ELSEVIER

Cell Transplantation, Vol. 7, No. 4, pp. 357–363, 1998

0963-6897/98 $19.00 + .00

PII S0963-6897(98)00027-X

Original Contribution

TRANSPLANTATION OF HEPATOCYTES FOR PREVENTION OF INTRACRANIAL HYPERTENSION IN PIGS WITH ISCHEMIC LIVER FAILURE

NIKOLAOS ARKADOPOULOS,* STEVE C. CHEN,* THEODORE M. KHALILI,* OLIVIER DETRY,* WINSTON R. HEWITT,* HELENE LILJA,* HIROFUMI KAMACHI,* LIDIJA PETROVIC,† CLAUDY J.P. MULLON,* ACHILLES A. DEMETRIOU,* AND JACEK ROZGA*[1]

*Liver Support Research Laboratory, Department of Surgery, Allen and Burns Research Institute and †Department of Anatomic Pathology, Cedars-Sinai Medical Center, UCLA School of Medicine, Los Angeles, CA 90048, USA

□ **Abstract — Intracranial hypertension leading to brain stem herniation is a major cause of death in fulminant hepatic failure (FHF). Mannitol, barbiturates, and hyperventilation have been used to treat brain swelling, but most patients are either refractory to medical management or cannot be treated because of concurrent medical problems or side effects. In this study, we examined whether allogeneic hepatocellular transplantation may prevent development of intracranial hypertension in pigs with experimentally induced liver failure. Of the two preparations tested—total hepatectomy ($n = 47$), and liver devascularization ($n = 16$)—only pigs with liver ischemia developed brain edema provided, however, that animals were maintained normothermic throughout the postoperative period. This model was then used in transplantation studies, in which six pigs received intrasplenic injection of allogeneic hepatocytes (2.5×10^9 cells/pig) and 3 days later acute liver failure was induced. In both models (anhepatic state, liver devascularization), pigs allowed to become hypothermic had significantly longer survival compared to those maintained normothermic. Normothermic pigs with liver ischemia had, at all time points studied, ICP greater than 20 mmHg. Pigs that received hepatocellular transplants had ICP below 15 mmHg until death; at the same time, cerebral perfusion pressure (CPP) in transplanted pigs was consistently higher than in controls (45 ± 11 mmHg vs. 16 ± 18 mmHg; $p < 0.05$). Spleens of transplanted pigs contained clusters of viable hepatocytes (hematoxylin-eosin, CAM 5.2). It was concluded that removal of the liver does not result in intracranial hypertension; hypothermia prolongs survival time in both anhepatic pigs and pigs with liver devascularization, and intrasplenic transplantation of allogeneic hepatocytes prevents development of intracranial hypertension in pigs with acute ischemic liver failure.** © 1998 Elsevier Science Inc.

□ **Keywords — Hepatocyte; Transplantation; Fulminant hepatic failure; Intracranial pressure.**

INTRODUCTION

Intracranial hypertension, secondary to brain edema, is the most common cause of brain injury and death in patients with FHF (13,24). It is life-threatening, because it may cause (a) brain stem herniation, (b) compression of the posterior cerebral artery leading to infarction, (c) obstructive hydrocephalus due to cerebral aqueduct and subarachnoid space compression, and (d) brain stem compression resulting in brain stem ischemia, hemorrhage, and death. Additionally, intracranial hypertension may cause a significant decrease in CPP and cerebral blood flow, and thus aggravate cerebral ischemia or infarction resulting in major neurological deficits.

The pathogenesis of intracranial hypertension is not fully understood. At present, both vasogenic (hypoxic) and cytotoxic factors have been etiologically implicated (6,11,33,34). Mannitol, barbiturates, and hyperventilation have been used in this setting, but most patients are either refractory to medical management or cannot be treated because of related complications (e.g., renal failure) or side effects (9,16,22). Orthotopic liver transplantation (OLT) remains the only definitive treatment for FHF and its neurologic complications. However, severe neurologic dysfunction precludes many FHF patients from being listed for a transplant. Furthermore, of those listed for urgent OLT (United Network for Organ

ACCEPTED 4/9/98.
[1]Correspondence should be addressed to: Jacek Rozga, M.D., Ph.D., Liver Support Research Laboratory, Department of Surgery, Cedars-Sinai Medical Center, 8700 Beverly Blvd., D-4018, Los Angeles, California 90048.

