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Construction of bone marrow mesenchymal cells-derived engineered hepatic tissue and its therapeutic effect in rats with 90% subtotal hepatectomy

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Abstract

Engineered hepatic tissue (EHT) is considered as a promising strategy for healing acute liver failure (ALF), therefore, in the present study we evaluated the therapeutic potential of the EHT which engaged with bone marrow mesenchymal cells (BMSCs) derived hepatocytes (BMSCs-Hepas) in ALF rats. After characterization of isolated BMSCs, we seeded passage 3 BMSCs which have being cultured in medium containing 20 ng/ml hepatocyte growth factor (HGF) and 10 ng/ml epidermal growth factor (EGF) for 14 days on three scaffolds individually in Transwell system, and then cultured for more than 3 days to construct three kinds of EHT named EHT1, EHT2, and EHT3. Based on morphology and urea production assays, we chose an optimal one and transplanted it into ALF rat with 90% subtotal hepatectomy and assessed its therapeutic potential by survival time, hepatic encephalopathy score (HES) and related liver function test. The remnant liver was acquired, sectioned and identified by con-focal scanning microscopy. The isolated cells possessed basic properties of BMSCs, when cultured in hepatogenic medium for 2 weeks, BMSCs would restore to the functional properties of primary rats' hepatocytes, expressing albumin (ALB) and alpha fetoprotein (AFP) simultaneously. Transplantation of EHT3 significantly prolonged the survival time, increased HES, and ameliorated the liver function. BMSC will be a newly cell source for the construction of EHT. Importantly, the EHT transplantation may be an effective strategy to treat ALF in clinic.

Key words: Bone marrow mesenchymal stem cells, Hepatocytes, Transplantation, Engineered hepatic tissue, Acute liver failure.

Introduction

Acute liver failure (ALF) remains a challenge in modern medicine with a mortality over 85 percent (1,2), till now; there are no effective means in curing it. Although orthodox liver transplantation gives the chance for curing (3), shortage of donor liver, high cost, and long-term immune suppression limit the clinical application of this therapy. Artificial liver support system (ALSS) including mechanical ALSS and bio-ALSS would be alternative choices at this moment. However, improved survival of patients with ALF is seldom documented after the application of mechanical ALSS (4,5). Additionally, acquiring vital hepatocytes, and high cost are all drawbacks of bio-ALSS. Furthermore, risk factors of ALSS include hemodynamic instability, bleeding, and etc. (6).

As a simple form of adjunct internal liver support, hepatocyte transplantation (7) confronts to shortage of donors' hepatocytes, cellular loss, and time lag. Thus, it is urgent to search a safe, effective, and low cost method. Engineered hepatic tissue (EHT) would be a promising substitute for its facilitated controlling, low cost, and safety. Nevertheless, series of issues are used to construct EHT (8), including acquisition of vital hepatocyte, choices of scaffold and culture system, and nutrition supply for the EHT. Among them, acquiring vital hepatocyte is of importance.

Due to the characteristics that bone marrow mesenchymal stem cells (BMSCs) can be readily isolated and expanded in the culture and their multi-differentiation potential, and the immunosuppressive properties of BMSCs make it probably allogeneic, as well as autologous, BMSC is becoming an attractive cellular tool in many clinical applications, including regenerative medicine, immune modulation and tissue engineering (9).

Recently, studies have demonstrated that *in vitro* BMSCs can differentiate into a variety types of cells, including myocytes (10), neurons (11) and hepatocytes (12,13), depending on the appropriate stimuli and microenvironment. In the present study we try to explore the possibility of constructing a BMSCs derived EHT *in vitro* and to evaluate its therapeutic efficiency in the treatment of ALF rats.

Materials and methods

Isolation and culture of BMSCs

Bone marrow cells were obtained from healthy male Sprague-Dawley rats weighing 250±25g as described previously (14). All the animal experiments were conducted in accordance with the national guidelines for the care and use of laboratory animals. This study was approved by the Ethnic Committee of Renmin Hospital of Wuhan University (Wuhan, China). The acquired BMSCs were confirmed when they differentiated into osteocytes and adipocytes after the additional adding of specific differentiation media as before (15), and the immunophenotype was assayed by flow cytometry after a co-incubation with fluorescein isothiocyanate (FITC)/phycoerythrin (PE)-conjugated monoclonal antibodies including CD29, CD34, CD44, CD45, CD80 and CD86

(BD Biosciences, Sparks, MD, USA) as described previously (15).

