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Phases I–II Matched Case-Control Study of Human Fetal Liver Cell Transplantation for Treatment of Chronic Liver Disease

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Fetal hepatocytes have a high regenerative capacity. The aim of the study was to assess treatment safety and clinical efficacy of human fetal liver cell transplantation through splenic artery infusion. Patients with end-stage chronic liver disease on the waiting list for liver transplantation were enrolled. A retrospectively selected contemporary matched-pair group served as control. Nonsorted raw fetal liver cell preparations were isolated from therapeutically aborted fetuses. The end points of the study were safety and improvement of the Model for End-Stage Liver Disease (MELD) and Child-Pugh scores. Nine patients received a total of 13 intrasplenic infusions and were compared with 16 patients on standard therapy. There were no side effects related to the infusion procedure. At the end of follow-up, the MELD score (mean ± SD) in the treatment group remained stable from baseline (16.0 ± 2.9) to the last observation (15.7 ± 3.8), while it increased in the control group from 15.3 ± 2.5 to 19 ± 5.7 ($p = 0.0437$). The Child-Pugh score (mean ± SD) dropped from 10.1 ± 1.5 to 9.1 ± 1.4 in the treatment group and increased from 10.0 ± 1.2 to 11.1 ± 1.6 in the control group ($p = 0.0076$). All treated patients with history of recurrent portosystemic encephalopathy (PSE) had no further episodes during 1-year follow-up. No improvement was observed in the control group patients with PSE at study inclusion. Treatment was considered a failure in six of the nine patients (three deaths not liver related, one liver transplant, two MELD score increases) compared with 14 of the 16 patients in the control group (six deaths, five of which were caused by liver failure, four liver transplants, and four MELD score increases). Intrasplenic fetal liver cell infusion is a safe and well-tolerated procedure in patients with end-stage chronic liver disease. A positive effect on clinical scores and on encephalopathy emerged from this preliminary study.

Key words: Human fetal liver cell transplantation (hFLCTx); Fetal hepatocytes; Chronic liver disease; Model for End-Stage Liver Disease (MELD) score; Portosystemic encephalopathy (PSE)

INTRODUCTION

Liver transplantation (LTx) is currently the only therapeutic option for patients with end-stage liver disease, but its applicability is limited because of the widening gap between the number of transplant candidates and available organs. As a consequence, the development of new and successful treatments for liver failure could have a significant clinical and social impact. Recent advances in the fields of regenerative medicine may provide a remedy for this emerging problem. In the last two decades, several studies on adult human hepatocyte transplantation (HTx), isolated from the patient's liver or from discarded

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transplant organs, have been conducted in patients with acute or chronic liver failure in an attempt to correct metabolic defects or support liver function as a bridge to LTx (2,3,8,10,11,30,36,41). Currently, the most successful outcome has been for patients with liver-based metabolic disorders. Intraportal injection is the preferred delivery method for clinical hepatocyte transplantation for treatment of acute liver failure (ALF) and liver-based metabolic diseases, while in cirrhotic patients the intrasplenic injection would minimize the risk of portal pressure increase, portal vein thrombosis (2), and microembolic liver damage and would potentially favor the formation of an auxiliary liver in the patient's spleen (5,42). Splenic transplantation of differentiated adult hepatocytes has been shown to control hyperammonemia, correct genetic defects in liver function, and bridge patients with ALF to LTx (42). Hepatocyte nests (and tight junctions on electron micrographs) have been found in the spleen in an autopsy case (42), and in one patient, up to 11 months after transplantation, indicating that human hepatocytes are capable of prolonged survival in the spleen (30). Preclinical studies have demonstrated that adult hepatocytes can survive and proliferate in a rat spleen for over 16 months, retain hepatocyte structure, and rebuild a hepatic sinusoidal architecture (14,15,23,29).

Most of the available studies have focused on adult hepatocytes for transplantation because they are considered a valid resource as a bridge to LTx. However, this emphasis should perhaps be tempered because donor liver tissue availability for cell isolation is scarce (discarded organs are generally used) and because adult hepatocytes have a low rate of liver cell engraftment (13,43), cell survival, and a limited proliferative capacity.

Human fetal hepatocytes offer several advantages over adult hepatocytes, including greater proliferative capacity and plasticity in vitro (25), greater resistance to cryopreservation, and greater capacity for significant repopulation in vivo (1,28,31). The spleen also retains these cells for a longer time after intrasplenic injection (5). An increased ratio of CYP3A4 to 3A7 gene expression, indicating acquisition of nonsynthetic phase I cell metabolic function and then differentiation into adult hepatocytes, was found in vitro (24,34).

To date, few studies have been published on the clinical use of human fetal hepatocytes, with different approaches (injection in peritoneal cavity or in hepatic artery) (16,20–22) and encouraging results. Our group has gained considerable experience in the last 5 years in fetal liver procurement, isolation, and characterization of human fetal liver cells through vascular perfusion (12). Our first patient treated with intrasplenic human fetal liver cell transplantation (hFLCTx) had a decrease in the Model for End-stage Liver Disease (MELD) score from 15 to 12 at 2 months and, following a second hFLCTx, recovered from portosystemic encephalopathy (PSE) (12).

This study reports our experience with the use of hFLCTx through the splenic artery in a series of patients with end-stage chronic liver disease and on the waiting list for LTx. The outcome of these patients is compared with a matched-pair group of similar, contemporary patients who did not undergo hFLCTx.

MATERIALS AND METHODS

Study Description

This is a phases I–II matched-pair case-control study on the safety and efficacy of hFLCTx through the splenic artery in patients with end-stage chronic liver disease and on the waiting list for LTx. The study was approved by ISMETT's Institutional Research Review Board (IRRB/01/2006) and Ethics Committee. The study was conducted in compliance with current Good Clinical Practice standards (1979) and in accordance with the principles of the Declaration of Helsinki (1975). All procedures were compliant with local and national legislations, regulations, and guidelines.

