CIR ESP. 2010;87(3):139–147

CIRUGÍA ESPAÑOLA

www.elsevier.es/cirugia

Special article

Hepatic cell transplantation. Technical and methodological aspects

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ARTICLE INFORMATION

Article history:
Received March 28, 2009
Accepted October 14, 2009
Online January 15, 2010

Keywords:
Liver cell transplantation
Cryopreservation
Isolation of hepatocytes
Cell therapy

ABSTRACT

Hepatic cell transplantation consists of grafting already differentiated cells such as hepatocytes. Human hepatocytes are viable and functionally active.

Liver cell transplantation is carried out by means of a 3-step method: isolation of hepatocytes from donor liver rejected for orthotopic transplantation, preparing a cell suspension for infusion and, finally, hepatocytes are implanted into the recipient. There are established protocols for the isolation of human hepatocytes from unused segments of donor livers, based on collagenase digestion of cannulated liver tissue at 37°C.

The hepatocytes can be used fresh or cryopreserved. Cryopreservation of isolated human hepatocytes would then be available for planned use.

In cell transplant, the important aspects are: infusion route, number of cells, number of infusions and viability of the cells. The cells are infused into the patient through a catheter inserted via portal vein or splenic artery.

Liver cell transplantation allows liver tissue to be used that would, otherwise, be discarded, enabling multiple patients to be treated with hepatocytes from a single tissue donor.

Trasplante celular hepático. Aspectos técnicos y metodológicos

Palabras clave:
Trasplante celular hepático
Criopreservación
Aislamiento de hepatocitos
Tratamiento celular

RESUMEN

El trasplante celular hepático (TCH) se basa en el empleo de células hepáticas adultas, hepatocitos humanos, viables y metabólicamente funcionales.

El TCH consta de 3 pasos: el aislamiento del hepatocito del hígado no válido para trasplante hepático, la preparación de las suspensiones celulares y, finalmente, su implante en el receptor. La obtención de los hepatocitos se realiza mediante digestión hepática con colagenasa a 37°C. Tras el aislamiento, las células pueden congelarse o administrarse en fresco. La criopreservación permite realizar el procedimiento de forma programada.

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Los aspectos clave del implante celular son la vía de infusión, el número de células por infundir, el número de infusiones y la viabilidad celular. La implantación es mediante catéter para infusión en la vena porta o la arteria esplénica.

El TCH permite utilizar los órganos desechados para trasplante convencional, y se tratan varios pacientes con hepatocitos del mismo donante.

Introduction

Currently, the only available effective treatment for terminal hepatic failure is an orthopic hepatic transplantation (HT). In spite of the constant increase in the number of HTs, the wait list continues to grow. Despite the broadening of donor selection criteria, the results obtained are unsatisfactory. This has motivated the development of various alternative methods to conventional HT, such as treatments to alleviate chronic or terminal liver illnesses, at least as a temporary procedure until organ procurement is possible.[1-6]

Among the proposed procedures, the most satisfactory results are obtained from hepatic cell transplantation (HCT) or human hepatocyte transplantation, since these allow the retention of hepatic functions until procuring an organ, and in some cases can achieve hepatic regeneration. The published results indicate that this can be a useful technique, provided that quality human hepatocytes are available with high viability and metabolic functioning.[1,2,6-13]

Procurement of organs donated for implantation, identification of the candidate patients for treatment, and the acquisition, processing, conservation, and finally, implantation of the isolated hepatocytes into the subsidiary patients are all necessary steps for this type of treatment.

HCT aims at transplanting hepatocytes, as these are the principal malfunctioning cells in poor liver function. A proper treatment uses fully differentiated adult cells (hepatocytes) on the basis of the high capacity of the liver to regenerate and the capacity of transplanted hepatocytes to develop all of the functions of a healthy liver.[2,14,15] Currently, the main problem with HCT is the lack of adequate sources of hepatocytes.

