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Isolated Hepatocyte Transplantation for Crigler-Najjar Syndrome Type 1

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Crigler-Najjar syndrome type 1 (CN1) is an inherited disorder characterized by the absence of hepatic uridine diphosphoglucuronate glucuronosyltransferase (UDPGT), the enzyme responsible for the conjugation and excretion of bilirubin. We performed allogenic hepatocyte transplantation (AHT) in a child with CN1, aiming to improve bilirubin glucuronidation in this condition. A 9-year-old boy with CN1 was prepared with plasmapheresis and immunosuppression with prednisolone and tacrolimus. When a graft was made available, 7.5×10^9 hepatocytes were isolated and infused into the portal vein percutaneously. After 2 weeks phenobarbitone was added to promote the enzymatic activity of UDPGT of the transplanted hepatocytes. Nocturnal phototherapy was continued throughout the studied period. Total bilirubin was considered a reliable marker of allogenic cell function. There was no significant variation of vital signs nor complications during the infusion. Mean ± SD bilirubin level was 530 ± 38 μmol/L before and 359 ± 46 μmol/L after AHT (*t*-test, $p < 0.001$). However, the introduction of phenobarbitone was followed by a drop of tacrolimus level with increase of alanine aminotransferase (ALT) and increase of bilirubin. After standard treatment of cellular rejection bilirubin fell again but from then on it was maintained at a greater level. After discharge the patient experienced a further increase of bilirubin that returned to predischarge levels after readmission to the hospital. This was interpreted as poor compliance with phototherapy. Only partial correction of clinical jaundice and the poor tolerability to nocturnal phototherapy led the parents to refuse further hepatocyte infusions and request an orthotopic liver transplant. After 24 months the child is well, with good liver function on tacrolimus and prednisolone-based immunosuppression. Isolated AHT, though effective and safe, is not sufficient to correct CN1. Maintenance of adequate immunosuppression and family compliance are the main factors hampering the success of this procedure.

Key words: Hepatocyte transplantation; Crigler-Najjar syndrome; Uridine diphosphoglucuronate glucuronosyltransferase; Enzyme replacement therapy

INTRODUCTION

Crigler-Najjar syndrome type 1 (CN1) is an autosomal recessive disorder characterized by a severe, chronic, nonhemolytic, unconjugated hyperbilirubinemia resulting from the absence of hepatic uridine diphosphoglucuronate glucuronosyltransferase (UDPGT), the enzyme responsible for the conjugation and excretion of bilirubin (4,5,7,16). The standard treatment of CN1 so far has been orthotopic liver transplantation (OLT) (10). The replacement of the whole organ by OLT is curative but carries surgical risks and precludes the benefits of new techniques hopefully available in the future, such as gene therapy. Allogenic hepatocyte transplantation (AHT) has been performed in inborn errors of metabolism (8,9,13,20). Despite that the technique has been shown to be safe and effective, isolated infusions have not been shown to be sufficient to avoid OLT. Following the demonstration that AHT is feasible, the challenge of the current study was to perform several hepatocyte infusions and better understand the reasons for failure to maintain in vivo viability of these cells in the long term. Our aim was therefore to restore UDPGT activity in a child with CN1 by AHT, focusing on events affecting cell function following the infusion.

MATERIALS AND METHODS

A 9-year-old boy with severe unconjugated hyperbilirubinaemia and a clinical diagnosis of CN1 was referred to our center to be considered for OLT. The child already had clinical signs of kernicterus, including developmental delay, slurred speech, and mild ataxia. Magnetic resonance imaging was compatible with kernicterus.

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The confirmation of the diagnosis of CN1 was based on the following findings: total bilirubin under 16 h a day of phototherapy of around 500 μmol/L, with a conjugated fraction lower than 20 μmol/L; lack of response to phenobarbitone; absent signs of hemolytic disorders.

After discussion with the parents who provided written informed consent and once approval was obtained from the ethical committee of the University of Padua, the child was considered for AHT.

Patient Preparation

After listing for liver transplantation, a percutaneous transhepatic portal catheter was inserted under sedation, followed by a portogram and a CT scan with contrast to check the correct positioning and flow pattern in the portal system. The CT scan showed that the left lobe was preferentially perfused by the stream of contrast although the catheter was correctly placed in the portal trunk. A continuous infusion of 3 ml/h of NaCl 0.9% with sodium heparin 1 U/ml was maintained in the portal catheter. The child was then started on plasmapheresis every 3–4 days through a subclavian venous line, intensive phototherapy, and calcium carbonate 20 mg/kg four times a day. Immunosuppression with tacrolimus (levels around 10 μmol/L) was commenced.

