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Original Article

Efficacy and limitation of bone marrow transplantation in the treatment of acute and subacute liver failure in rats

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Aim: Recent reports have shown that bone marrow cells (BMC) retain the potential to differentiate into hepatocytes. Thus, the BMC have been recognized as an attractive source for liver regenerative medicine. However, it has not been clarified whether BMC transplantation can be used to treat liver damage *in vivo*. In the present study, we explored whether BMC possess therapeutic potential to treat acute and/or subacute liver failure.

Methods: Fulminant hepatic failure (FHF) was induced by 70% hepatectomy with ligation of the right lobe pedicle (24% liver mass), followed by transplantation of BMC into the spleen. Dipeptidyl peptidase IV-positive (DPPIV⁺) BMC were then transplanted into DPPIV-negative (DPPIV⁻) recipients following hepatic irradiation (HIR) in which 70% of the liver was resected and the remnant liver irradiated.

Results: There was no benefit of BMC transplantation towards survival in the FHF model. DPPIV⁺ hepatocytes appeared in the liver tissues of the DPPIV⁻ HIR model rats, but DPPIV⁺ hepatocytes replaced less than 13% of the recipient liver.

Conclusion: BMC transplantation may have limitations in the treatment of fulminant or acute liver failure because they do not have sufficient time to develop into functional hepatocytes. Preparative HIR may be beneficial in help to convert the transplanted BMC into host hepatocytes, and provide a survival benefit. Although, However, the precise mechanism warrants further studies.

Key words: bone marrow, fulminant liver failure, irradiation, liver regeneration

INTRODUCTION

With an ever-increasing shortage of donor organs for orthotopic liver transplantation, there is a significant need for alternative therapies for liver disease. Isolated hepatocyte transplantation has been successfully reported in experimental animals and in some clinical human cases.¹⁻³ However, the procedure of hepatocyte transplantation requires a great number of hepatocytes, and it is still uncertain as to whether or not the transplanted cells can actually engraft in the liver. Recent reports have shown that bone marrow cells (BMC) retain the potential to differentiate into a variety of non-hematopoietic cell lineages,⁴⁻⁸ including hepatocytes. Thus, BMC have been recognized as an attractive cell source for liver regenerative medicine. For instance, Sakaida *et al.*⁹ reported that BMC transplantation exhibited therapeutic potential by reducing liver fibrosis. This therapeutic potential against liver damage is considered to be due to differentiation to mature hepatocytes as well as improvement of intrahepatic micro-conditions. In addition, fusion between BMC and hepatocytes has been reported,^{10,11} in which the fusion of host hepatocytes and donor BMC can give rise to mature hepatocytes without *trans*- or dedifferentiation. Such fusion is a new concept, but it is still unknown whether fusion is only a morphological phenomenon or whether it has a significant effect towards regeneration of the liver. On the other hand, previous reports have shown that *in vivo* cell fusion is an unlikely explanation for the “*trans*-differentiation” of bone marrow-derived cells into differentiated phenotypes.^{12,13} In any case, investigation of BMC may contribute to the resolution of stem cells or progenitor cells which proliferate to mature hepatocyte, and may result in the promotion of cell transplantation study.

For clinical application, BMC transplantation has an advantage over other cell sources, for example, hepatocytes, embryonic stem cells and hepatic stem cells (oval cell or small hepatocytes), because: (i) the extraction technique of BMC has already been established by hematologists through bone marrow transplantation therapy; (ii) autologous transplantation can avoid rejection; and (iii) BMC may have the potential for self replication and aggressive proliferation.

In the present study, we explored whether BMC could have therapeutic potential in the two distinct, well-established, diseased liver models. One was the surgically induced fulminant liver failure model (FHF model) in which liver failure was caused by 68% hepatectomy followed by occlusion of the remnant liver lobes. The other was the hepatic irradiation model (HIR model), in which 68% hepatectomy was performed followed by irradiation of the remnant liver resulting in inhibition of liver regeneration.