End Points

Safety. The safety was determined by the absence of any adverse event or complication occurring after hFLCTx, including, but not limited to, splenic artery thrombosis or wall dissection, uncontrollable bleeding from the puncture site, acute renal failure, splenic abscess, pulmonary embolism, acute thrombosis of the portal–splenic–mesenteric axis, and occurrence of infections and/or neoplasms.

Efficacy. Efficacy was determined by improvement in MELD and Child-Pugh scores.

Patients

The study population was composed of patients with end-stage chronic liver disease consecutively listed on the waiting list for LTx at our institute from February 2007 to May 2010. Inclusion criteria: 1) clinical diagnosis of cirrhosis (evidence of chronic liver disease, presence of ascites and/or esophageal varices at endoscopy, and/or evidence of portal hypertension on radiologic imaging) or histologic diagnosis of liver cirrhosis, 2) Child-Pugh score of ≥8 and/or MELD score of ≥14, and 3) informed consent to the study signed by the patient. Exclusion criteria: age <18 years, MELD score ≥25, hepatocellular carcinoma (HCC), portal vein thrombosis, serious cardiovascular or respiratory disease, hemodynamic instability, type-1 (acute) hepatorenal syndrome, serum creatinine level >2 mg/dl and/or creatinine clearance <30–40 ml/min, active infection, active gastrointestinal bleeding, alcohol abuse, pulmonary hypertension (>35 mmHg), history of neoplasm, positive HBV DNA, HIV infection, pregnancy, residence outside Sicily, transjugular intrahepatic portosystemic shunt (TIPS) placed in the previous month, and

vascular contraindications to the procedure (i.e., splenic artery aneurysm, thrombosis, splenorenal shunt, splenic angioma).

Both treated and control patients were on standard therapy, consisting of diuretics, β blocker, lactulose, and rifaximin.

After obtaining informed consent, the patient's name was inserted onto a new waiting list by blood type. As soon as a fetal liver was available, priority was given to the patient with a compatible blood type and the highest MELD score. The patient was kept on the official regional waiting list and transplanted according to organ availability and to our internal policy for organ assignment, which follows UNOS rules. No incentive was given to participate in the study.

Patients in the control group were retrospectively selected and had to satisfy the above-mentioned inclusion and exclusion criteria. They were matched to treated patients according to the following criteria: they had to be included on the waiting list in the same period (±2 months from the date on which treated patients received hFLCTx), age (±10 years with respect to treated patients), same gender, and severity of liver disease based on Child-Pugh and/or MELD score (±2 points with respect to treated patients).

Donors

Pregnant women between the 16th and 26th gestational weeks were consecutively recruited by a gynecologist not involved in the study. After obtaining informed consent to donate the aborted fetus, the donors underwent blood culture, serology, and polymerase chain reaction (PCR) tests for HIV 1 and 2, hepatitis B and C, cytomegalovirus, rubella, parvovirus B19, toxoplasma gondii, treponema pallidum, herpes simplex, varicella zoster, and herpes 6 and 8. A fetal blood sample was taken to determine the blood type. Soon after abortion, the fetus was placed in University of Wisconsin liver storage solution and transported on ice to our institute's laboratories. A blood sample from the umbilical cord or from a transthoracic intracardiac puncture was taken to determine the fetal blood type.

Fetal Liver Cell Isolation

The fetal liver was procured, and the fetal liver cells were isolated according to our previously described protocol (12). The obtained cell preparation was not expanded or cryopreserved in order to limit cell damage. Morphological assessment was made by phase-contrast microscopy and viability with the trypan blue (Lonza, Basel, Switzerland) exclusion test. Cell suspension with a viability of less than 70% was not used. Before transplantation, quality control of the human fetal liver cell isolation included a mycoplasma test (VenorGeM Advance; Minerva Biolabs, Berlin, Germany), an endotoxin test (ENDOSAFE-PTS Cartridges; Charles River Laboratories, Charleston, SC, USA), and Gram's stain (Aerospray microbiology slide stainer; Delcon, Milan, Italy), all done according to manufacturer's instructions. Only the cell preparation with a negative final microbiologic screening was considered for clinical application.

Fetal Liver Cell Characterization

Flow cytometry and reverse transcription-PCR (RT-PCR) methods have been previously described (12). The following antigenic markers were detected through flow cytometry: epithelial cell adhesion molecule (EpCAM; Becton Dickinson Biosciences, Franklin Lakes, NJ, USA), asialoglycoprotein (Hycult Biotechnology, Uden, the Netherlands), cytokeratin 18 (CK18; Abcam, Cambridge, UK), albumin (R&D Systems, Minneapolis, MN, USA), α-fetoprotein (AFP; R&D Systems), CK19 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD45 (Becton Dickinson Biosciences), CD34 (Becton Dickinson Biosciences), CD133 (Miltenyi Biotech Bisley, Surrey, UK), CD90 (Becton Dickinson Biosciences), HLA-I (Becton Dickinson Biosciences), and Ki-67 (Becton Dickinson Biosciences). Details on antibodies and dilutions used are listed in a previous study of ours (12). Flow cytometry data were acquired with a BD FACS ARIA II instrument and were analyzed with Diva 6.1.2 (Becton Dickinson Biosciences). The primer sequences for RT-PCR (Invitrogen, Carlsbad, CA, USA) are also listed in a previous study of ours (12). The genes for cytochrome P4501B1 (CYP1B1), cytochrome P4502B6 (CYP2B6), cytochrome P4501A1 (CYP1A1) and cytochrome P4503A4 (CYP3A4), hepatocyte nuclear factor 4 (HNF4), CK7, CK8, and albumin were analyzed with RT-PCR. The primer sequences, annealing temperatures, and sizes of the amplified fragments for CYP1B1, CYP2B6, HNF4, CK7, CK8, and albumin have been published (12). The primer sequence used for CYP1A1 was forward 5′-AGCACTACA AAA CCT TTG AGA AG-3′, reverse 5′-GCTGGACATTGGCGTTCT-3′ (annealing temperature 55°C; size: 101 bp); and for CYP3A4 was forward 5′-AAATCTGAGGCGGGA AGC-3′, reverse 5′-TTGG GATGAGGAATGGAA AG-3′ (annealing temperature 55°C; size: 217 bp).

