Int J Artif Organs 2014; 37 (6): 442-454 DOI: 10.5301/ijao.5000341

ORIGINAL ARTICLE

A single-center experience of non-bioartificial liver support systems among Chinese patients with liver failure

Qi Xia, Xiahong Dai*, Jianrong Huang, Xiaowei Xu, Qian Yang, Xiaoli Liu, Yuemei Chen, Lanjuan Li*

State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, First Affiliated Hospital, Zhejiang University, Hangzhou, Zhejiang - China

*Qi Xia and Xiahong Dai contributed equally to this work.

Introduction: *Liver failure is one of the most deadly, prevalent, and costly diseases worldwide. Non-bioartificial liver support systems (NBALs) have been shown to be effective in improving the clinical symptoms and laboratory parameters of patients with liver failure. The main aim of this large case series analysis was to investigate the status of NBALs and their effectiveness in improving survival in liver-failure patients.*

Methods: *In this retrospective study, 460 patients with liver failure who received NBAL treatment in addition to conventional medications were compared with 422 patients who were treated with conventional medications alone. Kaplan-Meier and life table analyses were used to estimate survival rates.*

Results: *Clinical outcomes were improved after NBAL treatment. The 30-day survival rates of subacute liver failure (SALF) patients were 63% among those who received NBALs and 21% among those who did not receive NBALs ($p<0.01$). Similarly, the 30-day survival rate of acute-on-chronic liver failure (ACLF) patients who received NBALs was 47%, significantly higher than that of the non-NBAL patients ($p<0.05$). The survival rates of ACLF patients with low Model for End-Stage Liver Disease (MELD) scores (MELD≤20) were 64% and 40% among whom received NBALs and those who did not, respectively ($p<0.01$).*

Conclusions: *NBAL treatment is helpful to improve the survival of patients with ALF, SALF or ACLF. ACLF patients with lower MELD scores showed improved outcomes relative to those with higher MELD scores.*

Keywords: *Liver failure, Prognosis, Survival, Artificial liver support*

Accepted: May 12, 2014

INTRODUCTION

Liver failure is one of the most prevalent, costly, and life-threatening clinical syndromes, and it has a complicated etiology, variable manifestations, and a high short-term mortality rate. The subtypes of liver failure include acute liver failure (ALF), subacute liver failure (SALF), acute-on-chronic liver failure (ACLF), and chronic liver failure (CLF) (1). Although advances in the medical management of intensive care patients have improved outcomes, the prognoses of ALF and SALF remain poor, with reported mortality rates of 60% to 90% (2-4). Compared with ALF and SALF, ACLF is more common in Asians because of the large population of hepatitis B patients in Asia. ACLF has a short-term mortality rate of 50% to 90% (5).

Although liver transplantation (LT) has significantly improved the prognosis of liver failure, its application has been restricted because of a shortage of organs, matching difficulties, and high cost. Therefore, artificial liver support systems are a potentially valuable treatment for liver failure patients. Artificial liver support systems consist of bioartificial liver support systems (BALs) and non-bioartificial liver support systems (NBALs). BALs are *in vitro* devices that contain cultured human liver cells, whereas NBALs are blood purification devices that temporarily perform the main functions of the liver by removing harmful substances and supplying beneficial substances. NBAL techniques include plasma exchange (PE), plasma perfusion (PP), plasma bilirubin adsorption (PBA), hemofiltration (HF), continuous hemodiafiltration (CHDF), and plasma diafiltration (PDF) as well as techniques using the molecular adsorbent recirculation system (MARS), continuous albumin purification system (CAPS), and the Prometheus system (6). BALs remain in the research stage, but NBALs have already been shown to be effective in improving the clinical symptoms and laboratory parameters of patients with liver failure (7, 8). To date, there have been few large studies on the prognosis of liver failure patients who undergo NBAL treatment, and the potential of NBAL to improve patient survival is controversial.

The main aim of this retrospective study was to investigate the status of NBALs and their effectiveness in improving survival of liver failure patients.

PATIENTS AND METHODS

Study design and ethics statement

Consecutive patients diagnosed with liver failure were included in this retrospective, observational case series analysis of survival. Eligible patients were treated at the First Affiliated Hospital, School of Medicine, Zhejiang University, during the period from December 1, 2008 to May 1, 2012. The study complied with the Standards for Reporting of Diagnostic Accuracy guidelines. Approval of the Ethics Committee was obtained. The research protocol was approved by the Human Ethics Committee of the First Affiliated Hospital of the School of Medicine at Zhejiang University. All patients participating in the study signed an informed consent form. Each patient with NBAL at our center was strictly selected according to the operating guide for artificial liver support systems (9). All the enrolled liver failure patients with stable hemodynamics had the choice to be treated with NBAL or not, a decision made with their clinical doctor. The home affordability and extent of disease were essential factors in their choice. Usually liver failure patients with hepatic encephalopathy (HE) were treated with plasma exchange (PE) in combination with plasma perfusion (PP). Liver failure patients with hepatorenal syndrome were treated with a combination of plasma exchange (PE) plus continuous hemodiafiltration (CHDF) or plasma exchange (PE) plus plasma diafiltration (PDF). In patients with hyperbilirubinemia, a therapy of plasma exchange (PE) plus plasma bilirubin adsorption (PBA) was used.

Study variables

The definition of liver failure used in this study was in accordance with those of the Guidelines for Diagnosis and Treatment of Liver Failure (1).

The ALF criteria included the following features: 1) acute onset of liver failure within two weeks in the absence of pre-existing liver disease; 2) encephalopathy ≥ stage II; 3) extreme fatigue with severe digestive symptoms, such as obvious anorexia, abdominal distension, nausea, or vomiting; 4) progressively worsening jaundice within a short period; 5) obvious hemorrhagic tendency with prothrombin activity (10) (PTA) ≤40% (approximate prothrombin time, [PT] ≥18.3 s; international normalised ratio [INR] >1.50); 6) progressive decrease of liver function. The absence of any of the above six criteria precluded a diagnosis of ALF.

The SALF criteria included the following features: (1) subacute onset of liver failure within 15 days to 26 weeks in the absence of pre-existing liver disease; 2) extreme fatigue with severe digestive symptoms, such as obvious anorexia, abdominal distension or nausea and vomiting; 3) progressively worsening jaundice within a short period (serum total bilirubin ≥10 mg/dl or a daily elevation ≥1 mg/dl); 4) obvious hemorrhagic tendency with PTA ≤40% (PT ≥18.3 s or INR >1.50). The absence of any of the above five criteria precluded a diagnosis of SALF.