METHODS

Animals

Adult male Sprague–Dawley (SD) rats and DPPIV-positive (DPPIV⁺) 344 rats were purchased from Japan SLC (Shizuoka, Japan), while DPPIV-negative (DPPIV⁻) 344 rats were purchased from Charles River Japan (Tokyo, Japan). All animal care and procedures were performed with the approval of the Nagasaki University Institutional Animal Care and Use Committee.

Surgical animal models

Induction of FHF model

Fulminant liver failure was induced as described previously by ourselves.^{14,15} Briefly, the abdomen of male SD rats weighing 270–350 g (7–9 weeks old) was entered through a midline incision. The common pedicle to the right lobes of the liver (24% of the liver) was ligated, and the two anterior liver lobes (68% of the liver) were removed using the standard Higgins and Anderson technique.¹⁶ The two omental liver lobes (8% of the liver) were left intact (Fig. 1). All surgical preparations and euthanasia were performed under general (diethyl-ether) anesthesia using sterile surgical technique. At the completion of the surgical procedure and every 12 h after the surgery, each FHF animal received a s.c. bolus of 10 mL of 5% dextrose in normal saline.

Induction of HIR model

The HIR model was generated as described previously.¹⁷ Anesthesia was induced by i.p. injection (0.5 mL/kg pentobarbital). After aseptic preparation, 68% partial hepatectomy was performed through a midline incision. Immediately after partial hepatectomy, the animals were placed in a supine position on a surgical board. A jig with a 5 cm × 7 cm irradiation port was aligned to this platform. Two 1 cm × 2 cm lead shields, each 2 mm in thickness, were wedged under the liver to protect the stomach, kidney, spine and intestines, taking care not to compress the hepatic and aortic vessels. In addition, two lead shields were placed above the chest and the lower abdomen. A Toshiba EXS-300-5 was used (200 kVP, 10 mA, 0.5 mm aluminum, filtration, 43.5 cm SSD; dose rate, 3.2 Gy/min). The abdomen was then closed in two layers.

Bone marrow transplantation

Bone marrow cells were obtained from DPPIV⁺ rats by flushing from the femurs with Dulbecco's modified Eagle medium (Sigma-Aldrich Japan, Tokyo, Japan) using a 21-G needle. The cells were filtered through a cell strainer (Falcon catalog no. 352350) and centrifuged at 200g for 5 min at 4°C, as previously described.⁴ After washing with phosphate-buffered saline (PBS), pH 7.4, the cell pellet was suspended in 10 mL of lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM ethylene diamine tetra acetate). After centrifugation, cells were washed twice in PBS, and the viability of BMC was confirmed to be more than 80% using Trypan blue dye exclusion.

A small left subcostal incision was made and the spleen was exposed. BMC (2×10^6 cells suspended in 0.5 mL of physiological saline) were injected under the fibrous capsule of the spleen. The numbers of transplanted BMC referred to the numbers of bone marrow and hepatocyte transplantation, as described previously.^{4,9,18-20} The abdomen was closed in two layers.

Experimental design

BMC transplantation to FHF model rats

Fulminant liver failure-induced rats underwent intrasplenic injection of BMC (group I; $n = 12$) or normal saline (group II; $n = 12$). Eight rats in each group were monitored until death to determine the survival time, while the other four rats in each group were killed at 12 h after the BMC transplantation for blood analysis. The experiment for the survival rate and the experiments for biochemical blood analysis were separately done.

BMC transplantation to HIR model rats

Two days after induction of HIR, BMC (group A; $n = 15$) or normal saline (group B; $n = 20$) were injected into the spleen. Long-living rats were killed at 1, 3 or 5 months after the operation.

Postoperative evaluation

Blood chemistry

Blood samples were analyzed for total bilirubin (T-Bil) levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the clinical laboratory.