Hepatic cell transplantation. Hepatic Cell Therapy Unit

Obtaining human hepatocytes is a complex process due to the scarcity of suitable sources for cell isolation, the infrastructure necessary to carry out this isolation, and the conservation and lability of the hepatocytes for their maintenance and identification in the recipient.

HCTs were performed at the Hepatic Cell Therapy Unit (HCTU) at the Hospital La Fe, Valencia, whose installations are accredited under the European standards EN-ISO 14.644 on clean rooms and adjoining premises; the clean rooms are classified as ISO class 6, and the adjoining rooms as ISO class 7.

The clinical focus of the unit is on patients with hepatopathy HCT subsidiaries. The unit collaborates with the National Transplant Organization to obtain donated organs that have been deemed unsuitable for transplantation.

The isolation, characterization, and cryopreservation of human hepatic cells are performed under good manufacturing practices, and optimized procedures have been established in order to obtain human hepatocytes of the highest quality.

Methodology for hepatic cell transplantation

Basically, HCT is made up of three phases: hepatocyte isolation from donated livers that had been rejected for whole-organ transplantation, preparation of the cell suspensions, and implantation into the recipient. Following isolation, the cells can be frozen and stored (cell cryopreservation), and analyzed for bacteriology and quality control. The implantation of the hepatocytes into the recipient is performed by infusion in the portal vein and splenic artery, and this procedure can be planned with anticipation.

1. *Sources for obtaining hepatocytes*

Currently, the principal problem in HCT is the lack of adequate sources for viable human hepatocytes. Hepatocytes are acquired from whole organs not valid for transplantation due to steatosis greater than 40-50%,[1] a prolonged ischaemia, traumatic damage to the graft,[9] capsular tear,[10,16] blood group incompatability[17] and vascular or biliary lesions. Occasionally, foetal hepatocytes have been used for infusion.[11]

New sources for obtaining hepatocytes include hepatic tissue resulting from hepatic reductions, split liver grafts and segment iv following split liver grafts for 2 recipients,[12,13,18,19] grafts from non-heart beating donors, and donors with arteriosclerosis. Other proposed sources are hepatic tissues resulting from hepatic resection due to benign illnesses, or following colorectal tumour resection[20] (Figure 1).

The HCTU procured 13 donor organs deemed unsuitable for HT, but fit for HCT. The hepatocytes were isolated from donors with arteriosclerosis, unconfirmed doubtful viral serology, non-heart beating donation, split liver grafts, hepatic fibrosis, and newborn cerebral anoxia.

2. *Hepatocyte isolation*

Berry and Friend (1969) were the first to develop a procedure for rat hepatocyte isolation using *in situ* liver perfusion

