• *BRIEF REPORTS* •

Hybrid artificial liver support system for treatment of severe liver failure

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Abstract

AIM: To construct a novel hybrid artificial liver support system (HALSS) and to evaluate its efficacy in patients with severe liver failure.

METHODS: Hepatocytes were isolated from suckling pig by the modified Seglen's method. Isolated hepatocytes were cultured in a spinner flask for 24 h to form spheroids before use and the functions of spheroids were detected. HALSS consisted of a plasma separator, a hemo-adsorba and a bioreactor with hepatocytes spheroids in its extra-fiber space. HALSS was applied to 10 patients with severe liver failure. The general condition and the biochemical indexes of the patients were studied just before and after the treatment.

RESULTS: The number of cells per liver was about 2-4×10^{10} (mean, $3.1\pm1.5\times10^{10}$). The cell viabilities were more than 95%. After 24 h of spheroid culture, most hepatocytes formed spheroids. The levels of albumin and urea in the medium of spheroid culture were higher than those in supernatant of petri dish culture ($P = 0.0015$ and 0.0001, respectively). The capacity of albumin production and urea synthesis remained stable for more than one wk and declined rapidly after two weeks *in vitro*. In HALSS group, the duration of HALSS treatment was 6-10 h each time. All patients tolerated the treatment well without any fatal adverse reaction. After HALSS treatment, the general condition, psychic state, encephalopathy and hepatic function of the patients were improved. The survival rate of the HALSS group, Plasmapheresis group and control group was 30% (3/10), 20% (2/10) and 0% (0/10), respectively ($P = 0.024$). Two weeks after treatment, Tbil and ALT decreased and the PTA level elevated in HALSS group and pasmapheresis group (P value: 0.015 *vs* 0.020, 0.009 *vs* 0.012 and 0.032 *vs* 0.041, respectively). But there was no significant change of blood albumin concentration before and after treatment in HALSS group and Plasmapheresis group.

CONCLUSION: The HALSS established by us is effective in supporting liver function of patients with severe liver failure.

INTRODUCTION

Liver is an important organ with complex functions, including gluconeogenesis, synthesis of blood proteins, amino acid metabolism, urea synthesis, lipid metabolism, drug detoxification, waste removal, and immune and hormonal modulation[1]. Many people suffer from liver diseases, especially severe hepatitis, in China, and most of the cases give rise to widespread hepatic necrosis with little hepatocyte regeneration. Currently, the only available treatment is liver transplantation. However, liver transplantation faces acute shortages of donors worldwide and the patients treated with liver transplantation are subjected to the lifetime risks of graft rejection and immunosuppression[2,3]. The liver has a remarkable capacity for regeneration. But a minimum critical mass of hepatocyte is required to support homeostasis while regeneration progresses after liver damage. Without this critical mass, liver failure supervenes and regeneration is impaired[4]. Artificial liver support systems, such as plasma exchange, hemodialysis and hemofiltration, have been proposed as a temporary liver support that allows the native liver to regenerate[5-7]. But these treatment do not perform many complex functions for survival. Hybrid artificial liver support system (HALSS) is an extracorporeal circulation system comprising of a bioreactor with highly differentiated hepatocytes in hollow fiber space[8]. The large number of highly differentiated hepatocytes is the key to construct HALSS. It has become increasingly evident that three-dimensional rather than monolayer growth is particulary important for maintaining differentiated hepatocyte function in culture. One means of establishing three-dimensional hepatocyte growth is the creation of multicellular spheroid aggregates[8-12]. Here we developed a HALSS that contained a hollow fiber bioreactor inoculated with porcine hepatocyte spheroids and applied it to 10 patients with severe liver

failure. The efficacy of the HALSS for treatment of severe liver failure was evaluated.

MATERIALS AND METHODS

Animals
Chinese suckling pigs weighing about 4.0 kg were supplied by Laboratory Animal Center of Chinese Academy of Sciences.

Isolation and spheroid culture of hepatocytes
Hepatocytes were isolated from suckling pigs by modified two-step *in situ* collagenase perfusion method described by Seglen[13]. The liver was perfused at 4 ℃ with Hanks solution containing 40 mg/L dexamethasone (Sigma-Aldrich, the USA) through the portal vein to rinse whole blood. At the end of the first perfusion, the liver was excised gently from the surrounding tissue and the isolated liver was placed on the stage of perfusion apparatus. Then the liver was circularly perfused with Hanks solution containing 0.5 g/L collagenase IV (Sigma-Aldrich, America) at 37 ℃, which was equilibrated with 950 mL/L O_2 and 50 mL/L CO_2. The softened liver was gently lifted and placed in ice-cold minimum essential medium (MEM) (GIBCO™, Invitrogen). After dissociation of the liver capsule by scissor, the liver was shaken gently in the medium until it was reduced to only connective tissue. The resulting cell suspension was filtered through 50 μm sterile stainless steel meshes. The hepatocytes were washed three times at 500 r/min for 1 min each.

