

## Full Length Research Paper

# Evaluation of a reversibly immortalized human hepatocyte line in bioartificial liver in pigs

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An appropriate cell source is essential for the clinical application of bioartificial liver (BAL) system. This study aimed to test a reversibly immortalized human hepatocyte line (HepLi-4) in our newly validated choanoid fluidized bed bioreactor based BAL in pigs with fulminant hepatic failure (FHF). 15 FHF pigs were allocated to three groups: a BAL group receiving BAL treatment with HepLi-4 cells; a sham BAL group receiving cell-free BAL treatment; and a FHF group receiving intensive care only. Expression of liver-specific genes in HepLi-4 cells before and after BAL was analyzed, adult human hepatocytes acted as a reference. In BAL group, Fischer index was higher and serum indirect bilirubin level was lower compared with two control groups. Survival time in BAL group was longer than that in two control groups, but the difference was not statistically significant. Gene expression analysis showed that the transcript levels of liver-specific genes in HepLi-4 were retained after BAL, but significant variations were observed between HepLi-4 and human hepatocytes. HepLi-4 showed beneficial metabolic effects on FHF pigs in BAL, but is still not an appropriate cell source for BAL. More insights into interpreting the conditions for hepatocyte differentiation are needed.

**Key words:** Reversible immortalization, hepatocytes, bioartificial liver, fulminant hepatic failure.

## INTRODUCTION

Facing the world-wide donor organ shortage, bioartificial liver (BAL) system has been anticipated to be a bridge to

liver transplantation or to provide a chance for the native liver to recover in case of liver failure (Strain and Neuberger, 2002; Carpentier et al., 2009). Bioreactor and cell source are two main issues of BAL (Pless, 2010). Fluidized bed bioreactor is promising for liver support (Dore and Legallais, 1999; Coward et al., 2009; Desille et al., 2001; David et al., 2004). We recently designed a BAL system based on a choanoid fluidized bed bioreactor filled with alginate-chitosan (AC) encapsulated primary porcine hepatocytes and proved it could prolong the survival of pigs with fulminant hepatic failure (FHF) induced by D-galactosamine injection (Lv et al., 2011). The potential risk of zoonotic transmission, however, might exist, which limits its clinical application (Patience et al., 1997). Researchers in our group also proved that porcine endogenous retroviruses (PERV) can pass

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**Abbreviations:** BAL, Bioartificial liver; FHF, fulminant hepatic failure; AC, alginate-chitosan; PERV, porcine endogenous retroviruses; SV40 LT, simian virus 40 large T antigen; BCAAs, branched chain amino acids; AAAs, aromatic amino acids; GS, glutamine synthetase; UGT1A1, uridine diphosphate glucuronyltransferase; ALB, albumin; GST-P, glutathione S-transferase P;  $\beta$ -actin, human beta-actin; HE, hepatic encephalopathy; RT-PCR, reverse transcription-polymerase chain reaction.

through the immune barrier in AC microbeads (Yang et al., 2010). Therefore, developing a safe cell source for BAL is necessary.

Widespread clinical use of primary human hepatocytes in BAL is almost impossible because healthy donor livers are so scarce that only organ or tissue discarded from transplantation are available. Among non-primary cell sources, several human liver tumor derived cell lines, including GS-HepG2 (glutamine synthetase, GS), HepG2-GS-3A4 and FLC-4, were used in BAL in large animal models. Prolongations of survival were achieved in these studies (Kanai et al., 2007; Enosawa et al., 2001, 2006; Wang et al., 2005). However, none of them has been so far applied to clinical trials. Poor differentiation and the potential risk of tumor transmigration might be the main hurdles (Nyberg et al., 1994). Only the C3A cell line, a subclone of HepG2, was used in a pilot-controlled clinical trial (Ellis et al., 1996). Unfortunately, no improvement in either survival or biochemical parameters is demonstrated.

For several years, we have been dedicated to establishing immortalized hepatocyte line (Pan et al., 2010; Li et al., 2005). Although these cell types have unlimited expansion capabilities *in vitro*, continuous expression of simian virus 40 large T antigen (SV40 LT) might be tumorigenic (Kobayashi et al., 2001; Hahn et al., 1999; Woodworth et al., 1988). To solve this problem, researchers in Japan established a reversibly immortalized human hepatocyte line (NKNT-3) in the year 2000 (Kobayashi et al., 2000). It shows that the immortalizing oncogene, e.g., SV40 LT, can be excised by a Cre/LoxP site-specific recombination. Then, liver-specific genes expression can be increased later. However, obtaining a large number of reverted hepatocytes was hindered by the secondary gene transfer until the drug-mediated Cre/LoxP recombination was applied (Totsugawa et al., 2007). With the drug-medicated Cre/LoxP recombination, we established another reversibly immortalized cell line (HepLi-4) by transfection of primary human hepatocytes with the retrovirus containing SV40 LT flanked by a pair of LoxP recombination targets. Thus, enough number of reversibly immortalized hepatocytes can be achieved to equip our newly validated choanoid fluidized bed bio-reactor based BAL system. In order to demonstrate the clinical potential of our hepatocytes in BAL, we carried out experiments in large animal models.

## MATERIALS AND METHODS

This study protocol was ratified by the Animal Ethics Committee of Zhejiang University and the Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine.

### Large-scale cultivation and encapsulation of HepLi-4 cells

The HepLi-4 cell line was developed by immortalization of primary human hepatocytes with the retrovirus containing SV40LT flanked

by a pair of LoxP recombination targets, and the HepLi-4 cells were subsequently transfected with the recombinant vector containing Cre-ERT2 fusion protein gene derived from pCAG-CreERT2 plasmid (kindly gifted by Addgene, USA). Reverted HepLi-4 cells were capable of expressing liver-specific genes *in vitro* and prolonging the survival of common bile duct ligated mice after intrasplenic transplantation (Pan et al., 2009). There was also no evidence for tumorigenesis of reverted HepLi-4 cells in nude mice within three months after subcutaneous transplantation.