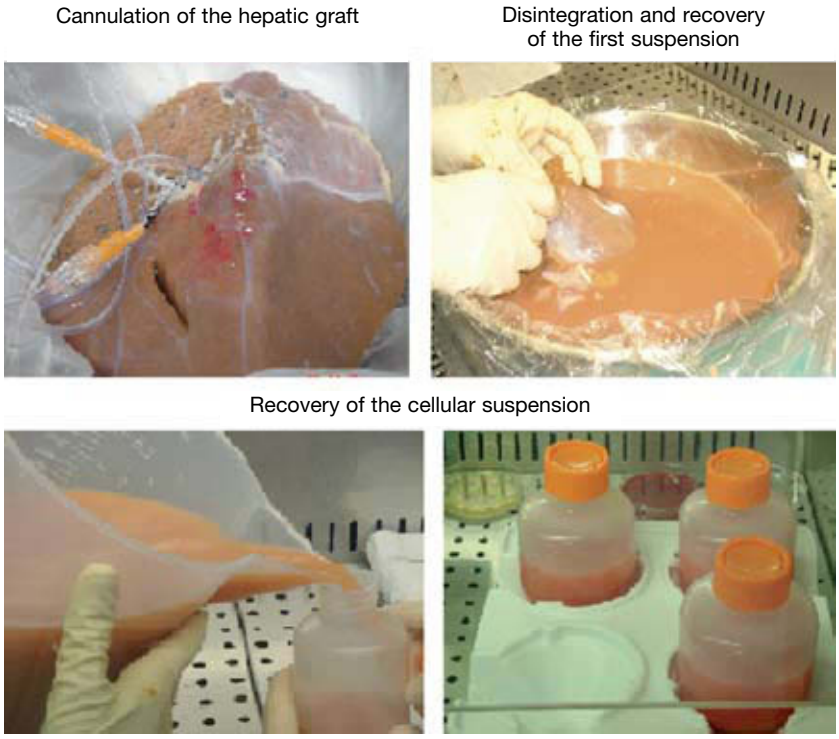

Figure 1 – Hepatocyte isolation: digestion of the hepatic parenchyma.

Table 1 – Hepatocyte isolation from whole organs or split liver grafts unsuitable for conventional liver transplantation

Sex	Age, years	Sample	Weight, g	Steatosis, %	Duration of ischaemia, h	Viability, %	Yield x 10^6 cells/g
F	80	LHL	350	< 30	12	73	2.8
F	65	WL	3500	40-50	16	85	1.6
F	69	WL	1142	30	13	85	2.6
M	75	WL	2200	40	9	53	1
M	32	RHL	1200	< 30	11	86	2.6
M	26	WL	2032	50	17	20	Discarded
M	58	WL	1550	40	15	20	0.11
M	67	WL	2354	10	6	20	Discarded

F: female; LHL: left hepatic lobe; M: male; RHL: right hepatic lobe; WL: whole liver.

using collagenase, and laid the foundation for current protocols. Collagenase is a proteolytic enzyme that allows digestion of the hepatic tissue and isolation of its cellular components.[18,21-28]

Initially, we used biopsies of livers preserved in Celsior® to investigate the effect of factors such as duration of cold ischaemia and hepatic steatosis determined by anatomopathological studies on the success of isolation and hepatocyte quality. Both cellular viability and performance decreased as the duration of cold ischaemia and steatosis increased.[29-32]

Based on these results, we attempted to optimize the process of hepatocyte isolation and cultivation from whole livers (1.5-3.5 kg) or complete lobes (0.5-1 kg) rejected for transplantation due to steatosis or other reasons. We initially performed 5 isolations[32] followed by 3 more, for a total of 8 (Table 1). All of the grafts were perfused with Celsior®, since our experience has shown that its use produces higher cell viability in the process of human hepatocyte isolation compared to other preserving liquids.[31] Organs with advanced steatosis (≥ 40%) showed lower cell yields during isolation. The older rejected donated organs showed tendencies of reduced viability (Table 1). In general, the disintegration process of the liver or whole lobes gives lower yields than those obtained through surgical biopsies of fatty liver tissue, although we must also highlight higher yields

from samples of a lower weight (single lobes) (Table 1). This could be attributed to the problems initially presented by enzymatic graft digestion.

Of all the organs rejected for implantation, 13 were sent to the HCTU for hepatocyte isolation. The criteria for acceptance of these grafts for cellular isolation are similar to those required for HT donation. Appropriate donors for consideration for HCT are all those grafts rejected for HT, with no age limit, that show no signs of sepsis or history of neoplasia, with negative serology for hepatitis C and B, HIV, HTLV, and syphilis, family consent for use of the organ for research purposes, steatosis less than 30%, no hepatic illnesses (cirrhosis, cholestasis, or haemophilia), and a duration of cold ischaemia less than 6 hrs.

When steatosis surpasses 30%, this invalidates the tissue for cell isolation. Our experience has allowed us to demonstrate that, compared to a normal liver, hepatocytes from fatty livers show a reduced viability, lower isolation yields (viable hepatocytes obtained/g of tissue processed), are very fragile in the process of cryopreservation, and the chances of a successful graft are lower, which is why these are unsuitable organs for cell treatment,[21,30-32] and this is a criteria for exclusion.

Of the 13 donors (8 women, 5 men), isolation was performed in split liver grafts in 3, complete organs in 2, and in the rest of the donors, the organ was divided into 2 lobes, left and right segments and segment iv for improved perfusion. The percentage of steastosis was determined by anatomopathological studies, and duration of ischaemia was under 4 hrs in all cases. Cell viability was between 70 and 89% with a yield of 1-17×10^6 cells/g (Table 2).

