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Original Article

Use an alginate scaffold-bone marrow stromal cell (BMSC) complex for the treatment of acute liver failure in rats

Jizong Lin[1*], Lili Meng[2*], Zhicheng Yao[3], Shuxian Chen[1], Jun Yang[4], Zhaofeng Tang[1], Nan Lin[1], Ruiyun Xu[1]

[1]Department of Hepatobiliary Surgery, The Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510630, China; [2]Department of Gynecology and Obstetrics, The Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University, Guangzhou 510630, China; [3]Department of General Surgery, The Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510630, China; [4]The Key Laboratory of Bioactive Materials, Mninistry of Education, College of Life Science, Nankai University, Tianjin 300071, China. [*]Equal contributors.

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Abstract: To evaluate the effects of alginate scaffold-bone marrow stromal cell (BMSC) in the treatment of acute liver failure in rats and provide a basis for in vivo application of artificial liver tissue. CM-DiI-labeled BMSCs were planted and grown on alginate scaffolds to form alginate scaffold-BMSC complex. Alginate scaffold-BMSC complex (the experimental group) or alginate scaffolds (the control group) were placed onto the surface of liver wound of rats after 70% of hepatectomy. The scaffold-BMSC complex and alginate scaffolds were removed after 4 weeks and fluorescence microscopy was used to track the growth and distribution of CM-DiI-labeled BMSCs. The liver tissues were stained for albumin and glycogen to investigate the differentiation of BMSCs on alginate scaffolds. The survival rate and liver function were also compared between the two groups of rats. BMSCs on alginate scaffolds and liver tissues were clearly demonstrated by CM-DiI labeling. BMSCs on alginate scaffolds secreted albumin and produced glycogen. The survival rate and liver function of the rats of the experimental group were significantly higher than that the control group rats. Alginate scaffold-BMSC complex promotes the regeneration of liver tissues in rats of acute liver failure.

Keywords: Alginate scaffold, bone marrow-derived mesenchymal stem cells, acute liver failure, artificial liver tissue

Introduction

Currently, liver transplantation is the only effective approach for the treatment of a variety of end-stage liver diseases. However, liver transplantation is not a commonly used in clinic due to the shortage of donor livers and lifelong use of immunosuppressive drugs.

Therefore, alternative approaches for the treatment of end-stage liver diseases are highlighted. In recent years, hepatic tissue engineering has become a promising strategy for the treatment of a variety of end-stage liver diseases [1].

In hepatic tissue engineering, appropriate cells were seeded onto supporting biomaterials, and differentiate and grow under specific stimuli to form a certain amount of liver parenchymal cells and tissues that are transplanted into the patients to repair or replace liver of failed function. The ultimate goal of hepatic tissue engineering is to establish a complete, functional, and transplantable liver for patient of end-stage liver diseases.

Identification of appropriate seeding cells and supporting biomaterials are critically important for successful hepatic tissue engineering. Currently, bone marrow stromal cells (BMSCs) are widely used in hepatic tissue engineering due to a number of advantages such as abundant source, easy performance, pluripotency, and low immunogenicity. It has been reported by many studies that BMSCs are able to be induced to differentiate into hepatocytes in vivo [2, 3].

Alginate scaffolds have been widely used as cell carriers in tissue engineering because of their hydrophilic properties, porous three-

dimensional structure, and excellent tissue compatibility. In our previous study, an alginate scaffold-BMSCs complex has been established and used in hepatic tissue engineering *in vivo*. Our results showed that the alginate scaffold-BMSCs complex may be a promising alternative approach for the treatment of end-stage liver diseases [4].

Currently, bioartificial liver based on the bioreactor technology is mainly used to complement liver function in vitro. However, bioartificial liver based on the bioreactor technology is not widely used in clinic due to technical limitations, high costs, and special requirements on healthcare. Therefore, hepatic tissue engineering based on three-dimensional and porous biomaterials as well as cell transplantation has been extensively investigated [1]. Whether alginate scaffold-BMSCs complex can be applied in vivo is not clear. In the present study, we developed and transplanted an alginate scaffold-BMSCs complex into the live in rats undergone 70% of hepatectomy. The differentiation and growth of BMSCs and the liver function were also examined. Our results provide basis for the study of alginate scaffold-BMSCs complex in the treatment of end-stage liver diseases.

