic capacity in order to reduce clinical complications and costs [2]. Since liver performs different kinds of functions, no single biochemical test can decipher the global function of the liver and hence there is a need for a battery of conventional liver function tests to be employed for management of patients [3-5]. Among these, only a few are capable of rapidly assessing the function of liver grafts but with lowered sensitivity [6, 7]. Consequently, the development of alternative techniques for assessment of graft function is desired.

One ideal situation might be to think of a method which shows the metabolic variations occurring due to graft dysfunction in minimum steps. This has increased interest in the development of metabolite profiling approaches such as metabolomics/ metabonomics using NMR spectroscopy [8, 9]. NMR spectroscopy has many advantages as it is non-destructive and does not require any chemical pre-treatment steps. In contrast to conventional techniques it is not pre-selective for target metabolite analysis and thus efficiently allows qualitative and quantitative information on a large number of metabolites in a single step. NMR spectroscopy of body fluids has emerged as an important tool for the differential diagnosis of various diseases and
in providing insights in understanding pathogenesis [8, 10, 11]. Blood, serum and urine specimens have been commonly used in NMR for the diagnosis of various liver diseases and assessment of graft function compared to other body fluids [12-18]. Studies involving blood are important as it is readily available. It perfuses all living cells and maintains a normal homeostasis of various metabolites by regulatory pathways. Liver is one of the major organs involved in such regulation. One of the key functions of liver is amino acid metabolism and urea cycle and hepatic failure results into disruption of these pathways [19, 20].

In our earlier communication, an attempt was made to use 1H NMR spectroscopy of serum and urine specimens from a single liver transplant recipient to monitor glutamine and urea levels as possible indicators of graft function [17]. An attempt has also been made to use blood for its use in metabolomic analysis for early detection of liver graft failure, however in order to evaluate different metabolites, the study involved an extraction step [18]. Our present study focuses on the 1H NMR studies of serum specimens in liver transplant patients, preoperatively and at various time points following transplantation in order to reveal distinct metabolites, mainly amino acids, as possible biomarkers which may provide snap shots of hepatocyte functions aimed at deciding appropriate therapeutic intervention in patient management.

Material and Methods

Patients and study protocol

The patients admitted for liver transplantation in one of the tertiary medical centres of northern India were included in this study. Detailed history, clinical evaluation, baseline investigation test for vital signs, admission laboratory analyses, routine liver function tests were recorded. For further diagnosis, the use of imaging modality (ultrasonography and/or computed tomography/MRI) or Doppler evaluation of vascular anatomy were also incorporated when necessary. All of the 9 patients [average age: 26 years, range (3-53) years] requiring liver transplantation had reached end stage chronic liver failure because of following aetiologies: biliary atresia (1), chronic auto immune hepatitis with primary biliary cirrhosis (1), hepatitis C virus related cirrhosis (1), alcoholic cirrhosis (1), pulmonary hypertension (2), fulminant hepatic failure (FHF) with encephalopathy (1), chronic Budd-Chiari syndrome (1) and cholestatic jaundice with chronic liver disease (1). Eight patients received living related orthotopic liver transplantation while one received cadaveric liver graft. The liver grafts were assessed as normal both by visual inspection by the surgeon and histological findings. After obtaining consent from the patients, blood samples (1 mL in a sterile vial) were collected in a fasting state as per standard protocol [21] at the following time points: preoperatively, immediately after transplant followed by every 24 hrs of post transplantation until patients were discharged from the hospital or expired. Of the nine patients, six were surviving with functional liver graft at the time of discharge while three met fatality with graft dysfunction. Patients had mean duration of 18 (range: 6-24) days of hospital stay. For NMR studies, blood collected in a serum separator tube was kept for 30 minutes to allow clotting. Thereafter, serum was separated from the blood clot and collected in a clean tube and again centrifuged for 5 min at 8,000 rpm and frozen in cryogenic vial at -80°C until NMR experiments were performed. Routine liver function tests such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), serum albumin, total bilirubin, direct bilirubin, prothrombin time (PT), and total leucocyte count (TLC) were also monitored at similar time points.

NMR experiments

NMR experiments were performed on a Bruker AVANCE DRX 400 spectrometer equipped with a broad-band inverse probe with shielded z-gradient (Bruker Biospin, Karlsruhe, Germany). Deuterium oxide (D$_2$O; 99.98 atom% D) and the sodium salt of 3-trimethylsilyl-(2,2,3,3-d$_4$)-propionic acid (TSP) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Measured volume of serum (0.5 ml) was taken in 5-mm NMR tube (Wilmad, Buena, USA) and a sealed co-axial glass capillary containing 35µl of 0.375% TSP in D$_2$O was inserted into the NMR tube before obtaining the NMR spectra. TSP served as chemical shift reference as well as reference signal for quantitative estimation of serum metabolites and D$_2$O served as “field-frequency lock”.