BMSCs trans-differentiation

After the labeling with CM-DiI, BMSCs were cultured in mesenchymal stem cell growth medium (BMSCs-GM, Osiris therapeutics company, USA) containing 10% FBS, 20 ng/ml HGF (Sigma company, USA), and 10 ng/ml EGF (Sigma, USA). Meanwhile, BMSCs-GM that only contained 10% FBS served as a control. Cells were cultured successively for 21 days, and then detected by the morphologic examination, immunocytochemical analysis, and RT-PCR at 7, 14, and 21 day respectively.

BMSCs were co-incubated with anti-rat monoclonal antibody ALB or AFP (1:100) (Abcam, Cambridge, UK) and followed by incubation with FITC-conjugated secondary antibody goat anti-rabbit IgG (1:1000) (Boster Biotech, Wuhan, China). Cells were analyzed using the con-focal scanning microscope. Specimen that incubated with 0.01M PBS (pH 7.4) instead of monoclonal antibody served as a negative control.

For RT-PCR analysis, total RNA was extracted from cells using Trizol reagent (Gibco Co., USA) according to the manufacturer's instructions, and was used for RT-PCR assay with the following primes: ALB, Forward: 5'-AAGGCACCCCGATTACTCCG-3', Reverse: 5'-TGCGAAGTCACCCATCACCG-3'; AFP, Forward: 5'-AGGCTGTACTCATCATTAAACT-3', Reverse: 5'-ATATTGTCCTGGCAT TTCG-3'; β-actin was served as an internal control, Forward: 5'-AGAGGGAAATCGTCGCTGAC-3', Reverse: 5'-AGGAGCCAGGGCCAGTAATC-3'.

The following PCR amplification was performed for 30 cycles. The reaction condition included denaturation at 95°C for 1 min and annealing at 58°C, 60°C, 55°C for ALB, AFP, and β-actin respectively; the rest of the conditions included denaturation at 94°C for 1 min followed by extension at 72°C for 1 min. The PCR products containing ethidium bromide were assayed using 2.0% agarose gel electrophoresis at 120 V for 30 min. Images of the gels were analyzed by Gel-1000 Single Wavelength Mini-Transluminitor (BioRad, USA).

Construction of EHT

EHT was respectively constructed by three scaffolds named scaffold 1, 2, and 3 including denuded amniotic membrane, DACRON PATCH cadio-vascular mesh (Braun Co., German) and bio-surgery mesh (Guanhao Bio-tech Co., Guangzhou, China). The consequential sterile denuded amniotic membrane was cut into pieces with a diameter of approximately 1.5cm and sutured onto a piece of PET membrane, with the basement membrane facing up, in the wells of the 24-well Transwell system. The DACRON PATCH cardiovascular mesh and bio-surgery mesh were washed with 0.01 M PBS (pH7.4) under sterile conditions and cut and sutured onto PET membrane in Transwell system for use as denuded amniotic membrane. Before the construction, the three scaffolds were examined by an H-9500 transmission electron microscopy (TEM) (Hitachi, Tokyo, Japan).

The BMSCs which had been cultured in BMSCs-GM containing HGF and EGF for 14 days were seeded in the Transwell system in which three scaffolds were placed in advance, and then the BMSCs were cultured in the medium for more than 3 days to construct three kinds of EHT. The consequential EHT were named EHT1, 2 and 3 according to the scaffold order.

After the construction, the three kinds of EHT were evaluated by con-focal scanning microscope, scan electronic microscopy (SEM) assays. For con-focal scanning microscope assay, three kinds of EHT were freeze-sliced and morphologically examined by con-focal scanning microscopy. Three kinds of scaffold were carried out SEM examination.

For assessment of urea production, EHTs were exposed to the medium containing 10 mM NH_4Cl for 24 h at 37°C in an atmosphere of 5% CO_2 (13). The rates of conversion of ammonia into urea nitrogen were determined by an automatic biochemically analyzer.

The therapeutic role of EHT for ALF rats

The model of ALF rat with 90% subtotal hepatectomy was established as following. Briefly, the rats were anaesthetized with a compound of chloral hydrate and ketamine (0.01 ml/kg), A 2-cm abdomen midline incision was made and the left and median lobes were exposed. The lower and upper parts of the right lobe were isolated, ligated, and resected, leaving the caudate lobe only. The incision was then closed. After the rats recovered from anesthesia, 20% glucose drinking water was given for 24 hours.