Sharing-UNOS status 1), 20 to 50% will develop severe neurologic dysfunction and brain death before an organ becomes available for transplantation. Still others will suffer from neurologic complications or even die after OLT (2,7,13). It is, therefore, necessary to develop new therapeutic modalities aimed at protecting FHF patients from brain edema.

Transplantation of hepatocytes has been shown to provide metabolic support and improve survival in various experimental models of acute and chronic liver failure (e.g., 90% hepatectomy, liver ischemia, D-galactosamine toxicity, porto-systemic encephalopathy) (5,8, 12,17,23,25,32). In this study, we examined whether allogeneic hepatocyte transplantation may delay, or prevent, development of intracranial hypertension in pigs with intracranial hypertension caused by experimentally induced liver failure.

MATERIALS AND METHODS

Animal studies were performed in compliance with institutional and National Institute of Health guidelines for humane care of experimental animals.

Animals

Female farm swine were purchased from SNS Farms (Ranchita, CA) and were placed in 5-day quarantine prior to use in the experiment. They were housed in a climate-controlled (25°C) room under a 12-h light/dark cycle, were given tap water and fed with a Southwest Farms Swine Finisher (Newco, CA). Animals weighing 40–60 kg were used as hepatocyte recipients, and younger pigs (15–20 kg) served as hepatocyte donors. All operations were performed under general anesthesia (ketamine 20 mg/kg, i.v.; endotracheal isoflurane 0.5–1.0%) using a sterile surgical technique.

Chemicals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Hepatocyte Isolation

Donor hepatocytes were isolated by in situ two-step ethylenediaminetetraacetic acid (EDTA)/collagenase digestion, as described previously (21). Hepatocyte viability was always greater than 90%, as determined by trypan blue exclusion.

Experimental Surgical Models

In all pigs, the femoral artery and vein were dissected and cannulated with a 14-gauge Angiocath (Becton Dickinson, Sandy, UT) and Silastic Medical Grade Tubing (Dow Corning Co., Midland, MI), respectively. For ICP monitoring, a subdural bolt (Codman ICP Kit, Codman & Shurtleff Inc., New Brunswick, NJ) was inserted through a hole burred in the right temporoparietal region of the cranium. Cystostomy was performed using a Bardex Lubricath temperature sensing Foley catheter (CR Bard Inc., Covington, GA).

Liver devascularization (LD). Ischemic liver failure was induced by total liver devascularization. This was achieved by an end-to-side porto-caval shunt and transection of all ligamentous attachments to the liver.

Total hepatectomy (TH). After creating an end-to-side porto-caval shunt, the hepato-duodenal ligament was transected and the liver removed between two clamps. Inferior vena cava continuity was reconstituted with an 18-mm polytetrafluoroethylene graft (Gore-Tex Stretch, W.L. Gore & Associates Inc., Flagstaff, AZ). Heparin administration and veno-venous bypass were not utilized. Using this technique, the two episodes of splanchnic venous stasis (portocaval shunt and vena cava grafting) lasted only 10–15 min each, and were separated by a 10-min interval during transection of hepatoduodenal ligament.

Hepatocyte transplantation (HcTx). The three main vascular pedicles of the pig spleen (main splenic, gastroepiploic, and short gastric) were identified and cross-clamped. Next, a suspension of fresh, viable isolated allogeneic hepatocytes (2.5×10^9 cells in 60 mL of normal saline) was slowly injected into the splenic pulp through multiple punctures using a 24-gauge catheter (Venocath, Becton Dickinson, Sandy, UT). Following transplantation, the clamps were removed, hemostasis was achieved, and the abdomen was closed. After recovery from anesthesia, animals were returned to the vivarium and offered free access to food and water. All transplant recipients were immunosuppressed with Cyclosporine A (Sandoz, NJ; 5 mg/kg/day, i.m.) starting at 24 h prior to transplantation.

