J Gastrointest Canc (2011) 42:11–19
DOI 10.1007/s12029-010-9218-0

ORIGINAL ARTICLE

Simultaneous Injection of Autologous Mononuclear Cells with TACE in HCC Patients; Preliminary Study

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Published online: 3 November 2010

Abstract
Background The discovery of the pluripotent stem cells made the prospect of cell therapy and tissue regeneration a clinical reality, especially with the evidence of contribution of the stem cells of bone marrow origin in hepatic regeneration. Infusion of bone marrow stem cells before trans-arterial chemoembolization may help to increase liver volume and consequently increase hepatic reserve in patients with HCC, and this may improve the outcome of this procedure.
Materials and Methods Four Child B class patients with unresectable hepatocellular carcinoma treated by trans-arterial chemoembolization were injected with autologous bone marrow mononuclear layer containing stem cell in the hepatic artery feeding the contralateral lobe of the liver in the same session, follow-up of the patients was done by doing liver profile and CT liver volumetry before the surgery and 3 months later.
Results We observed that patients receiving stem cell therapy simultaneously with TACE had shown a significant improvement in biological and volumetric parameters of liver function compared to those historically reported of patients receiving TACE only who usually shows deterioration of liver parameters.
Conclusion BMC infusion into the hepatic artery synchronized with TACE for patients with chronic liver disease complicated with HCC is safe, feasible, and demonstrated an improvement in both biological and radiological volumetric parameters.

Keywords Chemoembolization · Stem cell therapy · HCC · Autologous bone marrow stem cells

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Introduction

Hepatocellular carcinoma (HCC) is a highly malignant tumor with a very high morbidity and mortality rate worldwide, carrying a poor prognosis due to its rapid infiltrating growth besides its usual existence on top of a cirrhotic liver [1]. If left untreated, more than 90% of patients die within 5 years of diagnosis [2]. Surgical resection, liver transplantation, and cryosurgery are regarded as potentially curative treatment for HCC, but most patients are not suitable candidates [3]. Currently the local interventional therapy of liver tumor has been rapidly evolving, which includes transarterial chemoembolization (TACE), percutaneous ethanol injection (PEI), radiofrequency ablation, laser-induced thermotherapy, and microwave coagulation therapy [4–13].

TACE is one of the most common forms of interventional therapies and seems most effective against encapsulated small HCCs without extracapsular invasion, whereas

in large HCCs, viable residual tumor cells remain and the tumor frequently recurs [14–16].

Moreover, in patients with large lesions, multiple TACE sessions are necessary to control tumor growth but may increase the risk of worsening hepatic function through damage to non-cancerous liver parenchyma [15, 17]. TACE is a technique that utilizes intra-abdominal infusion of chemotherapy then blocking (embolizing) the small blood vessels with different types of compounds such as gel foam. This deprives the tumor of its needed blood supply, which can result in damage or death of the tumor cell and makes the disease less severe. Despite of being incurative, TACE can be applied only in patients with relatively preserved liver function as its use may lead to liver failure in individuals with poor liver function [18].

Thus, TACE was introduced as a palliative treatment in patients with unresectable HCC. Nowadays, it has become one of the most common forms of interventional therapies [19].

TACE is most effective for disease in which nodules are less than 5 cm in diameter, involve <50% of the liver, and are encapsulated [20]. Larger HCC lesions can be more effectively treated with a combination of TACE and PEI. The presence of a capsule significantly enhances the chances of success and is an important requirement when patients are selected for combined TACE and PEI therapy [21].

The patient also needs to have adequate liver and renal function as another important requirement for TACE therapy. Side effects such as abdominal pain, vomiting, and fever (associated with tumor necrosis) are frequently associated with TACE. This is also referred to as post-embolization syndrome. The side effects are short term and usually disappear within a few days [22]. Patients who also have cirrhosis are at risk of deterioration of liver function, which may result in development of ascites, jaundice, encephalopathy or, rarely, death. Furthermore, patients who undergo repeated procedures may develop progressive liver atrophy. If several courses of TACE are performed in a short period of time, the patient's quality of life may worsen. Complications of TACE that have been reported in the literature include gastrointestinal hemorrhage, ischemic cholecystitis, encephalopathy, ascites, arterial and portal thrombosis, bile leakage, dissection of the hepatic artery, and hepatic decompensation resulting in death [23].