Isolation of Hepatocytes

Once an ABO matched graft was made available it was retrieved and perfused with cold Belzer solution (3). The donor was a 47-year-old man who died from a cerebral vascular accident. A split liver technique was performed. The left lobe was dissected, with the left artery and left portal vein. The right lobe was transplanted into an adult patient with end-stage liver disease, leaving the left lobe for hepatocyte isolation that was started about 5 h following cross-clamping. The left liver lobe was maintained on ice and transported to the laboratory for cell isolation. Once in the laboratory, the graft was perfused with collagenase type IV at a concentration of 0.05 g/ml in phosphate buffer solution (PBS, Seromed) supplemented with 5 mmol of calcium chloride at a temperature of 37°C at a flow rate of 200 ml/min through the left branch of the portal vein and left hepatic artery. The two perfused segments were shred and passed through a digestion chamber. Then the liver cells (90% hepatocytes and approximately 10% nonparenchymal cells by morphological evaluation) were filtered through three silk mesh filters of different porosity from 500 down to 200 μm. Liver cells were suspended in cold Dulbecco's modified Eagle's medium (DMEM/Ham's F-12, Seromed) supplemented with 10% fetal calf serum (Sigma) and washed three times following $100 \times g \times 5$ min centrifugations. Fetal calf serum has been used safely for cell suspensions in human studies despite the concerns of immune reactions or cross-infections (2,11,19). The resulting suspension contained single cells along with small clusters of hepatocytes (1). The viability of the hepatocytes was determined by the trypan blue dye exclusion test before each infusion time and was 80% and 60%, respectively. Samples from the cell suspension were collected for microbiological tests, including bacteria, endotoxin, fungi, and mycoplasma. Aliquots of liver cells were stored for DNA analysis and future reference.

Infusion of Hepatocytes

The child was admitted to the Paediatric Intensive Care Unit and continuous monitoring of portal pressure, central venous pressure, arterial blood pressure, transcutaneous saturation of O_2, and ECG was commenced. Light sedation with midazolam 0.1 mg/kg/h and antibiotic prophylaxis with cephazolin and teicoplanin were started. Systemic heparinization with sodium heparin infusion (starting with 10 U/kg/h), adjusting the dose to achieve an activated partial thromboplastin time (APTT) of about 40 s or an activated clotting time (ACT) of 220–240 was initiated, followed by administration of methylprednisolone 10 mg/kg intravenously in 10 min, and then 20 mg/kg during the infusion of the hepatocytes. The hepatocytes were administered in two consecutive aliquots of 300 ml of Ringer lactate solution shaken and oxygenated continuously (Jatem-macchi; Mod. 1C-1000 oxygenator) containing 3.5×10^9 and 4.0×10^9 cells, respectively, and infused in 2 h each separated by a 2-h break. Cells for the second infusion were maintained on ice during the first infusion process.

After 6 h from the end of the infusion the portal catheter was removed under fluoroscopy, provided the visualization of the portal venous system at portogram. In order to avoid bleeding during the catheter extraction, an intraparenchymal embolization was performed (Spongostan, Johnson & Johnson-Medical Limited Gargrave, Skipton, UK).

Follow-Up

Daily Doppler ultrasound for 3 days to rule out portal vein thrombosis was performed. A week after the infusion oral phenobarbitone was added to enhance the enzymatic activity of the transplanted hepatocytes. Medical treatment and follow-up were otherwise the same as for patients receiving an OLT, as described previously (6). Daily serum bilirubin levels were measured to provide an estimate of the engraftment and function of the transplanted hepatocytes. Total bilirubin was considered a reliable marker of in vivo cell viability. Nocturnal phototherapy was continued throughout the study period. A liver biopsy was taken on day 30 and the liver fragment and saliva DNA were extracted with a forensic DNA kit (Biotek Omega, USA). PCR was carried out using an

AmpFISTR Profiler plus kit and short tandem repeat (STR) fragments were separated using the ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA) (15). We adapted the AmpFlSTR Profiler Plus kit commercialized as human identification system, which we used for chimerism analysis after allogenic bone marrow transplantation (BMT). For the interpretation of mixed pattern donor- and recipient-specific peaks a standardized approach was validated. Due to the high number of markers, the method is very helpful when a sex mismatch analysis is not possible.

