Experimental treatment modalities for liver failure
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Chapter 1

General introduction and outline of the thesis
1. General Introduction

Acute liver failure is a complex and devastating consequence of acute liver injury. It is characterized by hepatic encephalopathy, jaundice, coagulopathy and high mortality rates. Emergency liver transplantation is currently the only effective treatment for those patients who are unlikely to spontaneously recover. Donor shortages however remain a serious problem as the number of patients requiring orthotopic liver transplantation has far outpaced the number of donor livers and many patients die before a suitable organ is identified. This has generated interest in designing devices that would support or replace normal liver function until a donor liver became available for liver transplantation, or the patient’s own liver recovered. In this chapter, an overview is given of hepatic failure, liver transplantation and possible alternative treatment modalities including bioartificial liver support and hepatocyte-transplantation. In addition, complexities in storing hepatocytes, crucial for BAL-systems to proceed from laboratory to clinical practice, will be discussed.

2. Acute Liver Failure

A. Epidemiology

Liver failure is a major cause of mortality. About 30,000 patients die each year from end-stage liver diseases in the U.S. About 80% of these patients have decompensated chronic liver disease and are often too ill to tolerate a liver transplantation procedure. The other 20% die of acute liver failure primarily due to acetaminophen poisoning, viral hepatitis, severe sepsis, insufficient remnant liver function after liver surgery, and alcoholic liver disease. Less frequent causes include exposure to toxins (e.g. Amanita phalloides mushroom poisoning), idiosyncratic drug reactions, Wilson’s disease and pregnancy-related acute fatty liver. The nature of the initiating event is an important factor influencing both the rate of progression of the clinical syndrome and its
prognosis. Geographic differences are considerable in the etiology of acute liver failure. Acetaminophen poisoning (associated with either therapeutic or (para)suicidal intent) is now the most common cause in many Western countries, especially in the UK (accounting for 60%-70% in some series). Hepatitis B and hepatitis E are particularly prominent in France and India, respectively. Children have unique etiologies, with indeterminate causes accounting for more than 50% of pediatric cases.

B. Clinical syndrome

The most common clinical features of liver failure are abnormal liver chemistries, coagulation disorders, jaundice, susceptibility to bacterial and fungal infection and, by definition, hepatic encephalopathy. When these symptoms occur in previously healthy individuals with normal livers, it is termed acute liver failure (ALF). Fulminant hepatic failure (FHF) is a more severe form of ALF characterized by a rapid onset of hepatic encephalopathy (<2 weeks). The presence of hepatic encephalopathy is the essential clinical feature that differentiates ALF from acute severe hepatitis. Its pathophysiologic basis is poorly understood and multifactorial: ammonia accumulation, increased GABA-ergic tone, accumulation of false neurotransmitters and endogenous benzodiazepines are thought to play an important role.

Acute liver failure results in a sudden and profound decrease in the synthesis of coagulation proteins, which is manifested by the prolongation of prothrombin time. Recent studies highlight the prognostic value of vitamin-K dependent factor V. Factor VIII however, also synthesized in the vascular endothelium and kidney, can be markedly elevated in ALF cases. A factor VIII/V ratio of more than 30 has been reported as associated with poor prognosis. Coagulation disorders in ALF are complex as not only procoagulant, but also anticoagulation factors such as protein C and protein S are synthesized in the liver, and many of the activated clotting factors are removed from the circulation by the liver as well. Hypoglycemia also is frequently seen in ALF and is caused by a depletion of glycogen stores and impaired gluconeogenesis.

The leading cause of death for patients with ALF is progressive cerebral edema resulting in brain herniation. Irreversible brain injury occurs in nearly all cases of prolonged severe increase in intracranial pressure (more than 50 mmHg) and reduction in cerebral perfusion pressure below 40 mmHg for more than 2 hours. The high mortality rates associated with ALF are caused by the complications of ALF, which include the development of cerebral edema, renal failure, sepsis, and cardiopulmonary collapse that result in multisystem organ failure.

Liver failure in patients with pre-existing chronic disease (e.g. cirrhosis) is called acute-on-chronic liver failure. This acute decompensation is often induced by a superimposed complication, such as viral or bacterial infection, ischemia due to portal vein thrombosis or by variceal bleeding etc.

