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Effects of coencapsulation of hepatocytes with adipose-derived stem cells in the treatment of rats with acute-on-chronic liver failure

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Introduction: *Cell transplantation is an alternative to liver transplantation, which is hampered by short survival time and immunorejection. The goal of this study was to evaluate the therapeutic potential of hepatocytes coencapsulated with adipose-derived stem cells (ADSCs) in treating acute-on-chronic liver failure (ACLF).*

Methods: *Rat hepatocytes and ADSCs were isolated, and coencapsulated by alginate-poly-L-lysine-alginate microencapsulation. The morphological and functional changes of heterotypic interactions were characterized. ACLF in rats was induced by D-galactosamine administration following CCl4-induced cirrhosis. These rats were subjected to intraperitoneal transplantation of 5×10^7 coencapsulated hepatocytes with ADSCs, 5×10^7 encapsulated hepatocytes alone, or empty vehicles after 24 h, respectively. The survival rate and liver functions were assessed.*

Results: *Hepatocyte performance levels such as albumin secretion and urea synthesis induction were all significantly enhanced in the coencapsulation group compared with the homo-encapsulated hepatocytes group ($p<0.05$). The results of cell cycle analysis showed that larger populations of hepatocytes with ADSC treatment were accumulated in the G2-S phase, and there were fewer in the G0-G1 phase compared to encapsulation of hepatocytes alone. Intraperitoneal transplantation of coencapsulated hepatocytes with ADSCs not only increased the survival rate, but also improved liver functions in a rat model of ACLF.*

Conclusions: *Transplantation of coencapsulated hepatocytes and ADSCs might be a promising strategy for cell-based therapy of acute liver diseases.*

Keywords: *Hepatocytes, Adipose-derived stem cells, Cell transplantation, Acute-on-chronic liver failure, Encapsulation*

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INTRODUCTION

Acute-on-chronic liver failure (ACLF) is the most common type of liver failure in China. In a large-scale study from 2002 to 2007 in China, of 1977 patients with liver failure, 91.70% (1813/1977) were diagnosed as ACLF (1). The introduction of orthotopic liver transplantation has been a major advance, resulting in significant improvement in patient survival (2). However, the limited supply of transplantable organs has prevented this modality from being available to all. It has therefore become necessary to develop alternative approaches for the treatment of this disease. Considering that encapsulated hepatocyte transplantation can prevent immunologic damages thanks to the encapsulation membrane and does not require immunosuppression after transplantation, it represents a promising approach for the treatment of acute liver diseases. It is well known that primary mature hepatocytes are terminally

differentiated and have very poor proliferation potential once isolated. In order to be practical, the encapsulated hepatocytes must remain hepatocyte-specific functions for a sufficient length of time. Many studies have been performed to improve hepatocyte-specific functions by targeting the intrahepatic microenvironment through co-culturing hepatocytes with different cell types including liver-derived or non-liver derived mesenchymal cells (3-5). One such approach is coculture of hepatocytes with bone marrow mesenchymal stem cells (MSCs). Given that MSCs could provide the stromal supporting scaffold by secreting crucial cytokines and endogenous extracellular matrix components (6), they could be considered ideal candidates; however, the amount of available bone marrow is usually low and the procurement procedure is invasive. On the other hand, adipose-derived stem cells (ADSCs) display biological properties similar to BMSCs (7) and can also be induced to differentiate into various mesenchymal cell types (8-10), including hepatic cells. Unlike BMSC, ADSCs are present in abundance in the body; they can be repeatedly harvested, and the procedure is simple and minimally invasive. These cells can be easily expanded and manipulated *in vitro*.

In this study, we investigated the functional changes in hepatocytes by coencapsulation of hepatocytes with ADSCs, and further examined whether transplantation of coencapsulated hepatocytes and ADSCs could enhance the ability of hepatocytes to alleviate ACLF.