Materials and methods

Materials

Sprague Dawley (SD) rats were purchased from the Experimental Animal Center of Sun Yat-Sen University. Alginate and goat anti-mouse albumin (ALB) antibody were purchased from Sigma. DMEM/F12 medium was purchased from Gibco. Chloro-methylbenzamido dialkylcarbocyanine (CM-DiI) and Hoechst33258 were purchased from Invitrogen.

Preparation of alginate scaffolds

Preparation of alginate scaffolds, and isolation and culture of BMSCs were conducted according to the previous study [5]. Alginate (6%) prepared in 0.1% PBS was sterilized at 120°C and 0.15 Mpa, and stored at 4°C for follow-up experiment. Prior to BMSC seeding, the alginate solution was diluted using α-2MEM to reach a final concentration of 3%.

Seven hundred microliter alginate (3%) was added to each well of a 24-well culture plate to form an alginate plate of ~1 mm in thickness. Then, 2 ml $CaCl_2$ (0.1%) was added onto the surface of the alginate plate. After the alginate plate was placed at room temperature for 40 min, and transformed to be gel-like materials, $CaCl_2$ was removed and the alginate plate was soaked in 2 mL α-2MEM at 4°C. The α-2MEM was changed every 24 h, and the alginate was used for BMSC seeding after 72 h.

Isolation and labeling of BMSCs

CM-DiI (1 g/L) was prepared in dimethyl sulfoxide (DMSO). BMSC suspension (500 μL) was prepared by adding BMSCs (5×10^6) of the 3rd-6th generations to Dulbecco's phosphate-buffered saline (DPBS). Five microliter CM-DiI (1 g/L), which was prepared in dimethyl sulfoxide (DMSO), was added into 500 μL BMSC suspension and incubated at 37°C for 10 min and 4°C for 20 min. The CM-DiI-labeled BMSCs were washed using PBS for three times, re-suspended in 50 μL PBS, and seeded onto alginate scaffolds. The remaining CM-DiI-labeled BMSCs were inoculated in 24-well culture plates to evaluate CM-DiI labeling.

The growth of CM-DiI-labeled BMSCs seeded on alginate scaffolds were evaluated under a fluorescence microscope using ordinary light and light of a wavelength of 540-650 nm.

Rat hepatectomy and transplantation of alginate scaffold-BMSCs

A total of 20 adult SD rats were randomly divided into two groups, the experimental (n=10) and the control (n=10) groups. Animals were anesthetized by intraperitoneal injection of 5% chloral hydrate (6 mL/kg). Open abdominal surgery was conducted under successful anesthesia. Perihepatic ligaments were isolated and cut off. Liver resection of right upper and right lower lobes was conducted by completely ligating the hepatic artery at hepatic porta. About 70% of the liver was resected. After wound hemostasis, the alginate scaffold-BMSC complex was placed on the wound surface of liver resection. For the control group, the wound surface was covered by alginate scaffolds. The abdomen was closed and animals were kept warm at 25-28°C. The SD rats were observed in cages to evaluate their condition and survival rates for 4 weeks after the liver resection surgery.