Immunofluorescence on Cells

For immunofluorescence staining, the cells were fixed according to the antibody manufacturer's instructions. After being blocked in phosphate-buffered saline (PBS) 0.5% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA), 3% bovine serum albumin (BSA; Sigma-Aldrich), the cells were incubated with the primary antibodies and then incubated with the appropriate fluorescence-conjugated secondary antibodies (Alexa Fluor; Invitrogen). Cells

were mounted with Prolong Gold antifade (Invitrogen), which includes diamidino-2-phenylindole for the nuclear counterstaining. Cell imaging was done with a microscope (Nikon Eclipse 50i, Melville, NY, USA) coupled with a camera (Olympus XM10, Tokyo, Japan) and Cell F software for image acquisition (Olympus). The primary antibodies used were anti-albumin (Sigma-Aldrich), anti-AFP (Santa Cruz), anti-CK18 (Abcam), anti-Ki-67 (Abcam), and anti-OV-6 (R&D Systems).

Immunocytochemistry Analysis

Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), washed with PBS, and incubated with methanol (Sigma-Aldrich) 3% H_2O_2 (Sigma-Aldrich) to deactivate the endogenous peroxidase. After washing in PBS and blocking with PBS, 0.5% Tween 20, and 3% BSA, the cells were incubated with the primary antibodies anti-albumin (Sigma-Aldrich), anti-AFP (Santa Cruz), and anti-CK18 (Abcam). The cells were then washed with PBS, stained with the secondary antibody using the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA), and incubated with 3,3′-diaminobenzidine (DAB) chromogen substrate using the DAB Substrate Kit for peroxidase (Vector Laboratories).

Cell Function Assays

An aliquot of fetal liver cells was studied by assessing urea metabolism (urease glutamic dehydrogenase; Dimension RxLMax; Siemens Healthcare Diagnostics, Tarrytown, NY, USA), cytochrome p450 (CYP) activity (Promega kit; Promega, Madison, WI, USA), albumin secretion (ELISA kit; Abnova, Heidelberg, Germany), glycogen storage (periodic-acid Schiff staining, Sigma-Aldrich), glucose 6 phosphatase (G6Pase) assay (Sigma-Aldrich), and indiocyanine green uptake (Sigma-Aldrich). Clonetics™ primary human hepatocytes (Lonza, Basel, Switzerland) and HepG2 cell line culture ($n=3$ each) were used as controls for the in vitro characterization.

Fetal Liver Cell Transplantation

On admission, the patient underwent blood sample and antibiotic prophylaxis. The femoral artery was cannulated with a 5-Fr catheter (Cook, Inc., Bloomington, IN, USA) and a cell preparation, resuspended in lactated Ringer solution (Fresenius Kabi, Verona, Italy) and 2% human albumin (Grifolds, Pisa, Italy) at a concentration of $5–10\times10^8$ cells/50 ml, was placed in a 50-cc syringe (Mark V Provis 150 ml; Medrad, Indianola, PA, USA) and infused, through the splenic artery, at room temperature and at a rate of 5 ml/min. During infusion, an occlusion catheter was placed in the main splenic artery (26). No anticoagulation was used.

Candidates for hFLCTx could receive more than one cell infusion, each with a number of cells not exceeding 10×10^8, at intervals of not less than 15 days. Immunosuppression with tacrolimus (Prograf; Fujisawa Healthcare, Inc., Deerfield, IL, USA) was started in all patients within 24 h of the first hFLCTx (target blood level: 4–6 ng/ml) and was continued throughout the study.

Follow-Up

The observation period was 1 year from the first hFLCTx (treatment group) or from inclusion in the study (control group). Treated patients were visited weekly for 4 weeks after each infusion, then monthly. Control patients were visited every 3 months. The MELD and Child-Pugh scores were calculated at each visit. The West Haven criteria were used for semiquantitative grading of mental state (7), and all patients were evaluated by the same physician.

Each treated patient underwent MRI (Signa HDXt; GE Medical Systems, Milwaukee, WI, USA) with hepatospecific contrast agent (Gd-BOPTA, Multihance; Bracco, Milan, Italy) and hepatobiliary scintigraphy ^{99m}Tc-mebrofenin (Mallnkrodt, Milan, Italy) at time 0, 1 month, and every 6 months after hFLCTx.

Statistical Analysis

Continuous data are expressed as mean ± standard deviation (SD). For categorical data, we used counts and proportions, and differences were assessed by Fisher's exact test. The Student's *t*-test was used to assess any difference between baseline mean values in both groups. In case of unequal variances, the Mann–Whitney test was used. To evaluate differences between groups at 1 year, a change score analysis was done. For all analyses, a value of $p\le0.05$ was considered statistically significant. All analyses were completed with R 2.14 (R Core Team, Vienna, Austria) and STATA 13 (StataCorp LP, College Station, TX, USA).

RESULTS

Cell Preparation Analysis

The cellular yield of processed fetuses was 1.5×10^9 (0.54×10^9); the mean viability using the trypan blue test was 80.0% ± 5.9%. Of the isolated fetal cells, 51.5% ± 18.3% expressed the Ki-67 marker, while 1.5% ± 1.1% of the isolated cells were positive on flow cytometry for the apoptotic marker annexin V (12).