MATERIALS AND METHODS

ADSCs isolation and characterization

All animal procedures were approved by the Animal Care Ethics Committee of Nanjing Medical University and Nanjing DrumTower Hospital, as well as in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes for Health (NIH Publication 85-23, revised 1996). Sprague-Dawley female rats (180-200 g) fat tissue was washed extensively with phosphate buffered saline (PBS). It was cut and minced to small pieces, and digested with 0.1% type I collagenase (Sigma-Aldrich, USA) under gentle shaking for 60 min at 37°C. DMEM/F12 containing 10% FBS was yhen added to the reaction to neutralize the enzyme activity. Cell suspension was filtrated sequentially through a 100 μm and a 40 μm nylon mesh, and washed via three centrifugations (50 g), then resuspended in complete culture medium. Cells were incubated at 37°C in a humidified chamber containing 5% CO2 for 24 h. The adherent cells were further expanded with medium change at 3-day intervals. The phenotype of rat ADSCs was evaluated by flow cytometry analysis (FACS, Becton Dickinson, USA). Antibodies against following cell surface markers were used: phycoerythrin (PE)-conjugated CD45 (Antigenix America, USA), CD90, CD105, CD44, CD34 (Becton Dickinson, USA). Isotypic antibodies served as the control.

Hepatocytes isolation

Hepatocytes were harvested by a two-step *in situ* collagenase perfusion procedure. In brief, after being perfused with 0.05% Type IV collagenase (GIBCO, Grand Island, NY, USA), the liver was minced in cold DMEM-LG and digested to free hepatocytes. The hepatocytes were filtered through a sterile 100 μm nylon mesh and washed with DMEM-LG for three times by centrifugation (50 x *g*). Following the last wash, the hepatocytes were kept on ice. Cell viability was assessed by trypan blue exclusion.

Coencapsulation of hepatocytes with ADSCs

A total of 1 mL of freshly isolated hepatocytes (1×10^6 cells/ml) was mixed with 1 mL of ADSCs (5×10^5 cells/ml), followed by the addition of equal volume of sterile filtered 4% alginate (Sigma-Aldrich, USA) dissolved in Ca^{2+} free DMEM (GIBCO, USA). Alginic acid sodium salt was gradually dissolved in NaCl (pH 7.4) to give a 2% w/v solution and sterilized, and then was diluted with DMEM to yield 4% solution. The suspension was extruded through a droplet generator NISCO encapsulator (NISCO, Zurich, Switzerland), and sprayed into 100 mmol/l $CaCl_2$ solution (Sigma-Aldrich, St Louis, MO, USA). The resulting beads formed after 10 min were washed with 4°C 0.9% NaCl solution (Sigma-Aldrich, USA) and treated with 1% poly-L-lysine for 8 min. After two washes, the beads were immersed in a 0.15% alginate solution for 10 min and washed again. The beads were then incubated in 50 mmol/l sodium citrate solution (Sigma-Aldrich, USA) for 10 min to dissolve the alginate core. Microencapsules containing hepatocytes and ADSCs were obtained and cultured using DMEM supplemented with 10% fetal bovine serum. As a control, microencapsulated hepatocytes were also cultured in the absence of ADSCs. All medium samples were

collected every day and stored at -80°C for further analysis. However, for the *in vivo* study, since cells that comprise more than 5% of the original liver mass could be sufficient to complement the damaged liver mass, the coencapsulated cell density was increased from 1.5×10^6 cells/ml to 2.5×10^7 cells/ml.

Tests of hepatocyte-specific functions

The amount of albumin secreted into the culture medium was determined by enzyme-linked immunosorbent assay (ELISA) using purified goat anti-albumin and horseradish peroxidase-conjugated antibody (Bethyl Laboratories, Montgomery, TX, USA). The absorbance was measured at 490 nm with Cytofluor multiwell plate reader (Benchmark, Greenville, SC, USA). Urea synthesis was analyzed using a commercial kit (Biochain Institute, Neward, CA, USA) according to the manufacturer's instructions.