3. *Isolation technique*

Our laboratory works from a standardized protocol for hepatocyte isolation from surgical hepatic biopsies, which has been optimized and adapted for large scale isolations from organs unfit for whole-organ transplantation or from liver fragments from hepatectomies.[23]

Enzymatic perfusion is performed following cannulation of the hepatic artery and the portal vein from the donated liver tissue. When feasible, we also cannulate the biliary duct. In the first isolations we performed a perfusion of the entire organ; however, the digestion of the hepatic tissue was incomplete. This spurred a modification of the perfusion system, and we divided the hepatic parenchyma into left and right lobes, performing a separate perfusion of each lobe (Figure 1).

Enzymatic digestion was made up of 2 stages: in the first stage we used the non-recirculating perfusion system to treat the hepatic tissue with a buffered Krebs-Ringer saline solution with HEPES (20 mmol, pH 7.4) free of calcium but with EGTA (0.5 mmol) in order to cleanse the tissue of blood and weaken the intercellular unions; the second stage, with a recirculating perfusion system, used the same saline solution without EGTA and with 5mmol Cl_2Ca and collagenase at 0.05% for complete digestion of the hepatic parenchyma.[25,26] Both stages were carried out at 37ºC.[26] This was followed by mechanical disintegration of the hepatic tissue in order to obtain a suspension of hepatocytes in the Kebs-Ringer solution for filtration (Figure 1). This cellular suspension is washed several times, centrifuged, and the supernatant discarded and cellular sediment resuspended. In the end, we

Table 2 – Hepatocyte isolation in organs unsuitable for orthopic hepatic transplantation

Age, years	Sex	Group	Sample	Weight, g	M	VT, %	Yield x 10^6 cells/g	NV
53	F	0	LHL	420	78	67	1.3	Split
1	M	0	WL	420	75	68.5	6	Unconfirmed DS
14	F	0	RHL	774	75	74.7	6	Split
83	M	0	LHL	260	89	63.3	6.7	Hepatic FB
32	F	0	LHL	350	79.3	50	4.9	Hepatic FB
			RHL	832				
			IV	676				
52	M	A	LHL	376	87.5	37.8	9.9	NBH donor
			RHL	1154				
15	F	0	LHL	460	86.5	63.8	5.75	NBH donor
			RHL	1162				
73	M	AB	LHL	444	70	NA	3.3	Hepatic FB
76	F	A	LHL	325	88.5	NA	5.25	AE
			RHL	782				
82	M	0	LHL	488	80.5	41.7	4.95	AE
			RHL	1140				
15	F	0	RHL	1292	87	71	8.7	Hepatic TR split
4 D	F	A	WL	238	100	74	17	NR
53	F	0	RHL	998	95	67	6.58	CAA
			LHL	306				

AE: arteriosclerosis; CAA: celiac artery aneurysm; D: days; DS: doubtful serology; F: female; FB: fibrosis; LHL: left hepatic lobe; M: male; NBH: non-beating heart; NR: no recipient; NV: not valid; RHL: right hepatic lobe; TR: trauma; VT: viability after thawing; WL: whole liver.