30 rats were randomly divided into 3 groups (10/group): group 1, only ALF, served as a control; group 2, ALF in combination with scaffold transplantation; group 3, ALF in combination with EHT transplantation. Optimal EHT was transplanted onto the wound of liver or in omentum with induced cells (1×10^6) immediately after the establishment of ALF; and 1 ml of saline was injected through tail vein on the operation day and day 1 and 2 post the operation.

The survival and psychometric hepatic encephalopathy score (PHES) of each rat were evaluated on 8 a.m everyday. PHES is a battery of neuropsychological tests used in the diagnosis of minimal hepatic encephalopathy (MHE). Blood samples were collected via femoral artery prior to and after the surgery daily. Plasma samples were prepared and ALB, amino alanine transferase (ALT), and aspartate amino transferase (AST) were determined.

Data presentation and statistical analysis

All experiments were performed at least three times. Data were expressed as means ± standard deviations. ANOVA analysis was used to analyze the differences among groups, and Kaplan-meier analysis was used for the animal survival assay.

Results

Isolation, culture and labeling of BMSCs

BMSCs population originating from the bone marrow of the rats was successfully isolated, passaged in monolayer culture, and then characterized. The BMSCs were cultured for 3-5 passages and then the characters of BMSCs were further confirmed by immunophenotype determination and specific abilities of osteogenic

and adiogenic differentiation.

With the CM-DiI labelled, red autofluorescence was identified by using the con-focal laser scanning microscope. Staining efficacy with CM-DiI was good in terms of both quality (good intensity) and quantity, as more than 98% of cells were stained.

The ALB and AFP expressed by BMSCs were conformed by immunofluorescence microscopy on day 14, the induced cells would become round or oval shape with yellow fluorescence which combined with the red fluorescence of CM-Dil and green of ALB or AFP, while the staining of AFP or ALB was negative in untreated cells (Fig. 1).

Trans-differentiation of BMSCs

When passage 3 BMSCs were cultured in hepatic inducing medium for a week, cells could transform from fusiform shape to triangle or oval shape, and more adherent to flasks. On the second week, cells became round and enlarged, with poor proliferation, and assembled to form whirlpool structure, or to be concentrically arranged. On the third week, cells assemble became obscure, and apoptosis took place. On the contrary, BMSCs keep its characteristic elongated shape in the control group.