The ACLF criteria included the following features: 1) acute deterioration of pre-existing chronic liver disease; 2) extreme fatigue with severe digestive symptoms, such as obvious anorexia, abdominal distension or nausea and vomiting; 3) progressively worsening jaundice within a short period (serum total bilirubin ≥10 mg/dl or a daily elevation ≥1 mg/

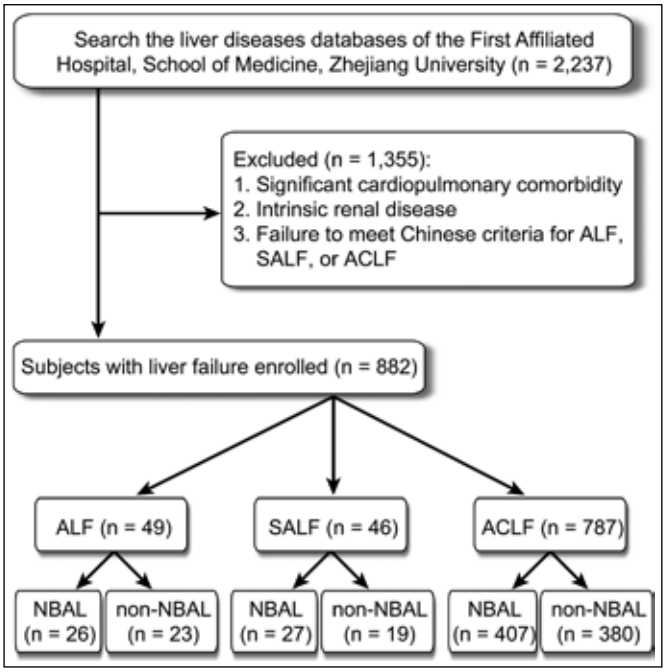

Fig. 1 - *Flowchart of this retrospective large case series analysis. ALF = acute liver failure; SALF = subacute liver failure; ACLF = acute-to-chronic liver failure; NBAL = patients treated with non-bioartificial liver support systems; non-NBAL = patients treated without non-bioartificial liver support systems.*

dl); 4) obvious hemorrhagic tendency with PTA ≤40% (PT ≥18.3 s or INR >1.50). The absence of any of the above four criteria precluded a diagnosis of ACLF.

Hepatic encephalopathy (HE) was defined as the presence of neuropsychiatric abnormalities during the course of liver disease, including the involvement of cognitive, affective/emotional, behavioral, or bioregulatory domains. Abnormal mental states were clinically assessed for classification into stages I to IV (11, 12). The diagnosis of hepatorenal syndrome (HRS) was based on the exclusion of other causes of renal failure. The presence of ascites was confirmed by abdominal ultrasonography and paracentesis. Three stages of ascites, i.e., mild, moderate, and massive, were differentiated using B-mode ultrasonography.

Management protocol and data collection

A flow chart for the study group selection process is presented in Figure 1. By searching the liver disease databases of the hospital, 2 237 cases of suspected liver failure occurring between December 1, 2008 and May 1, 2012 were found. Of these cases, 1 355 patients were excluded because of marked cardiopulmonary comorbidity, intrinsic renal disease or other comorbities such as diabetes or hypothyroidism, or because they did not meet the predefined liver failure criteria. The remaining 882 patients met the study criteria for ALF, SALF, or ACLF. The follow-up interval extended from the date of diagnosis until death or the end of the study (August 1, 2012). The direct causes of death in patients with liver failure include serious infections, gastrointestinal bleeding, hepatic encephalopathy, hepatorenal syndrome, HPS, water and electrolyte acid-base disorders. When patients are merged with serious underlying diseases, their survival is significantly affected. For example, diabetic patients are more likely to be complicated by severe infection; patients with heart disease are more likely to have heart failure and unstable hemodynamics; primary kidney disease patients are more prone to kidney failure; liver function in patients with hyperthyroidism is more likely to deteriorate. Thus we exclude these cases merged with serious underlying diseases and grouped by baseline MELD scores.

All of the patients were treated with conventional medications, which included intensive care unit (ICU) treatment; absolute bed rest; energy supplements; intravenous drip infusion of albumin or plasma; maintenance of electrolyte and acid-base equilibrium; administration of glutathione, adenosylmethionine, or branched-chain amino acids to nourish the liver cells; and the prevention and treatment of complications. Briefly, treatment of acute variceal bleeding was performed with terlipressin or somatostatin together with variceal band ligation. Ascites was treated with sodium restriction and with large-volume paracentesis combined with albumin administration. Aldosterone antagonists were given only if there were no contraindications such as renal failure or hyperkalemia. Hepatic encephalopathy was treated with standard therapy, including lactulose or lactitol and cleansing enemas. Spontaneous bacterial peritonitis was treated with cephalosporins. Approximately 49% of the patients underwent NBAL. Some patients from both groups underwent orthotopic liver transplantation (OLT) in this case series analysis.

The NBAL methods used included plasma exchange (PE), plasma perfusion (PP), plasma bilirubin adsorption (PBA), hemofiltration (HF), continuous hemodiafiltration (CHDF), and plasma diafiltration (PDF). All of the patients were treated with PE, and most of them were treated with one or more additional methods. The choice of therapy was based on each patient's condition. For example, we performed PE in combination with PP for HE. We chose

the combinations of PE + CHDF in patients with hepatorenal syndrome. Excluding patients who were deceased and those who underwent liver transplantation after the first NABL treatment, the remaining patients received two to three times more in the subsequent seven to ten days. Each session of PE therapy lasted for four to six hours, and each session of PBA lasted for two to three hours. Therapy using PE + CHDF, PE + PP, PDF, or HF usually took six to eight hours per session.

The NBAL treatment room was thoroughly sterilized by ultraviolet (UV) light before each treatment. The external circulation system and the separator were connected under sterile conditions, washed with a 0.9% saline solution to remove the microbubbles from the line and then filled with a 2 mg/500 ml heparin saline solution. In addition, we carefully adjusted the doses of heparin and protamine according to the PT and maintained the fluid balance to decrease complications such as bleeding, hemolysis, and hypotension.

Descriptive statistics for the patients' features were recorded within 24 h of diagnosis. The PT in seconds was converted to the INR using the international sensitivity index (ISI) for thromboplastin (10). Missing values were automatically deleted.

Statistical analysis

The actual survival rates of the patients with or without NBAL in the ALF, SALF, and ACLF groups were compared by a Kaplan-Meier analysis (13). The ACLF patients were divided into three subgroups based on their MELD scores, and the actual survival rates of the three subgroups were compared by a Kaplan-Meier analysis. The Mantel-Cox log-rank test was used to evaluate the differences between the Kaplan-Meier curves. The statistical analyses were performed using SPSS software, version 16.0 (SPSS, Chicago, IL, USA). Continuous variables are expressed as the mean ± standard deviation (SD) or as the median and interquartile ranges, and categorical variables are described by counts and proportions. For all of the analyses, $p<0.05$ was considered statistically significant.

RESULTS

Patient demographics and follow-up

As shown in Figure 1, this retrospective large case series analysis included 882 patients with liver failure, of whom 49 had ALF, 46 had SALF, and 787 had ACLF. All of the enrolled patients were treated with conventional medications. In addition, 26/49 ALF patients, 27/46 SALF patients and 407/787 ACLF patients were treated with NBAL.

The baseline characteristics of the patients with ALF, SALF, and ACLF are shown in Table I. The MELD scores of the NBAL and non-NBAL patients with ALF were 26.00 (16.00) and 24.00 (8.00), respectively. The MELD scores of the NBAL and non-NBAL patients with SALF were 24.00 (5.00) and 23.00 (8.00), respectively. The MELD scores of the NBAL and non-NBAL patients with ACLF were 25.00 (7.00) and 25.00 (10.00), respectively. No significant differences in the baseline MELD score were observed between the NBAL and non-NBAL patients in any group ($p>0.05$). Similarly, the baseline parameters, including age; sex; albumin, total cholesterol, potassium and sodium levels; INR; and PT, did not differ between the NBAL and non-NBAL patients with ALF, SALF or ACLF ($p>0.05$; Tab. I).