Postoperative Care

Following either liver devascularization or total hepatectomy, pigs were maintained on continuous intravenous infusion of 5% Dextrose in Lactated Ringer's solution (Abott Laboratories, Chicago, IL; 0.07 mL/kg/min). No other supportive measures were used, and no attempts were made to correct respiratory, hemodynamic, or metabolic abnormalities. In "normothermic" animals, body core temperature was maintained at 37°C through external heating (heating pad, blanket, and lamp)

Table 1. ICP (time of death) and survival in pigs with acute liver failure

	Normothermic LD ($n = 8$)	Hypothermic LD ($n = 8$)	Normothermic TH ($n = 27$)	Hypothermic TH ($n = 20$)
ICP (mm Hg)*	24 ± 8	10 ± 4†	11 ± 2†	9 ± 3†
Survival (h)*	21 ± 1.4	29 ± 1.6†	33 ± 1‡	44 ± 4§

Abbreviations: LD, liver devascularization; TH, total hepatectomy; ICP, intracranial pressure.
*Values are expressed as means ± SD.
†Significantly different from the Normothermic LD group ($p < 0.05$).
‡Significantly different from the Normothermic LD and Hypothermic LD groups ($p < 0.05$).
§Significantly different from all other groups ($p < 0.05$).

and through warming of intravenously administered fluids (Spectratherm, Cobe BCT, Inc., Lakewood, CO). "Hypothermic" animals were kept in a temperature-controlled environment at 21°C so that their body temperature was allowed to decrease below 30°C.

Experimental Design

In searching for a suitable experimental animal model of intracranial hypertension, we first monitored ICP in anhepatic pigs. Forty-seven pigs were rendered anhepatic. Twenty pigs were allowed to enter mild hypothermia, whereas the remaining 27 were maintained normothermic (>36°C). In another group of 16 pigs, liver necrosis was induced, and half of the animals were allowed to enter mild hypothermia and the other half were kept normothermic. In all pigs, ICP and CPP were recorded at frequent time intervals until the animals' death.

Hepatocyte transplantation studies were performed in normothermic pigs ($n = 6$) with liver devascularization. It has been reported that intrasplenically seeded isolated hepatocytes require a minimum of a few days to engraft and to undertake differentiated functions (20). Therefore, in this study hepatocyte transplantation was carried out 3 days prior to induction of ischemic liver failure. Each pig received 2.5×10^9 viable allogeneic hepatocytes, which corresponds to approximately 5% of the host liver mass.

Postoperative Monitoring

Vital signs, mean arterial blood pressure (MABP), and ICP were recorded using a Gould Windograf monitor (Gould Inc., OH). Because elevated ICP in FHF is thought to result from brain edema (3,4) and measurements of ICP with a subdural bolt are considered accurate (1), we used ICP as the sole index of brain edema. CPP was calculated by subtracting the ICP from the MABP. Core body temperature was determined using a Bard Urotrack Plus probe (CR Bard Inc., Covington, GA). Arterial blood samples were collected from both groups immediately following recovery from anesthesia. Hemoglobin (Hb) and serum sodium concentrations were determined in a clinical laboratory (Smith Kline & Beecham Clinical Laboratories, Los Angeles, CA). Arterial pH was measured using a blood gas analyzer (Radiometer Medical A/S, Copenhagen, Denmark). In all animals, autopsies were performed to verify absence of surgical complications (bleeding, shunt obstruction, pulmonary embolism) and total liver necrosis (pigs with LD).

Morphological Evaluation

Sections of transplant-bearing spleens were stained with hematoxylin-eosin. Additional sections were immunostained for a low molecular-weight keratin marker (CAM 5.2; Becton Dickinson, San Jose, CA), as described previously (14).

Statistical Analysis

Data are presented as means ± standard deviation (SD). Statistical analysis was performed using the Kaplan-Meier Survival test and one-way analysis of variance (ANOVA); p-values ≤ 0.05 were considered significant.

RESULTS

All animals recovered from surgery uneventfully and were hemodynamically stable. Anhepatic pigs lived longer than pigs with ischemic liver failure. When pigs were kept at ambient (21°C) temperature, their body temperature progressively decreased, reaching 25 ± 0.1°C at 20 h post-induction. In both experimental models, "hypothermic" pigs lived longer than "normothermic" ones (Table 1).