The hepatocyte suspension was adjusted to 5×10^6/mL in warmed dulbecco's modified eagle medium (D-MEM) culture medium supplemented with 0.1 μmol/L dexamethasone, 10 μg/L hepatocyte growth factor (HGF), 20 μg/L epidermal growth factor (EGF), 1×insulin-transferrin-selenium solution (GIBCO™, Invitrogen), 10 mg/L linoleic acid, 200 mmoL glutamine, 50 mL/L fetal bovine serum, 0.1 μg/L $CuSO_4.5H_2O$, 50 pmoL $ZnSO_4.7H_2O$, 15 mmoL HEPES, 125 mg/L penicillin and 100 mg/L streptomycin. The cells suspension was then placed in a 50 mL/L CO_2 and 950 mL/L air humidified incubator at 37 ℃ and immediately stirred with a magnetic stirrer at 120 r/min. Air from the incubator was blown into the flask from the open side of the spinner flask by an air pump. The medium was changed every 24 h. Albumin secretion and urea synthesis were measured in the medium of the spheroid culture according to the method described by Chen *et al*[14] with the petri dish culture at the same density as control.

Configuration of bioartificial liver
The HALSS was constructed as shown in Figure 1. The formed spheroid hepatocytes containing $1\text{-}2 \times 10^{10}$ highly differentiated primary porcine hepatocytes in DMEM medium were inoculated into the outer space of the hollow fibers of the bioreactor (Languna Hills). A hepatocytes reservoir was designed to connect to the extra-fiber compartment of the bioreactor just as described by Ding *et al*[15,16]. Blood was removed from the patient through a double lumen catheter in superficial femoral vein at a rate of 100 mL/min and run through a plasma separator. The

separated plasma passed a hemo-adsorba 160 for removing bilirubin and toxin, and then it was run through the intra-fiber compartment of bioreactor. The reacted plasma was then reconstituted with red blood cells and returned to the patient via the venous cannula.
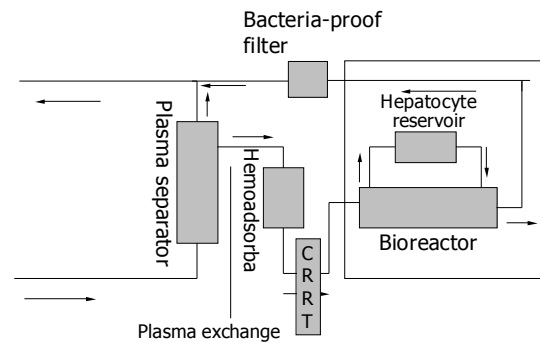

Figure 1 Schematic diagram of HALSS.

Clinical treatment
All patients were at the end stage of severe liver failure according to the diagnostic standard defined in Xi 'an meeting in China in 2000. Ten patients (including 7 males and 3 females, and 8 patients were infected with hepatitis B and 2 patients were infected with hepatitis E) with severe liver failure were assigned to HALSS group. Ten patients (2 females, 8 males) were assigned to Plasmapheresis group. And another ten patients were assigned to control group. There was no significant difference in symptoms and biochemical index between the two groups (Table 1).

Table 1 Clinical feature of patients

	HALSS group (n = 10)	Plasmapheresis group (n = 10)	Control group (n = 10)
Sex			
Male	3	2	3
Female	7	8	7
Average age (yr)	39.5	41.5	40.5
Range (yr)	35-56	27-60	30-58
Encephalopathy	4	2	1
Ascites	8	6	7
Hepatorenal syndrome	9	7	7
Biochemical index			
Tbil (μmol/L)	499.5±25.1	519.4±137.5	496.3±93.2
Dbil (μmol/L)	227.5±19.0	269.6±118.4	286.5±107.3
ALT (U/L)	347.6±126.5	303.2±139.4	336.2±139.6
ALB (g/L)	29.8±0.4	29.9±5.3	30.9±5.3
PTA	17.5±1.2	22.8±6.5	21.5±4.4

Tbil: Total bilirubin, Dbil: Direct bilirubin, ALT: Alanine aminotransferase, PTA: Prothrombin activity.

Biochemical analysis
Blood samples were obtained at the start and end of the treatment. Blood alanine aminotransferase (ALT), total bilirubin (TBil) and albumin (ALB) were determined with

an automatic biochemical analyzer. Prothrombin activity (PTA) was detected according to manufacturer's instructions.

Statistical analysis

Results were expressed as mean±SD. Paired t test was used (SPSS10.0 software) to compare the data. $P<0.05$ was considered statistically significant.