The ACLF patients were further divided into three subgroups based on their MELD scores: MELD≤20, 20<MELD ≤30 and MELD>30. The MELD scores of the NBAL and non-NBAL patients in the MELD≤20 subgroup were 18.50 (3.00) and 19.00 (3.00), respectively. The MELD scores of the NBAL and non-NBAL patients in the 20<MELD≤30 subgroup were 25.00 (4.00) and 25.00 (4.00), respectively. The MELD scores of the NBAL and non-NBAL patients in the MELD>30 subgroup were 37.00 (9.00) and 33.00 (6.00), respectively. In the ACLF patients in the MELD≤20 and 20<MELD≤30 subgroups, the MELD scores did not differ significantly between NBAL and non-NBAL patients (Tab. II). In the ACLF patients in the MELD >30 subgroup, the MELD score of NBAL patients was higher than that of non-NBAL patients ($p<0.05$).

All 882 patients were followed from the date of diagnosis until either death or the end of the study (August 1, 2012). As shown in Table III, 565 patients died during follow-up, and 69 were lost to follow-up. The remaining 248 patients were still alive at the end of the study. The deaths that occurred were related to complications of liver disease, such as HE, HRS, gastrointestinal bleeding, severe infection, and hepatopulmonary syndrome. Of the 882 patients included in the analysis, 424 were followed up for less than 4 weeks, and 458 were followed up for less than 24 weeks. The average duration of follow-up was 36.29 weeks (range 0.00 to 183.1 weeks). Overall, 72 patients underwent OLT, including 1 NBAL patient in the ALF group, 2 NBAL patients in the SALF group, 60 NBAL

TABLE I - BASELINE CHARACTERISTICS OF SUBJECTS WITH OR WITHOUT NBAL TREATMENT

Variable	ALF (n = 49)		SALF (n = 46)		ACLF (n = 787)	
	NBAL (n = 26)	non-NBAL (n = 23)	NBAL (n = 27)	non-NBAL (n = 19)	NBAL (n = 407)	non-NBAL (n = 380)
Demographic						
Age (y)	39 (28)*	49 (36)	44 (16)*	49 (18)	45 (17)*	46 (18)
Sex (male/female)	14/12*	13/10	10/17*	10/9	348/59*	305/75
Clinical						
Ascites (%)						
0 = None	46.15%	69.60%	51.90%	47.40%	25.10%	31.80%
1 = Detected only by ultrasonography	23.08%	21.70%	33.30%	36.80%	32.90%	33.20%
2 = Shifting dullness	26.92%	8.70%	14.80%	10.50%	30.70%	24.50%
3 = Tense ascites	3.85%	0.00%	0.00%	5.30%	11.30%	10.50%
Hepatic encephalopathy (%)						
Stage 0	-	-	59.30%	57.90%	64.20%	52.10%
Stage I	-	-	11.10%	26.30%	14.70%	16.50%
Stage II	61.54%	60.90%	18.50%	0.00%	9.20%	15.20%
Stage III	26.92%	13.00%	3.70%	0.00%	5.30%	9.30%
Stage IV	11.54%	26.10%	7.40%	15.80%	6.60%	6.90%
Hepatorenal syndrome (%)	15.38%	30.40%	7.40%	10.50%	10.30%	12.00%
Biochemical						
Albumin (g/dl)	3.41 ± 0.40*	3.13 ± 0.55	3.26 ± 0.40*	3.17 ± 0.52	3.33 ± 0.37*	3.24 ± 0.48
Serum bilirubin (mg/dl)	22.89 ± 12.95*	24.39 ± 17.49	29.44 ± 6.66*	25.90± 12.73	29.29 ± 9.19#	22.55 ± 11.37
Cholinesterase (U/ml)	3.55 ± 1.23*	3.32 ± 1.14	3.17 ± 1.17*	2.87 ± 1.24	2.76 ± 1.76#	2.21 ± 1.50
Serum creatinine (mg/dl)	0.60 (0.67)*	0.92 (2.14)	0.68 (0.29)*	0.83 (0.63)	0.77 (0.38)#	0.83 (0.82)
Triglycerides (mg/dl)	116.91 (119.57)*	91.23 (164.74)	107.61 (102.08)*	65.54 (72.63)	78.83 (55.80)#	67.31 (55.80)
Total cholesterol (mg/dl)	2.51 ± 0.54*	2.23 ± 0.94	2.57 ± 0.87*	2.09 ± 0.93	1.86 ± 0.94*	1.82 ± 1.19
Potassium (mEq/l)	4.11 ± 0.93*	4.41 ± 0.78	3.79 ± 0.65*	4.21 ± 0.69	3.98 ± 0.72*	4.20 ± 0.68
Sodium (mEq/l)	137.19 ± 5.33*	136.83 ± 8.91	135.63 ± 5.34*	139.16 ± 11.45	134 ± 7.00*	135 ± 8.01
INR for prothrombin time	2.60 (1.95)*	3.29 (2.95)	2.17 (0.99)*	2.09 (1.49)	2.06 (0.92)*	2.16 (1.29)
Prothrombin time (S)	30.15 (24.65)*	42.75 (35.63)	25.00 (11.60)*	24.2 (17.6)	24.5 (11.83)*	25.7 (15.7)
Platelets (10^9/l)	136.46 ± 80.17*	127.52 ± 51.03	143.07 ± 88.43*	136.00 ± 91.29	91.5 ± 71.25#	81 ± 71.75
MELD score	26.00 (16.00)*	24.00 (8.00)	24.00 (5.00)*	23.00 (8.00)	25.00 (7.00)*	25.00 (10.00)

ALF = acute liver failure; SALF = subacute liver failure; ACLF = acute-on-chronic liver failure; MELD = Model for End-Stage Liver Disease scoring system; NBAL = patients with non-bioartificial liver support systems; non-NBAL = patients without non-bioartificial liver support systems.
Normally distributed continuous variables are expressed as the means ± SD. Non-normally distributed continuous variables are expressed as the medians (interquartile range).
*Comparison of NBAL and non-NBAL patients, p>0.05.
#Comparison of NBAL and non-NBAL patients, *P*<0.05

patients in the ACLF group and 9 non-NBAL patients in the ACLF group.

Disease etiology

The etiology analysis shown in Figure 2 indicates that the etiology varied because of the presence of different subtypes of liver failure. The main cause of ALF was drug-induced hepatitis (21 cases, 42.86%), followed by hepatitis B (8 cases, 16.33%), surgery/trauma (5 cases, 10.20%), and unexplained liver damage (15 cases, 30.61%) (Fig. 2A). Similarly, SALF was primarily caused by drug-induced hepatitis (25 cases, 54.35%), followed by hepatitis B (5 cases, 10.87%), hepatitis E (2 cases, 4.35%), and unexplained