Morphological evaluation (DPPIV staining)

After death of the rats, tissues were rapidly cooled and stored at -80°C until analysis. To detect DPPIV enzyme expression, cryostat sections ($5\ \mu\text{m}$ thick) were made. DPPIV stain was performed as previously described.²¹ Briefly, sections were fixed for 5 min in 95% ethanol 5% glacial acetic acid (99:1 v/v) at 0°C to -10°C , followed by a 5-min wash in 95% ethanol at 4°C . Air-dried slides were incubated for 10–20 min at 37°C in the substrate solution: 2.5 mg Gly-Pro-4-methoxy-b-naphthylamide (Sigma-Aldrich Japan) dissolved in 150 mL of dimethylformamide and mixed with 5 mL of a solution of Fast Blue BB salt (Sigma-Aldrich Japan) in 0.1 M Tris maleate, 0.1 M NaCl, pH 6.5. The slides were rinsed two times in 0.14 M NaCl, incubated for 2 min in 0.1 M CuSO_4 , and rinsed again in 0.14 M NaCl. The slides were fixed for 10 min in cold 4% paraformaldehyde/PBS/5 μM MgCl_2 and washed in 0.14 M NaCl. The slides were washed in water and counterstained with Harris hematoxylin. Finally, the percentage of DPPIV⁺ cells were counted using Scion image software (Scion Image Beta 4.03 for Windows XP version; Scion, Baltimore, MD, USA) from three independent rats.

Statistical analyses

Data were analyzed statistically using Mann–Whitney *U*-test and Welch test where appropriate. $P < 0.05$ was considered significant. Data are presented as the mean \pm standard deviation.

RESULTS

BMC transplantation to the FHF model rats

Survival time

In FHF rats, more than 90% of them died within 48 h (survival time, 33 ± 9 h), but mature hepatocyte transplantation was able to prolong the survival time (73 ± 22 h).¹⁵ However, BMC transplantation did not prolong the survival time. The survival times of group I (30 ± 11 h) versus group II (28 ± 14 h) were not significantly different, and the 48-h survival rate for each group was 12.5% (Fig. 2).

Blood chemistry

The average serum T-Bil levels (group I, 2.93 ± 0.72 mg/dL; group II, 2.82 ± 0.95 mg/dL), AST (group I, 5484 ± 1627 IU/L; group II, 6803 ± 3700 IU/L) and ALT activities (group I, 3008 ± 1531 IU/L; group II, 4123 ± 3868 IU/L) were not significantly different (Fig. 3). As for the HIR model, data from group A was only available 24 h after treatment: T-Bil 0.93 ± 0.49 , AST 1007 ± 223 and ALT 457 ± 140 .

BMC transplantation to the HIR model rats

Survival rate

Thirteen of 15 rats in group A survived up to 1 month, but 13 of 20 rats in group B did not survive up to 1 month. The survival times of group A (127 ± 109 days) versus group B (35 ± 50 days) were significantly different. The 150-day survival rate of group A (74.2%) was significantly greater than group B (17.1%) (Fig. 4).

DPPIV staining

Morphologically, clusters of DPPIV⁺ hepatocytes were found in the liver of rats in group A (Fig. 5a) but there were no DPPIV⁺ cells in the liver in group B. The DPPIV⁺ hepatocytes in the rats in group A were considered to be bone marrow-derived hepatocytes that replaced a maximum of 13% of the recipient liver (Fig. 5b).

DISCUSSION

We tested whether BMC have the potential to support the diseased liver in two distinct, well-established animal models. One is the surgically induced FHF model, in which liver failure is caused by 68% hepatectomy followed by occlusion of the remnant liver lobes. The other is the HIR model, in which 68% hepatectomy is performed followed by irradiation of the remnant liver resulting in fatal liver failure associated with inhibition of liver regeneration. Of note, the HIR model does not emulate human liver disease, but an artificial model to show proliferative advantage to the transplanted cells. Radiation induces critical deterioration in the liver microenvironment that is conducive to the selective proliferation of normal, transplanted hepatocytes as demonstrated previously.^{17,20} The precise mechanisms underlying this phenomenon have yet to be elucidated. Radiation is known to inhibit liver growth for extended periods of time; thus, its effect would appear to be similar, at least in principle, to that exerted by retrorsine; blocking that would block of the endogenous hepatocyte cell cycle