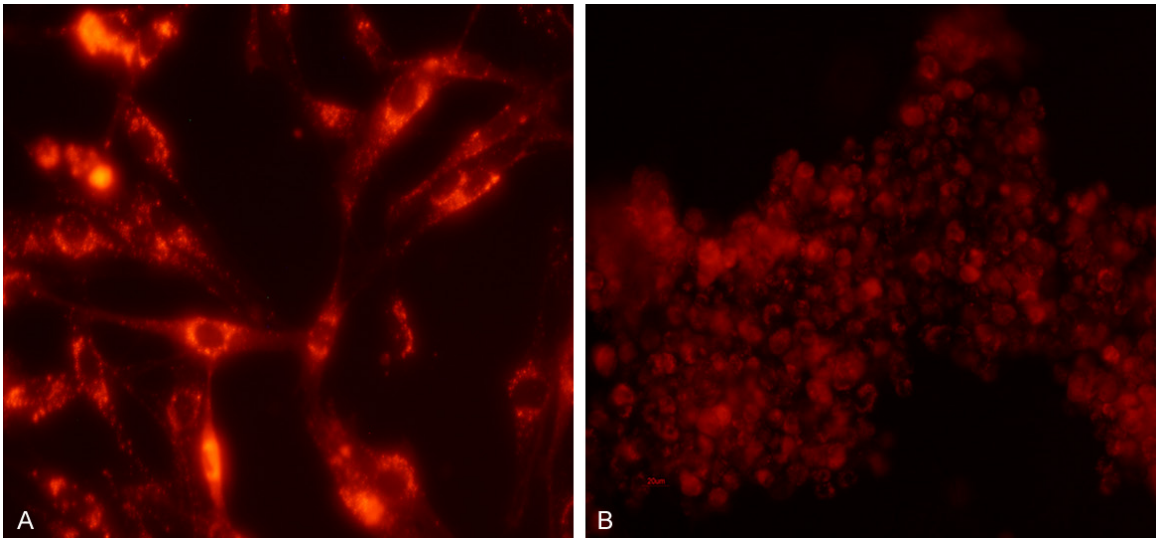

Figure 1. The morphology of CM-DiI-labeled BMSCs (fluorescence microscopy, ×200). A. Monolayer BMSCs. B. BMSCs on the alginate scaffolds.

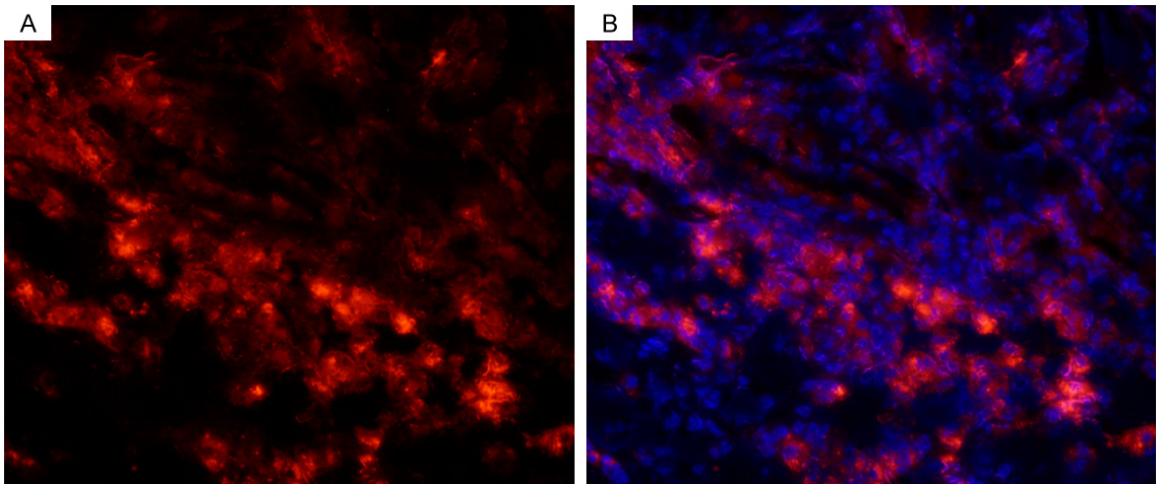

Figure 2. The morphology of alginate scaffold-BMSC complex 4 weeks after transplantations (fluorescence microscopy, ×200). A. CM-DiI-labeled BMSCs (red). B. CM-DiI (red) and Hoechst 33258 (blue)-labeled BMSCs.

Evaluation of serum levels of AST, ALT, and ALB, and the expression of ALB in the alginate scaffold-BMSC complex

Tail vein blood was collected on the 1st and 14th days after the liver resection surgery. Serum levels of aspartate transaminase (AST), alanine aminotransferase (ALT), and albumin (ALB) were determined using an automatic biochemical analyzer.

The animals were sacrificed 4 weeks after the liver resection surgery and the alginate scaffold-BMSC complex and alginate scaffolds were removed from the livers to prepare frozen sections of 4 μm. The frozen sections were examined under a fluorescence microscope. The cells morphology and distribution were evaluated by checking the Hoechst 33258-stained nuclei.