TABLE II - BASELINE CHARACTERISTICS OF ACLF PATIENTS WITH OR WITHOUT NBAL TREATMENT

Variable	ACLF MELD≤20 (n = 159)		ACLF 20<MEL≤30 (n = 434)		ACLF MELD >30 (n = 194)	
	NBAL (n = 76)	non-NBAL (n = 83)	NBAL (n = 242)	non-NBAL (n = 192)	NBAL (n = 89)	non-NBAL (n = 105)
Demographic						
Age (y)	46.13 ± 12.73*	47.61 ± 12.71	44.39 ± 11.46*	47.07 ± 12.50	47.24 ± 11.42*	46.98 ± 12.70
Sex (male/female)	20/56*	17/66	27/215#	38/154	12/77*	20/85
Biochemical						
Albumin (g/dl)	3.33 ± 0.38*	3.22 ± 0.46	3.34 ± 0.36*	3.24 ± 0.47	3.29 ± 0.39*	3.24 ± 0.50
Serum bilirubin (mg/dl)	24.20 (11.65)#	19.64 (17.51)	28.19 (10.97)#	23.25 (18.52)	34.31 (12.8)#	23.24 (20.82)
Cholinesterase (U/ml)	2.42 (1.17)#	1.96 (1.75)	2.82 (10.97)#	2.33 (1.85)	3.43 (1.28)#	2.32 (2.08)
Serum creatinine (mg/dl)	0.54 (0.13)#	0.78 (0.70)	0.77 (0.25)*	0.84 (0.63)	1.24 (1.03)#	0.92 (1.32)
Triglycerides (mg/dl)	96.54 (72.41)#	77.06 (73.51)	81.48 (51.37)#	69.08 (52.25)	58.01 (58.45)*	64.66 (57.57)
Total cholesterol (mg/dl)	2.25 ± 0.74*	2.35 ± 1.25	2.05 ± 0.69#	1.98 ± 0.98	1.48 ± 0.73*	1.62 ± 1.23
Potassium (mEq/l)	3.95 ± 0.73*	4.31 ± 0.60	3.94 ± 0.64*	4.15 ± 0.69	4.11 ± 0.88*	4.21 ± 0.72
Sodium (mEq/l)	134.74 ± 5.13*	134.98 ± 5.78	134.12 ± 5.32*	135.22 ± 5.75	132.70 ± 7.19*	134.64 ± 7.58
INR for prothrombin time	1.73 (0.4)*	2.04 (1.33)	2.04 (0.69)*	2.09 (1.33)	3.15 (1.71)#	2.29 (1.24)
Prothrombin time (S)	20.75 (6.65)*	24.70 (15.2)	23.65 (8.5) *	25.00 (16.82)	36.25 (19.63)#	27.5 (13.83)
Platelets (10^9/l)	96.45 ± 66.34*	96.43 ± 67.88	103.01 ± 52.48*	92.30 ± 62.08	84.51 ± 60.52#	67.21 ± 70.11
MELD score	18.50 (3.00)*	19.00 (3.00)	25.00 (4.00)*	25.00 (4.00)	37.00 (9.00)*	33.00 (6.00)
Orthotopic liver transplantation	16	1	27	6	17	2

ACLF = acute-on-chronic liver failure; MELD = Model for End-Stage Liver Disease scoring system; NBAL = patients with non-bioartificial liver support systems; non-NBAL = patients without non-bioartificial liver support systems.
Normally distributed continuous variables are expressed as the means ± SD. Non-normally distributed continuous variables are expressed as the medians (interquartile range).
*Comparison of NBAL and non-NBAL patients, p>0.05.
#Comparison of NBAL and non-NBAL patients, p<0.05.

TABLE III - FOLLOW-UP DATA FROM PATIENTS WHO UNDERWENT NBAL TREATMENT

	All patients (n = 882)	ALF (n = 49)		SALF (n = 46)		ACLF (n = 787)	
		NBAL (n = 26)	non-NBAL (n = 23)	NBAL (n = 27)	non-NBAL (n = 19)	NBAL (n = 407)	non-NBAL (n = 380)
Mean follow-up (weeks)*	36.29	32.31	10.63	48.83	16.43	42.11	31.97
Range of follow-up (weeks)	0.00-183.14	0.00-147.14	0.14-68.57	0.14-132.86	0.14-129.43	0.00-183.14	0.14-175.14
Deaths (n)	565	15	19	11	16	245	259
Patients lost to follow-up (n)#	69	4	1	6	1	22	35

ALF = acute liver failure; SALF = subacute liver failure; ACLF = acute-on-chronic liver failure.
*Among the 248 patients known to be alive at the last follow-up.
#Lost to follow-up at the end of the study.

liver damage (14 cases, 30.43%) (Fig. 2B). Of the 787 cases of ACLF, most were caused by chronic hepatitis B (718 cases, 91.24%), followed by alcohol abuse (29 cases, 3.69%), autoimmune factors (8 cases, 1.01%), cholestasis (8 cases, 1.01%) and other causes (24 cases, 3.05%) (Fig. 2C).

A further analysis of the 718 ACLF cases caused by chronic hepatitis B revealed that most patients had active replication

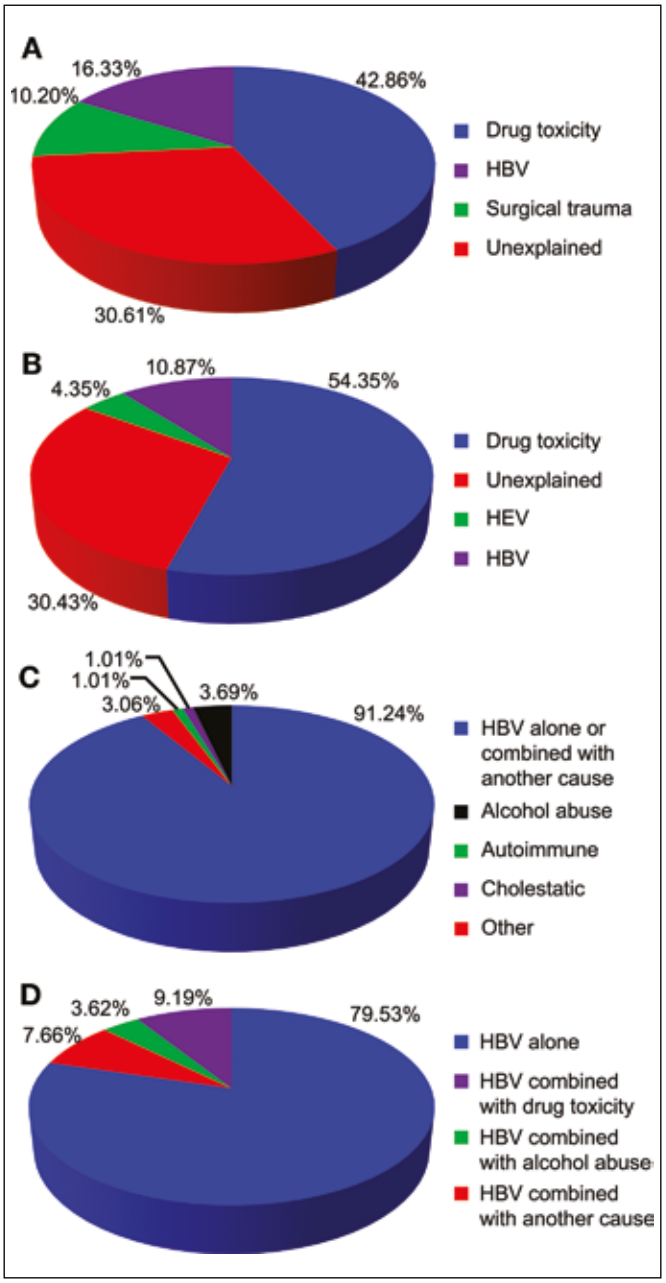

Fig. 2 - *Circle graph of liver failure aetiologies.*
A) *ALF patients;* ***B)*** *SALF patients;* ***C)*** *ACLF patients;* ***D)*** *combined causes in the 718 ACLF patients with hepatitis B. ALF = acute liver failure; SALF =subacute liver failure; ACLF = acuteon-chronic liver failure; HBV = hepatitis B; HEV = hepatitis E.*

of the hepatitis B virus alone (571 cases, 79.53%), followed by the coexistence of drug toxicity (66 cases, 9.19%), alcohol abuse (29 cases, 3.62%), and other causes (55 cases, 7.66%) (Fig. 2D).