The discovery of the pluripotent stem cells made the prospect of cell therapy and tissue regeneration a clinical reality, especially with the evidence of contribution of the stem cells of bone marrow origin in hepatic and pancreatic cell regeneration [24]. There are at least two types of stem cells in the human bone marrow: mesenchymal stem cells and hematopoietic stem cells (HSCs). HSCs are CD34+ and CD133+ and they can give rise to all lineages of blood cell differentiation. Furthermore, in vivo transdifferentiation of human HSCs to functional hepatocytes has been demonstrated [25]. Also, it has been shown that infusion of bone marrow stem cells to animal models of liver cirrhosis can lead to regression of liver fibrosis [26]. Recently, portal administration of autologous CD133+ HSCs was reported to accelerate liver regeneration [27].

We hypothesized that infusion of bone marrow stem cells (BMSC) before TACE may help to increase liver volume and consequently increase hepatic reserve in patients with HCC, and this may improve the outcome of this procedure through decreasing the possibility of decompensation which may follow TACE.

Materials and Methods

The study was approved by our faculty ethics committee. All patients included in this study were aware of their inclusion in either of the groups, and all signed an informed consent approved by the ethical committee; patients who refused to receive stem cell transplantation were included as a control group receiving only chemoembolization.

Both groups were matched together regarding age, Child–Pugh score, and tumor size. All fulfilled the following criteria: Child–Pugh class B and all of them possible candidates for TACE with future liver remnant volume (FLRV) below 25% of the total liver volume (TLV), exclusive of tumor volume.

Selection of Patient

Four consecutive new patients with diagnoses of unresectable hepatocellular carcinoma that were based on histology, cytology, or persistently elevated serum alpha-fetoprotein levels (≥400 ng/ml) with typical imaging findings were considered for entry into this single-center, open-label trial. Patients were not included if they refused to participate or had one or more of the following criteria: poor hepatic function (presence of hepatic encephalopathy, ascites not controlled by diuretics, history of variceal bleeding within the last 3 months, a serum total bilirubin level over 3 mg, a serum albumin level below 2.5 g/l, or a prothrombin time of more than 4 s over the control), serum creatinine level of over 1.5 mg/l, history of previous treatment for the tumor or acute tumor rupture, and presence of extrahepatic metastasis or vascular contraindications to chemoembolization (hepatic artery thrombosis, main portal vein thrombosis, or arteriovenous shunting).

Transarterial Chemoembolization TACE Technique

The patients underwent transarterial lipiodol chemoembolization after a standard protocol. The patients were prepared by fasting for 6 h and shaving of pubic hair.

Procedure was done via femoral puncture (usually the right side is used) while the patient is lying supine on the table of angiography machine. Then a thin catheter (usually 5 Fr in diameter) was advanced over a hydrophilic guidewire till we catheterize the hepatic artery supplying the lobe harboring the hepatocellular carcinoma. This was followed by water-soluble contrast injection through the catheter to identify the location of HCC blush and its feeder(s) which is then selectively catheterized. Then the cytotoxic lipiodol homogenous mixture was injected through the catheter into the feeder until the tumor blush is saturated by the mixture with an average dose of 1 mg/kg and maximum dose of 100 mg/session. Adriamycin was used as cytotoxic agent to which HCC is sensitive in all patients. After that, for selective embolization of the feeder by injection of embolizing material, injection is done slowly to avoid reflux into other unwanted branches, and injection is stopped when stasis is observed within the feeding artery. Discharge from the hospital was decided according to the clinical state and absence of complications.

