

Progress in human hepatocytes: isolation, culture & cryopreservation

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The quality of the hepatocytes used for clinical cell transplantation is very important, and depends to a large extent on the nature of the tissue used for isolation. The collagenase perfusion technique to isolate hepatocytes from animal livers has been further developed for isolation of human hepatocytes. As the donor organ pool is a scarce resource, marginal livers unsuitable for transplantation and segments from reduced grafts remain the main source of tissue for cell isolation. Use of livers from non-heart beating donors and foetal livers may further increase the tissue pool. With the limited supply of available tissue, improvements in the cryopreservation protocols are required to maintain cell viability on thawing and establish hepatocyte banks.

Key words: hepatocytes / collagenase / cryopreservation / transplantation

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Introduction

Orthotopic liver transplantation (OLT) is an established treatment for patients with severe acute and end-stage chronic liver disease. This technique requires removal of the entire native liver with transplantation of a high quality graft. Shortage of donor livers, and their timely availability continues to be a world-wide problem. Many preclinical studies into hepatocyte transplantation (HTx) have suggested it has potential clinical application (reviewed by Gupta and Chowdhury in this issue) and this has formed the basis for the few human studies of HTx as an alter-

native treatment for patients with either acute liver failure or metabolic liver disease. The technique may provide liver support while the native liver regenerates, or help in replacing the missing enzyme function in metabolic conditions, with the aim of avoiding liver transplantation.

This short review describes briefly the progress in the techniques used to isolate hepatocytes from unused/rejected donor livers for transplantation, the common techniques used for assessment of cell viability and metabolic activity of the isolated hepatocytes, and also cryopreservation of hepatocytes towards development of cell banks (Figure 1).

Techniques for isolation of hepatocytes

Hepatocyte isolation started in the mid-1960s, when Howard *et al.*¹ isolated rat hepatocytes using a combined mechanical/enzymatic digestion technique, subsequently modified by Berry and Friend.² This technique was further developed by Seglen³ to become the two-step collagenase perfusion technique, as widely used in today's practice.³ If a whole liver is to be perfused, cannulae are placed in the existing major blood vessels of the liver, and secured in place by sutures. For segments of liver, cannulae are placed in patent blood vessel openings on the cut surface, and secured by sutures. In this case, smaller blood vessel openings need to be sealed to prevent leakage of perfusion solutions from the cut surface. The liver tissue is perfused with a calcium-free buffer solution at 37 °C containing a cation-chelating agent to remove calcium (Ca²⁺) ions, leading to disruption of the desmosomal structures which hold cells together. The tissue is then perfused with the buffer solution containing Ca²⁺ and collagenase to digest the tissue. The hepatocytes are separated by low speed centrifugation, and the hepatocyte pellets obtained are washed with ice-cold buffer solution to purify the cells. Many groups are using this technique for isolation of hepatocytes from animal and human livers, with minor modifications.

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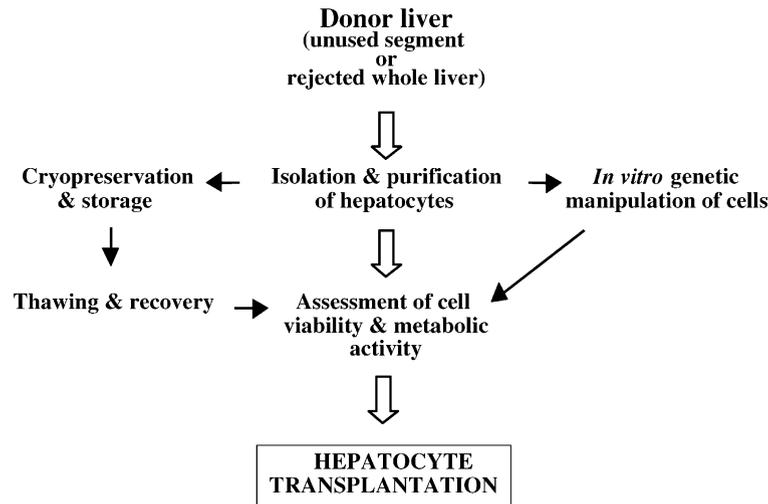


Figure 1. Flow diagram of the preparation of isolated hepatocytes for cell transplantation.