TABLE IV - OUTLINE OF NBAL METHODS

	ALF (n = 26)	SALF (n = 27)	ACLF (n = 407)
PE (times)	24	27	537
PE + CHDF (times)	16	15	304
PE + PP (times)	6	7	80
PDF (times)	1	5	113
PBA (times)	2	11	42
HF (times)	0	0	3
Total (times)	49	65	1079
Mean ± SD (times/person)	1.88 ± 1.07	2.41 ± 1.22	2.65 ± 1.32

ALF = acute liver failure; SALF = subacute liver failure; ACLF = acute-on-chronic liver failure; PE = plasma exchange; CHDF = continuous hemodiafiltration; PP = plasma perfusion; PDF = plasma diafiltration; PBA = plasma bilirubin adsorption; HF = hemofiltration.
Normally distributed continuous variables are expressed as the means ± SD.

NBAL methods

A combination of NBAL and conventional medications was administered to 460 patients overall, including 26 ALF patients, 27 SALF patients, and 407 ACLF patients. In total, 1 193 sessions of NBAL were performed, and the ALF, SALF, and ACLF patients had NBAL frequencies of 1.88 ± 1.07, 2.41 ± 1.22 and 2.65 ± 1.32 treatments/person, respectively (Tab. IV). All of the patients were treated with PE, and most were treated with one or more additional methods, including 13/26 (50.00%) ALF patients, 16/27 (59.26%) SALF patients, and 228/407 (56.02%) ACLF patients. The choice of therapy was based on each patient's condition: PE in combination with PP for HE was administered in 12.24% (6/49) of ALF patients, 10.77% (7/65) of SALF patients, and 7.41% (80/1079) of ACLF patients. In patients with HRS, we administered PE with CHDF in 32.65% (16/49) of ALF patients, 23.08% (15/65) sessions of SALF patients and 28.17% (304/1079) sessions of ACLF patients (Tab. IV).

Patient survival

The actual survival rates of patients with ALF, SALF, ACLF, and the three ACLF subgroups were compared using life table and Kaplan-Meier analyses. The survival rates at

TABLE V - SURVIVAL RATES OF PATIENTS WITH LIVER FAILURE WITH OR WITHOUT NBAL

Survival rate	ALF (n = 49)		SALF (n = 46)		ACLF (n = 787)	
	NBAL (n = 26)	non-NBAL (n = 23)	NBAL (n = 27)	non-NBAL (n = 19)	NBAL (n = 380)	non-NBAL (n = 407)
4 weeks	0.42#	0.17	0.63#	0.21	0.47#	0.4
8 weeks	0.42*	0.17	0.63#	0.21	0.43#	0.36
12 weeks	0.42*	0.17	0.59#	0.16	0.43*	0.35
24 weeks	0.42*	0.17	0.59#	0.16	0.42*	0.34
48 weeks	0.42*	0.17	0.59#	0.16	0.41*	0.33

ALF = acute liver failure; SALF = subacute liver failure; ACLF = acute-on-chronic liver failure; NBAL = patients with non-bioartificial liver support systems; non-NBAL = patients without non-bioartificial liver support systems.
*Comparison of NBAL and non-NBAL patients, p>0.05.
#Comparison of NBAL and non-NBAL patients, p<0.05.

TABLE VI - SURVIVAL RATES OF PATIENTS WITH ACLF WITH OR WITHOUT NBAL

Survival rate	ACLF MELD≤20 (n = 159)		ACLF 20<MELD≤30 (n = 434)		ACLF MELD>30 (n = 194)	
	NBAL (n = 76)	non-NBAL (n = 83)	NBAL (n = 242)	non-NBAL (n = 192)	NBAL (n = 89)	non-NBAL (n = 105)
4 weeks	0.64#	0.43	0.48*	0.42	0.29*	0.33
8 weeks	0.61#	0.40	0.44*	0.39	0.25*	0.30
12 weeks	0.61#	0.40	0.43*	0.37	0.25*	0.29
24 weeks	0.59#	0.39	0.43*	0.36	0.24*	0.26
48 weeks	0.59#	0.39	0.42*	0.35	0.21*	0.25

ACLF = acute-on-chronic liver failure; NBAL = patients with non-bioartificial liver support systems; non-NBAL = patients without non-bioartificial liver support systems.
*Comparison of NBAL and non-NBAL patients, p>0.05.
#Comparison of NBAL and non-NBAL patients, p<0.05.

various time points between 4 and 48 weeks in the ALF, SALF, and ACLF patients with and without NBAL are displayed in Tables V and VI.

The four-week survival rate of patients with ALF, SALF, and ACLF who underwent NBAL was distinctly higher than that of patients without NBAL. In particular, the ALF patients had four-week survival rates of 42% with NBAL and 17% without NBAL. The ALF patients with NBAL had a 25% higher chance of living for four weeks than the ALF patients without NBAL (Mantel-Cox log-rank test: p = 0.019) (Fig. 3A). Similarly, the 4-week and 48-week survival rates of SALF patients with NBAL were 63% and 59%, respectively, compared to 21% and 16% for SALF patients without NBAL. The SALF patients with NBAL had a 42% higher chance of living for four weeks than SALF patients without NBAL (Mantel-Cox log rank test: p = 0.003) (Fig. 3B). The 4-week and 48-week survival rates of ACLF patients with NBAL were 47% and 41%, respectively, compared to 40% and 33% for ACLF patients without NBAL. ACLF patients with NBAL had a 7% higher chance of living for four weeks than patients without NBAL (Mantel-Cox log-rank test: p = 0.008) (Fig. 3C).

In the ACLF subgroup analysis, the four-week survival rates of the MELD≤20, 20<MELD≤30 and MELD>30 patients after NBAL treatment were 64%, 48%, and 29%, respectively, whereas the survival rates of the non-NBAL

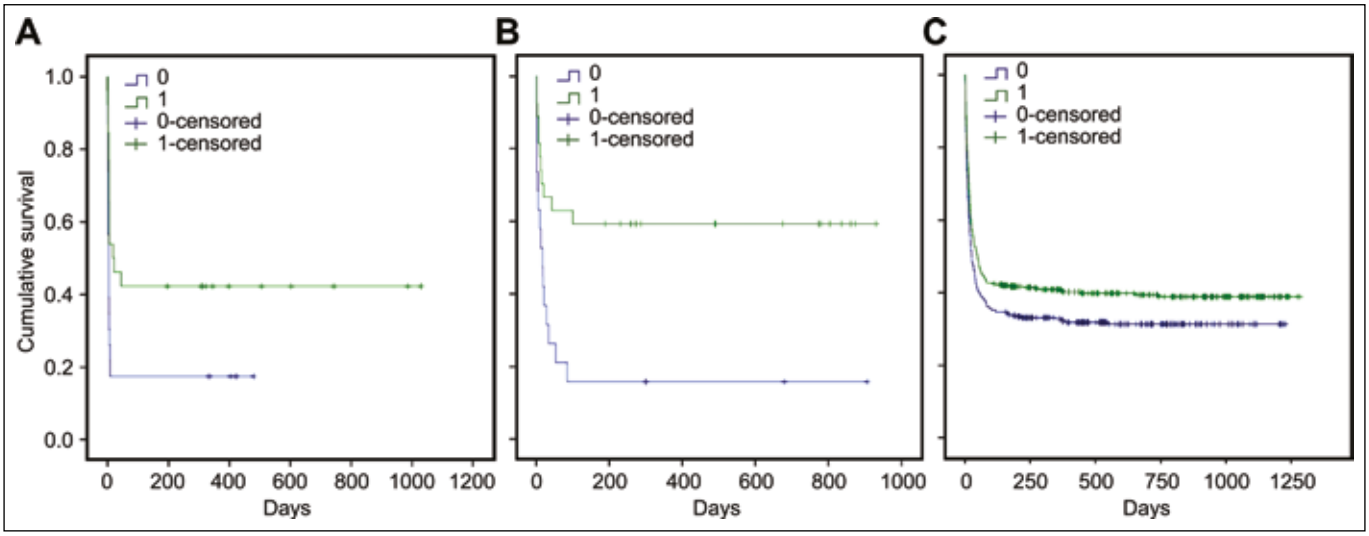

Fig. 3 - *Survival rates of patients with liver failure with or without NBAL.* ***A)*** *ALF patients;* ***B)*** *SALF patients;* ***C)*** *ACLF patients; 0: patients without NBAL; 1: patients with NBAL. There was a significant difference in survival between the patients with and without NBAL (p<0.05). ALF = acute liver failure; SALF = subacute liver failure; ACLF = acuteon-chronic liver failure; NBAL = non-bioartificial liver support system.*