The opening ICP value was 4.2 ± 0.3 mmHg. None of the anhepatic pigs developed intracranial hypertension (Table 1). Only four of eight of hypothermic pigs with liver ischemia had a transient rise in ICP at 18–22 h (33 ± 9 mmHg). In contrast, all normothermic pigs with liver ischemia developed significant and persistent intracranial hypertension (Table 1).

After hepatocyte transplantation, none of the pigs developed intracranial hypertension after subsequent in-

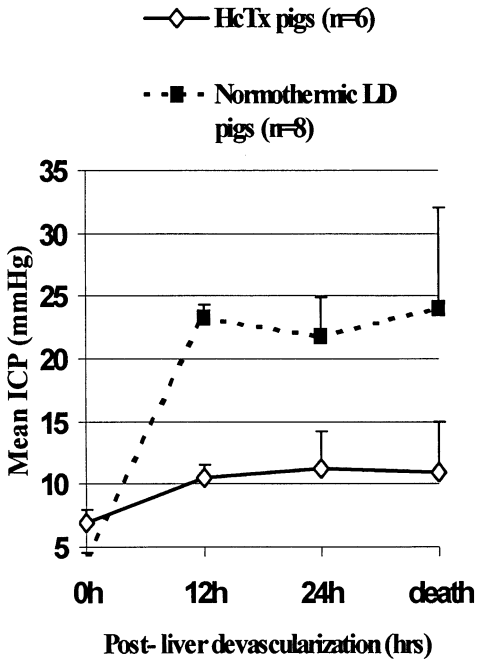

Fig. 1. Survival rate in normothermic LD and HcTx pigs.

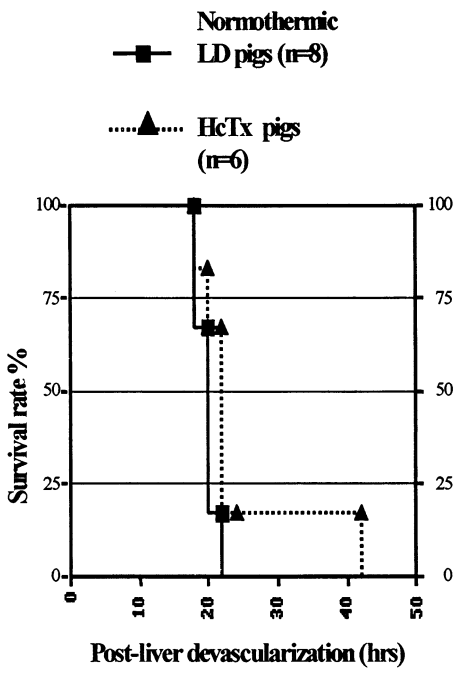

Fig. 2. ICP fluctuation in normothermic LD and HcTx pigs.

duction of total liver ischemia; in all pigs, the ICP was normal at all time points studied, with a peak value of 12 ± 3 mmHg. In keeping with these data, at the time of death, the ICP of transplanted pigs was significantly lower than that of nontransplanted animals (11 ± 4 vs. 24 ± 8 mmHg; $p < 0.05$), while CPP in transplanted pigs was significantly higher than in nontransplanted controls (45 ± 11 mmHg and 16 ± 18 mmHg, respectively; $p < 0.05$) (Fig. 1). Transplanted and nontransplanted pigs had similar baseline body core temperature, heart rate, MABP, pH, blood Hb, and serum Na values (Table 2). Finally, transplanted pigs survived longer, compared to normothermic pigs with liver ischemia (28.4 ± 7.6 vs. 21 ± 1.4 h; $p < 0.05$) (Fig. 2).

In transplanted pigs, spleen sections displayed numerous intact hepatocytes arranged in clusters (Fig. 3). Hepatocytes stained positive for CAM 5.2, an epithelial marker that is routinely used to characterize liver cells (14). Occasional mitoses were also present.