RESULTS

Cell morphology and viability

The number of cells per liver was about $2-4\times10^{10}$ (mean, $3.1\pm1.5\times10^{10}$). The cell viabilities were more than 95% (Figure 2). After 24 h of spheroid culture, most hepatocytes formed spheroids (Figure 2).

Metabolites secretion in culture

The functions about albumin production and urea synthesis are shown in Figure 3. The levels of albumin and urea in the medium of spheroid culture were higher than those in supernatant of petri dish culture ($P = 0.0015$, 0.0001, respectively). The capacity of albumin production and urea synthesis was constant for more than one week and declined rapidly after two weeks.

Clinical effect of HALSS

In HALSS group, the duration of HALSS treatment was 6-10 h each time. All patients tolerated the treatment well without any fatal adverse reaction. After HALSS treatment, general condition, psychic state, encephalopathy and hepatic function of the patients were improved (Table 2). The

survival rate of the HALSS group, Plasmapheresis group and control group was 30% (3/10) , 20% (2/10) and 0% (0/10), respectively ($P = 0.024$). Two weeks after treatment, TBil and ALT decreased and the level of PTA elevated in HALSS group and Plasmapheresis group (P value is 0.015 vs 0.020, 0.009 vs 0.012 and 0.032 vs 0.041, respectively). But there was no significant change of blood albumin concentration before and after the HALSS treatment (Figure 4).

**Table 2 General condition of patients at two weeks after treatment**

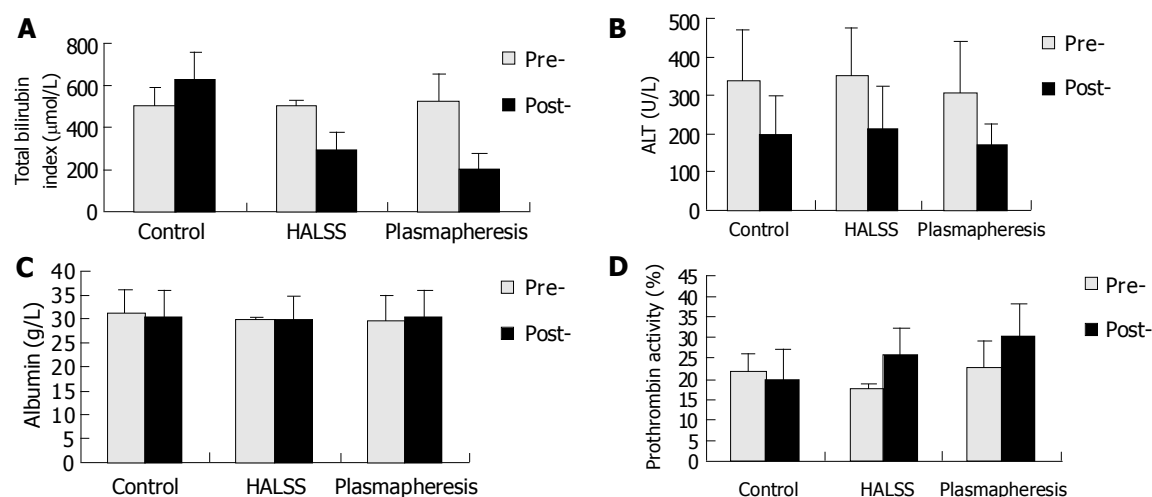|	HALSS group ($n=10$)		Plasmapheresis group ($n=10$)		Control group ($n=10$)	
	Pre-	Post-	Pre-	Post-	Pre-	Post-
Encephalopathy	4	2	2	2	1	2
Ascites	8	3	6	3	7	9
Hepatorenal syndrome	9	3	7	3	7	9