The cells were positive for albumin, AFP, CK18, and OV-6 (Fig. 1) and on immunohistochemistry and immunofluorescence cell staining. The fetal cell preparation was found heterogeneous on flow cytometry (Fig. 2), which included hepatocyte markers: CK18$^+$ cells (17.6% ± 12.0%), albumin$^+$ cells (15.4% ± 8.9%), AFP$^+$ cells (25.4% ± 15.1%), asialoglycoprotein receptor$^+$ cells (21.2% ± 12.3%), as well as E-cadherin$^+$ cells (11.7% ± 10.5%). The population also included bipotential

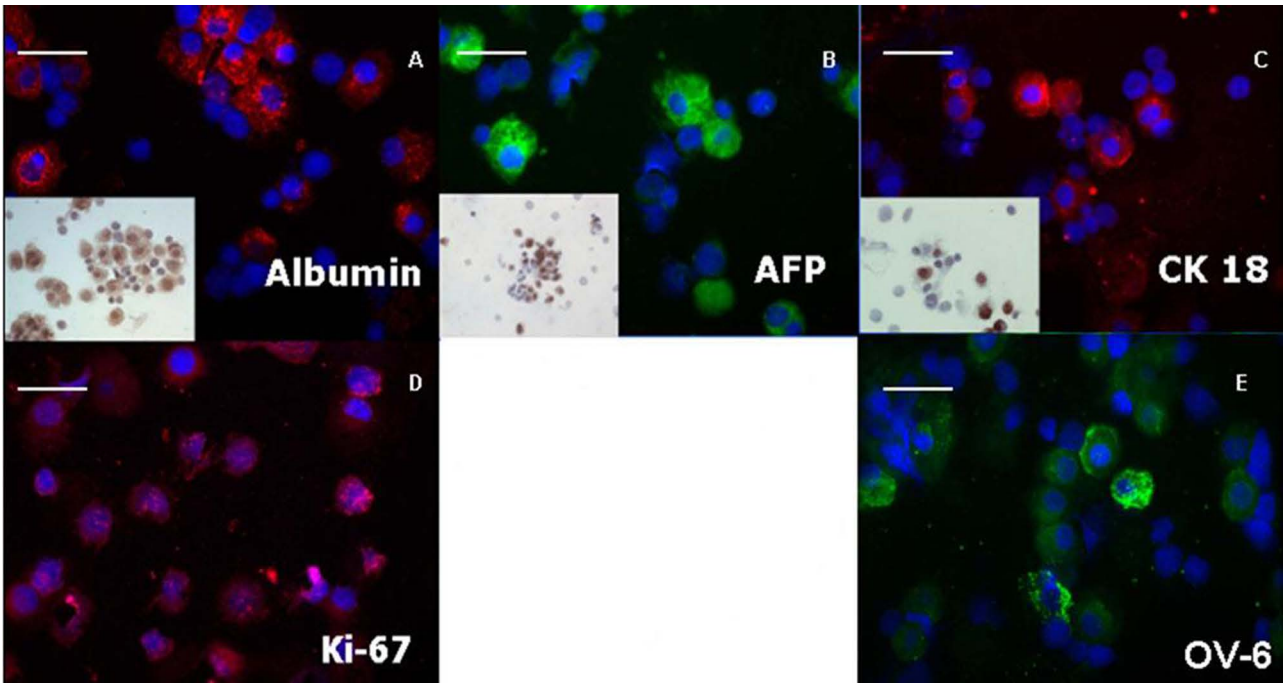

Figure 1. Immunofluorescence analysis of the isolated fetal liver cells. (A–E) Immunofluorescence analysis confirmed the positivity of the isolated fetal liver cells (A–E; scale bar: 50 μm) for albumin, AFP, CK18, OV-6, and for the proliferative index Ki-67. (A–C) Immunohistochemistry analysis confirmed the positivity of the isolated fetal liver cells for albumin, AFP, and CK18.

precursors of the hepatic and biliary lineages ($CK18^+$/$CK19^+$ cells, 12.2% ± 8.7%), $EpCAM^+$ cells (7.0% ± 4.4%), and Kupffer cells ($CD68^+$, 4.3% ± 1.9%). Further coexpression analysis showed cells positive for both AFP/CK18 (10.6% ± 12.7%) and AFP/CK19 (1.9% ± 1.9%). The cell preparation contained cells positive for mesenchymal markers: vimentin-positive cells (18.5% ± 10.6%), $CD90^+$ cells (1.8% ± 1.2%), $CD29^+$ cells (21.2% ± 16.6%), $albumin^+$/$CD90^+$ cells (1.8% ± 1.4%), and AFP^+/$CD90^+$ cells (1.8% ± 1.1%). Of the hematopoietic component of the isolated cells, 9.1% ± 5.0% were $CD45^+$; 7.5% ± 5.8% were $CD34^+$; 3.8% ± 5.3% were $CD45^+$/$CD34^+$, and 2.0% ± 1.9% were $CD34^+$/$CD133^+$.

The mean HLA-I/CK18 expression was 2.5% ± 1.8% compared with 41.0% ± 1.8% in the adult hepatocytes.

Fetal hepatocytes secreted albumin (113.7 ± 49.9 ng/ml per 1.5×10^6 cells/48 h), synthesized urea (14 ± 4 mg/dl per 1.5×10^6cells/48 h), and displayed activity of G6Pase (31% ± 14% of positive cells). Activity of CYP3A4, storage of glycogen, and ICG uptake were undetected. Expression of the CYP1B1, CYP1A1, and CYP3A4 was weak in fetal cells, strongly positive in adult cells, and negative in HepG2, while CYP2B6 gene expression was negative in the fetal cells, strongly positive in the adult cells, and negative in the HepG2 cells. All cell types analyzed were positive for HNF4, CK8, and albumin genes (data not shown).

Patients

From February 2007 to May 2010, 346 patients were on the LTx waiting list at our institute. Patients that satisfied the inclusion criteria were consecutively invited to participate in the study, and 13 gave informed consent. At the end of the study, only nine underwent hFLCTx, while four did not because of unavailability of fetal cells of compatible donor blood group.

We retrospectively selected 16 patients of the same timeframe and on the waiting list for LTx, with the same inclusion criteria and matched for age, gender, and severity of disease.

In the same period, 36 consecutive pregnant women donated their therapeutically aborted fetuses after signing informed consent. Twenty-eight fetuses were excluded because of active maternal infection or cellular viability of <70%. Finally, eight fetuses (17.6–22.4 weeks) satisfied the criteria for clinical application.