Using these models, we demonstrated that BMC may have the potential to morphologically *trans*-differentiate into hepatocytes in the HIR model. However, our study also demonstrated potential limitations of BMC transplantation in the treatment of fulminant liver failure. In the FHF model, previous studies have demonstrated that mature hepatocyte transplantation can provide survival benefit in liver failure.^{15,22} However, a large number of hepatocytes are necessary to adequately treat liver disease. On the other hand, some investigators have suggested that BMC transplantation can be used to treat chronic liver diseases through its regenerative potential.⁹ Therefore, we investigated the potential of BMC transplantation to treat FHF in an animal model of acute liver failure induced by the loss of a large amount of hepatocytes followed by lethal systemic inflammation.

As a result, BMC transplantation did not exhibit any survival benefit in rats in this FHF model. This may be because BMC require certain conditions and/or adequate time to differentiate into functional hepatocytes. Regarding the numbers of transplanted BMC, we injected 2×10^6 cells under the fibrous capsule of the spleen. In previous reports, the numbers of transplanted cells ranged from 10^6 to 10^7 cells. However, the injection of too many cells can sometimes cause venous thrombosis. Furthermore, our strategy required fewer cells to treat liver damage; namely, 2×10^6 BMC were considered sufficient. Accordingly, while BMC transplantation may be promising, acute liver failure may not be a good target for this treatment strategy.

Similar to previous reports that have indicated the potential of BMC to differentiate into various types of cells, DPPIV staining revealed that

several clusters of hepatocytes exhibited the features of BMC derived from transplanted donor cells in the HIR model. It remains unclear whether transplanted BMC were *trans*-differentiated into hepatocytes or were fused with the host hepatocytes. However, the presence of DPPIV⁺ cells in the HIR model indicates the possibility that these cells are committed stem cells of the liver. This is because the DPPIV⁺ hepatocytes are morphologically hepatocytes, but their potential may include self-replication and vigorous proliferation. Therefore, we anticipate the therapeutic potential of BMC against acute liver injury. In the present study, total replacement of the liver by BMC was not observed, however, several rats in the BMC transplantation group survived longer than those of the control group. Although the etiology of the survival benefit remains unknown, we speculate that BMC transplantation can contribute not only to the repopulation of hepatocytes but also to the improvement of the internal environment of the liver. For instance, it is hypothesized that BMC can contribute to the suppression or resolution of fibrosis in liver cirrhosis or can activate hepatic mesenchymal cells. The “fibrolysis” effect of BMC transplantation has been demonstrated.⁹ Further investigations of the contribution of BMC transplantation to improve the intrahepatic micro-condition are required.

Furthermore, these results suggest that the HIR model itself can be a useful model for cell transplantation in which high-dose irradiation of the liver can injure the host hepatocytes and impart a selective proliferative advantage to the unirradiated donor cells.^{21,23} Histologically, extensive loss of hepatocytes occurs in the irradiated liver, and various degrees of micro- and macrovesicular steatosis in the centrilobular areas. Furthermore, preparation of partial hepatectomy induces several chemical growth factors to regenerate the remnant liver. We speculate that this particular condition may lead to selective growth of transplanted cells. Thus, the HIR model could be applied to clinical cases, such as congenital or metabolic liver disease, by transplantation of normal cell sources. Cell transplantation therapy is expected to become a novel and effective therapy against liver diseases, as an alternative to liver transplantation. However, cell transplantation therapy requires a large number of donor cells that are able to survive in the host liver. In general, a sufficient number of mature hepatocytes cannot be provided, but stem cells may solve this problem. Among the currently available stem cell sources, BMC have several advantages, as previously described. To further elucidate this, further studies concerned with the evaluation of pure bone marrow stem cells and the etiology of *trans*-differentiation to hepatocytes are needed. Furthermore, it is still unknown whether “so called” bone marrow-derived hepatocytes are actually *trans*-differentiated from BMC or fused with BMC. We are currently investigating the cell fusion phenomenon to determine the contribution of BMC to support liver failure.