The expression of albumin in the alginate scaffold-BMSC complex was detected by immunohistochemistry (IHC). The frozen sections were fixed in 4% paraformaldehyde for 30 min, and washed with PBS containing 0.01% Triton for 3 times. After treated with 3% H_2O_2 for 30 min and rinsed by PBS, the frozen sections was

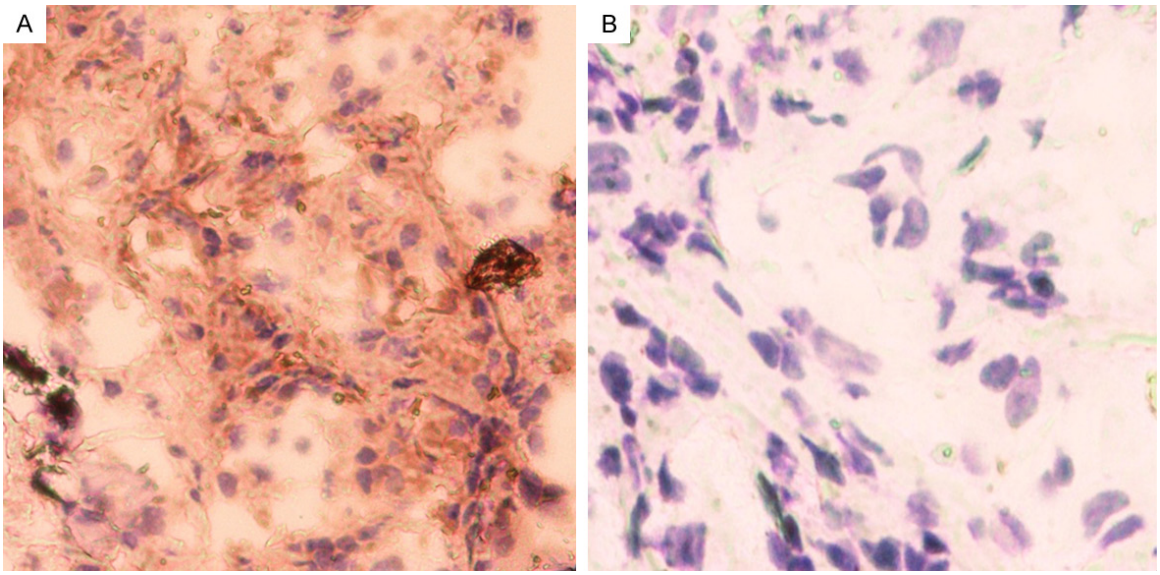

Figure 3. Albumin produced by BMSCs on the alginate scaffolds transplanted into the liver of rats undergone 70% of hepatectomy (immunohistochemical staining, ×200). A. Alginate scaffold-BMSC complex. B. Alginate scaffold alone without BMSCs.

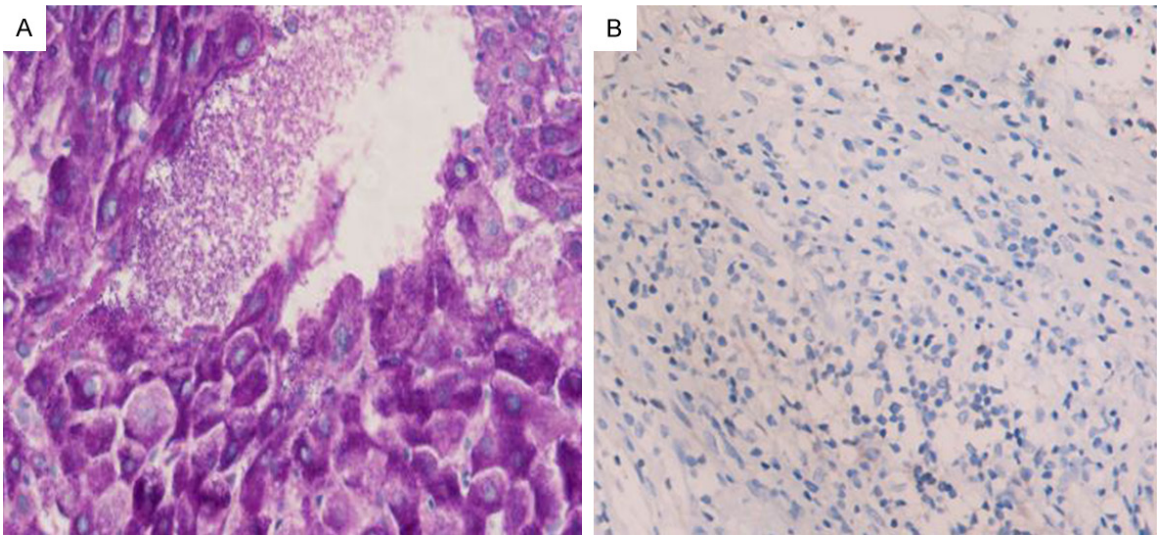

Figure 4. Glycogen produced by BMSCs on the alginate scaffolds transplanted into the liver of rats undergone 70% of hepatectomy (periodic acid-Schiff staining, ×400). A. Alginate scaffold-BMSC complex. B. Alginate scaffold alone without BMSCs.