controls were 43%, 42%, and 33%, respectively. In the MELD≤20 subgroup, ACLF patients with NBAL had a 21% higher chance of living for four weeks than patients without NBAL (Mantel-Cox log-rank test: p = 0.001) (Fig. 4B). In the 20<MELD≤30 subgroup, ACLF patients with NBAL had a 6% higher chance of living for four weeks than patients without NBAL (Mantel-Cox log-rank test: p = 0.039) (Fig. 4C). In contrast, in the MELD>30 subgroup, the survival rates of the patients with and without NBAL were not significantly different (Mantel-Cox log-rank test: p = 0.255) (Fig. 4D).

DISCUSSION

Liver failure profoundly affects a patient's quality of life, and it has a high incidence and high mortality worldwide (14, 15). The treatment of liver failure remains challenging, and breakthroughs in conventional medications have not been achieved. NBAL has been shown to be effective and practical for removing toxic substances (especially those bound to serum proteins), replacing them with essential substances such as coagulation factors, albumin, and immunoglobulin and creating an environment that promotes regeneration of the remaining hepatocytes (8, 16). Nearly 90% of patients experience an improvement in symptoms such as fatigue or abdominal distension following NBAL therapy, and liver function parameters have been shown to improve significantly in all tested subjects (17, 18). Additionally, NBAL can serve as a bridge to LT (19, 20). NBAL treatment prior to LT improves perioperative conditions, reduces the incidence of post-transplant complications, and reduces early mortality (7).

NBAL is effective for improving encephalopathy, INR, total bilirubin, creatinine, and endotoxin levels; proportions of amylopectin and aromatic amino acids; and electrolyte imbalances; it also decreases the MELD score (8, 16, 21, 22). However, the ability of NBAL to improve survival remains controversial. Our results show that NBAL treatment improved the survivals of patients with ALF, SALF, and ACLF with baseline MELD scores ≤30. Stutchfield et al noted that NBAL may improve the survival of ALF patients, but not ACLF patients (18). Kjaergard et al arrived at the opposite conclusion; they reported that NBAL reduced mortality in ACLF patients but not in ALF patients (23). The lack of consensus concerning the effectiveness of NBAL may be related to the choice of NBAL method. Each method has its own advantages and disadvantages. The Li-NBAL is a combination of different blood purification methods based on different patients' conditions. We chose the Li-NBAL method, whereas the Stutchfield and Kjaergard studies mainly used MARS. MARS usually provides alternative detoxification for the liver, clears protein-bound toxins, and corrects electrolyte and acid-base imbalances; however,

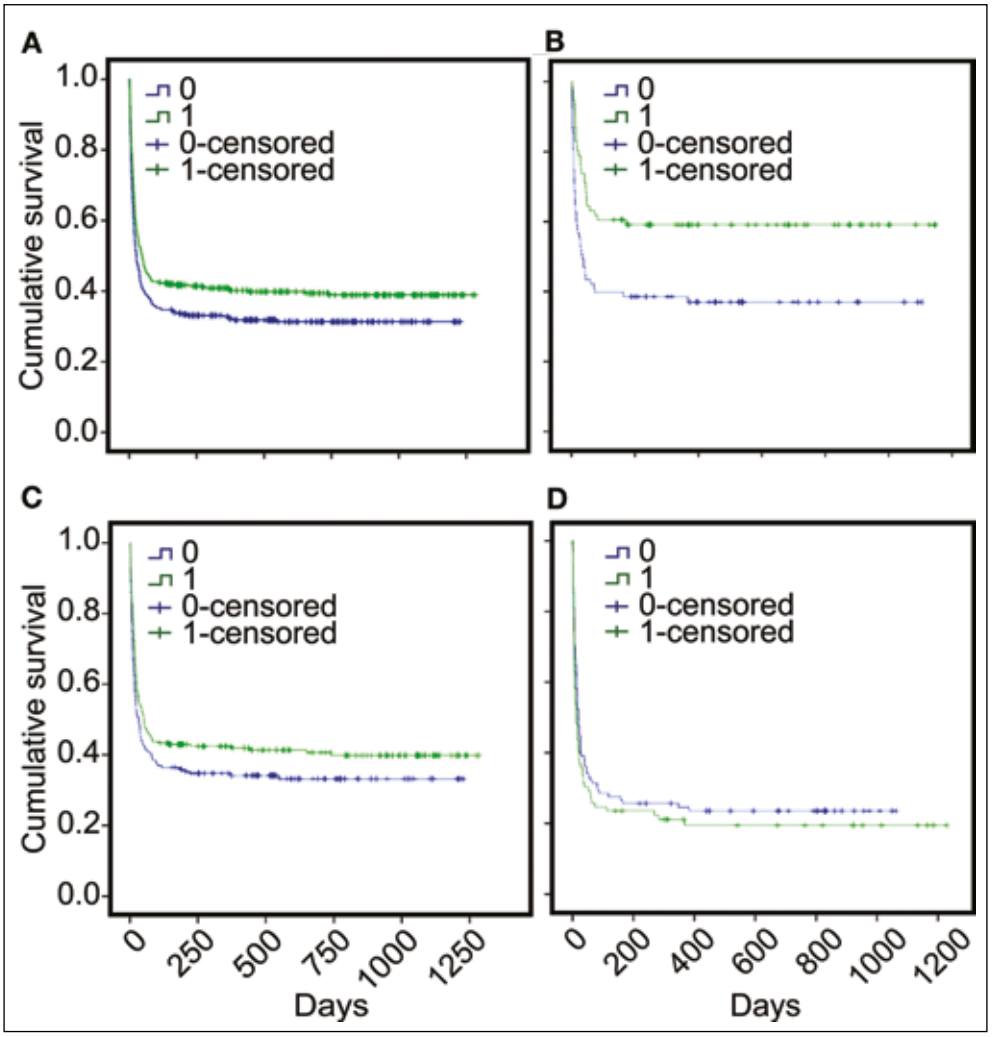

Fig. 4 - *Survival rates of patients with ACLF with or without NBAL.* ***A)*** *Total ACLF patients;* ***B)*** *MELD≤20 subgroup;* ***C)*** *20<MELD≤30 subgroup;* ***D)*** *MELD >30 subgroup; 0: patients without NBAL; 1: patients with NBAL. There was a significant difference in survival between the ACLF patients with and without NBAL. The survival rate of the ACLF patients with NBAL was higher than that of the ACLF patients without NBAL in the MELD≤20 and 20<MELD≤30 subgroups. However, the survival rates of the ACLF patients in the MELD >30 subgroup with and without NBAL were similar. ACLF = acute-to-chronic liver failure; NBAL = non-bioartificial liver support system; Cum Survival = Cumulative Survival.*

