ORIGINAL ARTICLE

Isolation of Hepatocytes from Livers from Non-Heart-Beating Donors for Cell Transplantation

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One of the limitations to hepatocyte transplantation is the restricted availability of donor liver tissue. The aim of this study was to evaluate livers from non-heart-beating donors (NHBDs) as a source of hepatocytes for cell transplantation. A total of 20 livers/segments obtained from NHBD were perfused under good manufacturing practices using a standard collagenase digestion method. The donor liver median warm ischemia time was 15 minutes (range, 11-40 minutes), and cold ischemia time was 13 hours (range, 6-30 hours) prior to cell isolation. The cell viability of the hepatocytes obtained was 52% (1-81%), with a yield of 2.2×10^6 ($0.2\text{-}29.7 \times 10^6$) cells per gram of tissue. There was a significant negative correlation between hepatocyte viability and length of both warm ischemia ($r = -0.544$, $P = 0.013$) and cold ischemia ($r = -0.510$, $P = 0.022$). Preliminary experiments were performed on the viability testing of NHBD livers based on digestion of needle biopsies with collagenase and assessment of the hepatocytes produced. Two of the NHBD cell preparations, which had been cryopreserved, were used as part of a series of cell infusions for hepatocyte transplantation. A 3.5-yr-old girl with Crigler-Najjar syndrome type I received 9.7×10^8 NHBD hepatocytes (viability on thawing, 65%), and a 4-month-old boy with inherited clotting factor VII deficiency received 5.0×10^8 hepatocytes (viability, 57%). In conclusion, hepatocytes suitable for cell transplantation can be obtained from NHBD livers. Higher viability values may be obtained if both warm and cold ischemia times of donor liver can be reduced prior to processing. ***Liver Transpl 12:713–717, 2006.*** © **2006 AASLD.**

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Hepatocyte transplantation is being evaluated as an alternative to orthotopic liver transplantation in the management of liver-based metabolic conditions and liver failure.[1,2] Experience of hepatocyte transplantation has been gained in patients with acute liver failure[3,4] and metabolic liver diseases such as Crigler-Najjar syndrome type I,[5] glycogen storage disease type 1a,[6] urea cycle defects,[7] and factor VII deficiency[8] for long-term, albeit partial, correction of the underlying metabolic defect.

For hepatocyte transplantation, cells isolated from donor livers are infused directly into the recipient liver or into the spleen so that they can migrate to the liver. The aim is to replace the missing liver function with transplanted hepatocytes and, particularly in the case of inborn errors of liver metabolism, repopulate the liver with normal donor cells. As with whole organ transplantation, hepatocyte transplantation is dependent on the availability of donor liver tissue, which is in limited supply. In this respect one of the potential advantages of hepatocyte transplantation, yet to be truly realized, is that organs currently not deemed suitable for transplantation, such as mild to moderately steatotic livers or where there is liver trauma, can be a source for isolation of hepatocytes. Livers from non-heart-beating donors (NHBDs), in which the organs are removed after the heart has stopped beating and respiration has ceased, are being assessed as an additional source of livers to increase the available supply for orthotopic transplantation.[9,10] The NHBD program was introduced at King's College Hospital in 2001. Strict selection of grafts was implemented to reduce the risks of

Abbreviation: NHBDs, non-heart-beating donors.

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TABLE 1. Details of Non-Heart-Beating Donors and Livers

No	Age (yr)	Gender	Lobe	Weight (g)	Cell yield $\times 10^6$	Cell viability (%)	Cause of death	Warm ischemia (min)	Cold ischemia (h)	Fatty or Transplant
1	39	M	Whole [Rt L]	1160	57000	22	Anoxia	40	12	
2	16	F	Whole	1600	47500	1.3	Trauma	25	30	Fatty
3	32	F	Whole [LLS]	409	6	50	Anoxia	17	17.5	Fatty
4	46	M	Whole	1545	464	25	Ischemia	12	14.5	Fatty
5	52	F	Whole	1498	430	40	IC bleed	12	12	
6	24	M	Whole	1740	380	50	Trauma	14	11	
7	49	F	Whole	1703	9000	64	Anoxia	12	12	Fatty
8	36	M	Seg IV and CL	73	10	52	Ischemia	12	12.5	TP
9	66	M	Whole [LLS]	356	2700	60	Ischemia	15	16	Fatty
10	57	M	Whole	-	690	65	Ischemia	14	14	Fatty
11	0.75	M	Whole [LLS]	323	5120	50	Ischemia	19	10.5	
12	54	F	Whole [LLS]	685	500	52	IC bleed	11	9	Fatty
13	32	F	Right lobe	1050	3800	65	Anoxia	11	7.5	TP
14	54	F	Whole [LLS]	870	600	66	IC bleed	14	17.5	Fatty
15	52	F	Whole	2093	1200	50	Ischemia	17	14.5	Fatty
16	7	M	Whole	595	379	22	Anoxia	20	13	
17	64	F	Right lobe	980	5870	79	IC bleed	16	6	TP + HTP
18	10	M	Right lobe	460	1003	81	Trauma	15	15.5	TP + HTP
19	15	M	Right lobe	1077	6480	60	Anoxia	14	13	TP
20	69	M	Whole [LLS]	640	430	51	IC bleed	18	12.5	Fatty