RESULTS

Safety and Tolerability of the Infusion

Hepatocyte infusion was carried out in 6 h with no significant variation of vital signs apart from an increase in portal pressure from 5 to a maximum and transient peak of 12 mmHg. Central venous pressure remained below 3 mmHg and heart rate, arterial blood pressure, and transcutaneous saturation of O_2 were constantly within the normal ranges throughout the procedure. There were no thrombotic complications. The child was always awake during the infusion and was discharged from the intensive care unit the following day. The child was never intubated and the only drugs used for catheter positioning and removal were midazolam and ketamine.

Engraftment and Efficacy of the Transplanted Hepatocytes

Total bilirubin dropped significantly following AHT from a mean ± SD level of 530 ± 38 μmol/L before AHT (in the period of maximized phototherapy during hospitalization) to values as low as 267 μmol/L. However, to further detail the effect of AHT and the complications observed during the follow-up, we deliberately identified five phases post-AHT (Fig. 1).

Phase 1 (days 0–14): Bilirubin dropped to a mean level of 362 μmol/L, showing a more pronounced decrease at around days 12–14 post-AHT.

Phase 2 (days 15–35): In this period bilirubin mean level was 395 μmol/L; at day 29 total bilirubin spiked to levels as high as 600 μmol/L (equivalent to pre-AHT values). This was interpreted as rejection and treated with pulsed steroids as previously described (6). Following steroids administration bilirubin started decreasing.

Phase 3 (days 36–60): Bilirubin remained stable at a mean level of 317 μmol/L (range 267–369).

Phase 4 (days 61–90): Bilirubin increased again to mean levels of 381 μmol/L (range 301–443). In this period the child had been discharged and was followed as an outpatient.

Phase 5 (days 91–146): Because of suspected poor compliance to nocturnal phototherapy the child was readmitted to the hospital and bilirubin dropped again to a mean level of 352 μmol/L (range 321–389).

Further Parameters During the Follow-Up

Tacrolimus. Tacrolimus levels were maintained at around 10 μmol/L in the first 10 days post-AHT (phase 1) and then dropped down to 6–8 μmol/L between day 15 and day 35 after AHT (phase 2). After day 35 tacrolimus levels were maintained between 10 and 12 μmol/L. This drop of tacrolimus followed the administration of phenobarbitone and was considered to be due to enzymatic induction. Tacrolimus levels returned to desired values after progressive and substantial increase of the administered dose (Fig. 2).

ALT. From day 35 post-AHT (phase 3) alanine aminotransferase (ALT, normal values <55 IU/L) went up to 355 IU/L at day 40, and then came back to normal after 1 month, approximately at day 85 post-AHT (phase 4). Such increase of ALT followed the drop of tacrolimus levels and the spike of bilirubin (Fig. 2). No evidence of infections or other complications during the 146-day follow-up after AHT were detected.

Only partial correction of clinical jaundice and the decreased tolerability to nocturnal phototherapy by the child led the parents to complain of a very poor quality of life and to request an OLT, performed successfully on day 146 post-AHT. Comparing the DNA of the donor liver before AHT, and saliva of the recipient, the hepatocytes of a needle liver biopsy taken at day 40 post-AHT and the native liver explanted 146 days after AHT chimerism could not be demonstrated.

After 12 months from OLT the child is well, with good liver function on tacrolimus- and prednisolone-based immunosuppression.

Statistical Analysis

The Student *t*-test between pre- and post-AHT bilirubin levels showed a significant drop of bilirubin ($p < 0.0001$). The same test applied to phase 3 versus phase 5 levels showed a significant increase of mean bilirubin levels ($p < 0.0001$).

DISCUSSION

Crigler-Najjar syndrome type 1 is a unique condition in which the liver architecture is completely normal and the enzymatic defect causes the accumulation of a single metabolite, unconjugated bilirubin. The jaundiced child with CN1 is managed easily with phototherapy as long as the body surface area, physiologically greater in infants, allows adequate exposure of skin to light with inactivation of toxic bilirubin. Typically, approaching adolescence, phototherapy becomes less effective and the child is committed to lie under the lamps not only over-