The cultivation and reversion procedures were previously described in detail (Totsugawa et al., 2007). Briefly, HepLi-4 cells at the 30th passage (Figure 1A) were expanded in roller bottles (Bellco, USA) with Dulbecco Modified Eagle Medium (DMEM; Gibco, USA) supplemented with 10% (v/v) fetal calf serum (Gibco, USA) using a cell production roller bottle apparatus (Bellco, USA) (Figure 1B). After achieving the required number of cells, SV40 LT genes were removed by keeping the cells in culture media containing 500 nM 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich, USA) for five to seven days. To verify the efficiency of excision of the SV40 LT gene, the mRNA expression of SV40 LT in immortalized and reverted HepLi-4 cells was analyzed by reverse transcription-polymerase chain reaction (RT-PCR). The primer sequence and amplification condition were described in our previous study (Pan et al., 2010). As shown in Figure 1C, SV40 LT gene was almost not detected in reverted HepLi-4 cells.

Thereafter, reverted HepLi-4 cells were trypsinized. The cell viability assessed by trypan blue exclusion was above 95%. We still adopted the single-stage AC encapsulation procedure which had previously been reported by us (Lv et al., 2011). Briefly, about  $3.0 \times 10^9$  hepatocytes were resuspended in 300 ml 1.7% sodium alginate (Sigma-Aldrich, USA) solution. The mixture was extruded through an electrostatic microencapsulator (300  $\mu$ m nozzle; Nisco, Switzerland) into a 0.7% chitosan solution (Jinan Haidebei Marine Bioengineering Co. Ltd, China). The gelation process lasted for 30 min, of which microbeads of 600 to 1000  $\mu$ m in diameter were obtained (Figure 1D).

### Experimental animals

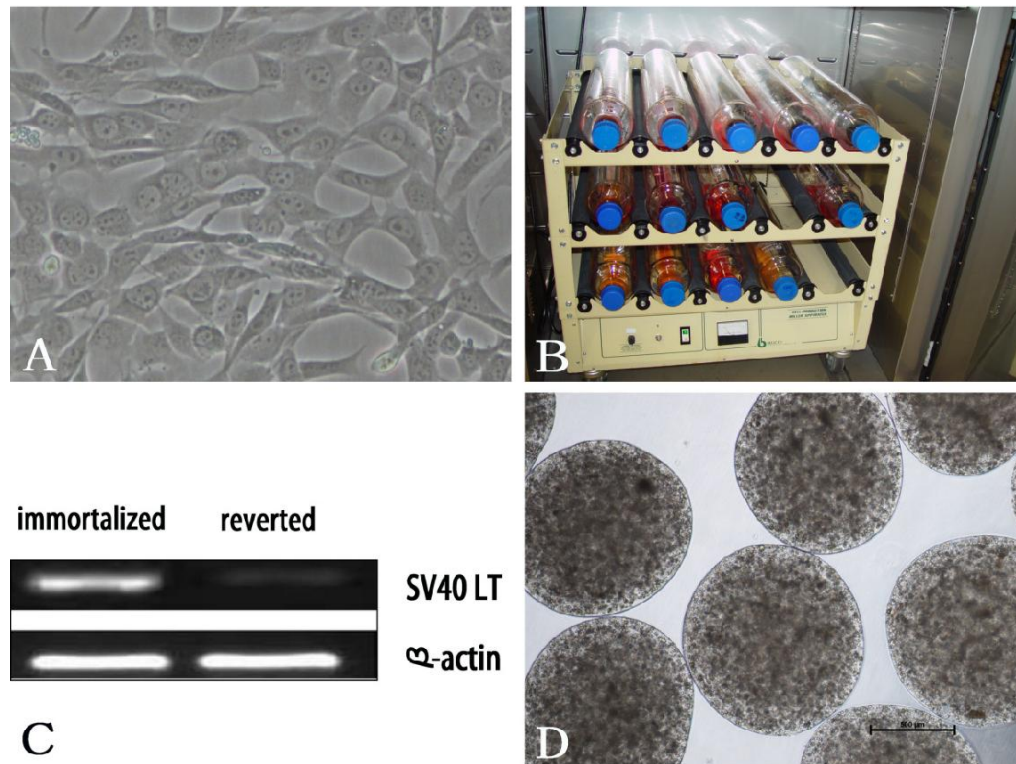
Male Chinese experimental miniature pigs weighting 10 to 15kg were purchased from China Agriculture University. All pigs were kept in separate cages under standard conditions and fed standard laboratory chow.