Treatment Group (Table 1). Nine patients (eight with HCV-related cirrhosis, one with alcohol-related cirrhosis) underwent a total of 13 intrasplenic hFLCTx from a total of eight donors (in five cases, the cell preparation of a single fetus was infused in two patients). Four patients had TIPS placed at a mean of 764.2 days before hFLCTx (range 300–1,902). Five patients had a history of chronic PSE, ranging between grades 1 and 3, and three of the

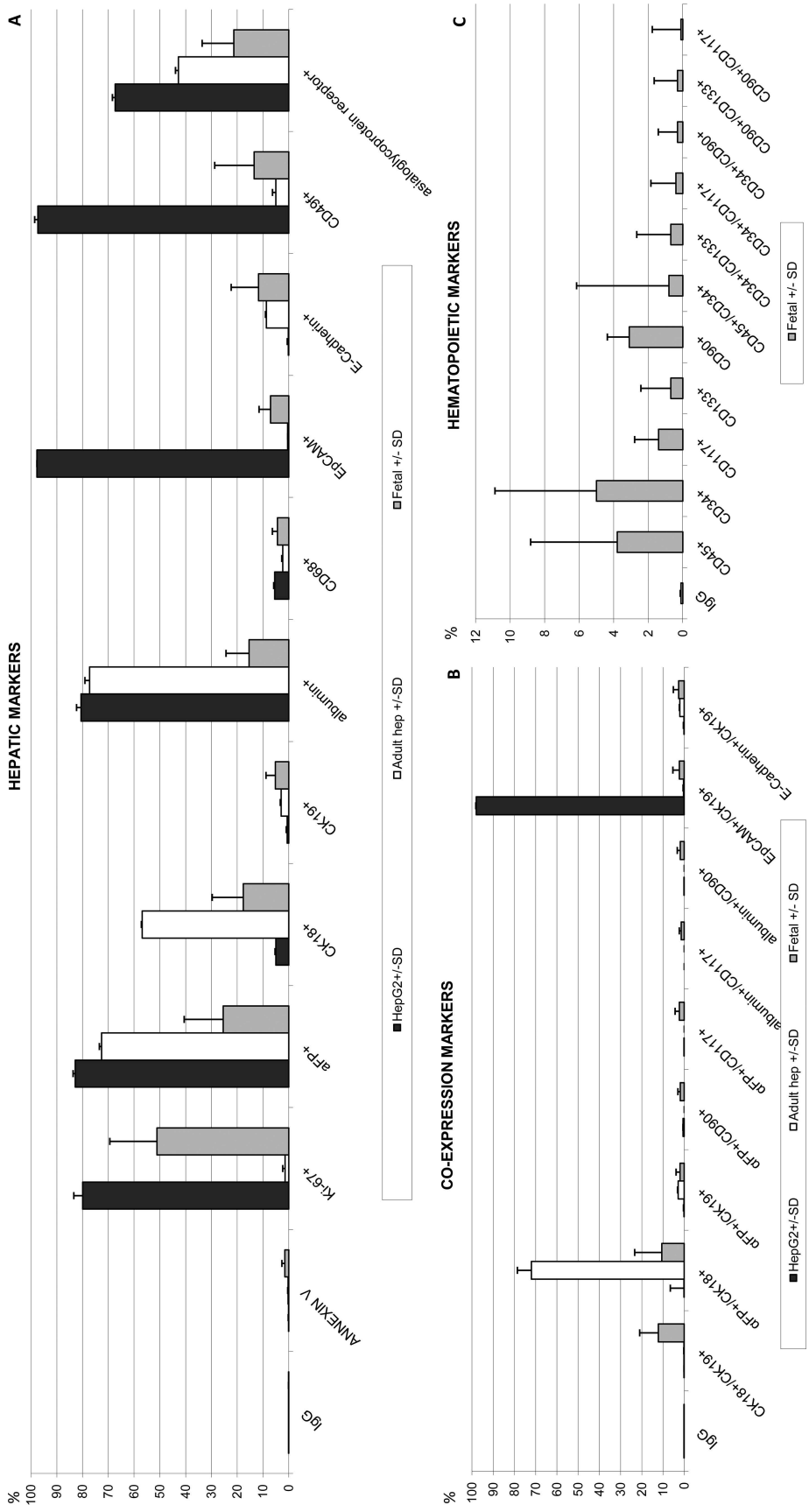

Figure 2. Flow cytometry for phenotypic cell markers of the different cell types. Flow cytometry data for (A) hepatic markers, (B) coexpression markers, (C) hematopoietic markers in the isolated cell population ($n = 8$), HepG2 ($n = 3$), and primary human hepatocytes ($n = 3$).

Table 1. Clinical Characteristic of the Treated and the Control Patients at Baseline and at End of Follow-Up

Patient Enrollment Data	Treated = 9	Controls = 16	*p* Value
Mean age (range)	53.1 (29–62)	53.3 (39–65)	0.8113§
Gender: male/female	6/3	13/3	0.4125¶
Etiology			0.9183¶
HCV	8	13	
Alcohol	1	2	
Both	/	1	
Ascites	5	14	0.3040¶
Mild/moderate/severe	1/1/3	1/5/8	
PSE	5	4	0.3940¶
Grade 1/2/3	5/–/–	4/–/–	
TIPS	4	5	0.5094¶
Follow-up months (SD)*	8.8 (4.8)	7.1(4.4)	0.3785#
Outcome Survival/death or LT	5/4	6/10	0.4340¶