Preparation and Characterization of BMSC

In accordance with the faculty ethical regulations and after obtaining patients' informed consent, the procedure of harvesting bone marrow for readministration of selected cells was performed in a closed system. Autologous bone marrow aspirated from the posterior iliac crest was drawn in heparin-coated syringes after the induction of anesthesia. The procedure is complete after 120 ml of bone marrow has been aspirated from four penetrations of the left ileum and four penetrations of the right ileum. Then the concentration procedure by Harvest centrifuge was performed outside the sterile field; upon completion of the processing cycle, excess plasma was removed by aspiration until no further volume could be aspirated.

Using the 20-ml syringe with the blunt cannula, the remaining volume was removed from the chamber; 2–3 ml was aspirated into the syringe and gently expressed back into the chamber in order to resuspend the cellular components back into the plasma. This action was repeated four or five times until the volume has a uniform consistence, and then the entire volume was aspirated into the syringe, and the cannula was removed and attached to a sterile syringe cap and saved in a syringe labeled "BMC". A blunt needle was attached to the syringe to transfer the BMC back into the sterile field for infusion. The enriched mononuclear cells were ready for intrahepatic application. Cells were resuspended in a total volume of 80 ml of phosphate-buffered saline solution.

Transplantation of BMSC

After local anesthesia, puncture of right femoral artery was performed and 5-Fr sheaths were inserted. A Simon catheter was advanced to the descending aorta, and catheterization of celiac axis and then hepatic artery was performed.

The mean duration of catheterization was 9.5 m (range 5–15 m). Nonionic low osmolal radiocontrast agent was used to visualize the hepatic artery. Bone marrow stem cells were selectively applied to the hepatic artery of other hepatic lobe free from tumors as equal aliquots of 10 ml, taking an average time of 10 min. After that, the catheter was flushed with 10 cm^3 of normal saline and the procedure was finished. After stem cell infusion, the catheter and the sheath were removed.

Volumetric Study

Patients underwent helical computed tomography (CT) to estimate liver volume prior to bone marrow cell injection, first time before the procedure and another time 3 months later, to determine the degree of induced hypertrophy.

CT volumetry was performed by an expert radiologist blinded to the patients' identity. First triphasic CT examination of the liver was done including arterial, porto-venous, and delayed phase, and then the source images are transferred to a workstation connected to the CT machine where the volumetric measurement was done using the porto-venous phase in which the hepatic veins were well demarcated so the right and left lobes could be separated using the plane of the middle hepatic vein as an anatomical landmark. The aim was to calculate the volume of the hepatic lobe free from tumor and in which stem cells will be injected; the margin of the targeted lobe was traced and marked section by section by means of an electronic cursor.

To enhance the accuracy of volumetric measurement, large vessels such as extrahepatic portal vein at the porta hepatic and the IVC as well as large fissures such as fissure for ligamentum teres were excluded from volumetric marking because they have no metabolic function and therefore cannot be added to the measured volume.

Then the workstation software calculated the number of pixels included within the traced contours in each section and provided the cross-sectional area of the measured lobe on a section-by-section basis. Then the circumscribed areas are multiplied by the CT section thickness, yielding the volume of each section, and the volumes of all sections were summed to give the total volume of the targeted lobe.

Table 1 Baseline characteristics of four patients who received stem cell therapy

	Patient 1	Patient 2	Patient 3	Patient 4
Age	47	53	61	51
Gender	Male	Male	Male	Male
Etiology of cirrhosis	Hepatitis C	Hepatitis C+bilharziasis	Hepatitis C	Hepatitis C
Medication	Spironolactone+frusemide	Spironolactone	Spironolactone+frusemide	Spironolactone+frusemide+DDB
Size of focal lesion	5×7 cm	5×6 cm	4×7 cm	4×8 cm
Lobe containing the focal lesion	Lt. lobe	Rt. lobe	Rt. lobe	Lt. lobe
Associated disease	Diabetes mellitus	Hypertension	None	Diabetes mellitus+hypertension

Results

Follow-up started immediately after the procedure. No side effects were observed except for mild pain at the bone marrow needle puncture site. Biochemical evaluation was done 1 month and 3 months after transplantation including liver enzymes AST, ALT, bilirubin, prothrombin time, and albumin level. Volumetric study was repeated 3 months after surgery using helical CT interpreted by the same radiologist.