### Induction of FHF

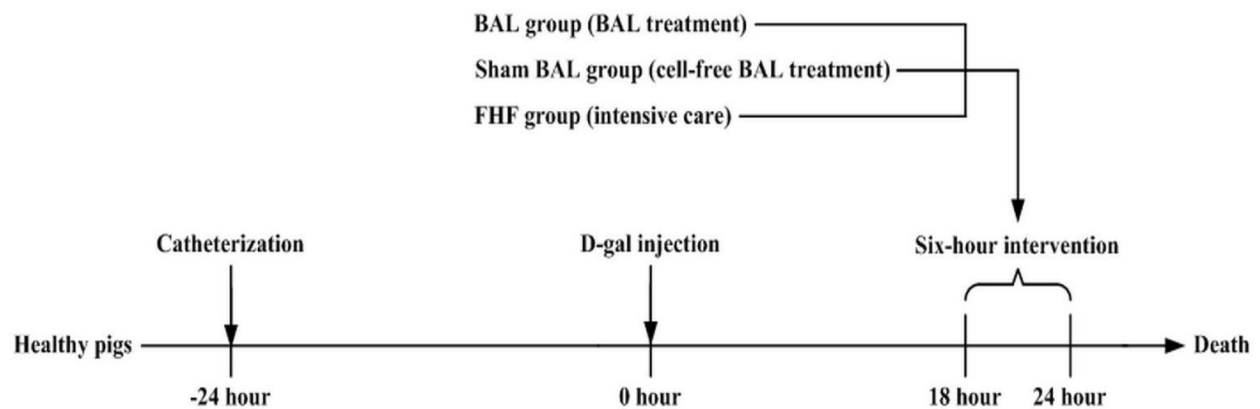
D-galactosamine (Shanghai Hanhong Chemical Co., Ltd, China) induced pig model of FHF was used in this experiment. The experiment work flow is shown in Figure 2. Catheterization, which had been previously described (Li et al., 2006), was carried out under general anesthesia achieved by continuous intravenous injection of Diprivan (AstraZeneca, Italy) at a rate of 2.5 mg/kg/h. Then, pigs awakened from anesthesia were sent back to their cages. Twenty-four hours after the catheterization, without anesthesia, D-galactosamine was delivered to pigs via the venous catheter at a dose of 1.5 g/kg body weight.

### Experimental groups

As shown in Figure 2, 15 FHF pigs were allocated to three groups: a BAL group (n = 5), receiving BAL treatment with HepLi-4 cells; a sham BAL group (device control, n = 5), receiving cell-free BAL treatment; and a FHF group (baseline control, n = 5), only receiving intensive care under general anesthesia. These three types of interventions were initiated 18 h after D-gal injection and lasted for



**Figure 1.** Large-scale cultivation and encapsulation of HepLi-4 cells. (A) Morphology of HepLi-4 cells under optical microscopy. (B) Large-scale cultivation of HepLi-4 cells in roller bottles. (C) The mRNA expression of SV40 LT in HepLi-4 cells before and after reversion. SV40 LT mRNA was detected in immortalized HepLi-4 cells, whereas almost not detected in the reverted cells by RT-PCR analysis. (D) Optical micrograph of alginate-chitosan (AC) microbeads containing HepLi-4 cells. Cell density was approximately  $1 \times 10^7$ /ml. Scale bar = 500  $\mu$ M.



**Figure 2.** Experimental flow chart.

6 h according to previous studies (Lv et al., 2011; Li et al., 2006).

### Bioreactor

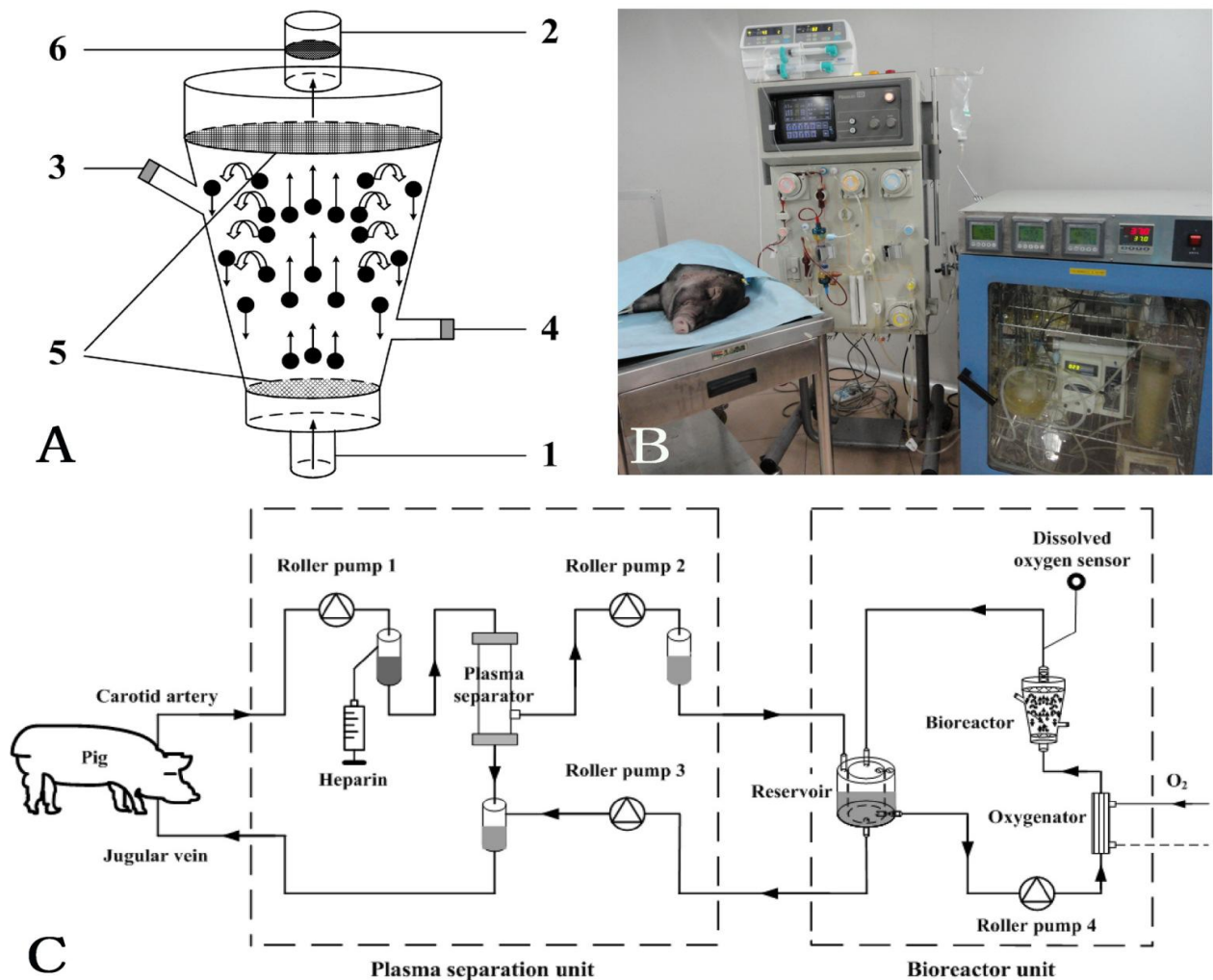
The bioreactor (Figure 3A; Chinese Patent no: ZL 200710070279.0) has a funnel-shaped structure with filters of 200 and 600 mesh/inch fixed to the bottom and top, respectively. To stop potential cell chips