The number and quality of the isolated hepatocytes vary depending on the quality of the tissue used, the composition of perfusion buffer solutions, and the type and concentration of collagenase. Collagenase is prepared from bacteria, and consists of a poorly purified blend of enzymes, which may have inconsistent enzymatic action, which varies from lot to lot.⁴ Some of these are proteases which may cause unwanted enzymatic reactions affecting the quality and quantity of viable/healthy cells. Liberase[®] is a new preparation of a highly purified blend of collagenase isoforms which have been used for preparation of pancreatic islets, and more recently for hepatocytes and has the advantage of lower bacterial contaminants. Donini *et al.*⁵ compared Liberase[®] HI to collagenase P for preparation of porcine hepatocytes, and showed that Liberase[®] HI was as effective in the isolation of hepatocytes, and resulted in a higher cell viability.⁵ More research is required to determine the optimum collagenase to be used for the isolation of human hepatocytes.

The sources of human hepatocytes include unused segments of donor liver obtained from split/reduced grafts, and whole livers considered unsuitable for transplantation for reasons such as excessive fat or trauma.^{6,7} Recent developments to increase the donor organ pool by using non-heart beating donors (NHBD) have also contributed towards increased availability of liver tissue for hepatocyte isolation. In our experience hepatocytes isolated from NHBD liver tissue had a higher cell viability and metabolic activity compared to the grafts rejected on the grounds of

being unsuitable for transplantation.⁸ Hepatocytes obtained from good quality donor liver segments are the most suitable for HTx, while cells obtained from fatty livers are difficult to purify, tend to have lower viability and are unlikely to be satisfactory for HTx. It may be possible to improve the quality of cells isolated from borderline donor tissue by incubation in modified culture media containing additives, for example growth factors such as hepatocyte growth factor (HGF). HGF has protective effects on cold-preserved fatty livers from rats⁹ and was also shown to be effective in treatment of alcohol-induced fatty liver in rats.¹⁰ On the other hand, cells from both normal and fatty livers can be used for *in vitro* studies of toxicology and metabolism. Due to the shortage of donor tissue, researchers have looked at other sources of human hepatocytes including tissue obtained from liver resections in patients with colorectal liver metastasis. In this case, hepatocytes are isolated from the nontumoural margin, however, there is a possibility of cancer cells being present along with the isolated hepatocytes. Another source of hepatocytes that is being evaluated is normal foetal liver obtained from unwanted pregnancies. It was shown that foetal hepatocytes possess metabolic (cytochrome P450) activities, and are able to synthesise hepatic marker proteins such as albumin.¹¹ Compared to adult hepatocytes, foetal hepatocytes are highly proliferative *in vitro*,¹² and this may potentially lead to enhancement of their engraftment and repopulation once transplanted into the liver.

In our experience of isolation of human hepatocytes from 35 livers, we have followed the collagenase perfusion technique of Strom *et al.*¹³ In the first 20 isolation procedures, hepatocytes were isolated from unused donor segments or whole livers rejected for transplantation. The median cell viability was 70% ($n = 13$; range: 13–95%) for split/liver segments where the other part was used for clinical transplantation and 35% ($n = 7$; range: 20–59%) for the rejected livers. These findings confirmed that the quality of the tissue used is important, with fatty livers rejected for transplantation giving lower cell viability.¹⁴ Improvements need to be made to the isolation and preparation of hepatocytes from fatty livers, as this is the most common cause of rejected whole livers. There was a significant correlation between age of the donor of the liver and *in vitro* viability of isolated hepatocytes obtained. There was no correlation of the cell viability with the cold ischaemia time (up to 24 h) of these livers. One method to improve the quality of the isolated hepatocytes is to separate the viable cells using a Percoll® centrifugation technique.^{6,7} However, there is the question whether Percoll® or other solutions such as Nycodenz® are acceptable for hepatocytes to be used in clinical transplantation, together with the problem of handling the large quantities required.