A group of four patients were randomly selected to show their baseline data (Table 1). Clinical and laboratory data before and after simultaneous injection of autologous bone marrow stem cells were recorded (Table 2). Clinically significant improvement in edema and ascites (Table 2) and Child score (Fig. 1) was observed. Improvements in liver function tests were also observed (35% average increase in serum albumin, 14% average decrease in PT, 31% average decrease in total bilirubin, 29% average decrease in ALT, and an average 55% decrease in AFT) (Tables 2 and 3). Radiological volumetric evaluation demonstrated an average 33% increase in injected lobes (Table 4) (Fig. 2a, b).

Discussion

Hepatocellular carcinoma is the sixth most common neoplasm in the world [28], and its incidence is increasing worldwide [29]. Overall, HCC is associated with liver cirrhosis in 80% of cases, and it is the leading cause of death among cirrhotic patients [30]. The prognosis of hepatocellular carcinoma is correlated with factors related both to the extent of tumor and hepatic function [31].

The treatment for hepatocellular carcinoma remains difficult because of the advanced tumor stage at diagnosis and the associated cirrhosis. No single available therapy is applicable to all patients, and the treatment should be catered according to the clinical state of the individual al patient. Hepatic resection offers a chance of cure for a minor proportion of patients with early tumor and preserved liver functions. In these patients, an anticipated future liver remnant volume below 25% of the total liver volume leads to an increased risk of postoperative morbidity and mortality [32].

The majority of the patients with unresectable hepatocellular carcinoma are treated by various palliative therapies. Transarterial chemoembolization is the most

Table 2 Clinical data of the patients at baseline and at the end of follow-up (3 months)

	Patient 1		Patient 2		Patient 3		Patient 4	
	Baseline	3 months	Baseline	3 months	Baseline	3 months	Baseline	3 months
Edema	+2	+1	+1	None	+2	+1	+2	+1
Ascites	Moderate	Mild	Moderate	US detected	Moderate	None	Moderate	Mild
Serum albumin (g/dl)	2.6	3.5	2.7	3.6	2.9	4	2.5	3.2
PT (s)	14	12	13	12	14	12	15	13
Cr (mg/dl)	1.4	1.2	1	0.9	1.2	0.8	2	1.7
Total bilirubin (mg/dl)	2.5	2.2	2.2	1	2	1.2	2.8	2.3
Direct bilirubin (mg/dl)	1.8	1.1	1	0.9	1.2	0.8	2	1.7
AST (IU/ml)	53	64	49	44	74	55	75	78
ALT (IU/ml)	45	44	66	42	64	35	78	54
AFP (μg/l)	320	110	189	87	432	312	45	12

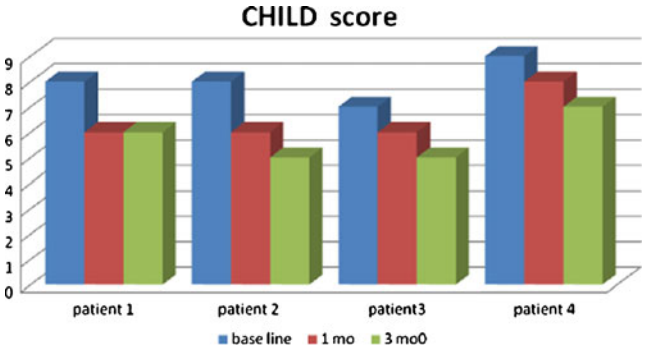

Fig. 1 Changes in Child score before and after stem cell injection. Significant score increase was observed 3 months after treatment ($P<0.0016$). Data were analyzed using paired Student's T test

widely used treatment in patients with HCC who are considered unsuitable candidates for surgery and/or ablative therapies [33]; it combines the effect of targeted chemotherapy [5, 6] with that of ischemic necrosis induced by arterial embolization [33].