Two sets of one-dimensional 1H NMR experiments were performed for all serum samples. A single pulse experiment with water presaturation during relaxation delay of 5 s was performed using 45º flip angle over a sweep width of 8000 Hz. The FID was acquired into 32K data points during 2 s acquisition time, resulting in a repetition time of 7 s; 64 transients were collected. Spin-echo experiments [Carr-Purcell-Meiboom-Gill (CPMG)] [22] were performed with water presaturation during relaxation delay of 18 s over a sweep width of 8000 Hz. The FID was acquired into 32K data points during 2 s acquisition time, resulting in a repetition time of 20 s; 64 transients were collected. T$_1$ filtering was obtained with an echo time of 640 µs repeated 420 times, resulting in a 269 ms effective echo time. Resulting data were Fourier transformed after multiplying by exponential window function using a line broadening function of 0.3 Hz. Spectral assignments were made using two-dimensional DQF-COSY and TOCSY experiments which were essentially in agreement with literature data [8]. Quantitative estimation of metabolites was performed using the CPMG spectra by a procedure as reported previously [23]. Briefly, by taking the integral area of metabolite signal relative to the integral area of TSP, variation in concentration of metabolites were obtained in survival and non-survival cases. Comparison of concentrations of metabolites between groups was analysed by Mann-Whitney U-test. The resulting p-Value for individual metabolites less than 0.05 was taken to indicate the significance. All these statistical analyses were carried out using SPSS 11.5 software.
Results

Mean concentrations of routine laboratory liver function tests in three patients who suffered graft failure and six who had successful liver grafts, with respect to time points (preoperatively, immediately after transplant followed by every 24 hrs of post transplantation until patients were discharged or expired) are presented in Fig. 1. It shows that liver function tests – albumin, PT and TLC were comparable in both groups. However, aminotransferases (ALT and AST) and ALP levels though appearing to increase in the later days (after about one week), were sometimes incoherent. The bilirubin values appeared comparable during initial days (3 days) and subsequently increased in non-survivors. However, up to that time the indication of the failed graft was already obtained using ultrasound and Doppler evaluation of vascular anatomy. A statistical comparison of biochemical and laboratory parameters was performed between survivors and non-survivors during all postoperative days. Mean concentrations [average of the mean concentrations (used for Fig.1) separately, in two groups] of ALT and AST were significantly elevated in non-survivors compared to survivors [ALT: 1055 vs 206 IU/L and AST: 1138 vs 137 IU/L (p<0.02 for both; Mann Whitney U test)]. Though the mean concentration of bilirubin was high in non-survivors compared to survivors, it did not reach a level of significance. The mean concentration of other parameters such as ALP, albumin, PT and TLC were comparable in both groups.

Thus, we were interested whether NMR based metabolomics of the serum may provide additional information for assessment of graft dysfunction following liver transplant in the patients. A longitudinal study was performed where $^1$H NMR spectra of serum at various time points were compared.

The spectrum of serum in a single pulse experiment is dominated by the broad resonances of lipoproteins and other plasma proteins such as albumin, which mask the signals from low molecular weight metabolites. Therefore, for

![Fig 1. Mean concentrations of different biochemical and laboratory parameters in survivors and non-survivors in liver transplant patients with respect to time (days) on x axis.](image-url)
quantitative estimation of various metabolites, we employed CPMG pulse sequence in order to attenuate or even eliminate resonances from macromolecules (or bound small molecules) with shorter $T_2$ relaxation times [24]. Indeed, the CPMG spectrum provides a much clearer representation of small metabolites as reported earlier [23]. Such spectra provide relative quantitative information of the metabolites with a fair degree of accuracy when the experiments are performed under identical conditions.

Still, the CPMG serum spectrum shows overlapping resonances of low molecular weight metabolites (spectral crowding), resulting in quantification problems as has been reported earlier [8, 23]. Signals of glucose between 3.0 to 4.0 ppm were overlapping with drugs in two patients: however, in all the patients glucose $\alpha$-anomeric proton which comes at 5.2 ppm, was not overlapping with any other signals. Therefore for estimation of glucose, $\alpha$-anomeric proton resonance was quantified, and total glucose concentration in the serum was calculated keeping in mind that $\alpha$-anomers and $\beta$-anomers are in a ratio of 36:64 [23]. Signals of branched chain amino acids, creatinine, creatine and phosphocreatine were not considered for quantitative estimation because of overlapping.