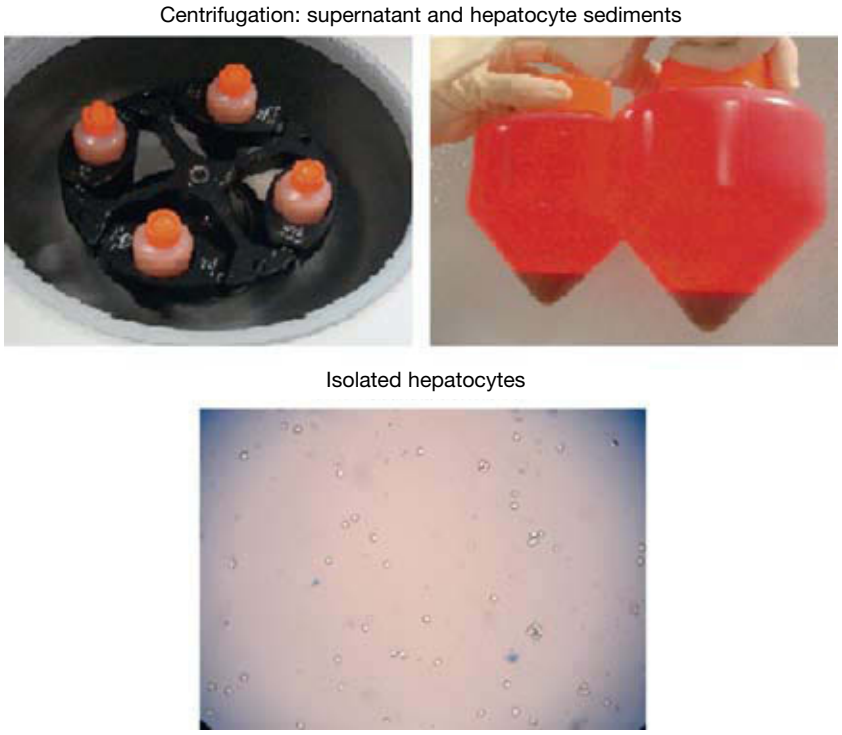

Figure 2 – Hepatocyte isolation: centrifugation of the cellular suspension and recovery of the hepatocytes.

measured cell viability using a trypan blue stain, cell yield and functionality through metabonomic studies of phase i and ii biotransformation enzymes, ureogenesis capabilities, and total number of cells[33] (Figure 2). Between 6×10^6 and 2×10^7 live hepatocytes/g of liver are obtained,[30,31,34] and the conditions for tissue isolation influence the procedure results.[34]

Once the preserving solution is applied, logistics for processing the entire graft in the least time possible, variability of the specific activity of the collagenase, the need to maintain the tissue at 37°C during the collagenase perfusion, and complete digestion of the tissue are all key aspects of an optimized procedure. Our recent studies have shown that the source of hepatic tissue, the type of ischaemia, and the perfusion liquid (Celsior®/Wisconsin) used all have an impact on the yield and quality of the hepatocytes obtained.[31,32]

4. *Preparation of cellular suspensions for implantation*

Following hepatocyte isolation, the cells are resuspended, either in the appropriate infusion medium[33] for fresh transplantation or in a freezing medium for cryopreservation and later use[18,25,30] (Figure 3). Cryopreservation of the obtained hepatocytes allows a semi-planned transplantation procedure. The samples are then submitted for microbiological study in order to dismiss any chance of contamination in the isolation process, along with the addition of prophylactic antibiotics.

5. *Hepatocyte implantation/infusion*

Cell transplantation requires an infusion of the human hepatocytes in suspension for a period that oscillates between minutes and hours (Figure 4). The composition of the infusion medium and the duration and storing of the hepatocytes all influence the quality of the cells obtained. Normally, the composition of the infusion is based on a plasmatic protein buffer with anticoagulating agents and glucose in hypothermic conditions. The addition of N-acetyl-cysteine has been shown to reduce oxidative stress and apoptosis.[27]

There are 4 key aspects for defining the implantation technique: route of infusion, number of cells to infuse, number of infusions, and cell viability.

Viability and functionality

Most authors assess cell viability using a the trypan blue exclusion test, which measures integrity of the cell membrane.[21,27] Live cells do not uptake trypan blue, while the dead ones are permeable to the colorant, and stain blue. Percent viability is then calculated using the following formula:

$$\%\ \text{cell viability} = \frac{(\text{mean total no. of cells mean no. dead})\text{x}100}{\text{mean total no. of cells}}$$