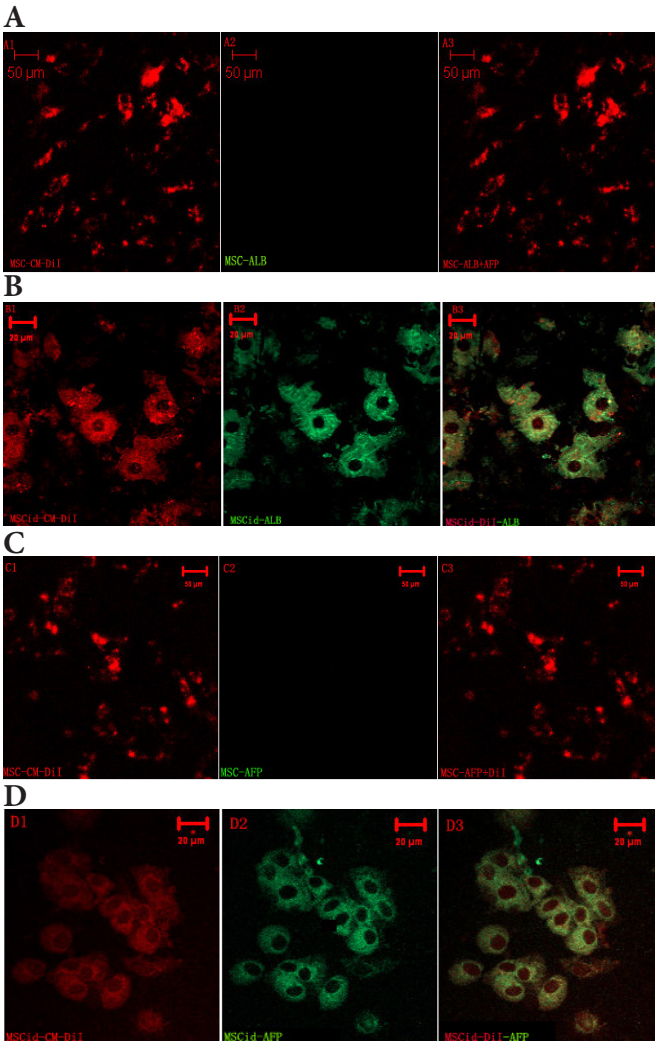

Figure 1. The expression of ALB/AFP in induced cells. The ALB and AFP expression was conformed by immunofluorescence microscopy on day 14, the induced cells would become round or oval shape with yellow fluorescence which combined with the red fluorescence of CM-Dil and green of ALB (B, Bar=20 μm) or AFP (D, Bar=20 μm). While the staining of AFP or ALB was negative in untreated cells (A, Bar=50 μm; C, Bar=50 μm).

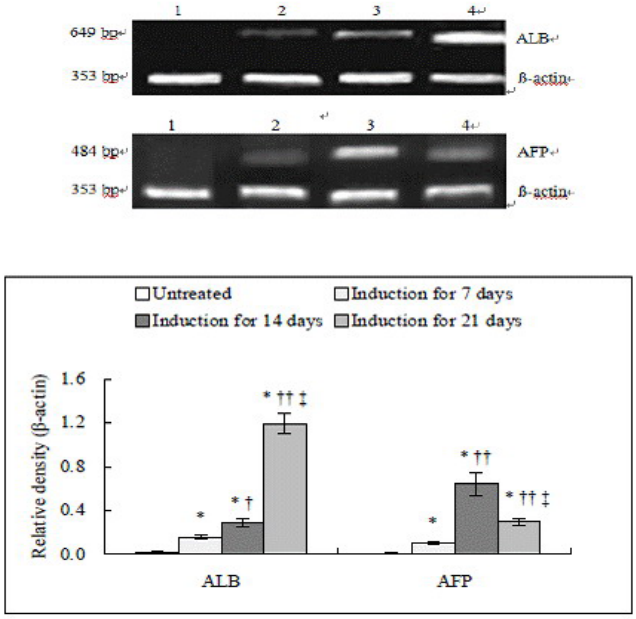

Figure 2. RT-PCR assays of ALF and AFP. Total RNA was extracted, and then RT-PCR was performed. The signals for ALF, AFP and β-actin were integrated on a Gel Doc 1000 Mini-Transilluminator (Bio-Rad, USA). The experiment was performed for three times. Lane 1: Untreated marrow mesenchymal cells; Lane 2: Marrow mesenchymal cells after the induction for 7 days; Lane 3: Marrow mesenchymal cells after the induction for 14 days; Lane 4: Marrow mesenchymal cells after the induction for 21 days. $^{*}P<0.01$ versus Untreated; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ versus Induction for 7 days; $^{\ddagger}P<0.01$ versus Induction for 14 days.

The ALB and AFP expressions were confirmed by immunofluorescence microscopy on day 14. The induced cells would become round or oval with yellow fluorescence, which combined with the red fluorescence of CM-Dil and green of AFP or ALB. The staining of ALB or AFP was negative in untreated cells (Fig. 1).

RT-PCR analysis revealed that on day 7, there is a weak expression of ALB, which increased along with with the prolongation of time and arrived a summit on day 21, however, AFP reached a peak on day 14 and began to slightly decrease on day 21 (Fig. 2), suggesting that the induced cells were immature ones between the fetal and mature hepatocytes.

EHT selection for transplantation

After construction for 3 days in the Transwell system, three kinds of EHTs were evaluated by morphologic and functional assays. After that, cells were found only on the surfaces of EHT1 and EHT2. Furthermore, compared with EHT1 (Fig. 4A and 4B), cells on the surface of the scaffold were loose, with lower affinity and poor cell morphology in EHT2 (Fig. 3 and Fig. 4C-4F). However, in EHT3, cells were not only on the surface of scaffold, but also in its pores (Fig. 3 and Fig. 4G-4J). In addition, the urea production was largest in EHT3 (Data not shown), suggesting the suitability of EHT3 for following studies.