Table 2. Hemodynamic and metabolic parameters in Normothermic LD and HcTx pigs

	Normothermic LD	HcTx
Temperature (°C)*	35.1 ± 1.3	34.9 ± 1
MABP (mmHg)*	64 ± 1	74 ± 15
Heart rate (beats/min)*	148 ± 15	130 ± 18
pH*	7.4 ± 0.03	7.4 ± 0.1
Hemoglobin (g/dl)*	11.5 ± 1	10.9 ± 1.3
Na (mEq/L)*	138 ± 3	141 ± 2

Values were measured immediately following recovery from anesthesia.
None of the differences between the two groups was statistically significant.
*Values are expressed as means ± SD.

DISCUSSION

Despite improved understanding of the pathophysiology of fulminant hepatic failure, management of brain edema remains a major clinical challenge (13,24). As mentioned earlier, mannitol, barbiturates, and hyperventilation have been used in this setting, but most patients show only transient response or cannot be treated because of side effects or concurrent medical problems (9,16,22). We have developed a bioartificial liver containing porcine hepatocytes and have shown its ability to provide detoxifying and synthetic functions in a series of in vitro and in vivo experiments as well as in a phase I clinical trial (35). During the trial, patients with FHF experienced remarkable neurologic improvement with reversal of the decerebrate state after bioartificial liver treatments. Additionally, brain stem function improved, and there was a significant reduction in ICP with a concomitant increase in CPP (35). Although further appropriately controlled trials are needed to confirm these observations, they, nonetheless, suggest that hepatocyte-based support can arrest the development of intracranial hypertension. The present experimental study was undertaken to determine whether support of the liver failure patient by means of hepatocyte transplantation could have a similar effect.