this system lacks the ability to synthesize complement proteins and coagulation factors, which may decrease its efficacy (24). One study found that the Prometheus system did not improve the four-week survival of patients with ACLF (25). Plasma exchange (PE), which has already been shown to be effective in ameliorating several aspects of liver failure over the last ten to fifteen years, was the basic method of our Li-NBAL; nearly all patients receiving NBALs got PE. However, Li-NBAL is different from traditional NBAL. Li-NBAL employs a reasonable combination of purification technologies such as PE, PP, PBA, HF, CHDF, and PDF depending on the patients' different conditions. The term "reasonable combination" refers to the variety of methods used together in a single NBAL session as well as in NBAL circulation sessions on different days. The reasonable combination of the various methods maximizes the advantages and minimizes the disadvantages of each approach. For example, plasma exchange (PE) was the basic method of our Li-NBAL. We performed PE in combination with PP for HE. We chose the combinations PE + CHDF and PE + PDF in patients with hepatorenal syndrome; in patients with hyperbilirubinaemia, PE with PBA was used (6). Due to the dissimilitude of the selected NABL method, the total amount of fluid exchange, individual exchange efficiency and cost, not only the duration of each session but also the completed times vary for each patient. In this study, excluding the patients who died and those who underwent liver transplantation after the first NABL treatment, the remaining patients received two to three times more in the following seven to ten days. Each session of

PE therapy lasts for four to six hours, and each session of PBA lasts for two to three hours. While for the therapy of PE + CHDF, PE + PP, PDF or HF, it usually takes six to eight hours per session. PE is efficacious for removing endotoxins and albumin-binding substances, while providing clotting factors and other biologically active substances. In contrast, CHDF is superior for removing toxins with relative molecular masses of 5 000 Da to 50 000 Da and for stabilizing the circulation. PE combined with CHDF maximizes the removal of liver failure-related hazardous substances and improves the microenvironment. In the present study, more than 50% of the patients underwent PE-based modular NBAL treatment, which we consider to be the most important reason for the improved survival in the NBAL group.

The etiology analysis revealed that causes varied according to different liver failure subtypes. Bernal et al have reported that in patients with ALF or SALF in the USA and much of Western Europe, the incidence of virally-induced disease has declined substantially in the past few years, with most cases now arising from drug-induced liver injury. In contrast, viral causes predominate in the developing world, with hepatitis E infection recognized as a common cause of liver failure in many countries (26, 27). In this study of Chinese patients, drug toxicity was the major cause of ALF and SALF, which is similar to the West but different from most of the developing world. The only difference was that Chinese herbal medicine was the most common drug-related cause of liver failure in our study, whereas acetaminophen induces most of drug-related liver failure in the USA and much of Western Europe (28, 29). The difference in the causes of ALF and SALF between this study and the study of Bernal et al may be related to the superior economic and local medical environment of our patient population, which are factors known to reduce the transmission risk of hepatitis E and other gastrointestinal diseases. However, approximately 30.61% of the ALF cases and 30.41% of the SALF cases had unclear etiology; these numbers are similar to those reported by other studies (26, 30). ACLF (89.23%) was more prevalent than ALF (5.56%) and SALF (5.22%) because of the high incidence of hepatitis B in China. We found that most of the ACLF cases (91.20%) were caused by chronic hepatitis B alone or hepatitis B combined with another cause. This finding contrasts with findings from Western countries, where hepatitis C is the major cause of ACLF. However, with the wide application of hepatitis B vaccines, the hepatitis B infection rate has decreased among young people in China (31). Thus, hepatitis B-related liver failure is likely to decline within days after a vaccine is administered, whereas the other causes of liver failure, such as alcoholism, autoimmunity, and cholestasis, may increase gradually.

Liver failure has many different definitions that provide equally satisfactory results in the existing guidelines. The AASLD published in 2005 noted that the most widely accepted definition of ALF includes evidence of coagulation abnormality, usually INR ≥1.5, and any degree of mental alteration (encephalopathy) in a patient without pre-existing cirrhosis and with an illness duration of <26 weeks (32). The ALF and SALF definitions in our study satisfy the AASLD guidelines with more stringent requirements in terms of hepatic encephalopathy and jaundice, i.e., encephalopathy ≥ stage II and progressively worsening jaundice within a short period. The 2009 APASL defined ACLF as acute hepatic insult manifesting as jaundice (serum bilirubin ≥5 mg/dl [85 umol/l]) and coagulopathy (INR ≥1.5 or prothrombin activity ≤40%), complicated within four weeks by ascites and/or encephalopathy in a patient with previously diagnosed or undiagnosed chronic liver disease (33). The ACLF definition in our study satisfies the APASL guidelines with more stringent requirements in terms of jaundice (progressively worsening jaundice within a short period, serum total bilirubin ≥10 mg/dl or a daily elevation ≥1 mg/dl). Considering that the unified MELD score was used to evaluate each patient's condition, we predict that this finding will be helpful to evaluate the survival of liver failure patients.

There are potential weaknesses in our approach. First, this is a large case series of NBAL interventions with contemporary controls from a single center. Prospective studies are needed from multiple centers in geographically diverse areas using larger and more heterogeneous groups of patients followed up for longer periods. Second, NBAL actually comprised six different interventions in our study, and it is possible that one of these interventions is better than the others. However, because of the limited sample size, the effectiveness of the different NBAL methods was not compared. The different interventions will be analyzed separately in a future study. Third, one of the reasons cited for lack of widespread use of NBAL is cost. It is noted that most patients who received liver transplants had previously received NBAL; thus, there were more OLT patients in the NBAL group than in the non-NBAL group. This finding may have been influenced by

socio-economic background. Fourth, this was not a randomized study because only the critically ill patients received timely NBAL treatment, which was limited by the availability of plasma and the high cost of the treatment. Fifth, the actual survival of patients with ALF, SALF, ACLF, and the three ACLF subgroups were compared using life table and Kaplan-Meier analyses in this study. As seen in Table V, the effect is mainly obtained within week 4 for ALF and within weeks 4 to 8 for ACLF, while for SALF the effect is present for 48 weeks. It seems that NBAL treatment only delays the initial mortality (at 4-8 weeks). The same survival rates from 4 weeks to 48 weeks in ALF are obtained while the p values are altered. The cause of the above contradiction may be the limited sample size. Moreover, after further dividing into three subgroups for ACLF based on their MELD scores (Tab. VI), the effect is obvious for 48 weeks in the MELD≤20 subgroup ($p<0.05$). Compared to life table analysis, the Kaplan-Meier analysis may reveal the relationship between time and survival more directly. As seen in Figure 3, significant differences in survivals of ALF, SALF, and ACLF patients with and without NBAL were obtained ($p<0.05$). As seen in Figure 4, the survival of ACLF patients in the MELD≤20 and 20<MELD≤30 subgroups who underwent NBAL were distinctly higher than that of those who did not. The survival of ACLF patients in the MELD>30 subgroup were similar in patients with and without NBAL. Both life table analysis and Kaplan-Meier analysis show that NBAL treatment has a powerful effect in improving the survival in the MELD≤20 subgroup, but it is powerless in the MELD >30 subgroup. However, there are conflicting findings in the 20< MELD≤30 subgroup, which may be involve the increase of the basic MELD scores.

We intend to validate and optimize the results in a prospective, multicenter cohort with a larger sample size in subsequent studies, and we plan to compare the effectiveness of different NBAL methods, including Li-NBAL, MARS, CAPS, and Prometheus, for treating liver failure. We propose a subsequent study to build a road map for reasonable NBAL combinations to tailor treatment to the individual patient's condition and disease duration.