The EHT3 transplantation prolongs the survival time and improved liver function of the ALF rats

ALF model was successfully induced in 90% hepatectomized rats and then confirmed by the clinical fea-

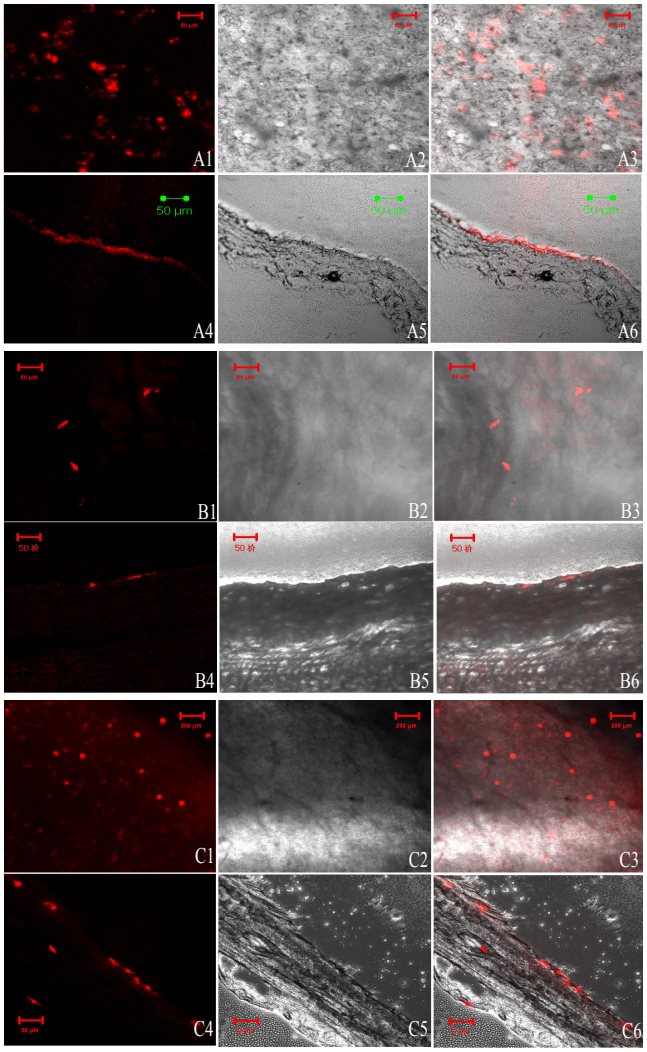

Figure 3. Con-focal scanning microscopy finding of three kinds of EHT. From con-focal scanning microscopy, cells were found only on the surface of EHT1 and EHT2, furthermore, compared with EHT I, cells on the surface of scaffold were loosen, with lower affinity and poor cell morphology in EHT II. In EHT III, cells were found to locate inside of the scaffold. (A: amniotic membrane, B: DACRON PATCH cadio-vascular mesh, C: Biosurgery mesh; cross-sectional (1–3) and longitudinal (4–6) images show BMSCs-Hepa in EHT I (A, bar=50 μm), II (B, bar=50 μm), and III (C, 1-3, bar=200 μm; 4-6, bar=50 μm). Images show CM-DiI labeling (images 1 and 4), phase-contrast microscopy (images 2 and 5), and both CM-DiI labeling and phase-contrast microscopy (images 3 and 6).