from entering the body, a 3  $\mu$ M strainer was installed in the outlet. The volume inside the bioreactor was about 500 ml. Other parameters were detailed in our previous report (Lv et al., 2011).

### BAL treatment

The BAL system (Figures 3B and C) which had previously been





**Figure 3.** Bioreactor and BAL treatment. (A) Schematic diagram of the choanoid fluidized bed bioreactor loaded with microbeads. 1, Plasma inlet; 2, plasma outlet; 3, microbeads inlet; 4, microbeads outlet; 5, filters; 6, strainer (pore size: 3  $\mu$ m). The solid arrows and the hollow arrows show the direction of plasma flow and the trajectory of microbeads in the bioreactor, respectively. (B) Photograph of the BAL treatment. (C) Structural diagram of the BAL system. BAL, Bioartificial liver.

reported by us consisted of a plasma separation unit and a bioreactor unit (Lv et al., 2011). Both were driven by a series of roller pumps. Under general anesthesia, arterial blood was drawn from pig's body at 20 to 25 ml/min and pumped into the plasma separator (OP-02W, Asahi-Kasei, Japan) through which plasma was separated from the cellular components at 8 to 10 ml/min into the reservoir. In the bioreactor unit, plasma was perfused through the bioreactor containing  $3.0 \times 10^9$  encapsulated HepLi-4 cells at 25 to 35 ml/min. At the same time, the purified plasma was returned to the pig's body at a rate equal to plasma separation. To prevent blood clotting, the first dose of heparin (100 U/kg) was administered intravenously and followed by sustained injection of heparin into the extracorporeal circulation at a rate of 40 U/kg per hour. In sham BAL treatment, bioreactor contained cell-free microbeads with the same volume.

#### Test of microbead integrity and cell viability

The percentage of intact beads before and after BAL treatment was determined under light microscope. 100 beads in five samples were

observed respectively. Trypan blue exclusion test was used to assess the viability of encapsulated hepatocytes before and after BAL treatment. Microbeads were lysed by a solution composed of 0.2 mol/L  $\text{NaHCO}_3$  and 0.06 mol/L  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$  (pH = 7.8) (Xue et al., 2004), then we used 0.4% trypan blue solution to dye dead cells according to the method described previously (David et al., 2004). Also, comparison between the viability of encapsulated HepLi-4 cells before and after BAL was performed by a previously reported MTT method with some modifications (Haque et al., 2005). In brief, 15 microbeads were incubated in a 96-well plate with 100  $\mu$ L media and 25  $\mu$ L MTT per well for 24 h. Thereafter, the supernatants were substituted for 100  $\mu$ L dimethyl sulfoxide (DMSO). Within 30 min, the absorbance of light at 595 nm was measured using a DTX 800 multi-mode detector (Beckman Coulter, USA). We repeated these experiments three times.

#### Characterization of liver-specific genes

Microbeads containing HepLi-4 cells before and after BAL treatment were lysed by the solution mentioned above. Total RNA was

**Table 1.** Microbead integrity and cell viability before and after BAL treatment.

Paramater	Pre-BAL	Post-BAL	P value
Integrity of microbeads (percentage of intact beads, %, n = 5)	99.2 ± 0.84	98.4 ± 1.14	0.24
Viability of encapsulated HepLi-4 cells (trypan blue exclusion, percentage of live cells, %, n = 5)	92.2 ± 1.79	90.8 ± 1.92	0.27
Viability of encapsulated HepLi-4 cells (MTT test, OD Value, n = 5)	0.73 ± 0.08	0.69 ± 0.08	0.45

Values are expressed as mean ± standard deviation. Difference between groups was analyzed using Student's t-test. The experiments were repeated three times.

extracted from HepLi-4 cells using Trizol reagent (Invitrogen, USA). To provide a reference, adult human liver samples obtained during the course of graft reduction for liver transplantation were included in the analysis. RT-PCR containing 1 µg total RNA was carried out using two-step RT-PCR kit (QIAGEN, USA) according to manufacturer's protocols. Primer sequences for amplification of liver-specific genes and internal control gene, including GS (535 bp), uridine diphosphate glucuronyltransferase (UGT1A 1,495 bp), albumin (ALB, 576 bp), glutathione S-transferase P (GST-P, 496 bp) and human beta-actin (β-actin, 610 bp), were detailed in previous report (Totsugawa et al., 2007). The thermal cycle involved one cycle of initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 40 s. Amplification was concluded with a one cycle of extension program at 72°C for 5 min. The amplification products were run on 1% agarose gels stained with ethidium bromide.

#### Measurement of parameters

The three blood sampling time points were the time just prior to the D-gal injection, before and after the 6-h intervention, respectively (0, 18 and 24 h after D-gal injection). Biochemical parameters, including serum bilirubin, albumin, aminotransferases, total cholesterol, lactate, glucose, creatinine, urea nitrogen and prothrombin time, were examined in clinical laboratories. Analysis of serum amino acids was performed by high-performance liquid chromatographic (HPLC, HITACHI, Japan). The moment of D-gal injection was the starting point for survival time. Death of animal was defined as the cessation of breathing, cardiac arrest and fixed pupil dilation.