In conclusion, BMC transplantation may have limitations in the treatment of these models of fulminant or acute liver failure because they do not have sufficient time to develop into functional hepatocytes. Preparative HIR may be beneficial and help convert the transplanted BMC into host hepatocytes to provide survival benefit, although the precise mechanism warrants further investigation.

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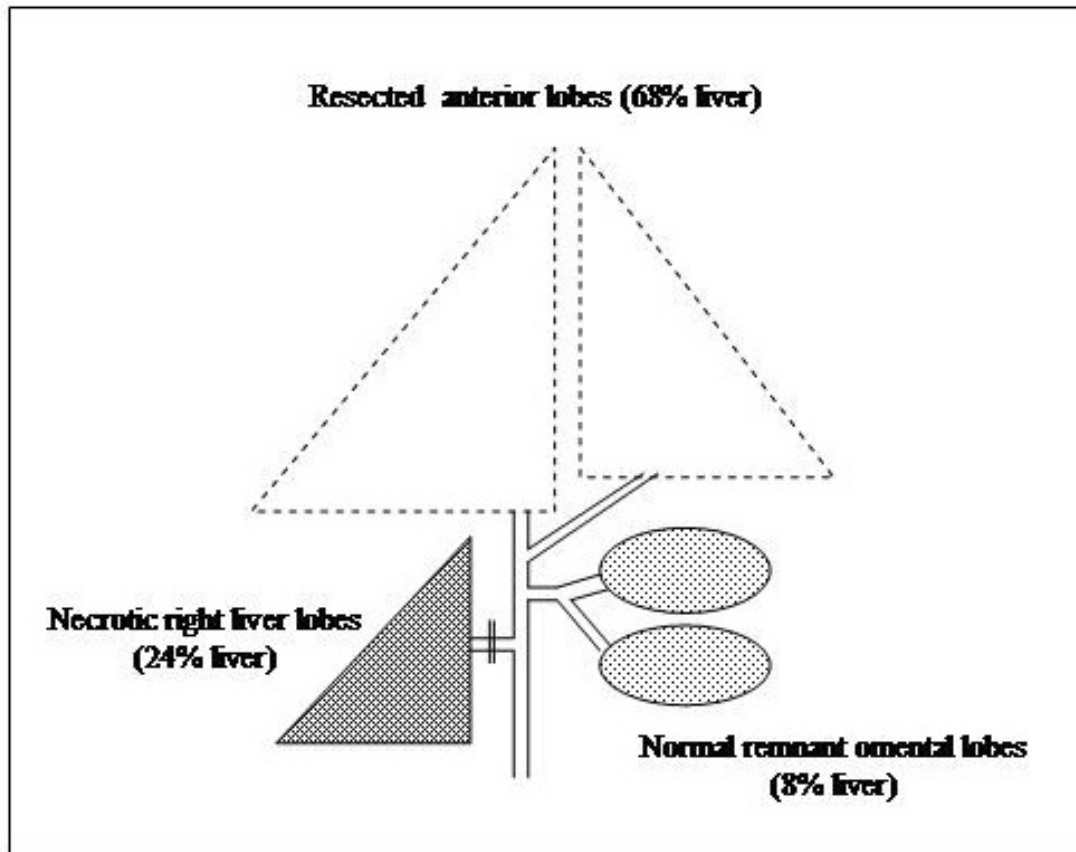


Figure 1 Schematic diagram depicting the technique used to induce fulminant hepatic failure in this model. Two anterior lobes (68%; median lobe and left anterior lobe) were resected and the right lobes (24%) were rendered necrotic. Only the omental lobes (8%) were left intact.