In summary, we found that NBAL treatment improved the survival of patients with ALF, SALF, or ACLF. We also compared the efficiency of NBAL therapy in ACLF patients with different MELD scores and concluded that lower MELD scores were associated with improved outcomes relative to higher MELD scores.

ACKNOWLEDGEMENTS

The authors would like to thank Hai-Yan Ma and Hai-Feng Ding from Hangzhou Normal University for their participation in the study.

Financial Support: This work was supported by Chinese National Science and Technology Major Project (2012ZX10002004) and the Science and Technology project of Zhejiang Province, China (2012C37071).

Conflict of Interest: The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interests with respect to this manuscript.

Address for correspondence:
Lanjuan Li
State Key Laboratory of Infectious Diseases
The First Affiliated Hospital
Zhejiang University
Hangzhou, Zhejiang, China
ljli@zju.edu.cn

REFERENCES

1. Liver Failure and Artificial Liver Group, Chinese Society of Infectious Diseases, Chinese Medical Association; Severe Liver Diseases and Artificial Liver Group, Chinese Society of Hepatology, Chinese Medical Association. [Diagnostic and treatment guidelines for liver failure] [Article in Chinese]. Zhonghua Gan Zang Bing Za Zhi. 2006;14(9):643-646.
2. Ostapowicz G, Fontana RJ, Schiødt FV, et al; U.S. Acute Liver Failure Study Group. Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. Ann Intern Med. 2002;137(12):947-954.
3. Schiodt FV, Atillasoy E, Shakil AO, et al. Etiology and outcome for 295 patients with acute liver failure in the United States. Liver Transpl Surg. 1999;5(1):29-34.
4. Lee WM. Acute liver failure in the United States. Semin Liver Dis. 2003;23(3):217-226.
5. Katoonizadeh A, Laleman W, Verslype C, et al. Early features of acute-on-chronic alcoholic liver failure: a prospective cohort study. Gut. 2010;59(11):1561-1569.
6. Li LJ, Wang ZH, Ye WJ. Artificial liver. Hangzou: ZheJiang University Press; 2012.
7. Ling Q, Xu X, Wei Q, et al. Downgrading MELD improves the outcomes after liver transplantation in patients with

acute-on-chronic hepatitis B liver failure. PLoS One. 2012;7(1):e30322.
8. Du WB, Li LJ, Huang JR, et al. Effects of artificial liver support system on patients with acute or chronic liver failure. Transplant Proc. 2005;37(10):4359-4364.
9. Artificial Liver Group Chinese Association of Infectious and Parasitic Diseases. [Operating guide for artificial liver support system] [Article in Chinese]. Zhonghua Gan Zang Bing Za Zhi. 2002;10(5):329-332.
10. van den Besselaar AM. Precision and accuracy of the international normalized ratio in oral anticoagulant control. Haemostasis. 1996;26(Suppl 4):248-265.
11. Ferenci P, Lockwood A, Mullen K, Tarter R, Weissenborn K, Blei AT. Hepatic encephalopathy—definition, nomenclature, diagnosis, and quantification: final report of the working party at the 11th World Congresses of Gastroenterology, Vienna, 1998. Hepatology. 2002;35(3):716-721.
12. Conn HO, Leevy CM, Vlahcevic ZR, et al. Comparison of lactulose and neomycin in the treatment of chronic portal-systemic encephalopathy. A double blind controlled trial. Gastroenterology. 1977;72(4 Pt 1):573-583.
13. Cox DR. Regression models and life-tables. J R Stat Soc Series B Stat Methodol 1972;34:187-220.
14. Shakil AO, Kramer D, Mazariegos GV, Fung JJ, Rakela J. Acute liver failure: clinical features, outcome analysis, and applicability of prognostic criteria. Liver Transpl. 2000;6(2): 163-169.
15. Mas A, Rodés J. Fulminant hepatic failure. Lancet. 1997;349(9058):1081-1085.
16. Chen YS, Wu ZW, He JQ, et al. The curative effect of ALSS on 1-month mortality in AoCLF patients after 72 to 120 hours. Int J Artif Organs. 2007;30(10):906-914.
17. Yoshiba M, Inoue K, Sekiyama K, Koh I. Favorable effect of new artificial liver support on survival of patients with fulminant hepatic failure. Artif Organs. 1996;20(11):1169-1172.
18. Stutchfield BM, Simpson K, Wigmore SJ. Systematic review and meta-analysis of survival following extracorporeal liver support. Br J Surg. 2011;98(5):623-631.
19. Mao W, Ye B, Lin S, Fu Y, Chen Y, Chen Y. Prediction value of model for end-stage liver disease scoring system on prognosis in the acute on chronic liver failure patients with plasma exchange treatment. ASAIO J. 2010;56(5):475-478.
20. Yu JW, Sun LJ, Zhao YH, Li SC. Prediction value of model for end-stage liver disease scoring system on prognosis in patients with acute-on-chronic hepatitis B liver failure after plasma exchange and lamivudine treatment. J Gastroenterol Hepatol. 2008;23(8 Pt 1):1242-1249.
21. Qian Y, Lanjuan L, Jianrong H, et al. Study of severe hepatitis treated with a hybrid artificial liver support system. Int J Artif Organs. 2003;26(6):507-513.
22. Li LJ, Yang Q, Huang JR, Xu XW, Chen YM, Fu SZ. Effect of artificial liver support system on patients with severe viral hepatitis: a study of four hundred cases. World J Gastroenterol. 2004;10(20):2984-2988.
23. Kjaergard LL, Liu J, Als-Nielsen B, Gluud C. Artificial and bioartificial support systems for acute and acute-on-chronic liver failure: a systematic review. JAMA. 2003;289(2): 217-222.
24. Vaid A, Chweich H, Balk EM, Jaber BL. Molecular adsorbent recirculating system as artificial support therapy for liver failure: a meta-analysis. ASAIO J. 2012;58(1):51-59.
25. Kribben A, Gerken G, Haag S, et al; HELIOS Study Group. Effects of fractionated plasma separation and adsorption on survival in patients with acute-on-chronic liver failure. Gastroenterology. 2012;142(4):782-789,e3.
26. Bernal W, Auzinger G, Dhawan A, Wendon J. Acute liver failure. Lancet. 2010;376(9736):190-201.
27. Reuben A, Koch DG, Lee WM; Acute Liver Failure Study Group. Drug-induced acute liver failure: results of a U.S. multicenter, prospective study. Hepatology. 2010;52(6):2065-2076.
28. Larson AM, Polson J, Fontana RJ, et al. Acetaminophen-induced acute liver failure: Results of a United States multicenter, prospective study. Hepatology. 2005;42:1364-1372.
29. Lee WM. Acetaminophen and the U.S. Acute Liver Failure Study Group: lowering the risks of hepatic failure. Hepatology. 2004;40(1):6-9.
30. Lee WM, Squires RH Jr, Nyberg SL, Doo E, Hoofnagle JH. Acute liver failure: Summary of a workshop. Hepatology. 2008;47(4):1401-1415.
31. Yang SG, Wang B, Chen P, et al. Effectiveness of HBV vaccination in infants and prediction of HBV prevalence trend under new vaccination plan: findings of a large-scale investigation. PLoS One. 2012;7(10):e47808.
32. Polson J, Lee WM; American Association for the Study of Liver Disease. AASLD position paper: the management of acute liver failure. Hepatology. 2005;41(5):1179-1197.
33. Sarin SK, Kumar A, Almeida JA, et al. Acute-on-chronic liver failure: consensus recommendations of the Asian Pacific Association for the study of the liver (APASL). Hepatol Int. 2009;3(1):269-282.