DISCUSSION

Acute or acute-on-chronic liver failure is a life-threatening condition. Most of the cases are caused by viral hepatitis, such as hepatitis B and C, in China. The mortality of liver failure is very high, more than 70%[17]. Liver transplantation is an effective therapy for many severe hepatic failure, but the number of patients who can benefit from this procedure is limited by the availability of donor organs[17,18]. Alternative therapy including liver cell
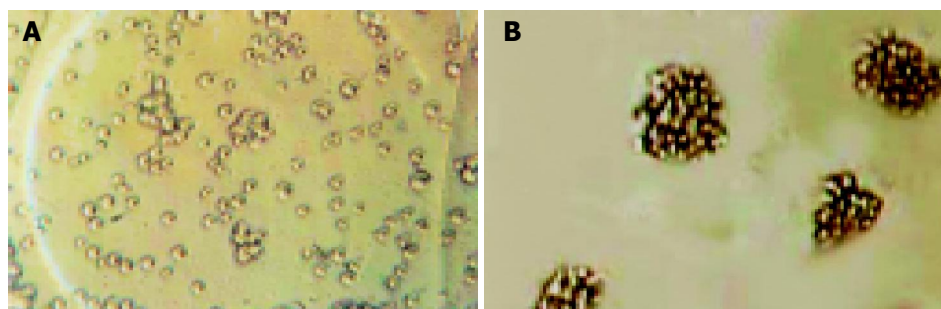
Figure 2 Morphology of hepatocytes. A: freshly isolated single hepatocytes stained with trypan blue (×200); B: spheroid formation after 24 h of spheroid culture (×100).
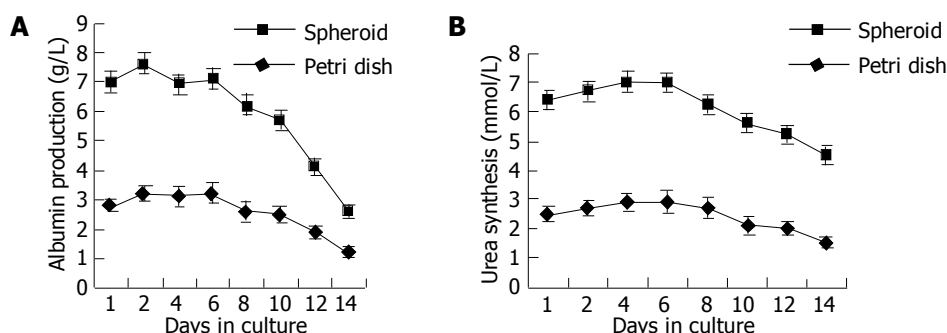
Figure 3 Spheroid cultivation of hepatocytes at a density of 5×10^6/mL cells. Control hepatocytes were cultivated in a petri dish at the same density. A: albumin production; B: urea synthesis.

Figure 4 Changes of total bilirubin, ALT, albumin and PTA index in patients in HALSS group, Plasmapheresis group and control group. Pre-: index before treatment. Post-: index at two weeks after treatment.

transplantation and artificial livers has been developed. The non-bioartificial livers consisted of haemofiltration, hemoperfusion, plasma exchange and molecular adsorbents recycling system[6,19-21]. These devices could remove toxic factors, including ammonia, endotoxin, mercaptans and endogenous inhibitor neurotansmitters. But some factors, which are involved in hepatic regeneration, such as interleukin-6, tumor necrosis factor-α, hepatocyte growth factor and albumin-bilirubin complex might also be removed[22]. And they cannot replace the complex functions of liver such as synthetic and metabolic functions. On this condition, bioartificial liver has been developed to support liver functions temporarily[4,15,16,23,24].

The large numbers of highly differentiated primary hepatocytes are demanded for configuration of bioartificial liver. Primary human hepatocytes divide much less readily *in vitro* even under optimal culture conditions[25]. Cell lines and stem cells are still under study, and they cannot be used for clinical treatment currently[11,26]. Primary porcine hepatocytes are the most common cellular components of current bioartificial liver devices[27]. Hepatocytes in monolayer culture lose their functions quickly *in vitro* and the density of heaptocytes in monolayer cannot satisfy the needs for configuration of bioreactor. Hepatocytes multicellular aggregates that have a tissue-like structure expressed highly differentiated functions[24,28]. Spheroidal aggregates of hepatocytes are known to exhibit better hepatocyte functions for a longer timer than hepatocytes produced by monolayer culture[10,29]. Our data showed that functions of albumin production and urea synthesis of spheroidal hepatocytes were better than that of monolayter cultured hepatocytes (*P*<0.05). The functions of spheroidal hepatocytes were stable more than one week in the medium supplemented with growth factors and hormones, but they declined quickly after two weeks *in vitro*. The density of spheroid culture was enough to meet the needs of large numbers of hepatocytes for configuration of bioartificial liver.

Blood from the patients with severe liver failure has many toxins in it, which are harmful to living hepatocytes[30]. In HALSS, the blood from the patients was detoxified with a hemo-adsorba 160 and continuous renal replacement therapy (CRRT) before it was run through a bioreactor. To ensure that enough hepatocytes were used, a hepatocyte reservoir was added to the system.

Data from clinical study showed that HALSS improved general condition, encephalopathy and biochemical indexes, including ALT, TBil and PTA of patients. But the albumin level was not changed, which was in agreement with the previous study by Ding *et al*[16]. The survival rate of HALSS treatment group was also higher than that of the Plasmapheresis group and control group (30% *vs* 0%). It suggest that the HALSS significantly prolong the survival time of the patients.

On the basis of the above mentioned results, the HALSS established by us is effective for the treatment of severe liver failure.

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