In fact, the benefits of the procedure should not be offset by treatment-induced liver damage as the possible impairment of liver function is a critical point when assessing TACE feasibility.

Severe side effects of TACE are related mainly to treatment-induced ischemic damage in the non-tumoral liver since it can precipitate or exacerbate liver failure, especially in cirrhotic patients of advanced Child–Pugh class. Acute hepatic decompensation has been estimated to occur in 20%, and about 3% of patients have irreversible hepatic decompensation associated with high doses of chemotherapy, high basal levels of bilirubin, prolonged prothrombin time, and advanced cirrhosis [34].

Based on the ability of stem cells to differentiate into specific cell types according to their environment, cell transplantation has become an attractive therapeutic method for the treatment of patients with liver disease. To further accelerate liver proliferation levels, we consequently followed accumulating evidence for the contribution of extrahepatic stem cells (SCs) like hematopoietic progenitor cells participating in the concert of liver regeneration [35].

Table 3 Statistical analysis of liver function tests

Parameter	Before (mean)	After (mean)	P value
Serum albumin (g/dl)	2.675	3.575	0.0008
Prothrombin time (s)	14	12.25	0.003
Total bilirubin (mg/dl)	2.37	1.67	0.037
Direct bilirubin (mg/dl)	1.5	1.12	0.05
AST (IU/ml)	62.75	60.25	0.72
ALT (IU/ml)	63.25	43.25	0.05
AFP (μg/l)	245.5	130.25	0.04

Data were analyzed using paired Student's T test

Table 4 Volumetric changes in liver lobes before and after stem cell injection

	Before		After	
	Lt. lobe	R. lobe	Lt. lobe	Rt. lobe
Patient 1			TACE	SCT
	550	800	550	1,280
Patient 2			SCT	TACE
	700	950	1,022	950
Patient 3			SCT	TACE
	762	750	786	750
Patient 4			SCT	TACE
	557	700	695	700

Statistical analysis (paired Student's T test) demonstrated significant size increase in lobes injected with stem cells ($P<0.04$). No changes were observed in TACE-treated lobes

Bone marrow (BM) cells have been shown experimentally to participate in liver proliferation after hepatic resection [27].

Furthermore, it has been postulated that hematopoietic progenitor cells are able to transdifferentiate into both hepatocytes and bile duct cells [36]. Mobilization of peripheral hematopoietic CD34+ SCs (known to bear the capacity for differentiation into a hepatic lineage) after liver resection in oncologic patients has been demonstrated and was 10-fold higher compared with liver-sparing abdominal surgery [37]. These data indicate a possible role for BM-derived SCs in liver proliferation after substantial loss of liver mass. Although as yet inconclusive, the therapeutic potential of hematopoietic SCs seems to be an attractive prospect for liver repair after acute or chronic hepatic injury [38].

Currently discussed mechanisms of the stem cell-induced hepatic regeneration are cell–cell fusion, stem cell conversion to liver cells as transdifferentiation without fusion, and endogenous hepatic regeneration triggered by stem cell-provided trophic factors such as interleukin-6 [39, 40].

Hepatic engraftment from extrahepatic progenitor cells is accelerated in cases of liver damage if contrasted with non-injured liver tissue [41]. So in our approach, hepatic homing of BMSC in the contralateral lobe of the liver is improved by chemoembolization of the lobe containing the tumor, and the latter may represent a strong stimulus for liver proliferation of non-embolized segments.