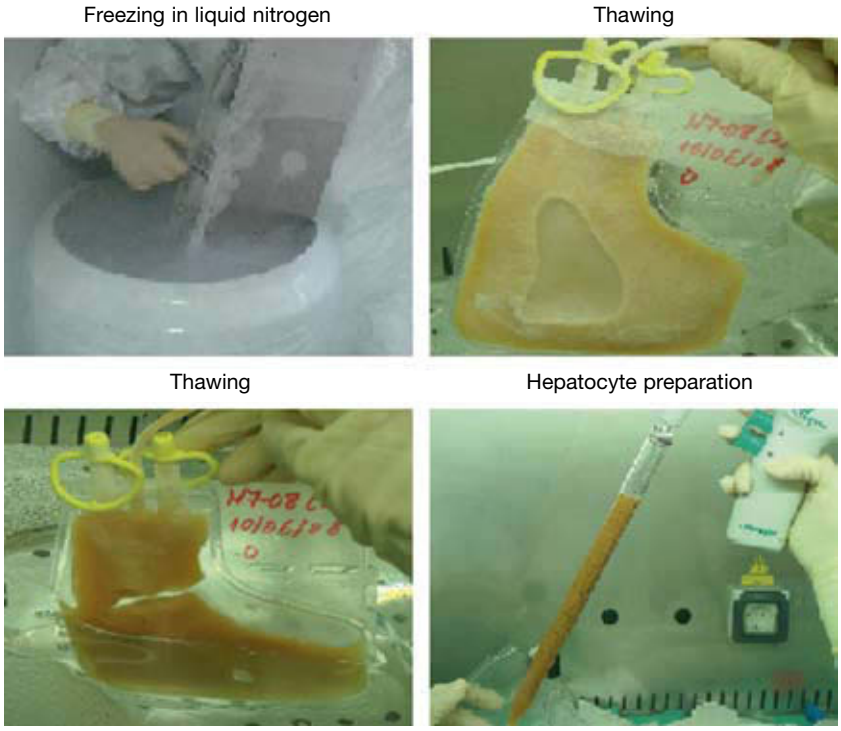

Figure 3 – Cryopreservation of the isolated hepatocytes and thawing of the cells for infusion.

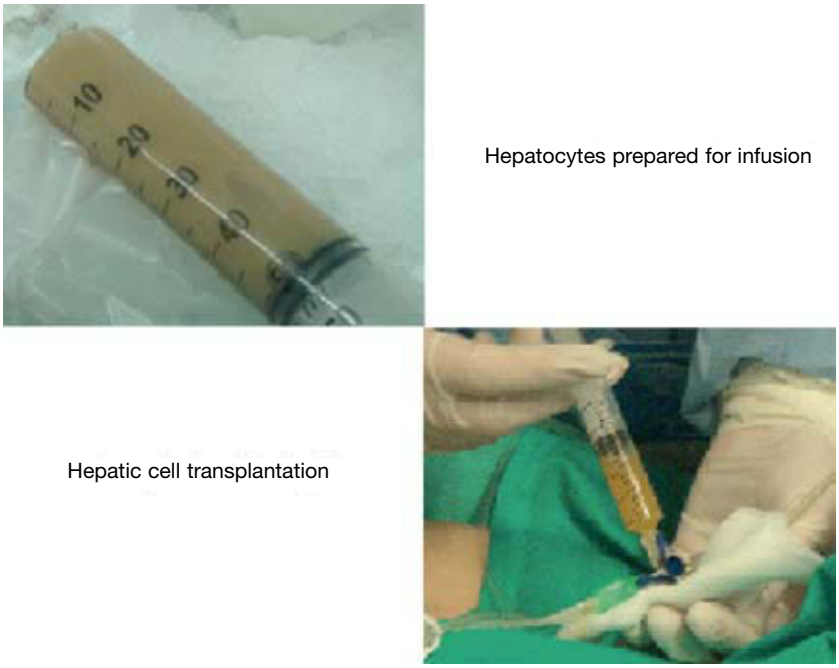

Figure 4 – Hepatic cell transplantation.