Patient Data Results [Mean (SD)]	Baseline			End Follow-Up		Change‡ Score
	Treated	Controls	*p* Value	Treated	Controls	*p* Value
Bilirubin (mg/dl)	2.88 (0.71)	3.68 (1.46)	0.1376#	3.00 (1.05)	4.88 (3.85)	0.4616§
Albumin (g/dl)	2.46 (0.42)	2.96 (0.46)	0.0127#	2.57 (0.35)	2.65 (0.50)	0.0989§
INR	1.46 (0.14)	1.42 (0.22)	0.6615#	1.53 (0.24)	1.63 (0.25)	0.0671#
Creatinine (mg/dl)†	1.00 (0.27)	0.88 (0.25)	0.4601#	0.91 (0.27)	1.01 (0.35)	**0.0411**#
Ascites (% yes)	55.6	87.5	0.3040¶	33.3	12.5	0.3121**
PSE (%)	44.4	25.0	0.3940¶	0.0	31.2	**0.0363****
Child-Pugh score	10.11 (1.54)	10.00 (1.26)	0.8469#	9.11 (1.45)	11.13 (1.63)	**0.0076**#
MELD score	16.00 (2.96)	15.31 (2.50)	0.5420#	15.67 (3.84)	19.06 (5.70)	**0.0437**#

PSE, portosystemic encephalopathy; TIPS, transjugular intrahepatic portosystemic shung; INR, international normalized ratio; MELD, Model for End-Stage Liver Disease.
*The mean value was calculated including patients who dies before 1 year of follow-up.
†None of the patients were on dialysis.
‡Difference between mean value of follow-up and baseline in the treatment group versus the differences between mean value of follow-up and baseline in the control group.
§Mann–Whitney test; ¶Fisher's exact test; #t-test; **z-test.

four had undergone TIPS placement. Five patients underwent one hFLCTx and four patients two hFLCTx, with the range of infused cells for each patient between 5×10^8 and 10×10^8 (Table 2). At the time of cell infusion, the mean MELD score was 16 (range 12–22), and the Child-Pugh class was B or C (B = 3; C = 6). Splenic arteriography after infusion showed a patent portal vein in all patients, as well as a patent splenic artery and vein.

Control Group (Table 1). Sixteen patients were enrolled. Disease etiology was HCV in 13 and alcohol abuse in 2, and both in 1; the mean MELD score was 15.3 (range 11–20), and the Child-Pugh class was B or C (B = 6; C = 10). Four patients had a history of chronic PSE, ranging between grades 1 and 2. Three patients had TIPS placed at a mean of 38.6 days (30–76 days) before being placed on the waiting list for LTx and two patients at a mean of 149.5 days (129–170 days) after being accepted.

Safety of Treatment. There were no adverse technical events related to the angiographic procedure (e.g., splenic infarction, splenic artery thrombosis). The average length of hospital stay after hFLCTx was 4.5 days (range 3–7 days). Two patients (patient 2 and patient 3) had rash at day 5 and day 19, respectively, which resolved rapidly after steroid treatment. Two patients experienced spontaneous bacterial peritonitis (patient 4 and patient 5, at 2 and 3 months, respectively) responsive to antibiotics; one patient (patient 8) experienced thrombophlebitis at 2 months, which was responsive to antibiotic therapy; and one patient (patient 1) had multifocal progressive leukoencephalitis (at 3 months) that, after extensive diagnostic and infective workup, was considered of unknown origin. In four patients (patients 1, 4, 5, and 8), tacrolimus (Prograf; Fujisawa Healthcare Inc.) was discontinued at the onset of the complication. Only patient 5 had a worsening of the MELD score at the end of follow-up (from 14 to 20), while the Child class remained the same. In the remaining patients, the MELD score improved (Table 3).

Treatment Efficacy. Analysis of the MELD score (mean ± SD) in the first 4 weeks after hFCLTx in the treatment

Table 2. Treated Patients: Cell Infusion Characteristics

Patients	1	2	3	4	5	6	7	8	9
No. of infusions	2*	1	2*	1	1	2*	2*	1	1
Total amount of injected cells	10×10^8	5×10^8	10×10^8	5×10^8	5×10^8	10×10^8	10×10^8	8×10^8	10×10^8
Fetal gestational weeks	21.3 and 22.2[a]	22.2[a]	22.3[b] and 18.3	22.3[b]	17.6	18.5[c] and 19[d]	18.5[c] and 19[d]	22.4[e]	22.4[e]
Cellular yield	15×10^8 (77%)	17.2×10^{8a}	19.2×10^{8b} (83%)	19.2×10^{8b}	1.8×10^9	1.3×10^{9c} (84%)	1.3×10^{9c} (84%)	2×10^{9e}	2×10^{9e}
(viability)	and 17.2×10^{8a} (87%)	(87%)	and 3×10^8 (70%)	(83%)	(80%)	and 1.6×10^{9d} (74%)	and 1.6×10^{9d} (74%)	(85%)	(85%)
Reason for tacrolimus discontinuation (months)	Multifocal progressive leukoencephalitis (3)	–	–	SBP (2)	SBP (3)	–	–	Thrombophlebitis (2)	–

SBP, spontaneous bacterial peritonitis.
*Cells obtained from two different fetuses.
[a,b,c,d,e]Cells obtained from the same fetus.

group showed a reduction from 16.0±2.9 to 14.0±1.5. In the treatment group (mean follow-up of 8.8±4.8 months), the MELD score remained stable from baseline (16.0±2.9) to the last observation (15.7±3.8), while in the same period the Child-Pugh score dropped from 10.1±1.5 to 9.1±1.4. In the control group (mean follow-up 7.1±4.4 months), the MELD score increased from baseline (15.3±2.5) to the last observation (19.0±5.7; $p=0.0437$) (Table 1). In the same period, the Child-Pugh score increased from 10.0±1.2 to 11.1±1.6 ($p=0.0076$) (Table 1).

PSE disappeared after hFCLTx in five of the patients with PSE at the time of inclusion, without recurrence at last follow-up; in two of them, the dosage of rifaximin was halved. No improvement was observed in the four control group patients with PSE at study inclusion, and a new case was recorded.