Natural history studies before the era of liver transplantation showed that with medical treatment alone, seventy-five percent of patients with ALF die due to life-threatening complications. Presently, survival rates are significantly higher as a result of better diagnostic and treatment options in specialized liver intensive care units.
3. REPLACEMENT OF LIVER FUNCTION

A. Liver transplantation

Orthotopic liver transplantation (OLT) has become an integral part of the management of acute liver failure, and currently is the only clinically proven effective treatment for patients with end-stage liver disease. Acute liver failure accounts for approximately 11% of all liver transplant activity in Europe, and 5% in the USA. Selection criteria for OLT are based on indices identifying the severely affected group, who have a poor prognosis with medical management alone. O'Grady and colleagues developed the King’s College prognostic criteria, a model for the selection of patients for liver transplantation based on early prognostic indicators, that has been adopted in most centers\textsuperscript{10}. It is based on pH, prothrombin time and serum creatinine in acetaminophen patients, and -in nonacetaminophen patients- on prothrombin time > 6.5 (INR), or any three of the variables: age, duration of jaundice, prothrombin time > 3.5 (INR) or serum bilirubin. Another set of criteria are the Clichy criteria, listing hepatic encephalopathy in combination with age and coagulation Factor V levels\textsuperscript{11}.

One of the largest published series on LT for FHF recently described the outcomes after liver transplantation for fulminant hepatic failure (n=204): 1- and 5-year survival rates were 73% and 67% (patient) and 63% and 57% (graft), respectively. The primary cause of patient death was sepsis, and the primary cause of graft failure was primary graft nonfunction\textsuperscript{11}.

While many patients’ lives are saved each year by OLT, this treatment is limited by the availability of organ donors and many patients die before an organ becomes accessible. In the western world, approximately 50% of the patients admitted with acute liver failure underwent an OLT, while up to 20% of patients died before an organ became available\textsuperscript{12}. The total number of deaths on the waiting list for liver transplantation in the USA were 1784, 2012, 1756 in the years 2000, 2001 and 2002 respectively (based on the Organ Procurement and Transplantation Network (OPTN) data as of August 3, 2003: www.optn.org). In The Netherlands, the number of cadaveric donor livers that were transplanted increased slightly from 89, 100, 95, 126 to 110 in the years from 1998 to 2001 respectively, while at the same time the number of patients on a waiting list for OLT grew from 27, 33, 47, 60 to 86 (Jaarcijfers Nederlandse Transplantatie Stichting: www.transplantatiestichting.nl). Apart from donor shortages, two other important problems associated with liver transplantation are high cost and the requirement of lifelong immunosuppressive drugs.

Living-related donor liver transplantation (LRLT) has changed the landscape of pediatric transplantation in the last years and is becoming an accepted procedure in adults. LRLT has the potential to improve survival rates and concurrently decrease the strain on the organ pool. In addition, it has gained rapid popularity in countries where retrieval of cadaveric organs is hampered by religious and cultural restraints. Nevertheless, it represents only about 3% of the total number of transplants performed in the United States\textsuperscript{14}. In addition, living donor methods are inherently limited because they represent a significant risk for the donor (mortality 0.5 – 1%)\textsuperscript{15}. Other ways of expanding the donor pool are by using marginal donors (e.g. non-heartbeating donors), split liver transplantation and domino transplantation. Although these options are all feasible and more frequently employed in clinical practice, their effect will be too limited to realistically solve the donor organ shortage\textsuperscript{16}.
B. Liver-assist devices and hepatocyte transplantation

Over the past 10 years, replacement of liver function in various forms has been promoted as temporary liver support in patients with ALF, with the goal of providing 'functional liver mass' until the native liver regenerates or a donor organ becomes available.

Bioartificial livers are techniques using porcine or human liver cells maintained in a bioreactor through which patient plasma or blood flows in a manner similar to hemodialysis. Transplantation of human hepatocytes directly into the spleen has also been performed with some success.