Abbreviations: [ ], tissue perfused; LLS, left lateral segment; Rt L, right lobe; CL, caudate lobe; TP, part orthotopic transplant; HTP, hepatocyte transplant.

liver graft primary nonfunction.[11] As a result, of 86 NHBD livers retrieved, only 44 were transplanted, with steatosis being the main reason for nonuse. As a result of this program, unused liver tissue from this source has been evaluated for isolation of hepatocytes for cell transplantation.

METHODS

Controlled NHBD livers were retrieved for clinical transplantation by the surgical donor organ retrieval team at King's College Hospital. Twenty liver tissues unsuitable or unused for orthotopic liver transplantation (15 whole livers, 5 liver segments) were delivered to the Cell Isolation Unit maintained in University of Wisconsin solution on ice. The other segments of the 5 split livers were used for orthotopic transplantation in 6 patients: 5 children and 1 adult. Warm ischemia time was taken as the time between development of a systolic blood pressure of <50 mmHg after withdrawal of life support and aortic perfusion with cold solution, this being the time of start of cold ischemia until commencement of the cell isolation procedure.

Isolation of Hepatocytes

Twenty isolation procedures were performed using 8 whole livers, 5 right lobes, 6 left lateral segments, and 1 segment IV plus caudate lobe under good manufacturing practices (Table 1). Hepatocyte isolation from NHBD tissue was carried out using a collagenase perfusion technique according to Strom et al.,[12] with modifications by Mitry et al.[13] The liver tissue was weighed and the major vessels were cannulated (1-4 cannulae). All perfusions were carried out at flow rates of between 20 and 80 mL/minute, and all buffers (Cambrex, UK) were maintained at 37°C. The first buffer consisted of Hank's Balanced Salt Solution containing 0.5 mmol/L ethylene glycol-bis (2-aminoethylether)-tetraacetic acid and 5 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; the second buffer consisted of Hank's Balanced Salt Solution only and then Minimum Essential Medium with Eagle's Balanced Salt Solution containing collagenase P (0.5 mg/mL, Roche Diagnostics, East Sussex, UK). Hepatocytes were purified by washing 3 times with ice-cold Minimum Essential Medium with Eagle's Balanced Salt Solution containing 2% human serum albumin (centrifugation at 50*g* for 5 minutes at 4°C). The cell number and viability were determined using a hemocytometer and trypan blue exclusion technique.

Cryopreservation of Hepatocytes

The freezing protocol used was based on that of Diener et al.,[14] with some modifications in a controlled rate freezer (Kryo 10, Series III; Planer PLC, Middlesex, UK). Freezing media consisted of University of Wisconsin solution to which dimethylsulfoxide was added to give a final concentration of 10% (v/v). After cryopreservation, hepatocytes were immediately transferred to the vapor phase of liquid nitrogen for storage until thawing.

Biopsy Test

Trucut needle biopsies were taken from 2 groups of NHBD livers. For the initial tests, samples were obtained from 7 cases (median age, 39 yr; range, 24-50 yr; gender, 4M:3F) in which the liver was used for orthotopic transplantation. This was performed after 10 to 12 hours of cold ischemia.

In a further 18 cases (median, 50 yr; range, 7-61 yr; gender, 10M:8F) liver biopsies were taken 2-3 hours after retrieval of the donor liver, and of these, 10 livers were subsequently used for orthotopic transplantation and 8 were rejected. Four of these were used for large-scale isolation of hepatocytes (numbers 13-16).