For clinical use, greater than 60% viability is recommended, and the hepatocyte quality must be evaluated.[27] To this end, our Unit performs assessments of metabolic capacity and measurements of biotransforming activity and functional expression of hepatic genes.[36,37] The cellular suspensions are tested for micoplasm, endotoxins, and bacterial and fungal contaminations.[21,24,25]

At our HCTU we have established criteria for functional quality control of the hepatocytes (fresh or cryopreserved) so that we can establish their suitability for cell transplantation in a reasonable time frame (1 hr). The activity of 5 P450 cytochrome isoenzymes (CYP1A2, CYP2A6, CYP3A4, CYP2C9 and CYP2E1) is determined along with phase ii conjugation enzymes (UGT and SULT) by incubation with a specific

substrate cocktail recently developed in our laboratory, and HPLC/MS/MS (or mass spectrometry) analysis.[38] We then determined ureogenic capacity by the conversion of ammonium into urea. In the future we hope to use metabonomic studies and establish a useful number of cellular metabolites.

Number of cells

From deductions taken from animal studies, we know that the cellular mass required to treat enzymatic malfunctioning is significantly lower than the amount needed to treat acute or chronic damage.[39] However, the results recently published by Sokal do not comply with this concept.[2]

In general terms, the number of fresh or cryopreserved cells required for transplantation represents 5-10% of the theoretical mass of the liver (200-400×10^6 cells/kg of body weight).[2,6-10] The lasting metabolic and clinical effects appear within 4-8 weeks of the infusion, and are maintained up to 26 weeks following the cell transplantation.[2]

Approximately 5-10% of hepatic cellular mass is transplanted in inherited metabolic disorders[2,6-9,17,40]; in patients with cirrhosis or acute liver failure (ALF), the cellular mass used is lower due to the increased risk of a portal vein thrombosis, with a limit of 30-100×10^6 cells/kg body weight.[2] Hence the need for alternative methods for administration that include the splenic artery and intraperitoneal duct.

Following cellular infusion, the percentage of hepatic cells that are grafted is limited, possibly due to apoptosis stemming from the loss of cellular adherence. As a result, our opinion is that the number of infused cells will be directly related to the estimated weight of the liver.

Route of infusion

At present, there are 3 generally used routes for cell transplantation: the portal vein, splenic vein, and intraperitoneal duct.[2,7,8,40-44]

Infused hepatocytes would block the portal spaces upon arrival, cause ischaemia-reperfusion phenomena, increase sinusoidal permeability, and permit access of the cells to the hepatic lobule. At the same time, the ischaemia-reperfusion causes the release of cytokines and stimulates Kupffer cells, which could produce destruction and phagocytosis of the infused hepatocytes. In cases of hepatic cirrhosis, portal hypertension facilitates the migration of infused hepatocytes to extrahepatic areas.[45]

In adults and cases of chronic hepatopathies or ALF, the spleen is considered to be the site of choice. Access through the splenic artery through a catheter inserted through the femoral artery for implantation in the spleen results in a combination of splenic hepatization and hepatocyte migration to the hepatic site.[1,3,4,8,10,11] Cell infusion by direct puncture in the spleen carries with it a high risk of haemorrhage, although it does facilitate integration and survival of the hepatocytes.[46]

In children with inherited metabolic disorders, a portal approach is preferred through a port-a-cath in the portal or superior mesenteric routes or through a percutaneous intervention.[2,7,9,15,17,41,42]

Other sites for infusion are the peritoneal cavity, the pancreas, and the pulmonary parenchyma, although it seems that the hepatocytes have poor survival with these approaches.

Portal venous pressure must be controlled during the infusion by an eco-Doppler, given that brief increases are common during infusion, so as to prevent haemorrhagic and infectious complications.