Purely artificial support systems remove toxins through hemodialysis, hemofiltration, or hemoperfusion. More recent systems combine hemodialysis with adsorption to charcoal or albumin (e.g., the MARS system). Although a recent systematic review suggested that artificial support systems reduced mortality in acute-on-chronic liver failure compared with standard medical therapy, these systems did not appear to affect mortality in acute liver failure. Conversely, purely biological approaches have shown encouraging results in some cases but have been difficult to implement in the clinical setting. These include whole organ perfusion, perfusion of liver slices, and cross hemodialysis. Xenotransplantation, using a transgenic pig modified to prevent hyperacute rejection, is also under investigation for the treatment of ALF. A more comprehensive discussion of purely artificial or biological supporting therapies is beyond the scope of this chapter.

**BAL devices**

The liver is one of the most complex and metabolically active organs in the body and has a number of crucial functions that are carried out by hepatocytes. It is generally believed that there is currently no other option than the application of viable hepatocytes in a bioreactor in order to replace the broad range of liver functions. The full spectrum of cellular functions required in BAL devices to effect positive clinical outcomes has not been determined. To address this problem, it is generally believed that the biological component of a BAL should express maximum levels of each known class of liver-specific functions that typically include phase I and phase II detoxification, synthesis of albumin and coagulation factors, gluconeogenesis, and (if possible) biliary excretion. The implicit assumption is that hepatocytes capable of a wide array of known functions will also express those unmeasured (or unknown) functions that are essential to their metabolic role.

Building a device to replace liver function is a formidable challenge that requires the interdisciplinary efforts of the fields of medicine, biology and engineering. Temporarily liver function support ideally could bridge patients until a liver graft has become available for transplantation, or could provide liver support allowing the diseased liver to recover through parenchymal regeneration. Several important issues need to be addressed in the design of BAL systems: (1) how to support a large cell mass without substrate limitations so that the cells function with maximum efficiency, (2) how to maintain long-term functional stability of hepatocytes in inhospitable environments, (3) how to promote biliary excretion, (4) how to scale up the system while keeping acceptable priming volumes, and (5) what is the best type of cell for a BAL bioreactor. Furthermore, efforts are made to maximize biocompatibility and facilitate logistics in preparation and...
Transport. Testing the clinical efficacy of bioartificial livers in clinical trials is a major challenge, as the proven efficacy of treatment with a liver transplantation will prohibit randomized allocation to either transplantation or BAL treatment when a donor organ is available for liver transplantation. Performing trials in patients with end-stage chronic liver disease could partially solve this problem, as these patients will spend more time on the waiting list for transplantation, and thus are more suitable for follow-up.

In the basic BAL device a large mass of viable hepatocytes is housed in an extracorporeal bioreactor, which is perfused with the patient's blood or plasma. The large number of bioreactor designs that has been described can be categorized in four main types: (1) hollow fiber, (2) flat plate and monolayer, (3) perfused beds/scaffolds, and (4) encapsulated and suspension. Most well-known bioartificial support systems that are currently under clinical evaluation are based on the hollow fiber design. Advantages of this design are the large attachment surface and matching cell mass, and the potential for immunoisolation or internal oxygenation. These four BAL systems will be discussed, as well as one particular design yet in a preclinical phase of development, but having undergone extensive characterization.

**Hepatassist** The HepatAssist is a porcine-hepatocyte based bioartificial liver, which was developed by Demetriou and coworkers and currently is the most widely clinically tested device. In this system containing five billion cryopreserved, microcarrier attached hepatocytes, a charcoal column is added to the extracorporeal perfusion circuit. Hollow fiber technology is used to separate cellular and perfusion compartments and to provide a basic scaffold for hepatocyte attachment. Dogs with complete liver ischemia treated with the HepatAssist showed significant neurological and biochemical improvements, but life could not be prolonged. In a temporary liver ischemia model in pigs however, BAL treatment was shown to lower intracranial pressure and prolong life.

In a phase I clinical trial, 31 patients in three groups were treated with the HepatAssist. In the first group of fulminant hepatic failure patients, 16 were successfully bridged to liver transplantation and one spontaneously recovered without a transplant; in the second group, all three patients with primary nonfunction of a transplanted liver were successfully bridged to retransplantation; in the third group of 10 patients with acute exacerbation of chronic liver diseases only two were supported to recovery and successful transplants at later dates, the other 8 patients were not eligible for transplantation and died. A large multicenter phase II/III clinical trial supported by Circe Biomedical is currently under way. An interim analysis (N=171) showed disappointing data with only in subgroups a significant improvement in 30-day survival and time-to-death over control. The results however were confounded by the impact of transplantation and variation in disease etiology.