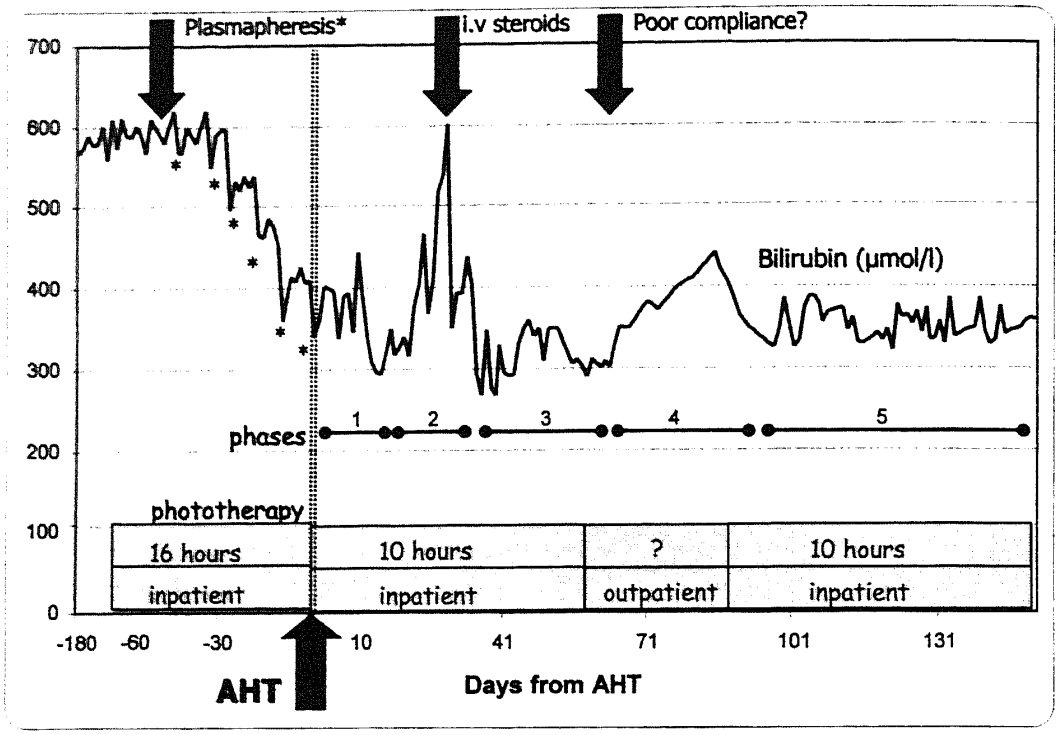

Figure 1. Bilirubin levels before and after allogenic hepatocyte transplantation (AHT). Plasmapheresis was performed as indicated by the asterisks (*). The follow-up has been divided into five phases: early postinfusion, tacrolimus drop, hepatocytolysis, discharge home, readmission.