In this study, we tried to evaluate the effect of autologous bone marrow mononuclear cell transplantation into the hepatic artery of patients with unresectable hepatocellular carcinoma treated by TACE. In the present study, we infused autologous BMCs into the hepatic artery and it was found to be feasible and not associated with serious local side effects except for mild pain at the bone

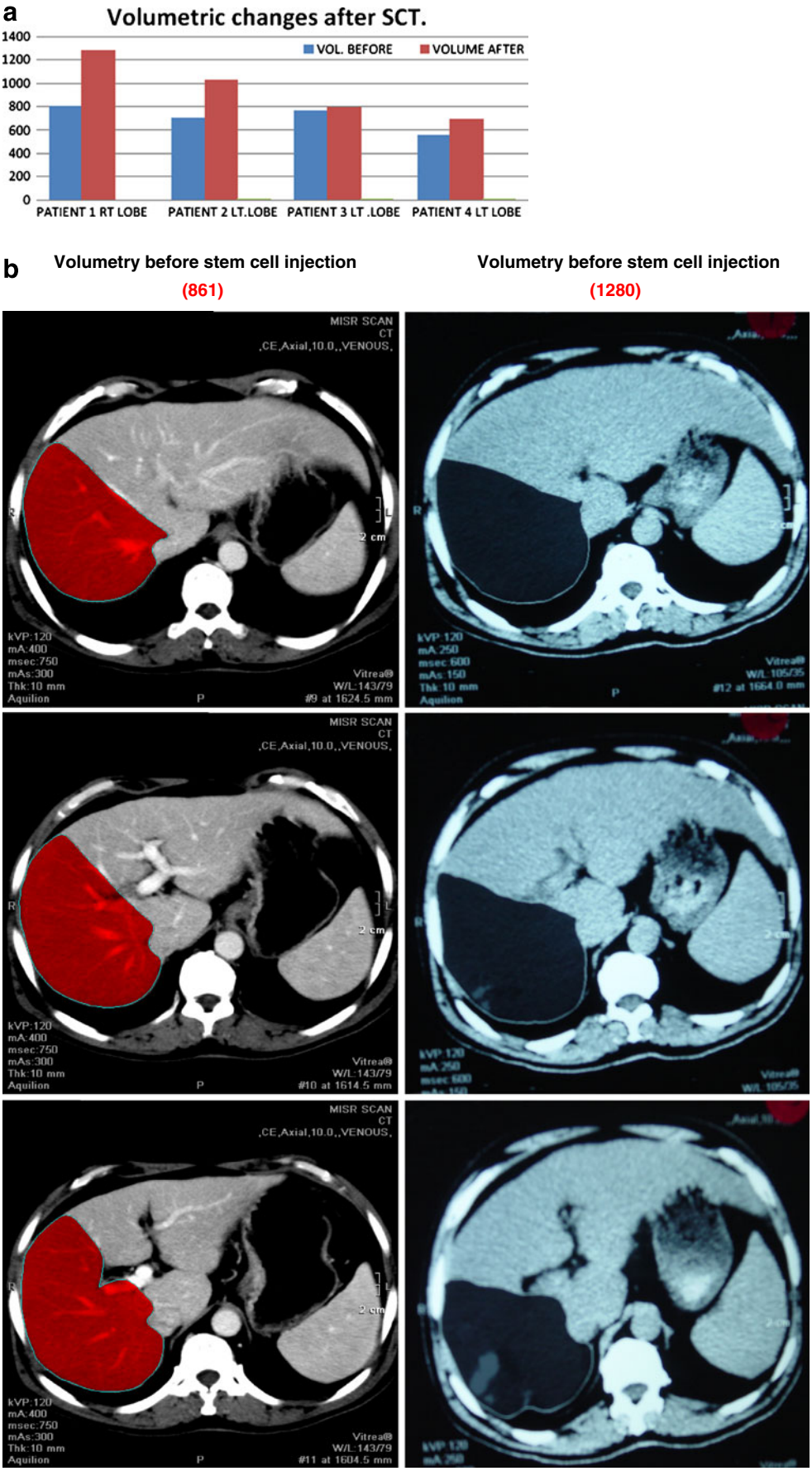

Fig. 2 **a**, **b** Volumetric changes in liver lobes volume before and after stem cell injection

marrow needle puncture site. We used the hepatic artery for cell infusion since the blood inflow to the liver could be mostly secured rather than the portal vein route, which may be complicated by embolization.