**ELAD** The Extracorporeal Liver Assist Device, introduced by Sussman et al, utilizes four hollow fiber cartridges each containing approximately 100 grams of hepatocytes from the C3A human hepatoblastoma cell line, or primary human hepatocytes. Animal studies in six anhepatic dogs showed improved metabolism of anesthetics in the animals, but there was no survival benefit. Several case reports showed safety of treatment with the ELAD, biochemical evidence of improved hepatic function and bridging to successful transplantation. A larger multicenter trial is currently underway in the US and UK under the direction of Vitagen.

The high oxygen uptake rate of hepatocytes and the relatively low solubility of oxygen in aqueous media deprived of oxygen carriers makes oxygen transport the most constraining parameter in the design of bioartificial liver devices. Thus, to improve
oxygen delivery, novel designs using fibers that carry gaseous oxygen straight into the device have been developed.

**MELS** The Modular Extracorporeal Liver System (MELS) developed in Berlin by Gerlach and colleagues comprises three sets of interwoven capillaries that create a three-dimensional extracapillary space to house hepatocytes. One set of capillaries provides an efficient internal oxygenation supply, and the remaining two sets control inflow and outflow of plasma. Using this approach, Gerlach and colleagues were able to demonstrate that hepatocytes could express differentiated functions over several weeks\(^{28,43}\). Treatment of anhepatic pigs with the MELS containing 10 billion pig hepatocytes showed an improvement in blood ammonia, phenylalanine and lactate levels, but there was no survival benefit compared to the control group\(^{44}\). The complexity of the Gerlach bioreactor seems to be its major disadvantage.

**AMC-BAL** In order to overcome the substrate transport limitations associated with bioreactors in which hepatocytes and plasma are separated by hollow-fiber membranes, Chamuleau, Flendrig and co-workers in Amsterdam developed the Academic Medical Center-BioArtificial Liver. The bioreactor consists of a polysulfon dialysis housing and incorporates a spirally wound polyester matrix sheet that includes an integrated hollow-fiber compartment for oxygenation (Fig. 1). The unique feature of this bioreactor is the direct contact between the perfusing plasma and the hepatocytes that are seeded on the matrix in high density in the extrafiber bioreactor space. Semipermeable filters (molecular weight cut-off of approximately 100 kDa) between the bioreactor and the patient prevent cells and cell fragments to enter the patients' circulation. It has been shown that the hepatocytes in the AMC-BAL generate metabolic function in *in vitro* studies\(^{45,46}\). It also has been shown that the AMC-BAL achieves prolonged survival in the total liver ischemia model in rats\(^{47}\), as well as in pigs\(^{48}\). Fourneau and coworkers in Leuven evaluated the AMC-BAL in a potentially reversible model of acute liver failure, based on transient ischemia of the liver. Evidence for the efficiency of the BAL was given by a significant prolongation of survival time, improvement of the neurological status and signs of regeneration on histological examination in the treated group (n=6) when compared to control (n=5)\(^{49}\).

**Flat plate BAL**. In the Center for Engineering in Medicine (Boston, USA) a microchannel flat-plate bioreactor with an internal membrane oxygenator was developed in which porcine or rat hepatocytes are cultured as a monolayer on a collagen coated glass surface in coculture with fibroblasts. Cell distribution and microenvironment are well controlled and have been extensively characterized in this system\(^{50,55}\). Rats with GalN-induced FHF that were treated with the BAL device seeded with porcine hepatocytes showed a significant reduction in plasma ammonia levels and prothrombin times, and a significantly higher survival (50.0%) than the control animal group treated with an unseeded BAL device (11.1%). Histologically, liver damage was reduced in the animal group treated with the hepatocyte-based BAL device\(^{56}\). The major advantage of flat plate bioreactors is that hepatocytes can be studied in detail in their artificial microenvironments, gaining valuable insights in maintaining hepatocyte phenotypes under different conditions. The complex scaling-up with a potential large dead volume appears a large barrier to overcome before the system can be applied clinically.
Biological Component