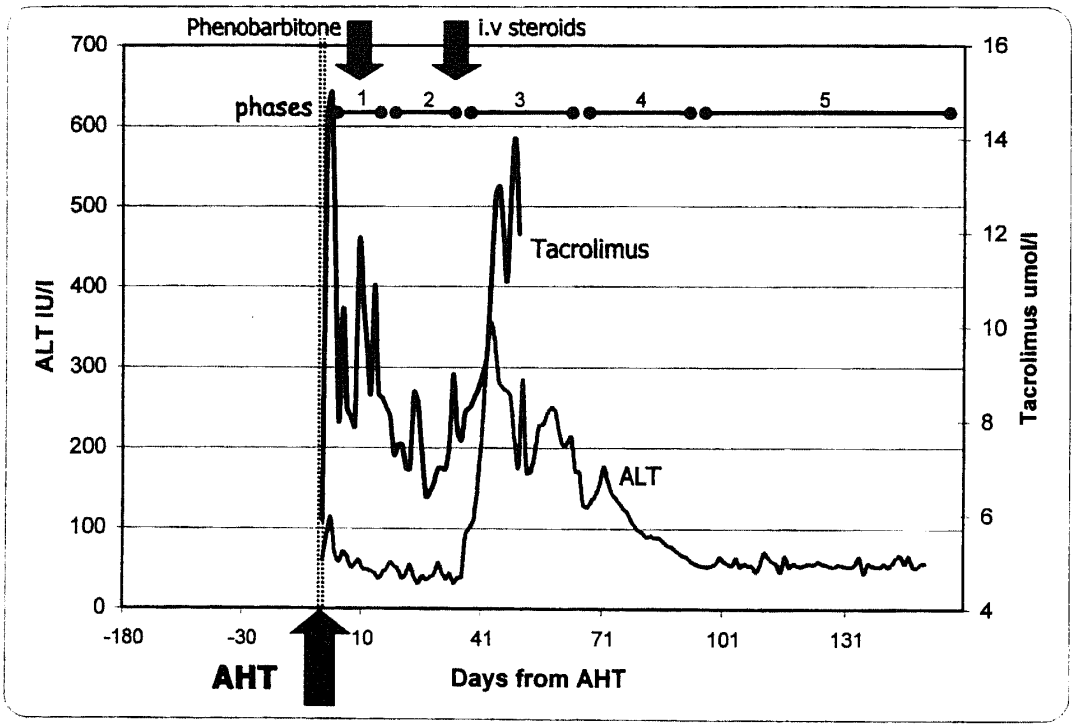

Figure 2. Alanine aminotrasferase (ALT) and tacrolimus levels after allogenic hepatocyte transplantation (AHT). The follow-up has been divided in five phases: early postinfusion, tacrolimus drop, hepatocytolysis, discharge home, readmission.

night while sleeping but also during the day. This leads to poor quality of life and also leads to underperformance of these subjects in physical and mental skills. However, the most worrying complication of such condition is the sudden rise of unconjugated bilirubin, often triggered by infections, with development of irreversible kernicterus (5). For these reasons liver transplantation has been advocated, which has been the treatment of choice for CN1 (21). OLT carries surgical and medical risks and condemns the patients to carry a graft for their whole life. Another option considered in CN1 has been performing auxiliary partial orthotopic liver transplant, a technique that, preserving a lobe of the native liver, offers the chance for gene therapy once available. This technique requires better surgical expertise and more careful follow-up than OLT and is not comparable to AHT as far as invasiveness and costs are concerned (17,22). All these reasons justify the attempt to decrease bilirubin levels by AHT.

With this study we have confirmed that AHT is a good alternative to OLT in CN1. Whereas OLT carries risks, is expensive and definitive, AHT in our experience is safe, effective, and inexpensive. Moreover, hepatocyte transplantation could be performed with a minimally invasive technique, without the need for general anesthesia or assistance for airway control.

Despite a significant and sustained reduction in serum bilirubin, the analysis of a biopsy taken during the study and the evaluation of the explanted liver after OLT did not demonstrate donor DNA in the liver tissue. A variety of methods have been used to evaluate cell engraftment following transplantation, including immunoglobulin allotyping, sex chromosome analysis, cytogenetic analysis, red blood cell phenotyping, and restriction fragment length polymorphisms (RFLP), each of which have limitations. The application of PCR-based technology has greatly enhanced the ability to detect small amounts of host or donor cells. We used a technique based on determination of chimeric status by short tandem repeat (STR) markers that was described as useful for monitoring minimal residual disease in patients after allogenic stem cell transplantation for hematological disorders. This technique has been described as able to detect as few as 1% of the chimeric cell population in the BMT setting (14,15). However, it is not validated for detection of chimerism in solid organ transplants and it is possible that the method might not have been sensitive enough in this setting. Moreover, in other experiments of hepatocyte transplantation the presence of a sex mismatch between donor and recipient could have favored the detection of chimerism in the recipient organ (8).

Assuming that 7.5 billion cells is approximately 5% of the hepatic cell mass and considering that, as previously suggested, probably less than half of the infused hepatocytes engraft, and also considering the flow-dependent distribution following the infusion in the portal vein, we were not surprised by the difficulty of locating donor cells within the native liver. Nevertheless, physicians dealing with CN patients know that such consistent and prolonged decrease in bilirubin cannot be attributed simply to fluctuations seen in this condition. It is possible that very few surviving cells can produce a noticeable biochemical difference of bilirubin conjugation in patients with CN1. Despite the initial success of the procedure, we faced several problems that led us to perform an OLT in this child. One of the difficulties we encountered was maintaining a level of tacrolimus steadily greater than 10 μmol/L. This was probably due to the enzymatic induction caused by phenobarbitone that was added to promote UDPGT activity in the transplanted hepatocytes. In fact, this was followed by a drop of the calcineurin inhibitor level despite progressive increase of the dose. This undesirable event allowed us to evaluate the effect of decreased immunosuppression in such a setting. The increase of bilirubin during phase 2 of the follow-up and of ALT shortly afterwards were, in this perspective, interpreted as cellular rejection that apparently responded to pulsed intravenous steroids and optimization of tacrolimus levels (Figs. 1 and 2). Interestingly, after the drop in tacrolimus levels, we observed first a remarkable increase of bilirubin and afterwards the increase of ALT (Figs. 1 and 2). Although this is not what is seen during rejection of a liver graft, also the previous reports on AHT referred to episodes of rejection lacking the typical biochemical features commonly expected after OLT (8,9,13,20). It cannot be excluded that rejection could present differently in AHT when compared to OLT. It has been shown in animal models that proinflammatory cytokines such as those observed during cellular rejection affect UDPGT activity, decreasing glucuronidation with a dose-dependent mechanism (12). If the amount of cells synthesizing UDPGT in the liver is 100%, such as after OLT, there is probably enough enzyme to overcome the inhibition of proinflammatory cytokines during cellular rejection. Conversely, if there is only 2% of cells synthesizing UDPGT a partial inhibition of its activity could be sufficient to worsen the jaundice in such patient. We speculated that the inflammatory response caused by cellular rejection initially inhibited UDPGT activity and later led to cell lysis, with loss of part of the transplanted hepatocytes. The relatively higher levels of bilirubin we were able to achieve thereafter (phase 5 vs. phase 3) support the hypothesis that some cells were lost after the increase of ALT, in keeping with cellular rejection. It is interesting to notice that in the published AHT literature, an episode of rejection is frequently suspected but concomitant elevation of transaminases has not been reported (8,9,13,