There were three deaths in the treatment group (33.3%) and six (37.5%) in the control group. Of the three patients in the treatment group who died (Table 3), the laboratory values at time of hFCLTx and at time of death were, respectively, as follows: patient 2, bilirubin 3.0 and 4.0 mg/dl, creatinine 0.8 and 0.9 mg/dl, international normalized ratio (INR) 1.4 and 1.6, albumin 2.3 and 2.3 g/dl; patient 4, bilirubin 3.5 and 2.5 mg/dl, creatinine 1.3 and 1.1 mg/dl, INR 1.7 and 1.5, albumin 2.9 and 2.6 g/dl; patient 9, bilirubin 3.1 and 2.2 mg/dl, creatinine 0.5 and 0.5 mg/dl, INR 1.6 and 1.3, albumin 2.5 and 2.4 g/dl.

It is worth noting that the three deaths in the treatment group were not related to liver failure (two cerebral hemorrhage and one acute myocardial infarction at 2, 10, and 2 months, respectively), Five of the six deaths in the control group were caused by liver failure, while one was of unknown cause. One patient of the nine (11%) in the treatment group received a LTx 3 months after cell infusion (MELD at baseline 22, MELD at transplant 21), while 4 of the 16 (25%) in the control group were transplanted at 3, 4, 1, and 5 months after inclusion in the study, with a MELD score at transplant of 17 (15 at baseline), 23 (20 at baseline), 16 (19 at baseline), and 16 (15 at baseline), respectively (Table 3).

Hepatobiliary scintigraphy ^{99m}Tc-mebrofenin (Mallnkrodt, Milan, Italy) and abdominal MRI (Signa HDXt; GE Medical Systems) detected no ectopic liver tissue during follow-up.

DISCUSSION

In this single-center, phases I–II matched case-control study, we found intrasplenic infusion of limited numbers of human fetal hepatocytes in patients with end-stage chronic liver disease and on the waiting list for LTx to be a safe bridging therapy. Because of the small sample size and the nonrandomized method for selection of the control group, we cannot make any claims, however preliminary, for the efficacy of this therapy in terms of liver

Table 3. Clinical Data of Treated and Control Patients

Patients	Follow-Up (Months)	Outcome 1 Year	Ascites			PSE			Child-Pugh Score					MELD Score				
			T0	T6	T12	T0	T6	T12	T0	T3	T6	T9	T12	T0	T3	T6	T9	T12
Treated																		
1*	12	Alive	Mild	No	No	Mild	No	No	B-9	B-9	B-7	B-7	B-7	15	15	11	10	11
2	2	Dead	No	–	–	Mild	–	–	C-10	–	–	–	–	14	–	–	–	–
3	12	Alive	No	No	No	Mild	No	No	C-10	C-10	B-9	B-9	B-9	16	16	18	17	18
4*	10	Dead	Severe	Moderate	–	No	No	–	C-11	C-10	C-11	C-10	–	19	15	15	16	–
5*	12	Alive	Severe	Mild	Severe	No	No	No	C-11	C-10	C-11	C-12	C-12	14	16	17	17	20
6	12	Alive	No	No	No	Mild	No	No	B-8	B-8	B-8	B-8	B-9	12	16	15	14	14
7	4	LTx	Severe	–	–	Mild	–	–	C-12	B-9	–	–	–	22	21	–	–	–
8*	12	Alive	No	No	No	No	No	No	B-8	B-8	B-7	B-7	B-8	16	12	11	12	11
9	2	Dead	Moderate	–	–	No	–	–	C-12	–	–	–	–	16	–	–	–	–
Control																		
1	3	Dead	Moderate	–	–	Mild	–	–	C10	C10	–	–	–	16	20	–	–	–
2	3	LTx	Moderate	–	–	Mild	–		C12	C12	–	–	–	15	17	–	–	–
3	12	Alive	Moderate	Moderate	Moderate	No	No	No	B9	B9	B9	B9	B8	14	11	14	13	14
4	3	Dead	Severe	–	–	Mild	–	–	C11	C13	–	–	–	13	38	–	–	–
5	4	LTx	Mild	–	–	No	–	–	C11	C11	–	–	–	20	23	–	–	–
6	3	Dead	Severe	–	–	No	–	–	C11	C12	–	–	–	13	16	–	–	–
7	8	Dead	Mild	Mild	–	Mild	Mild	–	C10	C13	C13	–	–	11	15	15	–	–
8	1	LTx	Severe	–	–	No	–	–	C12	–	–	–	–	19	–	–	–	–
9	11	Dead	Severe	Severe	–	No	Moderate	–	B9	C10	C12	C12	–	14	14	17	21	–
10	5	LTx	No	–	–	No	–	–	B9	C11	–	–	–	15	16	–	–	–
11	12	Alive	Moderate	Moderate	Moderate	No	Moderate	Moderate	C10	C10	C10	C10	B9	16	17	17	17	20
12	12	Alive	Moderate	Moderate	Moderate	No	No	No	B8	B9	B9	B9	B9	17	18	20	15	21
13	12	Alive	Severe	Moderate	Moderate	No	No	No	B9	B9	B9	B9	C12	15	14	12	17	16
14	11	Dead	Severe	Severe	–	No	No	–	C11	C12	C12	C12	–	18	17	17	20	–
15	12	Alive	No	No	No	No	No	No	C10	B7	A6	B9	B9	17	17	17	17	16
16	12	Alive	Mild	Moderate	Moderate	No	No	Moderate	B8	B8	C11	C12	C13	12	16	16	14	16

PSE, portosystemic encephalopathy; MELD, Model for End-Stage Liver Disease; LTx, liver transplantation.
*Patients who discontinued tacrolimus because of side effects.

function. Since our major concern was to avoid any harm to the patients, a fairly limited number of cells were transplanted in the spleen. The treated patients, affected with advanced cirrhosis, showed a stabilization of clinical scores and were free of encephalopathy, while the control patients showed a significant worsening of clinical conditions and no improvement in their mental status at the end of 1 year of follow-up.

Survival of the treated patients was not lower than that in the control group, and immunosuppression was well tolerated.