Critical Cell Mass. The minimum cell mass required to support an animal model of hepatic failure has not been systematically determined. Prior studies have shown significant improvements in various parameters using as low as 2-3% of the normal liver mass of the animal \(^{57}\). Devices which have undergone clinical testing have used \(6 \times 10^9 - 1 \times 10^{11}\) porcine hepatocytes \(^{58}\) or \(4 \times 10^{10}\) C3A cells \(^{29,36,59}\). In an ischemic model of hepatic failure, treatment with the AMC-BAL containing \(6 \times 10^9\) pig hepatocytes (about 3-5% of the liver mass) showed significantly improved survival times. \(^{49}\) Assuming that the cell mass necessary to support a patient undergoing acute liver failure is about 10% of the total liver weight (average 1500 grams), this yields a bioartificial liver containing approximately \(15 \times 10^9\) cells \(^{60}\).

Cell Source. When choosing the type of hepatocyte for use in a BAL system, it is important to be aware that several risks need to be balanced. The primary human hepatocyte seems to be the cell of choice for a BAL. However, time for sufficient quality control is limited, immune rejection and transmission of infection or malignancies are not ruled out and they are scarce due to a competing demand of livers for orthotopic transplantation. Proliferative capacities of human hepatocytes in vitro as well as the ability to cryopreserve these cells are inadequate up till now. When considering alternatives, important safety concerns include immune reactions to foreign antigens, xenozoonoses and escape of tumorogenic cells.

Xenogenic hepatocytes. Currently the first alternative is to use hepatocytes from other species, particularly pigs. Xenogenic hepatocytes offer no risk of transmitting malignancies to the patient, whereas a paramount advantage of utilizing porcine
hepatocytes is their nearly unlimited supply. Immune reactions to xenogenic antigens may only pose a significant role in repetitive applications of BAL therapy, as high titers are not generated for 1 week (IgM) to 3 weeks (IgG). Nonetheless, to date no adverse immunologic reactions have been reported with repeated use of these systems over a prolonged period of several days, possibly because patients with ALF are relatively immunosuppressed and are unable to mount significant immune responses. Although the risk of transmission of zoonosis in general may be substantially reduced using SPF (Specified Pathogen Free) herds, a number of studies also suggested a risk from agents such as PERV (porcine endogenous retrovirus), which is ubiquitous in the genome of bred pigs. In the many examples of xenotransplantation with porcine tissues though, induction of porcine retroviral expression has never been proven in humans. Two recent clinical studies on the use of porcine-hepatocyte based BAL systems indicated that humans may be nonpermissive for this infection through BAL treatment. On the other hand, the issue of possible cross-species PERV transmission is also relevant from the public point of view. This is the key argument at this moment for imposing a complete ban on xenotransplantation (including porcine-hepatocyte based BAL systems) for the treatment of patients in several countries including The Netherlands. A final consideration on the use of porcine hepatocytes is the potential mismatch between xenogenic and human liver functions. The limited number of studies available that compare pig and human hepatocytes tend to show similar or higher liver specific metabolic activities in pig hepatocytes.

Tumor-derived cell lines. The well-known cancer-derived C3A hepatocyte line, a clonal derivative of the hepatoblastoma-based HepG2 cell line, is the only human cell line that has been extensively used in clinical trials (the ELAD system). C3a cells offer an unlimited supply without the disadvantages associated with using xenogenic cells. Metabolic studies have shown high levels of such synthetic functions as albumin and alpha-fetoprotein production, but also demonstrated a crucial disadvantage as the C3a cells appear to underperform in P-450 IA1 activity, ammonia removal and amino-acid metabolism when compared to primary human-, pig- or rat hepatocytes.