Cell cycle analysis of hepatocytes

For cell cycle analysis, ADSCs were pre-labeled with 10 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Life Technologies, Carlsbad, CA, USA) for 10 min at 37°C followed by an additional incubation in fresh medium for 30 min prior to co-encapsulation with hepatocytes. Thus, cell cycling of hepatocytes as CFSE-negative cells in the co-encapsulation system could be distinguished from CFSE-labeled ADSCs by flow cytometry. Hepatocytes were stained with the CycleTEST PLUS DNA reagent kit (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. The cell cycle profiles were analyzed by FACScan flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

Rat model of ACLF

ACLF models were created in rats by CCl4 (Sigma-Aldrich, USA) intraperitoneal injection for ten weeks, followed by venous injection with D-galactosamine (D-gal) (Sigma-Aldrich, USA). The rats were injected with CCl4 dissolved in olive oil (10%) into the abdominal cavity twice every week. Doses of CCl4 were modified according to the index of liver function and the body weight. At ten weeks, two rats from each group were killed and the presence of cirrhosis was confirmed. Then the remaining rats were injected with D-gal at a dose of 0.70 g/kg BW to induce ACLF.

Transplantation of coencapsulated rat hepatocytes and ADSCs

Transplantation of coencapsulated rat hepatocytes and ADSCs was performed 24 h after D-galactosamine administration. The rats were randomly divided into three groups. In group 1 (n = 10), 2 mL empty capsules were injected into the peritoneum. In group 2 (n = 10), 5×10^7 encapsulated hepatocytes were injected into the peritoneum. In group 3 (n = 10), 5×10^7 coencapsulated hepatocytes with ADSCs were injected into the peritoneum. The animals were monitored for the following 7 days.

Blood biochemistry and survival rate

Blood samples were taken from the rat's angular vein. The blood samples were measured with an automatic analyzer (Hitachi 7600, Tokyo, Japan) for alanine aminotransferase (ALT), aspartate aminotransferase (AST), prothrombin time, plasma ammonia, and bilirubin levels. Survival was observed for 7 days.

Histopathology

Liver tissues collected from experimental rats 24 h after D-galactosamine injection and 7 days after transplantation were fixed by formalin, embedded in paraffin, and then cut and stained by hematoxylin-eosin (HE).

Statistical analysis

All values were expressed as the mean ± SD. The two-tailed unpaired Student's *t*-test or one-way analysis of variance (ANOVA) was used to evaluate the statistical significance of differences which was set with a p value <0.05. The survival rates were analyzed using Kaplan-Maier method and compared using a log-rank test.

RESULTS

Cell morphology

ADSCs showed spindle-shaped, fibroblast-like morphology at passage 5. Flow cytometric analysis revealed that

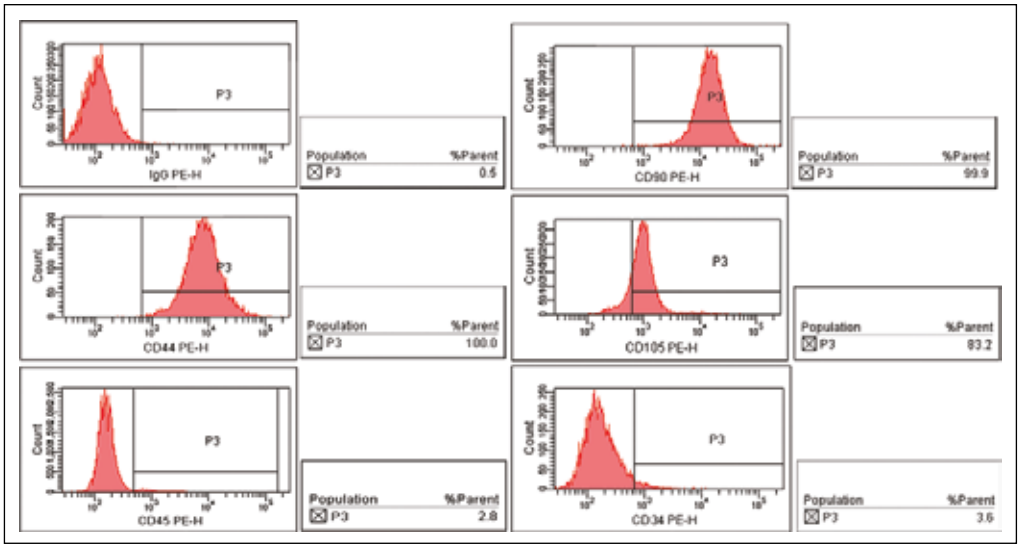

Fig. 1 - *The FACS results showed few ADSCs expressed CD45 and CD34, and that over 90% of cells expressed CD44, CD90 and CD105.*