The biopsies (~20 mg in weight) were minced into 2- to 3-mm^3 pieces and incubated at 37°C in 2 mL of Minimum Essential Medium with Eagle's Balanced Salt Solution containing collagenase P (0.5 mg/mL) for 30 minutes, with agitation every 10 minutes. The cells released were filtered through 70-μm nylon mesh. Eight milliliters of ice-cold Minimum Essential Medium with Eagle's Balanced Salt Solution was added and the hepatocytes pelleted by centrifugation (50*g* for 5 minutes at 4°C). Hepatocyte viability was determined using trypan blue exclusion.

Statistical Analysis

Results are expressed as median with range. The Mann Whitney U test and correlation analysis were used to determine relationship between parameters.

RESULTS

Hepatocyte Isolation

The median donor age was 43 yr (range, 1-69 yr), and 11 of them were male. The median warm ischemia time of the liver was 15 minutes (range, 11-40 minutes), and cold ischemia time was 13 hours (range, 6-30 hours). The median hepatocyte viability by trypan blue exclusion was 52%, (range, 1-81%), and median cell yield per isolation after purification was 0.85×10^9 cells (range, 6.0×10^6 to 5.7×10^{10} cells) (Table 1). The lowest viability result was for donor tissue number 2 (1%), for which the cold ischemia time was 30 hours.

Ten of 15 livers that had been rejected for clinical transplantation were steatotic, and in others there had been poor perfusion on retrieval. There was a statistically significant higher viability of cells isolated from liver segments in which part was used for orthotopic transplantation (median, 65%; range, 52-81%), compared to whole livers rejected for transplantation (50%; 1-66%; $P = 0.011$).

There was a significant negative correlation between the hepatocyte viability and the warm ischemia time ($r = -0.546$, $P = 0.016$) and also cold ischemia time ($r = -0.510$, $P = 0.026$). If the warm ischemia time in minutes and cold ischemia time in hours were added together without units to give an ischemia index, there was a highly significant correlation with the viability of the hepatocytes obtained (Fig. 1). There was no significant correlation between cell viability and cell yield, time of withdrawal of support to cardiac arrest, age of donor, stay in intensive care unit, or use of inotropes in the donor.

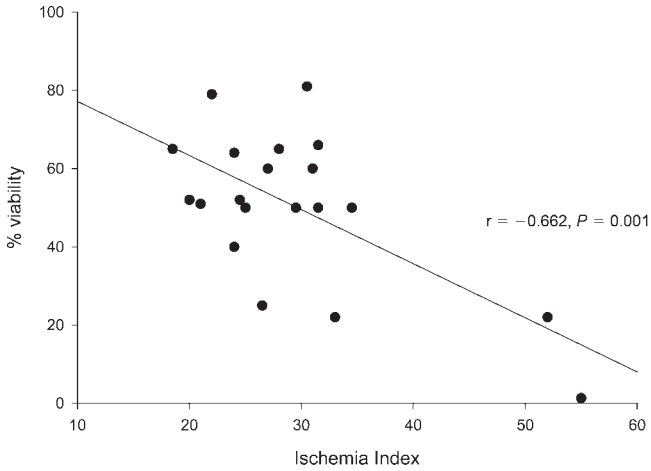

Figure 1. Correlation between in vitro viability of hepatocytes isolated from NHBD livers and the ischemia index (numerical sum of warm ischemia time in minutes and cold ischemia time in hours).

Use of Hepatocytes

For clinical transplantation hepatocytes should have a viability >60%, a cell number $>5 \times 10^8$ hepatocytes, together with an absence of microbiological contamination.[15] Overall, 7 of 20 (35%) NHBD livers processed met these criteria. Of these, 4 were from livers in which part was used for orthotopic liver transplantation, and 3 from livers rejected for orthotopic liver transplantation.

Hepatocytes from 2 of the cryopreserved NHBD preparations (numbers 17 and 18) that met the above criteria were used for hepatocyte transplantation in 2 children with liver-based metabolic liver disease as part of a series of cell infusions through a Hickman line placed surgically in the inferior mesenteric vein. A 3.5-yr-old girl with Crigler-Najjar syndrome type I received 9.7×10^8 hepatocytes (viability, 65% after thawing), and a 4-month-old boy with inherited clotting factor VII deficiency received 5.0×10^8 hepatocytes (viability, 57%). In both cases, this was performed without adverse clinical reaction, with beneficial effect in terms of lowered serum bilirubin and decrease in requirement for recombinant factor VII, respectively.