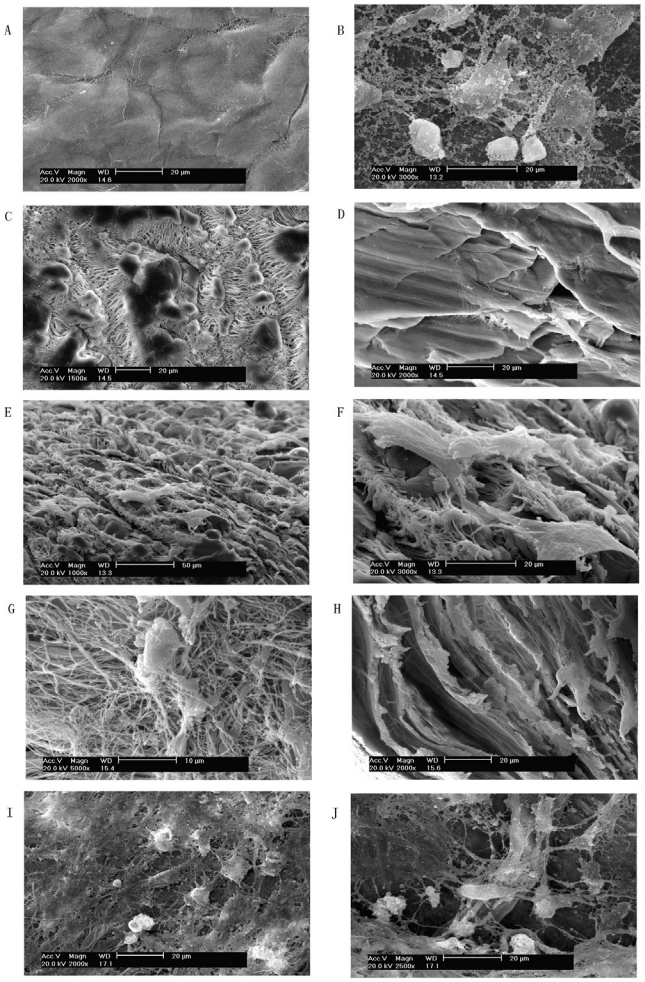

Figure 4. SEM finding of scaffold and EHT.
A: The surface of scaffold I (amniotic membrane) is made of filamentous collagen fibers (×2000, bar=20 μm). **B:** Round or polygonal cells with short and rough microvilli can be seen growing on the filamentous collagen fibers of scaffold I with high affinity, cells contact each other by cytoplasmic processes, with gauze-like or granular matrix peripherally (×3000, bar=20 μm). **C:** Cross-sectional image: the surface of scaffold II (DACRON PATCH cadio-vascular mesh) is made of the dense fibrous structure, with the wool-like appearance and small fiber micropore (×1500, bar=20 μm). **D:** Longitudinal section image: the normal fibrous structure of the scaffold II was broken and shows blade-like scratches (× 2000, bar=20 μm). **E-F:** Few polygonal cells with microvilli can be seen growing on the scaffold II with loose affinity (× 1000, bar=50 μm (E); × 3000, bar=20 μm (F)). **G:** Cross-sectional image: the surface of scaffold III (bio-surgery mesh) is made of the fibrous structure, with small fiber micropore (× 1500, bar=10 μm). **H:** Longitudinal section image: Obviously delamination with cross array fiber in scaffold III can be seen (×2000, bar=20 μm). **I:** Cross-sectional image: More round or polygonal cells with short microvilli can be seen growing on the filamentous collagen fibers of scaffold III with high affinity, cells contact each other by cytoplasmic processes, there is matrix existing peripherally (×2000, bar=20 μm). **J:** Longitudinal section image: round or polygonal cells with short microvilli can be seen filling in the cracks between the delamination of scaffold III, with cells contact each other by cytoplasmic processes, and matrix peripherally (× 2500, bar=20 μm)

ture of floppy, drowsiness, anorexia, and etc.

The EHT3 transplantation in the ALF rats could significantly prolong survival time. In brief, the rats in the EHT3 group died within 6 days, but the rats in ALF alone group and ALF in combination with Scaffold transplantation group died with in 3 days. Further, there were significant differences in survival rate between ALF plus EHT and ALF alone and ALF plus Scaffold transplantation (P<0.01, Kaplan-meier analysis) (Fig. 5a). Meanwhile, Transplantation of EHT3 increased PHES (Fig. 5b) and improved liver function including ALT, AST, and ALB (Fig. 5c-e).

Discussion

The unique characters of BMSCs including readily being harvested, isolated with plastic adherence, expanded in culture, genetically engineered, differentiated across germ layers, and handled in vitro makes it variable in regeneration medicine. Here, in case of the general manipulation would alter or influence BMSCs' natural phenotype, we selected a relatively primary population of 3- passage BMSCs and confirmed that the subsequent cells retained the characteristics of BMSCs