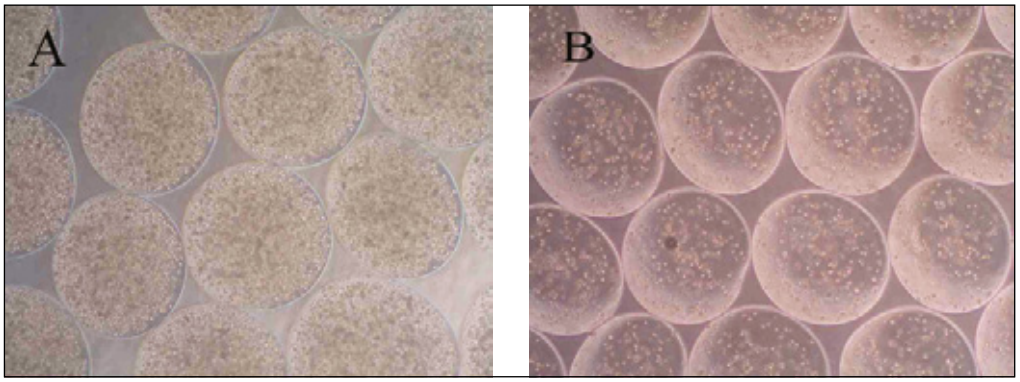

Fig. 2 - ***(A)*** *Hepatocytes and ADSCs were encapsulated in alginate-PLL-alginate microencapsulation on day 0 (magnification 100×).* ***(B)*** *Hepatocytes were encapsulated in alginate-PLL-alginate microencapsulation on day 0 (magnification 100×).*

few ADSCs expressed CD45 and CD34, and that over 90% of cells expressed CD44, CD90 and CD105 (Fig. 1). Figures 2A and 2B showed that each encapsulated bead was round in shape and identical in size with a mean diameter of 600 μm to 800 μm, containing 1500 cells and 1000 cells, respectively. The ratio of ADSCs to hepatocytes was 1:2. Immediately after encapsulation, the viability of encapsulated hepatocytes and co-encapsulated hepatocytes with ADSCs was 90% and 93%, respectively. After 7 days culture, viability of encapsulated hepatocytes and co-encapsulated hepatocytes with ADSCs was reduced to 30% and 61%, respectively.

Hepatocyte-specific functions

Hepatocyte-specific functions were evaluated by measuring albumin secretion and urea synthesis. Albumin secretion by hepatocytes in the coencapsulation group was about 3-fold the secretion by the encapsulated hepatocytes control ($p<0.05$). The production of albumin reached a peak on day 2 and then slowly decreased (Fig. 3A). In addition, the time-course profile of urea nitrogen level paralleled that of albumin secretion (Fig. 3B).

Cell cycle analysis of hepatocytes

To further investigate whether ADSCs increased the cell cycle progression of hepatocytes, cell cycle distributions of hepatocytes in coencapsulation as well as encapsulation of hepatocytes alone were analyzed by flow cytometry. As illustrated in Figures 4A and B, larger populations of hepatocytes with ADSC treatment were accumulated in the G2-S phase, and fewer in the G0-G1 phase compared to encapsulation of hepatocytes alone.