#### Statistics

Values are expressed as mean ± standard deviation. Group differences were analyzed using one-way analysis of variance (ANOVA) and Student's t-test. Survival time was compared by Kaplan-Meier analysis (log-rank significance test). P value <0.05 was defined as significant. All data were analyzed using SPSS for Windows version 15.0.

## RESULTS

#### Microbead integrity and cell viability

As shown in Table 1, having experienced the entire BAL treatment, few beads lost its integrity (percentage of intact beads reduced from 99.2 ± 0.84 to 98.4 ± 1.14%, n = 5) (P>0.05). Though the viability of encapsulated HepLi-4 cells showed a slight downward trend by both

trypan blue exclusion (%), from 92.2±1.79 to 90.8±1.92, n = 5) and MTT test (OD, from 0.73 ± 0.08 to 0.69 ± 0.08, n = 5) after BAL treatment, the differences were not statistically significant (P>0.05).

#### Expression of liver-specific genes

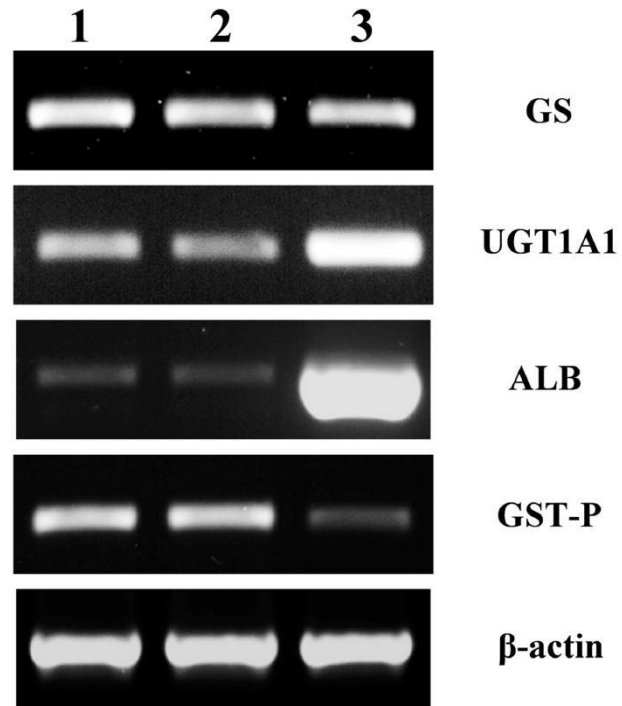
As shown in Figure 4, HepLi-4 cells that had experienced the BAL treatment still retained liver-specific gene expression. β-actin was an internal control, while the mRNA levels of mature human hepatocytes acted as a reference. In HepLi-4 cells, the mRNA level of GS was not less than that in adult human liver, while the mRNA level of UGT1A1 was somewhat lower. Moreover, the ALB mRNA level was extremely lower and GST-P mRNA level was extremely higher in HepLi-4 cells compared with those in human liver.

#### Treatment process and survival

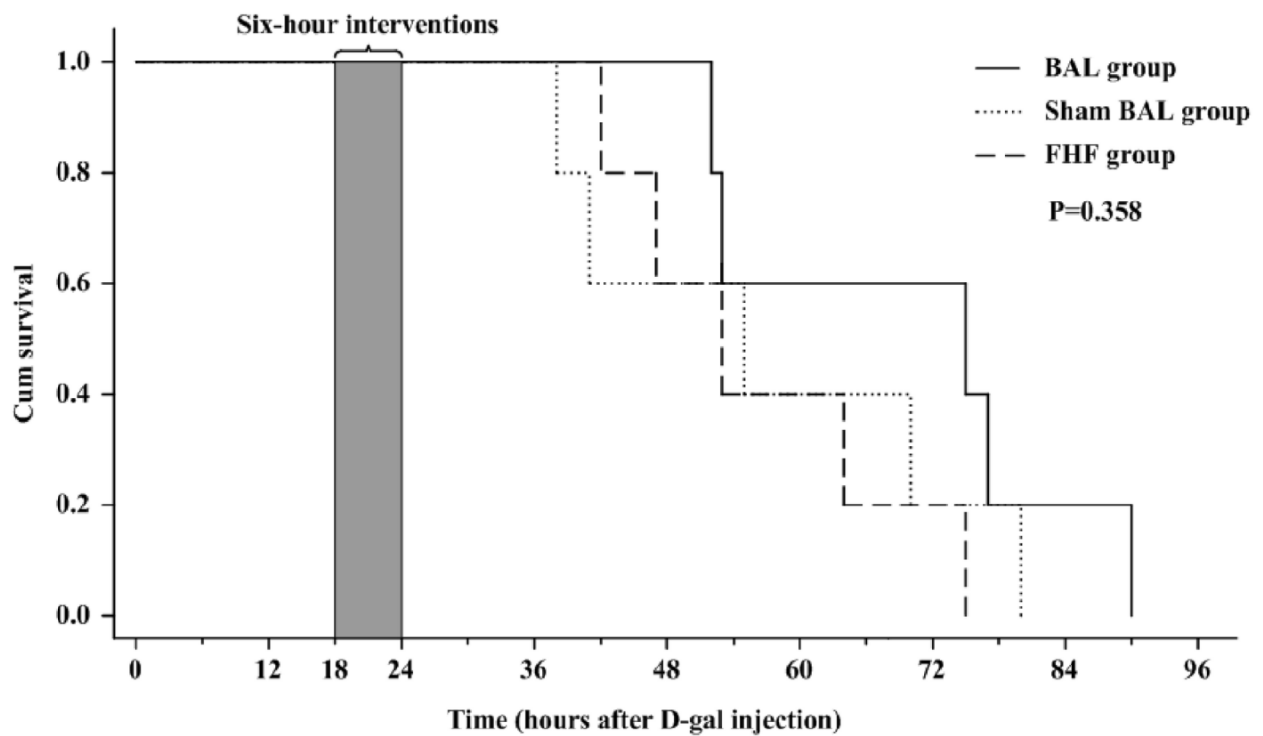
Throughout the 6-h BAL or sham BAL treatment process, no hemodynamic instability and bleeding episodes occurred in pig; no blood coagulation in extracorporeal circulation was observed. All pigs died of liver failure. As shown in Figure 5, survival time in BAL group (69.4 ± 16.5 h) was longer than that in FHF group (56.2 ± 13.3 h) and sham BAL group (56.8 ± 18.2 h), although the difference was not statistically significant (P>0.05).