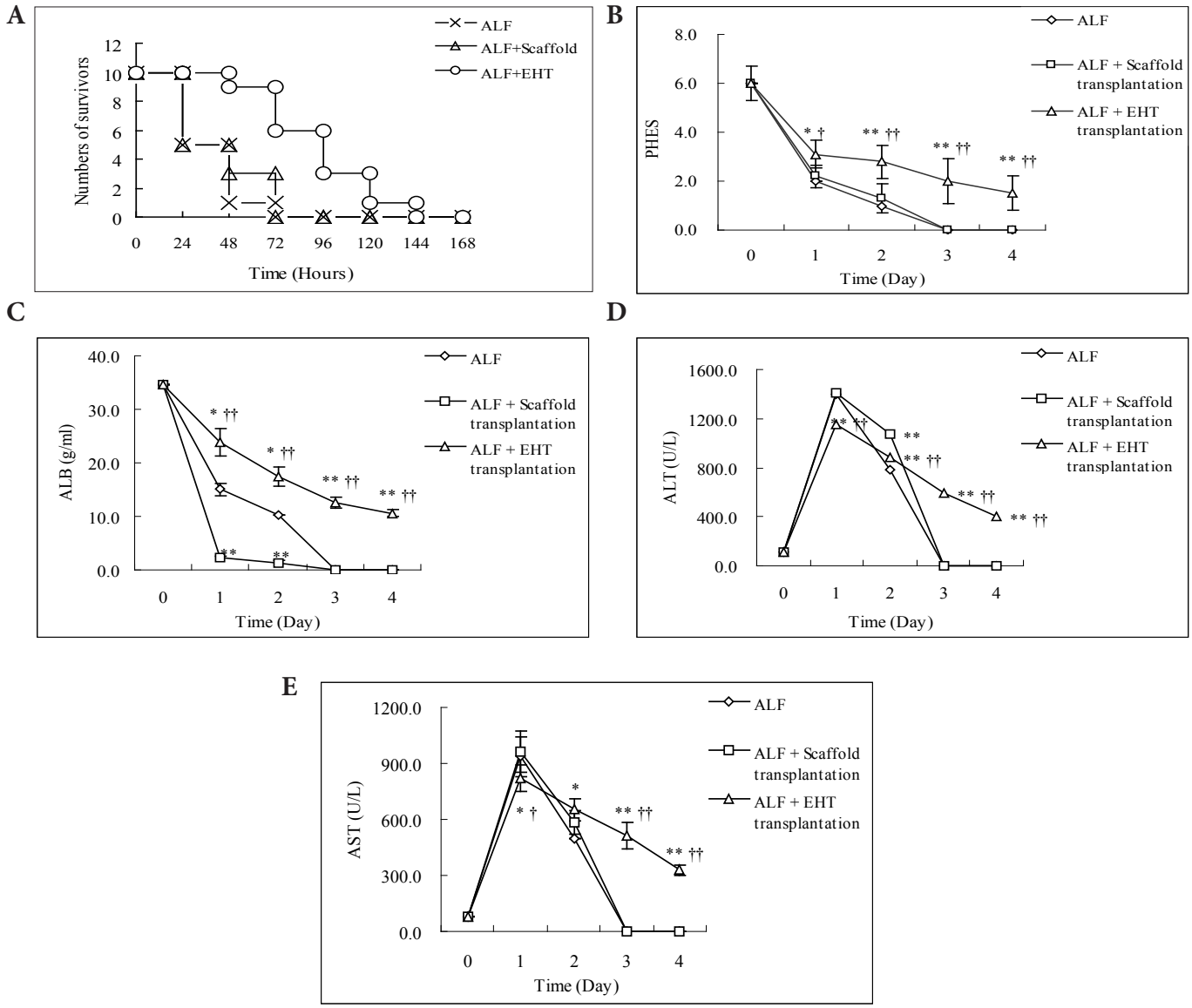

Figure 5. The therapeutic role of EHT in rats with ALF

A: Survival analysis of ALF rats in groups. The rats in ALF + EHT group were dead within five days, while counterpart in ALF and ALF + Scaffold transplantation were dead within three days, there was a significant difference after Kaplan-meier analysis; **B:** PHES of ALF rats among groups. On day 1, there is significant difference among HES of three group which indicates HES in ALF + EHT transplantation group is higher than the other two group; **C-E:** Liver function of ALF rats in groups. On day 1, there are significant differences among liver function of the three groups which indicates ALF + EHT transplantation group has better liver function compared with the other two. $^{*}P<0.05$, $^{**}P<0.01$ versus ALF, $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ versus ALF + Scaffold transplantation.

after their immunophenotyping profile and mesodermal differentiation potential in mesenchymal-supportive conditions containing osteogenic and/or adipogenic-specific agents (16).

The pivotal issue from BMSCs differentiating into hepatocytes is to create a suitable microenvironment for it, thus, we selected HGF and EGF as inducers for they modulate liver cellular proliferation, differentiation, and interactions among cell, mesenchyme, and extracellular matrix-related differentiation events that occurred (17). Here, we have shown that BMSCs can be differentiated into hepatocytes within 14 days and can obtain the functional properties of primary rats' hepatocytes, and our result is similar to the study of Stock (18), which displayed an acquired feature of the induced adult cell as well as fetal rats' hepatocytes.