The $^1$H NMR metabolic profile of serum of a typical representative patient from non-survivors and survivors are represented in Fig. 2A and 2B respectively for different time points: (a) pre-transplant, (b) post-transplant, thereafter (c-f) every alternate days and (g) represents the last day of the patient in the hospital. Metabolites were assigned unambiguously and the quantitative measurements were performed. In the case of the patient who did not survive, elevated signal intensities of lactate, alanine, lysine, glutamine, methionine, asparagine, histidine, tyrosine and phenylalanine were observed. Even the serum collected immediately after transplantation showed a substantial increase in the concentration of these metabolites. Distinctive elevated concentrations of these serum metabolites were observed during the critical state of the patient, leading to fatality within 24 hours. On the other hand, no increase in the concentration of these serum metabolites was observed between pre and post transplant days in the case of survivor.

Fig. 3 shows plots of mean concentrations (mean concentration of the metabolite is taken at each time point in three non-survivors and six survivors) of different metabolites with respect to time points (preoperatively, immediately after transplant followed by every 24 hrs of post transplantation until patients were discharged or expired) in non-survivors compared to survivors. Mean concentration [average of the mean concentrations (used for Fig. 3) separately, in two groups] of these metabolites during all postoperative days in non-survivors were significantly higher compared to that of survivors.
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of survivors: [lactate (9.8 vs 3.3), alanine (2.6 vs 0.5), lysine (1.7 vs 0.2), glutamine (3.9 vs 0.5), methionine (0.4 vs ND), asparagine (0.5 vs ND), histidine (0.6 vs 0.1), tyrosine (0.3 vs 0.1) and phenylalanine (0.2 vs ND) mM (p<0.02 for all; Mann Whitney U test)]. A consistent increase in concentration of these metabolites, starting since initial days, is clearly demonstrated in non-survivors compared to survivors with respect to time. Lactate was however ignored and not shown in Fig. 3, as it is well reported that its concentration alters during hypoxic conditions [25, 26]. The mean concentration of other metabolites viz. 1, 2 propandiol, β-hydroxy butyrate (BHB), acetate, acetone, acetoadetate, glutamate, citrate, glucose and formate were found to be comparable in both non-survivors and survivors. Fig. 4 shows plots of changes in concentration of a typical laboratory variable (ALT) and a typical NMR metabolite (glutamine) in all individual patients with respect to time. It is clear from the figure that glutamine shows a steadier, consistent and earlier increase than ALT (sometimes incoherent) in non-survivors compared to survivors.

Discussion

1H NMR metabonomics of serum provides a functional test that is able to monitor variation in different metabolites involved in various metabolic pathways, simultaneously. NMR spectroscopy bears the advantage of being non-selective providing information of various metabolites in a single experiment while the use of serum provides a readily available body-fluid which does not require a chemical pre-treatment/ extraction step for quantitative estimation of metabolites. However, in a clinical setting...
various biochemical conventional techniques have to be individually tested for various metabolites which will be time consuming.

The liver plays a central role in amino acid metabolism, protein synthesis, and its breakdown as well as in several detoxification processes [27]. Liver failure gives rise to multiple metabolic abnormalities which result in variation in metabolite concentration in serum. Such variations are independent of blood flow to liver and drug metabolism that usually influence other tests measuring hepatic functions [28, 29].

The present study has shown distinctive differences in serum amino acids in non-survivors compared to survivors on post transplant follow up days. Such results are conform with earlier reports performed on patients and experimental models of chronic liver failure indicating increased concentration of tyrosine, phenylalanine and methionine [30, 31]. The aromatic amino acids and methionine are catabolised mainly in the liver and their raised values in patients with failing liver graft probably result from impaired hepatic metabolism and portal systemic shunting of blood [32]. It is also reported that during acute hepatic necrosis all the amino acids increase in blood except branched chain amino acids [33]. Glutamine is found to be increased because of the impairment in the urea cycle, resulting in high levels of ammonia which triggers glutamine synthesis [17]. Under normal conditions, the liver is the site of anabolism and catabolism of amino acids in a dynamic state of control but a failing liver graft represents a catabolic state with decreased aromatic amino acid breakdown and thereby accumulation in serum [34, 35]. There has also been a report of increased concentration of tyrosine and phenylalanine in traumatic liver injury [16]. Hyperglucagonaemia and increased gluconeogenesis could also contribute to the abnormal amino acid pattern [36, 37].