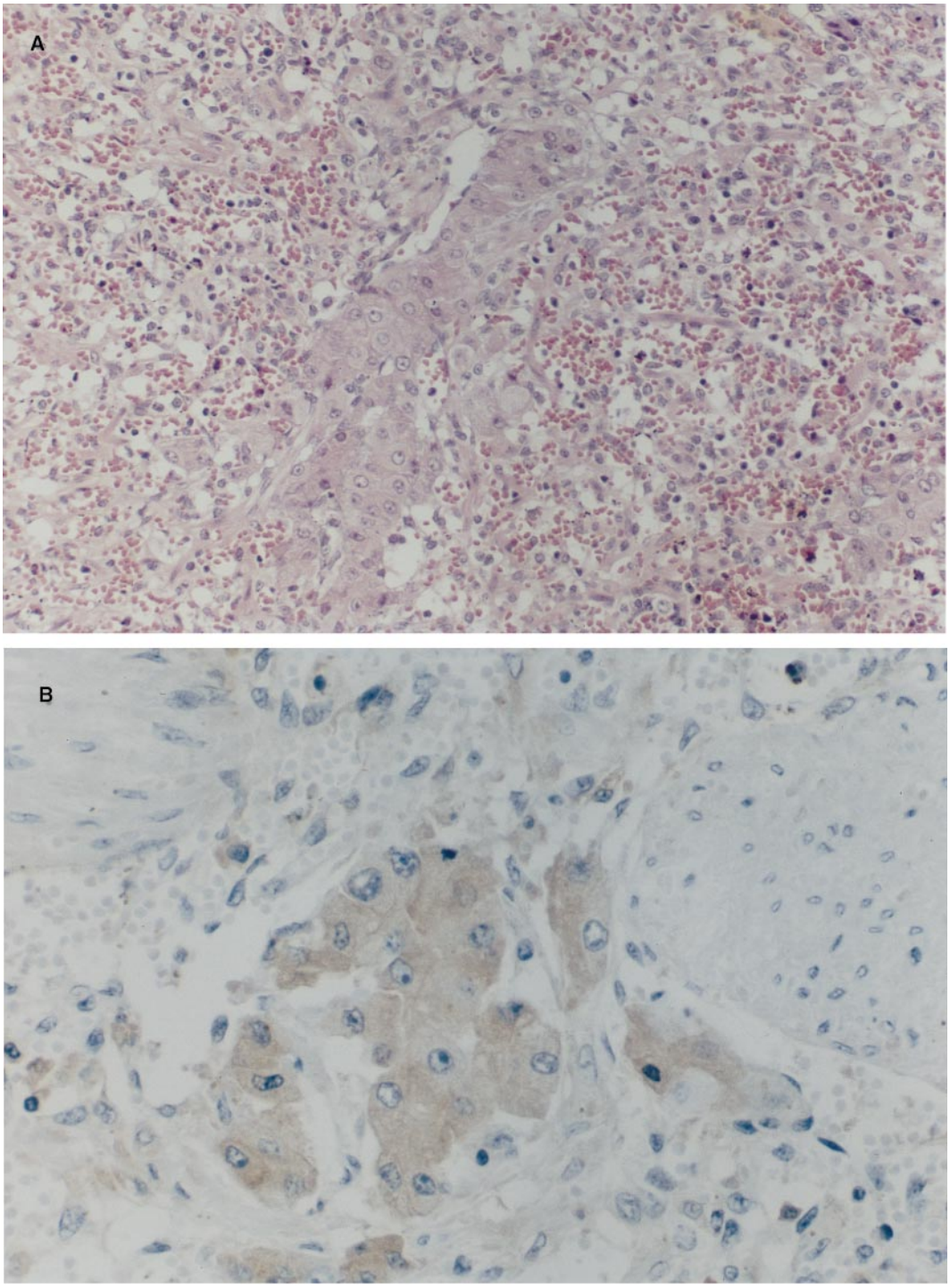

Fig. 3. Microscopic section of spleen from HcTx animals. A) Clusters of hepatocytes in the splenic pulp (hematoxylin-eosin ×75). B) Cluster of hepatocytes immunostained for a keratin marker (CAM 5.2 ×300).

To test this hypothesis, we first developed an appropriate large-animal experimental model of acute liver failure-induced brain edema. Initially, we studied an anhepatic animal model because in these animals there is a rapid accumulation in the blood of substances normally metabolized by the liver, including ammonia, bilirubin, bile acids, lactate, and aromatic amino acids, to name but a few (15,26,30). We found, however, that although survival time in our anhepatic pigs was twice as long as that reported by other investigators, none developed

intracranial hypertension. Intracranial pressure remained normal even at the terminal stages when CPP values were critically low. Maintaining animals normothermic has shortened survival time but had no effect on ICP, which remained below 10 mmHg throughout. We then tested pigs with ischemic liver failure. Here, again, we found that hypothermia prolonged survival when compared with pigs maintained normothermic through external heating. Additionally, only half of hypothermic animals developed episodes of intracranial hypertension. In contrast, normothermic pigs with liver necrosis showed early (12–14 h postoperatively) signs of brain edema and maintained elevated ICP and reduced CPP until death. Characteristically, ICP showed fluctuations exceeding 30 mmHg upon stimulation, which is widely seen clinically (4,22). As a result, this animal preparation was selected to examine the impact of cell therapy on brain edema.

Hepatocyte transplantation has been used by many investigators to demonstrate metabolic support and improve survival in animals with hepatic failure (5,12,19, 23,25,32). It has also been shown to reduce brain glutamine content and to improve abnormal behavior in rats with hepatic encephalopathy (17,29). This report demonstrates for the first time that hepatocellular transplantation can prevent development of brain edema in pigs with acute liver necrosis.

The mechanisms behind the observed effects are unknown, but likely to be due to transplanted cell function because in the recipients, the hepatized spleen was the only source of liver-specific support. Lack of brain edema in anhepatic pigs, including animals surviving for as long as 60 h on glucose supplementation alone, suggests that in the pathogenesis of liver-induced brain edema, liver necrosis plays a more important role than accumulation in the blood of substances metabolized by the liver.

This study, together with earlier reports in trauma and FHF-induced brain edema, suggests that hypothermia might find a role in the complex management of FHF (18,27). Review of the literature reveals that hypothermia lowers metabolic demands, slows accumulation of compounds normally metabolized by the liver and reduces neurotoxicity of ammonia (28). It also prevented increase in brain water content in rats with complete liver ischemia (33).

Recently, a limited number of patients with end stage liver failure underwent intrasplenic transplantation of small number of isolated hepatocytes with encouraging results (10,31). The first steps have thus been made, and this study provides further evidence in support of cell therapy in acute liver failure.

Acknowledgments — This study was supported in part by Circe Biomedical, Lexington, MA.

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