20). The same mechanism mediated by proinflammatory molecules may be responsible for the delayed drop of bilirubin following the procedure (phase 1, Fig. 1), possibly due to the cytokine storm caused by the infusion.

Although the reduction in bilirubin levels was precisely what would have been expected based on the mass of cells transplanted and the previously published results (8), the family became impatient with the persistent, though mild, jaundice and opted for an OLT, obviating an opportunity to correct the metabolic defect by further cell infusions. A report by Seppen et al. (18) suggests that as little as 10% of a normal amount of hepatic UDPGT activity might be sufficient to normalize bilirubin levels. An estimate of approximately 10% UDPGT activity being able to provide a near total correction of bilirubin levels is supported by the hepatocyte transplant studies. In the present case a transplantation of 5% of the liver mass resulted in a 50–65% correction in the bilirubin levels. These data suggest that if a second transplant was as effective as the first, one could estimate that it would require two or perhaps three separate cell infusions to provide normalization of bilirubin levels and relief of clinical symptoms of the disease, such as jaundice, which would avoid the need of continuing nocturnal phototherapy. Nevertheless, the family did not accept further infusions.

In future transplant attempts, the family of the patient should be carefully screened for their suitability to embark on this type of experimental procedure, to avoid problems of compliance. The increase of bilirubin we noticed while the child was followed as an outpatient (phase 4, Fig. 1) and the drop following readmission to the hospital (phase 5, Fig. 1) led us to consider decreased compliance to phototherapy at home. Before other pediatric patients are treated by hepatocyte transplants, the patient's family must be fully informed of the length of time the experimental procedure will require and the number of separate transplants that would most likely be needed before relief of symptoms such as jaundice could be expected.

The present results and those of Fox et al. (8) indicate that relatively few cells can have a profound effect on metabolic diseases of the liver. In a report by Muraca et al. (13) even fewer cells (2 billion) resulted in a substantial improvement in blood glucose levels in a patient with glycogen storage disease Type 1a. Horslen et al. performed hepatocyte transplantation in an infant with severe ornithine transcarbamilase deficiency (OTC) transplanting 4 billion viable hepatocytes (9). However, most of these cases ended up with the need for a traditional form of liver function restoration such as organ transplantation. It is clear that at this point the attention should be focused on the causes of failure to maintain the cells' efficacy in the long term and on the need to perform several hepatocyte infusions to succeed.

We have found that a common problem is the occurrence of what is likely an episode of rejection. This occurs often because of insufficient immunosuppression. Probably, in presence of inflammatory cytokines, the transplanted cells are inhibited and halt their function. If the insult is not removed the cells are destroyed and the efficacy of the procedure cannot be entirely rescued. Another problem we faced was the disappointment of the parents after partial correction of the defect, despite thorough explanation before the procedure, and the aversion to go on with further infusions of cells.

Additional studies will be required to determine if bilirubin levels can be normalized by cell therapy alone. The possibility to perform multiple hepatocyte infusions, a better preparation of the family, a more careful maintenance of adequate immunosuppression, and overall the effort to better understand the factors affecting the in vivo viability of transplanted allogenic hepatocytes are warranted to make this procedure more effective in the long term.

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