Scaffold is an important complementary element in regenerative medicine and tissue engineering, besides good biocompatibility and biodegradability, scaffold should meet the following criteria: (i) a stable 3-D spatial microenvironment to mimic the organized architectures of native liver; (ii) a pre-estblished vascular bed for sufficient nutrients and oxygen delivery, as well as timely elimination of wastes; and (iii) highly porous structures for organ regeneration (19). So far, two kinds of scaffold are well progressed: one is natural matrices, which have been used in liver tissue engineering from stem cells (20,21), these natural polymers are suitable for cellular interactions, however, scaffolds fabricated purely from these molecules exhibit poor mechanical strength and are not easy to handle; another is synthetic matrices that attributed to advance in polymer chemistry, however, well-organized scaffold with polymer chemistry is difficult to fabricate (19).

In the present study we displayed three kinds of scaffold, denuded amniotic membrane, DACRON PATCH cadio-vascular mesh, and bio-surgery mesh. Among them, denuded amniotic membrane is a natural macromolecule sheet, which is rich in nutritional factors, such as EGF, β-TGF, interleukins and collagens, widely

used in engineered cornea fabrication (22). DACRON PATCH cadio-vascular mesh is a proceeded filamentous tubular vascular prostheses, with highly compliant, porosity and a micropore size around 50 cc/min·cm^2, which shows wool-like structure from SEM, is widely used in cadio-vascular surgery, such as arterial reconstructions and heart repair. And the bio-surgery mesh, is the resilient tissue which is extracted from porcine and proceeded by a series of steps, such as patented technology, and low-temperature drying treatment, exhibiting excellent biocompatibility and low immunogenicity. To our amazement, the bio-surgery mesh exhibits a comparatively stronger mechanical strength to become an optimal substitute in hernia repair.

Our results show, there is degraded collagen in bio-surgery mesh from TEM, and BMSCs-Hepa exists not only on the surface, but also among the pore, furthermore, there are lots of cellular connections from SEM and immunofluorescence microscopy assay, which are the reasons for the more urea production compared with the former two kinds of EHT. All the results indicate that the EHT3 is the optimized one for the following transplantation.

As far as transplantation site was concerned, we selected the remnant liver as the first choice, which was different from other studies (23-26), in hopes of the inflammatory procedure would stimulate or maintain vigor of BMSCs-Hepa in EHT, as the results showed that compared with the control group, the survival time was significantly prolonged in EHT group, and it was also found that the BMSCs-Hepa with red fluorescence surrounding the remnant liver by immunofluorescence microscopy.

Although transplantation of EHT3 has effects on ameliorating liver function and increasing HES level on day 1, EHT prolongs the survival time of ALF few or no, which makes significant difference with former researches and also frustrates us. Navarro-Alvarez found that the engineered hepatic grafts provided life-saving support in models of acute, fulminant, and chronic liver failure (27). In addition, Soto-Gutierrez also reported that a kind of EHT prolonged the survival of liver failure-induced mice without adverse effects (26). We speculate the next three points should be responsible for this. The first one is the functional difference between the BMSCs-Hepa and hepatocytes. Although AT-MSC-Hepa was similar to liver with regard to gene expression with a round epithelial cell-like shape, BMSCs-Hepa could not be genuine hepatocyte functionally (28), besides, the amount of BMSCs-Hepa would be insufficient because cell apoptosis would take place on day 21 and cell loss would be exist during the transplantation. The second one is the culture system, although Transwell system is the optimal system in stationary culture, rotary cell culture system and perfusion culture system should be preferred, for the latter is genuine 3-D culture system which can make cells and scaffold contact completely. The third one is how to maintain the function of EHT, one is to induce neovascular formation in EHT, just like Yokoyama (25), whose research describes a pre-established vascular bed for EHT to facilitate sufficient nutrients and oxygen delivery, as well as timely waste removal. Another is to induce or maintain the high function of hepatocyte via co-culture (28) or some stimulator such as oncostatin M (29), although some results in EHT research are unsatisfactory (30), long-term studies are needed to reevaluate the potential efficacy of growth factor releasing system in maintenance of hepatocyte functions in EHT.

In conclusion, successful fabrication of BMSCs derived EHT and prolonging survival time of rats with ALF were demonstrated herein, which holds potential interest in the curing ALF in future. Next, great efforts should be devoted to the different parts of EHT including selecting scaffold, maintenance of BMSCs-Hepa, and optimizing culture system with a view to construct perfect EHT.

Acknowledgement

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