Our study confirmed the effect of BMC infusion into hepatic artery in patients with chronic liver disease due to hepatitis C complicated by liver cirrhosis and hepatocellular carcinoma. BMC transplantation was beneficial to all patients as there was apparent improvement in serum level of albumin and bilirubin. Also, there was a decrease in prothrombin time; this was collectively reflected in the improvement of Child score of the patients in spite of the process of TACE which was done in the same session for HCC in the contralateral lobe. BMSC infusion in our study made no chance for hepatic decompensation which commonly follows the technique of chemoembolization in such Child B patients. These results can be correlated to the significant increase of volume in the lobes injected with stem cell.

Recent progress in stem cell research and cell transplantation spurred our attempt to augment post-chemoembolization liver regeneration to minimize at least the effect of TACE on liver functions. In the same direction goes the work by Fürst et al. [32] who combined portal vein embolization (PVE) and CD133+ BMSC and compared this technique with PVE alone in patients prepared for hepatic resection to compare the effect of both techniques on liver volume and hepatic regeneration. The study showed that FLRV is significantly ($P<0.05$) higher in patients after administration of CD133+ cells than in patients without stem cell application [35].

Another study examined patients with liver cancer undergoing portal vein embolization to induce hypertrophy of the contralateral hepatic lobe in an attempt to increase the size and enhance the function of the future remnant liver volume before extensive partial hepatectomy [42]. In three treated patients, accelerated hepatic regeneration was observed after infusion of autologous CD133+ BM cells. By computed tomography, the left lateral segments showed 2.5-fold more hypertrophy than similar patients who had not been treated by BMC infusion.

Another preliminary uncontrolled study in five patients with cirrhosis showed a transient improvement in clinical parameters, such as serum bilirubin and albumin, at 60 days after portal vein or hepatic artery infusion of autologous CD34+ BM stem cells. In this study, feasibility and safety were clearly demonstrated [43]

Lazarus et al. were first to report a phase I trial to determine feasibility and safety of collection, ex vivo culture expansion, and intravenous infusion of human BM-derived MSCs (referred to as "mesenchymal progenitor cells" in this original report) [44]. Investigators collected and culture-expanded MSCs from 10 ml BM aspirates from 23 patients with hematologic malignancies in complete remission, 12 of them with a previous autologous or syngeneic bone marrow transplant. Autologous MSCs were reinfused intravenously after 4–7 weeks of ex vivo expansion into 15 patients. No adverse reactions were observed with infusion of MSCs. Thus, this study showed the feasibility of culturing MSCs from small-volume BM samples using clinically acceptable methodologies and their safety in infusion [44]. The same group subsequently conducted a phase I–II clinical trial with a therapeutic intent to determine feasibility, safety, and hematopoietic effects of culture-expanded autologous MSCs infused into 28 breast cancer patients after high-dose chemotherapy and autologous HSC transplantation [45] with the hypothesis that infusion of autologous MSCs after myeloablative conditioning would accelerate hematopoietic recovery. MSCs again were culture-expanded from a small bone marrow aspirate and were used after two to six passages at a dose of 1 to 2.2 million MSC/kg over 15 min. Again, autologous MSCs were infused without any toxicity, and hematopoietic recovery was rapid [45].

Led by the Lazarus group in a multi-center clinical trial, culture-expanded allogeneic MSCs derived from BM of HLA-identical sibling donors were infused 4 h before infusion of HSCs in 46 patients undergoing myeloablative HSC transplantation for various hematological malignancies using BM or peripheral blood HSCs. There were no infusion-related toxicities, ectopic tissue formation, or increase in the incidence or severity of graft-versus-host disease [46].

In summary, BMC infusion into the hepatic artery synchronized with TACE for patients with chronic liver disease complicated with HCC is safe and feasible. It demonstrated an improvement in both biological and radiological volumetric parameters. Our results warrant further studies in order to evaluate the effects of BMC transplantation in such patients to find possible roles of stem cell therapy to improve the outcome of different treatment modalities of HCC, to study in depth the effect of stem cell injection on liver regeneration, and to evaluate the mechanism involved in hepatic volume changes.

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