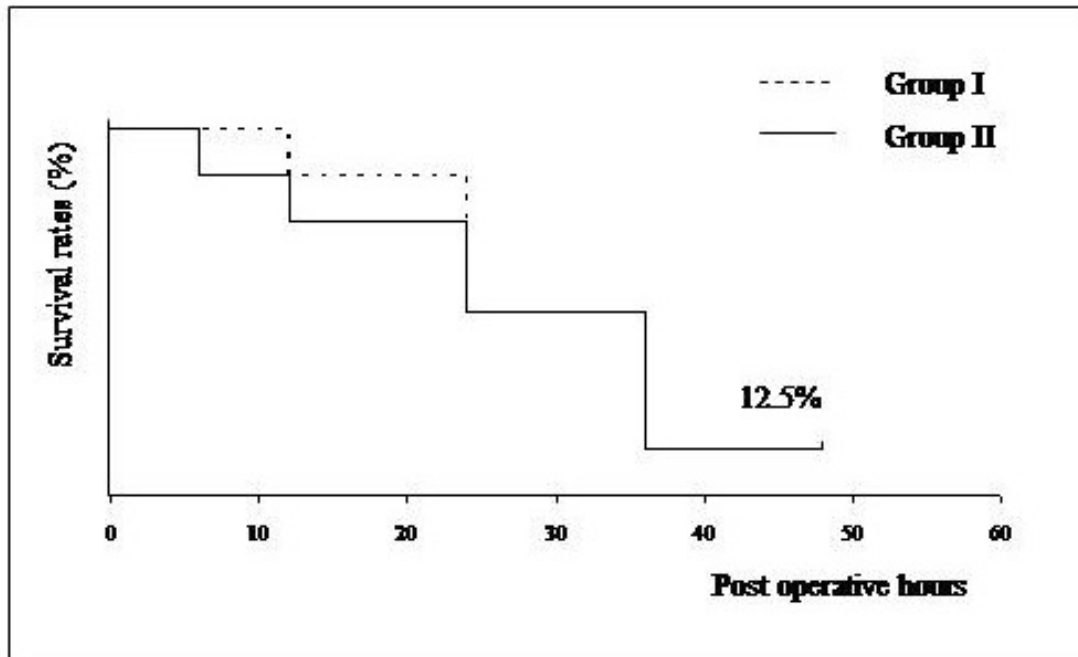


Figure 2 Effect of BMC in the treatment of FHF model. Comparison of the survival rate for Group I vs. Group II was not significant (12.5%). The mean survival time was 30 ± 11 and 28 ± 14 h for Group I and Group II, respectively. Group I; Acute liver failure with BMC, Group II; Acute liver failure without BMC.