One might hypothesize that the observed differences could be due to malnourishment which is also reported to influence amino acid concentration [38]. The patients in the present study, however, were of normal body weight, were not nutritionally depleted or anaemic as is clear from serum albumin values (pretransplant). This suggests that malnutrition is an unlikely cause for the differences observed. All the patients receiving transplants had reached the same metabolic stage i.e. end stage liver failure. Different aetiologies and age do not seem to be a likely cause, otherwise, it would have affected serum metabolite concentration values in pre-transplant stage. The spectra of the serum obtained preoperatively were similar in all the patients, therefore the changes in the metabolites that we have observed may simply represent altered cellular metabolism that occurs intrinsically with failure of liver function.

Metabolic profile as obtained from the NMR studies of serum in the survivors, was distinctively different compared to non-survivors. In all patients who did not survive the NMR spectra showed high concentrations of amino acids from the 2nd day onwards in contrast to the clinical symptoms where in one of the non-survival cases, the condition appeared to be stable up to six days. The patient died on the 10th day. These results are encouraging since the NMR data was indicative of liver dysfunction on the 2nd day itself. Corrective measures based on the NMR warnings could have possibly changed the situation. The results presented here demonstrate the potential of NMR spectroscopy in the assessment of graft function. However, more cases have to be studied in order to establish the significance of these results.

It is worthwhile to note that one patient, who was a recipient of cadaveric orthotopic liver transplantation, was one of the survivors indicating the significance of organ donation in transplant cases.

A unique non-survivor case of liver transplant: A post-operative infection leading to liver damage possibly

In this case also we studied the serum metabolic profile of the patient after every 24 hours as in other cases. The results are presented in Fig. 5. The metabolites lactate, alanine, lysine, glutamine, methionine, tyrosine, histidine and phenylalanine did not significantly increased up to day 23 and hence the results corresponded to the metabolic profile of survival cases till that day. However, on day 28 the concentration of the metabolites (lactate, alanine, lysine, glutamine, methionine, tyrosine, histidine and phenylalanine) increased. These metabolites have been found to be significantly elevated in non-survival cases compared to survivors. The patient died on day 29.

![Fig 4. Concentration values of ALT and glutamine in an individual patient falling in survivor and non-survivor group with respect to time.](image)
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final day when the patient expired, an increase in acetone and succinate was also observed. This was a matter of anxiety for us and therefore we examined the clinical data of the patient. This patient showed clinical symptoms such as those in the survivor patients in NMR data. After the operation he was extubated on day two. He was put on an oral diet on day three and tolerated well. However serum bilirubin, ALT and GGT showed an increasing trend. Liver biopsy was done on the 10th day due to a suspicion of graft rejection but no rejection was found. On day 23 due to oral ulcers the intake of patient decreased and subsequently he was maintained on nasogastric feeds. The patient’s condition was stable. Again on the 28th day the patient developed fever and bleeding from oral ulcers and also an upper gastrointestinal bleed. The TLC progressively increased and the platelet count also dropped. Chest X-ray showed fungal pneumonia. The patient’s condition deteriorated as also observed from the NMR data. The patient died on day 29 due to fungal pneumonia, septicemia and coagulopathy. Fig. 5 demonstrates that on the 28th day, the day before the patient died, the concentration of the same metabolites elevated as in non-survivor cases compared to survivor cases although to a much lesser extent. This may be interpreted in terms of the fact that the patient actually died because of the infections arising from fungal pneumonia and septicemia primarily, rather than due to graft dysfunction though some damage in the liver could have occurred secondarily due to the infections. Our results by NMR metabolic profile also demonstrate that a day before death there was an indication in increased concentration of amino acids possibly due to liver damage. This case has given us more confidence in our findings.

**Conclusion**

1H NMR analysis of metabolites in serum may be helpful in the assessment of status of liver transplant patients. It provides more time sensitive results when compared to other conventional liver function tests. Such assessment improves clinical prognosis by providing a wider treatment window. It allows monitoring graft function. The results unequivocally demonstrate a gradual increase in some of the marker metabolites ultimately leading to fatality. Hence, such techniques, when used complementarily to other biochemical methods, may provide prior information for possible intervention for reducing the risks leading to fatality. NMR spectroscopy provides a rapid and straightforward method which does not require any chemical pre-treatment step and is not pre-selective. The results also indicate the utility of cadaveric liver grafts emphasizing the importance of organ donation after death.

**Conflicts of interest**

None to declare.

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