6. Cell graft efficiency

Following cell infusion in the recipient, the percentage of hepatic cells implanted is limited, contrasting with the high viability obtained following isolation. This discordance could be related to the presence of apoptosis originating from the loss of cellular adherence (anoikis), and is characterized by the release of cytochrome C from the mitochondria, chromatin condensation, activation of caspases, and the formation of apoptotic bodies, and is implicated in the death of isolated and cryopreserved hepatocytes.[30,47]

These apoptosis phenomena have been detected 15 mins following isolation of human hepatocytes by the TUNEL test and annexin with flow cytometry, such that the presence of apoptosis could explain the discrepancies between the trypan blue test and the efficiency of the cell culture that indicates that the cells are incapable of adhering since they are apoptotic.[30,48]

Cryopreservation of hepatocytes: possibility for a planned cell treatment

The time lag between the isolation of hepatocytes and their utilization causes heavy losses of many resources due to the lack of a method for conservation during prolonged periods of time.

The option of freezing isolated hepatic cells makes cell transplantation possible at a better time, and provides stored cells available for repeated infusions on a periodic basis.

Cell freezing constitutes the best strategy for cryopreservation of the cells, and several key factors must be taken into account for this process.

The speed of freezing is a very important component; if the freezing velocity is too slow, the hepatocyte can suffer dehydration, but if it is too fast, the intracellular liquid fails to exit and freezes. The consequence of this process is the formation of crystals that, together with fluctuations in cellular volume, cause damage to the cellular membrane and rupture of the cell, causing cellular necrosis.

Apoptosis has been identified as one of the most important causes of cell death induced by cryopreservation.[27,30] Fisher found that the viability of hepatocytes decreased significantly within 60 min of freezing and describes reduced cellular damage in hepatocytes with normal function than in those derived from livers with steatosis greater than 40-50%.[49]

Cell density and volume of the stored cells also influences cell viability after freezing. A greater viability has been demonstrated when 10^6-10^7 cells are frozen together, and when using dimethylsulfoxide.

In freezing protocols, the temperature is lowered gradually, at 1 degree/min until reaching a temperature of –140ºC before transferring the cells to liquid nitrogen.[21] The damage caused by freezing results in a reduced cell viability (up to 50%) and a loss of function,[21,50] exemplifying the need for new and better methods of cryopreservation.[18]

In spite of obtaining positive results following cryopreservation, some authors prefer to use fresh cells, although no comparative studies exist for the two methods.

Cryopreservation protocol

For hepatocyte cryopreservation, the Wisconsin solution (UW) is used as the medium with 10% dimethylsulfoxide as a cryprotecting agent.

The suspension is frozen in 250ml bags; 50ml are frozen in each bag with a cell density of 20 million cells/ml using a programmed temperature reduction biological freezer (CM-2000, Metal Carbides) until reaching –140º C. The cells are then stored in liquid nitrogen at –196ºC.[10,12,35]

For each bag prepared, 2 criovials are frozen in order to characterize the functionality of the cells before thawing.

Cell thawing is performed by immersing the bags in a fixed-temperature bath at 37ºC. The cryopreservation solution is then diluted using the appropriate medium, centrifuged at –4º C for 5 min, and the supernatant is discarded. The cellular sediment is resuspended in an appropriate volume of the medium for infusion into the patient, and cellular viability is assessed (Figure 3).

Of the 13 isolations that took place at the HCTU, cell viability, which reached 78-89% following isolation, declined in all cases to percentages close to 20% (Table 2), and viability and functionality were preserved during prolonged periods of time when frozen in liquid nitrogen.[33]

Conclusion

The success of cell treatment is conditioned by the availability of viable and functional hepatic cells in sufficient number and of high functional quality. Those livers that are unsuitable for orthopic HT constitute the principal source of tissue for obtaining hepatocytes. The process of hepatocyte isolation from whole livers and separate lobes must be adjusted to the circumstances, and the optimal conditions for utilization of the hepatic tissue must be determined in order to ensure isolation and culture of high-quality hepatocytes.

In spite of the progresses made in cryopreservation of human hepatocytes, the protocols for freezing/thawing must be optimized in order to amass banks of viable and functional hepatocytes that will permit a better exploitation and planned use of the scarce current resources.

In recent years, the development of various hepatic cellular models has been attempted, allowing us to overcome hepatocyte shortages and constituting an alternative for cell treatment.

Conflict of interest

The authors affirm that they have no conflicts of interest.

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