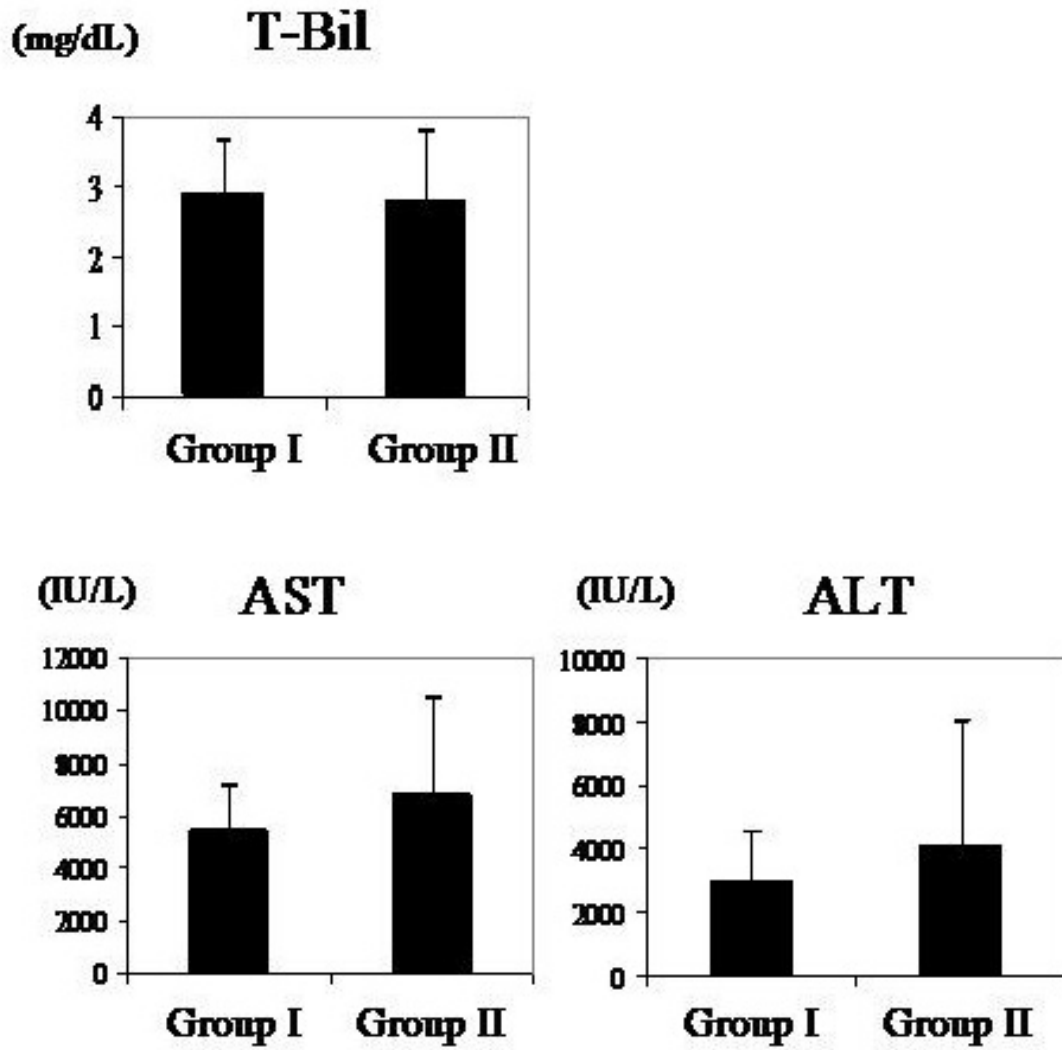


Figure 3 Biochemical changes after BMC treatment in the FHF model. Between groups I and II, the average serum T-Bil levels, and AST and ALT activities 12h after treatment were not significant.