Liver Biopsy Studies

With the liver biopsies taken from livers transplanted and tested after 10 to 12 hours cold ischemia, the median viability was 65%, (range, 50-90%) and the median cell yield per biopsy was 5.4×10^4 cells (range, 5.0×10^3 to 5.7×10^5 cells).

With the biopsies taken and tested earlier after 2 to 3 hours cold ischemia, there was no difference in the viability obtained between livers used for transplanta-

tion (76%; 50-87%) and those rejected for transplantation (75%; 50-87%). In the 4 livers used for isolation of hepatocytes, the viability (median, 58%) found on isolation after 7.5-17.5 hours cold ischemia) was lower than that in the early biopsy sample (median, 79%).

DISCUSSION

This study has shown that hepatocytes suitable for clinical hepatocyte transplantation can be isolated from NHBD livers, including those rejected for orthotopic liver transplantation. The viability obtained (median, 52%) was similar to that obtained with our initial experience[13] with 20 conventional donor livers (median, 56%). As previously, and not surprisingly, significantly higher cell viability was obtained with liver tissue in which the other part had been used for orthotopic transplantation. In some cases, whole livers were dissected to allow perfusion of segments, usually left lateral segments, as better yields and viability can often be obtained. One of the tissues processed was a segment IV plus caudate lobe from a split liver procedure. We have recently reported this to be a good source of isolated hepatocytes.[16]

Some of the NHBD hepatocytes isolated in this study, which had been cryopreserved, were used for hepatocyte transplantation in 2 children with liver-based metabolic liver disease, which is the first time hepatocytes from this source have been used clinically. These were part of a series of cell infusions mainly using hepatocytes from conventional donor livers, and it is not possible to determine their separate function after administration. There was no evidence of any different reaction on infusion of NHBD. In a recent study of cryopreservation of human hepatocytes from different liver tissue sources, hepatocytes isolated from a small number of NHBD livers did seem to be more vulnerable to the effects of freezing. This was seen by lower viability and albumin production rates on thawing compared to hepatocytes isolated from conventional donor livers.[17] Further infusions of cryopreserved NHBD cells alone in patient treatment or in experiments in animals will help determine their function in vivo.

The main difference between NHBD liver tissue and that from conventional heart-beating donors is the period of warm ischemia subsequent to the withdrawal of life support until the time of cardiac arrest. Warm ischemia in the liver is considered to be more harmful to hepatocytes than endothelial cells with cold ischemia, being the converse from experiments in rats.[18] In clinical organ transplantation, cold ischemia is a major concern in development of primary graft nonfunction and acute rejection. Thus, in terms of isolated hepatocytes, the length of warm ischemia is more likely to be a concern. Although the length of warm ischemia in the livers used was relatively short (<40 minutes), there still was a correlation with hepatocyte viability. There was also a correlation with the cold ischemia times, which were quite considerable by the time of processing (median, 13 hours). Cold ischemia times of >8 hours are reported to be harmful to outcome in transplantation of NHBD livers,[19] yet the hepatocyte quality from livers with greater cold ischemia times was good. If the warm and cold ischemia times were added together, the ischemia index gave a greater correlation with isolated hepatocyte viability. In terms of other differences between NHBD and conventional donor livers, one important factor is that they have not been exposed to high levels of inflammatory cytokines in the donor in association with brain death,[20] and this could be advantageous to their function after isolation.

Our preliminary experience with the biopsy test to rapidly assess the quality of liver tissue, which might be used to determine suitability for orthotopic transplantation[21] or isolation of hepatocytes for cell transplantation, was inconclusive. A simple test was established using digestion of biopsies with collagenase and assessment of the viability by trypan blue exclusion. This was applied prospectively to a series of NHBD livers with biopsies taken 2-3 hours after start of cold ischemia, a time when decisions are made about the use of the liver for clinical transplantation. At this time there was no difference in viability of cells obtained from the biopsies to help determine suitability for transplantation, which was better done as routinely by the surgical team on the basis of gross appearance, ease of perfusion, degree of steatosis, and donor characteristics. Of the livers from which biopsies were taken, large-scale isolation of hepatocytes was performed in 4 of the 8 livers rejected for whole organ transplantation, which was not sufficient number for comparison. Further studies are required using serial biopsies in the same livers and also consideration of other functional tests of the biopsy or cells isolated from it, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide metabolic activity or incorporation of ^{14}C-leucine into protein.

In conclusion, hepatocytes suitable for cell transplantation can be obtained from NHBD livers. Higher cell viability values may be obtained if both liver warm and cold ischemia times can be reduced prior to processing.