#### Biochemical parameters

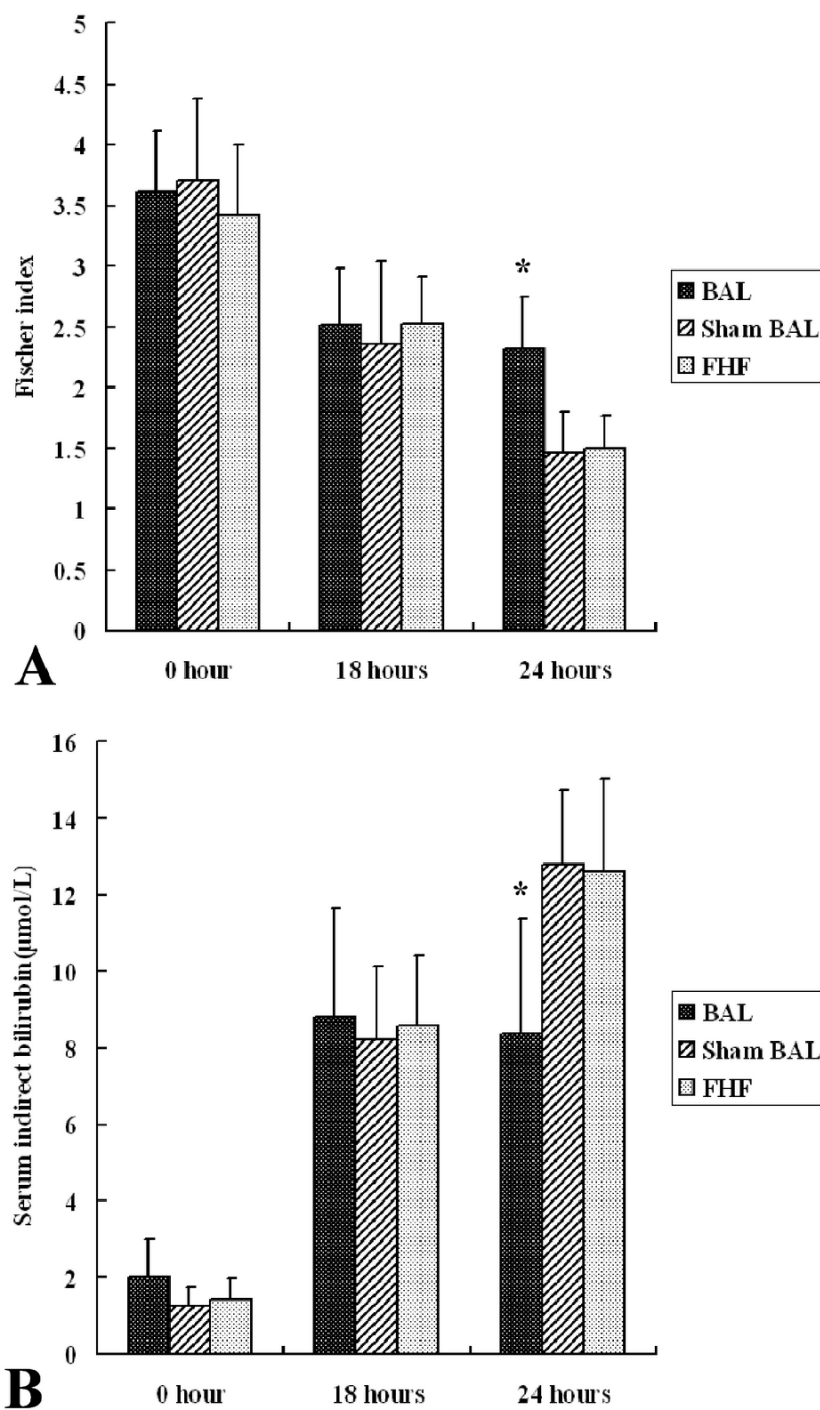
Fischer index is calculated as a ratio of branched chain amino acids (BCAAs; valine, isoleucine and leucine) to aromatic amino acids (AAAs; /tyrosine and phenyl-alanine) in plasma. As shown in Figure 6A, after D-galactosamine injection, Fischer index declined progressively in all groups. BAL treatment made the trend slow. At the end of the 6-h intervention (24 h after D- galactosamine injection), Fischer index in BAL group (2.32 ± 0.42) was significantly higher than those in sham BAL group (1.46 ± 0.34) and FHF group (1.49 ± 0.28) (P<0.05). As shown in Figure 6B, gradual increase of serum indirect bilirubin was observed in two control groups, while BAL treatment



**Figure 4.** Liver-specific genes expression in encapsulated HepLi-4 cells before BAL treatment (1), encapsulated HepLi-4 cells after BAL treatment (2) and adult human hepatocytes (3). BAL, Bioartificial liver.