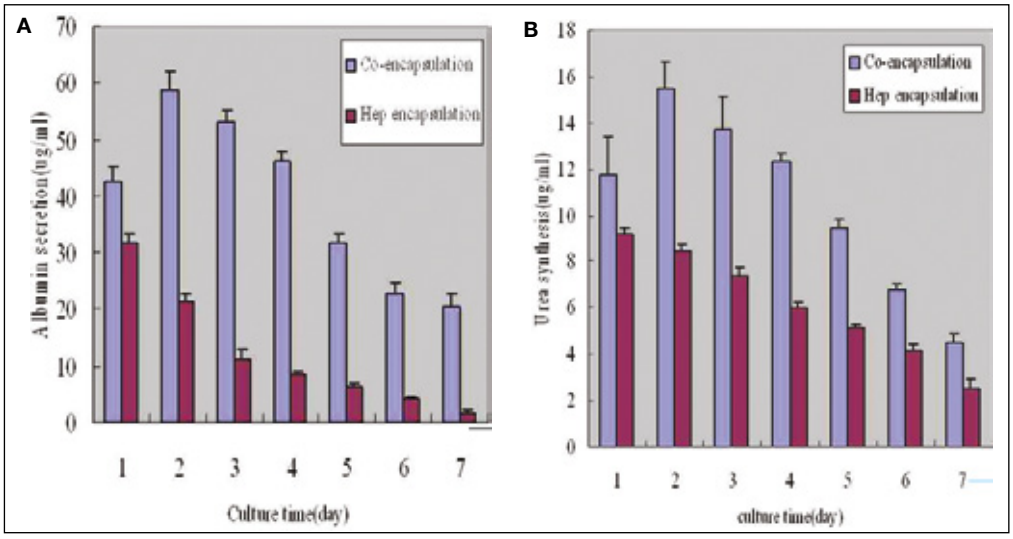

Fig. 3 - ***(A)*** *Albumin secretion rate in coencapsulation of hepatocytes and ADSCs group was significantly higher than encapsulation of hepatocytes group and gradually decreased over time (p<0.05).* ***(B)*** *Albumin secretion of hepatocytes in coencapsulation group was about 3 fold of the control.* ***(B)*** *Time-course profile of urea nitrogen levels paralleled that of albumin secretion.*

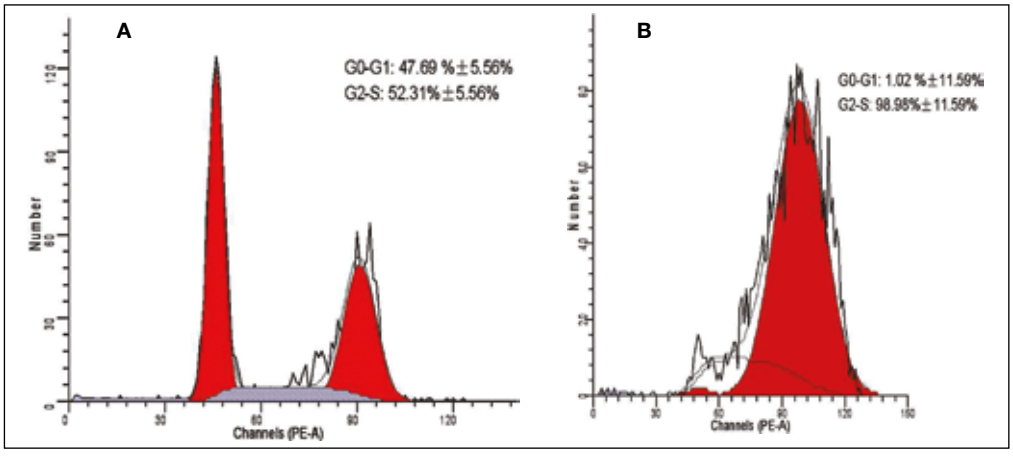

Fig. 4 - *Cell cycle distributions of hepatocytes were analyzed by flow cytometry for encapsulation of hepatocytes group* ***(A)*** *and co-encapsulation group* ***(B)****. Larger populations of hepatocytes from co-encapsulation group were accumulated in G2-S phase (98.98% ± 11.59%) compared with encapsulation of hepatocytes group (52.31 ± 5.56%).*

Survival rate in rats with acute liver failure

The animal survival rate for each group is shown in Figure 5A. No rat died in the first 24 h following D-galactosamine injection. The 72-h survival rates for group 1, 2, and 3 were 30%, 70%, and 80%, respectively. The 120-h survival rate was two or six times higher in group 3 than the other two groups. The 7-day survival rate was 60% (6/10) in group 3, 30% (3/10) in group 2, and 10% (1/10) in group 1 (control group). The survival rate of group 3 was significantly longer compared with the other groups (p<0.05).