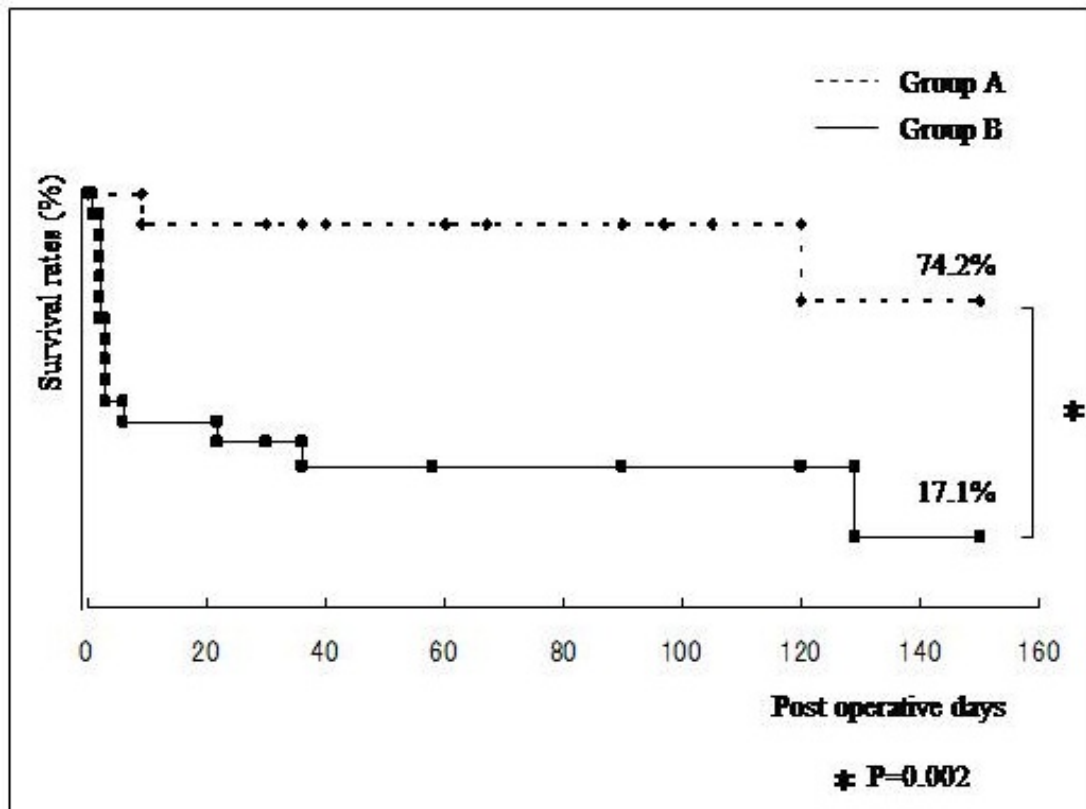


Figure 4 Effect of BMC in the treatment HIR model. The survival rate of group A (74.2%) vs. group B (17.1%) was significantly improved ($P = 0.002$).

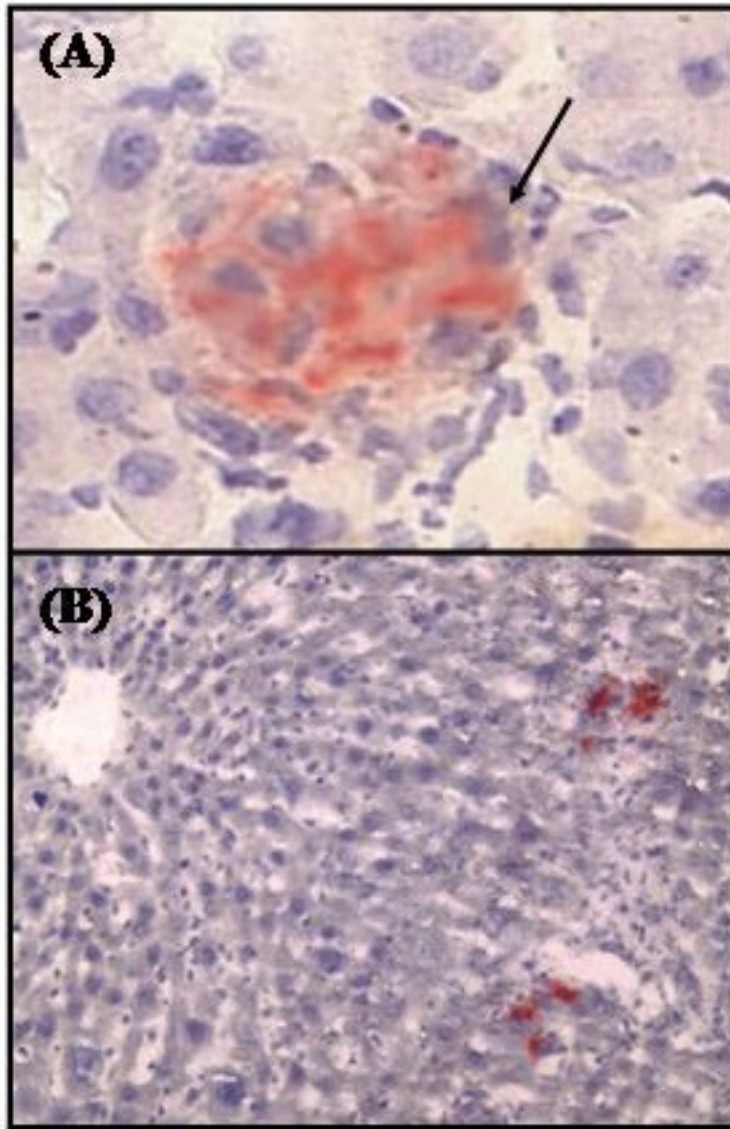


Figure 5 (a) Histological presence of bone marrow-derived hepatocytes. Clusters of DPPIV⁺ hepatocytes were found in the livers of rats from group A (arrow), but no DPPIV⁺ cells were found in the livers of group B rats. (b) DPPIV⁺ hepatocytes replaced the recipient liver to a maximum of 13%.