incubated with goat anti-mouse ALB antibody at 4°C overnight. The cells were shown using DAB and hematoxylin for 5 min. After rinsed with tap water, dehydrated, transparent, and mounted with neutral gum, the sections were examined under a light microscope.

To evaluate the glycogen storage activity of BMSCs in the alginate scaffold-BMSC complex, the frozen sections were fixed in AAF solution (5% of acetic acid, 15% of formaldehyde, and 85% of ethanol) for 15 min, and rinsed with PBS for 3 times (9 min). The sections were then oxidated with periodate (5 g/L) for 10 min, rinsed with distilled water, and stained with Schiff reagent for 20 min. Then, the sections were washed with sodium metabisulfite (5 g/L) for 3 times (9 min) and distilled water. The sections were stained using hematoxylin for 5 min. After rinsed with tap water, dehydrated, trans-

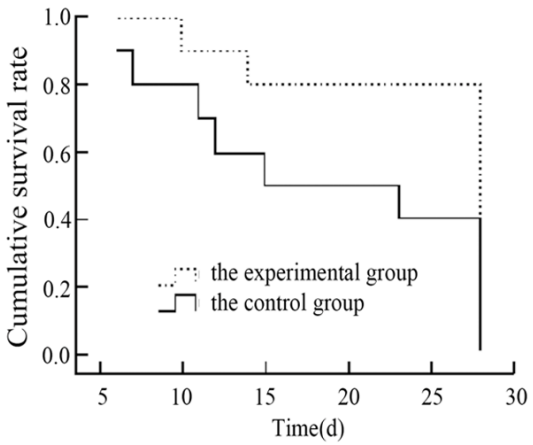

Figure 5. Comparison of the survival rate of the two groups of rats. The survival rate of the rats of the experimental group (transplanted with the alginate scaffold-BMSC complex) was significantly higher than that of the control group rats (transplanted with the alginate scaffolds) (n=10, $P<0.05$).

parent, and mounted with neutral gum, the sections were examined under a light microscope.

Statistical analyses

SPSS software was used for statistical analyses, measurement data were presented as mean ± standard deviation ($\bar{x} \pm s$). Kaplan-Meier and log-rank methods were used to compare the survival rate between the two groups of rats. The liver function was comparted between the two groups using ANOVA. A P value less than 0.05 was considered to be statistically significant.

Results

Morphology of BMSCs on the alginate scaffolds

The growth of BMSCs on the alginate scaffolds were examined under a microscope of a wavelength of 540-650 nm (**Figure 1**). Prior to the transplantation of alginate scaffold-BMSCs, BMSCs on the alginate scaffolds formed red spherical structure due to clustering growth along the alginate scaffolds. The membranes of fusiform-shaped BMSCs were stained in red with even distribution of fluorescence. The nuclei of BMSCs were complete without staining (**Figure 1**).

Four weeks after the surgery of hepatectomy and alginate scaffold-BMSC complex transplantation, the alginate scaffold-BMSC complex was removed to examine the growth of BMSCs *in vivo*. In the frozen sections of alginate scaffold-BMSC complex, strong red fluorescence was observed along the alginate scaffolds. With nuclear staining with Hoechst 33258, we found that BMSCs adhered the alginate scaffolds with even Hoechst 33258 staining in the nuclei (**Figure 2**). No red fluorescence and weak blue fluorescence of Hoechst 33258 were observed in the alginate scaffolds from the control animals.

Differentiation of BMSCs on the alginate scaffolds

Immunohistochemistry was used to evaluate the differentiation of BMSCs in the alginate scaffold-BMSC complex. Brown granules around BMSCs were observed in the alginate scaffold-BMSC complex from the experimental animals (**Figure 3A**). No brown granules were observed in the alginate scaffolds from the control animals in which only inflammatory cells were found (**Figure 3B**).