Liver functions in rats with acute liver failure

To determine the effect of transplantation of coencapsulated hepatocytes and ADSCs on acute liver failure in rats, we measured ALT, AST, prothrombin time, plasma ammonia and bilirubin levels. In group 1, venous ALT level reached its peak of 1910.5 ± 135.2 IU/l at 72 h after D-galactosamine administration, with most of mortality occurring in the first 72 h. In contrast, the ALT levels peaked at 36 h in groups 2 and 3 with the levels of 1573.1 ± 81.9 IU/l and 1113.2 ± 103.4 IU/l, respectively, and then gradually decreased to almost normal levels 7 days after transplantation, with slower decrease in group 2. In addition, the ALT levels of group 3 were significantly lower than group 1 and 2 (p<0.05) as shown in Figure 5B. Similar patterns were observed for AST (Fig. 5C). We found that in group 1, PT level reached its peak of 20 s at 72 h after D-galactosamine administration, with most of mortality occurring in the first 72 h. In contrast, the PT levels peaked at 48 h and 120 h in groups 2 and 3 with the levels of 16.7 s and 14.8 s, respectively, and then

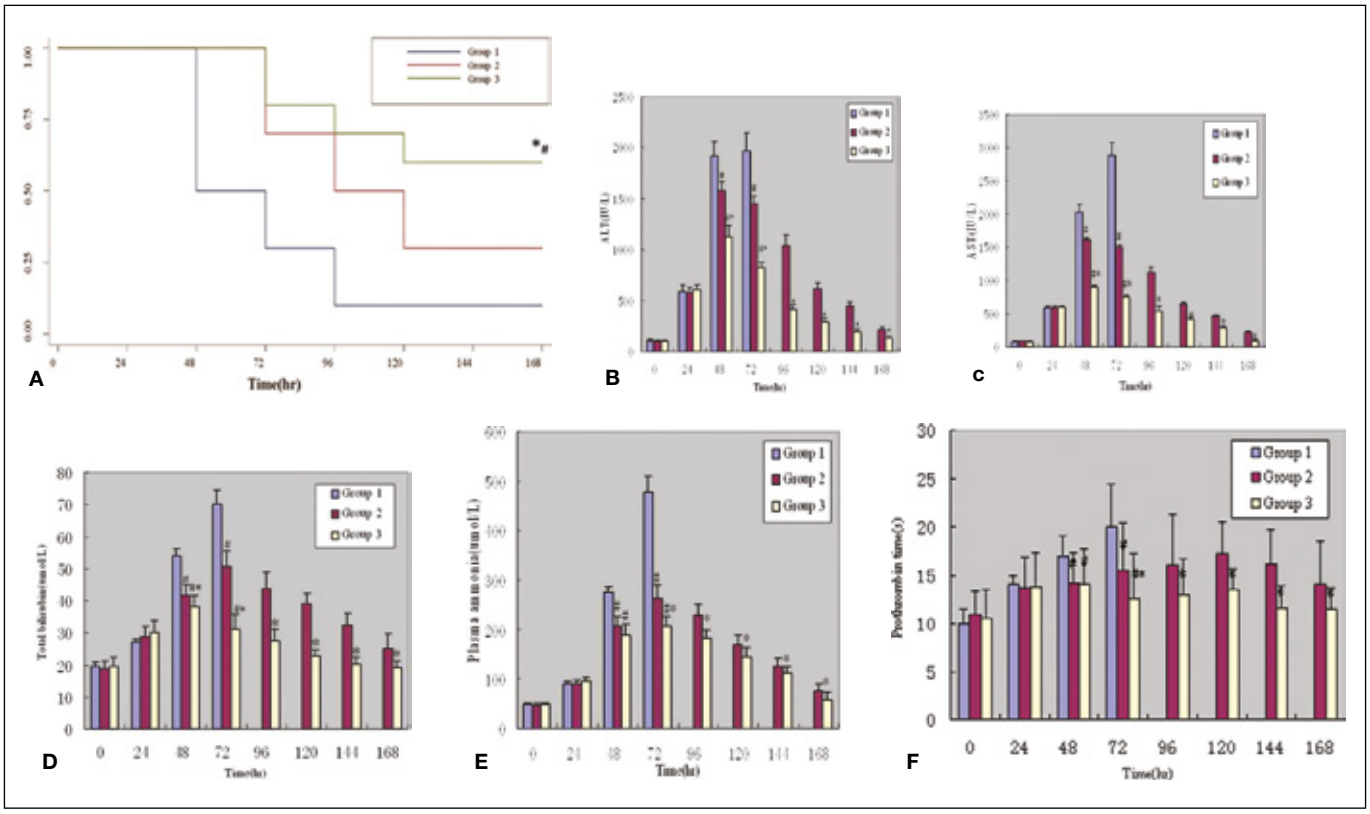

Fig. 5 - ***(A)*** *survival rate;* ***(B)*** *ALT level;* ***(C)*** *AST level;* ***(D)*** *total bilirubin level;* ***(E)*** *plasma ammonia level;* ***(F)*** *and prothrombin time in rats with acute liver failure. Date are means ± SD, n = 3. #p<0.05 compared to group 1. *p<0.05 compared to group 2. The animal survival rate in the first 24 h following D-galactosamine injection were 100%. The 7-day survival rate was 60% (6/10) in group 3, 30% (3/10) in group 2, 10% (1/10) in group 1 as a control. The survival rate of group 3 was significantly longer compared with the other groups. Due to rat death, some data points were missing for group 1.*