Another interesting result emerged from our study. Liver function improved early after injection (1 month) and remained stable in the long term (1 year), as evidenced by the significant differences in the MELD and Child-Pugh score trend between the treatment and the control groups. Analysis of the MELD score (mean ± SD) in the first 4 weeks after hFCLTx in the treatment group showed a reduction from 16.0 ± 2.9 to 14.0 ± 1.5. In addition, none of the treated patients with a history of recurrent PSE (grades 1–3) experienced further episodes during the entire follow-up period. Because we employed the West Haven scale, which is based only on clinical findings (7), we cannot rule out the possibility that minimal encephalopathy was still present. A positive effect on PSE has been already reported in two studies, the first with human fetal hepatocytes transplanted in the peritoneal cavity of patients with ALF (16), and the second reporting the injection of sorted $EpCAM^+$ fetal liver stem cells through the hepatic artery in patients with alcoholic liver cirrhosis (22).

Our results in terms of a decrease in the MELD score are in contrast with a study by Khan et al., which showed a decrease of 7 points in the MELD score at 6 months of follow-up. However, in this noncontrolled study, 23 of the 25 patients treated had acute decompensation on alcoholic liver cirrhosis. In addition, the authors did not state whether alcohol consumption was still active on admission, so it could reasonably be argued that the clinical improvement was due, in part, to suspension of alcohol consumption (22,32). Immunosuppression was well tolerated, and no neoplasm developed in 1-year follow-up, as already found in clinical experience (16). No oncogenic transformation at least 2 years after intrasplenic fetal HT has been reported in animal models, or in culture, despite acquisition of cytogenic aberrations (17,27).

The three deaths in our treatment group were not related to liver failure, while five deaths in the control group were. The high mortality rate in our study is related to the extremely small sample size. Waiting list mortality in our liver transplant program is around 25% per year and includes patients with HCC, an exclusion criterion in our study. Our high waiting list mortality rate is also the result of the vast discrepancy in our region between patients on the waiting list (between 150 and 200 per year) and the number of available organs. The donation rate for livers in Italy is 18.9 per million, while for our region, with a population of 5 million, it is 13 per million. At our institute, the overall waiting time for a liver transplant, including patients with HCC, who have a priority according to UNOS policy, is around 11 months. If we exclude patients with HCC, as we did in our study, the waiting time is longer, and this helps explain the low number of patients transplanted in the study period.

We arrived at a high fetal cell viability of 80% by using the vascular isolation method (12). The fetal cell preparation contained hepatic, hematopoietic, and mesenchymal progenitor marker-expressing cells. The interpretation of the MSC markers we investigated is hampered by the fact that liver-derived MSC markers are the subject of some debate. The fetal liver cell population that we characterized contained cells that were positive for vimentin, CD90, and CD29, and most of the $CD90^+$ cells expressed albumin or AFP. CD29 is often associated with MSC but has been also found on FACS analysis in $EpCAM^+$ cells from human fetal liver in the second trimester (33). Haruna et al. studied the expression of intermediate filaments during human liver development and proposed a temporary coexpression of vimentin with CK19 during hepatoblast differentiation toward the biliary phenotype (18). Dan et al. found that human fetal liver multipotent progenitor cells coexpress epithelial and mesenchymal markers and are able to generate both liver cells and mesenchymal lineages (6). Mesenchymal–epithelial transitional cells were found by Chagraoui et al. in human fetal livers and have been reported as supporting hematopoiesis (4). We decided not to sort liver progenitor cells in order to avoid losing cells expressing mesenchymal markers, which could have a cooperative role in cellular expansion. Joshi et al. (19), in a mouse model, found that adding human fetal liver-derived MSCs to fetal hepatocytes resulted in a higher rate of liver engraftment than when either class of cells was transplanted alone. Schmelzer et al. (35) found that in vitro colony expansion and cell outgrowth of human hepatic stem cells depended on the mesenchymal companion cells, identified as angioblasts or hepatic stellate cells. In order to avoid cell manipulation, the fetal liver cells were not labeled. This, and the limits of imaging resolution, made it impossible to follow their fate.

The most important factor preventing the use of adult hepatocyte transplants is the limited availability of hepatocytes, which are generally obtained from discarded organs. In our experience we faced a similar problem because of the low number of fetal donors that were suitable for cell transplantation (22%). Most of the organs were excluded because of active maternal infections or low cellular viability due to long ischemia time or occasionally to fetal liver damage secondary to abortion. To overcome this problem in the future, more gynecological centers should

be involved to increase the number of fetal donors, while the number of transplantable cells could be increased by pooling fetal liver cells of compatible blood groups.

The studies on adult HTx, though not comparable because of the means of infusion, amount of injected cells, and clinical variability among treated patients, have shown that HTx in patients with acute or chronic liver disease (20 cases) is safe and that 60% of the treated patients benefited from the hepatocyte infusion in terms of survival (9,30,37–40,42). In 23% of them, adult HTx had the role of providing temporary support as a bridge to LTx. We found a similar effect in terms of safety and stabilization of clinical scores, but to further test our preliminary data and establish if hFLCTx can be considered a potentially useful bridge therapy for decompensated cirrhotic patients, a prospective randomized clinical trial will be needed.

Our study has two significant limitations. First, the sample size was extremely small, though not by design. Using a single gynecology unit for donation, we had only a 22% rate of donation, necessarily limiting the availability of hFLCs. Second, our inclusion criteria for the control group, while aimed at clinical coherence, were also designed to guarantee an adequate number of patients for the purposes of the study. That said, these strict criteria, including residence on the island of Sicily, necessarily limited the pool of potential patients, as did the fact that ours is a single-center study, and carried out in a very small solid organ transplant center with only 77 beds.

On the other hand, Italy is one of the few countries in the West doing research in the field of fetal hepatocyte transplantation, a field we believe to have enough plausible potential to merit clinical study.

We are now designing a larger, prospective randomized controlled study, with an increase in the amount of cells to be injected. The aim will be to provide greater hepatic metabolic support.

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