REFERENCES

1. Horslen SP, Fox IJ. Hepatocyte transplantation. Transplantation 2004;77:1481-1486.
2. Fox IJ, Roy-Chowdhury J. Hepatocyte transplantation. J Hepatol 2004;40:878-886.
3. Strom SC, Fisher RA, Thompson MT, Sanyal AJ, Cole PE, Ham JM, et al. Hepatocyte transplantation as a bridge to orthotopic liver transplantation in terminal liver failure. Transplantation 1997;63:559-569.
4. Bilir BM, Guinette D, Karrer F, Kumpe DA, Krysl J, Stephens J, et al. Hepatocyte transplantation in acute liver failure. Liver Transplant 2000;6:32-40.
5. Fox IJ, Chowdhury JR, Kaufman SS, Goertzen TC, Chowdhury NR, Warkentin PI, et al. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. N Engl J Med 1998;338:1422-1426.
6. Muraca M, Gerunda G, Neri D, Vilei MT, Granato A, Feltracco P, et al. Hepatocyte transplantation as a treatment for glycogen storage disease type 1a. Lancet 2002;359: 317-318.
7. Horslen SP, McCowan TC, Goertzen TC, Warkentin PI, Cai

HB, Strom SC, et al. Isolated hepatocyte transplantation in an infant with a severe urea cycle disorder. Pediatrics 2003;111:1262-1267.

8. Dhawan A, Mitry RR, Hughes RD, Lehec C, Terry C, Bansal S, et al. Hepatocyte transplantation for inherited factor VII deficiency. Transplantation 2004;78:1812-1814.
9. Muiesan P. Can controlled non-heart-beating donors provide a solution to the organ shortage? Transplantation 2003;75:1627-1628.
10. Reddy S, Zilvetti M, Brockmann J, McLaren A, Friend P. Liver transplantation from non-heart-beating donors: current status and future prospects. Liver Transpl 2004;10: 1223-1232.
11. Muiesan P, Girlanda R, Jassem W, Vilca Melendez H, O'Grady J, Bowles M, et al. Single center experience with liver transplantation from controlled non-heart beating donors: a viable source of grafts. Ann Surg 2005;242:732-738.
12. Strom SC, Dorko K, Thompson MT, Pisarov LA, Nussler AK. Large scale isolation and culture of human hepatocytes. In: Franco D, Boudjema K, Varet B, eds. Îlots de Langerhans et hépatocytes. Paris: Les Editions INSERM; 1998:195.
13. Mitry RR, Hughes RD, Aw MM, Terry C, Mieli-Vergani G, Girlanda R, et al. Human hepatocyte isolation and relationship of cell viability to early graft function. Cell Transplant 2003;12:69-74.
14. Diener B, Utesch D, Beer N, Durk H, Oesch F. A method for the cryopreservation of liver parenchymal cells for studies of xenobiotics. Cryobiology 1993;30: 116-127.
15. Hughes RD, Mitry RR, Dhawan A. Hepatocyte transplantation for metabolic liver disease: UK experience. J R Soc Med 2005;98:341-345.
16. Mitry RR, Dhawan A, Hughes RD, Bansal S, Lehec C, Terry C, et al. One liver, three recipients—segment IV from split liver procedures as a source of hepatocytes for cell transplantation. Transplantation 2004;77:1614-1616.
17. Terry C, Mitry RR, Lehec SC, Muiesan P, Rela M, Heaton ND, et al. The effects of cryopreservation on human hepatocytes obtained from different sources of liver tissue. Cell Transplant 2005;14:585-594
18. Ikeda T, Yanaga K, Kishikawa K, Kakizoe S, Shimada M, Sugimachi K. Ischemic injury in liver transplantation: difference in injury sites between warm and cold ischemia in rats. Hepatology 1992;16:454-461.
19. Abt PL, Desai NM, Crawford MD, Forman LM, Markmann JW, Olthoff KM, Markmann JF. Survival following liver transplantation from non-heart-beating donors. Ann Surg 2004;239:87-92.
20. Jassem W, Koo DD, Muiesan P, Cerundolo L, Rela M, Fuggle SV, Heaton ND. Non-heart-beating versus cadaveric and living-donor livers: differences in inflammatory markers before transplantation. Transplantation 2003; 75:1386-1390.
21. Vilca Melendez H, Rela M, Murphy G, Heaton N. Assessment of graft function before liver transplantation: quest for the lost ark? Transplantation 2000;70:560-565.