BMSCs on the alginate scaffolds produced glycogen in vivo

The results of glycogen staining showed red staining of glycogen in the cytoplasm of BMSCs from the experimental group animals (**Figure 4A**), suggesting that the BMSCs along the alginate scaffold-BMSC complex had glycogen storage function. No red staining was observed in the alginate scaffolds from the control animal. Infiltration of inflammatory cells was observed in the alginate scaffolds from the control animal (**Figure 4B**).

Rats transplanted with alginate scaffold-BMSC complex had significantly higher survival rate than rats transplanted with alginate scaffolds

A total of 2 rats of the experimental group died after the surgery and the survival rate was 80%. The control group animals had a survival rate of 50%. Based on log-rank analysis, the survival rate of animals of the experimental group was significantly higher than that of the control group ($P<0.05$) (**Figure 5**).

Rats transplanted with alginate scaffold-BMSC complex had significantly better liver function than rats transplanted with alginate scaffolds

No significant difference in the liver function was identified between the two groups of ani-

Table 1. Comparison of the liver function between the two groups of rats ($\bar{x}$ ± s. *n*=10)

Time	Group	ALT (U/L)	AST (U/L)	ALB (g/L)
Before operation	Experimental	32.8±4.6	25.6±5.7	27.8±6.3
	Control	34.2±3.7	24.2±5.6	26.7±3.0
1 d after surgery	Experimental	175.3±28.4	156.7±30.1	15.8±6.5
	Control	168.2±18.7	171.3±12.8	16.1±5.1
14 d after surgery	Experimental	35.6±8.7	40.5±9.6	23.8±5.2
	Control	53.1±10.2*	45.2±7.4	27.9±6.7

*P<0.05 *vs* the control group. The experimental group rats were transplanted with alginate scaffold-BMSC complex. The control group rats were transplanted with alginate scaffolds alone.

mals before surgery and on the 1st day after surgery (P>0.05). The liver function of rats of the experimental group on the 14th day after surgery was significantly better than that of the control animals (P<0.05) (**Table 1**).

Discussion

Acute liver failure, cirrhosis, liver cancer, and other liver diseases are still serious challenges to public health with high mortality rates. Since the 1990s when the concept of tissue engineering was proposed, hepatic tissue engineering has been extensively studied for the treatment of end-stage liver diseases. Currently, artificial liver that can be used *in vivo* is highlighted in the study of hepatic tissue engineering [6].

Reliable source and sufficient count of cells are critically important in hepatic tissue engineering. BMSCs can differentiate into hepatocytes under certain condition *in vitro* due to its high plasticity. In addition, BMSCs that can differentiate into live stem cells and hepatocyte are also important source for liver repair and reconstruction after injury. Therefore, BMSCs are promising seeding cells used in hepatic tissue engineering.

Natural matrix materials have been widely used in tissue engineering because of their good biocompatibility and affinity [7, 8]. Alginic acid, a block polymer consisting of guluronic and mannuronic acid, is an acidic polysaccharide extracted from brown algae. Alginic acid can bind with divalent metal ions to form reticulated hydrogel, which is highly hydrophilic and permeable to nutrient diffusion [9]. In addition, the hydrolysates of alginic acid are not toxic to humans. Our previous studies have found that alginate scaffolds are a suitable base for the growth of BMSCs. BMSCs can adhere and expand well on alginate scaffolds. In addition, a number of growth factors such as HGF can induce BMSCs to differentiate into cells with characteristics of hepatocytes, suggesting that BMSCs and alginate scaffolds are promising seeding cells and carriers for hepatic tissue engineering.

Currently, the use of BMSCs for the treatment of liver dysfunction is mainly limited to cell transplantation through portal vein injection, spleen injection, intraperitoneal injection, or intrahepatic injection. These results showed that BMSCs transplantation significantly improved the liver function in animals of liver dysfunction. However, BMSCs transplantation can cause many problems such as portal vein thrombosis, pulmonary or hepatic artery embolization, splenic rupture when a large amount of cells are transplanted [10]. In the present study, we used a combination of three-dimensional porous scaffolds and cell transplantation technology to improve the liver function in animals undergone 70% of hepatectomy. The alginate scaffold-BMSC complex has the advantage of providing sufficient space for the adhesion, growth, and differentiation of a large amount of BMSCs. In addition, the efficiency of cell transplantation can be further increased by optimizing the composition and morphology of the scaffolds.