Immortalized cell lines. The prospect of an infinite source of safe and fully functional cultured human hepatocytes for BAL systems is very attractive and hopefully is only a matter of time to become reality. So far, one immortalized human hepatocyte cell line has been tested in BAL setting. A recent study reported a newly developed conditionally immortalized human cell line, NKNT-3, that was generated by retroviral transfer in normal primary adult human hepatocytes of an immortalizing gene that can be subsequently and completely excised by Cre/Lox site-specific recombination. These cells have been shown to express highly differentiated liver-specific function. When transplanted into the spleen of rats under transient immunosuppression, reversibly immortalized NKNT-3 cells provided life-saving metabolic support during acute liver failure induced by 90% hepatectomy. Replication of the reported in vitro studies in other laboratories appears to be complicated and laborious, and obviously more experience should be obtained before this cell line can be used in BAL devices.

Stem cells. Clearly, availability of embryonic or adult human stem cells that can proliferate yet retain the ability to differentiate would provide an ideal source for BAL devices. Although both processes recently have been described in literature, further research has to be undertaken to define the factors that efficiently induce hepatic differentiation of stem cells.
Hepatocyte transplantation

Hepatocyte transplantation (HcTx) has been proposed as an aid or as an alternative to whole-organ transplantation to support different forms of liver diseases including acute liver failure, as the HcTx procedure is much less invasive and less expensive for patients than OLT.

The scopes of liver cell transplantation and liver-directed gene therapy are different, but overlapping. Liver cells can be used as vehicles for introducing normal genes into the liver of a subject with inherited acquired liver diseases. On the other hand, gene therapy can be used effectively to aid liver cell transplantation by opposing allograft or xenograft rejection and promoting repopulation of the liver by the transplanted cells.

Acute or chronic liver failure As mentioned earlier, at least 10% of the normal hepatocyte mass is required to support a patient with ALF. Unfortunately, the number of hepatocytes that can be realistically transplanted in patients has been limited to less than 2-5% of the recipient original liver mass (vide infra). Regardless of these limitations, several clinical trials have been performed to ‘bridge’ patients with ALF to OLT81,82. A remarkable prospective controlled trial was reported by Strom in 1997, comparing transplantation of human hepatocytes with standard medical therapy in bridging FHF patients to OLT18. A splenic arterial infusion technique was used, administering approximately 3x10^7 hepatocytes. None of the four control subjects survived over three days, but all five hepatocyte-treated patients were successfully bridged to OLT, and 3/5 are doing well with more than 20 months of follow-up. Nevertheless, one must be careful in evaluating the clinical efficacy using this approach because the number of patients in these studies were insufficient and were not randomly assigned to treatment groups. Furthermore, in acute liver failure, regardless its cause, the efficiency of engraftment is generally quite low and a lag time, which may be as much as 48 hours, has to be awaited before any clinical benefit occurs83. This may be too long in a rapidly deteriorating patient. Important characteristics to be determined in further HcTx studies are amongst others the optimal number of hepatocytes to transplant, and the route of transplantation that would allow more cells to be infused84.

Metabolic disorders. For the treatment of inherited metabolic deficiencies, e.g. Crigler-Najjar Syndrome or β-Antitrypsin deficiency, the normal genes can be introduced simply by transplanting normal hepatocytes from allogeneic donors85. This, however, would require immunosuppression for the prevention of allograft rejection (similar issues are also involved in the treatment of liver failure). Alternatively, hepatocytes can be harvested surgically from an affected individual with an inherited metabolic disease. These cells can next be transduced in culture with a therapeutic gene and transplanted back in the subject84,86. It is important to understand how many cells are needed for achieving this therapeutic goal, how to solve the problem of repopulation of the liver, and how many cells can be transplanted safely at one time. Especially if the transplanted cells have a survival advantage (e.g. in inherited tyrosinemia type I) smaller numbers of transplanted hepatocytes—potentially, as few as 2% of the host hepatocyte cell mass—may be sufficient.