gradually decreased to almost normal levels 7 days after transplantation (Fig. 5F). Total serum bilirubin concentration increased in group 3 to 40 ± 3.9 μmol/l from 0 to 48 h and then returned to normal levels at 120 h (Fig. 5D). However, the bilirubin levels in groups 1 and 2 were significantly higher compared with group 3 ($p<0.05$). Additionally, the plasma ammonia levels in group 3 were significantly lower than those in group 2 from 72 h to 168 h ($p<0.05$) (Fig. 5E) and the levels of plasma ammonia in group 1 were significantly higher than the other groups ($p<0.05$).

Histopathology

Histopathology of native liver tissues demonstrated massive hemorrhage and extensive hepatocyte necrosis after D-galactosamine administration (Fig. 6A). In contrast, surviving rats started to recover native liver tissues 7 days after transplantation (Fig. 6B).

DISCUSSION

Cell transplantation is a promising therapy for acute liver disease (11). Unfortunately, this approach suffers from several drawbacks, including immune allograft rejection, short-term viability, and rapid phenotypic de-differentiation of cells in conventional culture system (12-13). Thus, encapsulated cell transplantation has been extensively studied. Hepatocyte encapsulation inside a membrane prevents hepatocytes from contacting with immune cells, immunoglobuins and complement factors (14). Buhler LH et al have demonstrated that transplantation of encapsulated hepatocytes or encapsulated cryopreserved hepatocytes could improve survival during acute liver failure (15-17). Moreover, coencapsulation of hepatocytes and MSCs would improve viability and function of hepatocytes *in vitro* (18).

In the present study, we successfully coencapsulated hepatocytes with ADSCs in alginate-PLL-alginate coated

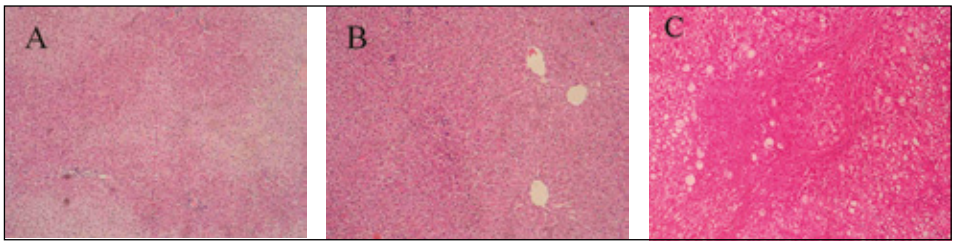

Fig. 6 - ***(A)*** *Histopathology of native liver tissue demonstrated massive hemorrhage and extensive hepatocytes necrosis after D-galactosamine administration.* ***(B)*** *In constrast, surviving rats started to recover native liver tissue 7 days after transplantation (hematoxylin-eosin, 100×).* ***(C)*** *Liver tissue sections from rats treated with CCl4 showed formation of pseudolobe (hematoxylin-eosin, 40×).*