Assessment of hepatocyte viability

Isolated hepatocytes are usually assessed immediately after isolation/purification using the standard trypan blue exclusion technique. Although this is commonly used, it does not allow detection of cells in the early stages of the cell death cascade, such as observed in apoptosis. *In vitro* cell viability may not reflect cell function after transplantation *in vivo*. *In vitro* assays/tests of cell viability and metabolic activity, are needed to characterise the quality of the isolated hepatocytes. The efficiency of plating or attachment of hepatocytes is considered to be a 'sign' of a good hepatocyte preparation. However, the attachment ability of transplanted cells may recover *in vivo* because of a favourable microenvironment. Other tests that can be used to detect viability and activity of the isolated hepatocytes include MTT assay, cytochrome P450 activity, protein synthesis using [¹⁴C]-leucine incorporation, DNA synthesis using [³H]-thymidine incorporation assays, and production of hepatic marker proteins such as albumin, transferrin, and apolipoproteins.¹⁵ Although these tests are more sensitive than trypan blue exclusion and reflect the cells activity and func-

tion they are usually applied to attached cells, and if cell suspension is used, the problem of determining which cells are dead remains. Ideally, viable cells should be separated e.g. by Percoll® centrifugation, then tested using the above assays; however, this is time consuming, and may lead to a decrease in the cell viability. Rapid tests need to be developed to assess the quality of liver tissue prior to hepatocyte isolation and for the hepatocytes produced to help determine their suitability for HTx.

Primary hepatocyte culture

One of the first requirements for both short-term and long-term culture of hepatocytes is good cell attachment to the culture vessel/flask. The culture vessels should be precoated with a suitable attachment factor/matrix such as gelatin, collagen type I or Matrigel®,^{16,17} which correspond to the *in vivo* extracellular matrix. Under standard *in vitro* cell culture conditions, mature hepatocytes usually do not survive for longer than 10–14 days, and do not proliferate. For longer term *in vitro* maintenance of differentiated hepatocytes and in order to promote intracellular metabolism, culture media used must be supplemented with foetal calf serum, and/or nonnutritional growth factors such as HGF, epidermal growth factor (EGF), insulin,^{18,19} and glucocorticoids such as dexamethasone.¹³ The majority of longer term hepatocyte cultures are used in pharmacological studies on the induction and analysis of cytochrome P450 isoforms (i.e. drug metabolism-related studies).^{20–23} In the context of HTx, cells are not usually cultured beforehand. However, this is important when hepatocytes are to be genetically modified *in vitro* to enhance or replace activities and then be transplanted *in vivo*.

Cryopreservation of hepatocytes

Shortage of donor liver tissue for the isolation of hepatocytes necessitates the development of improved cryopreservation techniques for long-term storage of human hepatocytes to make best use of available hepatocytes. Recently, it was shown that short-term storage of hepatocytes (up to 4 days) can be achieved by maintaining the cells in modified University of Wisconsin solution at 4 °C.²⁴ The ultimate goal of any improved cryopreservation protocol is to minimise sudden intracellular formation of ice crystals that could result in

ultrastructural damage, and thus maintains cell viability, attachment, and metabolic activity on thawing. Storage time of cryopreserved hepatocytes at temperatures well below -100°C (e.g. liquid nitrogen, and -140°C freezers) may play an important role in the quality of thawed cells. Most of the available protocols use dimethylsulfoxide (DMSO) in the cryopreservation medium, which currently appears to be the best cryoprotectant compared to other agents such as glycerol.²⁵ Hengstler *et al.*²⁶ developed a protocol for cryopreservation of human hepatocytes using a controlled-rate freezer, with rapid thawing followed by slow dilution of DMSO to avoid osmotic shock. The cells were shown to have a reasonable level of cell viability, and $\geq 60\%$ overall cytochrome P450 activity compared to freshly isolated hepatocytes, and these cryopreserved hepatocytes were used in short-term metabolism studies.²⁶

Cryopreservation of hepatocytes is essential for the emergency treatment of acute liver failure. The clinical use of such thawed cells has been reported by Strom *et al.*²⁷ and Bilir *et al.*²⁸ where between 10^7 and 10^{10} hepatocytes were infused into the splenic artery or portal vein of patients with liver failure. Soriano *et al.*²⁹ gave one to nine infusions of hepatocytes that had been cryopreserved over a period of 2–7 days to children with liver failure. These clinical reports certainly reflect the progress made to date in the isolation and handling of human hepatocytes, and it is hoped this will lead to a wider use of cellular therapies in liver disease.

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