Site of implantation. The success of transplantation strategies for isolated hepatocytes depends in large measure upon the site chosen for transplantation. In early studies, the choice of the transplantation site was dictated by accessibility and ease of the procedure, as well as by spatial considerations: the pulmonary vascular bed, dorsal and inguinal fat pads, and peritoneal cavity were preferred choices, due in large part to the availability of anatomical space within which transplanted hepatocytes might proliferate.
and survive. Later studies, however, demonstrated additional requirements for optimal function and differentiated gene expression of transplanted hepatocytes that are not met in some of the aforementioned ectopic sites. A microenvironment resembling that of liver was found to be optimal; such a microenvironment would include a basement substrate to promote hepatocyte anchorage and a venous blood supply mimicking the mechanical and biochemical environment of the hepatic sinusoids. The splenic pulp and the host liver itself thus came to be choice sites for transplantation of large numbers of hepatocytes in small rodents. Only a few studies have been performed on hepatocyte transplantation in large animals, such as in pigs, and these studies also mainly focus on intraportal and intrasplenic transplantation. With intraportal transplantation however, the infusion of large cell numbers is associated with portal vein thrombosis and pulmonary embolism, while intrasplenic transplantation has resulted in similar complications, with a large proportion migrating out of the spleen into the portal circulation.

Ectopic hepatocyte transplantation, defined here as a transplantation site for the hepatocytes other than the liver or the spleen, can potentially provide more space to transplant a greater number of donor hepatocytes, without the complications as described with intraportal or splenic transplantation. Another advantage of ectopic transplantation from an experimental point of view is that donor cells can be readily distinguished from host cells, which makes it easy to evaluate the status of the cells in biopsy specimens.

In summary, although extensively tested and developed in small animals, HCTx is still in the early stages of development to be used as a clinical procedure for liver disorders. Nevertheless, there have been several clinical reports of successful treatments of FHF or inherited liver disorders. Translating successful research in rodents to the clinical treatment of patients could be served by an evaluation of the HCTx procedure in a large animal model. One of the important issues in this field is finding the optimal route for transplantation and exploring the limits to the number of hepatocytes that can be transplanted.

4. Hepatocyte cryopreservation

For both BAL and hepatocyte transplantation to fully reach their clinical potential, isolated hepatocytes need to be successfully preserved for significant periods of time so that they can be isolated at convenient times, appropriately banked and distributed for on demand utilization at the clinical site. In addition, cellular preservation would allow for extensive testing and validation of cell sources to assess the safety and efficacy of the bioproduct, primary hepatocytes as well as cell lines. According to Eurotransplant data 20-25% of reported donor livers are not procured or transplanted, due to factors such as steatosis or cirrhosis. If primary human liver cells from transplant discards could be successfully cryopreserved after isolation, purification and extensive testing, they could play a substantial role as cell source in BAL-devices.

Cryopreservation and thawing In freezing, ice formation begins at a nucleation site that can be a randomly occurring cluster of molecules in the liquid phase. The nucleated ice crystal forms into an ice front expanding through the liquid until solidification is complete. Ice crystals preferably develop in the extracellular liquid, causing the partially frozen extracellular solution to be more concentrated than the intracellular compartment. Rates of freezing that are too fast or too slow contribute to cell
destruction by causing intracellular ice crystal formation\textsuperscript{91} and/or cellular dehydration while the cell attempts to maintain its osmotic balance during the freezing process. The optimum rate of cooling varies with the cell type and for hepatocytes is estimated to be between -1°C and -10°C per minute, preferably by computer-controlled stepwise freezing\textsuperscript{92}.

Dimethyl sulfoxide (DMSO) reduces intracellular ice formation and osmotic changes during freezing and is considered the most suitable cryoprotectant\textsuperscript{93}. Seeding is a technique used to deliberately induce extracellular ice formation in solutions that have already been cooled just below the solution melting point. Samples can frequently be damaged by 'supercooling' during freezing, as ice formation will occur at random, unpredictable temperatures. Seeding, typically by touching the sample with a needle, can minimize this damage and is, therefore, often essential for cell survival. To allow this crystallization process to complete, and to thermally and chemically equilibrate the sample after the release of solutes and heat of fusion resulting from ice formation, it is common to hold the specimen at the seeding temperature for some time before continuing the cooling protocol.

Although temperatures below -80°C are generally required for successful preservation of cells and tissues for extended periods of time, shelf life increases dramatically as the storage temperature is reduced. At -196°C (the boiling point of liquid nitrogen) there is insufficient energy for chemical reactions, and the only deterioration that can occur in a biological sample is DNA damage by background radiation and cosmic rays. The shelf-life of cells stored at liquid nitrogen temperatures has been estimated to be of the order of 10\textsuperscript{3} years\textsuperscript{94}.