beads. Our results indicated that hepatocyte-specific functions such as albumin secretion and urea synthesis were all significantly enhanced in the coencapsulation group. The best hepatic function levels were achieved on day 2 and then moderately decreased. However, decreased albumin secretion and urea synthesis were observed for the encapsulated hepatocytes group. These results are consistent with several other studies showing that hepatocyte functions were maintained by other models of coculture (19-20). Interestingly, hepatocytes in the coencapsulation group demonstrated a higher viability after 7 days compared with encapsulated hepatocytes group. The results of cell cycle analysis showed that larger populations of hepatocytes with ADSC treatment were accumulated in the G2-S phase, and fewer were accumulated in the G0-G1 phase compared to encapsulation of hepatocytes alone. However, this experiment only determined the relative distribution of the cell cycle phases and did not give a mitotic index. Although most cell lines are growing, the amount of time spent in each cell cycle phase is variable. Mimicking the cell environment *in vitro* is of vital importance for reconstruction of tissue architectures and restoration of isolated hepatocyte functions (21). Previous studies showed that heterotypic cell interaction between parenchymal cells and nonparenchymal neighbors modulated cell growth and migration (22). Furthermore, some studies suggested that stem cell-based cadherin presentation might be an effective tool to induce hepatotrophic differentiation by coculture (23-24). In the coencapsulation of hepatocytes with ADSCs, cell-cell and cell-matrix contact were successfully established. Therefore, the preservation of hepatocyte functions seemed to be dependent on cell-cell contact interaction.

To test whether the transplantation of coencapsulated hepatocytes and ADSCs could enhance the ability of the hepatocytes to alleviate ACLF *in vivo*, coencapsulated hepatocytes and ADSCs as well as encapsulated hepatocytes were transplanted into the peritoneal cavity of rats. The results showed that transplantation of coencapsulated hepatocytes and ADSCs significantly increased survival rate to 60%. However, transplantation of encapsulated hepatocytes alone increased survival rate only from 10% to 30%. The 7-day survival rate was two times higher in transplantation of coencapsulated hepatocytes and ADSCs group than in transplantation of encapsulated hepatocytes group ($p<0.05$). A consistent trend was observed for liver function. The ALT and AST, markers of hepatocyte injury, were much lower in the co-encapsulation group. The results indicated that the hepatocytes in the co-encapsulation group appeared to be capable of compensating the hepatic function of the damaged liver, allowing the native liver to regenerate, suggesting that transplantation of coencapsulated hepatocytes and ADSCs may be an ideal treatment for acute liver failure.

The mechanisms of improved liver function may be due to cytokine production from transplanted ADSCs. It has been reported that ADSCs can secret several potentially beneficial growth factors, such as granulocyte-macrophage colony-stimulating factor, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), basic fibroblast growth factor, transforming growth factor-β, and insulinlike growth factor-1 (IGF-1) (25). ADSCs have also been shown to produce significantly more bioactive factors than BMSCs (26), therefore they may have an equal or even stronger regenerative effect on implanted tissues than BMSCs. Also, research has shown that ADSCs can inhibit the inflammatory reaction to reduce injury by reprogramming macrophages to attenuate sepsis or inhibit the respiratory burst of neutrophils (27). Moreover, some studies have shown that the regenerating hepatocytes in transplant recipients may be derived from MSCs that fused with host hepatocytes (28-29). However, recent reports have indicated that bone marrow cells and hematopoietic stem cells can convert into hepatocytes without fusion (30-31). Therefore, we believe that these three elements

play important cooperative role in the treatment of the ACLF model. It is necessary to verify the mechanisms of this treatment in further study.

Although microencapsules have an immunoisolation effect, the outcome of long-term *in vivo* use of microencapsules is still unknown in this study. The cells would be exposed on the membrane surface if microencapsules were broken by pressure, resulting in immunological damages. Thus, an optimal encapsulation method, such as the two-step encapsulation method (32), is warranted to resolve this issue.

In conclusion, coencapsulation of hepatocytes and ADSCs could improve hepatocyte-specific functions *in vitro*. Furthermore, transplantation of coencapsulated rat hepatocytes and ADSCs could enhance the ability of the hepatocytes to alleviate ACLF *in vivo*. Transplantation of coencapsulated rat hepatocytes and ADSCs might be a promising approach for cell-based therapy of acute liver diseases, which serves as a bridge to liver transplantation.

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