**Figure 5.** Survival curves for the FHF pigs in BAL group, sham BAL group and FHF group. The time of D-gal injection was the starting point for survival time. Survival curves did not show significant prolongation of survival in BAL group compared with two control groups by Kaplan.



**Figure 6.** Biochemical parameter changes caused by BAL treatment. (A) Fischer index in all groups (\* $P = 0.005$  versus both control groups by one-way ANOVA. Fischer index = valine + isoleucine + leucine/tyrosine + phenylalanine). (B) Serum indirect bilirubin levels in all groups (\* $P = 0.025$  versus both control groups by one-way ANOVA). BAL, Bioartificial liver; FHF, fulminant hepatic failure.

prevented its further increase. The serum level of indirect bilirubin at 24 h after D-galactosamine injection in BAL group ( $8.40 \pm 2.97 \mu\text{mol/L}$ ) was significantly lower compared with sham BAL group ( $12.80 \pm 1.92 \mu\text{mol/L}$ )

and FHF group ( $12.60 \pm 2.41 \mu\text{mol/L}$ ) ( $P < 0.05$ ). Biochemical parameters with non-statistically significant differences ( $P > 0.05$ ) between three groups are listed in Table 2. Among them, after D-galactosamine injection,

**Table 2.** Biochemical parameters with non-statistically significant differences between three groups.

Parameter	Group	Baseline (0 h)	Pre-BAL (18 h)	Post-BAL (24 h)
Total bilirubin ( $\mu\text{mol/L}$ )	BAL	$3.26 \pm 1.02$	$22.68 \pm 6.48$	$34.24 \pm 8.52$
	Sham BAL	$2.8 \pm 0.45$	$24.6 \pm 6.07$	$38 \pm 13.82$
	FHF	$2.38 \pm 0.43$	$25.4 \pm 9.11$	$37.88 \pm 10.5$
Prothrombin time (s)	BAL	$11.56 \pm 1.15$	$21.6 \pm 2.75$	$65.98 \pm 33.27$
	Sham BAL	$12 \pm 2.32$	$23.16 \pm 4.56$	$65.22 \pm 32.75$
	FHF	$11.62 \pm 1.44$	$22.2 \pm 2.32$	$53.38 \pm 7.82$
Lactate (mmol/L)	BAL	$2.44 \pm 0.54$	$5.42 \pm 0.8$	$6.46 \pm 1.37$
	Sham BAL	$2.18 \pm 0.59$	$5.06 \pm 0.89$	$6.28 \pm 1.28$
	FHF	$2.66 \pm 0.41$	$5.88 \pm 0.91$	$7.25 \pm 1.25$
Alanine aminotransferase (IU/L)	BAL	$42.4 \pm 9.96$	$100.6 \pm 49.41$	$171.2 \pm 47.52$
	Sham BAL	$43.6 \pm 3.13$	$90 \pm 11.94$	$164.4 \pm 77.06$
	FHF	$46.4 \pm 9.56$	$92 \pm 25.44$	$170.6 \pm 40.16$
Glucose (mmol/L)	BAL	$5.88 \pm 1.15$	$4.54 \pm 0.58$	$3.61 \pm 1.59$
	Sham BAL	$5.66 \pm 0.51$	$4.7 \pm 1.16$	$2.53 \pm 0.4$
	FHF	$5.45 \pm 0.64$	$4.15 \pm 0.44$	$2.71 \pm 0.91$
Total cholesterol (mmol/L)	BAL	$2.18 \pm 0.16$	$1.37 \pm 0.39$	$0.94 \pm 0.3$
	Sham BAL	$1.89 \pm 0.31$	$1.24 \pm 0.4$	$0.81 \pm 0.24$
	FHF	$1.88 \pm 0.16$	$1.27 \pm 0.35$	$0.85 \pm 0.18$
Albumin (g/L)	BAL	$37.18 \pm 5.6$	$38.12 \pm 4.14$	$33.46 \pm 3.5$
	Sham BAL	$39.02 \pm 4.78$	$37.72 \pm 5.67$	$33.62 \pm 5.28$
	FHF	$38.76 \pm 3.15$	$39.52 \pm 4.36$	$37.58 \pm 4.24$
Creatinine ( $\mu\text{mol/L}$ )	BAL	$37.2 \pm 13.44$	$46 \pm 5.96$	$44.8 \pm 6.38$
	Sham BAL	$40.4 \pm 14.29$	$41.8 \pm 4.27$	$43 \pm 7.84$
	FHF	$39.4 \pm 5.22$	$46.2 \pm 11.65$	$44.6 \pm 10.97$
Urea nitrogen (mmol/L)	BAL	$3.35 \pm 0.66$	$3.01 \pm 0.44$	$2.98 \pm 0.52$
	Sham BAL	$3.91 \pm 1.37$	$3.75 \pm 0.52$	$3.58 \pm 0.94$
	FHF	$3.35 \pm 0.6$	$3.2 \pm 1.13$	$3.15 \pm 0.97$

Values are expressed as mean  $\pm$  standard deviation. Difference between groups was analyzed using one-way ANOVA.

the levels of serum total bilirubin, prothrombin time, lactate and alanine aminotransferases increased gradually. On the other hand, the levels of glucose and total cholesterol decreased gradually, while the levels of albumin, creatinine and urea nitrogen did not change significantly.