Large amounts of liver-derived cytokines, such as transforming growth factor-α (TGF-2α), endothelial cell growth factor (EGF), and hepatocyte growth factor (HGF) are secreted by the liver undergone 70% of hepatectomy. These growth factors are critically important for liver regeneration [11].

Enrichment of these growth factors in liver of dysfunction promotes the differentiation of BMSCs into hepatocyte. After BMSCs are transplanted into injured liver, the expression of a number of genes in BMSCs are induced by the growth factors, which promotes the differentiation of BMSCs into hepatocyte of appropriate function and improve the regeneration of injured liver [12]. The microenvironment in rats

of liver dysfunction induces BMSCs differentiation on artificial supporting materials and improves the liver function *in vivo*, which is the basis of the application of BMSCs-derived artificial liver tissues *in vivo*.

Our results showed that BMSCs on the alginate scaffolds transplanted into the liver produced albumin and glycogen, suggesting that these BMSCs have preliminary function of hepatocytes. In addition, the survival and liver function of the experimental group rats were significantly higher than that of the control group rats. These results suggest that BMSCs can differentiate into hepatocytes in vivo and BMSC-derived artificial liver tissue can improve the liver function in rats of liver dysfunction. Construction of artificial liver tissue that can be used in vivo is a major goal in hepatic tissue engineering. Our study provides basis for the application of BMSCs-derived artificial liver tissue in vivo.

Based on immunohistochemistry assay and glycogen staining, we detected the expression of albumin and the synthesis of glycogen in BMSCs on the alginate scaffolds after 4 weeks of the transplantation of alginate scaffold-BMSC complex into animal livers. While we observed that some cells attached onto alginate scaffolds in the control group rats, no expression of albumin and synthesis of glycogen were detected in these cells. Expression of albumin and synthesis of glycogen are two specific and important activities of hepatocytes, suggesting that the BMSCs on alginate scaffolds were able to differentiate into hepatocytes in rats. In addition, we observed that the survival rate and liver function of rats of the experimental group were significantly higher than the control group rats ($P<0.05$). The liver function of rats of the experimental group on the 14th day after hepatectomy and alginate scaffold-BMSC complex transplantation was even higher than that of the control group rats, suggesting that BMSCs on the alginate scaffold were able to differentiate into hepatocytes and improve the liver function in rats of liver dysfunction.

Stable and lasting labeling of BMSCs is important in studies of BMSC differentiation in vivo. In the present study, we used CM-DiI to label BMSCs. CM-DiI is a lipophilic fluorescent dye, which can be easily integrated and freely move in cell membranes to label whole cells. In addition, CM-DiI is a non-cytotoxic dye, having limited influence on the survival and growth of labeled cells [13]. In the present study, we detected strong fluorescence in CM-DiI-labeled BMSCs in vivo. In addition, the CM-DiI-labeled BMSCs proliferated well on alginate scaffolds. No significant cytotoxic effects of CM-DiI dying on BMSCs were found. Strong fluorescence was detected in CM-DiI-labeled BMSCs on alginate scaffolds were detected even for 4 weeks. The fluorescence distributed along the alginate scaffolds. Taken together, these results suggest that CM-DiI is an appropriate fluorescence dye for tracking the growth and differentiation of BMSCs *in vivo*.

In summary, we developed an alginate scaffold-BMSC complex, in which BMSCs adhered and grew on the alginate scaffolds. We transplanted the alginate scaffold-BMSC complex into liver wound in SD rats undergone 70% of hepatectomy and evaluated the growth and differentiation of BMSCs in vivo, as well as the liver function and survival of the rats. Our results suggest that the alginate scaffold-BMSC complex is an efficient artificial liver. Our study provides a basis for the application of alginate scaffold-BMSC complex in the treatment of end-stage of liver diseases. However, there are still a number of critical questions needed to be addressed, such the efficient delivery of alginate scaffold-BMSC complex into livers and differentiation of BMSCs into hepatocytes of full function.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ruiyun Xu, Department of Hepatobiliary Surgery, The Third Affiliated Hospital of Sun Yat-Sen University, Guangzhou 510630, China. Tel: +86-20-85253333; Fax: +86-20-85253336; E-mail: xuruiyun520@163.com

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