A second mechanism for cell damage during cryopreservation involves recrystallization. This is defined as the tendency of very small ice crystals that may have formed intracellularly during the cooling phase, to increase in size during the warming stage\textsuperscript{95}. Under such circumstances, slow warming rates may be harmful since they allow time for recrystallization to occur\textsuperscript{96}.

Hepatocytes in suspension Various strategies have been described over the last 25 years for cryopreserving hepatocytes. Reports on cryopreservation of isolated primary hepatocytes demonstrate high viability, ranging from 30-90% immediately post thaw\textsuperscript{97,97-102}, and reveal a continuous decline in cell number within a few hours\textsuperscript{103}. Additionally, many investigators have reported that despite high viability, few hepatocytes were able to attach to culture surfaces and survive for extended periods of time\textsuperscript{104}. Reports detailing assessment of cellular function (protein secretion, urea synthesis, P450 activity) have also focused on short-term (hours, 1-2 days) analysis. The use of post-thaw processing, such as centrifugation in Percoll gradients, has resulted in selected assessment of viable cell function and when unquantified, fails to provide an accurate evaluation of the efficacy of a given cryopreservation protocol. Overall, every study that carefully described survival and function of entire populations of cryopreserved isolated hepatocytes indicated low cell recovery and greatly impaired metabolic activity.

Cultured hepatocytes Though there are rather discouraging results for freshly isolated cells, a number of investigators has demonstrated the long-term survival of cultured hepatocytes after cryopreservation\textsuperscript{105,106}. Borel Rinkes and coworkers developed a cryopreservation protocol for rat hepatocytes cultured in a sandwich configuration, that resulted in a 75% recovery of long-term protein secretion, and morphology for at least 2 weeks post-thawing\textsuperscript{107}. Porcine hepatocytes are also cultured on microcarriers and frozen
for use in a BAL device. Immediate post-thaw viability of these hepatocytes ranged between 80-85%. Long-term viability and hepatospecific function were not reported. In summary, cryopreservation is a technology with potentially far reaching implications for the clinical use of bioartificial livers and hepatocyte transplantation. Although there has been reasonable progress in cryopreservation of cultured hepatocytes, the goal of freezing primary isolated hepatocytes while maintaining high viability and intact biological function has not yet been reached.

5. AIM AND OUTLINE OF THE THESIS

On its road to clinical application, the AMC-Bioartificial Liver had to pass many checkpoints on safety, feasibility and efficacy issues. The first studies in this thesis describe the final major, pre-clinical test: assessment of its activity in the anhepatic pig model of hepatic failure. Known total heptectomy procedures appeared not to answer the requirements for testing BAL devices, as the animals succumb to surgical bleeding problems instead of liver failure in itself. For this purpose, we designed and tested a new, rigid vascular prosthesis for 3-way portal-caval reconstruction. The surgical procedure is described in detail in chapter 2, including a characterization of the postoperative course of the animals until death. The actual assessment of benefit of the AMC-BAL in anhepatic pigs, focusing on survival and blood biochemistry, is set out in chapter 3. Served by the total absence of liver tissue in the animal, the same experimental setup is used in chapter 4 to study the synthetic function of the BAL itself in vivo, by extensive measurements in the animal of blood coagulation parameters produced inside the bioreactor.

In chapter 5, an attempt is made to explore new routes for the transplantation of isolated hepatocytes in the pig, serving as an intermediate stage between studies in rodents and utilization in clinical practice.

Successful cryopreservation of hepatocytes will greatly facilitate the widespread use of BAL devices as well as hepatocyte transplantation. Trying to push aside numerous reports of unsuccessful attempts, chapter 6 focuses on a newly developed way to cryopreserve hepatocytes, describing in detail long-term overall survival as well as hepatospecific function. In chapter 7, cryopreserved hepatocytes are further studied in coculture with fibroblasts. This is a culture system suited for use in an (experimental) BAL. The effects of cryopreservation on hepatocytes are taken from a functional to a genetic level in chapter 8, using microarray analysis to study gene expression changes following cryopreservation and thawing.
6. REFERENCES


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Introduction