## DISCUSSION

To test the efficiency of a human derived hepatocyte line, we chose pigs as the animal model due to the high metabolic similarities between human and pig hepatocytes

(Donato et al., 1999). Drug-induced FHF was applied to pigs because it met the criteria proposed by Terblanche and Hickman (1991). Also, it closely resembled human FHF (Kalpana et al., 1999). In this study, progressively prolonged prothrombin time increased serum total bilirubin and lactate levels and decreased serum glucose level, and Fischer index were observed in all pigs. Histological examination showed massive hepatic necrosis in all of them (data not shown). It was therefore confirmed that all pigs died of FHF. Studies related to the encapsulation of mammalian cells in AC microbeads have shown that AC membrane provides mechanical stability, mass transfer capacity and immunoisolation *in vitro* (Baruch and Machluf, 2006;



Yu et al., 2009; Haque et al., 2005). In this study, cell viability and microbead integrity were maintained at acceptable levels after the 6-h perfusion of FHF pig's plasma and this was consistent with our previous study (Lv et al., 2011). Also, the mRNA levels of GS, UGT1A1, ALB and GST-P in encapsulated HepLi-4 cells were all retained after BAL treatment.

In this study,  $3 \times 10^9$  encapsulated immortal hepatocytes were used to treat an FHF pig weighing 10 to 15 kg. The cell mass should be adequate assuming the cells were fully-functional (Hoekstra and Chamuleau, 2002). However, it was disappointing to note that the prolongation of survival by BAL treatment was not statistically significant. This problem might lie mainly in cell functions. The results from RT-PCR analysis could explain partially. Though we only adopted a semi-quantitative analysis, it could be intuitively seen in Figure 4 that the expression of liver-specific genes of HepLi-4 significantly varied with adult human hepatocytes, especially since the HepLi-4 had an extremely lower transcript level of mature hepatocytes marker (ALB) but a higher level of immature hepatocytes marker (GST-P) than human liver. Thus, we concluded that HepLi-4 cells were not fully differentiated after reversion, which was also found by other studies (Deurholt et al., 2009; Chamuleau et al., 2005). It was demonstrated by quantitative RT-PCR in these studies that the transcript levels of some mature hepatocytes markers (albumin,  $\alpha$ -1-antitrypsin, and transferrin) in reverted NKNT-3 cells were only equivalent to 0.1 to 1% of those in human liver, while the mRNA level of GST-P was much higher. Another reversibly immortalized human hepatocyte line, 16-T3, has never been compared with mature human hepatocytes at genetic level (Totsugawa et al., 2007). In our previous studies, BALs charged with primary porcine hepatocytes could reduce serum lactate and stabilize blood glucose in FHF pigs (Lv et al., 2011; Li et al., 2006). However, this phenomenon was not observed in the current experiment. This revealed that there are some other functional deficiencies in HepLi-4 cells.

Although obvious defects existed, the relatively appropriate expression of detoxification-related genes (GS and UGT1A1) in HepLi-4 cells prompted us to continue the study. GS, an enzyme responsible for ammonia elimination, was considered essential for liver function (Kobayashi et al., 2000). Some researchers had modified HepG2 cells to realize high expression of GS (Tang et al., 2008; Enosawa et al., 2000). Although the expression of GS in HepLi-4 cells was not weaker than that in human liver, we failed to reveal their ammonia removal efficiencies in FHF pigs due to the shortage of test equipments. UGT1A1 is the only physiological enzyme that can transform water-insoluble indirect bilirubin into water-soluble direct bilirubin by conjugating it with glucuronic acid (Strassburg et al., 2008). Thus, bilirubin can be excreted into bile duct and eliminated from the body. Though the mRNA level of UGT1A1 in HepLi-4

cells was somewhat lower than human liver, we did observe a decrease of serum indirect bilirubin after BAL treatment compared with two control groups. However, the levels of serum total bilirubin at 24 h after D-galactosamine injection showed no significant difference between three groups. This hurdle cannot be conquered because all of the current bioreactors lack a biliary system capable of collecting bile and moving it out of extracorporeal circulation. Nevertheless, we proved that encapsulated HepLi-4 cells could fulfill the function of bilirubin glucuronidation in our BAL system. Also, it can be inferred that AC membrane is permeable to pig serum albumin for bilirubin is an albumin-bound toxin (Falkenhagen et al., 1999). In addition to bilirubin, UGT1A1 has several other substrates, such as hormones and drugs (Strassburg et al., 2008). Perhaps these functions benefited on correcting the metabolic disorders in FHF pigs, but they were not reflected in routine biochemical tests and survival.

Studies have shown that hepatic encephalopathy (HE) is associated with an imbalance in plasma BCAAs and AAAs; Fischer index and the grade of HE are negatively correlated (Koivusalo et al., 2008; Fischer et al., 1976). In this study, BAL treatment tended to normalize the Fischer index in pigs with FHF. However, it is difficult to evaluate the mental status of an animal accurately, especially in the early stage of HE. The method of reversible immortalization was once encouraging (Kobayashi, 2009, 2000; Totsugawa et al., 2007). However, our studies showed that hepatocytes generated by this method were poorly differentiated *in vitro*. The newly established immortalized human fetal hepatocyte line, cBAL111, is facing the same dilemma (Poyck et al., 2008; Deurholt et al., 2009). Even the stem cell derived hepatocytes, which are now considered promising sources for liver replacement therapy, still have functional deficiencies which are difficult to overcome (Dalgetty et al., 2009; Kung and Forbes, 2009). No wonder some researchers hold the view that no cell source so far is suitable for BAL support (Pless, 2010; Chamuleau et al., 2005). Probably, clinical trials of BALs can be carried out only on the condition that great progress is made in the promotion of hepatocyte differentiation *in vitro*. Maybe future studies should focus on such issues as differentiation promoting factor, matrix and co-culture, so that an *in vivo*-like environment can be provided (Chamuleau et al., 2005).

In conclusion, HepLi-4 cells in the choanoid fluidized bed bioreactor based BAL system showed some beneficial metabolic effects on FHF pigs, but still cannot be called a suitable cell source for BAL. Future research should put more insights into interpreting the conditions for hepatocyte differentiation.

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